MÔNICA LARUCCI VIEIRA

**INTERAÇÃO DE *LEPTOSPIRA INTERROGANS* COM
O SISTEMA PROTEOLÍTICO PLASMINOGÊNIO /
PLASMINA: ANÁLISE, CARACTERIZAÇÃO E
POSSÍVEIS IMPLICAÇÕES NA INFECÇÃO**

Tese apresentada ao Programa de
Pós-Graduação Interunidades em
Biotecnologia USP/Instituto
Butantan/IPT, para obtenção do
Título de Doutor em Biotecnologia.

São Paulo
2012

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Área de concentração: Biotecnologia.

Orientadora: Prof. Dra. Ana Lúcia
Tabet Oller do Nascimento

Versão original

São Paulo
2012

DADOS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP)
Serviço de Biblioteca e Informação Biomédica do
Instituto de Ciências Biomédicas da Universidade de São Paulo

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Vieira, Mônica Larucci.

Interação de *Leptospira interrogans* com o sistema proteolítico do plasminogênio/plasmina: análise, caracterização e possíveis implicações na infecção / Mônica Larucci Vieira. – São Paulo, 2012.

Orientador: Profa. Dra. Ana Lúcia Tabet Oller do Nascimento.

Tese (Doutorado) – Universidade de São Paulo. Instituto de Ciências Biomédicas. Programa de Pós-Graduação Interunidades em Biotecnologia USP/IPT/Instituto Butantan. Área de concentração: Biotecnologia. Linha de pesquisa: Patogenicidade bacteriana

Versão do título para o inglês: *Leptospira interrogans* interactions with the plasminogen/plasmin proteolytic system: analysis, characterization and possible implications for the infection.

1. *Leptospira* 2. Leptospirose 3. Plasminogênio 4. Plasmina 5. Metaloproteases de matriz 6. Patogenicidade bacteriana I. Nascimento, Profa. Dra. Ana Lúcia Tabet Oller do II. Universidade de São Paulo. Programa de Pós-Graduação Interunidades em Biotecnologia USP/IPT/Instituto Butantan III. Título.

ICB/SBIB086/2012

UNIVERSIDADE DE SÃO PAULO
Programa de Pós-Graduação Interunidades em Biotecnologia
Universidade de São Paulo, Instituto Butantan, Instituto de Pesquisas Tecnológicas

Candidato(a): Mônica Larucci Vieira.

Título da Tese: Interação de *Leptospira interrogans* com o sistema proteolítico do plasminogênio/plasmina: análise, caracterização e possíveis implicações na infecção.

Orientador(a): Profa. Dra. Ana Lúcia Tabet Oller do Nascimento.

A Comissão Julgadora dos trabalhos de Defesa da Tese de Doutorado, em sessão pública realizada a/...../....., considerou

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Universidade Federal de São Paulo
Escola Paulista de Medicina

Comitê de Ética em Pesquisa
Hospital São Paulo

São Paulo, 16 de dezembro de 2005.

CEP 0664/05

Ilmo(a). Sr(a).

Pesquisador(a) MIRYAM PAOLA ALVAREZ FLORES

Co-Investigadores: Ana Marisa Chudzinski Tavassi, Marico Fritzen

Disciplina/Departamento: Biologia Molecular/Bioquímica da Universidade Federal de São Paulo/Hospital São Paulo

Patrocinador: Recursos Próprios.

PARECER DO COMITÊ DE ÉTICA INSTITUCIONAL

Ref: Projeto de pesquisa intitulado: **“Caracterização funcional do ativador de fator x purificado a partir do extrato bruto das cerdas da lagarta Ionomia oblíqua: ação sobre células endoteliais (HUVECs) e clonagem da proteína”.**

CARACTERÍSTICA PRINCIPAL DO ESTUDO: Experimental - categoria A.

RISCOS ADICIONAIS PARA O PACIENTE: Não se aplica.

OBJETIVOS: Estudar o sistema de ativação do ativador de Fator X purificado em nosso laboratório, sobre células endoteliais de cordão umbilical humano (HUVEC). Estudar o efeito do ativador sobre a produção de moléculas derivadas do endotélio envolvidas na modulação da função plaquetária (óxido nítrico, prostaciclina), na fibrinólise (ativador tissular do plasminogênio), na atividade procoagulante (FvW e fator tissular). Avaliar a atividade antiapoptótica e proangiogênica do ativador de Fator X. Clonar o cDNA responsável pelo ativador de Fator X utilizando a biblioteca já construída para determinação da sequência completa da proteína e possivelmente obtê-la na forma recombinante..

RESUMO: Serão utilizados 5 camundongos Swiss. Anestésico: Pentobarbital sódico. Proteína será obtida da larva, purificada e analisada. Diferentes concentrações de extrato bruto e das frações das diversas etapas de purificação, serão ensaiadas quanto a sua capacidade em ativar o Fator X e testado pela hidrólise de substrato cromogênico. A proteína será submetida à hidrólise e os fragmentos serão analisados e sequenciados. Serão realizados experimentos de biologia molecular, construindo oligonucleotídeos e realizar clonagem. Células endoteliais serão cultivadas in vitro e será testada a ação direta da proteína purificada e a possibilidade de modulação de respostas biológicas induzidas por moléculas como a trombina, TNF α e IL-1. Será analisado o efeito do fator ativador de Fator X sobre a expressão de FvW, prostaciclina, óxido nítrico, tPA e anti apoptótica. Será realizado também um estudo para determinar o efeito na microcirculação do ativador de FX, in situ, no músculo cremaster de camundongos machos do tipo Swiss, para visualizar a microcirculação..

FUNDAMENTOS E RACIONAL: Estudo fundamentado, visando caracterizar atividade do extrato de cerdas da lagarta Ionomia sobre atividade do fator X sobre células endoteliais..

MATERIAL E MÉTODO: Estão descritos os métodos, sendo de amplo domínio dos pesquisadores envolvidos.

DETALHAMENTO FINANCEIRO: FAPESP - R\$ 5562,00.

CRONOGRAMA: 48 meses.

OBJETIVO ACADÊMICO: Doutorado.

ENTREGA DE RELATÓRIOS PARCIAIS AO CEP PREVISTOS PARA: 11/12/2006 e 6/12/2007.

O Comitê de Ética em Pesquisa da Universidade Federal de São Paulo/Hospital São Paulo **ANALISOU** e **APROVOU** o projeto de pesquisa referenciado.

1. Comunicar toda e qualquer alteração do projeto.
2. Comunicar imediatamente ao Comitê qualquer evento adverso ocorrido durante o desenvolvimento do estudo.
3. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos para possível auditoria dos órgãos competente

Atenciosamente,

Prof. Dr. José Osmar Medina Pestana

Coordenador do Comitê de Ética em Pesquisa da
Universidade Federal de São Paulo/ Hospital São Paulo

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CERTIFICADO

Certificamos que o Projeto intitulado "Interação de *Leptospira interrogans* com sistema proteolítico plasminogênio / plasmina: análise, caracterização e possíveis implicações na infecção", protocolo nº 506/08, sob a responsabilidade de Ana Lúcia Tabet Oller do Nascimento e Mônica Larucci Vieira, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS DO INSTITUTO BUTANTAN (CEUAIB) em reunião de 13/08/2008.

We certify that the research entitled "*Leptospira interrogans* interaction with the proteolytic system plasminogen / plasmin: analysis, characterization and possible role during infection", protocol nº 506/08, under the responsibility of Ana Lúcia Tabet Oller do Nascimento and Mônica Larucci Vieira, is in agreement with the Ethical Principles in Animal Research, adopted by the Brazilian College of Animal Experimentation, and was approved by the ETHICAL COMMITTEE FOR ANIMAL RESEARCH of BUTANTAN INSTITUTE in the meeting of 08/13/2008.

Vigência do Projeto: 08/2008 – 08/2012	Nº de animais/espécie
	135 / camundongo Balb/c
	30 / hamster Golden

São Paulo, 19 de agosto de 2008.

Dra. Denise V. Tambourgi
Presidente da CEUAIB

De acordo:

Dr. Otávio Azevedo Mercadante
Diretor do Instituto Butantan

**Aos meus pais, Sandra e Glauco,
Pelas valorosas lições de vida e
confiança nas minhas escolhas.
Dedico.**

**À minha irmã Carolina, minha avó Alice
e meu marido Pedro,
por todo o apoio, compreensão e carinho.
Ofereço.**

AGRADECIMENTOS

Ao finalizar esta tese de Doutorado, aproveito a oportunidade de deixar registrados meus sinceros agradecimentos àqueles que, cada qual à sua maneira, contribuíram ao longo desta jornada.

Agradeço à Dra. Ana Lúcia Tabet Oller do Nascimento pela orientação, confiança depositada em mim e no meu trabalho, pelo apoio às minhas ideias, pela amizade, discussões e incentivo ao longo dos anos no Laboratório. Esta experiência incrementou muito na minha formação pessoal e profissional, e fomentou meu anseio de dedicação à pesquisa científica. Hoje eu tenho a certeza de um futuro dedicado à ciência!

Agradeço ao Laboratório de Bioquímica e Biofísica do Instituto Butantan, chefiado pela Dra. Ana Mariza Chudzinski-Tavassi, pela disponibilização de espaço, tempo e material para a realização dos experimentos com as HUVECs. Agradeço principalmente à Dra. Myriam Paola Alvarez-Flores pela ajuda com os experimentos, pela paciência durante as várias repetições e pelas valiosas discussões científicas (e não científicas), além do apoio e amizade.

Agradeço ao Dr. Sílvio Arruda de Vasconcellos e à Zenaide de Moraes pela colaboração com a cultura de leptospiros e manutenção da virulência dessas bactérias tão “temperamentais”.

Agradeço ao técnico do microscópio confocal do Laboratório de Parasitologia do Instituto Butantan, Alexander de Sousa, pela sua paciência, dedicação e, porque não, suas piadas ao ajudar na captura de imagens das leptospiros.

Agradeço ao laboratório de Biotecnologia Molecular III do Instituto Butantan na figura da Dra. Luciana Cezar de Cerqueira Leite e de seus alunos, que sempre estiveram abertos para o empréstimo de equipamentos e discussão de técnicas. Um obrigada especial ao Henrique Rofatto pela ajuda nos experimentos de PCR em tempo real.

Agradeço à Dra. Eliete Romero do Instituto Adolfo Lutz pelo fornecimento de soros de pacientes diagnosticados com leptospirose e à Dra. Karin Kirchgatter do Instituto de Medicina Tropical por ceder os soros de pacientes diagnosticados com dengue, malária, doença de Chagas e HIV.

Agradeço aos amigos de laboratório que por lá passaram ao longo de todos esses anos. Obrigada pela amizade, companheirismo e pelos momentos de descontração que tornaram esse caminho muito mais agradável.

Agradeço às técnicas que sempre deram seu suporte no preparo de soluções e de materiais básicos, e na organização do laboratório.

Agradeço principalmente aos meus pupilos queridos Luís Guilherme, Natalie e Renan, por quem tenho um carinho especial. Luís com sua curiosidade infinita, amizade e parceria para o que der e vier; Natalie com a sua distração e determinação; Renan com sua prestatividade e individualidade. Registro também meu agradecimento particular à amiga Marina, hoje minha madrinha, que sempre esteve disposta a ajudar e partilhar de momentos dentro e fora do laboratório.

Agradeço aos estudantes, pesquisadores e funcionários do Centro de Biotecnologia do Instituto Butantan pelo apoio e convivência durante os últimos anos, sempre dispostos a um “bom dia” e um sorriso.

Agradeço aos meus amigos do badminton da USP, que indiretamente ajudaram muito com seu companheirismo, cuidados e distração nos meus finais de tarde, principalmente Ricardo, Maurício, João e Clarissa. Nada como dar umas risadas e umas petecadas para encerrar um dia pós-laboratório!

Agradeço a amizade e o apoio dos meus eternos amigos da época do colégio, Marcelo, Gisele e Ana Paula. Não importa o tempo e a distância, o carinho permanece o mesmo!

Agradeço aos meus queridíssimos amigos da Biologia da USP, o grupo Purede. Meu padrinho querido Renato Ramos Massaro Batata, minha vizinha Ana Carolina Tuska,

Alice Brites e Joana Carvalho. Obrigada pela amizade, confiança, desabafos, conversas, preocupação, apoio, e cervejas partilhadas desde a graduação!

Agradeço aos amigos de Braga, Magno, Virgínia, Marília, Mayron e Matheus. Obrigada por fazerem essa experiência ter valido à pena! Foram meses resumidos em amizade, desabafos, trabalho, viagens e muitos finos!

Agradeço, porque não, às pedras no caminho deste percurso. Cada uma que foi chutada, tropeçada ou pulada trouxe muito aprendizado e me fez crescer pessoal e profissionalmente. Os arranhões e cicatrizes que ficaram fazem apenas me lembrar dos desafios contornados.

Agradeço à Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pela concessão da bolsa de doutorado. Agradeço também ao CNPq e à Fundação Butantan pelo apoio financeiro, sem os quais este trabalho não teria sido viável.

Impossível de nomear a todos, agradeço aos que cruzaram minha vida e meu caminho durante esta jornada! Obrigada!

Deixo o final agradecimento a toda a minha família. Tios, primos, avós, todos! Agradeço especialmente aos meus pais, Sandra e Glauco, minha irmã Carolina, minha avó Alice, meu marido Pedro e à Yolli e Jully. Sem o estímulo, ajuda e suporte de vocês não teria feito nada!

Muito obrigada!

**A espantosa realidade das coisas
É a minha descoberta de todos os dias.
Cada coisa é o que é,
E é difícil explicar a alguém quanto isso me alegra,
E quanto isso me basta.
Basta existir para se ser completo.
(A espantosa realidade das coisas, Alberto Caeiro – FP)**

**"O importante é não parar de questionar."
(Albert Einstein)**

RESUMO

Vieira ML. Interação de *Leptospira interrogans* com o sistema proteolítico do plasminogênio/plasmina: análise, caracterização e possíveis implicações na infecção. 253 páginas. Tese (Doutorado em Biotecnologia). Universidade de São Paulo, Instituto de Ciências Biomédicas, São Paulo, Brasil, 2012.

A leptospirose é uma importante zoonose, causada por bactérias espiroquetas do gênero *Leptospira*. No Brasil, a leptospirose representa um grave problema de saúde pública com surtos epidêmicos nas épocas de chuvas, além de acarretar grande impacto econômico na pecuária. Apesar da sua importância, os mecanismos moleculares da patogênese, virulência e processo infeccioso das leptospirosas permanecem não compreendidos. Recentemente, foram identificadas proteínas de leptospirosas com função de adesão aos componentes de matriz extracelular. Seguindo a adesão, as bactérias devem alcançar a corrente sanguínea para chegar aos tecidos e órgãos, e então colonizar sítios secundários de infecção e imunologicamente privilegiados, como os túbulos renais. A atividade proteolítica de degradação e desestruturação da matriz extracelular e membrana basal é crucial para essa etapa. No entanto, as leptospirosas, bem como a maioria dos micro-organismos patogênicos, não apresentam proteases conhecidas capazes de degradação dessas barreiras mecânicas. Como estratégia, muitos patógenos desenvolveram a capacidade de interagir com o sistema proteolítico do plasminogênio/plasmina dos hospedeiros, de modo que adquirem atividade proteolítica de degradação de matriz extracelular e membrana basal. Esse parece ser um mecanismo importante durante o processo de disseminação nos hospedeiros. Tendo isso em vista, propôs-se a investigação do mesmo sistema durante o processo infeccioso da leptospirose. Foi demonstrada pela primeira vez a interação de *Leptospira* com o sistema fibrinolítico via captura de plasminogênio na superfície e geração de plasmina por ativadores do hospedeiro. Foram identificadas diversas proteínas que podem atuar como receptores de plasminogênio em *Leptospira*, algumas aparentemente diferencialmente expressas em condição de virulência, o que sugere a importância desse mecanismo para a patogenicidade dessas bactérias. A plasmina associada à superfície bacteriana confere capacidade de degradação de componentes de matriz extracelular e maior habilidade de penetração por monocamadas de células endoteliais, o que pode favorecer a disseminação no hospedeiro. A plasmina ligada às leptospirosas beneficia a evasão do sistema imune inato, já que diminui a deposição de IgG e C3b, aumentando a

sobrevivência das bactérias ao ataque do sistema complemento. As leptospiros mostraram-se capazes de estimular o desbalanço na regulação de sistemas dependentes de proteases do hospedeiro, já que induzem a expressão de ativadores de plasminogênio e metaloproteases de matriz por células endoteliais. Além disso, pacientes com leptospirose confirmada exibem níveis mais elevados de ativadores de plasminogênio e metaloproteases de matriz circulantes no plasma, o que parece ser mais exacerbado no início da infecção. Os resultados obtidos são de grande contribuição para o conhecimento acerca dos aspectos moleculares do processo infeccioso das leptospiros. Nossos estudos descrevem uma nova atividade proteolítica que possivelmente é (representa) um dos mecanismos utilizados pelas leptospiros para facilitar a sua penetração, disseminação e evasão imune no hospedeiro.

Palavras-chave: *Leptospira*. Leptospirose. Plasminogênio. Plasmina. Metaloproteases de matriz. Patogenicidade bacteriana.

ABSTRACT

Vieira ML. *Leptospira interrogans* interactions with the plasminogen/plasmin proteolytic system: analysis, characterization and possible implications for the infection. 253 pages. Doctoral thesis (Biotechnology). Universidade de São Paulo, Instituto de Ciências Biomédicas, São Paulo, Brazil, 2012.

Leptospirosis is a globally important zoonosis, caused by pathogenic bacteria belonging to the order Spirochaetales, genus *Leptospira*. Leptospirosis is a disease with great social and economic impact in public health and livestock in Brazil. Despite its importance, the molecular mechanisms for leptospiral pathogenesis, virulence, and infectious process remain poorly understood. Recently, several leptospiral proteins were characterized as extracellular matrix components ligands. After adhesion, the organism has to reach the blood circulation in order to colonize target organs, immunologically safe environments, such as the renal tubules. The proteolytic activity is crucial for this stage, as it allows the extracellular matrix and basal membrane disruption. However, as the majority of pathogenic microorganisms, *Leptospira* lack proteases capable of degrading the components of these structural barriers. In an attempt to overcome this absence, many pathogens evolved the ability to interact with the host's plasminogen/plasmin system in order to acquire proteolytic activity. This ability seems to be important for the pathogens' spreading process along the hosts' tissues. Those facts prompted the investigation of the same mechanism during the infectious process of leptospirosis. This work reports for the first time the *Leptospira* interaction with the fibrinolytic system by capturing plasminogen on the surface and generation of plasmin by the host's activators. Several plasminogen binding proteins that may act as receptors were identified in *Leptospira*, some of them apparently differentially expressed in highly virulent conditions, suggesting the importance of this mechanism for the leptospiral pathogenesis. The plasmin associated to the bacterial surface was capable to degrade extracellular matrix components, facilitating the penetration through endothelial cells that may enhance the dissemination within the host. The plasmin bound to the leptospires contributes to the immune evasion, by decreasing IgG and C3b depositions, augmenting the bacterial survival upon complement system attack. Leptospires were shown to stimulate an imbalance in the fibrinolytic and proteases systems regulation by increasing the expression of plasminogen activators and matrix metalloproteases by endothelial cells. Furthermore, confirmed leptospirosis patients

show higher levels of circulating plasminogen activators and matrix metalloproteases, which appears to be more pronounced at the beginning of the infection. Altogether, the results obtained contribute to the knowledge of the molecular aspects of leptospirosis infection. These studies reveal a novel proteolytic activity that represents one possible mechanism used by the leptospire to enhance penetration, dissemination and immune evasion within the hosts.

Keywords: *Leptospira*. Leptospirosis. Plasminogen. Plasmin. Matrix metalloproteases. Bacterial pathogenesis.

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LISTA DE ABREVIATURAS E SIGLAS

2D – bidimensional

AAC – ácido aminocapróico

ACN – acetonitrila

ACTB – beta actina

AMPA – acetato p-aminophenyl mercúrico

BSA – albumina sérica bovina (*bovine serum albumin*)

C4BP – proteína ligante de C4 (*C4 binding protein*)

CCD – Coordenadoria de Controle de Doenças

cDNA – ácido desoxirribonucleico complementar

CEUAIB – Comitê de Ética no Uso de Animais Instituto Butantan

CT – ciclo limiar (*threshold cycle*)

CVE – Centro de Vigilância Epidemiológica

DAB – tetrahidrocloreto de 3,3-diaminobenzidina

DNA – ácido desoxirribonucleico

DP – desvio padrão

DTT – ditioneitol

EDTA – ácido etilenodiamino tetra-acético

ELISA – ensaio imunoenzimático (*enzyme-linked immunosorbent assay*)

EMJH – meio Ellinghausen-McCullough-Johnson-Harris

Estados Unidos – EUA

FH – fator H

FHL-1 – proteína similar ao fator H (*fator H-like*)

FIOCRUZ – Fundação Oswaldo Cruz

FITC – isotiocianato de fluoresceína

GAPDH – gliceraldeído 3-fosfato desidrogenase

HIV – vírus de imunodeficiência humana

HUVECs – células endoteliais de veia de cordão umbilical humano (*Human Umbilical Vein Endothelial Cells*)

IAA – iodoacetamida

IgG – imunoglobulina G

IMT – Instituto de Medicina Tropical

IPG – fitas de gel com pH imobilizado em gradiente (*Immobilized pH gradient gel strips*)

K_D – constante de dissociação

Len – proteína de leptospiroses similar à endostatina (*leptospiral endostatin-like*)

Lig – proteína de leptospiroses similar à imunoglobulina (*leptospiral immunoglobulin-like*)

LPHS – hemorragia pulmonar associada à leptospirose (*leptospirosis pulmonary haemorrhage syndrome*)

LPS – lipopolissacarídeos

lsPBS – solução salina-fosfato contendo baixa concentração de sal (*low-salt phosphate buffered saline*)

m/v – relação massa-volume

m/z – relação massa-carga

MALDI-TOF – Ionização e dessorção a laser assistida por matriz – Tempo de voo (*Matrix assisted laser desorption ionization – Time-of-flight*)

MAT – teste de aglutinação microscópica (*microscopic agglutination test*)

MB – membrana basal

Mce – proteína de entrada em células de mamíferos (*mammalian cell entry protein*)

MEC – matriz extracelular

MMP – metaloprotease de matriz (*matrix metaloprotease*)

MS – Ministério da Saúde

N-terminal – amino-terminal

OMP – proteína de membrana externa (*outer membrane protein*)

OMS – Organização Mundial da Saúde

OPD – *o*-phenylenediamina

PAI – inibidor de ativador de plasminogênio (*plasminogen activator inhibitor*)

PBS – solução salina-fosfato (*phosphate buffered saline*)

PBS-T – PBS adicionada de tween 20

PCR – reação em cadeia da polimerase (*polymerase chain reaction*)

pH – potencial de hidrogênio

pI – ponto isoelétrico

PLA – plasmina

PLG – plasminogênio

PMF – espectros de massas dos peptídeos (*peptide mass fingerprint*)

PMSF – fluoreto de fenilmetilsulfonil

qPCR – PCR em tempo real

RNA – ácido ribonucleico

RNA_m – ácido ribonucleico mensageiro

RPMI – meio *Roswell Park Memorial Institute*

S-2251 – substrato para plasmina D-Val-Leu-Lys dicloridrato 4-nitroanilida

SC – sistema complemento

SDS-PAGE – eletroforese em gel de poliacrilamida-dodecil sulfato de sódio (*Sodium Dodecyl Sulphate-Gel Electrophoresis*)

SES-SP – Secretaria da Saúde do Estado de São Paulo

SFB – soro fetal bovino

SINAN – Sistema de Informação de Agravos de Notificação

SVS – Secretaria de Vigilância em Saúde

TFA – ácido trifluoroacético

TIMP – inibidor tecidual de metaloproteases (*tissue inhibitor of metaloproteases*)

TLR – receptor do tipo Toll (*Toll-like receptor*)

tPA – ativador de plasminogênio tipo tecidual (*tissue-type plasminogen activator*)

TX-114 – triton X-114

uPA – ativador de plasminogênio tipo uroquinase (*uroquinase-type plasminogen activator*)

USP – Universidade de São Paulo

v/v – relação volume-volume

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1 INTRODUÇÃO

1.1 BACTÉRIAS ESPIROQUETAS

As espiroquetas constituem um distinto grupo de bactérias helicoidais móveis, amplamente distribuídas na natureza, que vivem desde como formas livres no ambiente, como em associação com diversos grupos de eucariotos (Charon e Goldstein, 2002). Embora as associações com os hospedeiros sejam em sua maioria benignas, algumas espiroquetas são patogênicas para animais e humanos. Muitas espécies causam doenças altamente prevalentes e relevantes como a leptospirose (*Leptospira* spp.), sífilis (*Treponema pallidum pallidum*), doença de Lyme (*Borrelia burgdorferi*) e periodontite (*Treponema denticola*).

O filo Spirochaetales contém uma única classe e ordem. Como grupo taxonômico, a ordem Spirochaetales foi definida com base nas características morfológicas únicas entre as bactérias que a compõem. Possuem corpo celular espiralado, envolto por membrana lipídica e parede celular de peptidoglicanos, também chamado de cilindro protoplasmático, correspondendo à estrutura típica de outras bactérias gram-negativas. O que as torna singulares é a presença de uma membrana de bicamada lipídica externa ao cilindro protoplasmático, definindo o periplasma, que contém o flagelo periplasmático, ou endoflagelo. Os endoflagelos podem variar em número e tamanho de acordo com a espiroqueta, ficando esses ancorados em uma das extremidades do corpo celular, estendendo-se até a extremidade oposta.

Embora as espiroquetas compartilhem características estruturais e morfológicas, constituem um grupo extremamente diversificado fisiologicamente. Variam desde anaeróbicas até aeróbicas de vida livre ou parasitas obrigatórios.

1.2 AS LEPTOSPIRAS

1.2.1 Taxonomia e classificação das leptospiiras

As leptospiiras pertencem à família Leptospiraceae, composta pelos gêneros *Leptospira* spp., *Turneria* spp. e *Leptonema* spp. (Haake et al., 2002). O gênero *Leptospira* é composto por espécies saprofíticas de vida livre e espécies patogênicas, sendo estas últimas parasitas obrigatórias ou transientes. Há duas formas de classificação de *Leptospira* spp.: uma

molecular, fundamentada em análises gênicas, e outra sorológica, baseada em determinantes antigênicos. Ambas reconhecem espécies patogênicas e saprofíticas.

A sorologia foi o método utilizado para a classificação das leptospiros até o final da década de 1980 (Dikken et al., 1978) sendo que, atualmente, as amostras ainda são comumente referidas pelo sorovar. A classificação sorológica baseia-se em testes de aglutinação microscópica, definindo a nomenclatura de sorovar. São agrupados no mesmo sorovar os isolados que apresentam reatividade cruzada com soros de animais infectados com sorovares determinados. Atualmente, as leptospiros são classificadas em mais de 250 sorovares distribuídos em 24 sorogrupos diferentes (Bharti et al., 2003; Faine et al., 1999; Farr, 1995; Levett, 2001). A diversidade de sorovares reside na heterogeneidade de carboidratos que compõem a cadeia lateral dos lipopolissacarídeos (LPS) da membrana bacteriana, que podem refletir um caráter adaptativo para a infecção de uma gama maior de novas espécies hospedeiras (Bharti et al., 2003; Faine et al., 1999; Farr, 1995).

Com o advento de técnicas moleculares, a classificação por sorovares começou a ser revisada. A classificação molecular do gênero *Leptospira* baseia-se no grau de hibridização por homologia de DNA (ácido desoxirribonucleico) (Brenner et al., 1999). Assim, o gênero divide-se em 17 espécies definidas, com pelo menos 70% de similaridade gênica, e com divergência de pelo menos 5% de bases não pareadas. Por esta classificação, os sorovares são encontrados em mais de uma espécie. Embora a reclassificação por genotipagem seja taxonomicamente mais confiável, ainda requer maior amplitude e refinamento, permanecendo motivo de controvérsia entre os microbiologistas por ser incompatível com o sistema sorológico adotado por clínicos e epidemiologistas (Bharti et al., 2003).

1.2.2 Aspectos biológicos e morfológicos das leptospiros

As leptospiros são células longas, finas e helicoidais, exibindo espiras curtas e regulares para o lado direito (Faine et al., 1999). Suas extremidades são encurvadas em forma de ganchos típicos. Apresentam dois flagelos periplasmáticos axiais, ancorados cada um em uma das extremidades da célula, que se prolongam em direção ao centro celular, sem sobreposição (Charon e Goldstein, 2002; Goldstein et al., 1994). Sua mobilidade se dá por rotação, impulsionada pelos flagelos periplasmáticos. Em geral, têm tamanho de 0,1 a 0,15 µm de diâmetro por 6,0 a 12,0 µm de comprimento. Não é possível observá-las em microscópio ótico comum, mas podem ser visualizadas em microscópio de campo escuro ou

contraste de fase (Faine et al., 1999). A maioria não é corada pela técnica de Gram, mas podem ser visualizadas por impregnação por sais de prata.

Na membrana externa, os LPS constituem os principais antígenos de leptospiras. São estruturalmente semelhantes aos LPS de organismos gram-negativos típicos, contudo relativamente pouco tóxicos para células ou animais (Faine et al., 1999; Werts et al., 2001). Os LPS são comumente compostos por três segmentos ligados covalentemente: lipídeo A, *core*, e antígeno O. O lipídeo A é a porção lipídica, que ancora o LPS à membrana externa celular, e possui características únicas em *Leptospira*, incluindo uma unidade dissacarídica de glucosamina metilada e fosforilada (Que-Gewirth et al., 2004). O *core* é a porção polissacarídica que forma a região intermediária e, mais externamente, localiza-se o antígeno O, que consiste em grupos polissacarídicos altamente variáveis. O antígeno O é considerado o principal agente antigênico do LPS das bactérias gram-negativas (Faine et al., 1999). Curiosamente, o LPS de *Leptospira* induz resposta imune inata em humanos via receptor do tipo Toll 2 (TLR2, *Toll-like receptor 2*), ao invés de TLR4, o receptor clássico de LPS bacterianos (Nahori et al., 2005; Que-Gewirth et al., 2004).

Os demais constituintes da membrana externa são proteínas de membrana (OMPs, *outer membrane proteins*), sendo em sua maioria proteínas lipidadas, as lipoproteínas (Haake e Matsunaga, 2010; Malmstrom et al., 2009). Estima-se que as leptospiras apresentem mais de 300 OMPs, entre lipoproteínas e proteínas transmembrana, sendo que a maior parte delas não apresenta homólogos em outros organismos nem função definida. Devido à sua localização, acredita-se que as OMPs sejam importantes para a patogênese, mediando interações patógeno-hospedeiro.

1.2.3 Metabolismo e cultivo das leptospiras

As leptospiras são micro-organismos quimio-organotróficos aeróbicos, que utilizam oxigênio como aceptor final da cadeia de transporte de elétrons. Em condições de cultivo em laboratório, não utilizam açúcares ou aminoácidos como fonte energética, embora possuam os genes codificadores para todas as enzimas da via glicolítica (Nascimento et al., 2004b). A fonte de carbono e energia utilizada são ácidos graxos de cadeia longa, metabolizados por β -oxidação (Faine et al., 1999). Os carboidratos podem ser sintetizados pelo ciclo do ácido tricarboxílico, via das pentoses-fosfato não oxidativa, e vias do glioxalato ou fosfogluconato (Faine et al., 1999). São catalase e oxidase positivas, mas não possuem superóxido dismutase (Nascimento et al., 2004b).

As leptospirosas podem ser cultivadas *in vitro* em meios de cultura artificiais. O meio de cultura mais utilizado é o Ellinghausen-McCullough-Johnson-Harris (EMJH), que possui ácidos graxos complexados a sorbitol como fonte de carbono e albumina sérica bovina (BSA, *bovine serum albumin*) como agente detoxificante. Geralmente, suplementa-se o meio EMJH com 8-12% de soro de coelho levemente hemolisado como fonte de ferro, cianocobalamina, albumina, ácidos graxos, e outros nutrientes (Faine et al., 1999). Em substituição ao soro de coelho, há disponíveis suplementos comerciais sintéticos.

As culturas crescem em condições ótimas de potencial de hidrogênio (pH) e temperatura de 7,2 a 7,6 e entre 28 a 30 °C, respectivamente (Levett, 2001). As densidades das culturas não alcançam as obtidas com outras bactérias, mas atingem concentrações de 10^7 a 10^8 células/mL, com turbidez visível a partir do quarto ou quinto dia (Faine et al., 1999). Isolados clínicos de mamíferos são dificilmente cultiváveis, podendo permanecer em fase estacionária por dias ou semanas, não apresentando bom crescimento nem em temperatura fisiológica de 37 °C.

1.3 A LEPTOSPIROSE

1.3.1 Distribuição e prevalência

A leptospirose é uma das zoonoses mais disseminadas mundialmente, causada por espécies patogênicas de *Leptospira* (Bharti et al., 2003; Faine et al., 1999; Vinetz, 2001). A leptospirose tem ampla distribuição geográfica, acometendo desde regiões tropicais e subtropicais, até zonas temperadas. É endêmica em diversos países, oferecendo potencial epidêmico. Epidemias podem estar associadas com mudanças de comportamentos humanos, contaminação da água, variações na densidade de animais carreadores ou a desastres naturais, como ciclones e enchentes (Organização Mundial de Saúde, *World Health Organization*, WHO) (WHO, 2003). A doença vem sendo considerada um importante problema de saúde pública devido à sua crescente incidência (Meites et al., 2004; WHO, 2003).

A incidência da leptospirose é baixa nos países desenvolvidos, estando principalmente associada às atividades recreativas envolvendo contato com água ou atividades ocupacionais consideradas de risco, como veterinários, fazendeiros, mineradores ou trabalhadores de abatedouros (Haake et al., 2002; Hartskeerl et al., 2011). Já nos países em desenvolvimento, a incidência parece ser crescente, sendo que nos últimos anos, várias epidemias vêm sendo relatadas (Hartskeerl et al., 2011), acometendo principalmente regiões metropolitanas

periféricas com precária rede sanitária. Essa prevalência está associada às condições climáticas (ambiente quente e úmido) que favorecem a sobrevivência das bactérias livres no ambiente durante seu ciclo de transmissão, e às deficitárias redes de saneamento básico, que propiciam a proliferação dos roedores, os principais vetores da doença. Assim, no contexto urbano, a leptospirose atinge principalmente pessoas de baixo nível socioeconômico, e o seu controle está diretamente relacionado a melhorias nas condições de saneamento básico (Reis et al., 2008).

Os índices da ocorrência anual de casos humanos da leptospirose não são precisamente conhecidos, já que esses dados são falhos ou ausentes em diversos países, especialmente na África. De acordo com as informações atualmente disponíveis, a incidência varia de aproximadamente 0,1 a 1/100.000 por ano em regiões temperadas, até 10 a 100/100.000 por ano em climas tropicais úmidos. Durante epidemias, a incidência pode chegar a 100/100.000 (WHO, 2003). Assim, estima-se que mais de 500.000 casos de leptospirose ocorram a cada ano no cenário mundial (WHO, 2003). Alguns estudos ainda mostram que a leptospirose pode representar 20 a 40% das doenças febris de origem desconhecida (Ko et al., 1999). Apesar de bastante significativos, acredita-se que esses números sejam subestimados devido à falha no diagnóstico ou falta de notificação dos órgãos competentes.

No Brasil, a leptospirose é uma doença de notificação compulsória desde o ano de 1985. Em todo o país, o número de casos confirmados no período de 1997 a 2010 foi de 47.782, com média anual de 3.413 casos. Desses pacientes, 5.054 foram a óbito (361 óbitos/ano) (Secretaria de Estado da Saúde/Secretaria de Vigilância de Saúde/Ministério da Saúde, SINAN/SVS/MS, 2012). No Estado de São Paulo, no ano de 2011, houve 5.713 casos notificados, sendo 958 casos confirmados laboratorialmente, atingindo a taxa de letalidade de 11,38%. No período de 1986 a 2011, o total de casos confirmados no Estado foi de 17.395, com 2.201 óbitos, consistindo em médias anuais de 669 casos/ano, 84 óbitos/ano, e taxa de letalidade média anual de 13% (SINAN – Divisão de Zoonoses/ Centro de Vigilância Epidemiológica/Coordenadoria de Controle de Doenças/Secretaria de Estado da Saúde de São Paulo, SINAN/CVE/CCD/SES-SP, 2012).

1.3.2 Impactos econômicos da leptospirose

No Brasil, em outros países da América Latina e em países asiáticos como a Índia, por exemplo, a leptospirose é um grave problema de saúde pública (Bharadwaj et al., 2002; Ko et al., 1999; Lomar et al., 2000). Quando diagnosticada, o tratamento envolve uma combinação

de antibióticos e medidas de suporte. Nos casos mais graves, o tratamento requer hospitalização com necessidade de diálise renal, tratamento dos quadros hemorrágicos e acompanhamento intensivo. Assim, além de sua alta letalidade, a leptospirose representa um problema econômico para o sistema de saúde pública, uma vez que o tratamento é de alto custo hospitalar (Faine et al., 1999).

Além do elevado impacto econômico social, essa zoonose ainda acarreta grande impacto econômico na pecuária, pois, além de humanos e roedores, as leptospirosas infectam mamíferos de valor comercial agregado. Deste modo, é uma importante doença infecciosa relacionada a transtornos da reprodução, acometendo rebanhos de bovinos, suínos, equinos e outros. Nos bovinos, as leptospiroses causam consideráveis perdas econômicas em decorrência de repetições de cio, infertilidade, mastites, abortos, natimortalidade, bezerros prematuros, nascimento de bezerros fracos, mortes, e decréscimo na produção leiteira e de carne (Faine et al., 1999; Sheldon e Dobson, 2003).

1.3.3 Ciclo de transmissão

O ciclo de transmissão da leptospirose envolve o meio ambiente, hospedeiros de manutenção assintomáticos e hospedeiros susceptíveis à doença. A transmissão entre os hospedeiros pode ser direta ou indireta. A transmissão direta ocorre quando leptospirosas presentes em tecidos, fluidos corporais ou urina de animais com infecção aguda ou carreadores assintomáticos crônicos entram em contato com novos hospedeiros, iniciando a infecção. A transmissão direta é comum entre grupos ocupacionais que trabalham com animais ou seus tecidos. Entre humanos, a transmissão direta é extremamente rara, embora já reportada (Bolin e Koellner, 1988).

A transmissão indireta ocorre quando animais ou humanos entram em contato com leptospirosas presentes no meio ambiente, oriundas geralmente da urina de hospedeiros de manutenção (Faine et al., 1999). Embora sejam vulneráveis aos fatores ambientais, em especial ao ressecamento, as leptospirosas podem sobreviver por longos períodos na água e solo, podendo inclusive multiplicar-se em condições favoráveis (Baker e Baker, 1970). Quase toda espécie de roedores, marsupiais e de mamíferos pode ser carreadora e excretora de leptospirosas (Faine et al., 1999). Esses animais albergam as bactérias nos rins e as eliminam vivas no meio ambiente, re-infectando constantemente o solo, a água e outros animais. Os principais reservatórios urbanos são os roedores sinantrópicos, como *Rattus norvegicus* (ratazana ou rato de esgoto), *Rattus rattus* (rato-preto) e *Mus musculus* (camundongo).

As principais vias de entrada das leptospiros nos hospedeiros são a pele e mucosas, por onde as bactérias parecem penetrar através de abrasões ou ativamente pelo tecido íntegro após exposição prolongada à água, por mecanismos ainda não compreendidos.

1.3.4 Leptospirose humana

Os humanos são hospedeiros acidentais dentro da cadeia de transmissão de *Leptospira*, sendo a principal fonte para a sua infecção o contato com águas contaminadas (Faine et al., 1999; Plank e Dean, 2000). Geralmente, a leptospirose humana não é uma doença fatal. A doença ocorre pela penetração ativa dos microrganismos através das mucosas (ocular, digestiva, respiratória e genital), ou por meio da pele lesada ou mesmo íntegra (Bharti et al., 2003; Faine et al., 1999; Levett, 2001). Após a penetração, as leptospiros percorrem as vias linfáticas e sanguíneas, atingindo sítios imunologicamente privilegiados, como túbulos renais, fígado, cérebro e olhos, onde persistem e se multiplicam (Faine et al., 1999). O tempo de incubação da doença até o surgimento dos sintomas pode ser de algumas horas até 28 dias, sendo em média de 7 a 15 dias.

A apresentação clínica da leptospirose é bifásica, sendo que a fase aguda ou septicêmica (inicial) dura cerca de uma semana, seguido pela fase imune, caracterizada pela produção de anticorpos e excreção de leptospiros na urina, a leptospiúria (Levett, 2001). A fase inicial, denominada leptospiemia, manifesta-se com sintomatologia ampla e inespecífica, como febre, calafrios, cefaleia e mialgia, podendo ser confundida com outras doenças como gripe, dengue e outras doenças virais. O amplo espectro de sintomas clínicos é o principal motivo de a doença ser subdiagnosticada ou não reportada na maioria das vezes (Plank e Dean, 2000) gerando, assim, um baixo índice de casos notificados.

A leptospirose pode evoluir para uma condição mais severa, conhecida como síndrome de Weil (descrita por Weil em 1886), que corresponde a aproximadamente 5% a 15% dos casos. Esta manifestação clínica pode desenvolver-se para quadros caracterizados por hemorragias, icterícia, falência renal, meningite e hipotensão (Faine et al., 1999; Levett, 2001; Vinetz, 2001), atingindo letalidade de 15%, geralmente associada à falência múltipla de órgãos e hemorragia generalizada (Bharti et al., 2003). A severidade da doença parece depender do sorovar infectante, a carga de leptospiros, bem como a idade e condição imunológica do indivíduo infectado, porém ainda não foi totalmente compreendida. Observa-se que a síndrome de Weil normalmente está associada à infecção pelos sorovares Icterohaemorrhagiae, Copenhageni e Lai (Adler e de la Pena Moctezuma, 2010).

Além da síndrome de Weil, outra forma severa da leptospirose, denominada síndrome de hemorragia pulmonar associada à leptospirose (LPHS, *leptospirosis pulmonary haemorrhage syndrome*), vem sendo foco de atenção ultimamente. Esta síndrome, primeiramente identificada na Coreia do Norte e na China, vem sendo cada vez mais reportada mundialmente (Gouveia et al., 2008; McBride et al., 2005). Estima-se que os índices de mortalidade desta manifestação respiratória da doença possam chegar a 70% (Toyokawa et al., 2011). Os fatores responsáveis pela LPHS e a sua emergência não são conhecidos.

1.3.5 Diagnóstico da leptospirose

Como os sintomas da leptospirose são amplos e inespecíficos para a doença, a experiência clínica, a avaliação do ambiente epidemiológico e história clínica do paciente podem contribuir para o diagnóstico médico. Todavia, a confirmação da leptospirose só é possível através de diagnóstico laboratorial. Os métodos de diagnóstico de leptospirose disponíveis atualmente incluem microscopia, histopatologia, cultivo e sorologia – como teste de aglutinação microscópica (MAT), aglutinação macroscópica, hemaglutinação indireta, aglutinação micro capsular, ou ELISA (ensaio imunoenzimático, *enzyme-linked immunosorbent assay*) (Thongboonkerd, 2008).

Entre os métodos sorológicos, o mais utilizado é o MAT, sendo a técnica padrão-ouro recomendada pela OMS (WHO, 2003). No entanto, é um teste de difícil padronização, controle e interpretação, sendo realizado apenas em determinados laboratórios de referência. É um método relativamente pouco sensível e subjetivo. Além disso, um dos maiores problemas do seu emprego é a necessidade de manutenção de culturas de diferentes sorovares de leptospirosas vivas e virulentas para a reação com soro de pacientes. O MAT baseia-se na composição dos LPS de membrana, e na reatividade dos anticorpos dos soros dos pacientes com esses antígenos de superfície (Bharti et al., 2003). Na fase inicial da doença, o título de anticorpos séricos ainda é muito baixo (Faine et al., 1999) e a detecção de anticorpos na evolução da infecção humana é tardia, sendo as sorologias inteiramente confiáveis apenas a partir do quinto ao sétimo dias de doença (Levett, 2003), quando já podem haver muitos danos causados pelas bactérias instaladas no paciente (Bharti et al., 2003). Além do diagnóstico tardio, o MAT pode apresentar inespecificidades geradas por reações cruzadas entre os sorovares.

Como alternativa ao MAT, outros métodos têm sido empregados para o diagnóstico de leptospirose. Testes de ELISA têm se mostrado sensíveis e reprodutíveis, mas ainda não há um antígeno suficientemente precoce e específico para detectar a doença em sua fase inicial (Ahmad et al., 2005; Faine et al., 1999). Entre os métodos moleculares, técnicas de PCR (reação da polimerase em cadeia – *polymerase chain reaction*) vêm sendo desenvolvidas para a detecção de leptospiras nos tecidos e fluidos dos pacientes. Por PCR pode-se amplificar, seletivamente, sequências-alvo de DNA a partir de líquido e urina de indivíduos suspeitos de estarem infectados com leptospirose (Fonseca et al., 2006; Levett et al., 2005; Merien et al., 1992). Porém, o uso do método de PCR é limitado, demanda técnicas e reagentes moleculares específicos, sendo de difícil implantação principalmente em países pobres e regiões sem recursos ou estrutura adequados.

Recentemente, foi divulgado o desenvolvimento de um novo método diagnóstico baseado na proteína de membrana LigA de leptospiras. A proteína recombinante purificada LigA é imobilizada em um cartão, e o contato com uma gota do soro de paciente com suspeita de leptospirose permite a detecção da presença de anticorpos para esse antígeno (Fundação Oswaldo Cruz, FIOCRUZ, 2010).

1.3.6 Medidas profiláticas

Devido à multiplicidade de sorovares, fontes de infecção e transmissão, o controle da leptospirose é dificultado e dependente de condições locais específicas. Medidas preventivas devem considerar a identificação de grupos com risco particular de infecção e os fatores epidemiológicos locais, focando a fonte de infecção, a rota de transmissão ou a infecção ou doença. O risco de infecção pode ser minimizado evitando-se o contato com urina, animais ou ambientes infectados. A população deve ser conscientizada sobre a doença, e devem ser adotadas medidas de melhora na higiene, cuidados pessoais e saneamento.

Estão disponíveis atualmente no mercado vacinas para uso veterinário, obtidas a partir de preparação de leptospiras atenuadas ou inativadas. No entanto, a resposta imune desencadeada a partir dessas preparações é ativada principalmente pela porção polissacarídica dos LPS, estimulando uma resposta timo independente. Desta forma, não induzem memória imunológica e proteção em longo prazo contra a infecção. Além disso, são sorovar-específicas, ou seja, não promovem proteção cruzada contra sorovares diferentes dos inclusos na preparação vacinal (Faine et al., 1999; Levett, 2001).

Até o momento, somente Cuba e China possuem vacinas licenciadas para uso em humanos, compostas de bactérias inativadas (Martinez et al., 2004; Yan et al., 2003). Essas vacinas apresentam problemas semelhantes às de uso veterinário, sendo sorovar-específicas e requerendo administração periódica. Portanto, não existem vacinas eficientes para uso humano nem veterinário.

Dada a grande dificuldade de preparar uma vacina de LPS multivalente e eficiente, torna-se crítica a identificação de antígenos proteicos conservados para o desenvolvimento vacinal. Por isso, a busca de proteínas relacionadas à patogenicidade ou virulência, e que tenham um amplo espectro de conservação dentre os principais sorovares patogênicos ao homem vem sendo perseguida por vários grupos de pesquisa no mundo (Branger et al., 2005; Haake et al., 1999; Palaniappan et al., 2006; Yan et al., 2010).

1.4 PATOGENICIDADE E VIRULÊNCIA DE *LEPTOSPIRA* SPP

1.4.1 Mecanismos de patogenicidade

Apesar da sua importância na saúde pública e animal, até hoje pouco se sabe sobre os mecanismos moleculares de patogenicidade e virulência das leptospiros. Contudo, é consenso que proteínas expostas na superfície, mantendo contato direto com o hospedeiro, são importantes durante o processo infeccioso. Nos últimos anos, os sequenciamentos genômicos de cinco cepas diferentes de *Leptospira* spp. revelaram um grande número de novas proteínas hipotéticas que podem estar envolvidas na patogenicidade (Bulach et al., 2006; Nascimento et al., 2004b; Picardeau et al., 2008; Ren et al., 2003). Ainda, mais recente, foram desenvolvidas técnicas de mutagênese direcionada e randômica por inserção de transposons, além da complementação genética de leptospiros transformadas com plasmídeos sintéticos, o que possibilitou o início da exploração do papel individual de genes e proteínas específicas na virulência (Bourhy et al., 2005; Croda et al., 2008; Murray et al., 2009a). No entanto, até o momento, foram identificadas poucas proteínas como fatores de virulência com contribuição para a patogênese da doença.

1.4.2 Adesão

Nos últimos anos, vem sendo bastante documentado que a interação de patógenos com proteínas de matriz extracelular (MEC) exerce um papel inicial importante na colonização do

hospedeiro. A aderência aos diferentes constituintes da MEC do hospedeiro é mediada por proteínas expostas na superfície dos micro-organismos (Boyle e Finlay, 2003). É bem estabelecido que a habilidade das bactérias espiroquetas de se aderirem às células eucarióticas e às proteínas de MEC seja crucial durante o início do processo de patogênese (Antonara et al., 2011; Bonazzi e Cossart, 2011), sendo que várias proteínas com características de adesinas já foram reportadas nos gêneros *Borrelia* e *Treponema* (Brown et al., 1999; Cameron, 2003; Cameron et al., 2004, 2005; Guo et al., 1998; Probert e Johnson, 1998; Probert et al., 2001).

Em leptospiros, o grupo de pesquisa no qual se realizou este trabalho caracterizou e descreveu diversas proteínas de membrana que têm função de adesão. Entre elas, Lsa24, a primeira proteína de leptospiros reportada que se liga à laminina (Barbosa et al., 2006), Lsa21, que interage com laminina, fibronectina plasmática e colágeno IV (Atzingen et al., 2008) Lsa27, ligante de laminina (Longhi et al., 2009) e Lsa63, que interage com laminina e colágeno IV (Vieira et al., 2010b), entre outras (Atzingen et al., 2010a). Outros grupos já demonstraram a capacidade de adesão a MEC das lipoproteínas LigA e LigB (proteína de leptospiros similar à imunoglobulina, *leptospiral immunoglobulin-like*) (Choy et al., 2007), das proteínas da família Len (proteína de leptospiros similar à endostatina, *leptospiral endostatin-like*) (Stevenson et al., 2007) e da lipoproteína majoritária de membrana de leptospiros, LipL32 (Hauk et al., 2008; Hoke et al., 2008). Em conjunto, esses dados indicam que as leptospiros apresentam múltiplas proteínas com função redundante de ligação aos componentes de MEC, sugerindo que o processo de adesão seja de fato importante para a virulência das leptospiros e início da infecção e colonização.

1.4.3 Evasão imune

Micro-organismos patogênicos desenvolveram inúmeras estratégias para camuflar sua presença e driblar o sistema imune dos hospedeiros. Aparentemente, utilizam múltiplas estratégias de escape combinadas para inibir o ataque pelas diversas etapas da resposta imune inata ou adaptativa. Assim, após sua entrada no hospedeiro, as leptospiros patogênicas devem ser capazes de contornar o sistema imune enquanto na circulação, para se propagarem aos órgãos e finalmente colonizar sítios imunologicamente privilegiados, como os túbulos renais proximais (Faine et al., 1999). Portanto, a eficiente colonização por leptospiros deve estar intimamente relacionada à sua destreza em modular o ataque pelo sistema imune, evadindo as

primeiras linhas de defesa do hospedeiro, consistindo na remoção por fagocitose ou destruição pelo sistema complemento (SC).

No que concerne a fagocitose, estudos demonstraram que as leptospiros são resistentes à fagocitose por neutrófilos (Cinco e Banfi, 1983; Wang et al., 1984), sendo fagocitadas apenas por macrófagos *in vitro* e *in vivo* (Cinco et al., 1981, 1982; Merien et al., 1997; Vinh et al., 1984). A interação de leptospiros com macrófagos parece ocorrer apenas na presença de anticorpos opsonizantes específicos (Banfi et al., 1982; Wang et al., 1984). No entanto, o destino dessas bactérias interior de macrófagos após a fagocitose ainda é controverso. Recentemente, foi demonstrado que, embora as leptospiros não sejam patógenos intracelulares clássicos, são capazes de invadir e induzir apoptose em macrófagos de algumas espécies hospedeiras e não de espécies carreadoras crônicas, o que parece estar ligado à susceptibilidade e severidade da doença (Li et al., 2010). Assim, sugere-se que as leptospiros utilizem a internalização em macrófagos como um mecanismo de escape do sistema imune, e que esta etapa seja essencial para o princípio da infecção (Li et al., 2010).

Além da resistência à fagocitose, a habilidade de invasão das leptospiros parece estar relacionada à aptidão de escape ao SC (Cinco, 2010). O SC é o principal componente da imunidade inata, e está envolvido na proteção contra micro-organismos invasores graças às suas habilidades opsonizantes, inflamatórias e líticas (Blom et al., 2009). Uma vez ativado, o SC destrói os micro-organismos eficientemente em minutos. No entanto, muitos patógenos desenvolveram a capacidade de evadir sua ação pela aquisição de proteínas reguladoras do sistema ou por expressão de proteínas inibitórias (Rautemaa e Meri, 1999). De fato, diversos patógenos expressam em sua superfície proteínas que podem sequestrar reguladores do SC do hospedeiro (FH, FHL-1 e C4BP; fator H, proteína similar ao fator H- 1 e proteína ligante de C4, respectivamente), suprimindo a cascata através das vias clássica ou alternativa (Blom et al., 2009; Lambris et al., 2008; Rooijakkers e van Strijp, 2007; Zipfel et al., 2007).

Estudos pioneiros com o gênero *Leptospira* demonstraram que diversas cepas patogênicas permanecem viáveis após a exposição ao SC, enquanto que cepas não patogênicas são suscetíveis (Cinco e Banfi, 1983). A resistência foi correlacionada à interação com os reguladores FH e FHL-1, interferindo na ativação da via alternativa do SC ao impedir a ligação do fator B ao C3b, acelerar o decaimento de C3-convertase C3Bb e agindo como cofator para a clivagem de C3b pelo fator I (Meri et al., 2005). Foram identificadas algumas proteínas de membrana que atuam como receptores de FH e FHL-1, indicando redundância de função e, portanto, refletindo a importância do mecanismo para a evasão imune e

sobrevivência das leptospiros no hospedeiro (Castiblanco-Valencia et al., 2012; Verma et al., 2006).

Recentemente foi relatada a capacidade das leptospiros de interferirem com a via clássica do SC por ligação ao componente C4BP (Barbosa et al., 2009; Domingos et al., 2012). O C4BP é um regulador do SC que promove a clivagem de C4b mediada por fator I (Cinco, 2010). Isso ressalta que leptospiros virulentos possuem diversos mecanismos para aumentar a sua sobrevivência no hospedeiro.

1.4.4 Lipoproteínas e proteínas de membrana

Nos sequenciamentos genômicos de *Leptospira*, foram identificadas inúmeras proteínas de membrana e lipoproteínas, sendo que a maioria foi classificada como hipotética, não apresentando homólogos em outros organismos (Nascimento et al., 2004a). Devido à sua localização, essas proteínas desempenham diversas funções relacionadas à adaptação e interação com o ambiente. Assim, permitindo contato direto com o hospedeiro, é consenso que as proteínas de membrana e lipoproteínas exerçam papel essencial na virulência e patogenicidade das leptospiros. No entanto, poucas já foram caracterizadas como fatores de virulência.

O primeiro fator de virulência geneticamente identificado em *Leptospira* foi a proteína de superfície Loa22 (Ristow et al., 2007). Mutantes construídos por inserção de *transposon* resultaram em atenuação da virulência em modelos de infecção aguda (hamsters e porquinhos da índia). Entretanto, à parte sua essencialidade para a virulência, sua função permanece desconhecida. Vale ressaltar que *L. biflexa*, uma espécie saprofítica e não patogênica, apresenta o gene homólogo para Loa22 (Picardeau et al., 2008).

Muitos outros estudos realizados por técnicas de manipulação genética de leptospiros sugerem que uma característica única e intrigante dessas bactérias seja o alto nível de proximidade de função, o que torna difícil a identificação de fatores de virulência por inativação de genes únicos. Por exemplo, LipL32 é a proteína mais abundante da superfície de leptospiros (Malmstrom et al., 2009), sendo altamente conservada entre espécies patogênicas e ausente em espécie saprofíticas (Haake et al., 2000). É envolvida na interação com componentes de MEC (Hauk et al., 2008; Hoke et al., 2008) e expressa durante a infecção. No entanto, não está envolvida na colonização de carreadores assintomáticos nem na virulência durante a leptospirose aguda em hamsters (Murray et al., 2009c). Da mesma forma, as proteínas Lig são presentes apenas em espécies patogênicas, sua expressão é concomitante à

perda de virulência por atenuação (Matsunaga et al., 2003), LigA e LigB ligam-se à componentes de MEC (Choy et al., 2007) e sua expressão é aumentada em condições fisiológicas de osmolaridade (Matsunaga et al., 2005); contudo, a inativação de LigB não afeta a virulência (Croda et al., 2008).

1.4.5 Outros mecanismos e fatores de virulência

1.4.5.1 LPS

Em leptospiros, o LPS é o principal antígeno gerador de resposta imune humoral durante a infecção. O *locus* gênico codificando para a biossíntese de LPS em leptospiros é extenso em comparação com o de outras bactérias gram-negativas, e acredita-se que tenha sido adquirido por transferência lateral, apresentando algumas características de ilha de patogenicidade (Barocchi et al., 2001; Bulach et al., 2000; de la Pena-Moctezuma et al., 1999). Estudos de mutagênese não direcionada em larga escala mostraram que esta região normalmente tem baixa inserção de transposons, sugerindo que os genes sejam essenciais para a viabilidade de *L. interrogans* (Murray et al., 2009a). Trabalhos posteriores descreveram dois mutantes com inserções no *locus* do LPS que não foram capazes de apresentar virulência em modelo de hamsters, sugerindo fortemente que o LPS seja essencial para a virulência das leptospiros (Murray et al., 2010).

1.4.5.2 Motilidade e quimiotaxia

Para sobreviver no meio ambiente, as bactérias devem ser capazes de detectar e moverem-se em direção às condições favoráveis e contrárias às condições desfavoráveis. O sistema de quimiotaxia integra os sinais ambientais às respostas comportamentais mediadas por cadeias de transdução de sinal. Deste modo, quimiotaxia e motilidade são críticas para a virulência das leptospiros, permitindo a penetração através das barreiras teciduais dos hospedeiros e migração para os órgãos preferenciais de colonização (Charon e Goldstein, 2002). Análises genômicas e transcritômicas evidenciaram que leptospiros possuem múltiplas cópias de genes relacionados à quimiotaxia, que são regulados em resposta a diversos fatores relacionados à entrada no hospedeiro (Lo et al., 2006; Nascimento et al., 2004a; Qin et al., 2006; Ren et al., 2003). Da mesma forma, possuem cerca de 50 genes envolvidos com a

motilidade, indicando que possuem um complexo sistema flagelar e de movimentação (Charon e Goldstein, 2002).

Suportando a ideia de que a motilidade seja importante para a virulência de leptospiros, estudos recentes demonstraram que mutantes da proteína FlaA, constituinte do endoflagelo, apresentam capacidade infectante reduzida em hamsters, sendo incapazes de disseminar-se para os órgãos do hospedeiros e iniciar a colonização (Lambert et al., 2012).

1.4.5.3 Captura de ferro no hospedeiro

Grupos heme constituintes da hemoglobina são a forma mais abundante de disponibilidade de ferro a patógenos durante a infecção (Andrews, 2000). Análises genômicas de *L. interrogans* revelaram que essas bactérias apresentam genes de aquisição de heme, consistente com relatos prévios de que leptospiros utilizam esses grupos como a principal fonte de ferro durante a infecção. Por técnicas de mutagênese por transposons, foi identificado e descrito um mutante para o gene hemoxygenase, hemO, de *L. interrogans*, que revelou-se inviável em meios de cultura onde a única fonte de ferro era a hemoglobina. Por estudos em modelo animal, este gene foi classificado como fator importante para a virulência em animais, porém não essencial (Murray et al., 2009b).

1.4.5.4 Proteínas Mce

As proteínas de entrada em células de mamíferos (Mce, *mammalian cell entry*) parecem contribuir para a invasão de células, incluindo macrófagos, por bactérias e vírus (Arruda et al., 1993; Senaratne et al., 2008). Essas proteínas possuem motivos RGD (arginina-glicina-aspartato), que medeiam a interação com integrinas nas superfícies celulares, possibilitando a adesão e desencadeando a invasão (Ruoslahti, 1996; Sato et al., 2009).

Como já revisado anteriormente, muitas evidências sugerem que a invasão de macrófagos seja uma etapa importante durante o estabelecimento da leptospirose (Li et al., 2007; Liu et al., 2007; Merien et al., 1997). Fundamentado nessas evidências, foi recentemente demonstrado que a proteína Mce de *Leptospira* é necessária durante as primeiras fases da infecção (Zhang et al., 2012). A expressão do gene aumenta quando em contato com macrófagos, e a proteína Mce é responsável pela aderência e invasão de macrófagos pelas leptospiros virulentas, sendo que sua deleção atenuou significativamente a

virulência dessas bactérias em modelo de hamsters. Propõe-se que a proteína Mce seja um novo fator de virulência de leptospiros (Zhang et al., 2012).

1.4.5.5 Transcritômica: identificação de fatores de virulência em potencial

Ao longo do seu ciclo de transmissão, que compreende desde órgãos de animais selvagens ou humanos, até vida livre no solo ou na água, as leptospiros são sujeitas a condições ambientais amplamente distintas e variáveis (Haake et al., 2002). Para se adaptarem a condições nutricionais, sinalizadoras, quimiotáticas e de virulência tão divergentes, as leptospiros necessitam de uma regulação do conjunto de genes expressos em cada uma dessas condições. Embora se saiba que as leptospiros adaptam-se e sobrevivem em muitos ambientes diferentes, pouco é sabido a respeito da natureza molecular dessas adaptações.

Anterior à disponibilidade de dados gerados por experimentos de micro arranjos, era sabido que estímulos mimetizando condições *in vivo* durante a infecção afetam a expressão proteica em *Leptospira*, como as proteínas Qlp42 e LipL53, reguladas por temperatura (Nally et al., 2001; Oliveira et al., 2010), e LigA e LigB, reguladas por osmolaridade (Matsunaga et al., 2005). Para identificar outros genes diferencialmente expressos durante a infecção e apontar possíveis fatores de virulência, diversos estudos de micro arranjos foram realizados utilizando leptospiros crescidas sob diferentes condições de temperatura, osmolaridade e disponibilidade de soro ou ferro (Lo et al., 2006, 2010; Matsunaga et al., 2007; Patarakul et al., 2010; Qin et al., 2006). Essas análises geraram um número considerável de genes diferencialmente expressos que podem desempenhar papéis importantes na patogenicidade e virulência das leptospiros, e constituem avanços para o direcionamento de estudos mais minuciosos de novos mutantes ou proteínas envolvidas na infecção.

1.5 SISTEMAS PROTEOLÍTICOS

1.5.1 Sistema fibrinolítico

O sistema fibrinolítico ou sistema plasminogênio/plasmina (PLG/PLA) é uma cascata enzimática composta por diversas proteases séricas e inibidores, envolvidas na geração de PLA e regulação de sua atividade. A PLA exerce o papel central na fibrinólise, tendo por função primordial a degradação de fibrina, mas atuando também em outros processos proteolíticos uma vez que é capaz de degradar diversos constituintes da MEC.

O PLG é uma glicoproteína de cadeia única de 791 resíduos de aminoácidos produzida no fígado e presente em altas concentrações no plasma ($\sim 2 \mu\text{M}$) (Lijnen, 2001) e na maioria dos fluidos extra vasculares. É um precursor (zimógeno) inativo que, após clivagem da ligação peptídica $\text{Arg}^{561}\text{-Val}^{562}$ por ativadores proteolíticos, é convertido à sua forma ativa, a PLA. A PLA é composta por duas cadeias ligadas por ponte dissulfeto, contendo 561 e 230 resíduos de aminoácidos, respectivamente. A cadeia leve origina-se da porção amino-terminal (N-terminal) do PLG e abriga o sítio ativo homólogo ao de proteases do tipo serina, como tripsina e elastase. A cadeia pesada contém cinco domínios estruturais em forma de triplo-loop, denominados *kringle* (Lahteenmaki et al., 2001; Lijnen, 2001). Os domínios *kringle* possuem sítios de ligação aos resíduos de lisina, e medeiam as interações do PLG e da PLA com os substratos, reguladores e inibidores.

Fisiologicamente, existem dois tipos conhecidos de ativadores de PLG: tipo uroquinase e tipo tecidual (uPA e tPA, *urokinase-type* e *tissue-type plasminogen activators*). O tPA é produzido sobretudo por células endoteliais e constitui o principal ativador da fibrinólise no sangue, estando presente no plasma em concentração de 5 a 10 ng/mL. Já uPA é produzido por diversos tipos celulares e é o principal ativador associado à membrana celular, sendo importante na degradação mediada por células, como remodelamento tecidual, metástase tumoral e cicatrização (Lahteenmaki et al., 2001).

As atividades de uPA e tPA são inibidas principalmente por interação com inibidores da família das serpinas, com os quais formam complexos covalentes estáveis. O inibidor PAI-1 (inibidor de ativador de PLG tipo 1, *plasminogen activator inhibitor type 1*) é produzido por células endoteliais ou outros tipos celulares e é encontrado no plasma na concentração de 10 a 30 ng/mL. PAI-1 inibe tanto uPA quanto tPA, enquanto que o inibidor do tipo 2, PAI-2, produzido apenas por monócitos e macrófagos e detectado no plasma somente durante a gravidez, é capaz de inibir apenas uPA (Lahteenmaki et al., 2001).

A PLA, uma vez ativada, tem como principal inibidor fisiológico a serpina α_2 -antiplasmina, que forma um complexo reversível com a PLA ligando-se aos domínios *kringle* de afinidade à lisina. Segue-se uma reação irreversível na qual uma ligação covalente é formada entre o resíduo de serina presente no sítio ativo da PLA e o sítio ativo da α_2 -antiplasmina. Entretanto, uma vez que o PLG ou a PLA ligam-se aos receptores através dos mesmos domínios *kringle* que interagem com a α_2 -antiplasmina, estes ficam protegidos da sua ação inibitória (Collen e Lijnen, 2005). Outro regulador de atividade da PLA é o inibidor irrestrito de serino-proteases α_2 -macroglobulina, presente abundantemente no plasma humano.

No entanto, sua atividade apenas se dá quando a concentração sistêmica ou local de α_2 -antiplasmina é significativamente diminuída (Travis e Salvesen, 1983).

Enquanto ativadores e inibidores regulam a formação de PLA, a atividade da PLA é ainda controlada pelo direcionamento para regiões onde a atividade proteolítica é necessária via receptores de PLG. Uma série de células apresentam receptores de PLG em sua superfície, sendo os mais caracterizados a α -enolase, anexina II e anfoterina (Lahteenmaki et al., 2001). Os sítios de ligação à lisina presentes nos domínios *kringle* do PLG reconhecem e ligam-se aos resíduos de lisina dos receptores.

1.5.2 Sistema de metaloproteases de matriz

As metaloproteases de matriz (MMPs, *matrix metaloproteases*) constituem uma família de endopeptidases cálcio-dependentes, estrutural e funcionalmente relacionadas. São secretadas pelas células na forma inativa (latente), chamadas de pró-MMPs ou zimógenos, requerendo uma etapa de ativação para aquisição de atividade proteolítica de degradação de componentes de MEC. A família de MMPs humanas consiste em 23 enzimas separadas em seis grupos, com base nas características estruturais e a especificidade a diferentes substratos (colagenases, gelatinases, tipo de membrana, estromelinas, matrilisinas, e um grupo não nomeado) (Sbardella et al., 2012).

A estrutura básica de MMPs contém um peptídeo sinal N-terminal de exportação seguido de um domínio pró-peptídeo, um domínio catalítico que contém três histidinas conservadas na sequência His-Glu-X-X-His-X-X-Gly-X-X-His de ligação de Zn^{2+} e uma região rica em prolinas (região articular) que define a transição ao domínio N-terminal, responsável pela especificidade de substrato (Figura 1A). Todas as MMPs apresentam uma região conservada rica em glutamina e aspartato entre o sítio de ligação de Zn^{2+} e a região articular, que constitui o sítio de ligação de íons Ca^{2+} (Brinckerhoff e Matrisian, 2002).

As MMPs estão envolvidas em uma série de eventos fisiológicos relacionados ao remodelamento tecidual, como desenvolvimento embrionário, migração celular e cicatrização. Assim, devido à sua importância e potencial destrutivo de proteólise generalizada, as MMPs são finamente reguladas em diversos níveis, sendo que um pequeno desbalanço pode causar inúmeros eventos patológicos, como invasão microbiana, inflamação e danos teciduais (Sbardella et al., 2012). A regulação da atividade dessas proteases pode se dar em quatro níveis principais:

- a) expressão gênica;
- b) acúmulo e compartimentalização;
- c) ativação da pró-enzima;
- d)) interação com inibidores (Chakraborti et al., 2003).

Figura 1 – Estrutura esquemática dos domínios de MMPs

(A) Estrutura básica de uma MMP hipotética; (B) estrutura básica dos domínios das gelatinases MMP-2 e MMP-9. SP: peptídeo sinal de exportação para membrana ou secreção, Pro: domínio pró-peptídeo, Catalítico: domínio contendo sítio de ligação de Zn^{2+} e Ca^{2+} , FN: inserto de domínios de fibronectina tipo II, RA: região articular; Cys: resíduos de cisteína.
 FONTE: Vieira (2012).

Na maior parte, a produção de MMPs é regulada ao nível transcricional por sinais específicos que são temporalmente limitados e espacialmente confinados, como citocinas e fatores de crescimento. Pós-transcricionalmente, as MMPs são reguladas pela latência conferida pelo pró-peptídeo. A latência de pró-MMPs é mantida por interações entre o grupo tiol de uma cisteína conservada presente no pró-domínio e o zinco ligado ao sítio catalítico (Ra e Parks, 2007). Neste estado, o pró-domínio encobre o domínio catalítico, impedindo a interação com o substrato. A ativação pode se dar por clivagem proteolítica do pró-domínio pelas enzimas PLA, furina ou outras MMPs, ou de forma independente de proteases por mudanças conformacionais, seguido de autólise do pró-peptídeo (Ra e Parks, 2007).

Uma vez ativadas, as MMPs podem ser reguladas por inibição da atividade. Os principais inibidores de MMPs são a α_2 -macroglobulina, um inibidor não específico de proteases, e os inibidores teciduais de MMPs (TIMPs, *tissue inhibitors of MMPs*). Os TIMPs formam complexos com as MMPs, retardando a ativação de pró-MMPs e inibindo a atividade

de MMPs ativas. Esses inibidores estão distribuídos em todos os tecidos e fluidos, sendo que sua concentração geralmente encontra-se em excesso em relação à disponibilidade de MMPs (Murphy, 2011; Ra e Parks, 2007).

1.5.2.1 Gelatinases

As gelatinases são as MMPs definidas de acordo com sua afinidade por colágeno desnaturado (gelatina). Essa classe inclui dois membros, nomeadamente gelatinase A e gelatinase B, ou MMP-2 e MMP-9, respectivamente. A estrutura das gelatinases difere das demais MMPs por apresentar a inserção de um domínio de afinidade ao colágeno no domínio catalítico, constituído por domínios repetidos de fibronectina (Sbardella et al., 2012) (Figura 1B). As gelatinases estão intimamente relacionadas aos processos inflamatórios e progressão tumoral, estando envolvidas na origem de diversas patologias (Hua et al., 2011; Sbardella et al., 2012). Diversas evidências indicam que MMP-2 possua efeito pró-homeostático, enquanto MMP-9, atividade pró-inflamatória (Ram et al., 2006; Van den Steen et al., 2002).

A MMP-2 é constitutivamente expressa por virtualmente quase todas as células, principalmente por fibroblastos, células endoteliais e epiteliais. É detectada em concentrações significantes no plasma em condições fisiológicas devido à sua função homeostática (Van den Steen et al., 2002), e degrada principalmente citocinas e fatores de crescimento. A MMP-2 é fracamente induzível por estímulos, e sua atividade é fortemente regulada por TIMPs, particularmente do tipo 2, 3 e 4.

MMP-9 apresenta atividade proteolítica sobre os mesmos substratos que MMP-2, porém com diferentes afinidades, sendo que os principais alvos são componentes de MEC e membrana basal (MB). Diferentemente de MMP-2, essa protease é altamente induzível, e sua expressão fisiológica é limitada a um grupo restrito de tipos celulares, principalmente do sistema imunológico (Van den Steen et al., 2002). Portanto, sua distribuição é bem definida e geralmente ocorre associada a diversas condições anti-homeostáticas.

1.5.3 Interação sistema fibrinolítico e MMPs

Diversas interações moleculares entre o sistema PLG/PLA e o sistema de MMPs indicam que estes atuam em conjunto na degradação de MEC, requisito primordial para a migração celular e remodelamento tecidual. Embora a PLA tenha como principal alvo a degradação de fibrina, também participa do processo de degradação e remodelamento de

MEC por ser capaz de clivar diretamente alguns componentes de MEC como fibronectina, laminina e proteoglicanas, e também indiretamente, estes e outros componentes, por ativação proteolítica de pró-MMPs latentes e heparanases. A PLA pode ativar diretamente pró-MMP-1, pró-MMP-3, pró-MMP-9, pró-MMP-10 e pró-MMP-13. Pró-MMP-2 também pode ser ativada diretamente pelo ativador de PLG uPA.

Por outro lado, MMPs ativas também interagem com componentes do sistema fibrinolítico, afetando a fibrinólise e atividade da PLA (Lijnen, 2002). Já foi demonstrado que diversas MMPs são capazes de clivar PLG tanto *in vitro* quanto *in vivo* (Brauer et al., 2011; Lijnen et al., 1998; O'Reilly et al., 1994; Patterson e Sang, 1997; Pozzi et al., 2000; Sang, 1998), gerando angiostatina, que compreende os quatro primeiros domínios *kringle* do PLG e é um inibidor da angiogênese e da resposta imune. MMP-3 pode clivar uPA, separando o domínio catalítico do domínio de ligação ao receptor, porém sem afetar a atividade catalítica, provavelmente resultando em menor acúmulo de uPA na superfície celular, consequentemente gerando menor conversão de PLG à PLA (Orgel et al., 1998; Ugwu et al., 1998). MMP-3 hidrolisa ainda α_2 -antiplasmina, impedindo que se forme o complexo estável inibidor-PLA, o que pode constituir um mecanismo de favorecimento de proteólise mediada por PLA localizada. MMP-3 também hidrolisa e inativa PAI-1, beneficiando a fibrinólise.

Adicionalmente, MMPs degradam citocinas e fatores de crescimento que afetam a geração de PLA, enquanto que a PLA favorece a liberação de fatores de crescimento latentes que por sua vez podem interferir na regulação de MMPs. Algumas das interações mencionadas entre os sistemas proteolíticos PLG/PLA e MMPs estão simplificada e esquematizadas na Figura 2.

1.5.4 Interação de patógenos com o sistema proteolítico PLG/PLA

Durante o processo infeccioso de diversos micro-organismos, a penetração pelos tecidos do hospedeiro para alcançar a circulação e sítios secundários de infecção é uma etapa crucial. Para isso, a atividade proteolítica tem se mostrado essencial na patogênese bacteriana ao que permite a transposição de barreiras mecânicas impostas pela MEC, MB, tecidos epiteliais e endoteliais (Lahteenmaki et al., 2001). A proteólise associada às infecções pode ter várias funções: aumento da permeabilidade vascular, invasão da corrente sanguínea ou de locais secundários de infecção, liberação de nutrientes e danos teciduais. Em resumo, a degradação de estruturas teciduais por proteases potencializa a disseminação através do hospedeiro seja por ultrapassar as barreiras físicas, seja por propiciar condições nutricionais.

Figura 2 – Representação esquemática da ativação de PLA e MMPs e as possíveis interações entre os dois sistemas proteolíticos

Os triângulos verdes indicam os inibidores de enzimas e pró-enzimas: $\alpha 2$ -antiplasmina e $\alpha 2$ -macroglobulina atuando sobre a plasmina, TIMPs ligando-se às pró-MMPs e PAIs inibindo a ação de uPA e tPA. Os retângulos vermelhos representam os substratos para as enzimas plasmina e MMPs. Fatores de crescimento e citocinas são regulados por MMPs e plasmina, ao mesmo tempo que influenciam na disponibilidade de atividade das mesmas.

FONTE: Vieira (2012).

As barreiras teciduais de mamíferos são principalmente formadas por MECs e MBs, as quais as bactérias devem penetrar para atingir a circulação sanguínea. As MECs e MBs contêm colágenos, laminina, fibronectina, proteoglicanos e elastina como principais componentes. No entanto, a produção de enzimas proteolíticas capazes de degradar componentes de MEC (principalmente collagenases) é limitada a um número restrito de patógenos bacterianos durante a infecção (Lahteenmaki et al., 2001).

Muitas bactérias invasoras são extracelulares e necessitam de mecanismos ativos de penetração nos tecidos. Esses mecanismos consistem muitas vezes na utilização, em seu favor, de cascatas de sistemas dependentes de proteases dos hospedeiros como coagulação, fibrinólise, ativação do SC e fagocitose (Bergmann e Hammerschmidt, 2007; Colman et al., 1998; Herwald et al., 1998; Pizarro-Cerda e Cossart, 2006; Rossmann et al., 2007; Severi et al., 2007; Travis et al., 1995). As bactérias podem regular estas cascatas diretamente através

de suas proteases ou componentes expostos na membrana, ou indiretamente, estimulando a liberação de moléculas efetoras pelo hospedeiro.

Pelo grande espectro de atividade da PLA e a alta concentração no plasma do seu precursor, o PLG, o sistema fibrinolítico de mamíferos oferece condições que possibilitam o seu uso por bactérias patogênicas. A PLA degrada diretamente diversos componentes de MEC e causa danos indiretos à MB e MEC ao ativar MMPs, resultando assim em potencial destrutivo de desestruturação de MEC e tecidos endoteliais (Gebbia et al., 2001; Lahteenmaki et al., 2005; Liotta et al., 1981a,b).

De fato, nos últimos anos, muitos estudos têm demonstrado esse mecanismo bacteriano ao fornecer entendimento acerca da função do sistema do PLG/PLA durante a infecção (Bergmann e Hammerschmidt, 2007; Degen et al., 2007; Lahteenmaki et al., 2005; Sun, 2006). A maioria das evidências *in vivo* demonstrando a importância do sistema do PLG/PLA na patogenicidade bacteriana vem de estudos em doenças que se iniciam subcutaneamente. Uma das visões emergentes é que as bactérias invasoras responsáveis por essas infecções compartilhem duas propriedades: expressão de receptores e/ou ativadores de PLG, bem como capacidade de adesão a componentes de MEC e MB.

Pela forma de interação com o sistema PLG/PLA por expressão de receptores de PLG, as bactérias conseguem imobilizá-lo em sua superfície e, após a ativação por conversão à PLA (seja por ativadores endógenos ou ativadores do próprio hospedeiro), passam a funcionar como organismos proteolíticos. A capacidade proteolítica dos micro-organismos ligados à PLA aumenta a sua virulência ao permitir a degradação de MEC e MB, incrementando a capacidade de invasão e colonização. A expressão de receptores de PLG já foi reportada em várias espécies de bactérias patogênicas gram-positivas e gram-negativas (Lahteenmaki et al., 2001). Entre as espiroquetas, grupo ao qual pertencem as leptospiros, essa habilidade foi demonstrada em *T. denticola* (Fenno et al., 2000) e diversas espécies do gênero *Borrelia* (Lagal et al., 2006). Os receptores caracterizados são, em sua maioria, proteínas expostas na superfície bacteriana, principalmente lipoproteínas, que, geralmente, têm outras funções como adesão, transporte, metabolismo e interação com o sistema imune (Lahteenmaki et al., 2001).

Considerando-se a espiroqueta *Borrelia*, estudos mostraram que o PLG liga-se a múltiplas proteínas nas diversas cepas analisadas, e que a captação de PLA na superfície dessas bactérias promove a degradação de componentes de MEC e a migração através de monocamadas endoteliais e MEC artificial *in vitro* (Coleman e Benach, 2000; Coleman et al., 1995, 1999). Ainda, foi demonstrado que bactérias virulentas ligam-se mais eficientemente à PLA do que bactérias atenuadas, possivelmente por expressarem maior número de receptores

ou receptores alternativos, reforçando a importância desse sistema para a patogênese e virulência (Coleman e Benach, 2000). Além de possuir receptores de PLG, *Borrelia* tem mecanismos de indução da sua ativação para a forma enzimaticamente ativa, a PLA. Dados indicam que não só bactérias viáveis, mas também lipoproteínas purificadas são capazes de induzir níveis elevados de ativadores de PLG em monócitos, que se acumulam em infiltrados inflamatórios na infecção (Coleman et al., 2001; Haile et al., 2006). Um fato ressaltado nos trabalhos é a capacidade de adesão de *Borrelia* a MEC, o que pode ser importante no direcionamento dessas bactérias para esses sítios.

Ainda, dois estudos independentes com *Borrelia* mostraram que a imobilização de PLA na superfície promove invasão tecidual e disseminação nos tecidos, lavando à espiroquetemia. A capacidade proteolítica eleva a passagem através da barreira hematoencefálica e aumenta a invasão de rins, cérebro e coração. Experimentos de infecção em camundongos deficientes em PLG indicam que o PLG é essencial para a migração das espiroquetas através de vasos sanguíneos e para a eficiente disseminação para outros órgãos (Coleman et al., 1997; Haile et al., 2006).

1.5.5 Micro-organismos e o sistema proteolítico de MMPs

No evento de uma invasão microbiana, o sistema imunológico é desafiado e deve primeiramente recrutar leucócitos para o sítio da infecção, erradicar o patógeno e, finalmente, suprimir a resposta para que se cesse a inflamação sistêmica ou local. As MMPs exercem papel primordial neste processo tanto por permitir a degradação de MEC, quanto pela modulação de citocinas e atividade quimiotática, culminando na atração e migração de células efetoras ao sítio de infecção. Portanto, a migração de células inflamatórias é dependente da atividade de MMPs (Elkington et al., 2005).

Embora sejam necessárias para a resposta imune, paradoxalmente, as MMPs podem causar imunopatologias relacionadas à infecção. Danos teciduais podem ser causados pelo excesso de atividade dessas proteases, resultando de secreção aumentada de MMPs ou diminuída de TIMPs. O número de infecções durante as quais a atividade de MMPs afeta negativamente o hospedeiro é vasto, compreendendo agentes virais, bacterianos, protozoários, fúngicos e parasitas (Elkington et al., 2005; Sbardella et al., 2012). Dentre as bactérias espiroquetas, é relatada a influência da atividade exacerbada de MMPs nas patologias da doença de Lyme (Behera et al., 2005; Heilpern et al., 2009; Hu et al., 2001) e periodontite

(Gaibani et al., 2010; Miao et al., 2011), causadas por espécies de *Borrelia* e *Treponema*, respectivamente.

Como revisado por Amalinei et al. (2010), o papel de MMPs em patologias pode ser agrupado em três grupos:

- a) destruição tecidual, como no câncer e metástase, artrite reumatóide, osteoartrite, doenças neuroinflamatórias e úlcera gástrica;
- b) fibrose, como na cirrose, arterosclerose e esclerose múltipla;
- c) enfraquecimento da matriz, como em aneurismas da aorta e cardiomiopatias de dilatação.

1.6 CONTEXTUALIZAÇÃO DA PESQUISA

Apesar da sua importância, os mecanismos moleculares da patogênese, virulência e processo infeccioso das leptospiros permanecem não compreendidos. Mesmo após os sequenciamentos genômicos, poucas proteínas foram caracterizadas como tendo papel direto na patogênese. Tendo em vista a breve literatura citada, relatando a capacidade de adesão de proteínas de leptospiros aos componentes de MEC, adicionado ao fato de a leptospirose iniciar-se na pele com posterior disseminação para sítios secundários de infecção, e a capacidade de outras espiroquetas e patógenos de fazerem uso do sistema do PLG/PLA, levou à proposição da investigação da participação desse mecanismo na patogenicidade das leptospiros. Como em outras bactérias patogênicas e, especialmente, nas espiroquetas *Borrelia* spp. e *T. pallidum*, esse sistema pode ser importante para a disseminação das leptospiros no hospedeiro ao degradar componentes de MEC e tecidos e promover a penetração para órgãos preferenciais de infecção como rins e fígado a despeito da ausência de proteases secretadas com capacidade proteolítica.

2 OBJETIVOS

O objetivo do trabalho foi avaliar e caracterizar a participação do sistema fibrinolítico na virulência e patogenicidade de leptospiros. Estudou-se a interação de *Leptospira* com PLG e PLA, bem como as implicações desta ligação para a infecção. Foram avaliados os efeitos de facilitação na degradação de componentes de MEC e penetração por barreira endotelial, evasão imune e ativação de vias de proteólise.

3 MATERIAIS E MÉTODOS

3.1 LEPTOSPIRAS E CONDIÇÕES DE CULTIVO

L. interrogans sorovar Pomona cepa LPF e *L. interrogans* sorovar Copenhageni cepa Fiocruz L1-130 virulentas são cultivadas rotineiramente no Laboratório de Zoonoses Bacterianas da Faculdade de Medicina Veterinária da Universidade de São Paulo (USP) mediante infecções periódicas em hamsters para a manutenção da virulência. Hamsters recém-desmamados foram infectados com 500 µL contendo $1,0 \times 10^4$ leptospiiras virulentas. Os animais foram sacrificados após a manifestação dos sintomas, como perda de peso e mobilidade (aproximadamente cinco dias após a infecção). O fígado e os rins dos animais foram retirados e macerados, sendo os extratos teciduais inoculados em meio de cultura. As leptospiiras provenientes dos órgãos foram cultivadas em meio líquido EMJH (Difco, Le Point de Claix, França) suplementado com 10% de enriquecimento para *Leptospira* (Difco), a 28 °C.

As leptospiiras atenuadas (não virulentas) são igualmente cultivadas rotineiramente por manutenção em meio EMJH suplementado com 10% de enriquecimento para *Leptospira*. As cepas não virulentas utilizadas foram: *L. interrogans* sorovar Canicola cepa Hound Utrech IV, *L. interrogans* sorovar Copenhageni cepa M 20, *L. interrogans* sorovar Icterohaemorrhagiae cepa RGA, *L. interrogans* sorovar Pomona cepa Pomona, *L. borgpetersenii* sorovar Castellonis cepa Castellon 3, *L. borgpetersenii* sorovar Whitcombi cepa Whitcomb, *L. kirschneri* sorovar Cynoptery cepa 3522 C, *L. kirschneri* sorovar Grippotyphosa cepa Moskva V, *L. santarosai* sorovar Shermani cepa 1342 K e *L. biflexa* sorovar Patoc cepa Patoc.

Para os experimentos de mudança de temperatura, as bactérias cultivadas a 28 °C foram transferidas para estufas a 37 °C ou 39 °C por 24 h. Para as análises de mudança de osmolaridade, 120 mM NaCl foram adicionados às culturas em meio EMJH suplementado com 10% de enriquecimento para *Leptospira*, seguido de incubação a 28 °C por 24 h.

3.2 SOROS DE PACIENTES DIAGNOSTICADOS COM LEPTOSPIROSE E TESTE DE AGLUTINAÇÃO MICROSCÓPICA

Amostras de soros de pacientes com diagnóstico laboratorial de leptospirose confirmado foram obtidas da soroteca do Instituto Adolfo Lutz, São Paulo, SP, Brasil. O MAT dos soros foi realizado conforme descrito por Faine et al. (1999). Brevemente, amostras

de 22 sorovares de *Leptospira* spp. foram empregadas: Andaman, Australis, Autumnalis, Bataviae, Butembo, Canicola, Castellonis, Celledoni, Copenhageni, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc Pomona, Pyrogenes, Shermani, Tarassovi e Wolffi. Todas as cepas foram mantidas em meio EMJH, a 28 °C. Alíquotas dos soros dos pacientes foram colocadas em contato com as leptospirosas, e quando a aglutinação das bactérias foi observada, o MAT foi estabelecido positivo. O MAT foi considerado negativo quando não foi detectada aglutinação mediante exposição a nenhum dos 22 sorovares testados.

Em adição aos soros MAT positivos e negativos de pacientes diagnosticados com leptospirose, foram utilizados soros de doadores saudáveis, sem histórico conhecido de leptospirose. Esses tiveram confirmação laboratorial de ausência de anticorpos específicos para a doença (MAT negativo).

3.3 SOROS DE DOENÇAS INFECCIOSAS NÃO RELACIONADAS À LEPTOSPIROSE

Amostras de soros de pacientes com doença de Chagas foram doadas pelo Laboratório de Protozoologia do Instituto de Medicina Tropical da USP (IMT/USP). Soros de pacientes diagnosticados com vírus de imunodeficiência humana (HIV) e dengue foram recebidos do Laboratório de Virologia do IMT/USP. Amostras de pacientes com malária foram cedidas pelo Núcleo de Estudos em Malária do IMT/USP. Todos os soros de pacientes foram doados para fins de pesquisa científica.

3.4 ENSAIO DE IMUNOFLUORESCÊNCIA EM FASE LÍQUIDA

L. interrogans virulentas ($2,5 \times 10^9$ /amostra) foram sedimentadas a $12.800 \times g$ por 15 min e lavadas duas vezes com solução salina-fosfato (PBS, *phosphate buffered saline*) contendo baixa concentração de sal (50 mM NaCl) (IsPBS, *low salt* PBS). As bactérias foram ressuspensas em 200 µL IsPBS contendo 8 µg PLG humano purificado (Calbiochem, Darmstadt, Alemanha) e incubadas por 45 min a 37 °C. Após, 6 µg/mL de iodeto de propídeo (Sigma-Aldrich, St. Louis, Estados Unidos - EUA) foram adicionados, seguido de incubação por 45 min a 37 °C. As leptospirosas foram gentilmente lavadas três vezes com IsPBS e incubadas por 45 min a 37 °C com anticorpos anti-PLG humano produzidos em camundongo (Calbiochem), na diluição de 1: 50 em IsPBS. Em seguida, as leptospirosas foram lavadas três vezes e incubadas por 45 min a 37 °C com anticorpos anti-imunoglobulina G (IgG) de

camundongo conjugados a isotiocianato de fluoresceína (FITC; Sigma-Aldrich) em diluição de 1: 50. Após incubação, as bactérias foram lavadas duas vezes e ressuspensas em IsPBS adicionado de *ProLong Gold* (Molecular Probes, Eugene, EUA). As leptospiros marcadas foram visualizadas com o uso de microscópio confocal de imunofluorescência LSM 510 META (Carl Zeiss, Alemanha) em aumento de 600 vezes. Como controles, foram utilizados anticorpos produzidos em camundongo contra as proteínas recombinantes LipL32 e GroEL, seguindo os mesmos procedimentos utilizados para PLG.

3.5 LIGAÇÃO DE LEPTOSPIRAS AO PLASMINOGÊNIO/PLASMINA

O total de $7,0 \times 10^9$ leptospiros foram centrifugadas a $6.000 \times g$ por 10 min a 25°C , ressuspensas em 1,4 mL de IsPBS e divididas em 7 alíquotas de 0,2 mL cada ($1,0 \times 10^9$ leptospiros) e re-centrifugadas. As bactérias foram lavadas duas vezes com IsPBS e cada um dos 7 tubos recebeu um tratamento diferente:

- a) 30% plasma humano e 3U urokinase (uPA, Sigma-Aldrich) em 100 μL IsPBS;
- b) 5 μg PLG e 3U uPA em 100 μL IsPBS;
- c) 2 μg PLG e 3U uPA em 100 μL IsPBS;
- d) 0,5 μg PLG e 3U uPA em 100 μL IsPBS;
- e) 5 μg PLG em 100 μL IsPBS;
- f) 3U uPA em 100 μL IsPBS;
- g) 100 μL IsPBS.

Todas as preparações foram incubadas por 1 h a 37°C sob leve agitação antes da adição de uPA, seguido por 1 h de incubação a 37°C após a adição do ativador. As leptospiros foram centrifugadas e lavadas três vezes com 0,7 mL IsPBS.

3.6 AVALIAÇÃO DA ATIVIDADE ENZIMÁTICA DE LEPTOSPIRAS COMPLEXADAS COM PLASMINA

As leptospiros tratadas como descrito na seção 3.5 foram ressuspensas em 300 μL IsPBS e divididas em 3 alíquotas. Cada alíquota recebeu 100 μL de 0,5 mg/mL do substrato cromogênico específico para plasmina D-Val-Leu-Lys dicloridrato 4-nitroanilida (S-2251, Sigma-Aldrich) em IsPBS, em concentração final de 0,25 mg/mL. As suspensões foram incubadas durante 1,5 h a 37°C , sob leve agitação, e centrifugadas a $6.000 \times g$ por 10 min a 25°C para sedimentação das leptospiros. Os sobrenadantes (150 μL) foram transferidos para

placas de micro diluição, e a degradação do substrato pela plasmina foi quantificada por leitura das absorbâncias no comprimento de onda de 405 nm.

Para verificar o efeito da aprotinina na inibição da atividade da plasmina associada às leptospiros, 1,0 µg de aprotinina bovina (Sigma-Aldrich) foi adicionada quando da adição do substrato S-2251 à reação. Amostras contendo somente 1,0 µg de aprotinina foram utilizadas como controle.

3.7 ENSAIO DE INIBIÇÃO POR ÁCIDO 6-AMINOCAPRÓICO

L. interrogans sorovar Copenhageni cepa Fiocruz L1-130 virulentas (10^9 leptospiros/amostra) foram tratadas com PLG (5 µg) e uPA (3U), conforme descrito anteriormente na seção 3.5, exceto pela adição de crescentes concentrações de ácido aminocapróico (AAC, Sigma-Aldrich) – 0 a 1.000 mM – concomitante à adição do PLG. Como controles, alíquotas receberam somente PLG, uPA ou AAC, ou apenas IsPBS sem adições. A atividade enzimática da plasmina foi avaliada conforme previamente relatado na seção 3.6.

3.8 CRESCIMENTO DE LEPTOSPIRAS LIGADAS AO PLASMINOGÊNIO

L. interrogans sorovar Copenhageni cepa Fiocruz L1-130 virulentas (10^9 bactérias/amostra) foram tratadas com PLG e uPA conforme previamente descrito. Após o tratamento, as bactérias foram sedimentadas por centrifugação e ressuspensas em 4 mL de meio EMJH suplementado com 10% de enriquecimento para *Leptospira*. Os inóculos foram incubados a 28 °C por 14 dias, sendo que o crescimento foi avaliado por medida da densidade óptica das culturas a 600 nm em intervalos de tempo determinados. Previamente ao uso, todas as soluções foram esterilizadas por filtração.

3.9 EXTRAÇÃO DAS FRAÇÕES CITOPLASMÁTICA E DE MEMBRANA DE LEPTOSPIRAS

Leptospiros foram lavadas em IsPBS contendo 5 mM MgCl₂ e tratadas com 2% Triton X-114 (TX-114; Sigma-Aldrich), 150 mM NaCl, 10 mM Tris-HCl pH 7.4, e 1 mM PMSF (fluoreto de fenilmetilsulfonil) em gelo por 4 h. O material insolúvel foi removido por centrifugação (17.000 x g por 10 min a 4 °C). Após, a fração solúvel em TX-114 foi

armazenada a -20°C por 16 h, seguido de descongelamento em gelo. O fracionamento foi obtido por centrifugação a $1.000 \times g$ por 10 min após aquecimento a 37°C , resultando em

- a) fase aquosa (citoplasmática);
- b) fase solúvel em TX-114 (proteínas de membrana);
- c) precipitado insolúvel.

A fase detergente foi lavada duas vezes com solução 0,06% Triton TX-114, 150 mM NaCl, 10 mM Tris-HCl pH 7.4, após incubação por 10 min em gelo seguido de 10 min a 37°C . Após as lavagens, a fase superior (TX-114) foi incubada a 37°C por 10 min, centrifugada, e precipitada com acetona. As frações foram quantificadas e aplicadas em gel de SDS-PAGE (eletroforese em gel de poliacrilamida-dodecil sulfato de sódio, *Sodium Dodecyl Sulphate-Gel Electrophoresis*) 12% para a análise da extração.

3.10 LIGAÇÃO DE PLG ÀS PROTEÍNAS FRACIONADAS DE LEPTOSPIRAS

A ligação de proteínas citoplasmáticas, de membrana ou lisado total de leptospiiras ao PLG foi avaliada através de ELISA modificado. Poços de placas de micro diluição (Costar High Binding, Corning, EUA) foram adsorvidas por 16 h a 4°C com $0,15 \mu\text{g/poço}$ das frações proteicas ou BSA, como controle negativo. Após adsorção, as placas foram lavadas com PBS suplementado com 0,05% (volume/volume, v/v) Tween 20 (PBS-T), seguido de bloqueio por 2 h a 37°C com PBS adicionado de 10% (massa/volume, m/v) leite desnatado desidratado. A solução de bloqueio foi desprezada e foram adicionados $100 \mu\text{L}$ de PBS contendo $10 \mu\text{g/mL}$ PLG, seguido de incubação por 2 h a 37°C . Os poços foram lavados quatro vezes com PBS-T e incubados por 1 h a 37°C com anticorpos anti-PLG humano produzidos em camundongo (1: 4.000 em PBS). As placas foram lavadas novamente e incubadas com anticorpos anti-IgG de camundongos conjugados com peroxidase, em diluição 1: 5.000, em PBS. Após três lavagens, foram adicionados $100 \mu\text{L/poço}$ de 1 mg/mL *o*-phenylenediamina (OPD) e $1 \mu\text{L/mL}$ H_2O_2 em tampão citrato fosfato (pH 5). As reações foram conduzidas por 5 min e paradas pela adição de $50 \mu\text{L/poço}$ de H_2SO_4 4N. As absorbâncias foram determinadas por leituras a 492 nm.

3.11 SDS-PAGE E IMMUNOBLOTTING DE AFINIDADE

Extratos proteicos totais de leptospiiras foram preparados a partir de 10 mL de cultura ($\sim 10^9$ bactérias) em meio líquido EMJH suplementado com 10% de enriquecimento para

leptospiras. As células foram sedimentadas por centrifugação, lavadas três vezes com 5 mM $MgCl_2$ em IsPBS e ressuspensas em 100 μ L PBS, sendo agitadas vigorosamente. Os extratos bacterianos foram separados por SDS-PAGE 10% e eletro transferidos para membranas de nitrocelulose (Hybond-ECL, GE Healthcare, Buckinghamshire, Reino Unido) em sistema semi-seco. As membranas foram bloqueadas por 2 h a 37 °C com 5% BSA (m/v) em PBS-T, lavadas (3 x 10 min) e incubadas por 16 h a 4 °C com solução 3 μ g/mL PLG adicionada ou não de 100 mM AAC, seguido de incubação por 2 h à temperatura ambiente. Após, as membranas foram lavadas (3 x 10 min) e incubadas com anticorpos anti-PLG humano (1: 750, em PBS-T) por 3 h à temperatura ambiente, seguido de mais três lavagens e incubação com anticorpos anti-IgG conjugados com peroxidase (1: 5.000, em PBS-T). Após lavagens, a ligação do PLG às proteínas imobilizadas nas membranas foi revelada por de reagente ECL (GE Healthcare) de acordo com as recomendações do fabricante. As membranas foram expostas a filmes de raios-X (Kodak, Rochester, EUA) por períodos adequados, seguido de revelação e fixação.

3.12 PREPARO DE EXTRATOS PROTEICOS TOTAIS DE *LEPTOSPIRA* PARA GEL BIDIMENSIONAL (2D)

Culturas de *L. interrogans* sorovar Pomona cepa LPF de primeira passagem foram centrifugadas (12.800 x g, 4 °C, 10 min), e os sedimentos bacterianos lavados cinco vezes com solução PBS contendo 5 mM $MgCl_2$. Posteriormente, ressuspendeu-se os sedimentos bacterianos em tampão comercial *DeStreak Rehydration Solution* (GE Healthcare), na proporção de 5 μ L por miligrama de bactérias. A lise celular mecânica foi feita por agitação vigorosa em vortex, seguido por sucessivas passagens em agulha de seringa de 1 mL. Em seguida, fez-se uma centrifugação a 20.800 x g por 10 min para a retirada do sedimento celular não solubilizado, e o sobrenadante foi coletado. A dosagem de proteínas dos extratos proteicos totais foi feita pelo kit comercial 2D *Quant Kit* (GE Healthcare), segundo as recomendações do fabricante.

3.13 ELETROFORESE EM GEL BIDIMENSIONAL DE EXTRATOS BACTERIANOS TOTAIS

A focalização isoeletrica foi realizada utilizando-se o sistema IPG (fitas de gel com pH imobilizado em gradiente, *Immobilized pH gradient gel strips*). Foram utilizadas fitas de 7 cm

de comprimento, com pH em gradiente linear imobilizado de 3 a 10 (GE Healthcare). A reidratação das fitas foi feita por no mínimo 16 h com os extratos protéicos totais ressuspensos em volume total ajustado para 150 µL de tampão *DeStreak Rehydration Solution*, adicionados de 0,8% (v/v) da mistura de anfólitos carreadores *IPG Buffer* (GE Healthcare) específicos para o pH utilizado. As fitas reidratadas foram acomodadas no isoeletrofocalizador *Ettan IPGphor II* (GE Healthcare), e cobertas com óleo mineral *DryStrip Cover Fluid* (GE Healthcare) para evitar evaporação e precipitação de uréia. A programação utilizada para a eletrofocalização está representada na Tabela 1.

Após a focalização isoeétrica, cada fita foi reduzida, sendo incubada por 15 min sob leve agitação em 10 mL de solução de equilíbrio contendo 50 mM Tris-HCl pH 8.8, 6 M uréia, 30% (v/v) glicerol, 2% (m/v) SDS e traços de azul de bromofenol, adicionada de 1% (m/v) ditioneína (DTT) (GE Healthcare). Posteriormente, seguiu-se a etapa de alquilação, com incubação por iguais 15 min em mesma solução de equilíbrio adicionada de 2,5% iodoacetamida (IAA) (GE Healthcare). Com as fitas focalizadas, reduzidas e alquiladas, seguiu-se a separação por massa molecular em gel de SDS-PAGE 12%. Os géis foram corados com solução de azul de coomassie R, e analisados no programa *ImageMaster* (GE Healthcare).

Tabela 1 – Protocolo de isoeletrofocalização de fitas para gel 2D

Etapa	Voltagem	Volts/h
S1 step	500	10
S2 grad	4.000	3.400
S3 step	5.000	4.600

Fitas de 7 cm com pH imobilizado em gradiente linear de 3 a 10. A amperagem máxima foi fixada em 50 µA/fita. O protocolo de focalização durou cerca de 4,5 h, e ao final foram acumulados aproximadamente 8.010 V/hr por gel.

FONTE: Vieira (2012)

3.14 PREPARAÇÃO DAS AMOSTRAS PARA ESPECTROMETRIA DE MASSAS

Os *spots* identificados foram excisados do gel com auxílio de pinças e bisturis, e colocados separadamente em micro tubos previamente lavados com metanol. Os *spots* foram fragmentados para facilitar o descoramento e posterior extração dos peptídeos. O

descoramento dos *spots* foi feito por lavagens com 400 μL de solução 25 mM bicarbonato de amônio (NH_4HCO_3) e 50% (v/v) acetonitrila (ACN) (Sigma-Aldrich) sob agitação em temperatura ambiente, até total extração do corante. Os *spots* foram então desidratados em 200 μL de ACN 100% por cerca de 10 min, até ficarem opacos. A ACN foi desprezada, e os *spots* submetidos à secagem em aparelho de secagem a vácuo por aproximadamente 15 min.

Para a digestão proteolítica *in gel* das proteínas em peptídeos, cada *spot* foi reidratado em 10 μL de solução 50 mM NH_4HCO_3 contendo 20 ng/ μL da enzima Tripsina modificada (Promega, Madison, EUA), por 10 min em banho de gelo. Após esse período, foram adicionados 20 μL de 50 mM NH_4HCO_3 , e os *spots* foram incubados a 37 °C por 16 a 24 h. Posteriormente, essa solução foi removida para micro tubos limpos devidamente identificados, e foram adicionados 30 μL de solução de extração contendo 50% (v/v) ACN + 5% (v/v) ácido trifluoroacético (TFA) (Sigma-Aldrich) aos pedaços de gel que foram mantidos nos tubos anteriores, sendo incubados por 30 a 60 min sob agitação à temperatura ambiente. Em seguida, a solução de extração de cada amostra foi retirada e adicionada à solução retirada anteriormente. O mesmo procedimento de extração foi repetido mais uma vez, e os pedaços de gel foram descartados. O volume resultante do processo de extração foi concentrado em aparelho de secagem a vácuo à temperatura de 30 °C, até atingir volume de aproximadamente 20 μL .

Para a purificação e concentração das amostras de mistura dos peptídeos resultantes de cada digestão proteolítica, foram utilizadas micro colunas de ponteira de pipeta ZipTipC18 (Millipore, Billerica, EUA). Cada ponteira foi lavada por várias pipetagens com 100% ACN, seguido de equilíbrio com 60% (v/v) ACN, e, posteriormente, com solução 0,1% (v/v) TFA. Com a resina equilibrada, foi realizada a ligação da amostra à micro coluna por pipetagens sucessivas. Após, a micro coluna foi lavada com solução 0,1% TFA, e a amostra eluída em um micro tubo limpo utilizando-se solução 60% ACN + 0,1% TFA.

3.15 DETERMINAÇÃO DA MASSA DOS PEPTÍDEOS POR MALDI-TOF

A espectrometria de massas das amostras foi realizada na central analítica do Departamento de Bioquímica da USP (IQ-USP). Foi utilizado o espectrômetro de massa modelo Ettan MALDI-TOF Pro (GE Healthcare), que emprega a tecnologia MALDI-TOF (Ionização e dessorção a laser assistida por matriz – Tempo de voo, *Matrix Assisted Laser Desorption Ionization – Time of Flight*), em modo de operação reflectivo. O volume de 1 μL de cada extrato concentrado e purificado de peptídeos proteolisados, presente em solução

0,1% TFA e 60% ACN, foi misturado com igual volume de 10 µg/mL matriz ácido α -ciano-4 hidroxicinâmico (Sigma-Aldrich) preparada em solução 0,1% TFA e 50% ACN. As amostras preparadas foram aplicadas em placa de amostragem para MALDI-TOF, secas ao ambiente e submetidas à análise para obtenção dos espectros de massas dos peptídeos (*peptide mass fingerprint*, PMF). Cada amostra foi aplicada em duplicata, e os PMF utilizados para a identificação foram resultantes da comparação e combinação dos dois dados obtidos. Para a calibragem dos valores de relações massa/carga (m/z) obtidas na espectrometria, foram utilizados como calibrantes internos os picos de autólise da tripsina, quando presentes. Quando ausentes, utilizou-se calibrantes externos de massas moleculares conhecidas, que foram aplicados em todas as primeiras posições de cada placa de amostragem para MALDI-TOF.

3.16 IDENTIFICAÇÃO DAS PROTEÍNAS EM BANCOS DE DADOS A PARTIR DA MASSA DOS PEPTÍDEOS IDENTIFICADOS POR MALDI-TOF

As listas contendo as relações m/z dos peptídeos frutos da digestão proteolítica das proteínas contidas nos diferentes *spots* dos géis foram submetidas à análise estatística pelo servidor MASCOT no modo de identificação de proteínas por PMF (Perkins et al., 1999). Os parâmetros utilizados para a análise dos dados derivados do processamento de todos os *spots* foram:

- a) banco de dados MSDB com as entradas de todos os organismos e/ou banco de genes de *L. interrogans* sorovar Copenhageni;
- b) tolerância de 1 sítio de clivagem perdido pela tripsina por peptídeo gerado;
- c) modificação constante por carbamidometilação nas cisteínas, (d) modificação aleatória por oxidação nas metioninas;
- e) valores de distribuição monoisotópica de m/z dos íons detectados;
- f) tolerância de erro de $\pm 0,5$ Da nos valores de m/z .

Foram consideradas identificadas significativamente as proteínas que obtiveram o algoritmo baseado em probabilidades *MOUSE scoring* superior ao nível de significância ($P < 0,05$).

A predição da localização celular das proteínas identificadas foi realizada pela submissão das sequências nos programas LipoP (Juncker et al., 2003) e CELLO (Yu et al., 2004).

3.17 CARACTERIZAÇÃO BIOINFORMÁTICA DAS PROTEÍNAS DE *LEPTOSPIRA* SELECIONADAS PARA CLONAGEM

A predição da localização celular das sequências codificadoras previstas na anotação do genoma de *L. interrogans* sorovar Copenhageni cepa Fiocruz L1-130 (Nascimento et al., 2004a,b) selecionadas para clonagem foi realizada pelo programa PSORT (Nakai e Horton, 1999; Nakai e Kanehisa, 1991). Os servidores SMART (Letunic et al., 2006; Schultz et al., 1998), PFAM (Finn et al., 2006), e LipoP (Juncker et al., 2003) foram utilizados para as predições de domínios funcionais e estruturais presentes nas sequências de aminoácidos das sequências codificadoras selecionadas.

3.18 CLONAGEM, EXPRESSÃO E PURIFICAÇÃO DAS PROTEÍNAS RECOMBINANTES

A clonagem, expressão e purificação das proteínas Lsa63, LIC10509, Lp29, Lp49 e Lsa27 foram previamente descritas (Gomez et al., 2008; Longhi et al., 2009; Neves et al., 2007; Vieira et al., 2010b). Os procedimentos para as proteínas MPL21, MPL17, MPL36, LipL40, LipL32, LIC10494, LIC12730 e LIC12922 estão apresentados em Vieira et al. (2010a). A clonagem, expressão e purificação das proteínas recombinantes foram realizadas em colaboração com integrantes do grupo de pesquisa em que foi realizado o presente trabalho.

3.19 PRODUÇÃO DE ANTICORPOS CONTRA AS PROTEÍNAS RECOMBINANTES

Brevemente, as proteínas purificadas foram adsorvidas em 0,2% (v/v) Al(OH)₃ (Alhydrogel, Brenntag Biosector, Frederikssund, Dinamarca), como adjuvante. Cada proteína recombinante (10 µg) foi injetada subcutaneamente em camundongos BALB/C fêmeas de 4 a 6 semanas de vida. Foram administradas duas doses subsequentes das mesmas preparações em intervalos de duas semanas. O grupo controle negativo foi injetado com PBS e Al(OH)₃. Duas semanas após a última imunização, os animais foram sangrados via plexo retro orbital, e os soros foram analisados por ELISA quanto aos títulos de anticorpos gerados contra as respectivas proteínas recombinantes. Os estudos com animais foram aprovados pelo Comitê de Ética no Uso de Animais Instituto Butantan (CEUAIB), São Paulo, SP, Brasil (protocolo 506/08).

3.20 LIGAÇÃO DE PROTEÍNAS RECOMBINANTES PURIFICADAS AO PLG

A ligação de proteínas ao PLG foi avaliada por ELISA modificado. Poços de placas de micro diluição (Costar High Binding, Corning) foram adsorvidas por 16 h a 4 °C com 100 µL de 10 µg/mL das proteínas recombinantes ou BSA, como controle negativo, seguido de uma lavagem com PBS-T e bloqueio por 2 h a 37 °C com PBS adicionado de 10% (m/v) leite desnatado desidratado. A solução de bloqueio foi desprezada, e 100 µL de 10 µg/mL PLG humano purificado foram adicionados, seguido de incubação por 2 h a 37 °C. Os poços foram lavados quatro vezes com PBS-T e incubados por 1 h a 37 °C com anticorpos anti-PLG humano produzidos em camundongo (1: 4.000 em PBS). As placas foram lavadas novamente e incubadas com anticorpos anti-IgG de camundongos conjugados com peroxidase, em diluição 1: 5.000 em PBS. Após três lavagens, foram adicionados 100 µL/poço de 1 mg/mL OPD e 1 µL/mL H₂O₂ em tampão citrato fosfato (pH 5). As reações foram conduzidas por 5 min e paradas pela adição de 50 µL/poço de H₂SO₄ 4N. As absorbâncias foram medidas em espectrofotômetro, em comprimento de onda de 492 nm.

3.21 SDS-PAGE E *IMMUNOBLOTTING* DE AFINIDADE DAS PROTEÍNAS RECOMBINANTES

As proteínas recombinantes purificadas foram separadas por SDS-PAGE 12% e transferidas para membranas de nitrocelulose em sistema semi-seco. As membranas foram bloqueadas por 2 h a 37 °C com 5% BSA (m/v) em PBS-T, lavadas (3 x 10 min) e incubadas por 16 h a 4 °C com solução 3 µg/mL PLG humano, seguido de incubação por 2 h à temperatura ambiente. Após, as membranas foram lavadas e incubadas com anticorpos anti-PLG humano (1: 750, em PBS-T) por 3 h à temperatura ambiente, seguido de mais lavagens e incubação com anticorpos anti-IgG conjugados com peroxidase (1: 5.000, em PBS-T). Em seguida, a ligação das proteínas ao PLG foi revelada por reagente ECL de acordo com as recomendações do fabricante. As membranas foram expostas a filmes de raios-X.

3.22 ENSAIO DE COMPETIÇÃO POR AAC NA LIGAÇÃO DO PLG ÀS PROTEÍNAS RECOMBINANTES

Para determinar o papel dos resíduos de lisina na interação do PLG com as proteínas recombinantes, o análogo de lisina AAC, foi adicionado aos poços imobilizados com as

proteínas recombinantes em concentração final de 2 mM, juntamente com o PLG. A detecção do PLG capturado foi realizada conforme anteriormente descrito na seção 3.20, utilizando-se anticorpos anti-PLG humano.

3.23 AVALIAÇÃO DE ATIVIDADE ENZIMÁTICA DA PLASMINA ASSOCIADA ÀS PROTEÍNAS RECOMBINANTES

Placas de ELISA foram adsorvidas a 4 °C com 10 µg/mL de proteínas recombinantes purificadas ou BSA, como controle negativo. As placas foram lavadas e bloqueadas com 10% leite em pó desnatado (m/v) por 2 h a 37 °C. A solução de bloqueio foi descartada, e 100 µL/poço de 10 µg/mL de PLG humano foram adicionados e incubados por 2 h a 37 °C. Após três lavagens, foram adicionados 100 µL/poço de solução PBS contendo 40 ng/mL de uPA e 0,4 mM do substrato para plasmina S-2251. As placas foram incubadas por aproximadamente 16 h a 37 °C, e a degradação do substrato foi determinada pela leitura da absorbância a 405 nm.

3.24 CURVAS DE DOSE-DEPENDÊNCIA DA LIGAÇÃO ENTRE PROTEÍNAS RECOMBINANTES E PLG

Placas de micro diluição foram adsorvidas por cerca de 16 h a 4 °C com 100 µL de 10 µg/mL de PLG diluído em PBS. Após bloqueio, as placas foram incubadas com concentrações crescentes (0 a 1 µM) das proteínas recombinantes em estudo. A detecção das proteínas ligadas ao PLG foi realizada por incubação por 1 h a 37 °C com as diluições apropriadas de anticorpos policlonais produzidos em camundongo contra cada proteína recombinante (1: 4.000 para LipL32; 1: 5.000 para rLIC12238, LipL40 e MPL36; 1: 1.000 para Lp29 e Lp49; e 1: 500 para rLIC12730), seguido de anticorpos anti-IgG de camundongo conjugados com peroxidase (Sigma-Aldrich), na diluição de 1: 10.000 em PBS. A ligação foi avaliada pela degradação de OPD com leitura a 492 nm. Os dados de ELISA foram utilizados para o cálculo das constantes de dissociação (K_D) das proteínas, segundo método descrito por Pathirana et al. (2006) e Lin et al. (2009), baseado na equação:

$$A = A_{\text{máx}} [\text{proteína}] / (K_D + [\text{proteína}]) \quad (1)$$

Onde A é a absorbância em dada concentração de proteína, e $A_{\text{máx}}$ é igual à absorbância no equilíbrio (saturação).

3.25 EFEITO DE SAL OU HEPARINA NAS INTERAÇÕES PLG-PROTEÍNA RECOMBINANTE

Para verificar o papel das interações iônicas ou de domínios de ligação de heparina na interação entre PLG e LipL32 ou rLIC12238, concentrações crescentes de NaCl (0 a 650 mM) ou heparina (0 a 500 IU) foram adicionadas ao tampão PBS contendo PLG incubado com as proteínas recombinantes imobilizadas em placas de micro diluição. A detecção do PLG ligado foi realizada conforme exposto anteriormente na seção 3.20.

3.26 INTERFERÊNCIA DE COMPONENTES DE MATRIZ EXTRACELULAR NA LIGAÇÃO DA PROTEÍNA LIPL32 AO PLG

Em placas de ELISA, 100 µL/poço de 10 µg/mL de LipL32 purificada foram adsorvidos por cerca de 16 h a 4 °C. Após uma lavagem, as placas foram bloqueadas por 2 h a 37 °C com solução 10% (m/v) de leite desnatado desidratado, seguido por incubação por 2 h a 37 °C com 200 nM PLG adicionado de concentrações crescentes (0 a 600 nM) de colágeno tipo IV (Sigma-Aldrich) ou fibronectina plasmática (Sigma-Aldrich). Após quatro lavagens, a ligação de PLG foi detectada e quantificada por anticorpos específicos conforme protocolo descrito anteriormente (seção 3.20).

3.27 DEPOSIÇÃO DE IGG E C3B NA SUPERFÍCIE DAS LEPTOSPIRAS

Aproximadamente 25×10^6 leptospiiras/poço (*L. interrogans* sorovar Pomona cepa LPF) foram diluídas em 50 µL IsPBS e adsorvidas em placas de micro diluição por 1 h a 37 °C, seguido de 16 h a 4 °C. As placas foram lavadas duas vezes com 200 µL IsPBS e bloqueadas com 5% (m/v) leite em pó desnatado e 2,5% (m/v) BSA por 2 h a 37 °C. Após adição de 50 µL/poço de soro humano (normal ou de pacientes com leptospirose MAT positivo ou MAT negativo) como fonte de IgG, as placas foram incubadas por 30 min em gelo e, posteriormente, lavadas por 3 vezes. As leptospiiras foram então tratadas por 2 h a 37 °C com solução contendo 50 µL/poço de 5 µg/mL uPA e 40 µg/mL PLG, ou, como controles negativos, somente com uPA ou PLG, ou sem adições. Depois de três lavagens, as placas foram incubadas com anticorpos anti-IgG humano específico para região Fc (Calbiochem) em diluição 1: 5.000 por 1 h a 37 °C, seguido de incubação com anticorpos secundários conjugados com peroxidase (1: 10.000), por 1 h a 37 °C. A deposição de IgG nas leptospiiras

foi avaliada pela degradação de substrato cromogênico para peroxidase, OPD, com medição das absorbâncias em comprimento de onda de 492 nm.

O protocolo para avaliação da deposição de C3b nas leptospiiras foi similar ao utilizado para deposição de IgG descrito acima, exceto pela condição de incubação com o soro humano, realizada a 37 °C por 50 min, e o uso de anticorpos anti-C3b humano (Calbiochem), na diluição de 1: 5.000, seguido de anticorpos secundários conjugados com peroxidase (1: 5.000).

3.28 DEGRADAÇÃO DE C3B HUMANO POR LEPTOSPIRAS LIGADAS À PLASMINA

Um total de 10^8 leptospiiras/amostra (*L. interrogans* sorovar Pomona cepa LPF) foram tratadas em 200 µL IsPBS com a adição de:

- a) 10 µg PLG e 3U uPA (plasmina);
- b) apenas 10 µg PLG;
- c) sem adições (não tratadas).

As células foram incubadas por 1 h com o PLG, e por mais 1 h após adição de uPA. As bactérias foram lavadas três vezes com IsPBS e ressuspensas em 100 µL IsPBS contendo 15 µg/mL de C3b humano purificado (Calbiochem), sendo incubadas por 5 ou 20 h a 37 °C. Como controle, amostras tratadas com PLG e uPA receberam 2 µg de aprotinina (Fluka) juntamente à adição do C3b. As leptospiiras foram removidas por centrifugação e 20 µL dos sobrenadantes foram separados por SDS-PAGE 10% e transferidos para membranas de nitrocelulose em sistema semi-seco. As membranas foram bloqueadas por incubação em tampão 5% (m/v) leite desnatado desidratado e 1% (m/v) BSA por 16 h a 4 °C. A detecção de C3b foi realizada por incubações com anticorpos anti-C3b humano e anticorpos secundários conjugados à peroxidase, seguido de revelação por solução contendo 0,05% (m/v) tetrahidroclorato de 3,3-diaminobenzidina (DAB, Fluka, Steinheim, Suíça) e 0,3% (v/v) H₂O₂.

3.29 ATIVIDADE ENZIMÁTICA DE LEPTOSPIRAS TRATADAS COM PLG E FATOR H

Aproximadamente 10^9 leptospiiras/amostra (*L. interrogans* sorovar Pomona cepa LPF) foram centrifugadas a 6.000 x g por 10 min e ressuspensas em 200 µL IsPBS. As bactérias receberam os seguintes tratamentos:

- a) 5 µg PLG humano purificado e 3U uPA humana purificada;
- b) 5 µg PLG, 3U uPA e 10 µg FH humano purificado (Calbiochem);
- c) 5 µg PLG, 3U uPA e 5 µg FH;
- d) 5 µg PLG, 3U uPA e 1 µg FH;
- e) 10 µg FH;
- f) somente lsPBS.

Incubou-se as preparações por 1 h a 37 °C com a adição de PLG e FH, e por mais 1 h após a adição de uPA. As leptospiplas foram centrifugadas e lavadas três vezes com 0,7 mL lsPBS. Após, foram ressuspensas em 200 µL lsPBS, sendo divididas em duas alíquotas. Cada alíquota recebeu 100 µL de solução contendo 0,5 mg/mL do substrato S-2251, para uma concentração final de 0,25 mg/mL. As suspensões foram incubadas por 2 h a 37 °C, sob leve agitação, e centrifugadas a 6.000 x g por 10 min. Os sobrenadantes (150 µL) foram transferidos para placas de micro diluição e a clivagem do substrato foi determinada pela leitura das absorbâncias a 405 nm.

3.30 DETECÇÃO DE FH HUMANO LIGADO ÀS LEPTOSPIRAS

Culturas de leptospiplas foram centrifugadas (6.000 x g por 10 min) e ressuspensas em 200 µL de lsPBS contendo:

- a) sem adições;
- b) 30 µg PLG, 4U uPA e 20 µg FH;
- c) 20 µg FH.

As amostras foram incubadas por 2 h a 37 °C com PLG e FH, e por mais 1 h após a adição de uPA. As bactérias foram lavadas duas vezes com lsPBS e posteriormente incubadas com anticorpos monoclonais anti-FH humano (Quidel, San Diego, EUA) (1: 300 em 150 µL de lsPBS) por 1 h a 37 °C. Após lavagens, incubou-se as bactérias com anticorpos secundários conjugados com peroxidase (1: 2.500) por 1 h a 37 °C. As leptospiplas foram lavadas duas vezes e ressuspensas em solução contendo 1 µg/mL de substrato OPD e 2 µL/mL H₂O₂. As absorbâncias foram lidas a 492 nm.

3.31 DETECÇÃO DE FH E PLG CAPTURADO POR LEPTOSPIRAS DO SORO HUMANO

L. interrogans sorovar Pomona cepa LPF (10^9 leptospiiras/amostra) foram centrifugadas e ressuspendidas em 200 μ L de lsPBS ou 100% soro humano de doadores saudáveis como fonte de PLG ou FH. As bactérias foram incubadas por 1 h a 30 °C, lavadas duas vezes e incubadas com anticorpos anti-FH ou anti-PLG humanos em diluição de 1: 300 em 200 μ L de lsPBS, por 2 h a 30 °C. Após três lavagens, as leptospiiras foram incubadas com anticorpos secundários conjugados à peroxidase (1: 1.000 em 200 μ L) por 1 h a 30 °C, seguido de mais uma etapa de lavagens. Dividiu-se as bactérias em quatro alíquotas, sendo cada uma ressuspendida em 50 μ L de solução contendo 2 μ g/mL de substrato OPD e 2 μ L/mL H_2O_2 . As ligações de PLG ou FH foram determinadas pela medida das absorbâncias a 492 nm.

3.32 TESTE DE SUSCEPTIBILIDADE AO SORO

A morte de leptospiiras mediada pelo SC foi avaliada por incubação das bactérias com soro humano de indivíduos saudáveis, baseado em método previamente descrito (Meri et al., 2005), com modificações. Aproximadamente $5,0 \times 10^8$ *L. interrogans* sorovar Pomona cepa LPF por amostra foram tratadas com 40 μ g PLG e 4U uPA em 200 μ L lsPBS, por 3 h a 37 °C. Como controle, uma amostra foi incubada somente com lsPBS, sem adições. Após, as amostras foram divididas em alíquotas contendo $1,0 \times 10^8$ células, às quais se adicionou 80 μ L de soro humano normal juntamente com igual quantidade de meio EMJH suplementado com 10% de enriquecimento para *Leptospira*. As suspensões bacterianas foram incubadas a 37 °C por 1 h, seguido de incubação por 5 min em gelo para bloquear a ativação do complemento. Em seguida, 20 μ L de cada amostra ($\sim 1,25 \times 10^7$ leptospiiras) foram transferidos, em quadruplicatas, para placas de micro diluição. Adicionou-se 180 μ L de EMJH a cada amostra. As placas foram seladas com filme adesivo estéril e incubadas em estufa a 30 °C por 4 dias. O crescimento bacteriano foi determinado por contagem das leptospiiras em câmara de Petroff-Hausser com o uso de microscópio de campo escuro.

3.33 DEGRADAÇÃO DE COMPONENTES PURIFICADOS DE MEC

Placas de 96 poços foram adsorvidas por 16 h a 4 °C com 0,5 µg/poço de componentes de MEC de origem humana purificados (fibronectina celular ou laminina – Sigma Aldrich), lavadas quatro vezes com PBS-T (200 µL/poço), bloqueadas com 2% BSA (m/v) em PBS-T, por 2 h a 37 °C, e lavadas mais duas vezes. As espiroquetas (10^8 leptospiras/amostra) foram tratadas conforme descrito anteriormente na seção 3.5 com 10 µg PLG e 3U uPA ou somente IsPBS (controle negativo). Após o tratamento, as bactérias foram lavadas, ressuspensas em 100 µL IsPBS e transferidas para as placas previamente adsorvidas com os componentes de MEC. As placas foram mantidas a 37 °C por 20 h e, após, lavadas 5 vezes para a remoção das leptospiras. A degradação dos componentes de MEC imobilizados foi detectada pela redução na absorbância após incubações com anticorpos específicos contra os componentes individuais (diluição 1: 5.000 em PBS-T; 45 min a 37 °C), anticorpos anti-IgG conjugados com peroxidase (diluição 1: 5.000 em PBS-T; 45 min a 37 °C) e 100 µL/poço de 1 mg/mL OPD adicionado de 1 µL/mL H₂O₂. A reação foi parada com 50 µL/poço H₂SO₄ 4N, e a absorbância foi medida a 492 nm. A porcentagem de degradação foi calculada através da fórmula:

$$(A-B)/A(100) \quad (2)$$

Onde A= média das absorbâncias das amostras PBS (controle positivo para a reatividade de MEC) e B= média das absorbâncias dos grupos experimentais (não-tratado ou PLA).

3.34 PREPARO DE CORDÃO UMBILICAL E EXTRAÇÃO DE CÉLULAS ENDOTELIAIS

As HUVECs (células endoteliais de veia de cordão umbilical humano, *Human Umbilical Vein Endothelial Cells*) foram obtidas por digestão de veias de cordões umbilicais humanos com collagenase, segundo o método de Jaffe et al. (1973). Brevemente, os cordões de umbilicais de parturientes foram coletados, colocados em papel alumínio e cuidadosamente lavados externamente com álcool 70%. Realizou-se um corte transversal na extremidade do cordão para melhor visualização da veia e das artérias. Foi inserida uma chave de três vias na veia do cordão para posterior injeção de solução salina e lavagem do interior da veia. Após

lavagem, a outra extremidade foi fechada com um *clamp*. Foi injetada solução de collagenase (0,3 mg/mL em meio de cultura RPMI - meio *Roswell Park Memorial Institute*). Após 15 min de incubação, o conteúdo foi colocado em um tubo de polipropileno e centrifugado durante 10 min a 1.000 x g, 4 °C. Após centrifugação, as células foram colocadas em garrafas de cultura contendo meio completo: meio RPMI 1640 (Cultilab, Campinas, Brasil), 10% (v/v) soro fetal bovino (SFB) (Cultilab), 45 µg/mL heparina (Roche), 1 mM piruvato de sódio (Spectrum Chemical, New Jersey, EUA), 2 mM L-glutamina (Sigma-Aldrich), penicilina 100 IU/estreptomicina 100 µg/mL (Cultilab), 50 µM 2-mercaptoetanol (Amersham Pharmacia Biotech, Piscataway, EUA), 25 µg/mL fator de crescimento endotelial (Sigma-Aldrich) e 0,75% (v/v) extrato de cérebro de camundongo.

Os cordões umbilicais foram coletados após o consentimento de parturientes no Amparo Maternal de São Paulo, conforme normas éticas e de assepsia condizentes. O uso das HUVECs para experimentação é aprovado como extensão de projeto contemplado pelo Comitê de Ética da Universidade Federal de São Paulo (UNIFESP), protocolo 0664/05.

3.35 CULTIVO DE CÉLULAS ENDOTELIAIS

Inicialmente, as HUVECs foram cultivadas a 37 °C em estufa com atmosfera de 5% CO₂ em garrafas de 25 ou 75 cm², contendo 2% (m/v) gelatina (Difco) e meio completo. Quando confluentes, a monocamada aderida foi liberada da superfície com solução 0,25% (m/v) tripsina e 0,02% (m/v) ácido etilenodiamino tetra-acético (EDTA, Cultilab), e as células semeadas em placas de cultura. Foram utilizadas HUVECs da primeira passagem em monocamada confluyente e subconfluyente, conforme o tipo de experimento realizado.

3.36 TRANSMIGRAÇÃO DE LEPTOSPIRAS ATRAVÉS DE MONOCAMADAS DE HUVECS

Em placas de 24 poços, câmaras *transwell* de 6,5 mm com membrana de policarbonato de poros de 5 µm (Corning) foram preparadas com 100 µL de 4% gelatina/poço. Sobre a camada de gelatina, foram semeadas 15.000 HUVECs/poço. As células foram incubadas com 1,5 mL de meio RPMI completo por cerca de 24 h, até total confluência da monocamada. *L. interrogans* sorovar Pomona (5 x 10⁸/amostra) foram tratadas com 50 µg PLG, em 200 µL lsPBS por 1,5 h, seguido de adição de 5 U uPA por 1 h a 37 °C. Como controle, amostras

foram tratadas apenas com PLG, apenas com uPA, ou sem adições (PBS). Após três lavagens com lsPBS, cada amostra de bactérias foi ressuspensa em 200 µL de meio RPMI completo sem a adição de SFB para excluir a interferência de PLG proveniente do soro nos experimentos. Após, 100 µL da suspensão de bactérias foram semeados sobre a monocamada de HUVECs, com a adição de 100 µL de meio RPMI na parte superior da câmara de transmigração, e 400 µL na parte inferior. Após 4 h de incubação em estufa com atmosfera de 5% CO₂ a 37 °C, os meios de cultura nas partes inferiores das câmaras foram coletados, centrifugados a 8.000 x g, e as leptospiros contadas em câmara de Petroff-Hausser, em microscópio de campo escuro.

3.37 ATIVAÇÃO DE HUVECS POR CONTATO COM LEPTOSPIRAS

Em placas de culturas de células de 96 poços (Corning), 15.000 HUVECs/poço foram semeadas e incubadas por 16 h a 37 °C para aderência. *L. interrogans* sorovar Pomona cepa LPF (5 x 10⁸/amostra) foram tratadas com 50 µg PLG (em 200 µL lsPBS) por 1,5 h, seguido de adição de 5 U uPA e incubação por 1 h a 37 °C. Amostras controle foram tratadas apenas com PLG, apenas com uPA, ou sem adições (PBS). Após três lavagens com lsPBS, cada amostra de bactérias foi ressuspensa em 500 µL de meio RPMI completo sem SFB. As suspensões bacterianas foram inoculadas (100 µL/poço + 100 µL meio RPMI) sobre as HUVECs previamente lavadas com PBS para a retirada de resquícios de SFB. HUVECs incubadas apenas com meio de cultura foram utilizadas como controle. Após 6 ou 24 h de incubação a 37 °C em atmosfera de 5% CO₂, os sobrenadantes foram coletados, centrifugados a 8.000 x g para a retirada das bactérias, e estocados em alíquotas a -80 °C até o uso.

3.38 DOSAGEM DE ATIVADORES DE PLG

As quantificações de uPA e tPA de soros humanos foram realizadas através dos kits comerciais *AssayMax Human Urokinase* e *AssayMax Human Tissue-Type Plasminogen Activator* (Assaypro, St. Charles, EUA). Os kits são baseados em ELISA de captura, e foram utilizados conforme recomendação do fabricante. As amostras foram diluídas a 25% previamente aos ensaios.

3.39 TESTE CROMOGÊNICO DE ATIVIDADE DE UPA

Soros humanos foram ensaiados quanto à atividade de uPA por método cromogênico direto (*uPA chromogenic activity direct assay*, Assaypro) de acordo com as recomendações do fabricante. As amostras foram diluídas 5 vezes para a realização dos experimentos.

3.40 QUANTIFICAÇÃO DA ATIVIDADE DE MMP-2 E MMP-9

Amostras de soros humanos ou sobrenadantes de HUVECs foram quantificadas quanto à presença de MMP-2 e MMP-9 por testes específicos (*Biotrak Activity Assay Systems*, GE Healthcare), segundo as recomendações do fabricante. Os testes são baseados na ativação proteolítica de pró-enzimas de detecção após captura de MMP-2 ou MMP-9 ativas, seguido de quantificação por degradação de substrato cromogênico. Acetato p-aminophenyl mercúrico (AMPA) foi adicionado às reações para a detecção de MMP-2 ou MMP-9 total (formas pro e ativa). As amostras de soros humanos normais ou de pacientes com leptospirose (MAT negativo ou MAT positivo) foram diluídas 20 e 50 vezes, respectivamente, para a determinação de MMP-9 e MMP-2. Sobrenadantes de culturas celulares foram analisados sem prévia diluição.

3.41 PCR EM TEMPO REAL (QPCR)

O ácido ribonucleico (RNA) total de HUVECs incubadas com leptospirose foi extraído utilizando-se o kit *RNAspin mini* (GE Healthcare), de acordo com as instruções fornecidas pelo fabricante. O RNA foi quantificado por espectrofotometria utilizando equipamento NanoDrop (Thermo). O ácido desoxirribonucleico complementar (cDNA) foi sintetizado a partir de 1.000 ng de RNA total utilizando-se o kit *SuperScript III Reverse Transcriptase* (Invitrogen, Carlsbad, EUA). O cDNA resultante foi submetido à reação de qPCR utilizando-se *TaqMan Master Mix* e *TaqMan Gene Expression Assays* (Applied Biosystems, Carlsbad, EUA). Os genes de interesse avaliados foram MMP-9, uPA e tPA, e beta actina (ACTB) (números de catálogo hs00957555_m1, hs01547050, hs00263492 e 4333762T, respectivamente). ACTB foi utilizado como controle de expressão constitutiva. As condições de cada reação foram: conjunto de *primers* e sondas específicas comerciais diluídas 20 vezes (uPA, tPA, MMP-9 e ACTB, Applied Biosystems), 50 ng cDNA, *TaqMan Master Mix* diluído 2X (Applied Biosystems), em volume total de 20 µL. As reações foram conduzidas no

equipamento Applied Biosystems 7300 Real-time PCR System (Applied Biosystems), utilizando-se o ciclo: 50 °C por 2 min, seguido de 10 min a 95 °C e 40 ciclos de 95 °C por 15s seguido de 60 °C por 60 s. Cada amostra foi avaliada em triplicatas, e os valores de ciclo limiar (CT, *threshold cycle*) foram calculados utilizando-se o *software 7300 System SDS Relative Quantification Study* (Applied Biosystems). Os níveis de transcrição relativa foram calculados pelo método comparativo $2^{-\Delta\Delta C_t}$ (Livak e Schmittgen, 2001), utilizando-se ACTB como controle constitutivo e as amostras de HUVECs incubadas na ausência de leptospiros por 6 h, como calibrante. A variação de expressão relativa (máximo e mínimo) foi calculada por $2^{-\Delta\Delta C_t}$ com $\Delta\Delta C_t + s$ e $\Delta\Delta C_t - s$, onde s = desvio padrão de $\Delta\Delta C_t$.

3.42 ZIMOGRRAFIA

Géis SDS-PAGE 8% foram copolimerizados com 0,1% (m/v) gelatina (Difco) ou colágeno tipo I (Sigma-Aldrich). Tampão de aplicação não desnaturante 5 vezes concentrado (250 mM Tris-HCl pH 6.8; 2% [m/v] SDS; 0,1 g/mL azul de bromofenol; 10% [v/v] glicerol) foi adicionado às amostras diluídas, que foram aplicadas no gel sem fervura. Após corrida dos géis, estes foram lavados com solução 2,5% (v/v) Triton X-100 (2 x 15 min), seguido de lavagens (2 x 5 min) com 50 mM Tris-HCl pH 8.4. Os géis foram então incubados por aproximadamente de 18 h a 37 °C em tampão de ativação (50 mM Tris-HCl pH 8.4, 5 mM CaCl_2 , 1 μM ZnCl_2), sendo posteriormente corados com solução de azul de coomassie (0,25% azul de coomassie R, 45% [v/v] metanol, 10% [v/v] ácido acético glacial). O excesso de corante foi retirado com solução descorante (45% [v/v] metanol, 10% [v/v] ácido acético glacial), até que as bandas de degradação (claras) fossem adequadamente visualizadas.

Experimentos controle foram realizados com solução de ativação sem CaCl_2 e ZnCl_2 , ou com a adição de 25 mM EDTA à solução de ativação completa.

Nos experimentos que foram utilizados soros humanos, as amostras foram diluídas 20 vezes, e 10 μL foram aplicados por poço, correspondendo a 0,5 μL de soro/poço. Os soros foram selecionados aleatoriamente da coleção disponível no laboratório, independentemente do título de MAT, sorovar envolvido na infecção ou progressão da doença em cada paciente. Para os experimentos de zimografia de sobrenadantes de culturas de células, foram utilizados 20 μL /poço das amostras sem diluição. Sobrenadantes de culturas de células HT1080 (linhagem celular de fibrosarcoma humano) foram utilizados como controle positivo para atividade de degradação de pró-MMP-2 e pró-MMP-9.

3.43 GEL BIDIMENSIONAL PARA ZIMOGRAFIA

Foram utilizados 8 μ L de *pool* de soros humanos por gel. A focalização isoelétrica foi realizada conforme previamente descrito na seção 3.13, com exceção da reidratação das fitas, que foi feita utilizando-se tampão não desnaturante (9M uréia, 4% [m/v] CHAPS, 0,05% [v/v] triton X-100 e traços de azul de bromofenol). Após a isoeletrofocalização, seguiu-se a separação por massa molecular em gel de SDS-PAGE 8%, excluindo-se as etapas prévias de redução e alquilação.

4 RESULTADOS E DISCUSSÃO

4.1 CARACTERIZAÇÃO DA LIGAÇÃO DE *LEPTOSPIRA* AO PLG

4.1.1 Ligação de plasminogênio humano à superfície de *L. interrogans*

A habilidade de *L. interrogans* de ligar PLG humano à sua superfície foi verificada através de ensaio de imunofluorescência em fase líquida. Foram utilizadas células vivas e intactas de *L. interrogans* sorovar Copenhageni cepa Fiocruz L1-130 virulentas. Após interação com PLG, as leptospiiras foram visualizadas por coloração do DNA por iodeto de propídeo (coloração do corpo bacteriano em vermelho), seguido de detecção de proteínas específicas por anticorpos policlonais na presença de anticorpos secundários conjugados a FITC. Fluorescência em cor verde pôde ser observada para o PLG (Figura 3B - PLG1, PLG2, PLG3) e LipL32 (Figura 3B – LipL32), uma proteína de membrana externa utilizada como controle positivo para localização na superfície (Nally et al., 2007), mas não para GroEL (Figura 3B – GroEL), uma proteína citoplasmática utilizada como controle negativo para localização na superfície (Haake e Matsunaga, 2002). A localização celular da marcação fluorescente verde foi observada pela superposição dos dois campos (iodeto de propídeo e FITC) (Figura 3C).

O padrão de fluorescência observado nas leptospiiras ligadas ao PLG sugere que essas bactérias apresentem receptores ao longo de toda sua superfície, como já descrito para *Borrelia* (Coleman et al., 1995). Este resultado é comparável com o padrão de distribuição resultante do experimento de localização celular da proteína de superfície Lsa63 (Vieira et al., 2010b) e, embora menos intenso, com o padrão da proteína majoritária de membrana, LipL32, mas contrastante com o obtido para outras proteínas menos expressas em leptospiiras, que apresentam padrão pontual de expressão (Longhi et al., 2009; Oliveira et al., 2008).

Este experimento ainda permitiu observar que as membranas externa e interna das leptospiiras provavelmente mantêm-se intactas após a ligação do PLG aos receptores. O tratamento com anticorpos anti-GroEL, uma proteína *heat shock* presente no cilindro protoplasmático, não gerou fluorescência nas bactérias, indicando a integridade das membranas pela inacessibilidade do citoplasma aos anticorpos. Adicionalmente, foi observado que as bactérias preservaram seu movimento rotacional normal após o tratamento com PLG, sugerindo que a ligação ao PLG não causa danos ao flagelo periplasmático.

Figura 3 – Reconhecimento da ligação de PLG à superfície de leptospiros por imunofluorescência em fase líquida

L. interrogans sorovar Copenhageni vivas e virulentas foram tratadas com PLG, e a ligação foi detectada por anticorpos específicos anti-PLG (**PLG 1 a 3**). Como controles, foram utilizadas leptospiros tratadas com PLG coradas para as proteínas **LipL32** (proteína de membrana externa) ou **GroEL** (proteína citoplasmática), e leptospiros não tratadas (**PBS**). (A) Coloração por iodeto de propídeo, (B) coloração por FITC e (C) imagens superpostas. A visualização foi feita em microscópio confocal, com aumento de 600X.

FONTE: Vieira (2012), (Vieira et al., 2009)

4.1.2 Conversão a plasmina do PLG ligado às leptospiros e determinação da atividade enzimática

Para a análise da ligação do PLG às leptospiros e determinação da atividade enzimática após a conversão para a enzima ativa PLA, as leptospiros tratadas com PLG e uPA foram incubadas na presença de substrato cromogênico específico para PLA, S-2251. Como demonstrado na Figura 4, tanto a cepa virulenta quanto a não virulenta de *L. interrogans* sorovar Copenhageni testadas foram capazes de interagir com o PLG, embora com diferentes intensidades. Como controles, foram utilizadas leptospiros tratadas somente com PLG, uPA ou PBS, e não geraram degradação do substrato em níveis significativos. O PLG capturado na superfície das bactérias foi convertido para a enzima ativa PLA apenas após a adição do ativador exógeno uPA, promovendo atividade proteolítica associada à membrana. Esse resultado sugere que, como já reportado para as espiroquetas *Borrelia* e *Treponema* (Lahteenmaki et al., 2001), bem como para outros micro-organismos patogênicos (Lahteenmaki et al., 2001), as leptospiros não possuem mecanismo endógeno de ativação do PLG, requerendo ativação pelo sistema fibrinolítico do próprio hospedeiro. Isso contrasta com alguns patógenos que apresentam sistema endógeno de ativação do PLG, como *Streptococcus* e *Staphylococcus*, que produzem e secretam streptoquinase e stafiloquinase, respectivamente, e *Yersinia pestis*, que expressa a protease Pla associada à membrana (Leytus et al., 1981; Li et al., 1999; Lijnen et al., 1994; McCoy et al., 1991; Okada et al., 1996; Schlott et al., 1998; Sodeinde et al., 1992; Suomalainen et al., 2007; Young et al., 1998). Estas, juntamente com as proteínas PauA e PauB de *Streptococcus uberis* (Johnsen et al., 1999; Leigh, 1994), a proteína Esk de *S. equisimilis* (Nowicki et al., 1994) e Pad de *S. dysagalactiae* (Leigh et al., 1998) constituem os ativadores de PLG de origem bacteriana conhecidos até o momento (Lahteenmaki et al., 2001, 2005).

A ligação ao PLG e, conseqüentemente, a atividade enzimática da PLA, ocorreram de forma dose-dependente na faixa de concentrações utilizadas (Figura 4). Foram necessárias baixas quantidades de PLG para a detecção de atividade enzimática, mostrando a especificidade e eficiência na captura de PLG pelas leptospiros. A cepa virulenta utilizada provou-se mais eficiente na captura de PLG do que a cepa não virulenta atenuada em cultura. O mesmo comportamento de maior ligação de PLG à superfície em condições de virulência já foi descrito para a espiroqueta *B. burgdorferi* (Coleman e Benach, 2000). Este resultado pode sugerir uma alteração nos receptores de PLG durante o processo de atenuação das leptospiros ou expressão de diferentes receptores, expressão dos mesmos receptores em maior ou menor

quantidade, ou ambos. A partir dessas observações, pode-se insinuar, ainda, que fatores de virulência atuem como receptores de PLG nas leptospiiras.

Figura 4 – Degradação de substrato cromogênico específico por leptospiiras complexadas à plasmina

L. interrogans sorovar Copenhageni virulentas e não virulentas foram tratadas em diferentes condições: sem adições (**PBS**), somente uPA (**uPA**), somente 5 µg PLG (**PLG**), PLG em concentrações crescentes (0,5, 2 e 5 µg) com a adição de uPA, e plasma humano diluído a 30% com a adição de uPA (**30% plasma**). As colunas representam as médias nas absorbâncias como medida da degradação do substrato para plasmina S-2251 ± o desvio padrão de três replicatas para cada grupo experimental e são representativas de três experimentos independentes. * e ** indicam, respectivamente, significância estatística de $P<0,0001$ e $P<0,001$ em comparação ao controle PBS. Os grupos experimentais de PLG + uPA e 30% plasma das leptospiiras virulentas apresentam significância estatística ($P<0,0001$) em relação aos mesmos grupos testados com leptospiiras não virulentas.

FONTE: Vieira (2012), (Vieira et al., 2009)

O PLG é uma proteína presente no plasma humano na concentração de aproximadamente $20,8 \pm 1,9$ mg/100 mL. Os dados obtidos mostram que a cepa virulenta de *L. interrogans*, mas não a atenuada em cultura, tem capacidade de capturar o PLG diretamente do plasma humano nas condições ensaiadas (aproximadamente 6 µg de PLG presentes em 100 µL de plasma diluído a 30%), visualizado indiretamente pela atividade da PLA (Figura 4). Pode-se atribuir esta diferença à maior habilidade das leptospiiras virulentas de ligarem o

PLG aos seus receptores, o que resulta na proteção da atividade de inibidores de ativadores de PLG preentes no soro, principalmente PAI-1. A atividade enzimática da PLA obtida por captura do PLG diretamente do plasma humano mostrou-se inferior quando comparada com quantia similar de PLG purificado (5 μ g), provavelmente devido à competição na reação de ligação à membrana pela complexa mistura de proteínas que compõe o plasma (α_2 -macroglobulina e α_2 -antiplasmina). De qualquer maneira, uma vez no hospedeiro, esse dado sugere que as leptospiros sejam capazes de se associar ao PLG livre, diretamente do fluído humano.

Quando o inibidor de serino-proteases, aprotinina, foi adicionada à reação de degradação do substrato S-2251 pelas bactérias previamente tratadas com PLG e uPA, não foi observada degradação significativamente diferente à dos controles negativos (Figura 5). Este dado sustenta a afirmação de que a atividade proteolítica observada é devido à atividade específica da PLA.

Figura 5 – Inibição por aprotinina da atividade da plasmina ligada às leptospiros

L. interrogans sorovar Copenhageni virulentas foram tratadas em diferentes condições: sem adições (PBS), somente aprotinina (Apr.), PLG+uPA+aprotinina (PLA+Apr.) ou PLG+uPA (PLA). As colunas representam as médias nas absorbâncias como medida da degradação do substrato para plasmina $\pm$ o desvio padrão de três replicatas para cada grupo experimental e são representativas de dois experimentos independentes. * $P < 0,0001$ em relação ao controle PBS.

FONTE: Vieira (2012)

4.1.3 Inibição da ligação de PLG e atividade proteolítica adquirida por *L. interrogans* pelo competidor AAC

Foi demonstrado para diversos patógenos bacterianos que a interação do PLG com receptores proteicos na superfície se dá através dos resíduos de lisina presentes nas proteínas, sendo mediada pelos domínios do tipo *kringle* presentes na estrutura do PLG (Attali et al., 2008; Candela et al., 2008; Coleman et al., 1995; Jobin et al., 2004; Kinnby et al., 2008; Magalhaes et al., 2007; Pantzar et al., 1998; Rojas et al., 2008; Xolalpa et al., 2007; Yavlovich et al., 2001). Sendo assim, para avaliar a participação dos domínios *kringle* do PLG na ligação aos receptores nas leptospiros, ensaios de ligação foram realizados na presença do competidor AAC, um derivado e análogo do aminoácido lisina. A adição de AAC reduziu a atividade proteolítica de *L. interrogans* sorovar Copenhageni virulenta de maneira dose-dependente, sendo estatisticamente significativa a partir da concentração de 1 mM, e atingindo quase que total inibição a 1.000 mM (Figura 6). Supõe-se que o AAC compita com os resíduos de lisina presentes nas proteínas na captura do PLG, impedindo que o último ligue-se à superfície bacteriana, o que acarreta a diminuição da atividade proteolítica da PLA.

4.1.4 Análise do crescimento em cultura de leptospiros associadas à plasmina

Para verificar se a presença de PLG ou PLA enzimaticamente ativa na superfície celular poderia implicar em algum dano na viabilidade ou crescimento das leptospiros, as bactérias foram tratadas e re-inoculadas em meio EMJH fresco, sendo monitoradas quanto ao crescimento por 14 dias. A presença de PLA ou PLG na superfície bacteriana parece não acarretar nenhum dano à divisão celular, já que o crescimento das leptospiros nos grupos tratados foi similar em relação ao crescimento do controle não tratado (PBS) (Figura 7).

Esse dado aliado ao resultado de imunofluorescência em fase líquida obtido com o anticorpo contra a proteína citoplasmática GroEL, que mostrou que as leptospiros com PLG em sua superfície conservam a membrana intacta (Figura 3), bem como dados de observação da motilidade das leptospiros em microscópio (dados não mostrados), indicam que as bactérias não sofrem danos devido à ligação de PLG ou atividade enzimática da serino-protease PLA em sua superfície, sendo a proteólise provavelmente direcionada para o meio extracelular.

Figura 6 – Inibição da ligação de PLG às leptospiros por AAC

L. interrogans sorovar Copenhageni virulentas foram tratadas conforme a seguir: PLG com a adição de uPA (**PLG + uPA**), PLG com a adição de uPA juntamente com concentrações crescentes de AAC (**0,01 a 1000 mM AAC**), somente **PLG**, **uPA** ou **AAC** e sem adições (**PBS**). A clivagem do substrato S-2251 foi medida pela absorbância a 405 nm. As colunas representam a média nas absorbâncias $\pm$ o desvio padrão de três replicatas para cada grupo experimental. Os dados são representativos de três experimentos independentes. Estão indicadas diminuições estatisticamente significativas da degradação do substrato por AAC em comparação com o controle positivo (PLG + uPA): ** $P < 0,01$ e * $P < 0,0001$. FONTE: Vieira (2012), (Vieira et al., 2009)

Figura 7 – Crescimento de leptospiros com plasmina ligada à superfície

L. interrogans virulentas foram tratadas somente com **PLG** ou com PLG juntamente com uPA (**PLG+uPA**) para a formação de plasmina enzimaticamente ativa na superfície, e reinoculadas em meio de cultura. Amostras controle (**PBS**) não receberam adições no tratamento. O crescimento foi monitorado por medida na absorbância das culturas (600 nm) em intervalos de tempo determinados. FONTE: Vieira (2012), (Vieira et al., 2009)

4.1.5 Efeito de fatores ambientais na geração de PLA na superfície de *L. interrogans*

A regulação da patogenicidade, refletida por indução ou inibição de fatores de virulência por fatores ambientais tais como temperatura e osmolaridade, já foi reportada para diversos patógenos (Mekalanos, 1992). Em *Leptospira*, foram descritos diversos genes que são expressos diferencialmente sob variações de temperatura (Lo et al., 2006), e genes que são susceptíveis à regulação osmótica (6% dos genes) (Matsunaga et al., 2007). Esses estudos têm mimetizado a transição de temperatura ou osmolaridade ambientais para condições de temperatura e osmolaridade corporais de mamíferos durante a infecção (Mekalanos, 1992).

Tendo em vista isso, investigou-se se esses condicionantes ambientais poderiam exercer influência sobre a ligação de PLG e ativação à PLA na superfície das leptospiplas. A atividade enzimática da PLA adquirida por leptospiplas tratadas com PLG e ativadas com uPA foram testadas em bactérias cultivadas a 28, 37 ou 39 °C, mimetizando, respectivamente, condições ambientais, temperatura normal e febril de hospedeiro mamífero. Não se observou diferença na atividade enzimática adquirida pelas leptospiplas mantidas nas diferentes condições testadas (Figura 8A). O efeito da osmolaridade foi avaliado pela adição de 120 mM NaCl às culturas, simulando condições fisiológicas quando da entrada das leptospiplas no hospedeiro mamífero (~300 mosmol/L) (Matsunaga et al., 2007). Igualmente, a ligação e ativação de PLG não foram afetadas pela osmolaridade, já que valores similares de degradação de substrato foram detectados nas situações controle e com adição de NaCl (Figura 8B).

Os dados obtidos neste trabalho indicam que a atividade enzimática em decorrência da associação e ativação do PLG às leptospiplas não seja afetada significativamente por osmolaridade fisiológica ou temperatura corporal de mamíferos. Isto sugere que proteínas participantes desta interação não sejam reguladas por tais condicionantes. Esta suposição é consistente com o fato de que os receptores de PLG são requeridos imediatamente após a entrada das leptospiplas no hospedeiro, não havendo tempo hábil de expressão dos receptores mais relevantes apenas por estímulo de condições ambientais no hospedeiro.

Figura 8 – Influência de temperatura e osmolaridade na ligação de PLG às leptospiros e atividade da plasmina

L. interrogans sorovar Copenhageni virulentas cultivadas a 28 °C em meio EMJH suplementado com 10% enriquecimento para leptospiros, foram translocadas para 37 °C ou 39 °C por 24 h (A), ou suplementadas com 120 mM NaCl e mantidas a 28 °C (B). As células foram tratadas com 5 µg PLG juntamente com uPA (**plasmina**) ou somente com **PBS**. As colunas representam a média das absorbâncias como medida de degradação do substrato $\pm$ o desvio padrão de quatro replicatas para cada grupo experimental. Os dados são representativos de dois experimentos independentes.

FONTE: Vieira (2012), (Vieira et al., 2009)

4.1.6 Envolvimento de proteínas de membrana externa na ligação das leptospiros ao plasminogênio

É bem estabelecido que proteínas expostas na superfície celular sejam importantes para a patogênese, já que medeiam diretamente a interação patógeno-hospedeiro. Assim sendo, foi examinada a capacidade de ligação ao PLG de proteínas de superfície fracionadas a partir do tratamento das leptospiros com o detergente não iônico TX-114. Como esperado, a interação do PLG com as proteínas imobilizadas, avaliada por ELISA modificado, evidenciou o envolvimento de proteínas de membrana externa fracionadas na fase detergente na ligação (Figura 9). No entanto, surpreendentemente, os dados apontaram para a superior contribuição das proteínas citoplasmáticas solúveis na fase aquosa após a extração, em detrimento das proteínas de membrana (Figura 9).

Figura 9 – Ligação de PLG a proteínas citoplasmáticas e de membrana fracionadas de leptospiros

Proteínas de membrana (fase detergente) e citoplasmáticas (fase aquosa) de *L. interrogans* sorovar Copenhageni virulentas foram fracionadas por extração com TX-114. Proteínas correspondentes ao lisado total (LT), fase detergente (FD) e fase aquosa (FA) foram imobilizadas em micro placas e incubadas com PLG. A interação do PLG com as diferentes frações foi quantificada por anticorpos específicos anti-PLG humano. Os resultados são representativos de dois experimentos independentes de extração e ligação. As colunas representam as médias das absorbâncias a 492 nm $\pm$ os desvios padrões de quatro replicatas para cada condição amostral.

FONTE: Vieira (2012), (Vieira et al., 2009)

Apesar de se assumir que as proteínas de membrana sejam as mais importantes na interação patógeno-PLG, corroborando os resultados obtidos, algumas proteínas citoplasmáticas já foram reportadas como ligantes de PLG. Entre essas, figuram principalmente as proteínas chaperona DnaK, GAPDH (gliceraldeído 3-fosfato desidrogenase) e enolase de diversos micro-organismos (Bergmann et al., 2001, 2004; Candela et al., 2009; Egea et al., 2007; Floden et al., 2011; Knaust et al., 2007; Matta et al., 2010; Nogueira et al., 2010; Toledo et al., 2012). É importante ressaltar que essas proteínas tidas como essencialmente citoplasmáticas foram demonstradas como integrantes da membrana de bactérias ou extracelulares, indicando sua secreção (Knaust et al., 2007; Matta et al., 2010; Toledo et al., 2012). Desta forma, as bactérias patogênicas parecem exibir essas proteínas em algum momento por mecanismos ainda não totalmente compreendidos, o que possibilita a interação das mesmas com o PLG.

Além disso, dados da literatura relatam que as lipoproteínas de membrana externa LipL48 e LipL32 sofrem degradação durante o processo de extração por TX-114, provavelmente por ação de alguma protease endógena (Cullen et al., 2004). Também,

algumas proteínas de membrana, como proteínas ligantes de penicilina, não se solubilizam em TX-114 (Haake et al., 1991). Talvez esses comportamentos possam ser estendidos para outras lipoproteínas e proteínas de membrana, o que contribuiria para a presença de um número reduzido de proteínas intactas solúveis na fração detergente após a extração por TX-114. Esses fatos podem contribuir para as diferenças observadas na ligação de PLG às proteínas solúveis na fase detergente ou aquosa.

4.1.7 Ligação de PLG a diferentes espécies de *Leptospira*

Para avaliar se a ligação de PLG e ativação à PLA na superfície de *L. interrogans* sorovar Copenhageni é um fenômeno comum a outras espécies e sorovares de *Leptospira*, foram realizados experimentos utilizando diferentes cepas patogênicas não virulentas atenuadas em cultura (*L. interrogans* sorovares Canicola, Interohaemorrhagiae e Pomona, *L. borgpetersenii* sorovares Castellonis e Whitcombi, *L. santarosai* sorovar Shermani e *L. kirschneri* sorovares Cynoptery e Grippotyphosa) e uma cepa saprofítica (*L. biflexa* sorovar Patoc). *L. interrogans* sorovar Copenhageni não virulenta foi inserida no ensaio para fins comparativos. Da mesma forma como para *L. interrogans* sorovar Copenhageni, o fenômeno de ligação ao PLG foi observado para as diversas cepas não virulentas atenuadas em cultura das diferentes espécies testadas, e também para a espécie não patogênica saprofítica, *L. biflexa*, exibindo atividade enzimática após tratamento com ativador exógeno, uPA (Figura 10). Os dados mostram que este fenômeno é amplamente difundido no gênero *Leptospira*, a despeito da capacidade infectante. De fato, como a interação com o PLG não se dá por domínios de ligação específicos, e sim por resíduos de lisina expostos na estrutura tridimensional dos receptores (Figura 6), é presumível que muitas proteínas sejam capazes de se ligar ao PLG e, portanto, que seja um fenômeno não restrito a algumas espécies de leptospiros, apenas.

Figura 10 – Aquisição de PLG e atividade proteolítica por diferentes espécies e cepas de *Leptospira*

Bactérias patogênicas não virulentas atenuadas em cultura (*L. interrogans* sorovar Canicola, *L. interrogans* sorovar Copenhageni, *L. interrogans* sorovar Icterohaemorrhagiae, *L. interrogans* sorovar Pomona, *L. Borgpetersenii* sorovar Castellonis, *L. Borgpetersenii* sorovar Whitcombi, *L. santarosai* sorovar Shermani, *L. kirschneri* sorovar Cynoptery e *L. kirschneri* sorovar Grippotyphosa) e saprofíticas (*L. biflexa* sorovar Patoc) foram incubadas somente com **PBS** ou tratadas com: **uPA**, 5 µg PLG (**PLG**) ou 5 µg PLG com a adição de uPA (**plasmina**). As barras representam a média das absorbâncias como medida da degradação do substrato cromogênico S-2251 de três replicatas para cada grupo experimental. Os dados são representativos de dois experimentos independentes.

FONTE: Vieira (2012), (Vieira et al., 2009)

4.2 RECEPTORES DE PLG EM *LEPTOSPIRA*

4.2.1 Análise de receptores de PLG em *L. interrogans* virulentas e não virulentas

A análise inicial da presença de receptores de PLG em leptospiiras foi realizada por *immunoblotting* de afinidade utilizando-se extratos proteicos totais de *L. interrogans* sorovar Copenhageni virulentas e não virulentas separados por tamanho molecular em SDS-PAGE. Após eletro transferência para membranas e incubação com PLG purificado, a detecção de reatividade foi feita por anticorpos específicos. Foi possível observar múltiplas regiões reativas ligantes de PLG nas condições ensaiadas tanto em extratos de leptospiiras de cepa virulenta quanto não virulenta (Figura 11A). Entretanto, notou-se a existência de três regiões

proteicas com diferenças de reatividade entre a cepa virulenta e a não virulenta atenuada em cultura (setas). A especificidade da reação foi determinada por ensaio de competição com AAC, no qual as bandas reativas foram quase que totalmente abolidas quando da adição do competidor à incubação de PLG com os extratos proteicos bacterianos (Figura 11B), e corrobora os resultados já apresentados de inibição da atividade proteolítica por AAC (Figura 6).

Figura 11 – *Immunoblotting* de afinidade da ligação de PLG humano a proteínas imobilizadas de lisado total de *L. interrogans*

Extratos proteicos totais foram separados por SDS-PAGE 10% e eletro transferidos para membranas de nitrocelulose. A membrana foi incubada com PLG, e a ligação foi detectada por anticorpos anti-PLG humano e anticorpos conjugados com peroxidase (A). Em B, AAC foi adicionado à incubação com PLG. As bandas reativas foram reveladas por ECL (GE Healthcare) seguido de exposição a filmes de raio-X. **NV**: *L. interrogans* sorovar Copenhageni cepa M 20 não virulentas atenuadas em cultura; **Vir**: *L. interrogans* sorovar Copenhageni cepa Fiocruz L1-130 virulentas de poucas passagens em cultura. As setas indicam bandas de proteínas reativas presentes no extrato da cepa virulenta, porém ausente no da não virulenta. As posições do padrão de massa molecular estão indicadas à esquerda.

FONTE: Vieira (2012), (Vieira et al., 2009)

Embora os ensaios de *immunoblotting* de afinidade não simulem as condições de ligação do PLG às bactérias intactas no hospedeiro *in vivo*, sugerem uma alteração nos receptores de PLG durante o processo de atenuação das leptospiros por expressão de diferentes receptores. Esse resultado ainda corrobora os dados previamente demonstrados indicando maior habilidade de captura do PLG pelas bactérias virulentas do que pelas não virulentas (Figura 4), o que provavelmente se dá por supressão da expressão de fatores de virulência que atuem como receptores de PLG durante a atenuação em cultura.

4.2.2 Identificação de receptores de PLG em leptospiros por gel 2D

Como o *immunoblotting* de afinidade a partir de SDS-PAGE convencional apontou para a presença de inúmeras proteínas capazes de se ligarem ao PLG (Figura 11), realizou-se ensaios baseados na separação de extratos proteicos por técnica de gel 2D seguido de identificação dos *spots* reativos por espectrometria de massas na tentativa de identificação dos receptores de PLG. Proteínas de extratos proteicos totais de *L. interrogans* sorovar Pomona virulentas foram separadas por ponto isoelétrico e massa molecular. Após eletro transferência para membranas, seguiu-se o *immunoblotting* de afinidade. Comparando-se os géis corados com azul de coomassie (Figura 12A) com os *spots* reativos ao PLG (Figura 12B), foi possível indicar individualmente os *spots* de proteínas que interagem com PLG. Os *spots* foram recortados, descorados, digeridos com tripsina e submetidos à identificação por espectrometria de massas MALDI-TOF. Foram identificadas sete proteínas capazes de se ligar ao PLG (Tabela 2): LIC12816 (proteína hipotética), proteína flagelar FlaA-1 (LIC10788), citrato-liase putativa (LIC11194), fator de alongamento Tu (LIC12875), acetil-CoA acetiltransferase (LIC12795), glutamina sintase putativa (LIC12407) e proteína de choque térmico DnaK (LIC10524). Dessas, segundo os programas de predição de localização celular CELLO e LipoP (Juncker et al., 2003; Yu et al., 2004), duas são preditas proteínas de membrana (LIC12816 e LIC10788) e as cinco demais, citoplasmáticas.

Os dados obtidos por esta técnica corroboram os obtidos a partir do fracionamento de proteínas de membrana e citoplasmáticas de leptospiros por TX-114, no qual se observou, por ELISA, a capacidade de ligação de proteínas citoplasmáticas ao PLG (Figura 9). Dentre as proteínas identificadas, já foram reportadas as capacidades de interação ao PLG em homólogos de outras espécies, como DnaK de *Bifidobacterium animalis* (Candela et al., 2009) e fator de alongamento Tu de *Pseudomonas aeruginosa* (Kunert et al., 2007). São necessários estudos adicionais para a verificação das implicações na patogênese da ligação de proteínas

citoplasmáticas de leptospira ao PLG: como já foi demonstrado que diversas proteínas definidas como essencialmente citoplasmáticas podem ser secretadas ou expostas na membrana pelos micro-organismos em certos momentos, é necessário analisar a localização celular das proteínas classificadas como citoplasmáticas que foram identificadas como receptores de PLG em leptospiras (Knaust et al., 2007; Matta et al., 2010; Toledo et al., 2012).

4.2.3 Seleção, expressão e purificação de proteínas recombinantes

No laboratório de pesquisa em que se realizou o presente trabalho, desenvolve-se a clonagem, expressão, purificação e caracterização de proteínas de membrana de *L. interrogans*. Já que proteínas de superfície entram em contato direto com o hospedeiro e, por isso, são receptores em potencial para o PLG, como uma estratégia alternativa para a identificação de receptores de PLG em leptospiras, foram selecionadas quatorze proteínas recombinantes para ensaios de interação com PLG purificado *in vitro*. São elas: doze proteínas previamente publicadas, LipL32 (Branger et al., 2001; Haake et al., 2000), LipL40, MPL36 (Gamberini et al., 2005), rLIC10509 (Gomez et al., 2008), Lp29, Lp49 (Neves et al., 2007), Lsa27 (Longhi et al., 2009), MPL21, MPL17 (Oliveira et al., 2008), rLIC10494, rLIC12730 (Barbosa et al., 2006), e Lsa63 (Vieira et al., 2010b), e duas novas proteínas, rLIC12922 e rLIC12238. A tabela com os dados das principais características das proteínas selecionadas, bem como a conservação entre os genomas sequenciados disponíveis, encontra-se no apêndice.

Em colaboração com o grupo de pesquisa, as sequências escolhidas foram amplificadas excluindo-se os peptídeos sinais, clonadas em vetor de expressão em sistema heterólogo de *E. coli*, e purificadas por cromatografia de afinidade a níquel. Todas as proteínas recombinantes purificadas resultaram em bandas majoritárias por análise em SDS-PAGE, embora em alguns casos, como para Lsa27 e MPL21, bandas menos intensas de contaminantes puderam ser observadas (Figura 13). As proteínas recombinantes foram utilizadas para imunizar camundongos BALB/c, gerando-se soros hiperimunes contra cada proteína (dados não mostrados).

Figura 12 – *Immunoblotting* de afinidade ao PLG em gel bidimensional de extrato total de *L. interrogans* sorovar Pomona cepa LPF

Géis 2D em SDS-PAGE 12% foram corados com azul de coomassie (A) ou submetidos a *immunoblotting* de afinidade ao PLG (B). Os spots correspondentes reativos no *immunoblotting* estão numerados em A. A detecção foi realizada por reagente ECL e exposição a filmes sensíveis. Os resultados são representativos de três experimentos realizados em duplicata.
 FONTE: Vieira (2012)

Tabela 2 – Proteínas ligantes de PLG identificadas por espectrometria de massas MALDI-TOF a partir de *immunoblotting* de proteínas fracionadas de *L. interrogans* sorovar Pomona cepa LPF

LIC identificada	MW	pI	Gene/descrição da proteína
LIC10524	69116	5,11	dnaK (Proteína de choque térmico DnaK)
LIC10788	35041	7,63	flaA-1 (Proteína da bainha do filamento flagelar)
LIC11194	37212	6,07	Citrato-liase putativa
LIC12407	55421	5,9	Glutamina-sintase putativa
LIC12795	47482	6,0	Acetil-CoA acetiltransferase
LIC12816	14751	9,47	Proteína hipotética
LIC12875	43662	5,74	tuf (Fator de alongamento Tu)

Estão listadas as proteínas identificadas de acordo com a nomenclatura genômica (LIC), bem como suas respectivas massas moleculares (MM), ponto isoeletrico teórico (pI) e nome/função da proteína.

FONTE: Vieira (2012)

Figura 13 – Análise por SDS-PAGE 12% da pureza das proteínas recombinantes selecionadas para ligação com PLG

As proteínas recombinantes foram expressas em *E. coli* e purificadas através de cromatografia de afinidade a metal, utilizando-se colunas de Sepharose carregadas com Ni^{2+} . M: marcador de massa molecular expresso em kDa.

FONTE: Vieira (2012), (Vieira et al., 2010a)

4.2.4 Ligação de proteínas recombinantes ao PLG

As proteínas recombinantes purificadas foram separadas por tamanho molecular em SDS-PAGE, eletro transferidas para membrana, e a ligação ao PLG foi avaliada por ensaios de *immunoblotting* de afinidade. As proteínas LipL32, Lp29, Lp49, LipL40, MLP36, rLIC10494, rLIC12730 e rLIC12238 mostraram-se reativas ao PLG, enquanto que não foi observada ligação para Lsa63, Lsa27, rLIC10509, MPL21, MPL17 e rLIC12922. Ressalta-se que as bandas reativas apresentam a massa molecular esperada para as proteínas recombinantes correspondentes (Figura 14A), refletindo a especificidade da reação.

A interação das proteínas recombinantes com o PLG foi adicionalmente avaliada por ELISA. As quatorze proteínas selecionadas e BSA, como controle negativo, foram imobilizados em placas de 96 poços, incubados com PLG humano purificado, e a interação detectada através de anticorpos específicos. Os resultados indicam que LipL32, Lp29, Lp49, LipL40, MLP36, rLIC10494, rLIC12730 e rLIC12238 apresentaram ligação estatisticamente significativa ao PLG, ao contrário de Lsa63, Lsa27, rLIC10509, MPL21, MPL17, rLIC12922 e BSA, que não geraram interação significativa (Figura 14B).

Os dados obtidos através de ambos os métodos foram consistentes, exceto por rLIC12730 e rLIC12238, que demonstraram discrepância nas intensidades de interação quando comparadas as duas metodologias. rLIC12730 apresentou maior intensidade de ligação por *immunoblotting* quando comparado ao ELISA, e rLIC12238 teve comportamento inverso (Figura 14). Esta discrepância pode se dar ao fato de o ELISA ser um método quantitativo, enquanto *immunoblotting*, qualitativo. Além disso, devido às condições desnaturantes do último, é possível que a exposição de certos epítomos tenha influenciado na ligação de rLIC12730 ao PLG, enquanto que o inverso tenha ocorrido para rLIC12238, que aparentemente necessita de uma conformação secundária para a interação.

Dentre as proteínas ligantes de PLG em estudo, Lp29, Lp49, LipL40 e MPL36 já foram publicadas como sendo reativas com soro de pacientes diagnosticados com leptospirose (Gamberini et al., 2005; Neves et al., 2007), enquanto que rLIC12238 e rLIC10494 são reativas com soro de hamsters experimentalmente infectados com *Leptospira* (Atzingen et al., 2010b; Oliveira et al., 2011). Estas informações sugerem que essas proteínas sejam expressas e expostas na superfície bacteriana ao longo da infecção, gerando resposta imune. Como essas proteínas são capazes de interagir com PLG humano, os dados insinuam que sejam possíveis fatores de virulência de leptospirosas.

A proteína LipL32 é o antígeno majoritário expresso pelas leptospirosas durante a infecção (Haake et al., 2000; Hoke et al., 2008; Malmstrom et al., 2009). Já foi demonstrado que LipL32 é reconhecida por soro de pacientes com leptospirose (Flannery et al., 2001; Hauk et al., 2008), e que tem a capacidade de se ligar a componentes de MEC (Hauk et al., 2008; Hoke et al., 2008). Estes dados sugerem que essa proteína tenha algum papel na patogênese dessas bactérias. No entanto, curiosamente, também foi demonstrado através de mutantes, que LipL32 não é essencial para a infecção aguda ou crônica (Murray et al., 2009c). Neste trabalho, foi proposta uma nova atividade para LipL32, como ligante de PLG, adicionando evidências para sua provável importância durante a infecção.

4.2.5 Papel dos resíduos de lisina na interação receptores-PLG

Como mostrado anteriormente pela competição por AAC na ligação de PLG às leptospirosas vivas e aos extratos proteicos totais, os domínios *kringle* do PLG medeiam as interações com os resíduos de lisina dos receptores (Figuras 6 e 11). Assim, a importância dos resíduos de lisina na ligação ao PLG foi igualmente avaliada para as proteínas recombinantes. Quando AAC foi adicionado, a interação das proteínas com PLG foi quase que totalmente abolida (Figura 15). Apesar disso, analisando-se as sequências primárias das proteínas, verificou-se que não há correlação entre o número de resíduos de lisina presentes nas proteínas e a intensidade da ligação ao PLG. As proteínas LipL32, Lp29, Lp49, LipL40, MPL36, LIC10494, LIC12238 e LIC12730, contêm 9,6%, 9,3%, 10,7%, 7,8%, 10,6%, 9,1%, 8,8% e 5,5% resíduos de lisina, respectivamente. A maior porcentagem de resíduos de lisina foi identificada na sequência da proteína Lsa63 (Vieira et al., 2010b), 16,2%, que não apresentou ligação ao PLG. Além disso, a posição dos resíduos de lisina na estrutura primária da proteína parece não se correlacionar com a capacidade de adesão, já que estão distribuídos ao longo de toda a extensão das proteínas em estudo, e não em um domínio ou região específica. Isso sugere que a conformação ou impedimento estérico possam ser fatores determinantes na ligação das proteínas ao PLG.

Figura 14 – Ligação de proteínas recombinantes purificadas ao PLG humano

(A) *Immunoblotting* de afinidade. As proteínas recombinantes foram separadas por SDS-PAGE 12% e transferidas para membrana de nitrocelulose, seguido por incubação com 3 µg/mL PLG e anticorpos específicos. As posições do padrão de massa molecular estão indicadas à esquerda, expressas em kDa. (B) ELISA de ligação das proteínas recombinantes ao PLG. Após imobilização em placas de ELISA (10 µg/mL), as proteínas recombinantes foram incubadas com PLG humano purificado (10 µg/mL). BSA foi utilizado como controle de ligação inespecífica (negativo). A ligação foi quantificada por anticorpos anti-PLG seguido de anticorpos secundários. As barras representam as médias das absorbâncias a 492 nm ± desvio padrão de quatro replicatas para cada proteína e são representativos de três experimentos independentes. * $P < 0,001$ e ** $P < 0,0001$.

FONTE: Vieira (2012), (Vieira et al., 2010a)

Figura 15 – Papel dos resíduos de lisina na interação PLG-proteínas recombinantes

A interação das proteínas recombinantes (10 µg/mL) ao PLG (10 µg/mL) foi avaliada por ELISA na presença do análogo de lisina (**PLG + AAC**) ou ausência (**PLG**). BSA foi utilizado como controle negativo. A ligação de PLG foi determinada por reação com anticorpos anti-PLG seguido de anticorpos conjugados à peroxidase. As barras representam a média das absorbâncias obtidas por leitura a 492 nm $\pm$ desvio padrão de quatro replicatas para cada grupo amostral. Os dados são representativos de dois experimentos independentes. Diminuições estatisticamente significativas da ligação do PLG quando da adição de AAC estão evidenciadas: * ($P < 0,001$) e ** ($P < 0,0001$).

FONTE: Vieira (2012), (Vieira et al., 2010a)

4.2.6 Atividade enzimática da plasmina associada às proteínas recombinantes

Foi demonstrado que o PLG ligado à superfície de *L. interrogans* é convertido à PLA enzimaticamente ativa após adição de ativador (Figura 4). Ensaios de ELISA modificado foram realizados a fim de determinar se o PLG ligado às proteínas recombinantes em estudo pode adquirir atividade proteolítica. As proteínas foram imobilizadas em placas de micro diluição e, após bloqueio e incubação com PLG, foi adicionado o ativador (uPA) juntamente com o substrato S-2251. A reação se deu por aproximadamente 16 h, e a atividade da PLA foi quantificada pela eficiência de clivagem do substrato. Observou-se que o PLG capturado pelas proteínas foi convertido à PLA, indicado indiretamente pela atividade proteolítica específica. Não foi observada atividade proteolítica para o controle negativo de ligação ao PLG (BSA), assim como para os controles em que não foi adicionado PLG, uPA ou o substrato cromogênico (Figura 16).

Figura 16 – Atividade enzimática da plasmina ligada às proteínas recombinantes

A clivagem de substrato S-2251 específico para PLA foi determinada por ELISA modificado. As proteínas imobilizadas receberam os tratamentos: PLG + uPA + substrato (**PLG + uPA + S**), ou controles com a ausência de um dos três componentes (**PLG + uPA**; **PLG + S**; **uPA + S**). BSA foi empregado como controle negativo. As barras representam as médias das absorvâncias a 405 nm como medida de clivagem do substrato $\pm$ desvio padrão de quatro replicatas para cada grupo, e são representativas de dois experimentos independentes. Diferenças estatisticamente significativas estão evidenciadas por * $P < 0,00001$, ** $P < 0,005$ e *** $P < 0,05$.

FONTE: Vieira (2012), (Vieira et al., 2010a)

4.2.7 Caracterização da ligação das proteínas recombinantes ao PLG

A especificidade e afinidade da interação entre as proteínas recombinantes e o PLG foram caracterizadas por curvas de dose-dependência. Ligações dose-dependentes e saturáveis foram observadas quando concentrações crescentes (0 a 1.000 nM) das proteínas recombinantes MPL36, LipL40, LipL32, rLIC10494 e rLIC12238 foram submetidas à interação com uma quantidade pré-fixada de PLG. O equilíbrio da reação não foi atingido para as proteínas Lp29, Lp49 a rLIC12730, mesmo na maior concentração testada (1.000 nM) (Figura 17). Com base nos dados de saturação por ELISA, foram calculadas as constantes de dissociação em equilíbrio (K_D) (Tabela 3). Para as proteínas que atingiram o equilíbrio, o maior e menor K_D observados foram, respectivamente, $25,87 \pm 5,09$ nM para LipL32 e $3,52 \pm 3,95$ nM para MPL36.

Figura 17 – Caracterização da ligação de proteínas recombinantes ao PLG em função da concentração

As proteínas recombinantes foram adicionadas em concentração crescente (0 a 1.000 nM) ao PLG previamente imobilizado (10 µg/mL) em placas de ELISA. As ligações foram detectadas e quantificadas através de anticorpos policlonais produzidos em camundongos nas diluições de 1: 4.000 para LipL32, 1: 5.000 para rLIC12238, LipL40 e MPL36, 1: 1.000 para Lp29 e Lp49, e 1: 500 para rLIC12730, seguido de anticorpos secundários conjugados com peroxidase. Os dados representam as médias das absorbâncias de cada condição $\pm$ desvio padrão de seis replicatas. Os resultados são representativos de dois experimentos independentes.

FONTE: Vieira (2012), (Vieira et al., 2010a)

Tabela 3 – Constantes de dissociação da ligação das proteínas recombinantes de leptospira ao PLG

Proteína recombinante	Constante de dissociação (K_D)
LipL32	$25,87 \pm 5,09$ nM
rLIC12238	$11,97 \pm 1,06$ nM
LipL40	$18,24 \pm 2,45$ nM
rLIC10494	$10,98 \pm 2,33$ nM
MPL36	$3,52 \pm 3,95$ nM
Lp49	ND
Lp29	ND
rLIC12730	ND

As constantes de dissociação (K_D) das proteínas que atingiram o equilíbrio até a concentração de 1.000 nM foram calculadas com base nos dados de ELISA, conforme descrito na seção 3.24. ND: não determinado.

FONTE: Vieira (2012), (Vieira et al., 2010a)

4.2.8 Efeito da adição de heparina e NaCl à ligação de LipL32 e rLIC12238 ao PLG

Como as proteínas LipL32 e rLIC12238 apresentaram os valores mais elevados de atividade da PLA associada (Figura 16), foram selecionadas para estudos adicionais de caracterização desta interação. Para verificar se a ligação das proteínas recombinantes ao PLG sofre interferência de íons Cl^- ou heparina, foram realizados ensaios na presença de diferentes concentrações desses componentes. A adição de NaCl não interferiu na interação de LipL32 ou rLIC12238 ao PLG em osmolaridade fisiológica ou inferior, somente causando um efeito positivo quando em altas concentrações (350 mM) (Figura 18A). Similarmente, a adição de heparina (até 500 IU) não resultou em interferência significativa na interação das proteínas com o PLG (Figura 18B).

Figura 18 – Efeitos da variação de osmolaridade e presença de heparina na ligação de LipL32 e rLIC12238 ao PLG

A ligação de LipL32 e rLIC12238 (10 µg/mL) ao PLG imobilizado (10 µg/mL) foi realizada na presença de concentrações crescentes de NaCl (0 a 650 mM) (**A**) ou heparina (0 a 500 IU) (**B**). A interação das proteínas recombinantes foi detectada e quantificada por anticorpos específicos anti-proteína recombinante e anticorpos secundários. As barras representam as médias das absorbâncias obtidas por leitura a 492 nm $\pm$ desvio padrão de quatro replicatas para cada grupo experimental e são representativas de dois experimentos independentes. * $P < 0,05$.

FONTE: Vieira (2012), (Vieira et al., 2010a)

Foi demonstrado que a interação e ativação de PLG são suprimidas pela presença de ânions Cl⁻ (Urano et al., 1987), enquanto que evidências sugerem que derivados de heparina dextran podem contribuir para a regulação da atividade da PLA pela inibição da ativação ao interagir com o zimógeno PLG e também por afetar diretamente a atividade catalítica da PLA (Ledoux et al., 2000). No entanto, os resultados obtidos não sugerem interferência fisiologicamente significativa de NaCl ou heparina na ligação e ativação de PLG associada às proteínas LipL32 ou LIC12238 (Figura 12). Resultados semelhantes foram obtidos por Brissette et al. (2009) ao estudar o efeito de ambos os componentes na interação entre a proteína ErpP recombinante de *B. burgdorferi* e PLG. Verma et al. (2010) demonstraram que heparina não teve efeito na ligação LenA-PLG, enquanto que adição de sal em altas concentrações levou a um aumento na interação.

4.2.9 Competição entre PLG e componentes de matriz extracelular na ligação à LipL32

Foi previamente demonstrado que a proteína LipL32 é capaz de se ligar aos componentes de MEC colágeno tipo IV e fibronectina (Hauk et al., 2008; Hoke et al., 2008). Foram realizados ensaios na presença de concentrações crescentes desses componentes para verificar se a interação do PLG com LipL32 compete pelos mesmos sítios de ligação. Os resultados indicam que a fibronectina plasmática não interfere na ligação de PLG à LipL32. No entanto, a adição de colágeno tipo IV diminuiu a ligação de PLG à LipL32 de maneira dose-dependente, sugerindo que ambas as moléculas competem pelo mesmo sítio de ligação ou ocasionam algum tipo de influência estrutural que interfira na interação da outra molécula (Figura 19).

A proteína LipL32 não é definida como um fator de virulência clássico, já que a sua deleção não afeta a capacidade infectante (Murray et al., 2009c). No entanto, esta versátil proteína abundantemente expressa na membrana de leptospiros patogênicas parece desempenhar papéis importantes para o início da infecção, ao que é capaz de interagir com MEC e PLG concomitantemente.

Figura 19 – Efeito de componentes de matriz extracelular na ligação de PLG à proteína LipL32

A interação PLG (10 µg/mL) com LipL32 imobilizada (10 µg/mL) foi realizada com a adição de concentrações crescentes de fibronectina plasmática ou colágeno tipo IV (0 a 600 mM). A detecção do PLG ligado à proteína recombinante foi feita através de anticorpos anti-PLG seguido de anticorpos secundários conjugados à peroxidase. As barras representam valores das médias das absorbâncias $\pm$ desvio padrão de quatro replicatas para cada condição e são representativos de dois experimentos independentes. Estão evidenciados resultados de interferência estatisticamente significativa em comparação ao controle positivo (sem adição de componentes de matriz extracelular): $P < 0,0003$ (*).

FONTE: Vieira (2012), (Vieira et al., 2010a)

4.3 IMPLICAÇÕES PARA A INFECÇÃO DA LIGAÇÃO DE PLG ÀS LEPTOSPIRAS

4.3.1 Evasão Imune

Do ponto de vista de uma bactéria, a inibição da opsonização por anticorpos ou proteínas do SC parece ser uma estratégia interessante para driblar a primeira linha de defesa do sistema imune humano após a infecção. Considerando os mecanismos enzimáticos de evasão, é conhecido que patógenos utilizam-se de proteases do hospedeiro, como a PLA, para escapar da opsonização. De fato, a literatura reporta que a PLA é capaz de degradar IgG humano (Harpel et al., 1989; Stanworth e Turner, 1986) e C3b, o componente central do SC (Seya et al., 1985). Assim, a formação de PLA na superfície das leptospiiras poderia conferir a essas bactérias a capacidade de modular a resposta imune inata do hospedeiro via atividade proteolítica da PLA, aumentando assim a sobrevivência bacteriana durante a infecção.

Fundamentando-se nessas afirmações, foi avaliado o efeito da PLA associada às leptospiiras na interação com componentes da imunidade inata, IgG e C3b.

4.3.1.1 Plasmina diminui deposição de IgG humano e C3b a *L. interrogans*

L. interrogans sorovar Pomona foram imobilizadas em placas de micro diluição e incubadas com soro humano de doadores saudáveis como fonte de C3b e IgG inespecífico. As bactérias foram tratadas com PLG e uPA, e as moléculas associadas às bactérias foram detectadas e quantificadas através de anticorpos anti-C3b e anti-IgG. O tratamento das leptospiiras com PLG e uPA, gerando PLA, resultou na diminuição da deposição de IgG e C3b na superfície dessas bactérias. PLG e uPA sozinhos não tiveram efeito nas deposições de IgG ou C3b (Figura 20).

Figura 20 – Efeito da plasmina na deposição de IgG e C3b na superfície de *L. interrogans*

Leptospiiras virulentas foram imobilizadas (25×10^6 leptospiiras/poço) e, a partir da incubação com soro humano normal como fonte de IgG (A) ou C3b (B), mediu-se as deposições desses componentes por ELISA. As bactérias foram tratadas somente com **PBS**, ou com adição de 40 $\mu\text{g/mL}$ **PLG**, 5 $\mu\text{g/mL}$ **uPA** ou PLG + uPA (**PLA**). As barras representam as médias das absorbâncias medidas a 492 nm $\pm$ desvio padrão de três replicatas para cada grupo experimental. Os dados são representativos de dois experimentos independentes. * $P < 0,001$.

FONTE: Vieira (2012), (Vieira et al., 2011)

Para averiguar se a atividade de degradação de IgG inespecífico (soros de doadores saudáveis) pela PLA também é observada para IgG imune proveniente de soros de pacientes diagnosticados com leptospirose, o mesmo experimento apresentado anteriormente foi conduzido utilizando-se soros MAT negativos (fase pré-imune da doença) e MAT positivos (fase imune convalescente). Como esperado, a incubação com *pool* de soros MAT positivos resultou em maior deposição de IgG na superfície bacteriana quando comparado com soros

MAT negativos ou de indivíduos saudáveis. Além disso, observa-se que a PLA foi capaz de diminuir significativamente a deposição de IgG específico (Figura 21), tendo o mesmo efeito observado quando utilizado soro de indivíduos não imunes (saudáveis).

Figura 21 – Efeito da plasmina na ligação de IgG de soros de pacientes diagnosticados com leptospirose a *L. interrogans*

Bactérias foram imobilizadas em placas de ELISA e incubadas com soro humano como fonte de IgG. A ligação de IgG às bactérias foi quantificada por reação com anticorpos anti-região Fc seguido de anticorpos secundários conjugados à peroxidase. As leptospirosas foram tratadas com **PBS**, 40 µg/mL **PLG**, 5 µg/mL **uPA** ou PLG + uPA (**PLA**). **Normal**: pool de soros de doadores saudáveis; **MAT-**: pool de soros de pacientes diagnosticados com leptospirose, porém negativos para MAT; **MAT+**: pool de soros de pacientes diagnosticados com leptospirose e com MAT positivo. As barras representam as médias das absorbâncias medidas a 492 nm ± desvio padrão de três replicatas para cada grupo experimental. O experimento foi realizado duas vezes com resultados semelhantes.

FONTE: Vieira (2012), (Vieira et al., 2011)

Pela atividade proteolítica de grande espectro da PLA, supõe-se que a diminuição dos agentes opsonizantes IgG e C3b na superfície das leptospirosas associadas à PLA ocorra por atividade de degradação desta enzima. Estudos prévios demonstraram que a opsonização por anticorpos específicos é essencial para que ocorra a fagocitose das leptospirosas por macrófagos durante a infecção (Banfi et al., 1982; Wang et al., 1984). Assim, a diminuição da deposição de IgG inespecífico ou específico para leptospirose pode constituir um eficiente e importante mecanismo auxiliando na evasão da fagocitose por essas bactérias. Ainda, a diminuição da deposição de C3b pode interferir na ativação da cascata do SC, somando efeito de inibição

desse sistema às atividades de ligação das leptospiplas aos reguladores do SC (FH, FHL-1 e C4BP) como previamente reportado por outros grupos (Barbosa et al., 2009; Meri et al., 2005). Com base nos resultados obtidos e nas estratégias de evasão imune já descritas na literatura, parece certo afirmar que as leptospiplas dispõem de múltiplos mecanismos de escape ao sistema imune do hospedeiro que, em conjunto, refletem na capacidade infectante dessas bactérias.

4.3.1.2 C3b humano é degradado pela PLA associada às leptospiplas

Como se observou que a PLA é capaz de diminuir a deposição de C3b na superfície das leptospiplas (Figura 20B), foram realizados experimentos para confirmar a hipótese de que isso ocorra via degradação. *L. interrogans* sorovar Pomona foram tratadas com PLG e uPA, adquirindo PLA enzimaticamente ativa em sua superfície, e então incubadas com C3b humano em fase fluida. Após incubação, a análise por *western blotting* do sobrenadante resultante permitiu a visualização de duas bandas de degradação, com concomitante diminuição nas massas das cadeias alfa (105 kDa) e beta (75 kDa) do C3b, o que ocorreu somente na amostra tratada com PLA (Figura 22 A e B). A clivagem foi tempo-dependente, com aparente total degradação das cadeias alfa e beta após 20 h de degradação. Não foi observada clivagem do C3b nas amostras tratadas somente com PLG, uPA ou não tratadas (PBS). Quando da adição de aprotinina, um inibidor de serino-proteases, às leptospiplas associadas à PLA, não ocorreu a formação de produtos de degradação de C3b (Figura 22B). Os dados confirmam que a diminuição da deposição de C3b na superfície das leptospiplas associadas à PLA ocorre devido à atividade proteolítica dessa enzima, via degradação.

4.3.1.3 Leptospiplas ligam-se independentemente à PLA e FH

Meri et al. (2005) reportaram a ligação de leptospiplas ao regulador do complemento FH. A fim de analisar se o FH compete pelos sítios de ligação do PLG ou interfere de alguma maneira na interação do PLG com as leptospiplas, foi realizado um ensaio de competição. Leptospiplas foram tratadas com PLG e uPA na presença ou ausência de FH, ou somente com FH. Após, as bactérias foram incubadas com substrato cromogênico para PLA, S-2251, e a atividade enzimática foi avaliada. O FH parece não exercer influência na ligação e ativação do PLG ou na atividade enzimática da PLA associadas às leptospiplas, já que não houve diferença na eficiência de clivagem do substrato entre as amostras tratadas na ausência ou presença de

concentrações crescentes de FH. As bactérias tratadas somente com FH exibiram a mesma absorbância de fundo para degradação de substrato do que o controle negativo, PBS (Figura 23).

Figura 22 – Degradação de C3b humano por plasmina associada à superfície de *L. interrogans*

Leptospiras virulentas (10^8 por amostra) foram tratadas com 10 μ g PLG e 3 U uPA (**Plasmina**), somente com **PLG** ou **uPA**, ou sem adições (**PBS**). As células foram incubadas com 15 μ g/mL de C3b humano purificado e os sobrenadantes separados por SDS-PAGE e transferidos para membranas de nitrocelulose. Como controle, amostras tratadas com PLG+uPA foram incubadas com C3b juntamente com o inibidor **Aprotinina**. As subunidades de C3b e seus produtos de degradação foram detectados por anticorpos específicos. A reação foi revelada por precipitação de DAB. Estão indicadas as massas moleculares das cadeias alfa (105 kDa) e beta (75 kDa) do C3b, bem como os produtos de degradação mais evidentes (setas).

FONTE: Vieira (2012), (Vieira et al., 2011)

Figura 23 – Influência de FH na ligação de PLG e atividade enzimática da plasmina associada às leptospiiras

L. interrogans sorovar Pomona (10^9 /amostra) foram tratadas com 5 µg PLG e 3U uPA (**PLA**), com adição massas crescentes de **FH** (1, 5 ou 10 µg). Como controles, amostras foram tratadas somente com 10 µg FH ou sem adições (**PBS**). As amostras foram incubadas com substrato cromogênico para plasmina, S-2251, sendo a atividade enzimática determinada como medida de clivagem do substrato. As barras representam as absorbâncias médias obtidas a 405 nm $\pm$ desvio padrão de três replicatas para cada grupo experimental. Os dados são representativos de dois experimentos independentes.
 FONTE: Vieira (2012), (Vieira et al., 2011)

O teste inverso também foi realizado, para a verificação da interferência da PLA enzimaticamente ativa na ligação do FH às bactérias. As bactérias foram tratadas com FH após associação com PLA ou somente tratamento com PBS. Não se observou mudança significativa na ligação de FH às leptospiiras nas duas condições (Figura 24), indicando que a PLA não influencia na ligação de FH, aparentemente não exercendo atividade de degradação deste componente.

Adicionalmente, foi verificada a capacidade das leptospiiras de capturarem PLG e FH concomitante e diretamente do soro humano. Leptospiiras foram incubadas com *pool* de soros humanos de indivíduos saudáveis, e a ligação ao PLG e FH foi quantificada utilizando-se anticorpos específicos. Os dados apresentados na Figura 25 sugerem que essas bactérias podem interagir concomitantemente com PLG e FH quando em contato com soro humano, o que pode ter grande implicação na evasão imune por essas bactérias.

Esses resultados adicionam evidências de que a evasão imune por interceptação de vias que influenciam o SC e fagocitose seja importante para a sobrevivência das leptospiiras

no início da infecção, sendo que essas bactérias empregam várias estratégias ao mesmo tempo para suprimir a atividade imune inata do hospedeiro.

Figura 24 – Influência de plasmina enzimaticamente ativa associada à superfície de leptospiros na ligação de FH

Leptospiros foram incubadas com PLG, uPA e FH (PLA+FH), ou somente com FH. A detecção da ligação do FH às bactérias foi realizada por ELISA, utilizando-se anticorpos monoclonais anti-FH, seguido de anticorpos secundários. Os dados estão expressos em percentagem de ligação de FH, sendo a amostra incubada na ausência de PLA considerada 100% de ligação. As barras representam as absorbâncias médias obtidas a 492 nm $\pm$ desvio padrão de três replicatas para cada grupo experimental. Os dados são representativos de dois experimentos independentes.

FONTE: Vieira (2012), (Vieira et al., 2011)

Figura 25 – Ligação de leptospiplas ao PLG e FH presentes no soro humano

L. interrogans virulentas foram incubadas com *pool* de soros humanos de doadores saudáveis, e a ligação de PLG e FH foi medida por incubação com anticorpos anti-FH ou anti-PLG humanos. Como controle negativo, bactérias foram tratadas somente com IsPBS, sendo utilizadas como absorbância de fundo do experimento. As barras representam as médias das absorbâncias a 492 nm, como medida da ligação de PLG ou FH, $\pm$ desvio padrão de oito replicatas para cada grupo experimental. O experimento foi conduzido duas vezes com resultados semelhantes.

FONTE: Vieira (2012), (Vieira et al., 2011)

4.3.1.4 PLA confere proteção contra a morte pelo complemento

L. interrogans sorovar Pomona foram descritas como parcialmente susceptíveis à morte pelo SC após incubação com soros humanos de indivíduos saudáveis (Meri et al., 2005). Como a PLA é capaz de diminuir a deposição de C3b na superfície das leptospiplas por degradação (Figuras 20 e 22), foi realizado um ensaio para verificar a consequência efetiva dessa propriedade na proteção contra morte pelo SC *in vitro*. *L. interrogans* sorovar Pomona virulentas foram tratadas com PLG e uPA (gerando PLA) ou somente com PBS, como controle negativo, e incubadas com soro humano de doadores saudáveis como fonte de componentes totais do SC. Após quatro dias de crescimento, as bactérias foram contadas em microscópio de campo escuro como forma de medida de sobrevivência ao ataque do SC presente no soro. O número de leptospiplas não tratadas foi aproximadamente quatro vezes menor que o de bactérias previamente ligadas à PLA ($0,35 \times 10^8 \pm 0,1 \times 10^8$ versus $1,28 \times 10^8 \pm 0,3 \times 10^8$ leptospiplas/mL). Esse resultado implica que a degradação de C3b por PLA acarreta na supressão da ativação da cascata do complemento, de fato protegendo as bactérias dos efeitos desse sistema.

Em resumo, conjuntamente, os dados mostram que a geração de PLA na superfície das leptospiros protege contra fagocitose e morte pelo SC. Até o momento, poucos estudos mostraram diretamente a degradação de anticorpos específicos ou opsoninas pela PLA e a consequência desta atividade na infecção (Crane et al., 2009; Rooijakkers et al., 2005). Considerando-se que já foi demonstrado que um grande número de patógenos interage com PLG e PLA, parece razoável especular que a estratégia de degradação de IgG e C3b via PLA possa ocorrer para diversos micro-organismos.

4.3.2 Penetração

Acredita-se que uma característica interessante das bactérias patogênicas seja a interação simultânea com componentes de MEC e PLG (Lahteenmaki et al., 2001), o que resultaria em direcionamento dos patógenos para a MEC ou MB do hospedeiro pelas adesinas, para que ocorra a desestruturação desses sítios pela PLA imobilizada. Isto facilitaria a penetração nos tecidos do hospedeiro e colonização de locais secundários de infecção. Como as leptospiros interagem tanto com componentes de MEC quanto PLG, este poderia ser um importante mecanismo de patogenicidade dessas bactérias, o que motivou a análise da capacidade de penetração por efeito da PLA adquirida.

4.3.2.1 *L. interrogans* complexadas à plasmina degradam componentes de matriz extracelular

Para avaliar a capacidade proteolítica de leptospiros ligadas a PLA na degradação de proteínas purificadas de MEC, foram empregadas fibronectina celular e laminina de origem humana como substratos. A degradação foi avaliada por meio da exposição dos componentes de MEC imobilizados às leptospiros virulentas previamente tratadas com PLG e uPA. A proteólise dos substratos foi quantificada por intermédio de anticorpos específicos: a degradação foi calculada com base na diminuição da absorbância em comparação com o controle positivo sem adição de bactérias (0% de degradação). As leptospiros complexadas com PLA foram capazes de degradar significativamente a fibronectina e laminina imobilizadas, quando comparado com bactérias não tratadas (Figura 26).

Dado que, até o momento, não são conhecidas proteases de leptospiros com a capacidade de degradação de componentes de MEC, a aquisição de PLA por contato com componentes do sistema fibrinolítico disponíveis na circulação do hospedeiro parece ser o único mecanismo descrito que possibilitaria a penetração ativa por essas bactérias, permitindo

a transposição das barreiras físicas impostas por MEC e MB. A propriedade de degradação de MEC por PLA associada à membrana já foi relatada para outros micro-organismos ligantes de PLG, sugerindo que deva ser uma vantagem comum conferida pela geração de PLA (Bergmann et al., 2005; Coleman et al., 1999; Virkola et al., 1996; Xolalpa et al., 2007).

Figura 26 – Degradação de componentes de matriz extracelular a por leptospiros com plasmina associada a sua superfície

L. interrogans virulentas foram incubadas somente com IsPBS (não tratadas, **NT**) ou com adição de PLG e uPA (**plasmina**). Placas de ELISA foram adsorvidas com fibronectina ou laminina humanas purificadas como substrato, e posteriormente incubadas por 20 h com as espiroquetas. Os substratos foram detectados por anticorpos específicos anti-fibronectina e anti-laminina, sendo que a degradação do substrato foi interpretada como redução nos valores de absorbância. A porcentagem de degradação foi calculada conforme descrito na seção 3.33. As barras representam a média percentual de degradação dos substratos em relação ao controle positivo (0% degradação) $\pm$ o desvio padrão de três replicatas. Foram realizados três experimentos independentes com resultados similares. * $P < 0,001$ e ** $P < 0,01$.

FONTE: Vieira (2012), (Vieira et al., 2009)

4.3.2.2 Transmigração de leptospiros através de monocamadas de HUVECs

Dado que as leptospiros ligadas à PLA degradam componentes de MEC (Figura 26), utilizou-se modelo de penetração *in vitro* através de monocamadas de células endoteliais para testar se esta propriedade poderia facilitar a invasão de barreiras mecânicas. Foram empregadas culturas primárias de HUVECs coletadas de cordões umbilicais humanos de

doadoras no pós-parto. *L. interrogans* sorovar Pomona cepa LPF virulentas, de primeira passagem em cultura, foram utilizadas para infectar as HUVECs em câmaras *transwell* de transmigração. Previamente à infecção das células, as leptospiros foram tratadas com PLG, PLG e uPA (PLA), ou sem adições (PBS). Após intervalo de contato das leptospiros com as HUVECs, os sobrenadantes dos compartimentos inferiores das câmaras de transmigração foram coletados, e as leptospiros que migraram através da monocamada celular foram contadas. As leptospiros migraram mais eficientemente quando tratadas com PLG ou com PLA, do que na ausência desses componentes (PBS). Adicionalmente, leptospiros com PLA em sua superfície resultaram em contagem significativamente maior na câmara inferior do que as tratadas apenas com PLG (Figura 27).

L. interrogans é um patógeno altamente invasivo, que promove rápida infecção sistêmica. A habilidade dessas espiroquetas de penetração através de monocamadas endoteliais parece estar associada à patogenicidade, já que estudos reportaram translocação mais rápida por cepas patogênicas do que por não patogênicas (Barocchi et al., 2002). Porém, os mecanismos envolvidos nesta atividade permanecem não compreendidos. Os resultados aqui apresentados indicam que a aquisição de PLG ou PLA na superfície das leptospiros facilita a penetração endotelial por essas bactérias. O efeito de facilitação de transposição da barreira endotelial provavelmente se dá a partir da atividade proteolítica da PLA ligada às bactérias, promovendo a desestruturação da monocamada celular pela degradação da MEC circundante. Em concordância com os resultados apresentados por Barocchi et al. (2002), como leptospiros patogênicos possuem maior capacidade de ligação ao PLG, isso pode refletir em maior habilidade de penetração. A ação da PLA na facilitação da penetração por monocamadas endoteliais ou invasão celular já foi demonstrada para outros micro-organismos patogênicos capazes de se ligarem ao PLG e à PLA (Coleman et al., 1995; Siemens et al., 2011; Stie et al., 2009).

Figura 27 – Influência da plasmina na migração de leptospiros virulentas através de monocamadas de HUVECs

L. interrogans sorovar Pomona cepa LPF virulentas foram tratadas com **PLG**, PLG+uPA (**PLA**), ou apenas com **PBS** como controle. HUVECs previamente semeadas em câmaras de transmigração para formação de monocamadas foram inoculadas com as leptospiros e incubadas por 4 h. As leptospiros que transpuseram as monocamadas de células foram contadas em microscópio de campo escuro. As barras representam as médias $\pm$ o desvio padrão de duas contagens independentes de cada duplicata experimental. Os dados são representativos de três experimentos independentes. * $P < 0,0025$ e ** $P < 0,03$.

FONTE: Vieira (2012)

4.3.3 Ativação de proteólise

O desbalanço na expressão ou regulação da atividade de MMPs pode levar a vários eventos patológicos. Sabe-se que inúmeros patógenos tem a habilidade de estimular a expressão de MMPs pelos hospedeiros. É conhecido, ainda, que a PLA induz direta ou indiretamente a expressão de MMPs, além de ser capaz de ativá-las pela clivagem proteolítica dos domínios pró-peptídeos (Gebbia et al., 2001; Lahteenmaki et al., 2005; Liotta et al., 1981a, 1981b). Fundamentando-se nessas afirmações, avaliou-se a capacidade de leptospiros associadas ou não à PLA de ativar o sistema proteolítico de MMPs. Além disso, outras formas de interação dessas bactérias com o sistema fibrinolítico foram investigadas.

4.3.3.1 Ativação de HUVECs por contato com leptospiiras

Dados descritos anteriormente neste trabalho apontaram para a inexistência de ativadores endógenos de PLG em leptospiiras (Figura 4). Como foi observado um aumento significativo na migração de leptospiiras tratadas com PLG em comparação às não tratadas (PBS) (Figura 27), foi levantada a hipótese de que as leptospiiras pudessem estimular a liberação de ativadores de PLG pelas HUVECs. Para testar esta suposição, foi extraído o RNA total de HUVECs incubadas com as leptospiiras, seguido de síntese de cDNA e avaliação por qPCR. As células foram avaliadas quanto aos níveis de transcrição de uPA e tPA; o gene de expressão constitutiva ACTB foi utilizado como controle endógeno para normalização dos transcritos em estudo. Os dados apresentados na Figura 28 indicam que as HUVECs em contato com as leptospiiras têm níveis mais altos de transcrição de ambos os ativadores de PLG testados, em comparação às células não expostas às bactérias (RPMI). Houve um pequeno aumento na expressão após 6 h de incubação enquanto que, após 24 h, um elevado incremento relativo de transcrição foi observado tanto para uPA, quanto tPA. Ademais, embora as leptospiiras submetidas aos diferentes tratamentos (PLG, uPA, PLA ou não tratado – PBS), tenham gerado aumento na expressão de uPA e tPA, as bactérias previamente revestidas com PLA ativa na superfície foram as que exibiram maior habilidade de estimular as HUVECs quanto aos parâmetros avaliados (mais de 10 vezes para uPA e 2 vezes para tPA). Os controles negativos (RPMI – células incubadas na ausência de leptospiiras) não apresentaram diferenças significativas na transcrição de ambos os ativadores.

Os níveis aumentados de transcritos de uPA e tPA revelaram que a expressão de ambos os ativadores é regulada positivamente quando as HUVECs são incubadas com as leptospiiras. Interessantemente, após 6 h de coincubação, o aumento na transcrição foi insignificante (Figura 28), enquanto que a facilitação na penetração foi detectada após 4 h (Figura 27). Provavelmente, logo nas primeiras horas de incubação das células endoteliais com as leptospiiras ocorre a liberação de grânulos de tPA pelas HUVECs, o que resulta na ativação do PLG associado às bactérias. De fato, é bem documentado que células endoteliais contêm grânulos de estocagem de tPA (Knipe et al., 2010), que são secretados durante eventos de injúria ou inflamação (Oliver et al., 2005). Da mesma forma como demonstrado para *Leptospira*, outros micro-organismos patogênicos estimulam a liberação de tPA por células endoteliais, como o vírus da dengue (Huang et al., 2003), *Prevotella intermedia* (Guan et al., 2011), entre outros (Mavrommatis et al., 2001).

Figura 28 – Transcrição de uPA e tPA por HUVECs incubadas com leptospiros avaliada por qPCR

Culturas primárias de HUVECs foram incubadas por **6 h** ou **24 h** com *L. interrogans* virulentas submetidas aos tratamentos: **PBS**, **PLG**, **uPA** ou PLG+uPA (**PLA**). Como controle, HUVECs foram incubadas na ausência de bactérias, somente com meio de cultura (**RPMI**). As HUVECs tiveram os seus RNAs extraídos, e a partir do cDNA foi realizado qPCR para cálculo da transcrição relativa dos genes uPA e tPA. A normalização foi feita utilizando-se o gene constitutivo ACTB, e as transcrições relativas estão expressas em função do controle incubado por 6 h, sem bactérias. As barras representam as variações de transcrições relativas calculadas a partir das triplicatas de cada amostra, como descrito na seção 3.41. Os dados são representativos de dois experimentos independentes.

FONTE: Vieira (2012)

A capacidade de estímulo de liberação de ativadores de PLG constitui uma nova estratégia de modulação do sistema fibrinolítico pelas leptospiros. Na falta de mecanismos endógenos de ativação do PLG, a indução de maior disponibilidade de ativadores pelo hospedeiro poderia levar à geração mais eficiente da PLA ligada às bactérias do que em níveis endógenos basais. Assim, além de serem capazes de capturar o PLG do hospedeiro, as leptospiros parecem interagir com o sistema fibrinolítico de modo a favorecer a sua ativação.

4.3.3.2 Secreção de MMPs por HUVECs incubadas com leptospiros

É sabido que muitos patógenos induzem a secreção e/ou produção de MMPs pelas células dos hospedeiros. É conhecido, também, que a PLA é uma enzima capaz de induzir direta ou indiretamente a atividade de MMPs (Lahteenmaki et al., 2005). Fundamentando-se nessas afirmações, foram realizados ensaios de zimografia a partir dos sobrenadantes celulares de HUVECs coincubadas *in vitro* com as leptospiros complexadas ou não à PLA para avaliar a liberação de MMPs por essas células. Sobrenadantes de cultura de células HT1080 foram empregados como controle de secreção constitutiva de pró-MMP-2 (~72 kDa) e pró-MMP-9 (~92 kDa). Bandas relativas à atividade de pró-MMP-2 em substrato de gelatina foram observadas em todas as amostras, tanto para os controles sem as leptospiros, como para as amostras incubadas com leptospiros não tratadas ou tratadas por 6 ou 24 h (Figura 29). Analisando-se os períodos de 24 h de coincubação, as bandas relativas à pró-MMP-9 e MMP-2 ativa (~66 kDa) tornaram-se presentes em todas as amostras, apesar de aparentemente com maior intensidade nas amostras incubadas com as bactérias. A banda de degradação correspondente à MMP-9 ativa (~78 kDa) tornou-se visível apenas nas amostras de HUVECs incubadas com leptospiros (tratadas ou não tratadas), sendo ausente no controle sem bactérias (Figura 29).

Quando os sobrenadantes celulares foram submetidos a testes de atividade de MMPs baseados em técnica de ELISA de captura (Figura 30), os resultados obtidos mostraram-se em conformidade com a análise por zimografia (Figura 29). A atividade de MMP-9 total apresentou tendência de aumento em sobrenadantes de HUVECs expostas às leptospiros por 24 h, enquanto que as atividades de MMP-2 (total e ativa) e MMP-9 ativa não apresentaram diferenças significativas (Figura 30 A e B).

Figura 29 – Zimografia em gel SDS-PAGE copolimerizado com gelatina de sobrenadantes de culturas de HUVECs incubadas com leptospiros

Culturas primárias de HUVECs foram incubadas por 6 ou 24 h com leptospiros sem tratamento (**PBS**) ou tratadas com **PLG**, **uPA** ou **PLG+uPA (PLA)**. Como controle negativo, células foram incubadas apenas com meio de cultura, sem bactérias (**HUVECs**). Sobrenadantes de culturas de células **HT1080**, foram utilizados como controles positivos para liberação constitutiva de pró-MMP-2 (~72 kDa) e pró-MMP-9 (~92 kDa). As amostras foram separadas em gel de 10% poliacrilamida + 0,1% gelatina. Após incubação em solução de ativação, os géis foram corados com azul de coomassie. As regiões sem coloração representam bandas de degradação do substrato. As bandas de degradação correspondentes às proteases pró-MMP-2, MMP-2 intermediária, MMP-2 ativa, pró-MMP-9 e MMP-9 ativa estão indicadas.

FONTE: Vieira (2012)

Figura 30 – Detecção e quantificação de níveis de MMP-9 e MMP-2 nos sobrenadantes de HUVECs coincubadas com leptospiros

Culturas primárias de HUVECs foram incubadas por **6 h** ou **24 h** com *L. interrogans* sorovar Pomona sem não tratadas (**PBS**), ou tratadas com **PLG**, **uPA** ou PLG+uPA (**Pla**). Como controle negativo, HUVECs foram incubadas com meio de cultura fresco, sem bactérias (**RPMI**). Os sobrenadantes livres de células e bactérias foram avaliados quanto à presença de MMP-9 (**A**) ou MMP-2 (**B**) por testes de atividade. As atividades de MMP-9 total ou MMP-2 total foram medidas por ativação das pró-MMPs com APMA. As barras representam as médias das duplicatas de dois experimentos independentes $\pm$ o desvio padrão.

FONTE: Vieira (2012)

Para melhor caracterizar o aumento da liberação e/ou atividade de MMP-9 por células HUVECs mantidas em contato com as bactérias, foram realizados ensaios quantitativos de qPCR a partir do cDNA total das células, para avaliar se MMP-9 é regulada ao nível transcricional. Os resultados indicam que MMP-9 tem níveis mais elevados quando as células são expostas às leptospiros pelo período de 24 h (Figura 31). Além disso, a maior transcrição relativa foi observada para leptospiros pré-tratadas com PLA (aumento de aproximadamente 7,5 vezes, em relação ao controle sem bactérias, com incubação por 6 h) (Figura 31).

Juntos, os resultados apresentados apontam para a regulação de MMP-9 mediada por contato das células endoteliais com as leptospiros. Foi evidenciado que a regulação é positiva e atua nos níveis transcricional e traducional, gerando maior atividade de MMP-9 nos sobrenadantes celulares. Adicionalmente, a presença de PLA na superfície das células parece exacerbar ainda mais a expressão de MMP-9.

Figura 31 – Transcrição de MMP-9 por HUVECs incubadas com leptospiros avaliada por qPCR

Culturas primárias de HUVECs foram incubadas por **6 h** ou **24 h** com *L. interrogans* virulentas submetidas aos tratamentos: **PBS**, **PLG**, **uPA** ou PLG+uPA (**PLA**). Como controle, HUVECs foram incubadas na ausência de bactérias, somente com meio de cultura (**RPMI**). As HUVECs tiveram seu RNA extraído, e a partir do cDNA foi realizado qPCR para cálculo da transcrição relativa do gene **MMP-9**, utilizando-se o gene constitutivo ACTB como normalizador. As transcrições relativas estão expressas em função do controle RPMI incubado por 6 h. As barras representam as variações de transcrições relativas calculadas a partir das triplicatas de cada amostra, como descrito na seção 3.41. Os dados são representativos de dois experimentos independentes.

FONTE: Vieira (2012)

4.3.3.3 Quantificação de ativadores de PLG em soro de pacientes com leptospirose

O estímulo da liberação de ativadores de PLG por células endoteliais humanas *in vitro* após contato com *L. interrogans* incitou a investigação dos níveis circulantes de uPA e tPA em soros humanos. Foi avaliado se soros de pacientes com leptospirose naturalmente adquirida apresentam quantidades alteradas desses ativadores em comparação aos indivíduos normais sem histórico conhecido da doença. Soros de pacientes com leptospirose (MAT negativos e MAT positivos) ou soros de doadores sadios foram analisados quanto à presença uPA ou tPA por kits específicos baseados em ELISA de captura. Observou-se o aumento tanto de uPA quanto de tPA nos soros de pacientes com leptospirose (Figura 32). A média da dosagem de uPA foi aproximadamente 4,5 vezes maior em indivíduos MAT negativos e 3,0 vezes maior em indivíduos MAT positivos em relação aos indivíduos normais. Ainda, os pacientes MAT negativos obtiveram dosagem de uPA 1,5 vezes maior do que MAT positivos. Já para tPA, soros MAT negativos resultaram em dosagem cerca de 2,2 maior do que soros normais, enquanto que MAT positivos 1,2 vezes maior. Comparando-se os soros MAT negativo e MAT positivo quanto à presença de tPA, o primeiro foi apenas 1,2 maior do que o segundo (Figura 32).

Os soros foram adicionalmente avaliados quando à atividade de uPA, o que evidenciou que o aumento das quantidades de uPA circulantes no soro são acompanhadas por proporcional aumento na atividade desta enzima (Figura 33). Isso indica que, muito provavelmente, a indução de expressão de uPA não é seguida por aumento de inibidores (PAIs). Em concordância com a dosagem de uPA, a atividade apresentou o nível mais elevado nas amostras de soro MAT negativo.

Juntos, estes dados sugerem que a liberação de ativadores de PLG deva ser condição importante durante a fase inicial da doença (MAT negativo), decaindo para valores mais próximos dos normais na fase final (MAT positivo). A disponibilização de soros de pacientes que sobreviveram à leptospirose após total restabelecimento e cura da doença seria necessária para a realização de testes que apontassem se os níveis de uPA e tPA voltam para os níveis normais. Os resultados indicam que as leptospirosas, além de capturarem PLG em sua superfície, provocam um desbalanço na regulação do sistema fibrinolítico, por indução de disponibilização de ativadores de PLG. A atividade de uPA nos soros de pacientes com leptospirose também é aumentada, indicando que o aumento nos níveis séricos de uPA não é concomitante ao aumento de inibidores de uPA, como PAI-1 e PAI-2, reforçando a suposição de que a leptospirose interfira na homeostase do sistema fibrinolítico. No entanto, testes

específicos fazem-se necessários para avaliar os níveis de PAIs em pacientes com leptospirose.

Figura 32 – Dosagem de ativadores de PLG em soros humanos normais ou de pacientes diagnosticados com leptospirose

Soros humanos normais (**Normal**), **MAT negativo** ou **MAT positivo** foram quantificados quanto à presença de uPA (**A**) ou tPA (**B**) através de kits comerciais específicos baseados em ELISA de captura. Os pontos representam as dosagens individuais de cada soro e as barras horizontais, as médias dos valores obtidos para cada grupo.

FONTE: Vieira (2012)

Figura 33 – Atividade de uPA avaliada em soro de indivíduos saudáveis e pacientes com leptospirose

Soros humanos de doadores saudáveis (**Normal**), pacientes com leptospirose confirmada na fase inicial (**MAT negativo**) ou convalescente da doença (**MAT positivo**), foram quantificados quanto à atividade da protease uPA por teste de atividade comercial específico. Os pontos representam as determinações individuais de atividade e as barras horizontais, as médias das atividades de uPA em cada grupo.

FONTE: Vieira (2012)

4.3.3.4 Comparação do perfil zimográfico de soros de pacientes com leptospirose e indivíduos saudáveis

Como as leptospirosas incitam o aumento na expressão e liberação de MMPs por HUVECs (Figuras 29, 30 e 31), foi investigado o perfil zimográfico de soros de pacientes com leptospirose para verificar se as leptospirosas são capazes de causar alteração na expressão de MMPs durante a infecção.

Uma quantidade padronizada de soros humanos foi aplicada em géis de SDS-PAGE copolimerizados com gelatina como substrato para proteases com atividade gelatinase, em condições não desnaturantes. Após tratamento, os géis foram incubados por cerca de 18 h em solução de ativação e corados com azul de coomassie. As bandas sem coloração representam as regiões onde houve degradação da gelatina, portanto ausência de substrato. Comparando-se os resultados obtidos para indivíduos saudáveis, sem histórico conhecido para leptospirose, e para pacientes MAT negativos e MAT positivos com leptospirose, foi possível distinguir bandas com diferenças visíveis de intensidade de atividade proteolítica, sendo a mais evidente

indicada por setas, com aproximadamente 90 kDa (Figura 34A), presumivelmente pró-MMP-9.

Aplicando-se o mesmo protocolo substituindo-se a gelatina por colágeno tipo I como substrato, observou-se que a região de degradação mais evidentemente aumentada no substrato gelatina (~90 kDa), também foi observada no substrato colágeno tipo I em amostras de soros de pacientes, indicando que se trata de uma protease com atividade tanto gelatinase quanto collagenase (Figura 34B). O tamanho molecular estimado, aliado à dupla atividade enzimática sustenta o fato de que se trate da pró-MMP-9 (Snoek-van Beurden e Von den Hoff, 2005). Além disso, quando foram realizados ensaios na ausência de íons divalentes ou na presença de agente quelante, EDTA, as atividades proteolíticas foram suprimidas, comprovando que provém de proteases dependentes de cálcio e zinco, ou seja, metaloproteases.

As amostras foram ainda avaliadas por zimografia em gel 2D com gelatina como substrato, o que reiterou as diferenças nas atividades gelatinolíticas presentes nos soros de pacientes, em comparação com indivíduos normais (Figura 35). Foram realizados experimentos de reconhecimento de MMP-9 por *immunoblotting* a partir de gel 2D dos soros humanos, porém os resultados foram inconclusivos (dados não mostrados). A zimografia é uma técnica extremamente sensível, sendo capaz de revelar a presença de MMPs na ordem de picogramas. Devido à mistura complexa de proteínas presentes no soro humano e à menor sensibilidade do *immunoblotting*, não foi possível confirmar a identidade de MMP-9 por este método.

Figura 34 – Zimografia de soros de pacientes com leptospirose ou indivíduos normais em gel SDS-PAGE copolimerizado com gelatina ou colágeno I como substrato

Soros humanos de doadores normais (**Normal**), pacientes com leptospirose na fase inicial da doença (**MAT negativo**) ou convalescente (**MAT positivo**), foram separados em gel de 10% acrilamida + 0,1% gelatina (**A**) ou 0,1% colágeno tipo I (**B**). Após incubação em tampão de ativação, os géis foram corados com azul de coomassie, e as regiões sem coloração representam bandas de degradação. As **setas** indicam a banda com maior aumento visível de expressão nas amostras de pacientes. **M**: marcador de peso molecular; **1 a 9**, amostras individuais.

FONTE: Vieira (2012)

Figura 35 – Zimografias a partir de géis 2D de soros de pacientes com leptospirose ou indivíduos saudáveis

A: soros de indivíduos normais, **B:** pacientes MAT negativos e **C:** pacientes MAT positivos. As amostras foram isoeletrofocalizadas em fitas de 7cm (pH 3-10) em duplicatas, sendo uma corada com azul de coomassie (**Gel 2D**) e outra utilizada para zimografia em gel de poliacrilamida copolimerizado com 0,1% gelatina (**Zimografia 2D**).

FONTE: Vieira (2012)

Para corroborar os dados obtidos por comparação dos perfis zimográficos, testes de atividade de MMP-9 e MMP-2 foram aplicados. Os valores de atividade apontam para maior taxa de pró-MMP-9 sérica circulante em pacientes diagnosticados com leptospirose, quando comparados com doadores normais. Os valores de pró-MMP-9 são representados pela atividade de MMP-9 total (Figura 36). Adicionalmente, maior concentração de pró-MMP-9 foi observada entre pacientes MAT negativos do que os MAT positivos, correspondendo a 2,5 vezes os valores médios obtidos para os doadores saudáveis. Em soros MAT positivos, os valores de MMP-9 aparentam haver regredido para níveis próximos dos normais. Assim, corrobora-se a maior expressão de MMP-9 na condição de leptospirose, embora dados adicionais sejam necessários para avaliar a expressão concomitante de inibidores de MMPs (TIMPs) durante a doença.

MMP-2 é constitutivamente expressa por quase todos os tipos celulares. Análises de MMP-2 indicam que a atividade dessa protease é diminuída em pacientes com leptospirose, tanto em MAT negativo quanto MAT positivo. Os níveis de MMP-2 quantificados em soros de pacientes normais (310 ± 44 ng/mL) foram similares aos reportados na literatura para plasma humano (300 a 500 ng/mL) (Kuyvenhoven et al., 2004; Zucker et al., 1992), o que valida os resultados obtidos. As possíveis razões e implicações para a diminuição do nível de MMP-2 em pacientes com leptospirose necessitam de investigações complementares.

Figura 36 – Detecção e quantificação de níveis de MMP-9 e MMP-2 em soros de pacientes com leptospirose e indivíduos normais

Soros de indivíduos normais (**Normal**) ou soros de pacientes diagnosticados com leptospirose na fase inicial (**MAT -**) ou convalescente da doença (**MAT +**) foram avaliados quanto à presença de MMP-9 ou MMP-2 por testes de atividade comerciais específicos. As atividades de MMP-9 total ou MMP-2 total foram medidas por ativação das pró-MMPs com APMA. As barras representam as médias das somatórias das amostras quantificadas em duplicatas $\pm$ o desvio padrão. * $P < 0,02$.

FONTE: Vieira (2012)

4.3.3.5 Comparação do perfil zimográfico de soros de pacientes com leptospirose e doenças não relacionadas

Como se observou alterações consistentes entre os soros de doadores normais e de pacientes com leptospirose, ensaios foram realizados para verificar se a alteração é comum para outras doenças infecciosas não relacionadas. Além dos soros de pacientes com leptospirose, foram selecionados aleatoriamente soros de pacientes diagnosticados com dengue, malária, HIV e doença de Chagas. As bandas com maior atividade gelatinolítica em soros de pacientes com leptospirose (~90 kDa, indicadas pelas setas) não são observadas nos soros dos pacientes com as demais doenças (Figura 37). Os perfis zimográficos dos soros dos pacientes com dengue, malária, e doença de Chagas são semelhantes aos obtidos para os doadores normais (Figura 35). Soros de pacientes diagnosticados com infecção por HIV apresentam uma região de degradação distinta, inferior à massa molecular de pró-MMP-9 (~90 kDa), ausente em soros normais e MAT negativos ou positivos para leptospirose (Figura 37).

Figura 37 – Zimografia em gel SDS-PAGE copolimerizado com gelatina de soros de pacientes com leptospirose e outras doenças

Soros humanos de pacientes na fase inicial (MAT negativo, linhas 1 e 2) ou fase convalescente (MAT positivo, linhas 3 e 4) diagnosticados com **Leptospirose**, ou de pacientes com **Dengue**, **Malária**, **HIV** e doença de **Chagas** foram separados em géis de 10% poliacrilamida + 0,1% gelatina como substrato. Após ativação das MMPs, os géis foram corados. As regiões claras representam as bandas de degradação. As **setas** indicam a região de maior degradação nas amostras de pacientes com leptospirose.

FONTE: Vieira (2012)

Os resultados das análises de meios de cultura de HUVECs por contato de leptospiros *in vitro* mostrando o estímulo de MMP-9 (Figuras 29, 30 e 31) são corroborados pelos ensaios que demonstram que o mesmo fenômeno ocorre *in vivo* durante infecção humana natural (Figuras 34 e 36). O aumento de MMP-9 circulante pode contribuir para a atividade de degradação e desestruturação da MEC e tecidos endoteliais. Isso soma efeito à atividade semelhante desencadeada pela ligação de PLG/PLA à superfície das leptospiros, o que gera atividade de proteólise de MEC e facilitação na penetração de monocamadas endoteliais (Figuras 26 e 27), favorecendo a penetração e invasão bacteriana. Desta forma, a leptospirose induz a expressão de MMPs pelos hospedeiros, o que não ocorre para as outras doenças infecciosas analisadas, indicando que não é consequência comum de infecção ou inflamação, mas sim devido à infecção por *Leptospira*. Ainda, como MMP-9 é induzível por fatores de crescimento e tPA, ambos secretados por células endoteliais (Cai et al., 2005; Lewalle et al., 1995; Ratel et al., 2007; Salo et al., 1991), o dado anteriormente relatado demonstrando que este ativador de PLG é secretado por HUVECs em contato com leptospiros (Figura 28) e encontra-se aumentado em soro de pacientes com leptospirose (Figura 32), pode contribuir para os maiores níveis de MMP-9 observados.

Da mesma forma como observado para *Leptospira*, é documentado que bactérias do gênero *Borrelia* também são capazes de induzir expressão exacerbada de metaloproteases, incluindo MMP-9, durante infecção em humanos (Gebbia et al., 2001, 2004; Hu et al., 2001). Essa atividade parece conferir maior habilidade de penetração e disseminação, além de contribuir para a patogênese da infecção por essas bactérias (Gebbia et al., 2001). Portanto, a capacidade de estímulo de expressão de MMPs pelos hospedeiros durante a infecção parece ser característica comum entre as espiroquetas.

Estudos adicionais são necessários para a compreensão dos efeitos patológicos do aumento de MMP-9 durante a leptospirose humana em adição os efeitos indiretos de facilitação de penetração e invasão de tecidos.

5 CONCLUSÕES

Ao longo deste trabalho de doutorado, foi reportada pela primeira vez a interação de leptospiros com o sistema fibrinolítico do hospedeiro, através da captura de PLG na superfície. Os dados sugerem que este mecanismo seja de grande importância para a virulência dessas bactérias, já que diferenças foram encontradas entre leptospiros virulentos e atenuados em cultura, indicando expressão diferencial. A presença de inúmeros receptores de PLG na superfície desses micro-organismos reforça a importância desta atividade para a virulência. Foram identificadas diversas proteínas de leptospiros que podem atuar como receptores de PLG durante a infecção, sendo possíveis fatores de virulência que merecem ser mais bem estudados.

Foi demonstrada por outros grupos a interação de leptospiros com reguladores do SC, resultando em proteção por conferir capacidade de escape ao sistema imune inato. Os resultados aqui discutidos adicionam a participação da PLA capturada pelas leptospiros na evasão imune dessas bactérias no hospedeiro. A PLA foi capaz de diminuir a deposição de IgG e C3b na superfície de *Leptospira*, o que pode ter implicações na diminuição da fagocitose por macrófagos mediada por anticorpos e na supressão da cascata de ativação do SC, e aumentou a resistência ao ataque pelo complemento. Estes dados reforçam a ideia da importância da atividade de escape ao sistema imune para o estabelecimento da leptospirose, ao que parece que as leptospiros apresentam diversas estratégias de inibição de mecanismos de defesa dos hospedeiros atuando em conjunto.

Até o momento, não foram identificadas proteínas de leptospiros capazes de degradarem componentes de MEC, o que pode ser contornado pela propriedade de degradação conferida pela PLA capturada na superfície das leptospiros. Por técnicas de cultura celular, foi demonstrado o efeito direto desta atividade proteolítica, de modo que houve a facilitação da penetração por monocamadas de células endoteliais. Este constitui o primeiro mecanismo descrito para a atividade de penetração ativa das leptospiros.

Além da capacidade de ligação ao PLG, foi apresentada outra forma de interação das leptospiros com o sistema fibrinolítico. As leptospiros mostraram-se capazes de induzir a produção de ativadores de PLG, demonstrado tanto pelo contato com células endoteliais *in vitro*, quanto durante a infecção natural, pela análise de soro de pacientes diagnosticados com leptospirose. Esse resultado implica no desbalanço da regulação do sistema fibrinolítico, culminando na formação de PLA. Esta atividade aumenta a disponibilidade de ativadores no hospedeiro, suprimindo a falta de mecanismos endógenos de conversão à PLA pelas leptospiros.

Adicionalmente, os maiores níveis de ativadores de PLG foram observados no início da infecção, reforçando a importância desse mecanismo para a penetração ativa das leptospiros nos primeiros estágios da leptospirose.

Com base nos dados obtidos e as interações diretas entre os sistemas PLG/PLA e de MMPs, hipóteses de que as leptospiros pudessem alterar a expressão e atividades de MMPs nos hospedeiros foram levantadas. Os resultados obtidos a partir de culturas de células humanas e soros de indivíduos infectados apontam para um fenômeno nunca antes descrito para a leptospirose: aumento da expressão de MMPs, mais precisamente, a MMP-9. A MMP-9 está envolvida em uma série de infecções e eventos patológicos. Sua maior expressão e atividade durante a leptospirose pode ter diversas implicações no estabelecimento e patologia da doença, e merece mais estudos para melhor caracterização e compreensão do processo.

Em conjunto, os resultados indicam a importância da aquisição de PLG e ativação da PLA durante a leptospirose. Além de capturar PLG, as leptospiros são capazes de estimular a ativação do sistema fibrinolítico, configurando um desbalanço na regulação desse sistema, de modo a favorecer a formação de PLA na superfície dessas bactérias. Adicionalmente, a presença de leptospiros e maior geração de PLA influenciam na expressão e ativação de MMPs. Os dois sistemas proteolíticos do hospedeiro são desregulados para atuar em benefício da disseminação das leptospiros. E, uma vez ativados, esses sistemas interagem, exacerbando ainda mais a ativação de proteólise no hospedeiro.

O entendimento de funções específicas da PLA durante as infecções bacterianas pode ser crítico para o desenvolvimento de novas terapias e vacinas. Com base no que foi apresentado, é possível afirmar que a interação com sistema proteolítico PLG/PLA deve ser importante durante o processo infeccioso e de invasão das leptospiros. Considerando-se a falta de conhecimento sobre a patogênese da leptospirose, os dados resultantes desta tese constituem um avanço na compreensão da patogenicidade e virulência das leptospiros. Acredita-se que essa nova característica seja de grande contribuição para o conhecimento dos aspectos moleculares do processo infeccioso e patogenicidade dessas bactérias e que possa colaborar na identificação de proteínas com papel na patogenicidade e, assim, antígenos em potencial para o desenvolvimento de uma vacina eficiente contra a leptospirose.

REFERÊNCIAS*

- Adler B e De la Pena Moctezuma A. *Leptospira* and leptospirosis. *Vet Microbiol.* 2010.140 (3-4): 287-296.
- Ahmad SN, Shah S e Ahmad FM. Laboratory diagnosis of leptospirosis. *J Postgrad Med.* 2005.51 (3): 195-200.
- Altschul SF, Gish W, Miller W, Myers EW e Lipman DJ. Basic local alignment search tool. *J Mol Biol.* 1990.215 (3): 403-410.
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W e Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997.25 (17): 3389-3402.
- Amalinei C, Caruntu ID, Giusca SE e Balan RA. Matrix metalloproteinases involvement in pathologic conditions. *Rom J Morphol Embryol.* 2010.51 (2): 215-228.
- Andrews NC. Iron homeostasis: insights from genetics and animal models. *Nat Rev Genet.* 2000.1 (3): 208-217.
- Antonara S, Ristow L e Coburn J. Adhesion mechanisms of *Borrelia burgdorferi*. *Adv Exp Med Biol.* 2011.715 35-49.
- Arruda S, Bomfim G, Knights R, Huima-Byron T e Riley LW. Cloning of an *M. tuberculosis* DNA fragment associated with entry and survival inside cells. *Science.* 1993.261 (5127): 1454-1457.
- Attali C, Frolet C, Durmort C, Offant J, Vernet T e Di Guilmi AM. *Streptococcus pneumoniae* choline-binding protein E interaction with plasminogen/plasmin stimulates migration across the extracellular matrix. *Infect Immun.* 2008.76 (2): 466-476.
- Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Moraes ZM, Lima DM, Abreu PA e Nascimento AL. Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC Microbiol.* 2008.8 70.
- Atzingen MV, Cerqueira GM, Vieira ML, Oliveira R, Oliveira TR, Domingos RF, Barros AT, Mendes RS e Nascimento ALTO (2010a). Identification and Characterization of Novel Adhesins in *Leptospira*. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology.* Vilas AM, Formatex Research Center. 1: 704-713.
- Atzingen MV, Goncales AP, de Moraes ZM, Araujo ER, De Brito T, Vasconcellos SA e Nascimento AL. Characterization of leptospiral proteins that afford partial protection in hamsters against lethal challenge with *Leptospira interrogans*. *J Med Microbiol.* 2010b.59 (Pt 9): 1005-1015.
- Baker MF e Baker HJ. Pathogenic *Leptospira* in Malaysian surface waters. I. A method of survey for *Leptospira* in natural waters and soils. *Am J Trop Med Hyg.* 1970.19 (3): 485-492.

* De acordo com:

International Committee of Medical Journal Editors. Uniform requirements for manuscripts submitted to Biomedical Journal: sample references. Available from: <http://www.icmje.org> [2011 Jul 15]

- Banfi E, Cinco M, Bellini M e Soranzo MR. The role of antibodies and serum complement in the interaction between macrophages and leptospires. *J Gen Microbiol.* 1982.128 (4): 813-816.
- Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, Vieira ML, Morais ZM, Vasconcellos SA e Nascimento AL. A newly identified leptospiral adhesin mediates attachment to laminin. *Infect Immun.* 2006.74 (11): 6356-6364.
- Barbosa AS, Abreu PA, Vasconcellos SA, Morais ZM, Goncales AP, Silva AS, Daha MR e Isaac L. Immune evasion of leptospira species by acquisition of human complement regulator C4BP. *Infect Immun.* 2009.77 (3): 1137-1143.
- Barocchi MA, Ko AI, Ferrer SR, Faria MT, Reis MG e Riley LW. Identification of new repetitive element in *Leptospira interrogans* serovar copenhageni and its application to PCR-based differentiation of *Leptospira* serogroups. *J Clin Microbiol.* 2001.39 (1): 191-195.
- Barocchi MA, Ko AI, Reis MG, McDonald KL e Riley LW. Rapid translocation of polarized MDCK cell monolayers by *Leptospira interrogans*, an invasive but nonintracellular pathogen. *Infect Immun.* 2002.70 (12): 6926-6932.
- Behera AK, Hildebrand E, Scagliotti J, Steere AC e Hu LT. Induction of host matrix metalloproteinases by *Borrelia burgdorferi* differs in human and murine lyme arthritis. *Infect Immun.* 2005.73 (1): 126-134.
- Bergmann S e Hammerschmidt S. Fibrinolysis and host response in bacterial infections. *Thromb Haemost.* 2007.98 (3): 512-520.
- Bergmann S, Rohde M, Chhatwal GS e Hammerschmidt S. alpha-Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. *Mol Microbiol.* 2001.40 (6): 1273-1287.
- Bergmann S, Rohde M, Chhatwal GS e Hammerschmidt S. Characterization of plasmin(ogen) binding to *Streptococcus pneumoniae*. *Indian J Med Res.* 2004.119 Suppl 29-32.
- Bergmann S, Rohde M, Preissner KT e Hammerschmidt S. The nine residue plasminogen-binding motif of the pneumococcal enolase is the major cofactor of plasmin-mediated degradation of extracellular matrix, dissolution of fibrin and transmigration. *Thromb Haemost.* 2005.94 (2): 304-311.
- Bharadwaj R, Bal AM, Joshi SA, Kagal A, Pol SS, Garad G, Arjunwadkar V e Katti R. An urban outbreak of leptospirosis in Mumbai, India. *Jpn J Infect Dis.* 2002.55 (6): 194-196.
- Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E, Vinetz JM e Peru-United States Leptospirosis C. Leptospirosis: a zoonotic disease of global importance. *Lancet Infect Dis.* 2003.3 (12): 757-771.
- Blom AM, Hallstrom T e Riesbeck K. Complement evasion strategies of pathogens-acquisition of inhibitors and beyond. *Mol Immunol.* 2009.46 (14): 2808-2817.
- Bolin CA e Koellner P. Human-to-human transmission of *Leptospira interrogans* by milk. *J Infect Dis.* 1988.158 (1): 246-247.

Bonazzi M e Cossart P. Impenetrable barriers or entry portals? The role of cell-cell adhesion during infection. *J Cell Biol.* 2011.195 (3): 349-358.

Bourhy P, Louvel H, Saint Girons I e Picardeau M. Random insertional mutagenesis of *Leptospira interrogans*, the agent of leptospirosis, using a mariner transposon. *J Bacteriol.* 2005.187 (9): 3255-3258.

Boyle EC e Finlay BB. Bacterial pathogenesis: exploiting cellular adherence. *Curr Opin Cell Biol.* 2003.15 (5): 633-639.

Branger C, Chatrenet B, Gauvrit A, Aviat F, Aubert A, Bach JM e Andre-Fontaine G. Protection against *Leptospira interrogans* sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. *Infect Immun.* 2005.73 (7): 4062-4069.

Branger C, Sonrier C, Chatrenet B, Klonjowski B, Ruvoen-Clouet N, Aubert A, Andre-Fontaine G e Eloit M. Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. *Infect Immun.* 2001.69 (11): 6831-6838.

Brauer R, Beck IM, Roderfeld M, Roeb E e Sedlacek R. Matrix metalloproteinase-19 inhibits growth of endothelial cells by generating angiostatin-like fragments from plasminogen. *BMC Biochem.* 2011.12 38.

Brenner DJ, Kaufmann AF, Sulzer KR, Steigerwalt AG, Rogers FC e Weyant RS. Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. *Int J Syst Bacteriol.* 1999.49 Pt 2 839-858.

Brinckerhoff CE e Matrisian LM. Matrix metalloproteinases: a tail of a frog that became a prince. *Nat Rev Mol Cell Biol.* 2002.3 (3): 207-214.

Brissette CA, Haupt K, Barthel D, Cooley AE, Bowman A, Skerka C, Wallich R, Zipfel PF, Kraiczy P e Stevenson B. *Borrelia burgdorferi* infection-associated surface proteins ErpP, ErpA, and ErpC bind human plasminogen. *Infect Immun.* 2009.77 (1): 300-306.

Brown EL, Guo BP, O'Neal P e Hook M. Adherence of *Borrelia burgdorferi*. Identification of critical lysine residues in DbpA required for decorin binding. *J Biol Chem.* 1999.274 (37): 26272-26278.

Bulach DM, Kalambaheti T, de la Pena-Moctezuma A e Adler B. Lipopolysaccharide biosynthesis in *Leptospira*. *J Mol Microbiol Biotechnol.* 2000.2 (4): 375-380.

Bulach DM, Zuerner RL, Wilson P, Seemann T, McGrath A, Cullen PA, Davis J, Johnson M, Kuczek E, Alt DP, Peterson-Burch B, Coppel RL, Rood JI, Davies JK e Adler B. Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proc Natl Acad Sci U S A.* 2006.103 (39): 14560-14565.

Cai G, Chen X, Fu B e Lu Y. Activation of gelatinases by fibrin is PA/plasmin system-dependent in human glomerular endothelial cells. *Mol Cell Biochem.* 2005.277 (1-2): 171-179.

Cameron CE. Identification of a *Treponema pallidum* laminin-binding protein. *Infect Immun*. 2003.71 (5): 2525-2533.

Cameron CE, Brouwer NL, Tisch LM e Kuroiwa JM. Defining the interaction of the *Treponema pallidum* adhesin Tp0751 with laminin. *Infect Immun*. 2005.73 (11): 7485-7494.

Cameron CE, Brown EL, Kuroiwa JM, Schnapp LM e Brouwer NL. *Treponema pallidum* fibronectin-binding proteins. *J Bacteriol*. 2004.186 (20): 7019-7022.

Candela M, Biagi E, Centanni M, Turrone S, Vici M, Musiani F, Vitali B, Bergmann S, Hammerschmidt S e Brigidi P. Bifidobacterial enolase, a cell surface receptor for human plasminogen involved in the interaction with the host. *Microbiology*. 2009.155 (Pt 10): 3294-3303.

Candela M, Miccoli G, Bergmann S, Turrone S, Vitali B, Hammerschmidt S e Brigidi P. Plasminogen-dependent proteolytic activity in *Bifidobacterium lactis*. *Microbiology*. 2008.154 (Pt 8): 2457-2462.

Castiblanco-Valencia MM, Fraga TR, Silva LB, Monaris D, Abreu PA, Strobel S, Jozsi M, Isaac L e Barbosa AS. Leptospiral immunoglobulin-like proteins interact with human complement regulators factor H, FHL-1, FHR-1, and C4BP. *J Infect Dis*. 2012.205 (6): 995-1004.

Chakraborti S, Mandal M, Das S, Mandal A e Chakraborti T. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem*. 2003.253 (1-2): 269-285.

Charon NW e Goldstein SF. Genetics of motility and chemotaxis of a fascinating group of bacteria: the spirochetes. *Annu Rev Genet*. 2002.36 47-73.

Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J e Haake DA. Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. *Infect Immun*. 2007.75 (5): 2441-2450.

Cinco M. New insights into the pathogenicity of leptospires: evasion of host defences. *New Microbiol*. 2010.33 (4): 283-292.

Cinco M e Banfi E. Activation of complement by leptospires and its bactericidal activity. *Zentralbl Bakteriol Mikrobiol Hyg A*. 1983.254 (2): 261-265.

Cinco M, Banfi E e Soranzo MR. Studies on the interaction between macrophages and leptospires. *J Gen Microbiol*. 1981.124 (2): 409-413.

Cinco M, Banfi E e Soranzo MR. Leptospires macrophage interactions. *Adv Exp Med Biol*. 1982.141 167-174.

Coleman JL e Benach JL. The generation of enzymatically active plasmin on the surface of spirochetes. *Methods*. 2000.21 (2): 133-141.

Coleman JL, Gebbia JA e Benach JL. *Borrelia burgdorferi* and other bacterial products induce expression and release of the urokinase receptor (CD87). *J Immunol*. 2001.166 (1): 473-480.

Coleman JL, Gebbia JA, Piesman J, Degen JL, Bugge TH e Benach JL. Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice. *Cell*. 1997.89 (7): 1111-1119.

Coleman JL, Roemer EJ e Benach JL. Plasmin-coated borrelia *Burgdorferi* degrades soluble and insoluble components of the mammalian extracellular matrix. *Infect Immun*. 1999.67 (8): 3929-3936.

Coleman JL, Sellati TJ, Testa JE, Kew RR, Furie MB e Benach JL. *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. *Infect Immun*. 1995.63 (7): 2478-2484.

Collen D e Lijnen HR. Thrombolytic agents. *Thromb Haemost*. 2005.93 (4): 627-630.

Colman RW, Sartor RB, Adam AA, DeLa Cadena RA e Stadnicki A. The plasma kallikrein-kinin system in sepsis, inflammatory arthritis, and enterocolitis. *Clin Rev Allergy Immunol*. 1998.16 (4): 365-384.

Crane DD, Warner SL e Bosio CM. A novel role for plasmin-mediated degradation of opsonizing antibody in the evasion of host immunity by virulent, but not attenuated, *Francisella tularensis*. *J Immunol*. 2009.183 (7): 4593-4600.

Croda J, Figueira CP, Wunder EA, Jr., Santos CS, Reis MG, Ko AI e Picardeau M. Targeted mutagenesis in pathogenic *Leptospira* species: disruption of the *LigB* gene does not affect virulence in animal models of leptospirosis. *Infect Immun*. 2008.76 (12): 5826-5833.

Cullen PA, Haake DA e Adler B. Outer membrane proteins of pathogenic spirochetes. *FEMS Microbiol Rev*. 2004.28 (3): 291-318.

de la Pena-Moctezuma A, Bulach DM, Kalambaheti T e Adler B. Comparative analysis of the LPS biosynthetic loci of the genetic subtypes of serovar Hardjo: *Leptospira interrogans* subtype Hardjoprajitno and *Leptospira borgpetersenii* subtype Hardjobovis. *FEMS Microbiol Lett*. 1999.177 (2): 319-326.

Degen JL, Bugge TH e Goguen JD. Fibrin and fibrinolysis in infection and host defense. *J Thromb Haemost*. 2007.5 Suppl 1 24-31.

Dikken H, Kmety E, de Geus A, Adinarayanan N e Timmer VE. Two new leptospira serovars belonging to the Hebdomadis serogroup. *Trop Geogr Med*. 1978.30 (4): 537-542.

Domingos RF, Vieira ML, Romero EC, Goncales AP, Morais ZM, Vasconcellos SA e Nascimento AL. Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions. *BMC Microbiol*. 2012.12 (1): 50.

Egea L, Aguilera L, Gimenez R, Sorolla MA, Aguilar J, Badia J e Baldoma L. Role of secreted glyceraldehyde-3-phosphate dehydrogenase in the infection mechanism of enterohemorrhagic and enteropathogenic *Escherichia coli*: interaction of the extracellular enzyme with human plasminogen and fibrinogen. *Int J Biochem Cell Biol*. 2007.39 (6): 1190-1203.

Elkington PT, O'Kane CM e Friedland JS. The paradox of matrix metalloproteinases in infectious disease. *Clin Exp Immunol*. 2005.142 (1): 12-20.

Faine S, Adler B, Bolin C e Perolat P (1999). *Leptospira* and Leptospirosis. Melbourne, Australia, MediSci.

Farr RW. Leptospirosis. Clin Infect Dis. 1995;21 (1): 1-6; quiz 7-8.

Fenno JC, Tamura M, Hannam PM, Wong GW, Chan RA e McBride BC. Identification of a *Treponema denticola* OppA homologue that binds host proteins present in the subgingival environment. Infect Immun. 2000;68 (4): 1884-1892.

Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M, Khanna A, Durbin R, Eddy SR, Sonnhammer EL e Bateman A. Pfam: clans, web tools and services. Nucleic Acids Res. 2006;34 (Database issue): D247-251.

FIOCRUZ. (2010). Acessado em 05 junho, 2012. Disponível no endereço <http://www.fiocruz.br/ccs/cgi/cgilua.exe/sys/start.htm?infoid=1110&sid=9&tpl=printerview>.

Flannery B, Costa D, Carvalho FP, Guerreiro H, Matsunaga J, Da Silva ED, Ferreira AG, Riley LW, Reis MG, Haake DA e Ko AI. Evaluation of recombinant *Leptospira* antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. J Clin Microbiol. 2001;39 (9): 3303-3310.

Floden AM, Watt JA e Brissette CA. *Borrelia burgdorferi* enolase is a surface-exposed plasminogen binding protein. PLoS One. 2011;6 (11): e27502.

Fonseca Cde A, Teixeira MM, Romero EC, Tengan FM, Silva MV e Shikanai-Yasuda MA. *Leptospira* DNA detection for the diagnosis of human leptospirosis. J Infect. 2006;52 (1): 15-22.

Gaibani P, Caroli F, Nucci C e Sambri V. Major surface protein complex of *Treponema denticola* induces the production of tumor necrosis factor alpha, interleukin-1 beta, interleukin-6 and matrix metalloproteinase 9 by primary human peripheral blood monocytes. J Periodontal Res. 2010;45 (3): 361-366.

Gamberini M, Gomez RM, Atzingen MV, Martins EA, Vasconcellos SA, Romero EC, Leite LC, Ho PL e Nascimento AL. Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis. FEMS Microbiol Lett. 2005;244 (2): 305-313.

Gebbia JA, Coleman JL e Benach JL. *Borrelia spirochetes* upregulate release and activation of matrix metalloproteinase gelatinase B (MMP-9) and collagenase 1 (MMP-1) in human cells. Infect Immun. 2001;69 (1): 456-462.

Gebbia JA, Coleman JL e Benach JL. Selective induction of matrix metalloproteinases by *Borrelia burgdorferi* via toll-like receptor 2 in monocytes. J Infect Dis. 2004;189 (1): 113-119.

Goldstein SF, Charon NW e Kreiling JA. *Borrelia burgdorferi* swims with a planar waveform similar to that of eukaryotic flagella. Proc Natl Acad Sci U S A. 1994;91 (8): 3433-3437.

Gomez RM, Vieira ML, Schattner M, Malaver E, Watanabe MM, Barbosa AS, Abreu PA, de Moraes ZM, Cifuentes JO, Atzingen MV, Oliveira TR, Vasconcellos SA e Nascimento AL. Putative outer membrane proteins of *Leptospira interrogans* stimulate human umbilical vein

endothelial cells (HUVECS) and express during infection. *Microb Pathog.* 2008.45 (5-6): 315-322.

Gouveia EL, Metcalfe J, de Carvalho AL, Aires TS, Villasboas-Bisneto JC, Queirroz A, Santos AC, Salgado K, Reis MG e Ko AI. Leptospirosis-associated severe pulmonary hemorrhagic syndrome, Salvador, Brazil. *Emerg Infect Dis.* 2008.14 (3): 505-508.

Guan SM, He JJ, Zhang M e Shu L. Prevotella intermedia stimulates tissue-type plasminogen activator and plasminogen activator inhibitor-2 expression via multiple signaling pathways in human periodontal ligament cells. *FEMS Immunol Med Microbiol.* 2011.62 (1): 91-100.

Guo BP, Brown EL, Dorward DW, Rosenberg LC e Hook M. Decorin-binding adhesins from *Borrelia burgdorferi*. *Mol Microbiol.* 1998.30 (4): 711-723.

Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, Matsunaga J, Levett PN e Bolin CA. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect Immun.* 2000.68 (4): 2276-2285.

Haake DA, Dundoo M, Cader R, Kubak BM, Hartskeerl RA, Sejvar JJ e Ashford DA. Leptospirosis, water sports, and chemoprophylaxis. *Clin Infect Dis.* 2002.34 (9): e40-43.

Haake DA e Matsunaga J. Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. *Infect Immun.* 2002.70 (9): 4936-4945.

Haake DA e Matsunaga J. *Leptospira*: a spirochaete with a hybrid outer membrane. *Mol Microbiol.* 2010.

Haake DA, Mazel MK, McCoy AM, Milward F, Chao G, Matsunaga J e Wagar EA. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. *Infect Immun.* 1999.67 (12): 6572-6582.

Haake DA, Walker EM, Blanco DR, Bolin CA, Miller MN e Lovett MA. Changes in the surface of *Leptospira interrogans* serovar grippityphosa during in vitro cultivation. *Infect Immun.* 1991.59 (3): 1131-1140.

Haile WB, Coleman JL e Benach JL. Reciprocal upregulation of urokinase plasminogen activator and its inhibitor, PAI-2, by *Borrelia burgdorferi* affects bacterial penetration and host-inflammatory response. *Cell Microbiol.* 2006.8 (8): 1349-1360.

Harpel PC, Sullivan R e Chang TS. Binding and activation of plasminogen on immobilized immunoglobulin G. Identification of the plasmin-derived Fab as the plasminogen-binding fragment. *J Biol Chem.* 1989.264 (1): 616-624.

Hartskeerl RA, Collares-Pereira M e Ellis WA. Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clin Microbiol Infect.* 2011.17 (4): 494-501.

Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Moraes ZM, Barbosa AS e Ho PL. In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. *Infect Immun.* 2008.76 (6): 2642-2650.

- Heilpern AJ, Wertheim W, He J, Perides G, Bronson RT e Hu LT. Matrix metalloproteinase 9 plays a key role in lyme arthritis but not in dissemination of *Borrelia burgdorferi*. *Infect Immun*. 2009.77 (7): 2643-2649.
- Herwald H, Morgelin M, Olsen A, Rhen M, Dahlback B, Muller-Esterl W e Bjorck L. Activation of the contact-phase system on bacterial surfaces--a clue to serious complications in infectious diseases. *Nat Med*. 1998.4 (3): 298-302.
- Hoke DE, Egan S, Cullen PA e Adler B. LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudoalteromonas tunicata*. *Infect Immun*. 2008.76 (5): 2063-2069.
- Hu LT, Eskildsen MA, Masgala C, Steere AC, Arner EC, Pratta MA, Grodzinsky AJ, Loening A e Perides G. Host metalloproteinases in Lyme arthritis. *Arthritis Rheum*. 2001.44 (6): 1401-1410.
- Hua H, Li M, Luo T, Yin Y e Jiang Y. Matrix metalloproteinases in tumorigenesis: an evolving paradigm. *Cell Mol Life Sci*. 2011.68 (23): 3853-3868.
- Huang YH, Lei HY, Liu HS, Lin YS, Chen SH, Liu CC e Yeh TM. Tissue plasminogen activator induced by dengue virus infection of human endothelial cells. *J Med Virol*. 2003.70 (4): 610-616.
- Jaffe EA, Nachman RL, Becker CG e Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest*. 1973.52 (11): 2745-2756.
- Jobin MC, Brassard J, Quessy S, Gottschalk M e Grenier D. Acquisition of host plasmin activity by the Swine pathogen *Streptococcus suis* serotype 2. *Infect Immun*. 2004.72 (1): 606-610.
- Johnsen LB, Poulsen K, Kilian M e Petersen TE. Purification and cloning of a streptokinase from *Streptococcus uberis*. *Infect Immun*. 1999.67 (3): 1072-1078.
- Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H e Krogh A. Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci*. 2003.12 (8): 1652-1662.
- Kinnby B, Booth NA e Svensater G. Plasminogen binding by oral streptococci from dental plaque and inflammatory lesions. *Microbiology*. 2008.154 (Pt 3): 924-931.
- Knaust A, Weber MV, Hammerschmidt S, Bergmann S, Frosch M e Kurzai O. Cytosolic proteins contribute to surface plasminogen recruitment of *Neisseria meningitidis*. *J Bacteriol*. 2007.189 (8): 3246-3255.
- Knipe L, Meli A, Hewlett L, Bierings R, Dempster J, Skehel P, Hannah MJ e Carter T. A revised model for the secretion of tPA and cytokines from cultured endothelial cells. *Blood*. 2010.116 (12): 2183-2191.
- Ko AI, Galvao Reis M, Ribeiro Dourado CM, Johnson WD, Jr. e Riley LW. Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group. *Lancet*. 1999.354 (9181): 820-825.

- Kunert A, Losse J, Gruszin C, Huhn M, Kaendler K, Mikkat S, Volke D, Hoffmann R, Jokiranta TS, Seeberger H, Moellmann U, Hellwage J e Zipfel PF. Immune evasion of the human pathogen *Pseudomonas aeruginosa*: elongation factor Tuf is a factor H and plasminogen binding protein. *J Immunol*. 2007.179 (5): 2979-2988.
- Kuyvenhoven JP, Molenaar IQ, Verspaget HW, Veldman MG, Palareti G, Legnani C, Moolenburgh SE, Terpstra OT, Lamers CB, van Hoek B e Porte RJ. Plasma MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 during human orthotopic liver transplantation. The effect of aprotinin and the relation to ischemia/reperfusion injury. *Thromb Haemost*. 2004.91 (3): 506-513.
- Lagal V, Portnoi D, Faure G, Postic D e Baranton G. *Borrelia burgdorferi* sensu stricto invasiveness is correlated with OspC-plasminogen affinity. *Microbes Infect*. 2006.8 (3): 645-652.
- Lahteenmaki K, Edelman S e Korhonen TK. Bacterial metastasis: the host plasminogen system in bacterial invasion. *Trends Microbiol*. 2005.13 (2): 79-85.
- Lahteenmaki K, Kuusela P e Korhonen TK. Bacterial plasminogen activators and receptors. *FEMS Microbiol Rev*. 2001.25 (5): 531-552.
- Lambert A, Picardeau M, Haake DA, Sermswan RW, Srikram A, Adler B e Murray G. FlaA proteins in *Leptospira interrogans* are essential for motility and virulence, but not required for the formation of flagella sheath. *Infect Immun*. 2012.
- Lambris JD, Ricklin D e Geisbrecht BV. Complement evasion by human pathogens. *Nat Rev Microbiol*. 2008.6 (2): 132-142.
- Ledoux D, Papy-Garcia D, Escartin Q, Sagot MA, Cao Y, Barritault D, Courtois J, Hornebeck W e Caruelle JP. Human plasmin enzymatic activity is inhibited by chemically modified dextrans. *J Biol Chem*. 2000.275 (38): 29383-29390.
- Leigh JA. Purification of a plasminogen activator from *Streptococcus uberis*. *FEMS Microbiol Lett*. 1994.118 (1-2): 153-158.
- Leigh JA, Hodgkinson SM e Lincoln RA. The interaction of *Streptococcus dysgalactiae* with plasmin and plasminogen. *Vet Microbiol*. 1998.61 (1-2): 121-135.
- Letunic I, Copley RR, Pils B, Pinkert S, Schultz J e Bork P. SMART 5: domains in the context of genomes and networks. *Nucleic Acids Res*. 2006.34 (Database issue): D257-260.
- Levett PN. Leptospirosis. *Clin Microbiol Rev*. 2001.14 (2): 296-326.
- Levett PN. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. *Clin Infect Dis*. 2003.36 (4): 447-452.
- Levett PN, Morey RE, Galloway RL, Turner DE, Steigerwalt AG e Mayer LW. Detection of pathogenic leptospires by real-time quantitative PCR. *J Med Microbiol*. 2005.54 (Pt 1): 45-49.
- Lewalle JM, Munaut C, Pichot B, Cataldo D, Baramova E e Foidart JM. Plasma membrane-dependent activation of gelatinase A in human vascular endothelial cells. *J Cell Physiol*. 1995.165 (3): 475-483.

- Leytus SP, Bowles LK, Konisky J e Mangel WF. Activation of plasminogen to plasmin by a protease associated with the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci U S A*. 1981.78 (3): 1485-1489.
- Li L, Ojcius DM e Yan J. Comparison of invasion of fibroblasts and macrophages by high- and low-virulence *Leptospira* strains: colonization of the host-cell nucleus and induction of necrosis by the virulent strain. *Arch Microbiol*. 2007.188 (6): 591-598.
- Li S, Ojcius DM, Liao S, Li L, Xue F, Dong H e Yan J. Replication or death: distinct fates of pathogenic *Leptospira* strain Lai within macrophages of human or mouse origin. *Innate Immun*. 2010.16 (2): 80-92.
- Li Z, Ploplis VA, French EL e Boyle MD. Interaction between group A streptococci and the plasmin(ogen) system promotes virulence in a mouse skin infection model. *J Infect Dis*. 1999.179 (4): 907-914.
- Lijnen HR. Elements of the fibrinolytic system. *Ann N Y Acad Sci*. 2001.936 226-236.
- Lijnen HR. Matrix metalloproteinases and cellular fibrinolytic activity. *Biochemistry (Mosc)*. 2002.67 (1): 92-98.
- Lijnen HR, De Cock F, Van Hoef B, Schlott B e Collen D. Characterization of the interaction between plasminogen and staphylokinase. *Eur J Biochem*. 1994.224 (1): 143-149.
- Lijnen HR, Ugwu F, Bini A e Collen D. Generation of an angiostatin-like fragment from plasminogen by stromelysin-1 (MMP-3). *Biochemistry*. 1998.37 (14): 4699-4702.
- Lin YP, Greenwood A, Yan W, Nicholson LK, Sharma Y, McDonough SP e Chang YF. A novel fibronectin type III module binding motif identified on C-terminus of *Leptospira* immunoglobulin-like protein, LigB. *Biochem Biophys Res Commun*. 2009.389 (1): 57-62.
- Liotta LA, Goldfarb RH, Brundage R, Siegal GP, Terranova V e Garbisa S. Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane. *Cancer Res*. 1981a.41 (11 Pt 1): 4629-4636.
- Liotta LA, Goldfarb RH e Terranova VP. Cleavage of laminin by thrombin and plasmin: alpha thrombin selectively cleaves the beta chain of laminin. *Thromb Res*. 1981b.21 (6): 663-673.
- Liu Y, Zheng W, Li L, Mao Y e Yan J. Pathogenesis of leptospirosis: interaction of *Leptospira interrogans* with in vitro cultured mammalian cells. *Med Microbiol Immunol*. 2007.196 (4): 233-239.
- Livak KJ e Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001.25 (4): 402-408.
- Lo M, Bulach DM, Powell DR, Haake DA, Matsunaga J, Paustian ML, Zuerner RL e Adler B. Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed by whole-genome microarrays. *Infect Immun*. 2006.74 (10): 5848-5859.

- Lo M, Murray GL, Khoo CA, Haake DA, Zuerner RL e Adler B. Transcriptional response of *Leptospira interrogans* to iron limitation and characterization of a PerR homolog. *Infect Immun*. 2010.78 (11): 4850-4859.
- Lomar AV, Diament D e Torres JR. Leptospirosis in Latin America. *Infect Dis Clin North Am*. 2000.14 (1): 23-39, vii-viii.
- Longhi MT, Oliveira TR, Romero EC, Goncalves AP, de Moraes ZM, Vasconcellos SA e Nascimento AL. A newly identified protein of *Leptospira interrogans* mediates binding to laminin. *J Med Microbiol*. 2009.58 (Pt 10): 1275-1282.
- Magalhaes V, Veiga-Malta I, Almeida MR, Baptista M, Ribeiro A, Trieu-Cuot P e Ferreira P. Interaction with human plasminogen system turns on proteolytic activity in *Streptococcus agalactiae* and enhances its virulence in a mouse model. *Microbes Infect*. 2007.9 (11): 1276-1284.
- Malmstrom J, Beck M, Schmidt A, Lange V, Deutsch EW e Aebersold R. Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. *Nature*. 2009.460 (7256): 762-765.
- Martinez R, Perez A, Quinones Mdel C, Cruz R, Alvarez A, Armesto M, Fernandez C, Menendez J, Rodriguez I, Baro M, Diaz M, Rodriguez J, Sierra G, Obregon AM, Toledo ME e Fernandez N. [Efficacy and safety of a vaccine against human leptospirosis in Cuba]. *Rev Panam Salud Publica*. 2004.15 (4): 249-255.
- Matsunaga J, Barocchi MA, Croda J, Young TA, Sanchez Y, Siqueira I, Bolin CA, Reis MG, Riley LW, Haake DA e Ko AI. Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. *Mol Microbiol*. 2003.49 (4): 929-945.
- Matsunaga J, Lo M, Bulach DM, Zuerner RL, Adler B e Haake DA. Response of *Leptospira interrogans* to physiologic osmolarity: relevance in signaling the environment-to-host transition. *Infect Immun*. 2007.75 (6): 2864-2874.
- Matsunaga J, Sanchez Y, Xu X e Haake DA. Osmolarity, a key environmental signal controlling expression of leptospiral proteins LigA and LigB and the extracellular release of LigA. *Infect Immun*. 2005.73 (1): 70-78.
- Matta SK, Agarwal S e Bhatnagar R. Surface localized and extracellular Glyceraldehyde-3-phosphate dehydrogenase of *Bacillus anthracis* is a plasminogen binding protein. *Biochim Biophys Acta*. 2010.1804 (11): 2111-2120.
- Mavrommatis AC, Theodoridis T, Economou M, Kotanidou A, El Ali M, Christopoulou-Kokkinou V e Zakyntinos SG. Activation of the fibrinolytic system and utilization of the coagulation inhibitors in sepsis: comparison with severe sepsis and septic shock. *Intensive Care Med*. 2001.27 (12): 1853-1859.
- McBride AJ, Athanazio DA, Reis MG e Ko AI. Leptospirosis. *Curr Opin Infect Dis*. 2005.18 (5): 376-386.

- McCoy HE, Broder CC e Lottenberg R. Streptokinases produced by pathogenic group C streptococci demonstrate species-specific plasminogen activation. *J Infect Dis.* 1991.164 (3): 515-521.
- Meites E, Jay MT, Deresinski S, Shieh WJ, Zaki SR, Tompkins L e Smith DS. Reemerging leptospirosis, California. *Emerg Infect Dis.* 2004.10 (3): 406-412.
- Mekalanos JJ. Environmental signals controlling expression of virulence determinants in bacteria. *J Bacteriol.* 1992.174 (1): 1-7.
- Meri T, Murgia R, Stefanel P, Meri S e Cinco M. Regulation of complement activation at the C3-level by serum resistant leptospires. *Microb Pathog.* 2005.39 (4): 139-147.
- Merien F, Amouriaux P, Perolat P, Baranton G e Saint Girons I. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. *J Clin Microbiol.* 1992.30 (9): 2219-2224.
- Merien F, Baranton G e Perolat P. Invasion of Vero cells and induction of apoptosis in macrophages by pathogenic *Leptospira* interrogans are correlated with virulence. *Infect Immun.* 1997.65 (2): 729-738.
- Miao D, Fenno JC, Timm JC, Joo NE e Kapila YL. The *Treponema denticola* chymotrypsin-like protease dentilisin induces matrix metalloproteinase-2-dependent fibronectin fragmentation in periodontal ligament cells. *Infect Immun.* 2011.79 (2): 806-811.
- Murphy G. Tissue inhibitors of metalloproteinases. *Genome Biol.* 2011.12 (11): 233.
- Murray GL, Morel V, Cerqueira GM, Croda J, Srikrum A, Henry R, Ko AI, Dellagostin OA, Bulach DM, Sermswan RW, Adler B e Picardeau M. Genome-wide transposon mutagenesis in pathogenic *Leptospira* species. *Infect Immun.* 2009a.77 (2): 810-816.
- Murray GL, Srikrum A, Henry R, Hartskeerl RA, Sermswan RW e Adler B. Mutations affecting *Leptospira* interrogans lipopolysaccharide attenuate virulence. *Mol Microbiol.* 2010.78 (3): 701-709.
- Murray GL, Srikrum A, Henry R, Puapairoj A, Sermswan RW e Adler B. *Leptospira* interrogans requires heme oxygenase for disease pathogenesis. *Microbes Infect.* 2009b.11 (2): 311-314.
- Murray GL, Srikrum A, Hoke DE, Wunder EA, Jr., Henry R, Lo M, Zhang K, Sermswan RW, Ko AI e Adler B. Major surface protein LipL32 is not required for either acute or chronic infection with *Leptospira* interrogans. *Infect Immun.* 2009c.77 (3): 952-958.
- Nahori MA, Fournie-Amazouz E, Que-Gewirth NS, Balloy V, Chignard M, Raetz CR, Saint Girons I e Werts C. Differential TLR recognition of leptospiral lipid A and lipopolysaccharide in murine and human cells. *J Immunol.* 2005.175 (9): 6022-6031.
- Nakai K e Horton P. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem Sci.* 1999.24 (1): 34-36.
- Nakai K e Kanehisa M. Expert system for predicting protein localization sites in gram-negative bacteria. *Proteins.* 1991.11 (2): 95-110.

Nally JE, Artiushin S e Timoney JF. Molecular characterization of thermoinduced immunogenic proteins Q1p42 and Hsp15 of *Leptospira interrogans*. *Infect Immun*. 2001.69 (12): 7616-7624.

Nally JE, Whitelegge JP, Bassilian S, Blanco DR e Lovett MA. Characterization of the outer membrane proteome of *Leptospira interrogans* expressed during acute lethal infection. *Infect Immun*. 2007.75 (2): 766-773.

Nascimento AL, Ko AI, Martins EA, Monteiro-Vitorello CB, Ho PL, Haake DA, Verjovski-Almeida S, Hartskeerl RA, Marques MV, Oliveira MC, Menck CF, Leite LC, Carrer H, Coutinho LL, Degraeve WM, Dellagostin OA, El-Dorry H, Ferro ES, Ferro MI, Furlan LR, Gamberini M, Giglioti EA, Goes-Neto A, Goldman GH, Goldman MH, Harakava R, Jeronimo SM, Junqueira-de-Azevedo IL, Kimura ET, Kuramae EE, Lemos EG, Lemos MV, Marino CL, Nunes LR, de Oliveira RC, Pereira GG, Reis MS, Schrieffer A, Siqueira WJ, Sommer P, Tsai SM, Simpson AJ, Ferro JA, Camargo LE, Kitajima JP, Setubal JC e Van Sluys MA. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. *J Bacteriol*. 2004a.186 (7): 2164-2172.

Nascimento AL, Verjovski-Almeida S, Van Sluys MA, Monteiro-Vitorello CB, Camargo LE, Digiampietri LA, Harstkeerl RA, Ho PL, Marques MV, Oliveira MC, Setubal JC, Haake DA e Martins EA. Genome features of *Leptospira interrogans* serovar Copenhageni. *Braz J Med Biol Res*. 2004b.37 (4): 459-477.

Neves FO, Abreu PA, Vasconcellos SA, de Moraes ZM, Romero EC e Nascimento AL. Identification of a novel potential antigen for early-phase serodiagnosis of leptospirosis. *Arch Microbiol*. 2007.188 (5): 523-532.

Nogueira SV, Fonseca FL, Rodrigues ML, Mundodi V, Abi-Chacra EA, Winters MS, Alderete JF e de Almeida Soares CM. Paracoccidioides brasiliensis enolase is a surface protein that binds plasminogen and mediates interaction of yeast forms with host cells. *Infect Immun*. 2010.78 (9): 4040-4050.

Nowicki ST, Minning-Wenz D, Johnston KH e Lottenberg R. Characterization of a novel streptokinase produced by *Streptococcus equisimilis* of non-human origin. *Thromb Haemost*. 1994.72 (4): 595-603.

O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Cao Y, Moses M, Lane WS, Sage EH e Folkman J. Angiostatin: a circulating endothelial cell inhibitor that suppresses angiogenesis and tumor growth. *Cold Spring Harb Symp Quant Biol*. 1994.59 471-482.

Okada K, Ueshima S, Takaishi T, Yuasa H, Fukao H e Matsuo O. Effects of fibrin and alpha2-antiplasmin on plasminogen activation by staphylokinase. *Am J Hematol*. 1996.53 (3): 151-157.

Oliveira R, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA e Nascimento AL. Characterization of novel OmpA-like protein of *Leptospira interrogans* that binds extracellular matrix molecules and plasminogen. *PLoS One*. 2011.6 (7): e21962.

Oliveira TR, Longhi MT, de Moraes ZM, Romero EC, Blanco RM, Kirchgatter K, Vasconcellos SA e Nascimento AL. Evaluation of leptospiral recombinant antigens MPL17 and MPL21 for serological diagnosis of leptospirosis by enzyme-linked immunosorbent assays. *Clin Vaccine Immunol*. 2008.15 (11): 1715-1722.

- Oliveira TR, Longhi MT, Goncales AP, de Moraes ZM, Vasconcellos SA e Nascimento AL. LipL53, a temperature regulated protein from *Leptospira interrogans* that binds to extracellular matrix molecules. *Microbes Infect.* 2010.12 (3): 207-217.
- Oliver JJ, Webb DJ e Newby DE. Stimulated tissue plasminogen activator release as a marker of endothelial function in humans. *Arterioscler Thromb Vasc Biol.* 2005.25 (12): 2470-2479.
- Orgel D, Schroder W, Hecker-Kia A, Weithmann KU, Kolkenbrock H e Ulbrich N. The cleavage of pro-urokinase type plasminogen activator by stromelysin-1. *Clin Chem Lab Med.* 1998.36 (9): 697-702.
- Palaniappan RU, McDonough SP, Divers TJ, Chen CS, Pan MJ, Matsumoto M e Chang YF. Immunoprotection of recombinant leptospiral immunoglobulin-like protein A against *Leptospira interrogans* serovar Pomona infection. *Infect Immun.* 2006.74 (3): 1745-1750.
- Pantzar M, Ljungh A e Wadstrom T. Plasminogen binding and activation at the surface of *Helicobacter pylori* CCUG 17874. *Infect Immun.* 1998.66 (10): 4976-4980.
- Patarakul K, Lo M e Adler B. Global transcriptomic response of *Leptospira interrogans* serovar Copenhageni upon exposure to serum. *BMC Microbiol.* 2010.10 31.
- Pathirana RD, O'Brien-Simpson NM, Veith PD, Riley PF e Reynolds EC. Characterization of proteinase-adhesin complexes of *Porphyromonas gingivalis*. *Microbiology.* 2006.152 (Pt 8): 2381-2394.
- Patterson BC e Sang QA. Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9). *J Biol Chem.* 1997.272 (46): 28823-28825.
- Perkins DN, Pappin DJ, Creasy DM e Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis.* 1999.20 (18): 3551-3567.
- Picardeau M, Bulach DM, Bouchier C, Zuerner RL, Zidane N, Wilson PJ, Creno S, Kuczek ES, Bommezzadri S, Davis JC, McGrath A, Johnson MJ, Boursaux-Eude C, Seemann T, Rouy Z, Coppel RL, Rood JI, Lajus A, Davies JK, Medigue C e Adler B. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PLoS One.* 2008.3 (2): e1607.
- Pizarro-Cerda J e Cossart P. Subversion of cellular functions by *Listeria monocytogenes*. *J Pathol.* 2006.208 (2): 215-223.
- Plank R e Dean D. Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. *Microbes Infect.* 2000.2 (10): 1265-1276.
- Pozzi A, Moberg PE, Miles LA, Wagner S, Soloway P e Gardner HA. Elevated matrix metalloprotease and angiostatin levels in integrin alpha 1 knockout mice cause reduced tumor vascularization. *Proc Natl Acad Sci U S A.* 2000.97 (5): 2202-2207.
- Probert WS e Johnson BJ. Identification of a 47 kDa fibronectin-binding protein expressed by *Borrelia burgdorferi* isolate B31. *Mol Microbiol.* 1998.30 (5): 1003-1015.

Probert WS, Kim JH, Hook M e Johnson BJ. Mapping the ligand-binding region of *Borrelia burgdorferi* fibronectin-binding protein BBK32. *Infect Immun*. 2001.69 (6): 4129-4133.

Qin JH, Sheng YY, Zhang ZM, Shi YZ, He P, Hu BY, Yang Y, Liu SG, Zhao GP e Guo XK. Genome-wide transcriptional analysis of temperature shift in *L. interrogans* serovar lai strain 56601. *BMC Microbiol*. 2006.6 51.

Que-Gewirth NL, Ribeiro AA, Kalb SR, Cotter RJ, Bulach DM, Adler B, Girons IS, Werts C e Raetz CR. A methylated phosphate group and four amide-linked acyl chains in *leptospira interrogans* lipid A. The membrane anchor of an unusual lipopolysaccharide that activates TLR2. *J Biol Chem*. 2004.279 (24): 25420-25429.

Ra HJ e Parks WC. Control of matrix metalloproteinase catalytic activity. *Matrix Biol*. 2007.26 (8): 587-596.

Ram M, Sherer Y e Shoenfeld Y. Matrix metalloproteinase-9 and autoimmune diseases. *J Clin Immunol*. 2006.26 (4): 299-307.

Ratel D, Mihoubi S, Beaulieu E, Durocher Y, Rivard GE, Gingras D e Beliveau R. VEGF increases the fibrinolytic activity of endothelial cells within fibrin matrices: involvement of VEGFR-2, tissue type plasminogen activator and matrix metalloproteinases. *Thromb Res*. 2007.121 (2): 203-212.

Rautemaa R e Meri S. Complement-resistance mechanisms of bacteria. *Microbes Infect*. 1999.1 (10): 785-794.

Reis RB, Ribeiro GS, Felzemburgh RD, Santana FS, Mohr S, Melendez AX, Queiroz A, Santos AC, Ravines RR, Tassinari WS, Carvalho MS, Reis MG e Ko AI. Impact of environment and social gradient on *Leptospira* infection in urban slums. *PLoS Negl Trop Dis*. 2008.2 (4): e228.

Ren SX, Fu G, Jiang XG, Zeng R, Miao YG, Xu H, Zhang YX, Xiong H, Lu G, Lu LF, Jiang HQ, Jia J, Tu YF, Jiang JX, Gu WY, Zhang YQ, Cai Z, Sheng HH, Yin HF, Zhang Y, Zhu GF, Wan M, Huang HL, Qian Z, Wang SY, Ma W, Yao ZJ, Shen Y, Qiang BQ, Xia QC, Guo XK, Danchin A, Saint Girons I, Somerville RL, Wen YM, Shi MH, Chen Z, Xu JG e Zhao GP. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. *Nature*. 2003.422 (6934): 888-893.

Ristow P, Bourhy P, da Cruz McBride FW, Figueira CP, Huerre M, Ave P, Girons IS, Ko AI e Picardeau M. The OmpA-like protein Loa22 is essential for leptospiral virulence. *PLoS Pathog*. 2007.3 (7): e97.

Rojas M, Labrador I, Concepcion JL, Aldana E e Avilan L. Characteristics of plasminogen binding to *Trypanosoma cruzi* epimastigotes. *Acta Trop*. 2008.107 (1): 54-58.

Rooijakkers SH e van Strijp JA. Bacterial complement evasion. *Mol Immunol*. 2007.44 (1-3): 23-32.

Rooijakkers SH, van Wamel WJ, Ruyken M, van Kessel KP e van Strijp JA. Anti-opsonic properties of staphylokinase. *Microbes Infect*. 2005.7 (3): 476-484.

- Rossmann E, Kraiczy P, Herzberger P, Skerka C, Kirschfink M, Simon MM, Zipfel PF e Wallich R. Dual binding specificity of a *Borrelia hermsii*-associated complement regulator-acquiring surface protein for factor H and plasminogen discloses a putative virulence factor of relapsing fever spirochetes. *J Immunol.* 2007.178 (11): 7292-7301.
- Ruoslahti E. RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol.* 1996.12 697-715.
- Salo T, Lyons JG, Rahemtulla F, Birkedal-Hansen H e Larjava H. Transforming growth factor-beta 1 up-regulates type IV collagenase expression in cultured human keratinocytes. *J Biol Chem.* 1991.266 (18): 11436-11441.
- Sang QX. Complex role of matrix metalloproteinases in angiogenesis. *Cell Res.* 1998.8 (3): 171-177.
- Sato Y, Uemura T, Morimitsu K, Sato-Nishiuchi R, Manabe R, Takagi J, Yamada M e Sekiguchi K. Molecular basis of the recognition of nephronectin by integrin $\alpha 8 \beta 1$. *J Biol Chem.* 2009.284 (21): 14524-14536.
- Sbardella D, Fasciglione GF, Gioia M, Ciaccio C, Tundo GR, Marini S e Coletta M. Human matrix metalloproteinases: an ubiquitous class of enzymes involved in several pathological processes. *Mol Aspects Med.* 2012.33 (2): 119-208.
- Schlott B, Guhrs KH, Hartmann M, Rocker A e Collen D. NH₂-terminal structural motifs in staphylokinase required for plasminogen activation. *J Biol Chem.* 1998.273 (35): 22346-22350.
- Schultz J, Milpetz F, Bork P e Ponting CP. SMART, a simple modular architecture research tool: identification of signaling domains. *Proc Natl Acad Sci U S A.* 1998.95 (11): 5857-5864.
- Senaratne RH, Sidders B, Sequeira P, Saunders G, Dunphy K, Marjanovic O, Reader JR, Lima P, Chan S, Kendall S, McFadden J e Riley LW. *Mycobacterium tuberculosis* strains disrupted in *mce3* and *mce4* operons are attenuated in mice. *J Med Microbiol.* 2008.57 (Pt 2): 164-170.
- Severi E, Hood DW e Thomas GH. Sialic acid utilization by bacterial pathogens. *Microbiology.* 2007.153 (Pt 9): 2817-2822.
- Seya T, Nagasawa S, Matsukura M, Hasegawa H e Atkinson JP. Generation of C3d,g and C3d by urokinase-treated plasma in association with fibrinolysis. *Complement.* 1985.2 (2-3): 165-174.
- Sheldon IM e Dobson H. Reproductive challenges facing the cattle industry at the beginning of the 21st century. *Reprod Suppl.* 2003.61 1-13.
- Siemens N, Patenge N, Otto J, Fiedler T e Kreikemeyer B. *Streptococcus pyogenes* M49 plasminogen/plasmin binding facilitates keratinocyte invasion via integrin-integrin-linked kinase (ILK) pathways and protects from macrophage killing. *J Biol Chem.* 2011.286 (24): 21612-21622.
- SINAN/CVE/CCD/SES-SP. Acessado em 05 junho, 2012. Disponível no endereço http://www.cve.saude.sp.gov.br/html/zoo/LEPTO_GVE11.htm.

SINAN/SVS/MS. Acessado em 05 junho, 2012. Disponível no endereço http://portal.saude.gov.br/portal/arquivos/pdf/casos_lepto_25_05_11.pdf.

Snoek-van Beurden PA e Von den Hoff JW. Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *Biotechniques*. 2005.38 (1): 73-83.

Sodeinde OA, Subrahmanyam YV, Stark K, Quan T, Bao Y e Goguen JD. A surface protease and the invasive character of plague. *Science*. 1992.258 (5084): 1004-1007.

Stanworth DR e Turner MW (1986). Immunochemical analysis of human and rabbit immunoglobulins and their subunits. *Handbook of experimental immunology*. DM W. London, UK., Blackwell Scientific: 12.11e12.45.

Stevenson B, Choy HA, Pinne M, Rotondi ML, Miller MC, Demoll E, Kraiczy P, Cooley AE, Creamer TP, Suchard MA, Brissette CA, Verma A e Haake DA. *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. *PLoS One*. 2007.2 (11): e1188.

Stie J, Bruni G e Fox D. Surface-associated plasminogen binding of *Cryptococcus neoformans* promotes extracellular matrix invasion. *PLoS One*. 2009.4 (6): e5780.

Sun H. The interaction between pathogens and the host coagulation system. *Physiology (Bethesda)*. 2006.21 281-288.

Suomalainen M, Haiko J, Ramu P, Lobo L, Kukkonen M, Westerlund-Wikstrom B, Virkola R, Lahteenmaki K e Korhonen TK. Using every trick in the book: the Pla surface protease of *Yersinia pestis*. *Adv Exp Med Biol*. 2007.603 268-278.

Thongboonkerd V. Proteomics in leptospirosis research: towards molecular diagnostics and vaccine development. *Expert Rev Mol Diagn*. 2008.8 (1): 53-61.

Toledo A, Coleman JL, Kuhlow CJ, Crowley JT e Benach JL. The enolase of *Borrelia burgdorferi* is a plasminogen receptor released in outer membrane vesicles. *Infect Immun*. 2012.80 (1): 359-368.

Toyokawa T, Ohnishi M e Koizumi N. Diagnosis of acute leptospirosis. *Expert Rev Anti Infect Ther*. 2011.9 (1): 111-121.

Travis J, Potempa J e Maeda H. Are bacterial proteinases pathogenic factors? *Trends Microbiol*. 1995.3 (10): 405-407.

Travis J e Salvesen GS. Human plasma proteinase inhibitors. *Annu Rev Biochem*. 1983.52 655-709.

Ugwu F, Van Hoef B, Bini A, Collen D e Lijnen HR. Proteolytic cleavage of urokinase-type plasminogen activator by stromelysin-1 (MMP-3). *Biochemistry*. 1998.37 (20): 7231-7236.

Urano T, Chibber BA e Castellino FJ. The reciprocal effects of epsilon-aminohexanoic acid and chloride ion on the activation of human [Glu1]plasminogen by human urokinase. *Proc Natl Acad Sci U S A*. 1987.84 (12): 4031-4034.

- Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA e Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol*. 2002.37 (6): 375-536.
- Verma A, Brissette CA, Bowman AA, Shah ST, Zipfel PF e Stevenson B. Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen. *Infect Immun*. 2010.78 (5): 2053-2059.
- Verma A, Hellwage J, Artiushin S, Zipfel PF, Kraiczy P, Timoney JF e Stevenson B. LfhA, a novel factor H-binding protein of *Leptospira interrogans*. *Infect Immun*. 2006.74 (5): 2659-2666.
- Vieira ML. Interação de *Leptospira interrogans* com o sistema proteolítico do plasminogênio/plasmina: análise, caracterização e possíveis implicações na infecção. Tese (Doutorado em Biotecnologia). Universidade de São Paulo, Instituto de Ciências Biomédicas, São Paulo, Brasil, 2012.
- Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, Vasconcellos SA e Nascimento AL. In vitro identification of novel plasminogen-binding receptors of the pathogen *Leptospira interrogans*. *PLoS One*. 2010a.5 (6): e11259.
- Vieira ML, de Moraes ZM, Goncales AP, Romero EC, Vasconcellos SA e Nascimento AL. Lsa63, a newly identified surface protein of *Leptospira interrogans* binds laminin and collagen IV. *J Infect*. 2010b.60 (1): 52-64.
- Vieira ML, de Moraes ZM, Vasconcellos SA, Romero EC e Nascimento AL. In vitro evidence for immune evasion activity by human plasmin associated to pathogenic *Leptospira interrogans*. *Microb Pathog*. 2011.51 (5): 360-365.
- Vieira ML, Vasconcellos SA, Goncales AP, de Moraes ZM e Nascimento AL. Plasminogen acquisition and activation at the surface of leptospira species lead to fibronectin degradation. *Infect Immun*. 2009.77 (9): 4092-4101.
- Vinetz JM. Leptospirosis. *Curr Opin Infect Dis*. 2001.14 (5): 527-538.
- Vinh T, Faine S e Adler B. Adhesion of leptospire to mouse fibroblasts (L929) and its enhancement by specific antibody. *J Med Microbiol*. 1984.18 (1): 73-85.
- Virkola R, Lahteenmaki K, Eberhard T, Kuusela P, van Alphen L, Ullberg M e Korhonen TK. Interaction of *Haemophilus influenzae* with the mammalian extracellular matrix. *J Infect Dis*. 1996.173 (5): 1137-1147.
- Wang B, Sullivan J, Sullivan GW e Mandell GL. Interaction of leptospire with human polymorphonuclear neutrophils. *Infect Immun*. 1984.44 (2): 459-464.
- Werts C, Tapping RI, Mathison JC, Chuang TH, Kravchenko V, Saint Girons I, Haake DA, Godowski PJ, Hayashi F, Ozinsky A, Underhill DM, Kirschning CJ, Wagner H, Aderem A, Tobias PS e Ulevitch RJ. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat Immunol*. 2001.2 (4): 346-352.
- WHO WHO (2003). Human Leptospirosis: Guidance for Diagnosis, Surveillance and Control. Geneva, Switzerland.

- Xolalpa W, Vallecillo AJ, Lara M, Mendoza-Hernandez G, Comini M, Spallek R, Singh M e Espitia C. Identification of novel bacterial plasminogen-binding proteins in the human pathogen *Mycobacterium tuberculosis*. *Proteomics*. 2007.7 (18): 3332-3341.
- Yan W, Faisal SM, McDonough SP, Chang CF, Pan MJ, Akey B e Chang YF. Identification and characterization of OmpA-like proteins as novel vaccine candidates for *Leptospirosis*. *Vaccine*. 2010.28 (11): 2277-2283.
- Yan Y, Chen Y, Liou W, Ding J, Chen J, Zhang J, Zhang A, Zhou W, Gao Z, Ye X e Xiao Y. An evaluation of the serological and epidemiological effects of the outer envelope vaccine to leptospira. *J Chin Med Assoc*. 2003.66 (4): 224-230.
- Yavlovich A, Higazi AA e Rottem S. Plasminogen binding and activation by *Mycoplasma fermentans*. *Infect Immun*. 2001.69 (4): 1977-1982.
- Young KC, Shi GY, Wu DH, Chang LC, Chang BI, Ou CP e Wu HL. Plasminogen activation by streptokinase via a unique mechanism. *J Biol Chem*. 1998.273 (5): 3110-3116.
- Yu CS, Lin CJ e Hwang JK. Predicting subcellular localization of proteins for Gram-negative bacteria by support vector machines based on n-peptide compositions. *Protein Sci*. 2004.13 (5): 1402-1406.
- Zhang L, Zhang C, Ojcius DM, Sun D, Zhao J, Lin X, Li L, Li L e Yan J. The mammalian cell entry (Mce) protein of pathogenic *Leptospira* species is responsible for RGD motif-dependent infection of cells and animals. *Mol Microbiol*. 2012.83 (5): 1006-1023.
- Zipfel PF, Wurzner R e Skerka C. Complement evasion of pathogens: common strategies are shared by diverse organisms. *Mol Immunol*. 2007.44 (16): 3850-3857.
- Zucker S, Lysik RM, Gurfinkel M, Zarrabi MH, Stetler-Stevenson W, Liotta LA, Birkedal-Hansen H e Mann W. Immunoassay of type IV collagenase/gelatinase (MMP-2) in human plasma. *J Immunol Methods*. 1992.148 (1-2): 189-198.

APÊNDICE A - Características das proteínas recombinantes de *L. interrogans* selecionadas para análise de interação com PLG purificado.

Locus gênico, nome dado à proteína, número de referência do banco de dados NCBI, características, conservação gênica, sequencia de oligonucleotídeos utilizados para amplificação por PCR, e massa molecular esperada. ¹ <http://aeg.lbi.ic.unicamp.br/world/lic/> (Nascimento et al., 2004a); ² <http://www.ncbi.nlm.nih.gov/protein/>; ³ Protein BLAST - <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi> (Altschul et al., 1990; Altschul et al., 1997); Previamente publicado por ^a(Gamberini et al., 2005); ^b(Vieira et al., 2010b); ^c(Gomez et al., 2008); ^d(Neves et al., 2007); ^e(Longhi et al., 2009); ^f(Oliveira et al., 2008); ^g(Barbosa et al., 2006); Lai: *L. interrogans* serovar Lai (Ren et al., 2003); LBH: *L. borgpetersenii* serovar Hardjo-bovis (Bulach et al., 2006); LBP: *L. biflexa* serovar Patoc (Picardeau et al., 2008).

Locus gênico ¹	Nome proteína recombinante	Número de referência NCBI ²	Descrição/Função	Conservação (identidade) ³	Sequencia de oligonucleotídeos para amplificação por PCR	Massa molecular recombinantes (kDa)
LIC11352	LipL32 ^a	YP_001316	Major outer membrane protein (MOMP), LipL32 lipoprotein	Lai (100 %); LBH (98 %)	F: 5' CACCGGTGCTTTCGGTGGTCTG 3' R: 5' ATTACTTAGTCGCGTCAGAAGC 3'	30.2
LIC10314	Lsa63 ^b	YP_000304	Conserved hypothetical protein with Borrelia_P83 domain	Lai (98 %); LBH (87 %) LBP (39 %)	F: 5' GGATCCTTATTTCTCAGGAAAG 3' (BamH I) R: 5' GGTACCTAAGGTTTAATTTTTT 3' (Kpn I)	63.0
LIC10509	rLIC10509 ^c	YP_000493	Putative lipoprotein	Lai (98%)	F: 5' CCGGGATCCAAAAGAGCAAGAAG 3' (BamH I) R: 5' GGTACCTACTCGAGACAGCCAGGACCTTC 3' (Kpn I)	22.0
LIC12892	Lp29 ^d	YP_002808	Putative lipoprotein		F: 5' CTCGAGGCGAGTACATTACAATCTTGCT 3' (Xho I) R: 5' CCATGGCTCTTAGGAGCCTGGAAA 3' (Nco I)	29.6
LIC10793	Lp49 ^d	YP_000772	Putative lipoprotein, Surface antigen	Lai (99%); LBH (86%)	F: 5' CTCGAGAGCGGAGACTTTTCTTACTT 3' (Xho I) R: 5' CCATGGTTAAAAACCATCTCTACGATAAAC 3' (Nco I)	49.1
LIC12895	Lsa27 ^e	YP_002811	Putative lipoprotein	Lai (79%)	F: 5' GGATCCCTGAAATATACGAA 3' (EcoRI) R: 5' GAATTCCTACTGTTCTCCTTC 3' (BamHI)	27.0
LIC13131	MPL21 ^{a,f}	YP_003039	Hypothetical protein with Ycel domain	Lai (98%); LBP (45%)	F: 5' CACCACGTCTCAAAGTTACGCTTCAG 3' R: 5' TTCTCACCATCCAGCTCGG 3'	21.9
LIC10765	MPL17 ^{a,f}	YP_000745	Conserved hypothetical protein	Lai (100%); LBH (80%) LBP (41%)	F: 5' CACCGAAAGTCCCGTAAGGTTCAAA 3' R: 5' TGCAGGAGTCCACATTTTA 3'	15.4
LIC10091	LipL40 ^a	YP_000088	Putative lipoprotein	Lai (100%); LBH (84%)	F: 5' CCATGGGACTCGAGACGCTCCTCCTAAAGATCC 3' R: 5' CTCCATGGTCATTTCAAACTTCTACGGGGC 3'	39.0
LIC10054	MPL36 ^a	YP_000054	Putative lipoprotein with Rare lipoprotein A (RplA) like domain	Lai (100%); LBH (88%) LBP (50%)	F: 5' CACCACGTCTTGTGCGTCGCTAGAG 3' R: 5' CCAAGTATTCTATTATACGTCCGAG 3'	35.1
LIC10494	rLIC10494 ^g	YP_000478	Putative lipoprotein	Lai (99%)	F: 5' CACCACTGCTAGGGCTGCAGAAA 3' R: 5' ACTTTGAGAGCTTCGTCTCGT 3'	25.8
LIC12730	rLIC12730 ^g	YP_002650	Hypothetical protein with TPR domain and 4 NHL repetition	Lai (100%); LBH (90%) LBP (37%)	F: 5' CACCACTTCTGACGGACTTCCCAA 3' R: 5' TCTTGCGAATGAGTTGATCC 3'	77.4
LIC12922	rLIC12922 ^h	YP_002837	Conserved hypothetical protein	Lai (100%); LBH (89%) LBP (48%)	F: 5' CACCGAATCACTCAACAGATCATTGC 3' R: 5' ATCAATCTAAATGAAACGTCTCTTC 3'	40.0
LIC12238	rLIC12238 ^h	YP_002173	Putative lipoprotein	Lai (100%); LBH (81%) LBP (39%)	F: 5' CTCGAGTGTTTTAAACCTACCGGAG 3' (Xho I) R: 5' AAGCTTCTACTTCATCGCTTTTCTATATC 3' (Hind III)	17.6

ANEXO A - Artigos publicados

- Artigo 1** - Vieira ML, Vasconcellos SA, Goncales AP, de Moraes ZM e Nascimento AL. Plasminogen acquisition and activation at the surface of leptospira species lead to fibronectin degradation. Infect Immun. 2009.77 (9): 4092-4101.
- Artigo 2** - Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, Vasconcellos SA e Nascimento AL. In vitro identification of novel plasminogen-binding receptors of the pathogen *Leptospira interrogans*. PLoS One. 2010a.5 (6): e11259.
- Artigo 3** - Vieira ML, de Moraes ZM, Vasconcellos SA, Romero EC e Nascimento AL. In vitro evidence for immune evasion activity by human plasmin associated to pathogenic *Leptospira interrogans*. Microb Pathog. 2011.51 (5): 360-365.
- Artigo 4** - Domingos RF, Vieira ML, Romero EC, Goncales AP, Moraes ZM, Vasconcellos SA e Nascimento AL. Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions. BMC Microbiol. 2012.12 (1): 50.

ANEXO B - Artigos aceitos para publicação

Artigo 1 - Vieira ML, Atzingen MV, Oliveira R, Mendes RS, Domingos RF, Vasconcellos SA e Nascimento ALTO. Plasminogen binding, protein receptors and plasmin generation on the surface of *Leptospira* spp. - the contribution to the bacteria-host interactions. (Review). Journal of Biomedicine and Biotechnology.

Artigo 2 - Souza NM, Vieira ML, Alves IJ, Morais ZM, Vasconcellos SA e Nascimento ALTO. Lsa30, a novel adhesin of *Leptospira interrogans* binds human plasminogen and the complement regulator C4bp. Microbial Pathogenesis.

ANEXO C - Artigos submetidos

Artigo 1 - Vieira ML, Alvarez-Flores MP, Kirchgatter K, Romero EC, Alves IJ, Morais ZM, Vasconcellos SA, Chudzinski-Tavassi AM and Nascimento ALTO. *Leptospira interrogans* interaction with human proteolytic systems enhances dissemination through endothelial cells and protease levels. PLoS Neglected Tropical Diseases.

Artigo 2 – Fernandes LG, Vieira ML, Kirchgatter K, Romero EC, Morais ZM, Vasconcellos SA, and Nascimento ALTO. OmpL1 is an extracellular matrix- and plasminogen-interacting protein of *Leptospira* spp. Infection and Immunity.

Plasminogen Acquisition and Activation at the Surface of *Leptospira* Species Lead to Fibronectin Degradation[▽]

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Received 26 March 2009/Returned for modification 29 April 2009/Accepted 26 June 2009

Pathogenic *Leptospira* species are the etiological agents of leptospirosis, a widespread disease of human and veterinary concern. In this study, we report that *Leptospira* species are capable of binding plasminogen (PLG) *in vitro*. The binding to the leptospiral surface was demonstrated by indirect immunofluorescence confocal microscopy with living bacteria. The PLG binding to the bacteria seems to occur via lysine residues because the ligation is inhibited by addition of the lysine analog 6-aminocaproic acid. Exogenously provided urokinase-type PLG activator (uPA) converts surface-bound PLG into enzymatically active plasmin, as evaluated by the reaction with the chromogenic plasmin substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride. The PLG activation system on the surface of *Leptospira* is PLG dose dependent and does not cause injury to the organism, as cellular growth in culture was not impaired. The generation of active plasmin within *Leptospira* was observed with several nonvirulent high-passage strains and with the nonpathogenic saprophytic organism *Leptospira biflexa*. Statistically significant higher activation of plasmin was detected with a low-passage infectious strain of *Leptospira*. Plasmin-coated virulent *Leptospira interrogans* bacteria were capable of degrading purified extracellular matrix fibronectin. The breakdown of fibronectin was not observed with untreated bacteria. Our data provide for the first time *in vitro* evidence for the generation of active plasmin on the surface of *Leptospira*, a step that may contribute to leptospiral invasiveness.

The spirochete *Leptospira interrogans* is a highly invasive pathogen and the causal agent of leptospirosis, one of the most widespread zoonoses of human and veterinary concern (7, 20, 25, 39, 76). The disease occurs mainly in peripheral metropolitan regions lacking adequate sanitary conditions during activities that involve direct contact with contaminated water, soil, or animals (25, 36, 76). Humans are accidental and terminal hosts in the transmission process of leptospirosis (20, 65). The leptospires enter the body via abrasions on skin or actively through mucosa, spreading to any tissue, but particularly colonizing kidneys and liver (39).

Despite its importance and the genomic sequencing of five strains of *Leptospira*, four pathogenic (9, 57, 66) and one saprophytic (64), molecular aspects of the pathogenesis, virulence, and invasion processes by which the leptospires infect the hosts and initiate tissue colonization are poorly characterized. To date, few virulence factors contributing to the pathogenesis of the disease have been identified (3, 48, 67).

It is known that one characteristic of leptospiral infection is the rapid dissemination within the host and colonization of renal tubules that constitute immunologically safe environments (20). The ability of the leptospires to adhere to extracellular matrix (ECM) macromolecules has been shown (4), and to date a few adhesins, ECM-binding proteins, have been

identified (4, 11, 29, 30, 72). After adherence, the next step must be to overcome the barriers imposed by epithelial tissues and ECMs. For this, the proteolytic activity achieved by subversion of host proteases by pathogens, such as plasmin, has been demonstrated to be important during several bacterial infections (37).

Plasmin is a broad-spectrum serine protease component of the fibrinolytic system, which has plasminogen (PLG) as the main component. It has been shown that several pathogens, including the spirochete *Borrelia burgdorferi*, bind PLG on the surface and convert it to plasmin by host activators (6, 13, 16, 19, 22, 31, 34, 37, 38, 60, 68, 73); this binding promotes degradation of ECM components and is essential for dissemination of the bacteria through the host tissues, suggesting its role during infection and pathogenesis (12, 14, 15, 28, 37, 58).

Based on these assertions, we were prompted to investigate the ability of pathogenic *L. interrogans* to bind PLG. We show in this work by *in vitro* assays that leptospires are capable of capturing PLG in its outer surface, that the conversion to enzymatically active plasmin could be achieved by an exogenous source, and that the active plasmin generated on the surface of *Leptospira* can degrade the fibronectin ECM component. Outer membrane proteins (OMPs) are involved with PLG acquisition, but aqueous soluble proteins also contribute to the binding. Neither temperature shift to the mammalian body, under normal and febrile conditions, nor physiologic osmolarity affected plasmin generation by leptospires. We also demonstrate a significant difference in the plasminogen acti-

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[▽] Published ahead of print on 6 July 2009.

vation system (PAS) between infectious and noninfectious leptospires, suggesting that this feature might have a role in leptospiral virulence.

MATERIALS AND METHODS

Bacterial isolates and culture conditions. Virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 is routinely cultured at Faculdade de Medicina Veterinária da Universidade de São Paulo by iterative passages in Golden Syrian hamsters for maintenance of virulence. Recently weaned hamsters were intraperitoneally infected with 500 μ l containing $\sim 1.0 \times 10^4$ virulent leptospires (50% lethal dose [LD_{50}] of $\sim 10^3$ leptospires/animal). The animals were sacrificed after appearance of symptoms such as loss of weight and mobility (~ 5 days postinfection). Kidney and liver were removed and macerated. The organ-derived leptospires were cultured at 28°C in semisolid modified Elinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with 10% rabbit sera. On the other hand, nonvirulent attenuated leptospires are equally routinely cultured by maintenance in culture in liquid modified EMJH medium supplemented with 10% rabbit sera. Animals infected with these high-passage attenuated-leptospirose-derived strains do not show any sign of the disease. The nonvirulent *Leptospira* strains used were: *L. interrogans* serovar Canicola strain Hound Utrecht IV, *L. interrogans* serovar Copenhageni strain M 20, *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. interrogans* serovar Pomona strain Pomona, *Leptospira borgpetersenii* serovar Castellonis strain Castellon 3, *L. borgpetersenii* serovar Whitcombi strain Whitcomb, *Leptospira kirshneri* serovar Cynoptery strain 3522 C, *L. kirshneri* serovar Grippotyphosa strain Moskva V, *Leptospira santarosai* serovar Shermani strain 1342 K, and *Leptospira biflexa* serovar Patoc strain Patoc.

For the experiments with PLG binding, to exclude PLG interference from the rabbit serum supplementing the culture medium, serum-free leptospires were obtained by three passages in liquid modified EMJH medium supplemented with 10% *Leptospira* enrichment EMJH medium (BD, Difco), cultured at 28°C. For the temperature shift experiments, the bacteria were incubated for 24 h at 37 or 39°C. For the osmolarity shift analysis, 120 mM NaCl was added to the EMJH medium supplemented with 10% *Leptospira* enrichment EMJH medium, followed by incubation at 28°C for 24 h.

Labeling of leptospires with plasmin. A total of 7.0×10^9 leptospires were centrifuged at $6,000 \times g$ for 10 min at 25°C, resuspended in 1.4 ml of EMJH culture medium supplemented with 10% *Leptospira* enrichment EMJH medium, divided into seven aliquots of 0.2 ml each (1.0×10^9 leptospires) in 2-ml microcentrifuge tubes, and recentrifuged. The seven tubes containing the leptospires received different treatments: (i) 30% plasma and 3 U urokinase-type uroplasminogen activator (uPA) (urokinase; Sigma) in 100 μ l low-salt phosphate-buffered saline (lsPBS; with 50 mM NaCl), (ii) 5 μ g PLG (native PLG purified from human plasma was from Merck), and 3 U uPA in 100 μ l lsPBS, (iii) 2 μ g PLG and 3 U uPA in 100 μ l lsPBS, (iv) 0.5 μ g PLG and 3 U uPA in 100 μ l lsPBS, (v) 5 μ g PLG in 100 μ l lsPBS, (vi) 3 U uPA in 100 μ l lsPBS, and (vii) 100 μ l lsPBS. All of the preparations were incubated for 1 h at 37°C, under gentle shaking, prior to the addition of the uPA, and followed by 1 h of incubation at 37°C. Leptospires were then centrifuged and washed three times with 0.7 ml lsPBS.

Measurement of enzymatic activity of plasmin-coated leptospires. The treated leptospires (1.0×10^9 per sample) were resuspended in 300 μ l lsPBS and divided into three aliquots. Each aliquot received 100 μ l of 0.5 mg/ml of the chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) in lsPBS, to a final substrate concentration of 0.25 mg/ml. The suspensions were incubated for 1.5 h at 37°C under gentle shaking and then centrifuged at $6,000 \times g$ for 10 min at 25°C. The supernatants (150 μ l) were transferred to 96-well microplates, and the cleavage of the specific plasmin substrate was quantified with a microplate reader set at a wavelength of 405 nm.

ACA binding-inhibition assay. Low-passage virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 leptospires (1.0×10^9 leptospires/sample) were treated with PLG and uPA, as described above, except for the addition of increasing concentrations of 6-aminocaproic acid (ACA; Sigma) ranging from 0 to 1,000 mM, added together with the PLG. The PLG quantity was set at 5 μ g and that of uPA was set at 3 U. Aliquots treated with only PLG, uPA, or ACA and those treated with just lsPBS were used as controls. The enzymatic plasmin activity was measured as described before.

Isolation of leptospiral OMPs and cytoplasmic fractions by TX-114 partitioning. Leptospires cultured as outlined before were washed in PBS-5 mM $MgCl_2$ and then extracted in the presence of 2% Triton X-114 (TX-114; Sigma-Aldrich), 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 1 mM phenylmethylsulfonyl fluoride on ice for 4 h (24). The insoluble material was removed by

centrifugation at $17,000 \times g$ for 10 min at 4°C. After centrifugation, the TX-114 soluble fraction was stored at $-20^\circ C$ overnight and then thawed on ice. Phase separation was performed by warming the supernatant at 37°C and subjecting it to centrifugation for 10 min at $1,000 \times g$. Three distinct fractions became apparent: the aqueous phase (AP), the TX-114 phase (detergent phase [DP]), and the insoluble pellet. The DP was washed with 0.06% TX-114, 150 mM NaCl, and 10 mM Tris-HCl (pH 7.4) by incubation for 10 min on ice followed by 37°C incubation and centrifugation. The washing step was performed one more time, and after incubation for 10 min on ice, the solution was centrifuged at $17,000 \times g$, and the upper phase (TX-114) was incubated at 37°C for 10 min. After centrifugation, the DP was precipitated with acetone. All protein fractions were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for analysis of the extraction.

PLG binding to extracted proteins. The binding of the proteins contained in the AP and DP or whole-cell lysate to PLG was evaluated by a modified enzyme-linked immunosorbent assay (ELISA), as follows. Ninety-six-well plates (Costar high binding; Corning) were coated overnight in PBS at 4°C with 0.15 μ g/well of proteins or bovine serum albumin (BSA), as a negative control. Plates were washed once with PBS supplemented with 0.05% (vol/vol) Tween 20 (PBS-T) and blocked for 2 h at 37°C with PBS with 10% (mass/vol) nonfat dry milk. The blocking solution was discarded, and 100 μ l of 10 μ g/ml of PLG in PBS was incubated for 2 h at 37°C. Wells were washed four times with PBS-T and incubated for 1 h at 37°C with mouse anti-human PLG (Sigma-Aldrich) (1:4,000 in PBS). Plates were washed again and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG), diluted 1:5,000 in PBS. After three washings, 1 mg/ml *o*-phenylenediamine (OPD) plus 1 μ l/ml H_2O_2 in citrate phosphate buffer (pH 5.0) was added at 100 μ l/well. The reactions were carried out for 5 min and stopped by addition of 50 μ l/well of 2 N H_2SO_4 . Readings were taken at 492 nm.

Growth monitoring of plasmin-coated leptospires in culture. Virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (1.0×10^9 bacteria/sample) were treated with the PAS as described above; after the treatment, the bacteria were centrifuged and the cell pellets were resuspended in 4 ml of fresh EMJH medium supplemented with 10% *Leptospira* enrichment EMJH medium. The bacterial cultures were incubated under aerobic conditions at 28°C. The growth was monitored by optical density measurements at 600 nm (OD_{600}), where an OD_{600} of 0.2 corresponds to $\sim 9.6 \times 10^8$ leptospires), at the 2nd, 6th, 10th, and 14th days. All of the solutions used were filter sterilized prior to use.

L-IFA. For the liquid-phase immunofluorescence assay (L-IFA), live bacterial suspensions (2.5×10^9) were harvested at $12,800 \times g$ for 15 min, washed twice with lsPBS, resuspended in 200 μ l lsPBS containing 8 μ g of human PLG, and incubated for 45 min at 37°C. After the incubation, 6 μ g/ml of propidium iodide (Sigma) was added to stain the nuclei, and the suspensions were incubated for another 45 min at 37°C. After this time, the leptospires were gently washed three times with lsPBS and incubated for 45 min at 37°C with mouse-produced anti-serum against human PLG at a 1:50 dilution. The leptospires were washed three times and incubated with goat anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC; Sigma) at a dilution of 1:50 for 45 min at 37°C. After this incubation, the leptospires were washed twice and resuspended in lsPBS-antifading solution (ProLong Gold; Molecular Probes). The immunofluorescence-labeled leptospires were examined by use of a confocal LSM 510 META immunofluorescence microscope (Zeiss, Germany). As a control for cell integrity, we used antibodies against recombinant *Leptospira* protein LipL32 or GroEL, following all of the previously mentioned procedures for PLG.

SDS-PAGE and affinity blotting. Total leptospiral protein extracts for SDS-PAGE were prepared from 10 ml of $\sim 10^9$ bacteria in EMJH serum-free cultures. The cells were harvested by centrifugation, washed three times with 5 mM $MgCl_2$ in lsPBS, and resuspended in 100 μ l PBS. The proteins were loaded for 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-ECL; GE Healthcare) in semidry equipment. The membranes were blocked for 2 h at 37°C with 5% BSA, washed three times (10 min for each wash) with PBS-T solution, and incubated overnight with 3 μ g/ml PLG or 3 μ g/ml PLG plus 100 mM ACA at 4°C, followed by a 2-h incubation at room temperature. Then, the membranes were washed three times and incubated with mouse anti-human PLG (1:750) for 3 h at room temperature, followed by more three washings and 1 h of incubation at room temperature with anti-mouse IgG (1:5,000). The membranes were washed, and the protein's reactivity was revealed by the ECL enhanced chemiluminescence reagent (GE Healthcare) with subsequent exposition to X-ray films.

Assay for the degradation of fibronectin. Ninety-six-well plates were coated overnight at 37°C with 0.5 μ g/well of cellular fibronectin (Sigma), washed four times at 200 μ l per wash with PBS-T, and blocked with 2% BSA in PBS-T for 2 h at 37°C, followed by two washings. The spirochetes (1.0×10^8 leptospires per sample) were treated as described above with 10 μ g PLG and 3 U uPA or lsPBS (untreated). Bacteria were washed, resuspended in 100 μ l lsPBS, and transferred

to the plate's previously coated wells. The plates were centrifuged at $180 \times g$ for 15 min to ensure the contact of the leptospires with the immobilized ECM component. The plates were incubated at 37°C for 20 h and washed five times to remove the bacteria. The degradation of fibronectin was detected by reduction in absorbance followed by incubation with anti-fibronectin IgG antibodies (1:5,000 dilution in 100 μl PBS-T for 45 min at 37°C), anti-IgG peroxidase-conjugated antibodies (1:5,000 dilution in 100 μl PBS-T for 45 min at 37°C) and 100 μl /well of 1 mg/ml OPD plus 1 μl /ml H_2O_2 . The reaction was stopped with 50 μl /well 4 N H_2SO_4 , and the absorbance was measured at 492 nm. Percentage degradation was calculated by the formula $(A - B)/A(100)$, where A is the mean PBS sample absorbance (positive control group for fibronectin reactivity) and B is the mean experimental group absorbance (untreated or plasmin).

Statistics. For the data that were tested for statistical significance, Student's two-tailed test was applied, considering the minimum significance at $P < 0.05$.

RESULTS

Binding of human PLG by *L. interrogans* cells. The ability of live *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 cells to bind human PLG was examined via L-IFA. Leptospirals were visualized by propidium iodide staining (Fig. 1A) (PLG1, PLG2, PLG3, LipL32, GroEL, and PBS) followed by protein detection with polyclonal mouse antiserum against each protein in the presence of anti-mouse IgG antibodies conjugated to FITC. In Fig. 1B, green fluorescence could be observed for PLG (PLG1, PLG2, and PLG3) and LipL32, an outer membrane protein used as a surface-positive control (56), but not with GroEL, a protoplasmic cylinder marker used as a negative control (26). The localization of the protein green light within the leptospires was achieved by superimposing both fields, and the results obtained are shown in Fig. 1C for PLG1, PLG2, PLG3, LipL32, GroEL, and PBS.

Activation and enzymatic activity of PLG-bound *Leptospira*. To analyze the *Leptospira* PLG binding and quantify the enzymatic activity after conversion to plasmin, we assayed the treated leptospires with a specific plasmin chromogenic substrate. As shown in Fig. 2, both the virulent and the nonvirulent strains of *L. interrogans* serovar Copenhageni tested were capable of capturing PLG and the bound PLG was converted to enzymatically active plasmin only by the addition of the exogenous activator uPA. The binding and consequently the plasmin enzymatic activity occurred in a dose-dependent manner with the PLG concentration range studied (Fig. 2). In addition to the purified PLG, low-passage virulent leptospires were also capable of binding PLG in 30% human plasma ($\sim 6 \mu\text{g}/\text{ml}$ of PLG). No proteolytic activity was detected with either intact *Leptospira* (Fig. 2) or cell lysate (not shown) or when the bacteria were treated with PLG or uPA alone.

Analysis of *L. interrogans* culture growth after PLG labeling. To analyze if the PLG binding to the surface of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 and its conversion to enzymatically active plasmin could cause any impairment in the growth of the leptospire culture, the bacteria were treated with PLG; activated by the addition of uPA; reinoculated into a fresh, serum-free EMJH medium; and monitored for the growth rate for 14 days. Cell division was not inhibited in plasmin-coated leptospires since bacterial growth was similar to that of untreated (PBS) or PLG- or PLG-uPA-treated *Leptospira* cells (data not shown).

Inhibition of proteolytic activity acquired by *L. interrogans* by ACA. It is known that PLG kringle domains frequently mediate interactions with lysine residues of the cellular recep-

tors (37). To evaluate the participation of these domains in the binding of PLG with *L. interrogans* serovar Copenhageni strain L1-130, a binding assay was carried out in the presence of ACA, a derivative and analogue of lysine amino acid. As shown in Fig. 3, ACA reduced the proteolytic activity of virulent *L. interrogans* serovar Copenhageni cells incubated with PLG in a dose-dependent manner, statistically significant from 1 mM ACA to almost total inhibition at 1,000 mM ACA, as a consequence of decreased PLG binding.

Involvement of leptospiral OMPs on PLG binding. OMPs that are surface exposed are expected to be relevant in pathogenesis. We thus examined the binding of proteins after treatment/extraction of leptospires with the nonionic detergent TX-114, as previously described (24). Experiments were performed with the virulent low-passage *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. The binding assay was performed with proteins from the soluble AP and DP and with whole-cell lysate. PLG acquisition, as evaluated by a modified ELISA, shows that OMPs partitioned in the DP contribute to PLG binding (Fig. 4), but a higher number of proteins contained in the soluble AP appear to be involved in the PLG acquisition by *Leptospira* (Fig. 4).

Effect of environmental factors on plasmin generation by *Leptospira*. Because some virulence factors are induced by salt and temperature (43, 49, 55), we decided to examine whether osmolarity and temperature could influence PLG binding and activation by leptospires. Assays were performed with the virulent low-passage *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. The transition of temperatures from ambient to mammalian body (37°C and later 39°C) has been correlated with changes in the expression of virulence determinants in many pathogens (53). Therefore, we compared plasmin generation in leptospires of cultures grown at 28°C , 37°C , and 39°C , reflecting growth under laboratory conditions and in the mammalian host under normal and febrile temperatures, respectively. Plasmin generation by *Leptospira* showed no difference under the temperatures tested (Fig. 5A). PLG binding/activation by osmolarity was assessed by addition of 120 mM NaCl to the EMJH medium supplemented with 10% *Leptospira* enrichment EMJH medium and incubation at 28°C . The addition of 120 mM NaCl to the medium mimics physiological conditions (~ 300 mosmol/liter) encountered by leptospires upon entry into the host (49). Cultures were incubated for 24 h and examined for PLG binding. As shown in Fig. 5B, PLG binding/activation is not affected by osmolarity because similar plasmin generation was detected upon incubation in normal growth medium and with additional NaCl.

Analysis of PLG binding to proteins of *L. interrogans*. The binding of leptospiral protein receptors to PLG was visualized by affinity blotting using low-passage virulent and high-passage nonvirulent bacterial protein extracts. Blotted membranes were probed with PLG followed by immune detection. Similar results were obtained from three independent experiments, and one representative affinity blotting is depicted in Fig. 6A. It is possible to observe at least 10 leptospiral proteins bind to PLG under the conditions employed with both the virulent and nonvirulent strains. The three arrows point out the existence of three distinct reactive protein regions between the protein extracts of the virulent and the nonvirulent attenuated *L. interrogans* strains. The protein reactive bands were almost to-

FIG. 1. Recognition of PLG binding to *Leptospira* by L-IFA. Live virulent *L. interrogans* serovar Copenhageni isolates were treated with PLG, and the recognition was assessed through polyclonal anti-PLG antibodies under a confocal immunofluorescence microscope (PLG1 to -3). PLG-treated leptospires were stained for the protein LipL32 (outer surface protein marker) or GroEL (a protoplasmic cylinder marker); untreated leptospires (PBS) are shown as controls. (A) DNA propidium iodide-stained DNA; (B) FITC-stained DNA; (C) composite images of panel A plus B.

FIG. 2. Cleavage of the plasmin-specific chromogenic substrate by plasmin-bound leptospires. Live low-passage virulent and high-passage nonvirulent *L. interrogans* serovar Copenhageni cells received the following treatments: PBS only (PBS), uPA alone (uPA), 5 μ g PLG alone (PLG), PLG at 0.5, 2, and 5 μ g together with uPA; and 30% human plasma together with uPA (30% plasma). Bars represent mean absorbance as a measure of relative substrate degradation $\pm$ the standard deviation of three replicates for each experimental group and are representative of three independent experiments. *, virulent leptospire experiments statistically significant ($P < 0.0001$) in comparison to the PBS control; **, nonvirulent leptospire experiments statistically significant ($P < 0.01$) in comparison to the PBS control. The 30% plasma (as the PLG source) and all of the PLG-uPA samples of the virulent leptospires were statistically significant ($P < 0.001$) in comparison to the same samples of nonvirulent bacteria tested.

tally abolished when the lysine analog ACA was added to both leptospiral protein extracts (Fig. 6B).

PAS among different species of *Leptospira*. In order to evaluate if the PLG binding and activation are extended to other *Leptospira* species, we performed experiments with different pathogenic high-passage nonvirulent strains of *Leptospira* (*L. interrogans* serovars Canicola, Interohaemorrhagiae, and Pomona; *L. borgpetersenii* serovars Castelonis and Whitcombi; *L. santarosai* serovar Shermani; and *L. kirshneri* serovars

Cynoptery and Grippotyphosa) and one saprophytic strain (*L. biflexa* serovar Patoc). Nonvirulent *L. interrogans* serovar Copenhageni was added in these experiments for comparison. As shown in Fig. 7, our results indicate that all of the strains tested are capable of binding PLG and generating active plasmin in the presence of uPA at the surface of bacteria, including, to some extent, the nonpathogenic saprophytic species *L. biflexa*.

Plasmin-coated *Leptospira* degrades immobilized fibrinectin. Binding and activation of PLG to generate the proteolytic

FIG. 3. Inhibition of PLG binding to *L. interrogans* by ACA. Low-passage virulent *L. interrogans* serovar Copenhageni cells were treated with the following: PLG together with uPA (PLG + uPA), PLG together with uPA with the addition of crescent concentrations of ACA (0.01 to 1,000 mM ACA), PLG alone (PLG), uPA alone (uPA), ACA alone (ACA), and no additions (PBS). The cleavage of the plasmin-specific substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride by the treated spirochetes was measured by absorbance readings at 405 nm. Bars represent mean absorbance $\pm$ the standard deviation of three replicates for each experimental group and are representative of three independent experiments. Statistically significant substrate degradation inhibition results in comparison to the positive control (PLG + uPA) are depicted: $P < 0.01$ (*) and $P < 0.0001$ (**), as assessed by Student's two-tailed *t* test.

FIG. 4. PLG binding to extracted fractions of leptospira. Low-passage virulent *L. interrogans* serovar Copenhageni proteins were fractionated by TX-114 extraction. Whole-cell lysate (WCL), DP, and AP were coated onto 96-well plates and allowed to bind to PLG. The interaction was quantified by specific anti-human PLG antibodies. The results are representative of two independent TX-114 partitioning and binding experiments. The bars represent the mean absorbance at 492 nm $\pm$ the standard deviation of four replicates.

enzyme plasmin have been correlated with the invasive potential of certain bacteria (77). In order to determine whether plasmin-coated leptospires were able to degrade the ECM component fibronectin, as previously demonstrated with *B. burgdorferi* (15), purified soluble human fibronectin was adhered onto 96-well plates. Degradation was evaluated by exposing the ECM proteins to low-passage virulent *L. interrogans* cells pretreated with PLG and/or uPA. The substrate breakdown was measured with polyclonal antibodies. Degradation of fibronectin was calculated by decreased absorbance compared to that of the positive control without bacteria. As shown in Fig. 8, the plasmin-coated leptospires were able to significantly break down the immobilized fibronectin compared to the untreated bacteria.

DISCUSSION

Several invasive gram-positive and gram-negative bacteria have shown the ability to interact with the host PLG system (6,

FIG. 6. Binding of human PLG to *L. interrogans* proteins by affinity blotting. Total protein extracts were resolved by 10% SDS-PAGE and electroblotted into nitrocellulose membranes. The membranes were incubated with PLG (A) or PLG plus ACA (B), and the binding was detected by anti-PLG antibodies and peroxidase-conjugated antibodies. The reactivity was revealed by ECL (GE Healthcare) and exposure to X-ray films. NV, high-passage nonvirulent *L. interrogans* serovar Copenhageni strain M 20; Vir, low-passage virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. The arrows indicate reactivity protein regions seen in the virulent bacterial extract but absent in the nonvirulent bacterial extract.

13, 19, 37, 73), a phenomenon that has been extended to viruses (22, 38, 60) and parasites (68), either by expression of PLG receptors or by stimulation of host activators. It has been demonstrated by in vitro and/or in vivo animal model of patho-

FIG. 5. Influence of temperature and osmolarity on the binding of PLG to leptospires. Low-passage virulent *L. interrogans* serovar Copenhageni cells cultured at 28°C in EHJM medium supplemented with 10% *Leptospira* enrichment EMJH medium were shifted to 37 or 39°C for 24 h (A) or maintained at 28°C but supplemented with 120 mM NaCl (B). The cells were then treated with 5 μ g PLG together with uPA or only with PBS. Bars represent the mean absorbance as a measure of relative substrate degradation $\pm$ the standard deviation of four replicates for each experimental group.

FIG. 7. Acquisition of plasmin activity by different strains of *Leptospira*. Live high-passage pathogenic *L. interrogans* serovar Canicola, *L. interrogans* serovar Interohaemorrhagiae, *L. interrogans* serovar Pomona, *L. borgpetersenii* serovar Castelonis, *L. borgpetersenii* serovar Whitcombi, *L. santarosai* serovar Shermani, *L. kirshneri* serovar Cynoptery, *L. kirshneri* serovar Grippotyphosa, and saprophytic *L. biflexa* serovar Patoc strains were treated with the following: no additions (PBS), uPA alone (uPA), 5 μ g PLG alone (PLG), and 5 μ g PLG together with uPA (plasmin). Bars represent the mean absorbance as a measure of relative substrate degradation $\pm$ the standard deviation of three replicates for each experimental group and are representative of two independent experiments.

genesis and infection that the PAS has a critical function for invasiveness and establishment of the infection for several microorganisms such as *Streptococcus* spp., *Leishmania mexicana*, and *Staphylococcus aureus* (23, 41, 46, 47, 69, 74). In the case of spirochetes, the PAS was studied with several species of *Borrelia* and with *Treponema denticola* and was suggested to have an important role during infectiveness (14, 16, 21, 35, 58).

L. interrogans serovar Copenhageni is capable of binding PLG in its outer surface as visualized by indirect immunofluorescence with living leptospires. The bound PLG is activated to plasmin by addition of human activator uPA, promoting a surface-associated plasmin activity within the bacteria. Plasmin bound to the surface of *L. interrogans* is enzymatically active, as measured by the degradation of chromogenic specific plasmin substrate. Small amounts of PLG were required for the detection of statistically significant plasmin activity compared to that of the controls. The fluorescence observed from the binding of leptospires with PLG suggests that the bacteria have receptors along their surfaces. This is comparable to, although less intense than, the binding pattern observed with LipL32, but it is in contrast with our previous data with two proteins, MPL17 and MPL21 (61), that presented a dot pattern of fluorescence. The leptospiral membrane probably remains intact as treatment with mouse antibody to GroEL, the protoplasmic-cylinder heat shock protein, and goat anti-mouse FITC-conjugated antibody did not exhibit any fluorescence within the bacteria. In addition, we have observed that the bacterial cells preserved the ordinary rotational movement after the treatment. As reported for the spirochetes *Borrelia* and *Treponema* (37), *Leptospira*, either as whole cells or lysate, is dependent on the host

FIG. 8. Fibronectin degradation by plasmin-coated leptospires. Spirochetes were incubated in IsPBS with no additions (UT) and with addition of PLG and uPA (plasmin). ELISA plate wells coated with fibronectin (three replicates) were incubated for 20 h with the spirochetes from each experimental group. Undegraded substrate was detected, and percent degradation (with a reduction in absorbance value interpreted as substrate degradation) was calculated as described in Materials and Methods. Bars represent the mean percent substrate degradation relative to the positive control (0% degradation) $\pm$ the standard deviation of three replicates. The experiment was independently performed three times, and significance was assessed by Student's two-tailed *t* test. *, $P < 0.005$.

PLG activation system because the labeling with PLG alone did not generate proteolytic activity, in contrast to some pathogens that present a PLG endogenous activation system (40–42, 52, 59, 70, 71, 79).

PLG is present in plasma at a concentration of $\sim 20.8 \pm 1.9$ mg/100 ml (17). Our data show that virulent *L. interrogans*, but not the attenuated strain, has the ability to bind PLG from human plasma (~ 6 μ g of PLG contained in 30% of human plasma) under the conditions assayed. The plasmin activity measured is lower than that with a similar concentration of added purified PLG (5 μ g), probably due to the presence of a complex mixture of proteins in human fluid that could compete for the binding reaction. In any event, once in the host, the leptospires can acquire PLG when they reach the blood circulation. Despite the proteolytic activity associated with the outer surface conferred by bound PLG activated to plasmin, *L. interrogans* was probably not damaged because cellular growth was not impaired.

The process for binding to bacterial receptors on the cell surface has been shown for several pathogens to be mediated by the PLG kringle domains through the contained lysine-binding sites (2, 10, 16, 32, 33, 46, 63, 68, 77, 78). Likewise, *Leptospira* probably interacts with PLG through the lysine residues because the binding and generation of active plasmin on the bacterial surface were inhibited by the presence of the lysine analogue ACA in the reaction. The inhibition was dose dependent on the ACA concentration, with the plasmin activity almost totally abolished by the addition of 100 mM ACA.

The involvement of OMPs in the acquisition of PLG by *Leptospira*, as expected, is clearly shown in the experiments with DP partitioning proteins. Surprisingly, our data shows that the contribution of aqueous soluble proteins in the binding process is higher than with proteins in the detergent fraction (OMPs). It has been shown that the major leptospiral antigens LipL32 and LipL48 are digested, probably by an endogenous protease, during TX-114 solubilization (18). Moreover, some subsurface proteins, such as penicillin-binding proteins, did not solubilize in the TX-114 phase (27). These events may account for the differences observed with proteins from the DP and AP. In any event, our data suggest that there are several proteins that may act as a PLG receptor in *Leptospira* (Vieira et al., unpublished data). It has been demonstrated that GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is a major surface protein on group A streptococci with multiple binding activities, including plasmin receptor (8, 44, 45, 62). GAPDH or members of the GAPDH-related family are conserved among several bacteria, including spirochetes, and it has been suggested that borrelial GAPDH homolog may contribute to plasmin(ogen) acquisition (1). In *Leptospira*, there is one gene coding for GAPDH that is predicted to be located at the cytoplasm of the bacteria, according to the PSORT program (54). Thus, GAPDH may be one of the proteins involved in PLG acquisition observed with AP proteins.

Regulation of virulence by environmental cues, such as temperature and osmolarity, has been reported in a variety of pathogens (53). In *Leptospira*, several genes that are differentially expressed under different temperature conditions were identified by whole-genome microarrays (43). Moreover, 6% of the leptospiral genes were susceptible to osmoregulation (49). Intriguingly, plasmin generation was not affected when

Leptospira cultivated at 28°C was shifted to temperatures found in the mammalian host under normal and febrile conditions. Likewise, the shift to physiologic osmolarity had no influence on PLG binding and plasmin generation by leptospires. Thus, virulence factors identified in *Leptospira*, such as LigA/LigB (50, 51) and Lsa21 (3), and known to be upregulated by temperature and osmolarity are probably not involved in PLG acquisition by leptospires.

The phenomenon of binding to PLG was observed with pathogenic high-passage nonvirulent strains from different species of the genus *Leptospira* and was also extended to the nonpathogenic saprophytic organism *L. biflexa*. However, as observed with the spirochete *B. burgdorferi* (12), the infectious low-passage *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was more efficient in capturing PLG ($P < 0.001$) on its surface than the noninfectious high-passage *L. interrogans* serovar Copenhageni strain M 20, a result that suggests an alteration of PLG protein receptors during the attenuation process.

The affinity blotting experiments using leptospire whole-cell lysates corroborate with these data, although the conditions assayed do not simulate the ones from the PLG binding with the intact bacteria. Comparable to the spirochete *B. burgdorferi* (16), the affinity blotting was inhibited by the presence of the lysine analog ACA. It is noteworthy that with the whole-cell lysates of the virulent strain, a cluster of proteins are involved in the PLG binding different from the profile of the attenuated strain. Whether the binding observed with the virulent strain of *Leptospira* is due to the expression of diverse protein receptors, a higher number of the same receptors, or both, deserves to be further studied.

L. interrogans is a highly invasive pathogen. The bacteria are thought to penetrate the skin or a break in the skin to initiate infection and then rapidly disseminate via the bloodstream to cause multisystem infection, targeting the liver and kidney (5, 20). ECM-binding proteins such as LenA (72), formerly LfhA/Lsa24 (4, 75), LigA/LigB (11), and LipL32 (29, 30) have been identified, but thus far, ECM degradation by leptospires has not been described. The spirochete *B. burgdorferi* was demonstrated to be capable of degrading ECM macromolecules when bound to plasmin (15), and this activity has been suggested to contribute to bacterial invasion (12, 15, 16). Plasmin-coated leptospires were also capable of employing this proteolytic activity to in vitro degrade the immobilized ECM component fibronectin.

Although more studies are required to identify leptospiral protein receptors involved in plasmin activation as well as to fully investigate the degradation of ECM components, our data show for the first time that *Leptospira* can borrow the PAS from the host and that this activity might be employed for the invasion process. We trust that the description of this new feature of *Leptospira* presented in this study would help to identify proteins of importance in pathogenesis and hence potential antigens for the development of an efficient vaccine against leptospirosis.

ACKNOWLEDGMENTS

This work has benefited from grants from FAPESP, CNPq, and Fundação Butantan. M.L.V. has a Ph.D. fellowship from FAPESP (Brazil).

REFERENCES

- Anda, P., J. A. Gebbia, P. B. Backenson, J. L. Coleman, and J. L. Benach. 1996. A glyceraldehyde-3-phosphate dehydrogenase homolog in *Borrelia burgdorferi* and *Borrelia hermsii*. *Infect. Immun.* **64**:262–268.
- Attali, C., C. Durmort, T. Vernet, and A. M. Di Guilmi. 2008. The interaction of *Streptococcus pneumoniae* with plasmin mediates transmigration across endothelial and epithelial monolayers by intercellular junction cleavage. *Infect. Immun.* **76**:5350–5356.
- Atzingen, M. V., A. S. Barbosa, T. De Brito, S. A. Vasconcellos, Z. M. de Moraes, D. M. Lima, P. A. Abreu, and A. L. Nascimento. 2008. Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC Microbiol.* **8**:70.
- Barbosa, A. S., P. A. E. Abreu, F. O. Neves, M. V. Atzingen, M. M. Watanabe, M. L. Vieira, Z. M. Moraes, S. A. Vasconcellos, and A. L. T. O. Nascimento. 2006. A newly identified leptospiral adhesin mediates attachment to laminin. *Infect. Immun.* **74**:6356–6364.
- Barocchi, M. A., A. I. Ko, M. G. Reis, K. L. McDonald, and L. W. Riley. 2002. Rapid translocation of polarized MDCK cell monolayers by *Leptospira interrogans*, an invasive but nonintraepithelial pathogen. *Infect. Immun.* **70**:6926–6932.
- Bergmann, S., and S. Hammerschmidt. 2007. Fibrinolysis and host response in bacterial infections. *Thromb. Haemos.* **98**:512–520.
- Bharti, A. R., J. E. Nally, J. N. Ricaldi, M. A. Matthias, M. M. Diaz, M. A. Lovett, P. N. Levett, R. H. Gilman, M. R. Willig, E. Gotuzzo, and J. M. Vinetz. 2003. Leptospirosis: a zoonotic disease of global importance. *Lancet Infect. Dis.* **3**:757–771.
- Border, W. A., S. Okuda, T. Nakamura, L. R. Languino, and E. Ruoslahti. 1991. Role of TGF-beta 1 in experimental glomerulonephritis. *Ciba Found. Symp.* **157**:178–193.
- Bulach, D. M., R. L. Zuerner, P. Wilson, T. Seemann, A. McGrath, P. A. Cullen, J. Davis, M. Johnson, E. Kuczek, D. P. Alt, B. Peterson-Burch, R. L. Coppel, J. I. Rood, J. K. Davies, and B. Adler. 2006. Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proc. Natl. Acad. Sci. USA* **103**:14560–14565.
- Candela, M., G. Miccoli, S. Bergmann, S. Turrone, B. Vitali, S. Hammerschmidt, and P. Brigid. 2008. Plasminogen-dependent proteolytic activity in *Bifidobacterium lactis*. *Microbiology* **154**:2457–2462.
- Choy, H. A., M. M. Kelley, T. L. Chen, A. K. Møller, J. Matsunaga, and D. A. Haake. 2007. Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. *Infect. Immun.* **75**:2441–2450.
- Coleman, J. L., and J. L. Benach. 2000. The generation of enzymatically active plasmin on the surface of spirochetes. *Methods* **21**:133–141.
- Coleman, J. L., and J. L. Benach. 1999. Use of the plasminogen activation system by microorganisms. *J. Lab. Clin. Med.* **134**:567–576.
- Coleman, J. L., J. A. Gebbia, J. Piesman, J. L. Degen, T. H. Bugge, and J. L. Benach. 1997. Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice. *Cell* **89**:1111–1119.
- Coleman, J. L., E. J. Roemer, and J. L. Benach. 1999. Plasmin-coated *Borrelia burgdorferi* degrades soluble and insoluble components of the mammalian extracellular matrix. *Infect. Immun.* **67**:3929–3936.
- Coleman, J. L., T. J. Sellati, J. E. Testa, R. R. Kew, M. B. Furie, and J. L. Benach. 1995. *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. *Infect. Immun.* **63**:2478–2484.
- Collen, D., G. Tytgat, H. Claeys, M. Verstraete, and P. Wallen. 1972. Metabolism of plasminogen in healthy subjects: effect of tranexamic acid. *J. Clin. Invest.* **51**:1310–1318.
- Cullen, P. A., D. A. Haake, and B. Adler. 2004. Outer membrane proteins of pathogenic spirochetes. *FEMS Microbiol. Rev.* **28**:291–318.
- Degen, J. L., T. H. Bugge, and J. D. Goguen. 2007. Fibrin and fibrinolysis in infection and host defense. *J. Thromb. Haemost.* **5**(Suppl 1):24–31.
- Faine, S., B. Adler, C. Bolin, and P. Perolat. 1999. *Leptospira* and leptospirosis, 2nd ed, p. 259. *MediSci*, Melbourne, Australia.
- Fenno, J. C., M. Tamura, P. M. Hannam, G. W. K. Wong, R. A. Chan, and B. C. McBride. 2000. Identification of a *Treponema denticola* OppA homologue that binds host proteins present in the subgingival environment. *Infect. Immun.* **68**:1884–1892.
- Goto, H., and Y. Kawaoka. 1998. A novel mechanism for the acquisition of virulence by a human influenza A virus. *Proc. Natl. Acad. Sci. USA* **95**:10224–10228.
- Guo, Y., J. Li, E. Hagstrom, and T. Ny. 2008. Protective effects of plasmin (ogen) in a mouse model of *Staphylococcus aureus*-induced arthritis. *Arthritis Rheum.* **58**:764–772.
- Haake, D. A., G. Chao, R. L. Zuerner, J. K. Barnett, D. Barnett, M. Mazel, J. Matsunaga, P. N. Levett, and C. A. Bolin. 2000. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect. Immun.* **68**:2276–2285.
- Haake, D. A., M. Dundoo, R. Cader, B. M. Kubak, R. A. Hartskeerl, J. J. Sejvar, and D. A. Ashford. 2002. Leptospirosis, water sports, and chemoprophylaxis. *Clin. Infect. Dis.* **34**:e40–e43.
- Haake, D. A., and J. Matsunaga. 2002. Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. *Infect. Immun.* **70**:4936–4945.
- Haake, D. A., E. M. Walker, D. R. Blanco, C. A. Bolin, M. N. Miller, and M. A. Lovett. 1991. Changes in the surface of *Leptospira interrogans* serovar grippityphosa during in vitro cultivation. *Infect. Immun.* **59**:1131–1140.
- Haile, W. B., J. L. Coleman, and J. L. Benach. 2006. Reciprocal upregulation of urokinase plasminogen activator and its inhibitor, PAI-2, by *Borrelia burgdorferi* affects bacterial penetration and host-inflammatory response. *Cell. Microbiol.* **8**:1349–1360.
- Hauk, P., F. Macedo, E. C. Romero, S. A. Vasconcellos, Z. M. de Moraes, A. S. Barbosa, and P. L. Ho. 2008. In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. *Infect. Immun.* **76**:2642–2650.
- Hoke, D. E., S. Egan, P. A. Cullen, and B. Adler. 2008. LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudoalteromonas tunicata*. *Infect. Immun.* **76**:2063–2069.
- Hu, L. T., G. Perides, R. Noring, and M. S. Klempner. 1995. Binding of human plasminogen to *Borrelia burgdorferi*. *Infect. Immun.* **63**:3491–3496.
- Jobin, M. C., J. Brassard, S. Quessy, M. Gottschalk, and D. Grenier. 2004. Acquisition of host plasmin activity by the swine pathogen *Streptococcus suis* serotype 2. *Infect. Immun.* **72**:606–610.
- Kinby, B., N. A. Booth, and G. Svensater. 2008. Plasminogen binding by oral streptococci from dental plaque and inflammatory lesions. *Microbiology* **154**:924–931.
- Klempner, M. S., R. Noring, M. P. Epstein, B. McCloud, R. Hu, S. A. Limentani, and R. A. Rogers. 1995. Binding of human plasminogen and urokinase-type plasminogen activator to the Lyme disease spirochete, *Borrelia burgdorferi*. *J. Infect. Dis.* **171**:1258–1265.
- Klempner, M. S., R. Noring, M. P. Epstein, B. McCloud, and R. A. Rogers. 1996. Binding of human urokinase type plasminogen activator and plasminogen to *Borrelia* species. *J. Infect. Dis.* **174**:97–104.
- Ko, A. I., M. Galvao Reis, C. M. Ribeiro Dourado, W. D. Johnson, Jr., L. W. Riley, et al. 1999. Urban epidemic of severe leptospirosis in Brazil. *Lancet* **354**:820–825.
- Lahteenmaki, K., P. Kuusela, and T. K. Korhonen. 2001. Bacterial plasminogen activators and receptors. *FEMS Microbiol. Rev.* **25**:531–552.
- LeBouder, F., E. Morello, G. F. Rimmelzwaan, F. Bosse, C. Pécoux, B. Delmas, and B. Riteau. 2008. Annexin II incorporated into influenza virus particles supports virus replication by converting plasminogen into plasmin. *J. Virol.* **82**:6820–6828.
- Levett, P. N. 2001. Leptospirosis. *Clin. Microbiol. Rev.* **14**:296–326.
- Leytus, S. P., L. K. Bowles, J. Konisky, and W. F. Mangel. 1981. Activation of plasminogen to plasmin by a protease associated with the outer membrane of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **78**:1485–1489.
- Li, Z., V. A. Ploplis, E. L. French, and M. D. Boyle. 1999. Interaction between group A streptococci and the plasmin(ogen) system promotes virulence in a mouse skin infection model. *J. Infect. Dis.* **179**:907–914.
- Lijnen, H. R., F. De Cock, B. Van Hoef, B. Schlott, and D. Collen. 1994. Characterization of the interaction between plasminogen and staphylokinase. *Eur. J. Biochem.* **224**:143–149.
- Lo, M., D. M. Bulach, D. R. Powell, D. A. Haake, J. Matsunaga, M. L. Paustian, R. L. Zuerner, and B. Adler. 2006. Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed by whole-genome microarrays. *Infect. Immun.* **74**:5848–5859.
- Lottenberg, R., C. C. Broder, and M. D. P. Boyle. 1987. Identification of a specific receptor for plasmin on a group A streptococcus. *Infect. Immun.* **55**:1914–1918.
- Lottenberg, R., C. C. Broder, M. D. P. Boyle, S. J. Kain, B. L. Schroeder, and R. Curtiss III. 1992. Cloning, sequence analysis, and expression in *Escherichia coli* of a streptococcal plasmin receptor. *J. Bacteriol.* **174**:5204–5210.
- Magalhaes, V., I. Veiga-Malta, M. R. Almeida, M. Baptista, A. Ribeiro, P. Trieu-Cuot, and P. Ferreira. 2007. Interaction with human plasminogen system turns on proteolytic activity in *Streptococcus agalactiae* and enhances its virulence in a mouse model. *Microbes Infect.* **9**:1276–1284.
- Maldonado, J., C. Marina, J. Puig, Z. Maizo, and L. Avilan. 2006. A study of cutaneous lesions caused by *Leishmania mexicana* in plasminogen-deficient mice. *Exp. Mol. Pathol.* **80**:289–294.
- Matsunaga, J., M. A. Barocchi, J. C. Croda, T. A. Young, Y. Sanchez, I. Siqueira, C. A. Bolin, M. G. Reis, L. W. Riley, D. A. Haake, and A. I. Ko. 2003. Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. *Mol. Microbiol.* **49**:929–945.
- Matsunaga, J., M. Lo, D. M. Bulach, R. L. Zuerner, B. Adler, and D. A. Haake. 2007. Response of *Leptospira interrogans* to physiologic osmolarity: relevance in signaling the environment-to-host transition. *Infect. Immun.* **75**:2864–2874.
- Matsunaga, J., M. A. Medeiros, Y. Sanchez, K. F. Werneid, and A. I. Ko. 2007. Osmotic regulation of expression of two extracellular matrix-binding proteins and a haemolysin of *Leptospira interrogans*: differential effects on LigA and Sph2 extracellular release. *Microbiology* **153**:3390–3398.

51. Matsunaga, J., Y. Sanchez, X. Xu, and D. A. Haake. 2005. Osmolarity, a key environmental signal controlling expression of leptospiral proteins LigA and LigB and the extracellular release of LigA. *Infect. Immun.* 73:70–78.
52. McCoy, H. E., C. C. Broder, and R. Lottenberg. 1991. Streptokinases produced by pathogenic group C streptococci demonstrate species-specific plasminogen activation. *J. Infect. Dis.* 164:515–521.
53. Mekalanos, J. J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.* 174:1–7.
54. Nakai, K., and M. Kanehisa. 1991. Expert system for predicting protein localization sites in gram-negative bacteria. *Proteins* 11:95–110.
55. Nally, J. E., J. F. Timoney, and B. Stevenson. 2001. Temperature-regulated protein synthesis by *Leptospira interrogans*. *Infect. Immun.* 69:400–404.
56. Nally, J. E., J. P. Whitelegge, S. Bassilian, D. R. Blanco, and M. A. Lovett. 2007. Characterization of the outer membrane proteome of *Leptospira interrogans* expressed during acute lethal infection. *Infect. Immun.* 75:766–773.
57. Nascimento, A. L., S. Verjovski-Almeida, M. A. Van Sluys, C. B. Monteiro-Vitorello, L. E. Camargo, L. A. Digiampietri, R. A. Harstkeerl, P. L. Ho, M. V. Marques, M. C. Oliveira, J. C. Setubal, D. A. Haake, and E. A. Martins. 2004. Genome features of *Leptospira interrogans* serovar Copenhageni. *Braz. J. Med. Biol. Res.* 37:459–477.
58. Nordstrand, A., A. Shamaei-Tousi, A. Ny, and S. Bergstrom. 2001. Delayed invasion of the kidney and brain by *Borrelia crocidurae* in plasminogen-deficient mice. *Infect. Immun.* 69:5832–5839.
59. Okada, K., S. Ueshima, T. Takaishi, H. Yuasa, H. Fukao, and O. Matsuo. 1996. Effects of fibrin and alpha2-antiplasmin on plasminogen activation by staphylokinase. *Am. J. Hematol.* 53:151–157.
60. Okumura, Y., M. Yano, M. Murakami, S. Mori, T. Towatari, and H. Kido. 1999. The extracellular processing of HIV-1 envelope glycoprotein gp160 by human plasmin. *FEBS Lett.* 442:39–42.
61. Oliveira, T. R., M. T. Longhi, Z. M. de Morais, E. C. Romero, R. M. Blanco, K. Kirchgatter, S. A. Vasconcellos, and A. L. Nascimento. 2008. Evaluation of leptospiral recombinant antigens MPL17 and MPL21 for serological diagnosis of leptospirosis by enzyme-linked immunosorbent assays. *Clin. Vaccine Immunol.* 15:1715–1722.
62. Pancholi, V., and V. A. Fischetti. 1992. A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. *J. Exp. Med.* 176:415–426.
63. Pantzar, M., A. Ljungh, and T. Wadström. 1998. Plasminogen binding and activation at the surface of *Helicobacter pylori* CCUG 17874. *Infect. Immun.* 66:4976–4980.
64. Picardeau, M., D. M. Bulach, C. Bouchier, R. L. Zuerner, N. Zidane, P. J. Wilson, S. Creno, E. S. Kuczek, S. Bommezzadri, J. C. Davis, A. McGrath, M. J. Johnson, C. Boursaux-Eude, T. Seemann, Z. Rouy, R. L. Coppel, J. I. Rood, A. Lajus, J. K. Davies, C. Medigue, and B. Adler. 2008. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PLoS ONE* 3:e1607.
65. Plank, R., and D. Dean. 2000. Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. *Microbes Infect.* 2:1265–1276.
66. Ren, S. X., G. Fu, X. G. Jiang, R. Zeng, Y. G. Miao, H. Xu, Y. X. Zhang, H. Xiong, G. Lu, L. F. Lu, H. Q. Jiang, J. Jia, Y. F. Tu, J. X. Jiang, W. Y. Gu, Y. Q. Zhang, Z. Cai, H. H. Sheng, H. F. Yin, Y. Zhang, G. F. Zhu, M. Wan, H. L. Huang, Z. Qian, S. Y. Wang, W. Ma, Z. J. Yao, Y. Shen, B. Q. Qiang, Q. C. Xia, X. K. Guo, A. Danchin, I. Saint Girons, R. L. Somerville, Y. M. Wen, M. H. Shi, Z. Chen, J. G. Xu, and G. P. Zhao. 2003. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. *Nature* 422:888–893.
67. Ristow, P., P. Bourhy, F. W. da Cruz McBride, C. P. Figueira, M. Huerre, P. Ave, I. S. Girons, A. I. Ko, and M. Picardeau. 2007. The OmpA-like protein Loa22 is essential for leptospiral virulence. *PLoS Pathog.* 3:e97.
68. Rojas, M., I. Labrador, J. L. Concepcion, E. Aldana, and L. Avilan. 2008. Characteristics of plasminogen binding to *Trypanosoma cruzi* epimastigotes. *Acta Trop.* 107:54–58.
69. Sanderson-Smith, M. L., K. Dinkla, J. N. Cole, A. J. Cork, P. G. Maamary, J. D. McArthur, G. S. Chhatwal, and M. J. Walker. 2008. M protein-mediated plasminogen binding is essential for the virulence of an invasive *Streptococcus pyogenes* isolate. *FASEB J.* 22:2715–2722.
70. Schlott, B., K. H. Guhrs, M. Hartmann, A. Rocker, and D. Collen. 1998. NH₂-terminal structural motifs in staphylokinase required for plasminogen activation. *J. Biol. Chem.* 273:22346–22350.
71. Sodeinde, O. A., Y. V. Subrahmanyam, K. Stark, T. Quan, Y. Bao, and J. D. Goguen. 1992. A surface protease and the invasive character of plague. *Science* 258:1004–1007.
72. Stevenson, B., H. A. Choy, M. Pinne, M. L. Rotondi, M. C. Miller, E. Demoll, P. Kraicz, A. E. Cooley, T. P. Creamer, M. A. Suchard, C. A. Brissette, A. Verma, and D. A. Haake. 2007. *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. *PLoS ONE* 2:e1188.
73. Sun, H. 2006. The interaction between pathogens and the host coagulation system. *Physiology* 21:281–288.
74. Svensson, M. D., U. Sjöbring, F. Luo, and D. E. Bessen. 2002. Roles of the plasminogen activator streptokinase and the plasminogen-associated M protein in an experimental model for streptococcal impetigo. *Microbiology* 148:3933–3945.
75. Verma, A., J. Hellwege, S. Artiushin, P. F. Zipfel, P. Kraicz, J. F. Timoney, and B. Stevenson. 2006. LfhA, a novel factor H-binding protein of *Leptospira interrogans*. *Infect. Immun.* 74:2659–2666.
76. Vinetz, J. M. 2001. Leptospirosis. *Curr. Opin. Infect. Dis.* 14:527–538.
77. Xolalpa, W., A. J. Vallecillo, M. Lara, G. Mendoza-Hernandez, M. Comini, R. Spallek, M. Singh, and C. Espitia. 2007. Identification of novel bacterial plasminogen-binding proteins in the human pathogen *Mycobacterium tuberculosis*. *Proteomics* 7:3332–3341.
78. Yavlovich, A., A. A.-R. Higazi, and S. Rottem. 2001. Plasminogen binding and activation by *Mycoplasma fermentans*. *Infect. Immun.* 69:1977–1982.
79. Young, K. C., G. Y. Shi, D. H. Wu, L. C. Chang, B. I. Chang, C. P. Ou, and H. L. Wu. 1998. Plasminogen activation by streptokinase via a unique mechanism. *J. Biol. Chem.* 273:3110–3116.

Editor: A. Camilli

In Vitro Identification of Novel Plasminogen-Binding Receptors of the Pathogen *Leptospira interrogans*

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Abstract

Background: Leptospirosis is a multisystem disease caused by pathogenic strains of the genus *Leptospira*. We have reported that *Leptospira* are able to bind plasminogen (PLG), to generate active plasmin in the presence of activator, and to degrade purified extracellular matrix fibronectin.

Methodology/Principal Findings: We have now cloned, expressed and purified 14 leptospiral recombinant proteins. The proteins were confirmed to be surface exposed by immunofluorescence microscopy and were evaluated for their ability to bind plasminogen (PLG). We identified eight as PLG-binding proteins, including the major outer membrane protein LipL32, the previously published rLIC12730, rLIC10494, Lp29, Lp49, LipL40 and MPL36, and one novel leptospiral protein, rLIC12238. Bound PLG could be converted to plasmin by the addition of urokinase-type PLG activator (uPA), showing specific proteolytic activity, as assessed by its reaction with the chromogenic plasmin substrate, D-Val-Leu-Lys 4-nitroanilide dihydrochloride. The addition of the lysine analog 6-aminocaproic acid (ACA) inhibited the protein-PLG interaction, thus strongly suggesting the involvement of lysine residues in plasminogen binding. The binding of leptospiral surface proteins to PLG was specific, dose-dependent and saturable. PLG and collagen type IV competed with LipL32 protein for the same binding site, whereas separate binding sites were observed for plasma fibronectin.

Conclusions/Significance: PLG-binding/activation through the proteins/receptors on the surface of *Leptospira* could help the bacteria to specifically overcome tissue barriers, facilitating its spread throughout the host.

Citation: Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, et al. (2010) In Vitro Identification of Novel Plasminogen-Binding Receptors of the Pathogen *Leptospira interrogans*. PLoS ONE 5(6): e11259. doi:10.1371/journal.pone.0011259

Editor: Holger Bruggemann, Max Planck Institute for Infection Biology, Germany

Received: March 15, 2010; **Accepted:** May 31, 2010; **Published:** June 22, 2010

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Funding: This research was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo - www.fapesp.br), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico - www.cnpq.br) and Fundação Butantan, Brazil. MLV, MVA, TRO and RO have fellowships from FAPESP (Brazil). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Leptospirosis is an emerging infectious disease with worldwide distribution and a zoonosis that is of human and veterinary concern. Pathogenic *Leptospira* are the etiological agents of leptospirosis, a disease with greater incidence in tropical and subtropical regions [1,2,3,4,5]. Leptospirae cause chronic infection in domestic and wild mammalian species that harbor the bacteria in their renal tubules, shedding them through the urine into the environment, thus constituting a source of re-infection in other animals. Humans are accidental and terminal hosts in the transmission process of leptospirosis [3,6]. Leptospirosis represents a great economic burden as it affects the public health system and livestock [3].

The molecular pathogenesis of leptospirosis is poorly understood. To date, few virulence factors contributing to the pathogenesis of the disease have been identified [7,8,9]. It is well documented that the interaction of pathogens with the extracellular matrix (ECM) may play a primary role in the colonization of host tissues, as long-lasting infections may occur if microorganisms

reach the sub-epithelial tissues [10]. Recently, the ability of the leptospirae to adhere to ECM macromolecules has been shown, and some adhesins, ECM-binding proteins, have been identified [9,11,12,13,14,15,16,17,18,19]. However, under normal conditions, ECM is not exposed to bacteria. The proteolytic activity achieved by subversion of host proteases by pathogens, such as plasmin, has been demonstrated to be important in various bacterial infections [20]. Plasmin is a broad-spectrum serine protease component of the fibrinolytic system, which has the zymogen plasminogen (PLG) as its main component.

In our previous work, we found that *Leptospira* species are able to bind PLG and generate plasmin, in the presence of activator, on the outer surface *in vitro* [21], an activity already shown for the pathogenic spirochetes *Borrelia* and *Treponema* [22,23,24,25,26]. We have also revealed that leptospirae express multiple PLG-binding proteins, as reported for other microorganisms. Recently, Verma et al. [27] have shown that the protein LenA of *L. interrogans* [14], formerly LfhA/Lsa24 [12,27], is a surface receptor for human plasminogen.

In the present study, we further evaluated the ability of 14 recombinant proteins of *Leptospira* to mediate binding to PLG *in vitro*. Eight of the 14 assayed were identified as novel PLG-receptor proteins, including the major outer membrane protein LipL32 [28], the previously published rLIC12730, rLIC10494, Lp29, Lp49, LipL40 and MPL36 [29,30,31], and one novel leptospiral protein rLIC12238. Bound PLG could be converted to plasmin by the addition of urokinase-type PLG activator (uPA), showing specific proteolytic activity. We also show that PLG interaction with LipL32, also an ECM adhesin, was affected by collagen type IV but not plasma fibronectin. Our data provide an array of novel leptospiral receptors for human plasminogen, which constitutes an important step for the understanding of the molecular pathogenesis and infection process of these bacteria.

Results

Selection of putative surface proteins from genome sequences

The rationale for protein selection was mostly based on cellular localization, since surface proteins are potential receptors for PLG. Fourteen proteins available in our laboratory were selected: twelve were previously published, LipL32 [28,32], LipL40, MPL36 [29], rLIC10509 [33], Lp29, Lp49 [30], Lsa27 [11], MPL21, MPL17 [31], rLIC10494, rLIC12730 [12], and Lsa63 [19], and two are novel proteins, rLIC12922 and rLIC12238. Table 1 summarizes the features of the selected proteins and gene conservation within the sequenced genomes [34,35,36,37].

Expression and purification of recombinant proteins

The amplified coding sequences, excluding the signal peptide tags, were cloned and expressed as full-length proteins in *E. coli*. Gene locus, protein reference number, given name, sequences of primers used for PCR amplifications and molecular mass of recombinants are depicted in Table 1. The recombinant proteins were purified by nickel affinity chromatography, and an aliquot of each protein was analyzed by SDS-PAGE (Fig. 1). All purified proteins were represented by major bands, although in some cases such as Lsa27 and MPL21, other less intense protein bands were also observed. Structural integrity of the purified proteins not previously published, LipL40, MPL36, rLIC12922 and rLIC12238, was assessed by CD spectroscopy. The minima at 208 and 222 nm, and the maximum at 192 nm in the CD spectrum showed the high α -helical secondary structure content of the recombinant proteins LipL40, MPL36 and rLIC12922, while rLIC12238 showed a predominant signal of β -strands, with minimum ellipticity around 215 nm (data not shown).

Cellular localization of the recombinant proteins

To determine whether the proteins not previously characterized, namely Lp29, Lp49, LipL40, MPL36, rLIC10494, rLIC12730, rLIC12922 and rLIC12238, are surface-exposed proteins, we set out to analyze the corresponding protein location on the bacteria using living *L. interrogans* serovar Copenhageni cells and the liquid-phase immunofluorescence method. Leptospire were visualized by propidium iodide staining followed by protein detection with polyclonal mouse antiserum raised against the protein in the presence of anti-mouse IgG antibodies conjugated to FITC. The localization of the protein-green light lying on the leptospire was achieved by superimposing the two fields, and the results obtained are shown in Fig. 2. For each protein, two fields are depicted where we can see, in general, green fluorescence spots along the bacteria. An intense green fluorescence could be observed for LipL32, a well-known major outer membrane

protein of *Leptospira*, used as a positive control [38], but not with GroEL, a protoplasmic-cylinder marker used as a negative control [39]. The PBS control showed only red fluorescence of leptospire stained with propidium iodide.

Recombinant leptospiral proteins bind human plasminogen

We have reported that *L. interrogans* binds PLG and that several proteins could act as receptors [21]. These data prompted us to investigate whether the selected surface-exposed proteins are capable of binding human PLG *in vitro*. We have employed Western blotting assay as a first screening to identify PLG-binding proteins. The results show that the proteins LipL32, Lp29, Lp49, LipL40, MPL36, rLIC10494, rLIC12730 and rLIC12238 were interacting with PLG, while no binding was seen with Lsa63, Lsa27, rLIC10509, MPL21, MPL17, rLIC12730 and rLIC12922 (Fig. 3A). In addition, the data clearly indicate that binding only occur with the expected molecular mass corresponding to the recombinant proteins (Fig. 3A). The binding of the proteins to PLG was also evaluated by ELISA. The fourteen recombinant leptospiral proteins, and BSA, used as negative control, were individually immobilized onto 96-wells plates, incubated with human PLG and the results obtained from three independent experiments are shown in Fig. 3B. Proteins LipL32, Lp29, Lp49, LipL40, MPL36, rLIC10494 and rLIC12238 presented statistically significant binding to PLG ($*P<0.001$ and $**P<0.0001$), while proteins, Lsa63, Lsa27, rLIC10509, MPL21, MPL17, rLIC12730, rLIC12922 and BSA did not show significant binding activity (Fig. 3B). The ELISA data are in agreement with the one obtained with Western blotting except for rLIC12730 and rLIC12238 that show discrepancy in binding with the two methodologies. The rLIC12730 depicted a stronger binding by Western blotting when compared to ELISA data, while rLIC12238 showed an opposite behavior (Fig. 3A, 3B).

Comparison of encoding sequences of the detected leptospiral plasminogen-binding proteins and those of published plasminogen-binding proteins

To investigate whether the identified PLG-binding proteins showed sequence similarity with other published spirochetal PLG-binding proteins [40,41,42,43,44], we carried out sequence alignment analysis using the Clustal X program and a tree-display NJ plot [45,46]. The constructed dendrogram showed that leptospiral PLG-binding proteins do not display sequence similarity or share conserved domains with other reported spirochetal PLG-binding proteins (data not shown).

Role of lysine residues in the plasminogen binding activity

It is known that PLG kringle domains frequently mediate interactions with lysine residues of the bacterial receptors [20]. These domains participate in the binding of PLG with intact live *L. interrogans* serovar Copenhageni strain L1–130, since the derivative and analogue of lysine, ACA, almost totally inhibited binding [21]. Sequence analysis showed that the native PLG binding proteins, LipL32, Lp29, Lp49, LipL40, MPL36, LIC10494, LIC12238 and LIC12730, contain 9.6, 9.3, 10.7, 7.8, 10.6, 9.1, 8.8 and 5.5% lysine residues, respectively. Based on these findings, the participation of lysine residues in the binding of the recombinant proteins was evaluated by the addition of ACA to the assay. As depicted in Fig. 4A, when 2 mM ACA was added to the reaction, the binding of the proteins to PLG was almost completely abolished.

Table 1. Gene locus, protein name, NCBI reference sequence, features, gene conservation, sequence of the primers employed for DNA amplification, and molecular mass of expressed recombinant proteins.

Gene locus ¹	Recombinant protein given name	NCBI reference sequence number ²	Description/ Function	Conservation (identity) ³	Sequence of primers for PCR amplification	Recombinant protein molecular mass [70]
LIC11352	LipL32 ^a	YP_001316	Major outer membrane protein (MOMP), LipL32 lipoprotein	Lai (100%); LBH (98%)	F: 5' CACCGGTGCTTCGGTGGTCTG 3' R: 5' ATTACTTAGTCGCGTCAGAAGC 3'	30.2
LIC10314	Lsa63 ^b	YP_000304	Conserved hypothetical protein with Borrelia_P83 domain	Lai (98%); LBH (87%) LBP (39%)	F: 5' GGATCCTTATTTTCTCAGGAAAG 3' (BamH I) R: 5' GGTACCCTAAGGTTTAATTTTTT 3' (Kpn I)	63.0
LIC10509	rLIC10509 ^c	YP_000493	Putative lipoprotein	Lai (98%)	F: 5' CCGGGATCCAAAAGAGCAAAGAAG 3' (BamH I) R: 5' GGTACCCTACTCGAGACGCCAGGA-CCTTC 3' (Kpn I)	22.0
LIC12892	Lp29 ^d	YP_002808	Putative lipoprotein		F: 5' CTCGAGGCAGTACATTACAATC-TTGCT 3' (Xho I) R: 5' CATGGCTCTTAGGAGCCTGGAAA 3' (Nco I)	29.6
LIC10793	Lp49 ^d	YP_000772	Putative lipoprotein, Surface antigen	Lai (99%); LBH (86%)	F: 5' CTCGAGAGCGGAGACTTTTCTTACTT 3' (Xho I) R: 5' CCATGGTTAAAAACCATCTCTACG-ATAAAC 3' (Nco I)	49.1
LIC12895	Lsa27 ^e	YP_002811	Putative lipoprotein	Lai (79%)	F: 5' GGATCCCTGAAATATACGAA 3' (EcoRI) R: 5' GAATTCCTACTGTTCTCTCTC 3' (BamHI)	27.0
LIC13131	MPL21 ^{a,f}	YP_003039	Hypothetical protein with Ycel domain	Lai (98%); LBP (45%)	F: 5' CACCACGTCTCAAAGTTACGCTTCAG 3' R: 5' TTCTCACCATCCAGCTCGG 3'	21.9
LIC10765	MPL17 ^{a,f}	YP_000745	Conserved hypothetical protein	Lai (100%); LBH (80%) LBP (41%)	F: 5' CACCGAAAGTCCCGTAAGGTTCAAA 3' R: 5' TGCAGGAGTCCACATTTTA 3'	15.4
LIC10091	LipL40 ^a	YP_000088	Putative lipoprotein	Lai (100%); LBH (84%)	F: 5' CCATGGGACTCGAGACGCCTCTCC-TAAAGATCC 3' R: 5' CTCCATGGTCATTTCAAACCTT-CTACGGGGC 3'	39.0
LIC10054	MPL36 ^a	YP_000054	Putative lipoprotein with Rare lipoprotein A (RplA) like domain	Lai (100%); LBH (88%) LBP (50%)	F: 5' CACCACGTCTTGTCGCTCGGTAGAG 3' R: 5' CCAAGTATTCTATTATACGTCCGAG 3'	35.1
LIC10494	rLIC10494 ^g	YP_000478	Putative lipoprotein	Lai (99%)	F: 5' CACCACTGCTAGGGCTGCAGAAA 3' R: 5' ACTTTGAGAGCTTCGTCTCGT 3'	25.8
LIC12730	rLIC12730 ^g	YP_002650	Hypothetical protein with TPR domain and 4 NHL repetition	Lai (100%); LBH (90%) LBP (37%)	F: 5' CACCAGTCTGACGGAATCCCAA 3' R: 5' TCTTGCAGATGAGTTGATCC 3'	77.4
LIC12922	rLIC12922 ^h	YP_002837	Conserved hypothetical protein	Lai (100%); LBH (89%) LBP (48%)	F: 5' CACCGAATCACTCAACAGAGTCATTGC 3' R: 5' ATCAATCTAAATGAAACGTCTCTTC 3'	40.0
LIC12238	rLIC12238 ^h	YP_002173	Putative lipoprotein	Lai (100%); LBH (81%) LBP (39%)	F: 5' CTCGAGTGTTTTAAACCTACCGGAG 3' (Xho I) R: 5' AAGCTTCTACTTCATCGCTTTTCTATATC 3' (Hind III)	17.6

¹<http://aeg.lbi.ic.unicamp.br/world/lic/> [35].²<http://www.ncbi.nlm.nih.gov/protein/>.³Protein BLAST - <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi> [71,72].^aPreviously published by Gamberini et al. [29].^bVieira et al. [19].^cPreviously published by Gómez et al. [33].^dPreviously published by Neves et al. [30].^ePreviously published by Longhi et al. [11].^fPreviously published by Oliveira et al. [31].^gPreviously published by Barbosa et al. [12].^hThis work; Lai: *L. interrogans* serovar Lai [34]; LBH: *L. borgpetersenii* serovar Hardjo-bovis [36]; LBP: *L. biflexa* serovar Patoc [37].

doi:10.1371/journal.pone.0011259.t001

Figure 1. SDS-PAGE analysis of purified recombinant proteins. Recombinant proteins were expressed in *E. coli* BL21 (DE3) or SI and purified from the bacterial cell lysate using Ni^{2+} -charged chelating Sepharose columns. M: molecular mass protein marker.
doi:10.1371/journal.pone.0011259.g001

Activation of plasminogen-bound proteins

PLG bound to the surface of *L. interrogans* is converted to enzymatically active plasmin by the addition of activator [21]. To determine if PLG bound to recombinant proteins can acquire proteolytic activity, 96-well plates were coated with the test proteins, blocked, and then incubated with PLG. Unbound PLG was washed away and a plasmin-type PLG activator was added together with a plasmin-specific chromogenic substrate. The reaction was carried out overnight and the plasmin activity was evaluated by measuring the cleavage of the plasmin-specific chromogenic substrate (absorbance at 405 nm). As shown in Fig. 4B, the PLG captured by the proteins could be converted into plasmin, as demonstrated indirectly by specific proteolytic activity. The negative control BSA did not bind PLG (see Fig. 4) and did not show any proteolytic activity, as with the controls lacking PLG, uPA or the chromogenic substrate.

Characterization of the binding of recombinant proteins to PLG

The interactions between the recombinant proteins and PLG were assessed on a quantitative basis as illustrated in Fig. 5A. Dose-dependent and saturable binding was observed when increasing concentrations (0 to 1,000 nM) of the recombinant proteins MPL36, LipL40, LipL32, rLIC10494 and rLIC12238 were allowed to individually adhere to a fixed PLG amount (1 μg). For the proteins Lp29, Lp49 and rLIC12730, the saturation level was not reached, even at the highest concentration tested (1,000 nM). Based on the ELISA data, the calculated dissociation equilibrium constants (K_D) for the recombinant proteins with PLG are depicted in Fig. 5B; for the ones that reached equilibrium, the highest and the lowest K_D values were for LipL32 (25.87 ± 5.09 nM) and MPL36 (3.52 ± 3.95 nM), respectively.

Effects of salt and heparin on LipL32- and rLIC12238 - plasminogen interactions

As the proteins LipL32 and rLIC12238 showed the highest PLG binding and plasmin activity (Fig. 3B and Fig. 4B), they were chosen for further studies. To verify whether binding of these recombinant proteins to PLG is affected by added Cl^- [47] or negatively charged heparin [48], binding assays were performed in

the presence of different concentrations of NaCl or heparin. As shown in Fig. 6A, the increase in Cl^- anions to physiological conditions did not affect binding, and interference was only detected at a higher salt concentration, 350 mM, for both proteins. Similarly, the addition of heparin (up to 500 IU) did not have any significant effect on the interaction of the proteins with PLG (Fig. 6B).

Collagen type IV, but not plasma fibronectin, competes with LipL32 for binding to PLG

LipL32 has been previously demonstrated to be a leptospiral adhesin capable of binding collagen type IV and plasma fibronectin [15,16]. To see if the extracellular matrix components compete with LipL32 for the binding site of PLG or interfere somehow in the interaction with PLG, the binding assay was performed in the presence of increasing concentrations of collagen type IV or plasma fibronectin. The results show that plasma fibronectin had no effect on the binding of LipL32 to PLG (Fig. 7). On the other hand, the addition of collagen type IV decreased LipL32 binding to PLG in a dose-dependent fashion (Fig. 7), suggesting that both molecules compete for the same binding site.

Discussion

The interaction of the human plasminogen system has been suggested to be a feature that significantly contributes to the virulence of many bacterial pathogens by facilitating the initial anchoring to the endothelium [20]. Through capturing the host PLG on its surface, followed by activation to enzymatically active plasmin, the bacteria are turned into proteolytic organisms, a characteristic that has been shown to contribute to the degradation of ECM components, tissue penetration and invasion. In our previous work, we showed that leptospires are capable of capturing PLG on its outer surface and that the addition of exogenous activator converted it into enzymatically active plasmin capable of degrading ECM fibronectin [21]. In addition, multiple PLG-binding proteins present in the *L. interrogans* serovar Copenhageni whole cell lysates were detected, but remained unidentified [21]. Moreover, as expected, our data suggest the involvement of outer membrane proteins in the binding. Indeed, Verma and colleagues [27] have recently demonstrated that LenA, a surface leptospiral

Figure 2. Cellular localization of proteins in *L. interrogans* by L-IFA. Protein recognition by L-IFA. Proteins in live *L. interrogans* serovar Copenhageni isolates were recognized by FITC-conjugated antibodies against Lp29, Lp49, LipL40, MPL36, LIC10494, LIC12730, LIC12922, LIC12238 and LipL32 (surface-exposed lipoprotein, positive control), but not against GroEL (a protoplasmic cylinder marker) and PBS (negative control) under confocal microscopy. The leptospires were identified using the DNA counterstain propidium iodide. Panels are composite images of propidium iodide- and FITC-stained slides. Magnification, 600 $\times$.
doi:10.1371/journal.pone.0011259.g002

protein [14] is a bacterial receptor for human plasminogen. The availability of several putative surface-exposed recombinant proteins of *L. interrogans* in our laboratory prompted us to investigate the ability of these proteins to interact with PLG *in vitro*, in order to reveal and extend the array of plasminogen receptors in *Leptospira*.

The fourteen proteins selected for the screening of PLG binding were cloned and expressed in *E. coli* as full-length recombinant proteins. The purified proteins exhibited major bands in SDS-PAGE making them suitable for PLG binding assays. The structural integrity of the unpublished purified proteins was attested by CD spectroscopy revealing a mixture of α -helix and β -strand structures. The other recombinant proteins had their CD

spectra confirmed and were similar to the ones previously reported [11,12,19,30,31,33]. The cellular localization of the proteins Lp29, Lp49, LipL40, MPL36, rLIC10494, rLIC12730, rLIC12922 and rLIC12238, was shown to be the cell surface, similar to findings for the previously published LipL32 [28], Lsa63 [19], rLIC10509 [33], Lsa27 [11], MPL21 and MPL17 [31].

Several invasive gram-positive and gram-negative bacteria have been shown to interact with the host PLG system [20,49,50,51,52], a phenomenon that has also been observed with viruses [53,54,55] and parasites [56], either by expression of PLG receptors or stimulation of host activators. In the case of spirochetes, the plasminogen activation system was studied with several species of *Borrelia* with *Treponema denticola* and suggested to have an important role during infection [22,23,24,25,26].

The human plasminogen heavy chain contains five triple-disulfide-linked peptide regions of ~ 80 amino acids, called kringles [57]. The process of binding to bacterial receptors on the cell surface has been shown for several pathogens to be mediated by the PLG kringle domains through the lysine-binding sites present [20]. Similarly, *Borrelia* [24] and *Leptospira* [21] also bind PLG via kringle domains. We have identified eight leptospiral plasminogen-binding proteins: LipL32 [28,32], rLIC10494, rLIC12730 [12], Lp29, Lp49 [30] and LipL40 and MPL36 [29] and one novel protein rLIC12238. The specificity of the binding of these recombinant proteins was attested by Western blotting. Interestingly, two proteins showed disparate binding intensity by Western blotting and ELISA. This could be explained by the fact that ELISA is a quantitative method but Western blotting is not. In addition, due to the denaturing condition of this latter method, it is possible that the exposure of some epitopes led to better binding for rLIC12730, while the inverse situation was seen with rLIC12238 which apparently needs a more native condition for PLG binding.

Plasminogen-binding proteins of *B. burgdorferi*, including the outer-surface lipoprotein OspA, have been identified [41]. Recently, Brisette and coworkers [44] identified three proteins of *B. burgdorferi*, ErpP, ErpA and ErpC, as being PLG-binding proteins. Interestingly, an evaluation of the primary sequence of spirochete PLG-binding proteins show that the proteins do not share similarity, with the exception of the Erp family protein of *B. burgdorferi* [44], BbCRASP-1 of *B. hermsii* [43] and HcpA of *B. recurrentis* [40].

Our data with the PLG-binding proteins point out to a primary role for lysine residues in plasminogen binding, as the lysine analog 6-aminocaproic acid (ACA) significantly inhibited binding [21,27,58]. However, no correlation between the number of lysine residues and PLG binding was detected. Indeed, the highest percentage of lysine residues was identified in Lsa63 [19], a protein that did not bind PLG. Moreover, lysine residue position does not seem to correlate with binding because they were distributed among all the protein sequences studied. This suggests that protein conformation and steric hindrance should be important factors in the binding of the protein/receptor to PLG. Plasminogen-binding proteins were able to generate active plasmin in the presence of urokinase-type PLG activator, as measured by the cleavage of plasmin-specific chromogenic substrate.

The recombinant proteins bind to PLG in a concentration-dependent and saturable fashion, indicating the specificity of the binding, for the proteins LipL32, LipL40, MPL36, rLIC12238 and rLIC10494. The calculated dissociation equilibrium constants (K_D) for the recombinant proteins with PLG varied from 25.87 ± 5.09 nM for LipL32 to 3.52 ± 3.95 nM for MPL36. The plasminogen interaction/activation has been shown to be suppressed by the presence of Cl anions [47], while there is

Figure 3. Binding of leptospiral recombinant proteins to human plasminogen. **A.** The proteins were electrophoresed in 12% SDS-PAGE gels and transferred to nitrocellulose membranes, followed by overnight incubation with 3 μ g/mL PLG; after washing, the membrane was incubated with mouse anti-human PLG (1:750), followed by incubation with anti-mouse IgG (1:5,000). Protein reactivity was revealed by ECL reagent/exposure to X-ray films. **B.** The recombinant proteins LipL32, Lsa63, rLIC10509, Lp29, Lp49, Lsa27, MPL21, MPL17, LipL40, MPL36, rLIC10494, rLIC12730, rLIC12922 and rLIC12238 were coated onto 96-well ELISA plates (10 μ g/ml) and allowed to interact with purified human PLG (10 μ g/ml). BSA was used as a negative control for nonspecific binding. Binding was detected and quantified by specific antibodies. Bars represent the mean absorbance values at 492 nm $\pm$ the standard deviation of four replicates for each protein and are representative of three independent experiments. * $P < 0.001$; ** $P < 0.0001$. doi:10.1371/journal.pone.0011259.g003

Figure 4. Role of lysine residues in recombinant protein-PLG interaction and activation to enzymatically active plasmin. A: Binding of the immobilized proteins LipL32, Lp29, Lp49, LipL40, MPL36, rLIC10494, rLIC12730 and rLIC12238 (10 μ g/ml) to PLG (10 μ g/ml) was carried out in the presence (PLG+ACA) or absence (PLG) of the lysine analogue 6-aminocaproic acid. BSA was used as a negative control for nonspecific binding. The bound PLG was detected and quantified by specific antibodies. Bars represent the mean absorbance at 492 nm $\pm$ the standard deviation of four replicates for each sample and are representative of two independent experiments. Statistically significant differences are shown by * ($P < 0.001$) and ** ($P < 0.0001$). **B:** Cleavage of specific plasmin substrate by PLG bound to recombinant proteins was assayed by modified ELISA as immobilized proteins received the following treatment: PLG+uPA+specific plasmin substrate (PLG+uPA+S), or controls lacking one of the three components (PLG+uPA; PLG+S; uPA+S). BSA was employed as a negative control. Bars represent the mean absorbance values at 405 nm, as a measure of relative substrate cleavage, $\pm$ the standard deviation of four replicates for each experimental group and are representative of two independent experiments. Statistically significant differences are shown by * ($P < 0.00001$), ** ($P < 0.005$) and *** ($P < 0.05$). doi:10.1371/journal.pone.0011259.g004

evidence that heparin dextran derivatives could contribute to the regulation of plasmin activity, not only by impeding plasmin generation, as a result of their binding to plasminogen but also by directly affecting the catalytic activity of the enzyme [48]. The

addition of Cl^- did not inhibit LipL32 or rLIC12238-PLG interactions, showing in fact, a positive effect with increasing sodium chloride concentration. The presence of heparin had no influence on the binding of either protein to PLG. Similar results

Figure 5. Characterization of recombinant proteins binding to PLG. A. PLG (10 μ g/ml) was immobilized in 96-well ELISA plates, and each recombinant protein at 0 to 1,000 nM was added for interaction. The binding was detected using antiserum raised in mice against each protein at appropriate dilutions (1:4,000 for LipL32; 1:5,000 for rLIC12238, LipL40 and MPL36; 1:1,000 for Lp29 and Lp49; 1:500 for rLIC12730), followed by horseradish peroxidase-conjugated anti-mouse IgG. Data represent the mean absorbance values $\pm$ the standard deviation of six replicates for each experimental group. The results are representative of two independent experiments. B. The dissociation constant (K_D) was calculated based on ELISA data for the recombinant proteins that reached equilibrium up to a concentration of 1,000 nM.
doi:10.1371/journal.pone.0011259.g005

were obtained by Brissette and coworkers [44] studying the effect of both compounds on the interactions of ErpP recombinant protein of *B. burgdorferi* and plasminogen. Furthermore, Verma et al. [27] showed that heparin had no effect on LenA-PLG interactions but that the addition of salt led to an increase in binding.

The identified PLG-binding proteins Lp29, Lp49, LipL40 and MPL36 have been previously reported to be reactive with sera from leptospirosis patients [29,30] while rLIC12238 and rLIC10494 react with sera from hamsters infected with *Leptospira* (Nascimento, AL et al., unpublished results). These data suggest that the proteins are expressed and exposed during the course of infection. We confirmed here the localization of these four proteins and presented their PLG-binding properties. Taken together, our results implicate that these proteins play a possible role in virulence.

The protein LipL32 is a major outer membrane antigen expressed during mammalian infection [16,28]. It has been shown

that LipL32 is recognized by patients' sera [15,59] and has the ability to bind to ECM macromolecules [15], activities that suggest a role in the pathogenesis of leptospires. Intriguingly, it has also been demonstrated that LipL32 is not essential for either acute or chronic infection with *L. interrogans* [60]. Here, we provide another activity for LipL32, as a PLG-binding protein, giving further support to its importance during infection. Additionally, our results suggest that collagen type IV and PLG interact with the same site of LipL32, a situation that does not occur with plasma fibronectin. The dual activity of LipL32 in binding PLG and ECM macromolecules together with our previous findings of fibronectin degradation by plasmin-coated leptospires [21], may have implications on the mechanisms of the invasion process.

In conclusion, we have identified and characterized eight proteins as being PLG-binding proteins of *Leptospira*. There is evidence that at least five of them (LipL32, Lp29, Lp49, LipL40

A

B

Figure 6. Effects of ionic strength and heparin in PLG binding by LipL32 and rLIC12238. Binding of LipL32 and rLIC12238 (10 μ g/ml) to immobilized PLG (10 μ g/ml) was performed with the addition of increasing concentrations of NaCl (0 to 350 mM) (A) or heparin (0 to 500 IU) (B). The detection of bound-PLG was assayed by specific antibodies. Bars represent the mean absorbance values $\pm$ the standard deviation of four replicates for each experimental group and are representative of two independent experiments. The data show statistically significant differences in **A**, shown by * ($P<0.05$), but not in **B**.
doi:10.1371/journal.pone.0011259.g006

and MPL36) are expressed during natural infection [28,29,30]. PLG-binding/activation through the proteins/receptors on the surface of *Leptospira* could help the bacteria to specifically overcome tissue barriers, facilitate invasion and colonize mammalian tissues. We believe that these studies provide new insights into leptospiral pathogenesis and infection.

Materials and Methods

Bioinformatics characterization of the proteins

Predicted coding sequences (CDSs) were analyzed as their cellular localization predictions by PSORT program, <http://psort.nibb.ac.jp> [61,62]. The web servers SMART, [http://smart.](http://smart.embl-heidelberg.de/)

[embl-heidelberg.de/](http://smart.embl-heidelberg.de/) [63,64], PFAM, <http://www.sanger.ac.uk/Software/Pfam/> [65], and LipoP, <http://www.cbs.dtu.dk/services/LipoP/> [66] were used to search for predicted functional and structural domains within the amino acid sequences of the CDSs.

Cloning, expression and purification of recombinant proteins

Amplification of the CDSs was performed by PCR from *L. interrogans* serovar Copenhageni genomic DNA using complementary primer pairs. The gene sequences were amplified without the signal peptide tag, predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). The final constructs were verified by DNA sequencing on an ABI Prism 3730_L sequencer (Seq- Wright,

Figure 7. Effect of extracellular matrix macromolecules on PLG binding by LipL32. Binding of LipL32 (10 μ g/ml) to immobilized PLG (10 μ g/ml) was performed with the addition of increasing concentrations of plasma fibronectin or collagen type IV (0 to 600 mM). The detection of bound-PLG was assayed by specific antibodies. Bars represent the mean absorbance values $\pm$ the standard deviation of four replicates for each condition and are representative of two independent experiments. Results of statistically significant interference with binding in comparison with the positive control (no addition of purified ECM macromolecules) are shown: $P<0.0003$ (*).
doi:10.1371/journal.pone.0011259.g007

Houston, TX) with appropriate vector-specific T7 (F: TAATAC-GACTCACTATAGGG) and pAE (R:CAGCAGCCAACT-CAGTTCCT) primers. Cloning, expression and purification of the recombinant proteins Lsa63, LIC10509, Lp29, Lp49 and Lsa27 have been previously described [11,19,30,33]. The sequences coding for MPL21, MPL17, MPL36, LipL40, LipL32, LIC10494, LIC12730 and LIC12922 were cloned into the *E. coli* Gateway cloning (pENTR) and expression (pDEST17) system (Invitrogen), according to the protocol described elsewhere [29]. The expression and purification of MPL21 and MPL17 were performed as reported elsewhere [31], and rLIC10494 and rLIC12730, as previously described [12]. The recombinant proteins MPL36 and LipL40 were purified from *E. coli* BL21 (DE3) and *E. coli* BL21-SI, respectively. Bacteria were grown in Luria-Bertani (LB) broth with or without NaCl, at 37°C or 30°C, and at log-phase they were induced by 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) or 3 mM NaCl for 3 h at 37°C or 30°C, respectively. The bacteria cell lysates were cleared by centrifugation at 3,000 $\times$ g for 15 min, and the supernatants were loaded onto Ni²⁺-charged Sepharose beads. The beads were washed with increasing concentrations of imidazole (20 to 60 mM) and the proteins eluted with 0.5 M imidazole. The proteins LipL32 and rLIC12922 were expressed in *E. coli* BL21-SI for 3 h at 30°C with the addition of 150 mM NaCl to the log-phase culture grown in LB broth without NaCl. The proteins were expressed in the soluble form, as the above protocol described for the proteins MPL36 and LipL40. The LIC12238 CDS was cloned into the pGEM-T-Easy vector (Promega), and transferred into the pAE expression vector [67]. The construct was transformed into *E. coli* BL21-SI, and the log-phase culture, grown at 30°C, was induced by incubation in the presence of 300 mM NaCl for 3 h. The protein rLIC12238 was expressed in the soluble form and purified as described above.

Circular dichroism spectroscopy

Purified recombinant proteins were dialyzed against sodium phosphate buffer (pH 7.4). Circular dichroism (CD) spectroscopy measurements were performed at 20°C using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo) equipped with a Peltier unit for temperature control. Far-UV CD spectra were measured using a 1 mm-path length cell at 0.5 nm intervals. The spectra were presented as an average of five scans recorded from 180 to 260 nm. The molar ellipticity (Φ) is expressed in deg.cm².dmol⁻¹.

Antisera production against recombinant proteins

The recombinant proteins were adsorbed onto 10% (vol/vol) of Alhydrogel (2% Al (OH)₃, Brenntag Biosector, Denmark), used as adjuvant. Each recombinant protein (10 μ g) was subcutaneously injected in female BALB/c mice (4–6 weeks old). Two subsequent booster injections were given at two-week intervals with the same protein preparations. Negative-control mice were injected with PBS/Alhydrogel. Two weeks after each immunization, the mice were bled from the retro-orbital plexus, and the pooled sera were analyzed by enzyme-linked immunosorbent assay for the determination of antibody titers. All animal studies were approved by the Ethics Committee of Instituto Butantan, under protocol 506/08, São Paulo, SP, Brazil.

Liquid-phase immunofluorescence assay (L-IFA)

The localization of the recombinant proteins by L-IFA was performed as previously described [31]. In brief, 2.5-ml suspensions of live leptospires (*L. interrogans* serovar Copenhageni strain M 20, routinely cultured as described elsewhere [21] were harvested

at 10,000 rpm for 15 min, washed twice with PBS (with 50 mM NaCl), resuspended in 200 μ l of PBS with 6 μ g/ml propidium iodide to stain the nuclei, and incubated for 45 min at 37°C. After incubation, the leptospires were gently washed with PBS and incubated for 30 min at 4°C with polyclonal mouse antiserum against each recombinant protein or GroEL at a 1:50 dilution. The leptospires were washed and incubated with goat anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC, Sigma) at a dilution 1:50 for 30 min at 4°C. After incubation with secondary antibody, the leptospires were washed and resuspended in PBS-antifading solution (ProLong Gold, Molecular Probes). The immunofluorescently labeled leptospires were examined with the use of a confocal LSM 510 META immunofluorescence microscope (Zeiss, Germany).

Plasminogen binding

The binding of the recombinant proteins to PLG was evaluated by a modified ELISA as follows: 96-well plates (Costar High Binding, Corning) were coated overnight in PBS at 4°C with 100 μ l of 10 μ g/ml of the recombinant proteins or bovine serum albumin (BSA) as negative control. Plates were washed once with PBS supplemented with 0.05% (vol/vol) Tween 20 (PBS-T) and blocked for 2 h at 37°C with PBS with 10% (wt/vol) non-fat dry milk. The blocking solution was discarded and 100 μ l of 10 μ g/ml human plasminogen in PBS was incubated for 2 h at 37°C. Wells were washed four times with PBS-T and incubated for 1 h at 37°C with mouse anti-human plasminogen (Sigma-Aldrich) (1:4,000 in PBS). Plates were washed again and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG), diluted 1:5,000 in PBS. After three washings, 100 μ l/well of 1 mg/ml *o*-phenylenediamine (OPD) plus 1 μ l/ml H₂O₂ in citrate phosphate buffer (pH 5.0) were added. The reactions were carried out for 5 min and stopped by the addition of 50 μ l/well of H₂SO₄ (2 N). Readings were taken at 492 nm.

Effects of salt and heparin on recombinant proteins-plasminogen interactions

To assess the role of ionic interactions or of heparin-binding domains in LipL32 or rLIC12238 binding to plasminogen, increasing concentrations of NaCl (0–350 mM) or heparin (0–500 IU) (Roche) were added to the PBS-based buffer with plasminogen to the recombinant protein-coated wells. In both experiments, the detection of bound-plasminogen was performed as described above.

SDS-PAGE and affinity blotting

The purified recombinant proteins were electrophoresed in 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare) in semi-dry equipment. The membranes were blocked with 5% BSA for 2 h at 37°C, washed three times (10 min for each wash) with PBS-0.5% Tween-20 solution and incubated overnight with 3 μ g/mL PLG 4°C, followed by 2h incubation at room temperature. The membranes were then washed three times and incubated with mouse anti-human PLG (1:750) for 3 h at room temperature, followed by three more washings and 1 h incubation at room temperature with anti-mouse IgG (1:5,000). The membranes were washed and the protein's reactivity was revealed using the ECL reagent (GE Healthcare) with subsequent exposure to X-ray films.

Characterization of the binding

To determine the role of lysines in plasminogen-recombinant protein interactions, the lysine analog 6-aminocaproic acid (ACA)

(Sigma-Aldrich) was added together with plasminogen at a final concentration of 2 mM to the recombinant protein-coated wells. To assess the role of ionic interactions or of heparin-binding domains in LipL32 or rLIC12238 binding to plasminogen, increasing concentrations of NaCl (0–350 mM) or heparin (0–500 IU) (Roche) were added to the PBS-based buffer with plasminogen which was then added to the recombinant protein-coated wells. In both experiments, the detection of bound-plasminogen was performed as described above.

Plasmin enzymatic activity assay

First, 96-well ELISA plates were coated overnight with 10 µg/ml recombinant proteins or BSA in PBS at 4°C. Plates were then washed once with PBS-T and blocked with PBS with 10% (wt/vol) non-fat dry milk for 2 h at 37°C. The blocking solution was discarded and 100 µl/well of 10 µg/ml human plasminogen was added, followed by incubation for 2 h at 37°C. Wells were washed three times with PBS-T, and then 4 ng/well of human uPA (Sigma-Aldrich) were added. Subsequently, 100 µl/well of the plasmin-specific substrate D-valyl-leucyl-lysine-*p*-nitroanilide dihydrochloride (Sigma-Aldrich) was added at a final concentration of 0.4 mM in PBS. Plates were incubated overnight at 37°C and substrate was measured by taking readings at 405 nm.

Dose-response curves

First, 96-well ELISA plates were coated overnight in PBS at 4°C with 100 µl of 10 µg/ml plasminogen. Plates were then blocked and increasing concentrations of the purified recombinant proteins (0–1 µM) were added (100 µl/well in PBS). The assessment of bound proteins was performed by incubation for 1 h at 37°C with the antiserum raised against each protein at appropriate dilutions (1:4,000 for LipL32; 1:5,000 for rLIC12238, LipL40 and MPL36; 1:1,000 for Lp29 and Lp49; 1:500 for rLIC12730), followed by horseradish peroxidase-conjugated anti-mouse IgG (Sigma) (1:10,000 in PBS). The binding was evaluated by the peroxidase

substrate OPD and readings were taken at 492 nm. The ELISA data were used to calculate the dissociation constant (K_D) according to the method described by Pathirana et al. [68] and Lin et al. [69], based on the equation: $A = A_{max} [\text{protein}] / (K_D + [\text{protein}])$, where A is the absorbance at a given protein concentration, A_{max} is the maximum absorbance for the ELISA plate reader (equilibrium), $[\text{protein}]$ is the protein concentration and K_D is the dissociation equilibrium constant for a given absorbance at a given protein concentration (ELISA data point).

Interference of collagen type IV and plasma fibronectin with PLG binding by LipL32

First, 96-well plates were coated overnight at 4°C with 100 µl of 10 µg/ml recombinant LipL32 in PBS, washed once and blocked with 10% (w/v) non-fat dry milk for 2 h at 37°C. The plates were then incubated for 2 h at 37°C with 200 nM PLG together with increasing concentrations of collagen type IV (Sigma-Aldrich) or plasma fibronectin (Sigma-Aldrich) (both from 0 to 600 nM). After four washings, PLG binding was quantified by specific antibodies as described above.

Acknowledgments

We are deeply indebted to Dr. Toshie Kawano and Alexsander Seixas de Souza (Departamento de Parasitologia, Instituto Butantan, São Paulo, Brazil) for use of the confocal microscope facilities and helpful discussion. We thank Dr. Gustavo M. Cerqueira for a critical reading of the manuscript and Dr. A. Leyva for help with English editing.

Author Contributions

Conceived and designed the experiments: MV ALTON. Performed the experiments: MV MA TO RO DA. Analyzed the data: MV MA TO RO DA SV ALTON. Contributed reagents/materials/analysis tools: MV MA TO RO DA SV ALTON. Wrote the paper: MV ALTON.

References

- Haake DA, Dundoo M, Cader R, Kubak BM, Hartskeerl RA, et al. (2002) Leptospirosis, water sports, and chemoprophylaxis. *Clin Infect Dis* 34: e40–43.
- Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, et al. (2003) Leptospirosis: a zoonotic disease of global importance. *Lancet Infect Dis* 3: 757–771.
- Faine S, Adler B, Bolin C, Perolat P (1999) *Leptospira* and *Leptospirosis*. Second ed. Melbourne, Australia: MediSci. 259 p.
- Levett PN (2001) Leptospirosis. *Clin Microbiol Rev* 14: 296–326.
- Vinetz JM (2001) Leptospirosis. *Curr Opin Infect Dis* 14: 527–538.
- Plank R, Dean D (2000) Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. *Microbes Infect* 2: 1265–1276.
- Matsunaga J, Barocchi MA, Croda J, Young TA, Sanchez Y, et al. (2003) Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. *Mol Microbiol* 49: 929–945.
- Ristow P, Bourhy P, da Cruz McBride FW, Figueira CP, Huerre M, et al. (2007) The OmpA-like protein Loa22 is essential for leptospiral virulence. *PLoS Pathog* 3: e97.
- Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Moraes ZM, et al. (2008) Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC Microbiol* 8: 70.
- Patti JM, Allen BL, McGavin MJ, Hook M (1994) MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu Rev Microbiol* 48: 585–617.
- Longhi MT, Oliveira TR, Romero EC, Goncalves AP, Moraes ZM, et al. (2009) A novel identified protein of *Leptospira interrogans* mediates binding to laminin. *J Med Microbiol*.
- Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, et al. (2006) A newly identified leptospiral adhesin mediates attachment to laminin. *Infect Immun* 74: 6356–6364.
- Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J, et al. (2007) Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. *Infect Immun* 75: 2441–2450.
- Stevenson B, Choy HA, Pinne M, Rotondi ML, Miller MC, et al. (2007) *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. *PLoS ONE* 2: e1188.
- Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Moraes ZM, et al. (2008) In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. *Infect Immun* 76: 2642–2650.
- Hoke DE, Egan S, Cullen PA, Adler B (2008) LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudoalteromonas tunicata*. *Infect Immun* 76: 2063–2069.
- Atzingen MV, Gomez RM, Schattner M, Pretre G, Goncalves AP, et al. (2009) Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells. *J Infect*.
- Carvalho E, Barbosa AS, Gomez RM, Cianciarullo AM, Hauk P, et al. (2009) Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity. *FEBS Lett* 583: 1381–1385.
- Vieira ML, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, et al. Lsa63, a newly identified surface protein of *Leptospira interrogans* binds laminin and collagen IV. *J Infect* 60: 52–64.
- Lahteenmaki K, Kuusela P, Korhonen TK (2001) Bacterial plasminogen activators and receptors. *FEMS Microbiol Rev* 25: 531–552.
- Vieira ML, Vasconcellos SA, Goncalves AP, de Moraes ZM, Nascimento AL (2009) Plasminogen acquisition and activation at the surface of *Leptospira* species lead to fibronectin degradation. *Infect Immun* 77: 4092–4101.
- Nordstrand A, Shamaei-Tousi A, Ny A, Bergstrom S (2001) Delayed invasion of the kidney and brain by *Borrelia burgdorferi* in plasminogen-deficient mice. *Infect Immun* 69: 5832–5839.
- Coleman JL, Gebbia JA, Piesman J, Degen JL, Bugge TH, et al. (1997) Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice. *Cell* 89: 1111–1119.
- Coleman JL, Sellati TJ, Testa JE, Kew RR, Furie MB, et al. (1995) *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. *Infect Immun* 63: 2478–2484.

25. Fenno JC, Tamura M, Hannam PM, Wong GW, Chan RA, et al. (2000) Identification of a *Treponema denticola* OppA homologue that binds host proteins present in the subgingival environment. *Infect Immun* 68: 1884–1892.
26. Klempner MS, Noring R, Epstein MP, McCloud B, Rogers RA (1996) Binding of human urokinase type plasminogen activator and plasminogen to *Borrelia* species. *J Infect Dis* 174: 97–104.
27. Verma A, Brissette CA, Bowman AA, Shah ST, Zipfel PF, et al. (2000) Leptospiral endostatin-like protein A (LenA) is a bacterial cell-surface receptor for human plasminogen. *Infect Immun*.
28. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, et al. (2000) The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect Immun* 68: 2276–2285.
29. Gamberini M, Gomez RM, Atzingen MV, Martins EA, Vasconcellos SA, et al. (2005) Whole-genome analysis of *Leptospira* interrogans to identify potential vaccine candidates against leptospirosis. *FEMS Microbiol Lett* 244: 305–313.
30. Neves FO, Abreu PA, Vasconcellos SA, de Moraes ZM, Romero EC, et al. (2007) Identification of a novel potential antigen for early-phase serodiagnosis of leptospirosis. *Arch Microbiol* 188: 523–532.
31. Oliveira TR, Longhi MT, de Moraes ZM, Romero EC, Blanco RM, et al. (2008) Evaluation of leptospiral recombinant antigens MPL17 and MPL21 for serological diagnosis of leptospirosis by enzyme-linked immunosorbent assays. *Clin Vaccine Immunol* 15: 1715–1722.
32. Branger C, Sonrier C, Chatrenet B, Klonjowski B, Ruvoen-Clouet N, et al. (2001) Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira* interrogans by adenovirus-mediated vaccination. *Infect Immun* 69: 6831–6838.
33. Gomez RM, Vieira ML, Schattner M, Malaver E, Watanabe MM, et al. (2008) Putative outer membrane proteins of *Leptospira* interrogans stimulate human umbilical vein endothelial cells (HUVECs) and express during infection. *Microb Pathog* 45: 315–322.
34. Ren SX, Fu G, Jiang XG, Zeng R, Miao YG, et al. (2003) Unique physiological and pathogenic features of *Leptospira* interrogans revealed by whole-genome sequencing. *Nature* 422: 888–893.
35. Nascimento AL, Verjovski-Almeida S, Van Sluys MA, Monteiro-Vitorello CB, Camargo LE, et al. (2004) Genome features of *Leptospira* interrogans serovar Copenhageni. *Braz J Med Biol Res* 37: 459–477.
36. Bulach DM, Zuerner RL, Wilson P, Seemann T, McGrath A, et al. (2006) Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proc Natl Acad Sci U S A* 103: 14560–14565.
37. Picardeau M, Bulach DM, Bouchier C, Zuerner RL, Zidane N, et al. (2008) Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PLoS ONE* 3: e1607.
38. Nally JE, Whitelegge JP, Bassilian S, Blanco DR, Lovett MA (2007) Characterization of the outer membrane proteome of *Leptospira* interrogans expressed during acute lethal infection. *Infect Immun* 75: 766–773.
39. Haake DA, Matsunaga J (2002) Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. *Infect Immun* 70: 4936–4945.
40. Grosskinsky S, Schott M, Brenner C, Cutler SJ, Kraiczky P, et al. (2009) *Borrelia recurrentis* employs a novel multifunctional surface protein with anti-complement, anti-opsonic and invasive potential to escape innate immunity. *PLoS One* 4: e4858.
41. Fuchs H, Wallich R, Simon MM, Kramer MD (1994) The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. *Proc Natl Acad Sci U S A* 91: 12594–12598.
42. Lagal V, Portnoi D, Faure G, Postic D, Baranton G (2006) *Borrelia burgdorferi* sensu stricto invasiveness is correlated with OspC-plasminogen affinity. *Microbes Infect* 8: 645–652.
43. Rossmann E, Kraiczky P, Herzberger P, Skerka C, Kirschfink M, et al. (2007) Dual binding specificity of a *Borrelia hermsii*-associated complement regulator-acquiring surface protein for factor H and plasminogen discloses a putative virulence factor of relapsing fever spirochetes. *J Immunol* 178: 7292–7301.
44. Brissette CA, Haupt K, Barthel D, Cooley AE, Bowman A, et al. (2009) *Borrelia burgdorferi* infection-associated surface proteins ErpP, ErpA, and ErpC bind human plasminogen. *Infect Immun* 77: 300–306.
45. Perriere G, Gouy M (1996) WWW-query: an on-line retrieval system for biological sequence banks. *Biochimie* 78: 364–369.
46. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25: 4876–4882.
47. Urano T, Sator de Serrano V, Chibber BA, Castellino FJ (1987) The control of the urokinase-catalyzed activation of human glutamic acid 1-plasminogen by positive and negative effectors. *J Biol Chem* 262: 15959–15964.
48. Ledoux D, Papy-Garcia D, Escartin Q, Sagot MA, Cao Y, et al. (2000) Human plasmin enzymatic activity is inhibited by chemically modified dextrans. *J Biol Chem* 275: 29383–29390.
49. Degen JL, Bugge TH, Goguen JD (2007) Fibrin and fibrinolysis in infection and host defense. *J Thromb Haemost* 5 Suppl 1: 24–31.
50. Bergmann S, Hammerschmidt S (2007) Fibrinolysis and host response in bacterial infections. *Thromb Haemost* 98: 512–520.
51. Coleman JL, Benach JL (1999) Use of the plasminogen activation system by microorganisms. *J Lab Clin Med* 134: 567–576.
52. Sun H (2006) The interaction between pathogens and the host coagulation system. *Physiology (Bethesda)* 21: 281–288.
53. Goto H, Kawaoka Y (1998) A novel mechanism for the acquisition of virulence by a human influenza A virus. *Proc Natl Acad Sci U S A* 95: 10224–10228.
54. Okumura Y, Yano M, Murakami M, Mori S, Towatari T, et al. (1999) The extracellular processing of HIV-1 envelope glycoprotein gp160 by human plasmin. *FEBS Lett* 442: 39–42.
55. LeBouder F, Morello E, Rimmelzwaan GF, Bosse F, Pechoux C, et al. (2008) Annexin II incorporated into influenza virus particles supports virus replication by converting plasminogen into plasmin. *J Virol* 82: 6820–6828.
56. Rojas M, Labrador I, Concepcion JL, Aldana E, Avilan L (2008) Characteristics of plasminogen binding to *Trypanosoma cruzi* epimastigotes. *Acta Trop* 107: 54–58.
57. Castellino FJA, A.V. (2003) Plasminogen: structure, activation, and regulation Waisman D, ed. New York: Kluwer Academic/Plenum Publishers.
58. Crowe SD, Sievwright JK, Auld GC, Hoore NR, Gow NA, et al. (2003) *Candida albicans* binds human plasminogen identification of eight plasminogen-binding proteins. *Mol Microbiol* 47: 1637–1651.
59. Flannery B, Costa D, Carvalho FP, Guerreiro H, Matsunaga J, et al. (2001) Evaluation of recombinant *Leptospira* antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. *J Clin Microbiol* 39: 3303–3310.
60. Murray GL, Srikrum A, Hoke DE, Wunder EA, Jr., Henry R, et al. (2009) Major surface protein LipL32 is not required for either acute or chronic infection with *Leptospira* interrogans. *Infect Immun* 77: 952–958.
61. Nakai K, Horton P (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem Sci* 24: 34–36.
62. Nakai K, Kanehisa M (1991) Expert system for predicting protein localization sites in gram-negative bacteria. *Proteins* 11: 95–110.
63. Letunic I, Copley RR, Pils B, Pinkert S, Schultz J, et al. (2006) SMART 5: domains in the context of genomes and networks. *Nucleic Acids Res* 34: D257–260.
64. Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains. *Proc Natl Acad Sci U S A* 95: 5857–5864.
65. Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, et al. (2006) Pfam: clans, web tools and services. *Nucleic Acids Res* 34: D247–251.
66. Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, et al. (2003) Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci* 12: 1652–1662.
67. Ramos CR, Abreu PA, Nascimento AL, Ho PL (2004) A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. *Braz J Med Biol Res* 37: 1103–1109.
68. Pathirana RD, O'Brien-Simpson NM, Veith PD, Riley PF, Reynolds EC (2006) Characterization of proteinase-adhesin complexes of *Porphyromonas gingivalis*. *Microbiology* 152: 2381–2394.
69. Lin YP, Lee DW, McDonough SP, Nicholson LK, Sharma Y, et al. (2009) Repeated domains of leptospira immunoglobulin-like proteins interact with elastin and tropoelastin. *J Biol Chem* 284: 19380–19391.
70. Viratyosin W, Ingsriswang S, Pacharawongsakda E, Palittapongarnpim P (2008) Genome-wide subcellular localization of putative outer membrane and extracellular proteins in *Leptospira* interrogans serovar Lai genome using bioinformatics approaches. *BMC Genomics* 9: 181.
71. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389–3402.
72. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215: 403–410.

In vitro evidence for immune evasion activity by human plasmin associated to pathogenic *Leptospira interrogans*

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ARTICLE INFO

Article history:

Received 18 April 2011

Received in revised form

20 June 2011

Accepted 22 June 2011

Available online 23 July 2011

Keywords:

Leptospira

Plasminogen

Immune evasion

ABSTRACT

Leptospirosis is a widespread re-emerging zoonosis of human and veterinary concern. It has been shown that virulent leptospires protect themselves against the host's innate immune system, a strategy that allows the bacteria to reach immunologically safe environments. Although extensive studies on host–pathogen interactions have been performed, little is known on how leptospires deal with host immune attack. In a previous work, we demonstrated the ability of leptospires to bind human plasminogen (PLG), that after treatment with activators, conferred plasmin (PLA) activity on the bacteria surface. In this study, we show that the PLA activity associated to the outer surface of *Leptospira* could interfere with the host immune attack by conferring some evasion advantage during infection. We demonstrate that PLA-coated leptospires interfere with complement C3b and IgG depositions on the bacterial surface, probably through the degradation of these components, thus diminishing opsonization process. Similar decrease on the deposition was observed when normal and immune sera from patients diagnosed with leptospirosis were employed as a source of IgG. We believe that decreasing opsonization by PLA generation might be an important aspect of the leptospiral immune escape strategy and survival. To our knowledge, this is the first proteolytic activity of plasmin associated-*Leptospira* related to anti-opsonic properties reported to date.

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1. Introduction

Leptospirosis is a worldwide spread re-emerging infectious disease of human and veterinary concern. Pathogenic *Leptospira* are the etiological agents of leptospirosis, a disease with greater incidence in tropical and subtropical regions [1–5]. Leptospires cause chronic infection in domestic and wild mammalian species that harbor the bacteria in their renal tubules, shedding them into the environment through the urine. Humans are accidental and terminal hosts in the transmission process of leptospirosis [3,6]. Leptospirosis represents a great economic burden as it affects the public health system and livestock [3].

The molecular mechanisms of leptospiral pathogenesis are poorly understood. A better understanding of host–pathogen interaction is an important approach to identify new virulence

factors. The first line of human defense against bacterial infections is the innate immunity. The complement system represents a major host attack for invading microorganisms [7], being C3 the central component of the complement cascade, generating C3a and C3b [8]. C3b binds to microbial surfaces where it acts as an opsonin and mediates recognition by host immune phagocytic cells [9]. Natural antibodies like immunoglobulin G (IgG) can also function as opsonins and complement activators, being important factors for innate defense.

It is not surprising that pathogenic microorganisms have developed a number of strategies to overcome the protective effects of antibodies or complement in the host. Apparently, pathogens utilize multiple escape strategies to obstruct or inhibit the host complement attack, such as capturing or mimicking complement regulators, modulation of complement proteins by direct interactions and inactivation by enzymatic degradation [7,10–12]. It has already been demonstrated that *Leptospira interrogans* can bind complement regulators FH (factor H), FHL-1 (FH-like 1) and C4BP (C4b-binding protein) from human plasma to their surface

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[11,13,14]. Considering the enzymatic mechanisms of evasion, there are well-known strategies for pathogens, like production and secretion of endogenous proteases or subversion of host proteases, such as plasmin. Indeed, the literature reports that plasmin is able to degrade human IgG [15], and can also cleave immobilized IgG in physiological conditions [16]. Plasmin has also been shown to interfere with C3b, as treatment of human plasma with urokinase lead to the cleavage of this central complement protein [17].

In a preceding work, we have found that *Leptospira* species are able to bind PLG and generate PLA on its outer surface [18], an activity previously shown for the pathogenic spirochetes *Borrelia* and *Treponema* [19–23]. Furthermore, we have revealed that leptospires express multiple PLG-binding proteins [18], and we have identified and characterized eight proteins as being PLG-binding receptors of *Leptospira*, including the major outer membrane protein, LipL32 [24]. PLG-binding/activation through the proteins on the surface of *Leptospira* could help the bacteria to surmount tissue barriers, facilitate invasion and colonize mammalian tissues. Because the formation of PLA occurs on the surface of *Leptospira* [18], this could give leptospires the opportunity to modulate the innate host immunity and increase bacterial survival upon infection through this protease-associated activity.

In the present study, we demonstrate *in vitro* evidence for the participation of PLA generated by leptospires on the immune evasion during infection. We show that bacteria-associated PLA is able to decrease IgG and C3b deposition on the leptospiral surface, which probably occurs via degradation.

2. Materials and methods

2.1. Bacteria isolates and culture conditions

Virulent *L. interrogans* serovar Pomona strain LPF is routinely cultured by iterative passages in Golden Syrian hamsters for maintenance of virulence. Recent-weaned hamsters were intraperitoneally infected with 500 μ L containing approximately 1.0×10^4 virulent leptospires. The animals were sacrificed after appearance of symptoms such as loss of weight and mobility (approximately 5 days post-infection). Kidney and liver were removed and macerated. The organs-derived leptospires were cultured at 28 °C in modified Elinghausen–McCullough–Johnson–Harris (EMJH) medium supplemented with 10% *Leptospira* enrichment EMJH (BD, Difco).

2.2. Human sera and microscopic agglutination test

Confirmed-leptospirosis serum samples were obtained from Instituto Adolfo Lutz collection, Sao Paulo, Brazil. The microscopic agglutination test (MAT) of the serum samples was performed according to Faine et al. [3]. In brief, an array of serovars of *Leptospira* spp. as antigens were employed: Australis, Autumnalis, Bataviae, Canicola, Castellonis, Celledoni, Copenhageni, Cynopteri, Djasiman, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc, Pomona, Pyrogenes, Sejroe, Shermani, Tarassovi and Wolffi. All the strains were maintained in EMJH liquid medium (Difco, USA) supplemented with 10% rabbit sera, at 29 °C. A laboratory-confirmed case of leptospirosis was defined by the demonstration of a four-fold microagglutination titer rise between paired serum samples. The probable predominant serovar was considered the titer that was the highest sample dilution with 50% of agglutination. MAT was considered negative when the titer was below 1:100. In addition of the MAT negative and MAT positive paired samples of the same individuals with laboratory and clinic leptospirosis confirmations, we also employed sera from normal healthy donors (normal human sera – NHS),

without a known history of leptospirosis, with confirmed negative MAT.

2.3. Human IgG and C3b deposition on leptospires

L. interrogans serovar Pomona strain LPF (2.5×10^7 cells/well) were diluted in 50 μ L low salt PBS (IsPBS – phosphate buffered saline with 50 mM NaCl), coated onto microplates and allowed to stand at 37 °C for 1 h, followed by overnight at 4 °C. The plates were washed twice with 200 μ L IsPBS and blocked with 5% non-fat dry milk and 2.5% BSA for 2 h at 37 °C. After addition of 50 μ L of pooled sera/well (NHS, MAT negative or MAT positive) as a source of IgG, the plates were allowed to incubate for 30 min on ice, and then washed three times. The coated leptospires were treated with 50 μ L/well of 5 μ g/mL uPA (urokinase, Sigma) and 40 μ g/mL PLG (Calbiochem) for 2 h at 37 °C, or only with uPA, only with PLG, or no additions, as negative controls. After three washings, the plates were incubated with anti-human IgG Fc region specific antibodies produced in mice (Calbiochem) at a dilution of 1:5000 for 1 h at 37 °C, and then with anti-mouse IgG antibodies conjugated with peroxidase at a dilution of 1:10,000 for 1 h at 37 °C. The IgG depositions on leptospires were evaluated by the peroxidase substrate o-phenylenediamine (OPD) and readings were taken at 492 nm. When dilutions of NHS were used, they were performed with IsPBS.

The protocol of C3b deposition on leptospires was similar to the IgG deposition described before, with exception of the incubation with NHS, placed at 37 °C for 50 min, and use of anti-human C3b primary antibodies produced in goat (Calbiochem) (1:5000), followed by anti-goat IgG antibodies conjugated with peroxidase (1:5000).

2.4. Human C3b degradation by leptospires

L. interrogans serovar Pomona strain LPF (10^8 leptospires/sample) were treated in 200 μ L IsPBS with the addition of 10 μ g PLG and 3U uPA (plasmin), 10 μ g PLG, 3U uPA or no additions (untreated). The cells were incubated for 1 h at 37 °C with the PLG, and for one more hour after the addition of uPA. The cells were washed three times with IsPBS, and then resuspended in 100 μ L IsPBS containing 15 μ g/mL human purified C3b (Calbiochem), being incubated for 5 h or 20 h at 37 °C. As a control, samples treated with PLG + uPA were added by 2 μ g aprotinin (Fluka) together with the C3b. The leptospires were removed by centrifugation, 20 μ L of the supernatants were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes in a semi-dry equipment. The membranes were blocked by incubating overnight at 4 °C with 5% non-fat dry milk and 1% BSA. The C3b detection was made by incubations with anti-human C3 polyclonal antibodies (Calbiochem) and secondary antibodies conjugated with peroxidase, followed by 3,3'-diaminobenzidine tetrahydrochloride hydrate (Fluka) development.

2.5. Enzymatic activity of plasmin and factor H-coated leptospires

L. interrogans serovar Pomona LPF strain ($\sim 10^9$ leptospires/sample) were centrifuged at $6000 \times g$ for 10 min at 25 °C and resuspended in 200 μ L of IsPBS. The tubes containing the leptospires received different treatments: (a) 5 μ g purified human PLG and 3U purified human uPA, (b) 5 μ g PLG, 3U uPA and 10 μ g purified human FH (Calbiochem), (c) 5 μ g PLG, 3U uPA and 5 μ g FH, (d) 5 μ g PLG, 3U uPA and 1 μ g FH, (e) 10 μ g FH and (f) no addition. All the preparations were incubated for 1 h at 37 °C, under gentle shaking, prior to the addition of the uPA, and followed by 1 h incubation at 37 °C. Leptospires were then centrifuged and washed three times

with 0.7 mL lPBS. The pellets of the treated leptospires were resuspended in 200 μ L lPBS and divided into 2 aliquots. Each aliquot received 100 μ L of 0.5 mg/mL of the chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) in lPBS, to a final substrate concentration of 0.25 mg/mL. The suspensions were incubated for 2 h at 37 °C, under gentle shaking, and then centrifuged at 6000 $\times$ g for 10 min at 25 °C. The supernatants (150 μ L) were transferred to 96-well microplates and the cleavage of the specific plasmin substrate was quantified with set at 405 nm.

2.6. Detection of purified human factor H bound to bacteria

Leptospires were centrifuged at 6000 $\times$ g for 10 min at 25 °C and resuspended in 200 μ L of (a) lPBS alone or containing (b) 30 μ g PLG, 4U uPA and 20 μ g FH or (c) only 20 μ g FH. The samples were incubated for 2 h at 37 °C with PLG and FH, and then for one more hour after addition of uPA. The bacteria were washed twice with lPBS, and then incubated with anti-FH monoclonal antibodies (Quidel) (1:300) for 1 h at 37 °C. After washings, the cells were incubated with secondary antibodies conjugated to peroxidase (1:2500) for 1 h at 37 °C. The leptospires were washed two times and resuspended in solution containing 1 μ g/mL of OPD substrate. Optical density readings were taken at 492 nm.

2.7. Detection of FH and PLG captured by leptospires from human sera

L. interrogans serovar Pomona strain LPF (10^9 leptospires/sample) were centrifuged and resuspended in 200 μ L of lPBS or 100% NHS pool. The samples were incubated for 1 h at 30 °C, leptospires washed twice with lPBS, and then incubated with anti-FH or anti-PLG specific antibodies in a dilution of 1:300, for 2 h at 30 °C. After three washings, the cells were incubated with secondary antibodies conjugated to peroxidase (1:1000 in 200 μ L) for 1 h at 30 °C. The leptospires were washed three times and divided in four aliquots. Each aliquot was resuspended in 50 μ L of solution containing 2 μ g/mL of OPD substrate and 2 μ L/mL H_2O_2 . The absorbance was taken at 492 nm. The background medium absorbance of lPBS-treated leptospires was subtracted from bacteria incubated with NHS.

2.8. Serum susceptibility testing for treated leptospires

Complement-mediated killing of leptospires was evaluated after incubation with non-immune NHS, as described by [13], with some

modifications. Approximately 5×10^8 *L. interrogans* serovar Pomona strain LPF per sample were treated with 40 μ g PLG and 4U uPA in 200 μ L lPBS for 3 h at 37 °C. As a control, one aliquot of *L. interrogans* serovar Pomona strain LPF was incubated only with PBS. Then, the samples were divided into aliquots containing 1.0×10^8 cells, which were added by 80 μ L NHS and 80 μ L EMJH medium supplemented with 10% *Leptospira* enrichment. The bacteria suspensions were incubated at 37 °C for 1 h, followed by a incubation of 5 min on ice for stopping complement activation. After, 20 μ L of each sample ($\sim 1.25 \times 10^7$ leptospires), in four replicates, were transferred to microplates filled with 180 μ L of EMJH medium/well. The plates were sealed with a sterile adhesive film and incubated at 30 °C for 4 days. Bacterial growth was determined by counting leptospires in a Petroff–Hausser chamber under dark-field microscopy.

3. Results and discussion

Pathogenic microorganisms have developed strategies to camouflage their presence in the host. Apparently, they use several and combined escape mechanisms to evade the multiple steps of innate and adaptive immune defense. After entrance in the host, pathogenic leptospires might be able to circumvent the host immune system while in circulation, in order to propagate to multiple organs and finally reach immunologically privileged sites, such as the proximal renal tubules [3]. Therefore, efficient leptospiral colonization might succeed by their ability to modulate host immune attack while in the circulation, as well as the pathogen–host interactions [25–28].

Plasmin associated to *L. interrogans* may facilitate the bacteria to directly degrade opsonizing IgG and C3b molecules, and consequently have a role in immune evasion. Virulent *L. interrogans* serovar Pomona were coated onto microtiter plates and incubated with NHS, as a source of C3b and nonspecific IgG molecules. Bacteria were then treated with PLG and uPA, and the resulting surface-associated opsonins were quantified by specific antibodies. As shown in Fig. 1, incubation of bacteria with PLG and uPA resulted in a decrease in IgG Fc regions (Fig. 1A) and C3b (Fig. 1B) depositions at the leptospiral surface. PLG and uPA alone had no effect on IgG or C3b binding to *L. interrogans*.

To assess whether the same PLA activity observed against nonspecific human IgG bound to leptospires could be applied to immune sera from patients diagnosed with leptospirosis, the same experiment was conducted but using MAT negative (pre-immune phase) and MAT positive (convalescent immune phase) pools of

Fig. 1. Human IgG and C3b bind to *L. interrogans*. Bacteria were coated onto microtiter plates (2.5×10^7 leptospires/well) and IgG (A) or C3b (B) were bound by incubating bacteria with 100% NHS. Bacteria were treated with PBS, 40 μ g/mL PLG (PLG), 5 μ g/mL uPA (uPA) or PLG + uPA (Pla). Presence of human IgG or C3b deposited on leptospires was determined by ELISA. Bars represent the mean absorbance values at 492 nm $\pm$ the standard deviation of three replicates for each experimental group and are representative of two independent experiments.

human sera. As depicted in Fig. 2, as expected, MAT positive sera resulted in significant higher IgG depositions on bacterial surface when compared to NHS and MAT negative sera. PLA – generated leptospires was able to decrease significantly the amount of IgG deposition from both negative and positive MAT sera.

Prevention of opsonization by antibodies or complement proteins seems an interesting approach to evade the first line of human immune defense upon infection. Indeed, several pathogens express on their surfaces proteins that can sequester host complement regulators FH, FHL-1 or C4BP, suppressing the complement cascade by the alternative or classical pathways [7,10–12]. Furthermore, another well-known strategy is the proteolytic cleavage of antibodies, what also prevents the activation of complement classical pathway or Fc receptor-mediated phagocytosis [7,10–12].

The ability of *Leptospira*-bound factor H (FH) acting as a cofactor for the serum protease factor I in cleaving C3b has been previously reported [13]. We thus decided to evaluate whether the decrease in C3b deposition by *Leptospira*-generated PLA was due to C3b cleavage. *L. interrogans* serovar Pomona were coated with PLA by treatment with PLG and uPA, and then incubated with human purified C3b in fluid-phase. Western blot analysis of the resulting supernatant with anti-C3 specific antibodies revealed the appearance of lower molecular mass bands, concomitantly with the decrease in the masses of the alpha (105 kDa) and beta (75 kDa) C3b chains (Fig. 3), which occurred only with PLA-coated leptospires. No cleavage was observed with untreated or only PLG or uPA treated leptospires. The C3b degradation activity seems to be due to plasmin, since control incubated with aprotinin, a serine protease inhibitor, exhibited the same profile as controls lacking active plasmin. The data clearly show that the human C3b is cleaved by leptospires-coated PLA, indicating that the observed decrease in deposition is probably due to degradation.

It has been reported that pathogenic *Leptospira* can bind the host complement regulators FH [13] and C4BP [14], what inhibits complement attack and confers protection upon exposure to human sera, enhancing bacterial survival, activity already shown for a number of pathogens [29–31]. We have hence performed

Fig. 3. Detection of human C3b. Leptospires (10^8 /sample) were treated with 10 µg PLG and 3U uPA (**Plasmin**), only with PLG (**PLG**), only with uPA (**uPA**), or left untreated (**UT**). The cells were incubated with 15 µg/mL human purified C3b for 5 h (A) or 20 h (B) at 37 °C. As a control, plasmin treated leptospires were incubated with C3b with the addition of serine protease inhibitor **aprotinin**. The supernatants were resolved by SDS-PAGE and transferred to membranes. The C3b was detected by specific antibodies. The arrows indicate the native human C3b **alpha chain** (105 kDa) and **beta-chain** (75 kDa), as well as the most significant degradation products.

a competition assay in order to evaluate if FH competes for the same binding sites of PLG or if its presence could interfere somehow in the interaction of PLG with the leptospires. Leptospires were treated with PLG and uPA in the presence or absence of FH, or only with FH. The leptospires were then incubated with a specific

Fig. 2. IgG from human leptospirosis immune serum binds to *L. interrogans*. Bacteria were coated onto microtiter plates (2.5×10^7 leptospires/well) and IgG was bound by incubating bacteria 50% non-immune human sera (NHS) or confirmed paired leptospirosis patients sera: MAT negative (MAT-) or MAT positive (MAT+). Bacteria were treated with PBS, 40 µg/mL PLG (PLG), 5 µg/mL uPA (uPA) or PLG + uPA (Pla). Presence of human IgG deposited on leptospires was determined by ELISA using Fc-specific anti-human IgG antibodies. Bars represent the mean absorbance values at 492 nm $\pm$ the standard deviation of three replicates for each experimental group and are representative of two independent experiments.

Fig. 4. Influence of factor H in the PLG-binding and plasmin activity associated to leptospires. 10^9 *L. interrogans* serovar Pomona strain LPF/sample were treated with 5 µg PLG and 3U uPA (**Pla**), added by 10, 5 or 1 µg **FH**, or just with 10 µg FH. As negative control, one sample received no additions (**PBS**). The samples were incubated with specific plasmin substrate, and the plasmin activity was measured as cleavage of the chromogenic substrate. Bars represent mean absorbance at 405 nm, as a measure of relative substrate degradation $\pm$ the standard deviation of three replicates for each experimental group and are representative of two independent experiments.

PLA chromogenic substrate, and the enzymatic activity was measured by the proteolysis of the PLA substrate. As shown in Fig. 4, the presence of FH does not seem to interfere with PLG-binding, PLG activation or PLA activity associated to the bacteria, as no significant difference in enzymatic activity was observed between samples in the absence or in the presence of increased concentrations of FH. Bacteria incubated only with FH exhibited the same absorbance background for substrate degradation as the PBS negative control.

As the binding of PLG/PLA and FH does not interfere with the PLA activity associated to the leptospires, we assessed if the reverse situation also occurred. *L. interrogans* serovar Pomona strain LPF were treated with purified human PLG and FH, and the FH binding was quantified by specific antibodies. As shown in Fig. 5A, no differences in the FH interaction to leptospires was observed when comparing leptospires treated only with FH and those treated with

FH in the presence of PLG and uPA (PLA). We have also investigated this binding in a more physiological condition: leptospires were treated with NHS pool, as a source of FH and PLG. The captured components on leptospiral surface were probed by specific anti-PLG or anti-FH antibodies. As suggested by data presented on Fig. 5B, leptospires can bind PLG and FH concomitantly and directly from human plasma, a condition that may happen during the infection process.

L. interrogans serovar Pomona have been described to be partially susceptible to complement killing upon incubation with normal human sera [13]. To assess if the PLG/PLA binding to leptospires confers protection upon exposure to NHS, *L. interrogans* serovar Pomona strain LPF were treated with PLG + uPA (Pla) or with PBS, as negative control, and incubated for 1 h with pooled NHS. After four days, the bacteria were counted in a dark-field microscopy using Petroff–Hausser chamber. The results show

Fig. 5. Binding of PLG and FH to leptospires. (A) Bacteria were treated with 30 µg PLG, 4U uPA and 20 µg FH (**PLA + FH**) or only 20 µg FH (**FH**), and the interaction of FH to leptospires was measured by monoclonal anti-FH antibodies, followed by secondary antibodies conjugated to peroxidase. The absorbances of bacteria treated only with IsPBS were subtracted from FH and PLG-incubated leptospires and the mean absorbance of only FH-treated leptospires was considered 100% FH binding. The bars indicate the mean percentage of FH binding $\pm$ the standard deviation of eight replicates for each group. (B) *L. interrogans* were incubated with 100% NHS, and the binding of **PLG** and **FH** was measured by specific antibodies. The background medium absorbance of IsPBS-treated leptospires was subtracted from bacteria incubated with NHS. Bars represent mean absorbance at 492 nm, as a measure of FH or PLG-binding $\pm$ the standard deviation of eight replicates for each experimental group and are representative of two independent experiments.

that the number of untreated leptospires were almost 4 times less than the plasmin-coated leptospires ($0.35 \times 10^8 \pm 0.1 \times 10^8$ versus $1.28 \times 10^8 \pm 0.3 \times 10^8$ leptospires/mL) suggesting that the plasmin at the surface of bacteria renders them less vulnerable to NHS.

The majority of the studies examining the role of the PLG and PLA on bacterial infections have focused on the contribution of the PLA-bacteria interaction to the spread of pathogens by the degradation of extracellular matrix proteins [18] and some have showed the degradation of specific antibodies or opsonins by PLA and the consequences of this activity in infection [32,33]. In this work, we expand this type activity to leptospires, as one step to elude the immune system.

4. Conclusion

Altogether the data here presented through the generation of enzymatically active PLA on the bacterial surface that lead to the decrease of C3b and IgG depositions, therefore opsonization and phagocytosis, may constitute a novel mechanism by which leptospires could escape the immune system until reach immunologically safe environments. We believe that our results represent an important contribution to the understanding of leptospiral pathogenesis and to the interaction with the hosts.

References

- [1] Haake DA, Dundoo M, Cader R, Kubak BM, Hartskeerl RA, Sejvar JJ, et al. Leptospirosis, water sports, and chemoprophylaxis. *Clin Infect Dis* 2002;34:e40–43.
- [2] Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, et al. Leptospirosis: a zoonotic disease of global importance. *Lancet Infect Dis* 2003;3:757–71.
- [3] Faine S, Adler B, Bolin C, Perolat P. *Leptospira* and leptospirosis. Melbourne, Australia: MediSci; 1999. p. 259.
- [4] Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001;14:296–326.
- [5] Vinetz JM. Leptospirosis. *Curr Opin Infect Dis* 2001;14:527–38.
- [6] Plank R, Dean D. Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. *Microbes Infect* 2000;2:1265–76.
- [7] Zipfel PF, Wurzner R, Skerka C. Complement evasion of pathogens: common strategies are shared by diverse organisms. *Mol Immunol* 2007;44:3850–7.
- [8] Zipfel PF, Mihlan M, Skerka C. The alternative pathway of complement: a pattern recognition system. *Adv Exp Med Biol* 2007;598:80–92.
- [9] van Lookeren Campagne M, Wiesmann C, Brown EJ. Macrophage complement receptors and pathogen clearance. *Cell Microbiol* 2007;9:2095–102.
- [10] Blom AM, Hallstrom T, Riesbeck K. Complement evasion strategies of pathogens: acquisition of inhibitors and beyond. *Mol Immunol* 2009;46:2808–17.
- [11] Lambris JD, Ricklin D, Geisbrecht BV. Complement evasion by human pathogens. *Nat Rev Microbiol* 2008;6:132–42.
- [12] Rooijakkers SH, van Strijp JA. Bacterial complement evasion. *Mol Immunol* 2007;44:23–32.
- [13] Meri T, Murgia R, Stefanel P, Meri S, Cinco M. Regulation of complement activation at the C3-level by serum resistant leptospires. *Microb Pathog* 2005;39:139–47.
- [14] Barbosa AS, Abreu PA, Vasconcellos SA, Morais ZM, Goncales AP, Silva AS, et al. Immune evasion of leptospira species by acquisition of human complement regulator C4BP. *Infect Immun* 2009;77:1137–43.
- [15] Stanworth DR, Turner MW. Immunochemical analysis of human and rabbit immunoglobulins and their subunits. In: Wier DM, editor. *Handbook of experimental immunology*. London, UK: Blackwell Scientific; 1986. pp. 12.1–12.45.
- [16] Harpel PC, Sullivan R, Chang TS. Binding and activation of plasminogen on immobilized immunoglobulin G. Identification of the plasmin-derived Fab as the plasminogen-binding fragment. *J Biol Chem* 1989;264:616–24.
- [17] Seya T, Nagasawa S, Matsukura M, Hasegawa H, Atkinson JP. Generation of C3d, g and C3d by urokinase-treated plasma in association with fibrinolysis. *Complement* 1985;2:165–74.
- [18] Vieira ML, Vasconcellos SA, Goncales AP, de Morais ZM, Nascimento AL. Plasminogen acquisition and activation at the surface of leptospira species lead to fibronectin degradation. *Infect Immun* 2009;77:4092–101.
- [19] Nordstrand A, Shamaei-Tousi A, Ny A, Bergstrom S. Delayed invasion of the kidney and brain by *Borrelia crocidurae* in plasminogen-deficient mice. *Infect Immun* 2001;69:5832–9.
- [20] Coleman JL, Gebbia JA, Piesman J, Degen JL, Bugge TH, Benach JL. Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice. *Cell* 1997;89:1111–9.
- [21] Coleman JL, Sellati TJ, Testa JE, Kew RR, Furie MB, Benach JL. *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. *Infect Immun* 1995;63:2478–84.
- [22] Fenno JC, Tamura M, Hannam PM, Wong GW, Chan RA, McBride BC. Identification of a *Treponema denticola* OppA homologue that binds host proteins present in the subgingival environment. *Infect Immun* 2000;68:1884–92.
- [23] Klemperer MS, Noring R, Epstein MP, McCloud B, Rogers RA. Binding of human urokinase type plasminogen activator and plasminogen to *Borrelia* species. *J Infect Dis* 1996;174:97–104.
- [24] Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, Vasconcellos SA, et al. In vitro identification of novel plasminogen-binding receptors of the pathogen *Leptospira interrogans*. *PLoS One* 5: e11259.
- [25] Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, Vieira ML, et al. A newly identified leptospiral adhesin mediates attachment to laminin. *Infect Immun* 2006;74:6356–64.
- [26] Vieira ML, D'Atri LP, Schattner M, Habarta AM, Barbosa AS, de Morais ZM, et al. A novel leptospiral protein increases ICAM-1 and E-selectin expression in human umbilical vein endothelial cells. *FEMS Microbiol Lett* 2007;276:172–80.
- [27] Gomez RM, Vieira ML, Schattner M, Malaver E, Watanabe MM, Barbosa AS, et al. Putative outer membrane proteins of *Leptospira interrogans* stimulate human umbilical vein endothelial cells (HUVECS) and express during infection. *Microb Pathog* 2008;45:315–22.
- [28] Breiner DD, Fahey M, Salvador R, Novakova J, Coburn J. *Leptospira interrogans* binds to human cell surface receptors including proteoglycans. *Infect Immun* 2009;77:5528–36.
- [29] Kraiczky P, Wurzner R. Complement escape of human pathogenic bacteria by acquisition of complement regulators. *Mol Immunol* 2006;43:31–44.
- [30] Zipfel PF, Hallstrom T, Hammerschmidt S, Skerka C. The complement fitness factor H: role in human diseases and for immune escape of pathogens, like pneumococci. *Vaccine* 2008;26(Suppl. 8):I67–74.
- [31] Zipfel PF, Skerka C, Hellwage J, Jokiranta ST, Meri S, Brade V, et al. Factor H family proteins: on complement, microbes and human diseases. *Biochem Soc Trans* 2002;30:971–8.
- [32] Rooijakkers SH, van Wamel WJ, Ruyken M, van Kessel KP, van Strijp JA. Antipsonic properties of staphylokinase. *Microbes Infect* 2005;7:476–84.
- [33] Crane DD, Warner SL, Bosio CM. A novel role for plasmin-mediated degradation of opsonizing antibody in the evasion of host immunity by virulent, but not attenuated, *Francisella tularensis*. *J Immunol* 2009;183:4593–600.

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RESEARCH ARTICLE

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"Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions"

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Abstract

Background: Leptospirosis is considered a re-emerging infectious disease caused by pathogenic spirochaetes of the genus *Leptospira*. Pathogenic leptospires have the ability to survive and disseminate to multiple organs after penetrating the host. Leptospires were shown to express surface proteins that interact with the extracellular matrix (ECM) and to plasminogen (PLG). This study examined the interaction of two putative leptospiral proteins with laminin, collagen Type I, collagen Type IV, cellular fibronectin, plasma fibronectin, PLG, factor H and C4bp.

Results: We show that two leptospiral proteins encoded by LIC11834 and LIC12253 genes interact with laminin in a dose - dependent and saturable mode, with dissociation equilibrium constants (K_D) of 367.5 and 415.4 nM, respectively. These proteins were named Lsa33 and Lsa25 (Leptospiral surface adhesin) for LIC11834 and LIC12253, respectively. Metaperiodate - treated laminin reduced Lsa25 - laminin interaction, suggesting that sugar moieties of this ligand participate in this interaction. The Lsa33 is also PLG - binding receptor, with a K_D of 23.53 nM, capable of generating plasmin in the presence of an activator. Although in a weak manner, both proteins interact with C4bp, a regulator of complement classical route. *In silico* analysis together with proteinase K and immunofluorescence data suggest that these proteins might be surface exposed. Moreover, the recombinant proteins partially inhibited leptospiral adherence to immobilized laminin and PLG.

Conclusions: We believe that these multifunctional proteins have the potential to participate in the interaction of leptospires to hosts by mediating adhesion and by helping the bacteria to escape the immune system and to overcome tissue barriers. To our knowledge, Lsa33 is the first leptospiral protein described to date with the capability of binding laminin, PLG and C4bp *in vitro*.

Background

Leptospirosis is a zoonosis caused by pathogenic species of the genus *Leptospira*. Greater incidence of human infection occurs in tropical and subtropical countries [1,2]. The transmission of leptospirosis has been correlated with exposure of individuals in close proximity to wild or farm animals [1,3]. Recently, the disease became prevalent in cities with sanitation problems and large urban rodent reservoirs that contaminate the environment through their urine [4].

Pathogenic *Leptospira* spp. have ability to adhere and rapidly disseminate within the host during the early stage

of infection. Surface - associated proteins are potential targets to mediate host - pathogen interactions, and therefore are likely to elicit several activities, including adhesion. The adhesion of leptospires to ECM components of the host was considered to be essential in the initial stage of the infection [5]. Indeed, we have reported that pathogenic leptospires are capable of binding several ECM molecules [6]. To date, several leptospiral ECM binding adhesins have been described [6–19]. After the adhesion, pathogens have to overcome tissue barriers in order to reach blood circulation and organs. We have reported that leptospires have the ability of binding PLG at their surface and that plasmin (PLA) can be generated in the presence of activator [20]. In addition, Verma and colleagues [21] and our group have described several leptospiral proteins as PLG - binding receptors [18,19,22]. More recently, we have reported that PLA generation on

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Leptospira decreased opsonization and that it might be an important aspect of the immune escape strategy and survival [23].

L. interrogans serovar Copenhageni genome annotation identified many unknown coding sequences predicted to be surface exposed proteins. Characterization of these proteins, with no previously assigned function, should increase our understanding of this intriguing pathogen's biology. In this work, we present our studies with two leptospiral coding sequences, LIC11834 and LIC12253, named Lsa33 and Lsa25, respectively. The genes were cloned and the proteins expressed using *E. coli*. The recombinant proteins were purified and their ability to bind various ECM and serum components was evaluated. We report that these proteins are novel surface adhesins capable of binding to laminin. In addition, Lsa33 can also interact to PLG and both proteins bind the complement regulator of the classical pathway C4bp. We believe that these proteins are likely to be involved in *Leptospira* - host interactions.

Results

Bioinformatic analysis

The selected coding sequences, LIC11834 and LIC12253, are genome annotated as hypothetical proteins, and one of them, LIC11834, is a putative lipoprotein, having lipoprotein signal peptide (signal peptidase II) and a cleavage site between amino acids 17–18. According to SMART web server, LIC11834 has a signal peptide from 1 to 21 amino acids and a FecR domain from amino acid 60 to 162. PFAM predicts that this domain is involved in regulation of iron dicitrate transport and that FecR is probably a sensor that recognizes iron dicitrate in the periplasm. LIC12253 presents a signal peptide from amino acid 1 to 21 and a DUF1566 (Domain of Unknown Function) from amino acid 58 to 164 [24,25]. The LIC11834 coding sequence can be classified as alpha - beta protein, being the percentage of 36.57 for

alpha-helix and 29.13 for beta strands secondary structure. In the case of coding sequence LIC12253, the protein can be classified as mixed, having a predicted secondary structure composition percent of 11.01, 19.38 and 69.60 for alpha - helix, beta strands and others, respectively. Cellular localization predicts as extra - cellular (non-cytoplasmic branch) for both proteins. The solvent accessibility composition (core/surface ratio) for the CDs LIC11834 and LIC12253 is expected to be 59.87 and 66.52% of amino acid residues exposed with more than 16% of their surface, respectively. All the predictions above were performed with PrecitedProtein web server [26,27]. The presence and identity of both coding sequences among *Leptospira* sequenced genomes are depicted in Table 1.

Distribution and expression of LIC11834 and LIC12253 genes among *Leptospira* strains

The presence of LIC11834 and LIC12253 genes in pathogenic strains and in one saprophytic strain was examined by PCR with a pair of primers designed according to *L. interrogans* serovar Copenhageni genome sequences. The gene LIC11834 was amplified by PCR in all strains belonging to the pathogenic species excluding in *L. santarosai* serovar Shermani (Figure 1A). No DNA amplification was detected in the non - pathogenic *L. biflexa* serovar Patoc. In the case of LIC12253 gene, DNA band was amplified in all pathogenic strains and a less intense band was detected in the saprophytic strain (Figure 1A). The expression of LIC11834 and LIC12253 genes was evaluated by PCR amplification of reversely transcribed total RNA. LIC11834 gene product was detected only in *L. interrogans* specie serovars Canicola, Pomona, Copenhageni, Icterohaemorrhagiae and Hardjo. No expression was observed in non-pathogenic strain. LIC12253 gene expression could be identified in all pathogenic strain tested (Figure 1B). Integrity of total RNA used in RT - PCR experiments was assured by the

Table 1 Gene locus, given names, features, gene conservation, sequence of the primers employed for DNA amplification, and molecular mass of expressed recombinant proteins

Gene locus ¹	Given name ²	Description/Function	Conservation (identity) ³	Sequence of primers for PCR amplification	Molecular mass
LIC11834	Lsa33	Putative lipoprotein	Lai (99%) LBH (87%) LBP (31%)	F:5'CTCGAGGATCTACAAGGTGGGGTTTTTAC3' XhoI R:5'CCATGGTTACTGAGGTTTACTTGGTCC3' NcoI	33.1 kDa
LIC12253	Lsa25	Conserved hypothetical protein	Lai (100%) LBH (77%) LBP (39%)	F:5'CTCGAGGAGGAGAAACCGGACGATAC 3' XhoI R:5' CCATGGTTAGGGAAGACTTCTAACACATC3' NcoI	24.07 kDa

¹http://aeg.lbi.ic.unicamp.br/world/lic/; LIC: *Leptospira interrogans* Copenhageni
²Lsa: Leptospiral surface adhesin of 33 and 24 kDa; we have named the latter as Lsa25 because Lsa24 has been already described (Barbosa et al., 2006)
³http://blast.ncbi.nlm.nih.gov/Blast.cgi/

Figure 1 Analysis of the LIC11834 and LIC12253 genes and their transcripts among different leptospiral strains. (A) Analysis by PCR of the LIC11834 (2) and LIC12253 (3) genes in pathogenic serovars (*L. interrogans*, *L. borgpetersenii*, *L. kirshnery*, *L. noguchi* and *L. santarosai*) and in the non - pathogenic *L. biflexa* strain. 16 S rRNA gene expression was used as an internal control (1). The negative control contained no DNA, indicated by (-). **(B)** Analysis of the LIC11834 (2) and LIC12253 (3) transcripts by RT - PCR using 2 µg total RNA extracted from different serovars belonging to the pathogenic species and the saprophytic *L. biflexa* serovar Patoc strain Patoc. 16 S rRNA gene expression was used as an internal control (1). Reverse transcriptase present, +; reverse transcriptase omitted, -. The negative control contained no cDNA, indicated by (-)

129 presence of a 1,042 - bp 16 S ribosomal cDNA fragment
130 in all samples (Figure 1B).

131 **Cloning and characterization of recombinant proteins**
132 The amplified DNA sequences of LIC11834 and LIC12253
133 were cloned into an *E. coli* pAE vector [28] and the corre-
134 sponding proteins were expressed as full-length with 6X
135 His sequence tag at their N - terminal. Expression of
136 recombinant proteins was elicited from cultures of *E. coli*
137 BL21 SI after addition of NaCl (300 mM). Recombinant
138 protein Lsa33 is expressed in its soluble form, while Lsa25
139 is expressed in its insoluble form, as inclusion bodies (data
140 not shown). Protein Lsa25 was recovered from inclusion
141 bodies after solubilization with 8 M urea. The purifica-
142 tion was performed by metal chelating chromatography
143 under normal (Lsa33) or denaturing condition, followed
144 by refolding by gradually removal of urea (Lsa25). The
145 proteins were recovered with 1.0 M imidazol. Evaluation
146 of protein purification has shown that most of the

contaminants were washed away and proteins were repre- 147
sented as single major bands. The recombinant protein 148
bands were further confirmed by western blotting probed 149
with monoclonal anti - His tag antibodies and with poly- 150
clonal antiserum raised against each protein (data shown). 151
The calculated 33.1 kDa and 24.07 kDa molecular masses 152
of the recombinant proteins comprise the vector fusion 153
plus the encoded amino acids. Structural integrity of the 154
purified proteins was assessed by circular dichroism (CD) 155
spectroscopy. The minima at 208 and 222 nm, and the 156
maximum at 192 nm in the CD spectrum showed the 157
high α - helical secondary structure content of both 158
recombinant proteins (data not shown). 159

Recognition of the LIC11834 and LIC12253 coding 160
sequences by immunofluorescence confocal microscopy 161
The assessment of the selected CDSs on the bacterial 162
cell membrane was performed using living organisms 163
and the liquid - phase immunofluorescence method. 164

Leptospire were visualized by propidium iodide staining (Figure 2, column A) followed by protein detection with polyclonal mouse antiserum raised against each protein in the presence of anti - mouse IgG antibodies conjugated to FITC. Green fluorescence could be observed in Figure 2 column B, for LIC11834, LIC12253 and LipL32, an outer membrane protein used as a positive control [29], but not with GroEL, a protoplasmic - cylinder marker, used as a negative control [30]. The localization of the protein - green light lying on the leptospire was achieved by merging both fields and the results obtained are shown in Figure 2, column C.

Cellular localization of the LIC11834 and LIC12253 coding sequences by protease assay
We have performed proteinase K accessibility assay by using the previously described assay (37, 41) with some modifications. Live leptospire were treated with 25 µg/ml of proteinase K and aliquots of the bacterial suspensions were taken at time 0, 1, 3 and 5 h; the

suspensions were sedimented and the resuspended bacteria were used to coat microplates, followed by incubation with polyclonal antibodies against each protein, including the controls, LipL32 and DnaK, for outer [29] and cytoplasmic [31] protein. The reactions proceeded as described in Methods. The leptospiral coding sequences LIC11834 and LIC12253 were both susceptible to protease treatment after 1 h incubation, similar to the positive control LipL32 (Figure 3). Almost no reduction was observed with DnaK cytoplasmic protein (Figure 3).

Recombinant protein Lsa25 is recognized by antibodies of confirmed cases of leptospirosis
To examine whether LIC11834 and LIC12253 leptospiral coding sequences are able to elicit an immune response from an infected host, we evaluated the reactivity of the recombinant proteins Lsa25 and Lsa33 with antibodies present in serum samples of early (MAT -) and convalescent (MAT +) phases of leptospirosis patients. ELISA was performed using 24 and 33 serum samples of negative MAT and of positive MAT, respectively. The recombinant protein Lsa33 was almost non-reactive with samples from both phases of the disease (Figure 4A), while Lsa25 showed 46 and 48% reactivity for negative and positive MAT, respectively (Figure 4B). When the two proteins were assayed together, a small increment was

Figure 2 Recognition of coding sequences LIC11834 and LIC12253 proteins in *L. interrogans* by their respective antibodies. Liquid - phase Immunofluorescence Assay (L - IFA) was performed with live *L. interrogans* under confocal microscopy using antiserum for LIC11834, LIC12253, LipL32 (surface - exposed lipoprotein) and GroEL (protoplasmic cylinder marker). Leptospire were identified by propidium iodide staining of the DNA (A). FITC - conjugated secondary antibodies were used to detect the surface - bound antibodies (B). Co - localization is shown in the merged images (C).

Figure 3 Protease accessibility assay of LIC11834 and LIC12253 encoded proteins of *L. interrogans*. Viable leptospire were incubated with 25 µg/ml of proteinase K at the indicated times. The suspensions were sedimented, washed, resuspended in PBS and coated in a microplate. Antibodies against recombinant proteins Lsa33, Lsa25, LipL32 and DnaK were added. After incubation, anti-IgG peroxidase conjugated was added and the reaction was developed with OPD peroxidase substrate. Blanks were run in parallels but antibodies against the proteins were omitted. Readings were taken at 492 nm. Bars represent the mean of absorbance ± the standard deviation of three replicates for each protein and are representative of three independent experiments. For statistical analyses, the signal was compared between 0 hour and hours of treatment with PK by two-tailed t test (**P* < 0.05)

Figure 4 Reactivity of the recombinant antigens Lsa33 and Lsa25 with serum samples of individuals diagnosed with leptospirosis. Responders were determined by ELISA with the recombinant proteins and serum samples from patients of both phases of the disease. The reactivity was evaluated as IgG antibodies. Serum was considered MAT positive or MAT negative if agglutination was detected when the sera were tested for their reactivity's with isolates of the 22 serovars (see Methods). The cutoff values are depicted as horizontal bars and were defined as the mean plus 3 standard deviations obtained for sera from 12 healthy individuals. (A) shows the data for Lsa33, (B) for Lsa25, and (C) depicts the data when both proteins were employed (Lsa33 plus Lsa25).

209 observed for positive MAT samples (58%) (Figure 4C).
210 Our data suggest that Lsa25 might be an interesting
211 protein for early diagnose of leptospirosis.

212 **Recombinant proteins adhesion to ECM components**
213 The ability of Lsa33 and Lsa25 proteins to mediate host
214 colonization by adhering to extracellular matrix proteins
215 was examined by ELISA. Laminin, collagen Type I,
216 collagen Type IV, cellular fibronectin, plasma fibronectin,
217 and the control proteins fetuin and gelatin were immo-
218 bilized on microdilution wells and recombinant protein
219 attachment was assessed by ELISA using antibodies

against the proteins. As shown in Figure 5A, both
220 recombinant proteins exhibited adhesiveness to laminin,
221 while no statistically significant binding was observed
222 with these proteins when wells were coated with collagen
223 Type I and IV, cellular and plasma fibronectin, gelatin or
224 with the highly glycosylated control protein fetuin. The
225 interaction of recombinant proteins with laminin was
226 also observed when anti - polyhistidine monoclonal anti-
227 bodies were employed to probe the ligands (Figure 5B).
228 The binding between Lsa33 and Lsa25 with laminin was
229 also evaluated on a quantitative basis as depicted in
230 Figure 5C. A dose - dependent and saturable binding
231

was observed when increasing concentrations of the recombinant proteins (0–6 µM) were allowed to adhere to a fixed laminin concentration (1 µg) (Figure 5C). Binding saturation level was achieved by protein concentration of ~4 and 5 µM for Lsa33 and Lsa25, respectively. Based on ELISA data, the calculated dissociation equilibrium constants (*K_D*) for the recombinant protein Lsa33 and Lsa25 with laminin is 367.5 and 415.4 nM, respectively. The role of laminin sugar moieties in the binding with Lsa33 and Lsa25 was assessed with laminin previously oxidized by increasing concentrations of sodium metaperiodate, ranging from 5 to 100 mM. The effect of metaperiodate concentration on the interaction is displayed in Figure 5D. Laminin oxidation had some effect on the interaction with Lsa25, being the reduction of 40% achieved at the highest metaperiodate concentration employed (100 mM). However, the attachment of Lsa33 to metaperiodate - treated laminin had no interference

on the binding. These results indicate that the binding of the proteins to laminin occur in a different manner and that sugar residues contribute to some extent for the interaction of Lsa25 with this ECM glycoprotein.

Interaction of recombinant proteins to serum components Our group has recently reported that leptospires interact with PLG and that several proteins could act as PLG - receptors [18–20,22]. Protein binding to complement regulators factor H and C4bp have also been shown [32,33]. Therefore, we set out to evaluate whether the recombinant proteins Lsa33 and Lsa25 were capable of binding human PLG, factor H and C4bp *in vitro*. The components, human PLG, factor H and C4bp and the control proteins, gelatin and fetuin, were individually immobilized onto 96 - wells plates followed by incubation with the recombinant leptospiral proteins. The results obtained using polyclonal antibodies against each

protein to probe the reactions showed that Lsa33 and Lsa25 interact with C4bp while only Lsa33 appears to bind to PLG (Figure 6A). No reaction was observed with factor H and the control proteins (Figure 6A). Similar results were achieved when binding was performed using monoclonal anti - his tag antibodies (Figure 6B). Both data show that while Lsa33 protein depicted a statistically significant absorption value for the interaction with PLG, the Lsa25 appears to have only a weak or no adherence to this component. These data were further confirmed when the reaction between the recombinant proteins and PLG were assessed on a quantitative basis as illustrated in Figure 6C. Dose - dependent and saturable binding was observed when increasing concentrations (0 to 1.0 μ M) of the recombinant protein Lsa33 were allowed to adhere to a fixed PLG amount (1 μ g), while very low absorption was detected with Lsa25 (Figure 6C). Based on the ELISA data, the calculated K_D for the recombinant protein Lsa33 with PLG is 23.53 ± 4.66 nM (Figure 6C). This K_D value is in the same order of magnitude with the ones obtained with several recombinant proteins in our laboratory [22].

PLG bound to leptospires and to several recombinant proteins, acting as PLG receptor, can acquire proteolytic activity in the presence of an activator, as we have previously shown [18–20,22]. Therefore, we investigated whether Lsa33 bound to PLG could also generate the enzymatically active plasmin. As a negative control, we have included the recombinant protein Lsa63, previously shown to be non-reactive with PLG [22]. Microplates were coated with the test protein, blocked, and then incubated with PLG. Unbound PLG was washed away and the urokinase - type PLG activator (uPA) was added together with a plasmin - specific chromogenic substrate. The reaction was carried out overnight and the plasmin activity was evaluated by measuring the cleavage of the substrate (absorbance at 405 nm). As shown in Figure 6D, the PLG captured by the Lsa33 protein could be converted into plasmin, as demonstrated indirectly by specific proteolytic activity. The negative controls Lsa63 and BSA did not show any proteolytic activity, similar to the controls lacking PLG, uPA or the chromogenic substrate.

The interaction of recombinant proteins with C4bp was studied in function of protein concentration. We have employed anti -Lsa33 and anti-Lsa25 polyclonal (Figure 6E) and anti-His tag monoclonal antibodies (Figure 6F) to probe the binding. Dose - response curves were obtained with both antibodies but the best response was achieved with anti-His tag monoclonal (Figure 6F), probably because of their homogeneous nature. However, C4bp was not saturated with the protein concentration range employed and therefore the K_D could not be calculated. Lsa63, a His - tag recombinant protein that does

not bind C4bp was also included, as a negative control, showed very low interaction and did not respond to increase protein concentration.

Inhibition of *L. interrogans* attachment to laminin or to PLG by Lsa33 and Lsa25

It has been reported that the several recombinant proteins with adhesin activity revealed an inhibitory effect on the binding of leptospires to ECM macromolecules [6]. We therefore performed experiments to assess whether the recombinant proteins had an effect on the binding of *Leptospira* to laminin or PLG by employing ELISA to detect the interaction in function of protein concentration (0–10 μ g). The results demonstrate that the addition of increasing concentrations of Lsa33 reduced the leptospiral binding to laminin and to PLG molecules in a dose - dependent manner (Figure 7A). Binding decrease in the number of leptospires interacting to laminin and PLG was statistically significant with 1.25 μ g of Lsa33 (*, $P < 0.05$). This interference was also evaluated with the binding of leptospires to laminin in the presence of increasing concentrations of Lsa25 (0–10 μ g), resulting in a similar effect as obtained with Lsa33 (*, $P < 0.05$) (Figure 7B). We have also assessed the competition for the binding of Lsa33 to PLG by increasing laminin concentration (0 to 1.0 μ g). The results revealed that both ligands compete for the binding with Lsa33 as a decrease of 40% in the binding was already detected with 0.25 μ g of laminin (*, $P < 0.05$) (Figure 7C). These experiments were performed in triplicate and Figure 7 shows one representative data of two independent experiments.

Discussion

Complement is a key component of the innate immune system responsible for protection against pathogenic microorganisms [34]. Factor H is a host fluid - phase regulator of the alternative complement pathway. Pathogenic leptospiral complement - resistant strains were found to bind factor H from human serum and this interaction seems to be associated to their serum resistance [32,35]. C4b - binding protein is an inhibitor of complement classical pathway system. This protein controls the complement classical pathway by interfering with the formation and regeneration of C3 convertase and acting as a cofactor to the serine proteinase factor I in the proteolytic inactivation of C4b [34,36]. It has been shown that pathogenic leptospiral strains can obtain C4bp from the host and that this acquisition preserves its cofactor activity [37]. The surface bound C4bp elicits factor I mediated cleavage of C4b, an interaction that appears to contribute to complement resistance by *Leptospira*, through the classical route [38]. Leptospiral binding proteins to C4bp, factor H and factor H - like

Figure 6 Recombinant proteins binding to serum components. (A) Human purified PLG, factor H and C4bp (10 µg/ml) were coated onto ELISA plates and allowed to interact with the recombinant proteins Lsa33 and Lsa25 (10 µg/ml). Gelatin and fetuin were used as negative controls for nonspecific binding. The binding was detected by antibodies raised against each recombinant protein (1:750). Bars represent the mean of absorbance at 492 nm ± the standard deviation of three replicates for each protein and are representative of three independent experiments. For statistical analyses, the binding of Lsa33 and Lsa25 was compared to its binding to gelatin by two - tailed t test (* $P < 0.05$ and ** $P < 0.005$). (B) Similar as described in (A) but the binding of the recombinant proteins was detected by anti - polyhistidine monoclonal antibodies (1:200). Included is a His - tag recombinant protein Lsa63 that does not bind C4bp. (C) Recombinant proteins dose - dependent binding experiments with PLG. The binding was detected by polyclonal antibodies against each protein; each point was performed in triplicate and expressed as the mean absorbance value at 492 nm ± standard error for each point. Gelatin was included as a negative control. The dissociation constant (K_D) is depicted and was calculated based on ELISA data for the recombinant protein that reached equilibrium. (D) Plasmin generation by PLG bound to recombinant proteins was assayed by modified ELISA as immobilized proteins received the following treatment: PLG + uPA + specific plasmin substrate (PLG + uPA + S), or controls lacking one of the three components (PLG + uPA; PLG + S; uPA + S). Lsa63 and BSA were employed as negative controls. Bars represent mean absorbance at 405 nm, as a measure of relative substrate degradation ± the standard deviation of four replicates for each experimental group and are representative of three independent experiments. Statistically significant binding in comparison to the negative control (BSA) are shown: * $P < 0.05$. (E) Recombinant proteins dose - dependent binding experiments with C4bp. The binding was detected by polyclonal antibodies raised against each protein (1:750); each point was performed in triplicate and expressed as the mean absorbance value at 492 nm ± standard error for each point. Gelatin was included as a negative control. (F) Similar to (E) but the protein binding was detected by anti-polyhistidine monoclonal antibodies (1:200); included is a His - tag recombinant protein Lsa63 that does not bind C4bp; each point was performed in triplicate and expressed as the mean absorbance value at 492 nm ± standard error for each point. Gelatin was included as a negative control.

Figure 7 Inhibition of *L. interrogans* attachment to immobilized laminin and PLG by recombinant proteins; The effect of laminin concentration on the binding of PLG to Lsa33. (A) Laminin or PLG (1 µg/well) was adsorbed onto microtiter plates followed by incubation with increasing concentrations of Lsa33 (0 to 10 µg) and in (B) laminin was adsorbed onto microtiter plates followed by incubation with increasing concentrations of Lsa25 (0 to 10 µg). In (A) and (B) the incubations were allowed to proceed for 90 min at 37°C. Live leptospires (100 µl/well of 4×10^7 *L. interrogans* serovar Copenhageni strain M20 leptospires) were added and incubated for another 90 min at 37°C. The unbound leptospires were washed away, and the quantification of bound leptospires was performed indirectly by anti - LipL32 antibodies produced in mice (1: 4,000 dilution) followed by horseradish peroxidase - conjugated antimouse IgG antibodies. Each point represents the mean absorbance value at 492 nm $\pm$ standard deviation of three replicates. Data are representative of two independent experiments (* $P < 0.05$). (C) The effect of laminin on the binding of PLG (10 µg/ml) to immobilized rLIC11834 (10 µg/ml) was assessed with the addition of increasing concentrations of laminin (0 to 1.0 µg). The detection of rLIC11834-bound PLG was performed by use of specific antibodies anti - PLG. Bars represent the mean absorbance values $\pm$ standard deviation of four replicates for each condition and are representative of two independent experiments. Results of statistically significant interference on the binding in comparison with the control (no addition of laminin) are shown: * $P < 0.05$

373 have also been identified in *Leptospira* [9,32,33]. Inter-
374 action of C4bp and of factor H with other pathogens has
375 been described, including the spirochetes *Borrelia* spp.
376 [34,38–42].

377 The capacity of the leptospires to adhere to extra-
378 cellular matrix components has been reported and to
379 date, several leptospiral adhesins have been identified.
380 These include 36 - kDa fibronectin - binding protein
381 [43], LfhA/Lsa24 [6,32], LigA and LigB proteins [7,8],
382 Len-family proteins [9], Lsa21 [10], LipL32 [12,44],
383 Lsa27 [13], Lp95 [11], TlyC [14], LipL53 [45], Lsa63 [16],
384 OmpL37 [46], Lsa66 [18] and Lsa20 [19]. We have
385 reported that *Leptospira* species were also capable to
386 bind PLG and generating plasmin, in the presence of
387 host activator, on the outer surface *in vitro* [20]. In
388 addition, we have described that plasmin - coated
389 virulent *L.interrogans* bacteria were capable to degrade
390 purified extracellular matrix components fibronectin [20]
391 and laminin (Vieira et al., unpublished data), a step that
392 may contribute for dissemination of the bacteria through
393 the host tissues. More recently, we have shown that
394 plasmin generation on the bacterial surface decreases the

deposition of C3b and IgG and hence, opsonization and
phagocytosis, a process that could facilitate leptospires to
evade the immune system [23]. Several PLG-receptor
proteins in *Leptospira* have been identified [18,19,21,22].

By data mining the genome sequences of *L. interrogans*,
searching for surface exposed proteins that could mediate
host - pathogen interactions, we have identified two
proteins annotated as *Leptospira* conserved hypothetical, one
of them, predicted to be a novel lipoprotein, LIC11834,
and the other, LIC12253, has recently been shown to be
non-protective in leptospiral challenge assay [47]. Both
selected coding sequences were cloned and the recombinant
proteins expressed in *E. coli*. We report that these proteins,
Lsa33 and Lsa25, are laminin - binding adhesins and in the
case of Lsa33, capable to bind PLG generating enzymatically
active plasmin. Although weak, both proteins showed the ability
to bind human purified C4bp, suggesting that these proteins have
the potential to participate in leptospiral immune evasion by
interfering with the complement classical pathway.

Due to the high degree of antigenic variation among
leptospires, we examined the gene/protein conservation

among important species of *Leptospira*. The LIC11834 and LIC12253 genes are conserved in five serovars of *L. interrogans* and in other species tested but in the case of *L. santarosai* serovar Shermani the gene LIC11834 is absent. However, LIC11834 transcripts were detected only in serovars of *L. interrogans*, while LIC12253 appears to be expressed in all strains evaluated. None of the proteins seems to be expressed in the saprophytic strain, *L. biflexa* serovar Patoc.

The recombinant proteins Lsa33 and Lsa25 were expressed with molecular mass of 33 - and 24 - kDa, respectively, having a 6XHis tag at N - terminal. The purified proteins exhibited single major bands in SDS - PAGE and were recognized by anti - His tag monoclonal antibodies and by homolog sera from mice immunized with each recombinant protein. Secondary structure of the recombinant proteins after the purification process was evaluated by CD spectroscopy and showed a predominance of alpha helices in both cases, similar to the data predicted by bioinformatics, indicating the suitability of recombinant proteins for further studies. The LIC12253 coding sequence is probably higher immunogenic than LIC11834 because it was recognized by approximately 45% of serum samples of both phases, initial and convalescent, of confirmed leptospirosis's cases. Interestingly, the LIC11834 protein although presented almost no reactivity among these serum samples, showed a slightly augment effect on serum reactivity when was assayed together with LIC12253. Immunofluorescence using live leptospires showed LIC11834 and LIC12253 coding sequences at the surface of bacteria, as a result of antiserum recognition raised against each protein. *In silico* analysis, proteinase K accessibility and immunofluorescence data together suggest that these proteins are likely to be surface exposed. In addition, the recombinant proteins partially inhibited leptospiral adherence to immobilized laminin and PLG.

Merien and colleagues [43] identified a 36-kDa fibronectin-binding protein expressed by a virulent variant of *Leptospira*. Our group described the first leptospiral laminin - binding protein, named Lsa24 [6]. These studies were followed by the identification of several extracellular matrix binding proteins [7,9-19]. The recombinant proteins Lsa33 and Lsa25 exhibited extracellular matrix - binding properties, and are laminin - binding proteins. The binding affinity dissociation constants estimated for both proteins to laminin showed similar K_D value of that reported for OmpL 37 (410 ± 81 nM) and the same ECM molecule [17]. Thus, it is possible that these proteins have a role in the adhesion of leptospires to hosts.

The PLG activation system with generation of plasmin was described for virus, parasites and bacteria, including the spirochetes *Borrelia* spp. and with *Treponema denticola* [48-51]. Plasmin is a serine protease with the

capacity to degrade a broad spectrum of substrates, including fibrin clots, connective tissue and components of extracellular matrices [52-54]. We have reported that *Leptospira* spp. bind PLG at their surface generating plasmin, when host activator is available, making the bacteria capable to degrade fibronectin [20] and laminin (Vieira, M.L., unpublished results). Verma et al. [21] have demonstrated that the protein LenA of *L. interrogans* [9] is a surface receptor for human PLG. Moreover, we have reported several novel PLG - receptor proteins of *Leptospira* [18,19,22]. We now describe Lsa33 as a novel PLG - binding protein. Similar to the previously reported proteins, bound PLG could be converted to plasmin by the addition of urokinase - type PLG activator (uPA), showing specific proteolytic activity. It is thus possible that the Lsa33 besides playing a role in the attachment to host and acting as PLG - receptor, may also help leptospires to surmount tissue barriers.

The inhibitory effect exerted on the binding of leptospires to laminin and PLG by the recombinant proteins was statistically significant with both, in the case of Lsa33, and with laminin for the Lsa25. The intensity of the interference upon the binding is expected given the presence of several ECM - or PLG-binding proteins in *Leptospira*. These data are comparable to the ones already reported in the literature [6,7,17-19,22]. Partial inhibitory effect was observed by laminin on the binding of Lsa33 to PLG, suggesting a competition for the same binding site.

Conclusions

We report in these studies a characterization of two leptospiral proteins, genome annotated as proteins of unknown function. The recombinant proteins Lsa33 and Lsa25 are laminin binding proteins that might be involved in the attachment to host. Moreover, both proteins showed the ability to bind C4bp, a feature suggesting their possible involvement in the immune evasion of leptospires. The recombinant Lsa33 is also PLG - binding protein that could help the bacteria during the infection process. Thus, it appears that Lsa33 and to a lesser degree, Lsa25, are multifaceted proteins that might have multiple functions in the leptospiral pathogenesis. To date, Lsa33 is the first described laminin -, PLG - and C4bp - leptospiral binding protein.

Methods

Leptospira strains and sera

The pathogenic *Leptospira* strains used were: *L. interrogans* serovar Canicola strain Hound Utrech IV, *L. interrogans* serovar Copenhageni strain M 20, *L. interrogans* serovar Hardjo strain Hardjoprajitno, *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. interrogans* serovar Pomona strain Pomona, *L. borgpetersenii* serovar Whitcombi strain

Whitcomb and serovar Grippothyphosa strain Moskva V, *L. kirshneri* serovar Cynoptery strain 3522 C, *L. santarosai* serovar Shermani strain 1342 K, *L. noguchi* serovar Panama strain CZ 214 and *L. biflexa* serovar Patoc strain Patoc, were cultured at 28°C under aerobic conditions in liquid EMJH medium (Difco®) with 10% rabbit serum, enriched with L - asparagine (wt/vol: 0.015%), sodium pyruvate (wt/vol: 0.001%), calcium chloride (wt/vol: 0.001%), magnesium chloride (wt/vol: 0.001%), peptone (wt/vol:0.03%) and meat extract (wt/vol: 0.02%) (Turner LH. *Leptospirosis. 3. Maintenance, isolation and demonstration of leptospires*. Trans R Soc Trop Med Hyg 1970; 64: 623–646). *Leptospira* cultures are maintained in Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil. Confirmed - leptospirosis serum samples were from Instituto Adolfo Lutz collection, São Paulo, Brazil.

539 Microscopic agglutination test (MAT)

The microscopic agglutination test was performed according to [1]. In brief, an array of 22 serovars of *Leptospira* spp. as antigens were employed: Australis, Autumnalis, Bataviae, Canicola, Castellonis, Celledoni, Copenhageni, Cynopteri, Djasiman, Grippothyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc, Pomona, Pyrogenes, Sejroe, Shermani, Tarassovi and Wolffi. All the strains were maintained in EMJH liquid medium (Difco, USA) at 29°C. A laboratory - confirmed case of leptospirosis was defined by the demonstration of a four - fold microagglutination titer rise between paired serum samples. The probable predominant serovar was considered to be the one with the highest dilution that could cause 50% of agglutination. MAT was considered negative when the titer was below 100.

556 Characterization of the protein *in silico*

Predicted coding sequence (CDSs) LIC11834 and LIC12253 were identified on *L. interrogans* serovar Copenhageni and selection was based on cellular localization; cellular localization prediction was performed by PSORT, <http://psort.nibb.ac.jp> [55] and PredictProtein web server, <https://www.predictprotein.org/> [26]. The SMART [24] <http://smart.embl-heidelberg.de/> and PFAM [56] <http://www.sanger.ac.uk/Software/Pfam/> web servers were used to search for predicted functional and structural domains. The presence of lipobox putative sequence was evaluated by use of the Lipop program [57] <http://www.cbs.dtu.dk/services/LipoP/>. The predicted sequence of the lipobox was also assessed by use of the SpLip program, as described by Setubal et al. [58]. Secondary structure, solvent accessibility and cellular localization predictions were also performed by using PredictProtein web server, <https://www.predictprotein.org/> [26].

574 DNA isolation and PCR analysis

Leptospira cultures were harvested by centrifugation at 11,500 g for 30 min and gently washed in sterile PBS twice. Genomic DNA was isolated from the pellets by guanidine - detergent lysing method using DNAzol® Reagent (Invitrogen), according to the manufacturer's instructions. Primers were designed according to *L. interrogans* serovar Copenhageni genome sequences (GenBank accession AE016823) and are listed in Table 1. PCR was performed in a reaction volume of 25 µl containing 100 ng of genomic DNA, 1 × PCR buffer (20 mM Tris - HCl, pH 8.4, 50 mM KCl), 2 mM MgCl₂, 20 pmol of each specific primer, 200 µM of each dNTP, and 2.5 U Taq DNA Polymerase (Invitrogen). Cycling conditions were: 94 ° C - 4 min, followed by 40 cycles at 94°C - 50 sec, 57°C (LIC11834) or 56°C (LIC12253) - 50 sec, 72°C - 90 sec, and a final extension cycle of 7 min at 72°C. PCR amplified products were loaded on a 1% agarose gel for electrophoresis and visualization with ethidium bromide.

593 RNA extraction and RT-PCR analysis

For reverse transcription RT - PCR, total RNA was isolated by the acid guanidinium thiocyanate phenol - chloroform method using TRIzol® Reagent (Invitrogen) according to the manufacturer's recommendations. 1 µg of RNA from each sample was treated with 1 U of DNase I Amplification Grade (Invitrogen) for 15 min at room temperature. DNase I was inactivated by the addition of 1 µl of 25 mM EDTA solution followed by an incubation at 65 ° C for 10 min. DNase - treated RNAs were reversely transcribed using the SuperScript™ III First - Strand Synthesis System for RT-PCR (Invitrogen). One tenth of RT products were amplified in a 25 µl reaction mix using oligonucleotides LIC11834 - F/LIC11834 - R or LIC12253 - F/LIC12253 - R, as described above. Samples quantity and integrity were verified by amplification of a 1,042 bp 16 S ribosomal cDNA fragment using oligomers:

16S - F 5'
CAAGTCAAGCGGAGTAGCAATACTCAGC 3' and
16S - R 5'GATGGCAACATAAGGTGAGGGTTGC 3'.

615 DNA recombinant techniques, protein expression and purification

Predicted CDSs LIC11834 and LIC12253, without signal peptides, were amplified by the PCR from *L. interrogans* serovar Copenhageni strain Fiocruz L1 - 130 genomic DNA using the primer pairs depicted in Table 1. The PCR products obtained for each corresponding gene were cloned into pGEM-T easy vector (Promega) and subcloned into the pAE expression vector [28] at the restriction sites shown in Table 1. The pAE vector allows the expression of recombinant proteins with a minimal

6X His - tag at the N - terminus. All cloned sequences were confirmed by DNA sequencing with an ABI 3100 automatic sequencer (PE Applied Biosystems, Foster city, CA). Protein expression of rLIC11834 and rLIC12253 was achieved in *E. coli* BL21 (SI) strain by the action of T7 DNA polymerase under control of the osmotically induced promoter proU [59]. *E. coli* BL21 (SI) containing recombinant plasmids were grown at 30°C in Luria - Bertani broth without NaCl and with 100 µg/ml ampicillin with continuous shaking until an optical density at 600 nm of 0.6 to 0.8 was reached. Recombinant protein synthesis was induced by the addition of 300 mM NaCl. After three hours, the cells were harvested by centrifugation and the bacterial pellets resuspended in lysis buffer (200 µg/ml of lysozyme, 1% Triton X - 100, 2 mM phenylmethylsulfonyl fluoride [PMSF]). The bacterial cell pellets were lysed on ice with the aid of a sonicator (Ultrasonic Processor; GE Healthcare). The insoluble fractions were washed with 20 ml of buffer (20 mM Tris - HCl, pH 8.0, 500 mM NaCl, 1 M urea and 1% Triton X-100) and resuspended in a buffer containing 20 mM Tris - HCl, pH 8.0, 500 mM NaCl, 5 mM Imidazole, 1 mM β - mercaptoethanol and 8 M urea. The proteins were then purified through metal chelating chromatography in a Sepharose fast flow column (GE Healthcare) and fractions were analyzed in 12% SDS-PAGE. The rLIC12253 protein was refolded by 500 times dilution with 20 mM Tris - HCl, pH 8.0, and 500 mM NaCl before chromatographic purification. The purified recombinant proteins were extensively dialyzed against phosphate - buffered saline (PBS), pH 7.4, 0.1% (wt/vol) glycine solution (1:100), pooled and stored at -20°C.

658 Circular dichroism spectroscopy

659 Purified recombinant proteins were dialyzed against sodium phosphate buffer (pH 7.4). Circular dichroism (CD) spectroscopy measurements were performed at 20°C using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo) equipped with a Peltier unit for temperature control. Far-UV CD spectra were measured using a 1 mm - path - length cell at 0.5 nm intervals. The spectra were presented as an average of five scans recorded from 185 to 260 nm. The molar ellipticity (Φ) is expressed in deg.cm.dmol⁻¹.

669 Antiserum

670 Five female BALB/c mice (4-6 weeks old) were immunized subcutaneously with 10 µg of the recombinant proteins. The recombinant proteins were adsorbed in 10% (vol/vol) of Alhydrogel (2% Al(OH)₃, Brenntag Biosector, Denmark), used as adjuvant. Two subsequent booster injections were given at two - week intervals with the same preparation of 10 µg of the proteins. 677 Negative - control mice were injected with PBS. One

week after each immunization, the mice were bled from the retro - orbital plexus and the pooled sera were analyzed by enzyme -linked immunosorbent assay (ELISA) for determination of antibody titers. All animal studies were approved by the Ethics Committee of the Instituto Butantan, São Paulo, SP, Brazil. The Committee in Animal Research in Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation.

Immunoblotting assay

686 The purified recombinant proteins were loaded into 12% SDS - PAGE and transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare) in semi - dry equipment. Membranes were blocked with 5% non-fat dried milk and 2.5% BSA in PBS containing 0.05% Tween 20 (PBS - T) and then incubated with anti - rLIC11834 (1:500), anti - rLIC12253 (1:500) mouse serum or anti - his antibody (1:1,000) (GE Healthcare) for 2 h at room temperature. After washing, the membranes were incubated with horseradish peroxidase (HRP) - conjugated anti - mouse IgG (1:5,000; Sigma) in PBS - T for 1 h. The protein's reactivity was revealed by ECL reagent kit chemiluminescence substrate (GE Healthcare) with subsequent exposition to X - Ray film.

ELISA for detection of human antibodies

701 Human IgG antibodies against Lsa33 or Lsa25 were detected by ELISA as previously described [60]. In brief, serum samples of negative (24) and positive (33) MAT from confirmed - leptospirosis patients were diluted 1:400 and evaluated for total IgG using goat HRP - conjugated anti-human IgG antibodies (1:5,000, Sigma). Cutoff values were set at three standard deviations above the mean OD_{492nm} of sera from 11 health individuals, unexposed to leptospirosis, from the city of São Paulo, Brazil and one pool of normal serum samples from USA (Sigma).

Proteinase K accessibility assay

713 Suspensions of 5 ml live leptospires/per treatment (~10⁸ cells/ml) were harvested at 10,000 rpm for 15 min at room temperature, washed once with PBS (with 50 mM NaCl), resuspended in 5 ml of PBS (with 50 mM NaCl) plus with 25 µg/ml of proteinase K (PK) (Sigma Aldrich). Four similar test tubes were then incubated for 0 to 5 h at 37°C and aliquots were taken at 0, 1, 3 and 5 h before the addition of 100 mM of phenylmethylsulfonyl fluoride (PMSF) to stop PK activity. The suspensions were subsequently pelleted by centrifugation at 10,000 rpm for 5 min, washed twice with PBS (with 50 mM NaCl) and resuspended in 1 ml PBS (with 50 mM NaCl) for ELISA analysis using antibodies against Lsa33, Lsa25, Lip32 and DnaK, as described

below. LipL32 and DnaK are membrane and cytoplasmic leptospiral proteins that were employed in our experiment as positive and negative control, respectively.

ELISA for detection cellular localization of the proteins

Leptospire were coated onto microplates and allowed to stand at room temperature for 16 h. The plates were washed three times with PBS (with 50 mM NaCl) and blocked with 5% non-fat dry milk and 1% BSA for 2 h at 37°C. After incubated for 2 h at 37°C with polyclonal mouse anti - serum against Lsa33, Lsa25, LipL32 or DnaK (dilution of an OD equal to 1). The leptospire were washed three times with PBS (with 50 mM NaCl) and incubated with 50 µL of a 1:5,000 dilution of HRP - conjugated goat anti - mouse IgG (Sigma) in PBS (with 50 mM NaCl) for 1 h at 37°C. The wells were washed three times with PBS (with 50 mM NaCl), and o - phenylenediamine (OPD) (1 mg/mL) in citrate phosphate buffer (pH 5.0) plus 1 µL/mL H₂O₂ was added (100 µL per well). The reaction proceeded for 5 min and was interrupted by the addition of 50 µL of 4 N H₂SO₄. The absorbance at 492 nm was determined in a microplate reader (TP - reader, Thermo) against the O.D. of blanks, containing all the reaction mixture but antibodies against the proteins. For statistical analyses, the binding of polyclonal mouse anti - serum against Lsa33, Lsa25, LipL32 or DnaK at 0 h incubation was compared with other incubations by Student's two - tailed *t* test.

Binding of recombinant proteins to ECM and to serum components

Protein attachment to individual macromolecules of the extracellular matrix was analyzed according to a previously published protocol [6] with some modifications. Briefly, 96 - well plates (Costar High Binding, Corning) were coated with 1 µg of laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, human PLG, factor H, C4bp, or gelatin (negative control) and fetuin (highly glycosylated attachment - negative control protein) in 100 µL of PBS for 3 h at 37°C. The wells were washed three times with PBS - T and then blocked with 200 µL of 10% (wt/vol) non-fat dry milk (overnight at 4°C). One microgram of each recombinant protein was added per well in 100 µL of PBS, and protein was allowed to attach to the different substrates for 2 h at 37°C. After washing six times with PBS - T, bound Lsa33 or Lsa25 was detected by adding mouse anti - recombinant proteins (1:750) in 100 µL of PBS or anti - His tag monoclonal (1:200) in 100 µL of PBS. Incubation proceeded for 1 h at 37°C. After three washings with PBS - T, 100 µL of a 1:5,000 dilution of HRP - conjugated goat anti - mouse IgG (Sigma) in PBS was added per well for 1 h at 37°C. The wells were washed three times, and o - phenylenediamine (OPD) (1 mg/mL) in

citrate phosphate buffer (pH 5.0) plus 1 µL/mL H₂O₂ was added (100 µL per well). The reaction proceeded for 10 min and was interrupted by the addition of 50 µL of 4 N H₂SO₄. The absorbance at 492 nm was determined in a microplate reader (TP - reader, Thermo). For statistical analyses, the binding of recombinant proteins to ECM macromolecules and to serum components were compared to its binding to gelatin and by Student's two - tailed *t* test.

Metaperiodate treatment of laminin

Microtitre wells were coated with 1 µg of laminin in 50 mM sodium acetate buffer, pH 5.0, and incubated for 16 h at 4°C. Wells were washed three times with the same buffer, and laminin was treated with different sodium metaperiodate concentrations (0-100 mM) in the same buffer for 15 min at 4°C in the dark. After three washes with 50 mM sodium acetate buffer, wells were blocked with 200 µL of 1% BSA for 1 h at 37°C. Binding of recombinant proteins (1 µg in PBS per well) to periodate - treated laminin was evaluated as described above.

Dose-response curves

First, 96 - well plates were coated overnight in PBS at 4°C with 100 µL of 10 µg/ml PLG, laminin or C4bp. Plates were then blocked and increasing concentrations of the purified recombinant proteins (0-6 µM) were added (100 µL/well in PBS). The assessment of bound proteins was performed by incubation for 1 h at 37°C with the antiserum raised against each protein at the dilution of 1:750, followed by HRP - conjugated goat anti-mouse IgG (Sigma) (1:5,000 in PBS). The reactions were detected with OPD as describe above. The ELISA data were used to calculate the dissociation constant (K_D) according to the method described by Pathirana et al. [61] and Lin et al. [62], based on the equation:
$$\frac{A}{A_{\max}} = \frac{[K]_{\text{protein}}}{[K]_{\text{protein}} + [K_D]}$$
 where A is the absorbance at a given protein concentration, $A_{\max}$ is the maximum absorbance for the ELISA plate reader (equilibrium), $[K]_{\text{protein}}$ is the protein concentration and K_D is the dissociation equilibrium constant for a given absorbance at a given protein concentration (ELISA data point).

Plasmin enzymatic activity assay

96 - well ELISA plates were coated overnight with 10 µg/ml recombinant proteins in PBS at 4°C. Lsa63, which does not bind PLG [22] and BSA were employed as negative control. Plates were washed once with PBS - T and blocked for 2 h at 37°C with PBS with 10% (wt/vol) non - fat dry milk. The blocking solution was discarded and 100 µL/well of 10 µg/ml human PLG was added, followed by incubation for 2 h at 37°C. Wells

were washed three times with PBS - T, and then 4 ng/
well of human uPA (Sigma - Aldrich) were added. Subse-
quently, 100 µl/well of plasmin - specific substrate D -
valyl - leucyl - lysine - p - nitroanilide dihydrochloride
(Sigma - Aldrich) were added at a final concentration of
0.4 mM in PBS. Plates were incubated overnight at 37°C
and substrate degradation was measured by readings at
405 nm.

Inhibition of live leptospires binding to laminin or to PLG by recombinant proteins

ELISA plates were coated with laminin or PLG (1 µg/
well). The plates were washed and blocked with 10% non
- fat dry milk in PBS - T for 2 h at 37°C. The blocking
solution was discarded, and the wells were incubated for
90 min at 37°C with increasing concentrations of pro-
teins (0 to 10 µg). After three washings, 50 µL/well of
4 × 10⁷ live low - passage virulent *L. interrogans* serovar
Copenhageni strain M20 were added for 90 min at 37°C.
The unbound leptospires were washed and the quantifi-
cation of bound leptospires was performed indirectly by
anti - LipL32 antibodies produced in mice (1:4,000),
given the fact that LipL32 is a major outer membrane
leptospiral protein [29]; the procedure was followed by
horseradish peroxidase - conjugated anti - mouse IgG
antibodies, essentially as described in Barbosa et al. [6].
The detection was performed by OPD as previously
described.

Liquid-phase immunofluorescence assay (L - IFA)

The localization of LIC11834 and LIC12253 encoded
proteins by L - IFA was performed as described by Oli-
veira et al. [15]. In brief, suspensions of 2.5 ml live lep-
tospires (~10⁹ cells/ml) were harvested at 10,000 rpm for
15 min, washed twice with PBS (with 50 mM NaCl),
resuspended in 200 µl of PBS with 6 µg/ml propidium
iodide to stain the nuclei, and incubated for 45 min at
37°C. After incubation, the leptospires were washed gen-
tly with PBS and incubated for 30 min at 4°C with poly-
clonal mouse anti - serum against Lsa33, Lsa25, LipL32
or GroEL at a 1:50 dilution. The leptospires were washed
and incubated with goat anti - mouse IgG antibodies
conjugated to fluorescein isothiocyanate (FITC, Sigma) at
a dilution 1:50 for 30 min at 4°C. After incubation with
secondary antibody, the leptospires were washed and
resuspended in PBS - antifading solution (ProLong Gold,
Molecular Probes). The immunofluorescence - labeled
leptospires were examined by employ of a confocal LSM
510 META immunofluorescence microscope (Zeiss,
Germany).

Nucleotide sequence accession numbers

GenBank accession numbers for protein sequences
LIC11834 and LIC12235 are AAS70420 and AAS70825,

respectively. The protein can also be accessed by the
genome nomenclature for the gene locus, LIC number
(*Leptospira interrogans* serovar Copenhageni).

ECM and biological components

The control proteins fetuin and gelatin, were purchased
from Sigma Chemical Co. (St. Louis, Mo.) and Difco®,
respectively. Laminin - 1 and collagen Type IV were
derived from the basement membrane of Engelbreth -
Holm-Swarm mouse sarcoma, cellular fibronectin was
derived from human foreskin fibroblasts, plasma fibro-
nectin was isolated from human plasma and collagen
Type I was isolated from rat tail. PLG, purified from
human plasma, was purchased from Merck. Human Fac-
tor H was from Calbiochem. C4bp was from Comple-
ment Technology, INC.

Acknowledgments

We are deeply indebted to Alexsander Seixas de Souza (Departamento de
Parasitologia, Instituto Butantan, Sao Paulo, Brazil) for use of Confocal facilities
and helpful discussion. This work was supported by FAPESP, CNPq and
Fundação Butantan, Brazil; RFD and MLV have fellowships from FAPESP.

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Author's contributions

RFD performed the molecular cloning studies, protein expression, ECM assays
and animal immunizations. MLV carried out the PLG assays and help with the
manuscript. ECR evaluated MAT of the collection serum samples. APG and
ZMM were responsible for bacteria growth, identification and virulence strain
maintenance. SAV participated in the design of the study and help drafted
the manuscript. ALTON conceived of the study, and participated in its design
and coordination and helped to draft the manuscript. All authors read and
approved the final manuscript.

Received: 18 November 2011 Revised: 30 March 2012

Accepted: 23 March 2012 Published: 30 March 2012

References

- SFaineBADlerCBolinPPerolat1999Leptospira and LeptospirosisAustralia
MediSciMelbourneFaine S, Adler B, Bolin C, Perolat P: *Leptospira and
Leptospirosis*. Melbourne: Australia MediSci; 1999.
- Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN,
Gilman RH, Willig MR, Gotuzzo E, et al: *Leptospirosis: a zoonotic disease of
global importance*. *Lancet Infect. Dis.* 2003, **3**(12):757-771.
- Levett PN: *Leptospirosis*. *Clin. Microbiol. Rev.* 2001, **14**(2):296-326.
- Ko AI, Galvao Reis M, Ribeiro Dourado CM, Johnson WD Jr, Riley LW: *Urban
epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study
Group*. *Lancet* 1999, **354**(9181):820-825.
- Merien F, Truccolo J, Rougier Y, Baranton G, Perolat P: *In vivo apoptosis of
hepatocytes in guinea pigs infected with Leptospira interrogans serovar
icterohaemorrhagiae*. *FEMS Microbiol. Lett.* 1998, **169**(1):95-102.
- Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, Vieira ML,
Morais ZM, Vasconcellos SA, Nascimento AL: *A newly identified leptospiral
adhesin mediates attachment to laminin*. *Infect. Immun.* 2006, **74**
(11):6356-6364.
- Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J, Haake DA:
Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA

- 941 and LigB bind extracellular matrix proteins and fibrinogen. *Infect. Immun.* 2007, **75**(5):2441–2450.
- 942 8. Lin YP, Chang YF: A domain of the *Leptospira* LigB contributes to high
- 943 affinity binding of fibronectin. *Biochem. Biophys. Res. Commun.* 2007, **362**
- 944 (2):443–448.
- 945 9. Stevenson B, Choy HA, Pinne M, Rotondi ML, Miller MC, Demoll E, Kraicz P,
- 946 Cooley AE, Creamer TP, Suchard MA, et al: *Leptospira* interrogans
- 947 endostatin-like outer membrane proteins bind host fibronectin, laminin
- 948 and regulators of complement. *PLoS One* 2007, **2**(11):e1188.
- 949 10. Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Moraes ZM, Lima
- 950 DM, Abreu PA, Nascimento AL: Lsa21, a novel leptospiral protein binding
- 951 adhesive matrix molecules and present during human infection. *BMC*
- 952 *Microbiol.* 2008, **8**:70.
- 953 11. Atzingen MV, Gomez RM, Schattner M, Pretre G, Goncalves AP, de Moraes ZM,
- 954 Vasconcellos SA, Nascimento AL: Lp95, a novel leptospiral protein that
- 955 binds extracellular matrix components and activates e-selectin on
- 956 endothelial cells. *J. Infect.* 2009, **59**(4):264–276.
- 957 12. Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Moraes ZM, Barbosa AS,
- 958 Ho PL: In LipL32, the major leptospiral lipoprotein, the C terminus is the
- 959 primary immunogenic domain and mediates interaction with collagen IV
- 960 and plasma fibronectin. *Infect. Immun.* 2008, **76**(6):2642–2650.
- 961 13. Longhi MT, Oliveira TR, Romero EC, Goncalves AP, de Moraes ZM,
- 962 Vasconcellos SA, Nascimento AL: A newly identified protein of *Leptospira*
- 963 *interrogans* mediates binding to laminin. *J. Med. Microbiol.* 2009, **58**(Pt
- 964 10):1275–1282.
- 965 14. Carvalho E, Barbosa AS, Gomez RM, Cianciarullo AM, Hauk P, Abreu PA,
- 966 Fiorini LC, Oliveira ML, Romero EC, Goncalves AP, et al: Leptospiral TlyC is an
- 967 extracellular matrix-binding protein and does not present hemolysin
- 968 activity. *FEBS Lett.* 2009, **583**(8):1381–1385.
- 969 15. Oliveira TR, Longhi MT, Goncalves AP, de Moraes ZM, Vasconcellos SA,
- 970 Nascimento AL: LipL53, a temperature regulated protein from *Leptospira*
- 971 *interrogans* that binds to extracellular matrix molecules. *Microbes and*
- 972 *infection/Institut Pasteur* 2010, **12**(3):207–217.
- 973 16. Vieira ML, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA,
- 974 Nascimento AL: Lsa63, a newly identified surface protein of *Leptospira*
- 975 *interrogans* binds laminin and collagen IV. *J. Infect.* 2010, **60**(1):52–64.
- 976 17. Pinne M, Choy HA, Haake DA: The OmpL37 surface-exposed protein is
- 977 expressed by pathogenic *Leptospira* during infection and binds skin and
- 978 vascular elastin. *PLoS Negl. Trop. Dis.* 2010, **4**(9):e815.
- 979 18. Oliveira R, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA,
- 980 Nascimento AL: Characterization of novel OmpA-like protein of
- 981 *Leptospira interrogans* that binds extracellular matrix molecules and
- 982 plasminogen. *PLoS One* 2011, **6**(7):e21962.
- 983 19. Mendes RS, Atzingen MV, Moraes ZM, Goncalves AP, Serrano SM, Asega AS,
- 984 Romero EC, Vasconcellos SA, Nascimento AL: The Novel Leptospiral
- 985 Surface Adhesin Lsa20 Binds Laminin and Human Plasminogen and Is
- 986 Probably Expressed during Infection. *Infect. Immun.* 2011, **79**(11):4657–
- 987 4667.
- 988 20. Vieira ML, Vasconcellos SA, Goncalves AP, de Moraes ZM, Nascimento AL:
- 989 Plasminogen acquisition and activation at the surface of *leptospira*
- 990 *species* lead to fibronectin degradation. *Infect. Immun.* 2009, **77**(9):4092–
- 991 4101.
- 992 21. Verma A, Brissette CA, Bowman AA, Shah ST, Zipfel PF, Stevenson B:
- 993 Leptospiral endostatin-like protein A is a bacterial cell surface receptor
- 994 for human plasminogen. *Infect. Immun.* 2010, **78**(5):2053–2059.
- 995 22. Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, Vasconcellos SA,
- 996 Nascimento AL: In vitro identification of novel plasminogen-binding
- 997 receptors of the pathogen *Leptospira interrogans*. *PLoS One* 2010, **5**(6):
- 998 e11259.
- 999 23. Vieira ML, de Moraes ZM, Vasconcellos SA, Romero EC, Nascimento AL: In
- 1000 vitro evidence for immune evasion activity by human plasmin associated
- 1001 to pathogenic *Leptospira interrogans*. *Microb. Pathog.* 2011, **51**(5):360–365.
- 1002 24. Schultz J, Milpetz F, Bork P, Ponting CP: SMART, a simple modular
- 1003 architecture research tool: identification of signaling domains. *Proc. Natl.*
- 1004 *Acad. Sci. U.S.A.* 1998, **95**(11):5857–5864.
- 1005 25. Letunic I, Doerks T, Bork P: SMART 6: recent updates and new
- 1006 developments. *Nucleic acids research* 2009, **37**(Database issue):D229–D232.
- 1007 26. Rost B, Yachdav G, Liu J: The PredictProtein Server. *Nucleic acids research* 2004,
- 1008 **32**(Web Server issue):W321–W326.
- 1009 27. Rajesh N, Burkhard R: Mimicking cellular sorting improves prediction of
- 1010 subcellular localization. *J. Mol. Biol.* 2005, **348**(1):85–100.
- 1011 28. Ramos CR, Abreu PA, Nascimento AL, Ho PL: A high-copy T7 *Escherichia*
- 1012 *coli* expression vector for the production of recombinant proteins with a
- 1013 minimal N-terminal His-tagged fusion peptide. *Braz. J. Med. Biol. Res.* 2004,
- 1014 **37**(8):1103–1109.
- 1015 29. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, Matsunaga J,
- 1016 Levett PN, Bolin CA: The leptospiral major outer membrane protein
- 1017 LipL32 is a lipoprotein expressed during mammalian infection. *Infect.*
- 1018 *Immun.* 2000, **68**(4):2276–2285.
- 1019 30. Haake DA, Matsunaga J: Characterization of the leptospiral outer
- 1020 membrane and description of three novel leptospiral membrane
- 1021 proteins. *Infect. Immun.* 2002, **70**(9):4936–4945.
- 1022 31. Stamm LV, Gherardini FC, Parrish EA, Moomaw CR: Heat shock response of
- 1023 *spirochetes*. *Infect. Immun.* 1991, **59**(4):1572–1575.
- 1024 32. Verma A, Hellwage J, Artiushin S, Zipfel PF, Kraicz P, Timoney JF, Stevenson
- 1025 B: LfhA, a novel factor H-binding protein of *Leptospira interrogans*. *Infect.*
- 1026 *Immun.* 2006, **74**(5):2659–2666.
- 1027 33. Barbosa AS, Monaris D, Silva LB, Moraes ZM, Vasconcellos SA, Cianciarullo
- 1028 AM, Isaac L, Abreu PA: Functional characterization of LcpA, a surface-
- 1029 exposed protein of *Leptospira* spp. that binds the human complement
- 1030 regulator C4BP. *Infect. Immun.* 2010, **78**(7):3207–3216.
- 1031 34. Blom AM: Structural and functional studies of complement inhibitor C4b-
- 1032 binding protein. *Biochem. Soc. Trans.* 2002, **30**(Pt 6):978–982.
- 1033 35. Meri T, Murgia R, Stefanel P, Meri S, Cinco M: Regulation of complement
- 1034 activation at the C3-level by serum resistant leptospires. *Microb. Pathog.*
- 1035 2005, **39**(4):139–147.
- 1036 36. Gigli I, Fujita T, Nussenzweig V: Modulation of the classical pathway C3
- 1037 convertase by plasma proteins C4 binding protein and C3b inactivator.
- 1038 *Proc. Natl. Acad. Sci. U.S.A.* 1979, **76**(12):6596–6600.
- 1039 37. Barbosa AS, Abreu PA, Vasconcellos SA, Moraes ZM, Goncalves AP, Silva AS,
- 1040 Dahan MR, Isaac L: Immune evasion of *leptospira* species by acquisition of
- 1041 human complement regulator C4BP. *Infect. Immun.* 2009, **77**(3):1137–1143.
- 1042 38. Cinco M: New insights into the pathogenicity of leptospires: evasion of
- 1043 host defences. *New Microbiol.* 2010, **33**(4):283–292.
- 1044 39. Ram S, Mackinnon FG, Gulati S, McQuillen DP, Vogel U, Frosch M, Elkins C,
- 1045 Guttormsen HK, Wetzler LM, Oppermann M, et al: The contrasting
- 1046 mechanisms of serum resistance of *Neisseria gonorrhoeae* and group B
- 1047 *Neisseria meningitidis*. *Mol. Immunol.* 1999, **36**(13–14):915–928.
- 1048 40. Kraicz P, Skerka C, Brade V, Zipfel PF: Further characterization of
- 1049 complement regulator-acquiring surface proteins of *Borrelia burgdorferi*.
- 1050 *Infect. Immun.* 2001, **69**(12):7800–7809.
- 1051 41. Carroll MC: The complement system in regulation of adaptive immunity.
- 1052 *Nat. Immunol.* 2004, **5**(10):981–986.
- 1053 42. Hovis KM, McDowell JV, Griffin L, Marconi RT: Identification and
- 1054 characterization of a linear-plasmin-encoded factor H-binding protein
- 1055 (FhbA) of the relapsing fever spirochete *Borrelia hermsii*. *J. Bacteriol.* 2004,
- 1056 **186**(9):2612–2618.
- 1057 43. Merien F, Truccolo J, Baranton G, Perolat P: Identification of a 36-kDa
- 1058 fibronectin-binding protein expressed by a virulent variant of *Leptospira*
- 1059 *interrogans* serovar icterohaemorrhagiae. *FEMS Microbiol. Lett.* 2000, **185**
- 1060 (1):17–22.
- 1061 44. Hoke DE, Egan S, Cullen PA, Adler B: LipL32 is an extracellular matrix-
- 1062 interacting protein of *Leptospira* spp. and *Pseudomonas tunicata*.
- 1063 *Infect. Immun.* 2008, **76**(5):2063–2069.
- 1064 45. Oliveira TR, Longhi MT, Goncalves AP, de Moraes ZM, Vasconcellos SA,
- 1065 Nascimento AL: LipL53, a temperature regulated protein from *Leptospira*
- 1066 *interrogans* that binds to extracellular matrix molecules. *Microb Infect*
- 1067 *Institut Pasteur* 2010, **12**(3):207–217.
- 1068 46. Pinne M, Choy HA, Haake DA: The OmpL37 surface-exposed protein is
- 1069 expressed by pathogenic *Leptospira* during infection and binds skin and
- 1070 vascular elastin. *PLoS neglected tropical diseases* 2010, **4**(9):e815.
- 1071 47. Félix SR, Hartwig DD, Argondizzo AP, Silva EF, Seixas FK, Seixas Neto AC,
- 1072 Medeiros MA, Lilienbaum W, Dellagostin OA: Subunit approach to
- 1073 evaluation of the immune protective potential of leptospiral antigens.
- 1074 *Clin Vaccine Immunol* 2011, **18**(12):2026–30.
- 1075 48. Fenno JC, Tamura M, Hannam PM, Wong GW, Chan RA, McBride BC:
- 1076 Identification of a *Treponema denticola* OppA homologue that binds
- 1077 host proteins present in the subgingival environment. *Infect. Immun.*
- 1078 2000, **68**(4):1884–1892.
- 1079 49. LeBouder F, Morello E, Rimmelzwaan GF, Bosse F, Pechoux C, Delmas B,
- 1080 Riteau B: Annexin II incorporated into influenza virus particles supports
- 1081

- 1082 virus replication by converting plasminogen into plasmin. *J. Virol.* 2008,
1083 82(14):6820–6828.
- 1084 50. Rojas M, Labrador I, Concepcion JL, Aldana E, Avilan L: **Characteristics of**
1085 **plasminogen binding to Trypanosoma cruzi epimastigotes.** *Acta Trop.*
1086 2008, **107**(1):54–58.
- 1087 51. Klemperer MS, Noring R, Epstein MP, McCloud B, Rogers RA: **Binding of**
1088 **human urokinase type plasminogen activator and plasminogen to**
1089 **Borrelia species.** *J. Infect. Dis.* 1996, **174**(1):97–104.
- 1090 52. Ponting CP, Marshall JM, Cederholm-Williams SA: **Plasminogen: a structural**
1091 **review.** *Blood Coagul. Fibrinolysis* 1992, **3**(5):605–614.
- 1092 53. Angles-Cano E: **Overview on fibrinolysis: plasminogen activation**
1093 **pathways on fibrin and cell surfaces.** *Chem. Phys. Lipids* 1994, **67**–68:353–
1094 362.
- 1095 54. Angles-Cano E, de la Pena Diaz A, Loyau S: **Inhibition of fibrinolysis by**
1096 **lipoprotein(a).** *Annals of the New York Academy of Sciences* 2001, **936**:261–
1097 75.
- 1098 55. Nakai K, Kanehisa M: **Expert system for predicting protein localization sites**
1099 **in gram-negative bacteria.** *Proteins* 1991, **11**(2):95–110.
- 1100 56. Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, Lassmann T,
1101 Moxon S, Marshall M, Khanna A, Durbin R, et al: **Pfam: clans, web tools and**
1102 **services.** *Nucleic acids research* 2006, **34**(Database issue):D247–D251.
- 1103 57. Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, Krogh A:
1104 **Prediction of lipoprotein signal peptides in Gram-negative bacteria.**
1105 *Protein Sci.* 2003, **12**(8):1652–1662.
- 1106 58. Setubal JC, Reis M, Matsunaga J, Haake DA: **Lipoprotein computational**
1107 **prediction in spirochaetal genomes.** *Microbiology (Reading, England)* 2006,
1108 **152**(Pt 1):113–121.
- 1109 59. Bhandari P, Gowrishankar J: **An Escherichia coli host strain useful for**
1110 **efficient overproduction of cloned gene products with NaCl as the**
1111 **inducer.** *J. Bacteriol.* 1997, **179**(13):4403–4406.
- 1112 60. Oliveira TR, Longhi MT, de Moraes ZM, Romero EC, Blanco RM, Kirchgatter K,
1113 Vasconcellos SA, Nascimento AL: **Evaluation of leptospiral recombinant**
1114 **antigens MPL17 and MPL21 for serological diagnosis of leptospirosis by**
1115 **enzyme-linked immunosorbent assays.** *Clin. Vaccine Immunol.* 2008, **15**
1116 (11):1715–1722.
- 1117 61. Pathirana RD, O'Brien-Simpson NM, Veith PD, Riley PF, Reynolds EC:
1118 **Characterization of proteinase-adhesin complexes of Porphyromonas**
1119 **gingivalis.** *Microbiology (Reading, England)* 2006, **152**(Pt 8):2381–2394.
- 1120 62. Lin YP, Lee DW, McDonough SP, Nicholson LK, Sharma Y, Chang YF:
1121 **Repeated domains of leptospira immunoglobulin-like proteins interact**
1122 **with elastin and tropoelastin.** *J. Biol. Chem.* 2009, **284**(29):19380–19391.

1123 doi:10.1186/1471-2180-12-50

1124 **Cite this article as:** Domingos et al.: "Features of two proteins of
1125 *Leptospira interrogans* with potential role in host-pathogen interactions".
1126 *BMC Microbiology* 2012 **12**:50.

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Plasminogen binding proteins and plasmin generation on the surface of *Leptospira* spp. - the contribution to the bacteria-host interactions

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Leptospirosis is considered a neglected infectious disease of human and veterinary concern. Although extensive investigations on host-pathogen interactions have been pursued by several research groups, mechanisms of infection, invasion and persistence of pathogenic *Leptospira* spp. remain to be elucidated. We have reported the ability of leptospires to bind human plasminogen (PLG) and that after treatment with activators, generated enzymatically active plasmin (PLA) on the bacteria surface. Several species of *Leptospira*, including the saprophytic strain, were competent of PLG binding/PLA generation, but the virulent strains were far more effective, and the only ones capable to acquire PLG from normal human plasma. PLA-coated *Leptospira* can degrade immobilized ECM molecules, an activity with implications in host tissue penetration. Moreover, we have identified and characterized several proteins that may act as PLG-binding receptors, each of them competent to generate active plasmin. More recently, we have shown that the PLA activity associated to the outer surface of *Leptospira* could hamper the host immune attack by conferring the bacteria some benefit during infection. The PLA-coated leptospires obstruct complement C3b and IgG depositions on the bacterial surface, most probably through degradation. The decrease of leptospiral opsonization might be an important aspect of the immune evasion strategy. We believe that the presence of PLA on the leptospiral surface may (i) facilitate host tissue penetration, (ii) help the bacteria to evade the immune system and, as a consequence, (iii) permit *Leptospira* to reach secondary sites of infection.

The spirochete *Leptospira interrogans* is a highly invasive pathogen and the causal agent of leptospirosis, one of the most widespread zoonosis of human and veterinary concern. The disease represents a great economic burden because it has an effect on the public health system and the livestock (1-4). The disease occurs mainly in urban regions lacking adequate sanitary conditions, associated with activities that involve direct contact with contaminated water, soil or animals (3, 5, 6). The leptospires chronically infect mammal species, which harbor the bacteria in their renal tubules, shedding them through the urine into the environment, thus constituting a source of re-infection to other animals. Humans are accidental and terminal hosts in the transmission process of leptospirosis (1, 7). The leptospires enter the body via abrasions on skin or actively through mucosa, spreading to any tissue, but specially colonizing kidneys and liver (2).

The understanding of molecular aspects of the pathogenesis, the virulence and invasion processes by which the leptospires infect the hosts and initiate tissue colonization remains to be elucidated, despite the availability of genomic sequencing of five strains of *Leptospira* that have identified more than 200 putative membrane proteins that might be involved in pathogenesis (8-12). Yet, only few virulence factors related to the pathogenesis of the disease have been reported (13-16).

The ability of the leptospires to adhere to extracellular matrix (ECM) proteins is assumed to be crucial during the first steps of pathogenesis (17). Indeed, this ability has been recently and firstly described by our group (18) and a number of adhesins, ECM-binding proteins, have been identified (15, 18-26). After leptospiral adherence, the next fundamental step must be to overcome the barriers imposed by epithelial tissues and extracellular matrixes, to reach the bloodstream and secondary sites of infection within the hosts. Proteolytic activity has been demonstrated to be important during the penetration of several pathogenic micro-organisms (27). Because the presence of ECM degrading enzymes is limited to few bacterial pathogens, one of the strategies employed to surmount this deficiency is the interaction with protease-mediated systems of the hosts (27).

Plasmin is a broad-spectrum serine protease component of the fibrinolytic system, which has PLG as main component. PLG is a circulating single-chain zymogen that is converted to plasmin by cleavage of the peptide bond between Arg-560 and Val-561, mediated by PLG activators-like uPA (urokinase-type PLG activator) and tPA (tissue-type PLG activator). Once activated to plasmin the bacteria are endowed with membrane-associated proteolytic activity, a characteristic that have been demonstrated to contribute to the degradation of ECM components, tissue

penetration and invasion. It has been reported that several pathogens, including the spirochetes *Borrelia* spp. and *Treponema* spp., bind PLG on the surface and converts it to plasmin by host activators (28-30). The binding of PLG and plasmin generation promotes ECM molecules degradation and it is essential for dissemination of the bacteria through the host tissues, thus suggesting its importance in invasiveness process (27, 31-35).

In this review, we highlight and compile the recent studies performed by our group describing and characterizing the leptospiral binding to PLG (36), the identification of possible PLG receptors (37-40) and the aspect of the leptospiral immune escape strategy associated to plasmin proteolytic activity (41). The disclosure of this proteolytic system on the surface of *Leptospira* spp. and the implications associated to bacterial infectivity are discussed.

MATERIALS AND METHODS

Bacteria isolates and culture conditions. Virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 is routinely cultured at Faculdade de Medicina Veterinária da Universidade de São Paulo by iterative passages in Golden Syrian hamsters for maintenance of virulence (1). The organs-derived leptospires were cultured at 28 °C in semi-solid modified Elinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with 10 % rabbit sera. Non-virulent culture-attenuated *L. interrogans* serovar Copenhageni strain M20 are equally routinely cultured by maintenance in liquid modified EMJH medium supplemented with 10 % rabbit sera (42). For the experiments of PLG binding, to exclude the PLG interference from the rabbit serum supplementing the culture medium, serum-free leptospires were obtained by three passages in liquid modified EMJH medium supplemented with 10% *Leptospira* enrichment EMJH (BD, Difco), cultured at 28 °C.

Labeling of leptospires with plasmin. The leptospires were treated with PLG and uPA based in the protocol described by Coleman et al. , with modifications. A total of 7.0×10^9 leptospires were centrifuged at $6,000 \times g$ for 10 min at 25 °C, resuspended in 1.4 mL of EMJH culture medium supplemented with 10% *Leptospira* enrichment EMJH, divided into 7 aliquots of 0.2 mL each (1.0×10^9 leptospires) in 2 mL microcentrifuge tubes and re-centrifuged. The 7 tubes containing the leptospires received different treatments: (a) 30 % plasma and 3 U uPA (urokinase, Sigma) in 100 µL low-salt PBS (IsPBS - with 50 mM NaCl), (b) 5 µg PLG (native PLG purified from human plasma was from Calbiochem) and 3U uPA in 100 µL IsPBS, (c) 2 µg PLG and 3 U uPA in 100 µL IsPBS, (d) 0.5 µg

PLG and 3 U uPA in 100 µL IsPBS, (e) 5 µg PLG in 100 µL IsPBS, (f) 3 U uPA in 100 µL IsPBS and (f) 100 µL IsPBS. All the preparations were incubated for 1 h at 37 °C, under gentle shaking, prior to the addition of the uPA, and followed by 1 h incubation at 37 °C. Leptospires were then centrifuged and washed three times with 0.7 mL IsPBS.

Measurement of enzymatic activity of plasmin-coated leptospires. The treated leptospires (1.0×10^9 per sample) were resuspended in 300 µL IsPBS and divided into 3 aliquots. Each aliquot received 100 µL of 0.5 mg/mL of the chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) in IsPBS, to a final substrate concentration of 0.25 mg/mL. The suspensions were incubated for 1.5 h at 37 °C, under gentle shaking, and then centrifuged at $6,000 \times g$ for 10 min at 25 °C. The supernatants (150 µL) were transferred to 96-well microplates and the cleavage of the specific plasmin substrate was quantified with a microplate reader set at a wavelength of 405 nm.

6-Aminocaproic acid (ACA) binding-inhibition assay. Low-passage, virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (1.0×10^9 leptospires/sample) were treated with PLG and uPA, as described above, except for the addition of increase concentrations of ACA (Sigma) ranging from 0 – 1,000 mM, added together with the PLG. PLG quantity was set at 5 µg, and uPA at 3 U. Aliquots treated with only PLG, uPA or ACA, and just IsPBS-treated, were used as controls. The enzymatic plasmin activity was measured as described before.

Liquid-phase immunofluorescence assay (L-IFA). Live bacteria suspensions (2.5×10^9) were harvested at $12,800 \times g$ for 15 min, washed twice with IsPBS, resuspended in 200 µL IsPBS containing 8 µg of human PLG and incubated for 45 min at 37 °C. After the incubation, 6 µg/mL of propidium iodide (Sigma) were added to stain the nuclei, and the suspensions were incubated for more 45 min at 37 °C. After this time, the leptospires were gently washed three times with IsPBS and incubated for 45 min at 37 °C with mouse-produced antiserum against human PLG (Sigma) at a 1:50 dilution. The leptospires were washed three times and incubated with goat anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC; Sigma) at a dilution of 1:50 for 45 min at 37 °C. After this incubation, the leptospires were washed twice and resuspended in IsPBS-antifading solution (ProLong Gold; Molecular Probes). The immunofluorescence-labeled leptospires were examined by use of a confocal LSM 510 META immunofluorescence microscope (Zeiss, Germany). As control for cell integrity, we used

antibodies against recombinant leptospira proteins LipL32 or GroEL, produced in mice, following all the mentioned procedures for PLG.

SDS-PAGE and affinity blotting. Total leptospiral protein extracts for SDS-PAGE were prepared from 10 mL of $\sim 10^9$ bacteria in EMJH serum-free cultures. The cells were harvested by centrifugation, washed three times with 5 mM MgCl_2 in IsPBS and resuspended in 100 μL PBS. The proteins were loaded into 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare) in semi-dry equipment. The membranes were blocked for 2 h at 37 °C with 5 % BSA, washed three times (10 min for each wash) with PBS-0.5 % Tween-20 solution (PBS-T) and incubated overnight with 3 $\mu\text{g/mL}$ PLG or 3 $\mu\text{g/mL}$ PLG + 100 mM ACA at 4 °C, followed by a 2 h incubation at room temperature. Then, the membranes were washed three times and incubated with mouse anti-human PLG (1:750, Sigma) for 3 h at room temperature, following by more three washings and 1 h incubation at room temperature with anti-mouse IgG-peroxidase conjugated (1:5,000, Sigma). The membranes were washed and the protein's reactivity was revealed by ECL reagent (GE Healthcare) with subsequent exposition to X-Ray films (Kodak).

Assay for the degradation of immobilized ECM macromolecules. 96-well plates were coated overnight at 37 °C with 0.5 $\mu\text{g/well}$ of cellular fibronectin (Sigma) or laminin (Sigma), washed four times with 200 μL per washing with PBS-T, and blocked with 2 % BSA in PBS-T for 2 h at 37 °C, followed by two washings. The spirochetes (1.0×10^8 leptospores/sample) were treated as described above with 10 μg PLG and 3 U uPA or IsPBS (untreated). Bacteria were washed, resuspended in 100 μL IsPBS and transferred to the plate previously coated wells. The plates were centrifuged at 180 X g for 15 min to ensure the contact of the leptospores with the immobilized ECM components. The plates were incubated at 37 °C for 20 h and washed 5 times to remove the bacteria. The degradation of the ECM components were detected by reduction in absorbance followed by incubation with anti-fibronectin or anti-laminin IgG antibodies (1:5,000 dilution in 100 μL PBS-T for 45 min at 37 °C, Sigma), anti-IgG peroxidase conjugated antibodies (1:5,000 dilution in 100 μL PBS-T for 45 min at 37°C, Sigma) and 100 $\mu\text{L/well}$ of 1 mg/mL *o*-phenylenediamine-OPD- in citrate phosphate buffer, pH 5.0 plus 1 $\mu\text{L/mL}$ H_2O_2 . The reaction was stopped with 50 $\mu\text{L/well}$ 4N H_2SO_4 , and the absorbance was measured at 492 nm. Percentage degradation was calculated by the formula $(A-B)/A(100)$, where A = the mean PBS samples absorbance (positive control group) and B = the mean experimental group absorbance (untreated or plasmin).

Bioinformatics characterization of the proteins. Predicted coding sequences (CDSs) were analyzed as their cellular localization predictions by PSORT program, <http://psort.nibb.ac.jp> (44, 45). The SMART, <http://smart.embl-heidelberg.de/> (46, 47), PFAM, <http://www.sanger.ac.uk/Software/Pfam/> (48), and LipoP, <http://www.cbs.dtu.dk/services/LipoP/> (49) web servers were used to search for predicted functional and structural domains within the amino acid sequences of the CDSs.

Cloning, expression and purification of recombinant proteins. Amplification of the CDSs was performed by PCR from *L. interrogans* serovar Copenhageni genomic DNA using complementary primer pairs. The gene sequences were amplified without the signal peptide tag, predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). The final constructs were verified by DNA sequencing on an ABI Prism 3730_L sequencer (Seq- Wright, Houston, TX) with appropriate vector-specific T7 (F: TAATACGACTCACTATAGGG) and pAE (R: CAGCAGCCAACTCAGTTCCT) primers. Cloning, expression and purification of the recombinant proteins have been previously described as summarized in Table 1.

Plasminogen binding to recombinant proteins. The binding of the recombinant proteins to PLG was evaluated by a modified ELISA, based in the protocol described by Brissette et al. (50), as follows: 96-well plates (Costar High Binding, Corning) were coated overnight in PBS at 4 °C with 100 μL of 10 $\mu\text{g/mL}$ of the recombinant proteins or bovine serum albumin (BSA, Sigma) as negative control. Plates were washed once with PBS-T and blocked for 2 h at 37 °C with PBS with 10 % (wt/vol) non-fat dry milk. The blocking solution was discarded and 100 μL of 10 $\mu\text{g/mL}$ human PLG (Calbiochem) in PBS was incubated for 2 h at 37 °C. Wells were washed four times with PBS-T and incubated for 1 h at 37 °C with mouse anti-human PLG (1:4,000 in PBS, Sigma). Plates were washed again and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG, Sigma), diluted 1:5,000 in PBS. After three washings, 100 $\mu\text{L/well}$ of 1 mg/mL OPD plus 1 $\mu\text{L/mL}$ H_2O_2 in citrate phosphate buffer (pH 5.0) were added. The reactions were carried out for 5 min and stopped by the addition of 50 $\mu\text{L/well}$ of H_2SO_4 (4 N). Readings were taken at 492 nm.

Enzymatic activity assay of plasmin bound to recombinant proteins. ELISA plates were coated overnight with 10 $\mu\text{g/mL}$ recombinant proteins or BSA in PBS at 4 °C. Plates were then washed once with PBS-T and blocked with PBS with 10 % (wt/vol) non-fat dry milk for 2 h at 37 °C. The blocking solution was

discarded and 100 μ L/well of 10 μ g/mL human PLG (Calbiochem) was added, followed by incubation for 2 h at 37°C. Wells were washed three times with PBS-T, and then 4 ng/well of human uPA (Sigma) were added. Subsequently, 100 μ L/well of the plasmin-specific substrate *D*-valyl-leucyl-lysine 4-*p*-nitroanilide dihydrochloride (Sigma) was added at a final concentration of 0.4 mM in PBS. Plates were incubated overnight at 37 °C and substrate was measured by taking readings at 405 nm.

Dose-response curves and dissociation constant of the proteins-PLG interactions. ELISA plates were coated overnight in PBS at 4°C with 100 μ L of 10 μ g/mL PLG. Plates were then blocked and increasing concentrations of the purified recombinant proteins (0 - 1 $\square$ M) were added (100 μ L/well in PBS). The assessment of bound proteins was performed by incubation for 1 h at 37 °C with the antiserum raised against each protein at appropriate dilutions, followed by horseradish peroxidase-conjugated anti-mouse IgG (1: 10,000 in PBS, Sigma). The binding was evaluated by the peroxidase substrate OPD and readings were taken at 492 nm. The ELISA data were used to calculate the dissociation constant (K_D) according to the method described by Pathirana et al. (51) and Lin et al. (52), based on the equation: $A = A_{max} [\text{protein}] / (K_D + [\text{protein}])$, where A is the absorbance at a given protein concentration, A_{max} is the maximum absorbance for the ELISA plate reader (equilibrium), [protein] is the protein concentration and K_D is the dissociation equilibrium constant for a given absorbance at a given protein concentration (ELISA data point).

Two-dimensional gel electrophoresis (2-DE gels). Virulent low passage leptospires culture was harvested by centrifugation at 12,800 X g at 4 °C for 10 min. Pellet was washed (X 5) by resuspension in PBS containing 5 mM MgCl₂. The pellet was resuspended in DeStreak Rehydration Solution (GE Healthcare, USA) and lysed by vigorous vortexing. The cellular debris was separated by centrifugation at 20,800 X g for 10 min, and the supernatant was collected. Total protein content was determined according to the Bradford method (Pierce Biotechnology, USA). Samples containing 200 μ g of protein were adjusted to 125 μ L with DeStreak Rehydration Solution (GE Healthcare, USA), along with 0.8 % (v/v) IPG buffer, with a pH range of 3–10 (GE Healthcare).

First-dimension isoelectric focusing was performed using the IPGphor-System (GE Healthcare, USA), and the second dimension was conducted on the Ettan DALTsix system (GE Healthcare). The IPG gel strips (7 cm) with a linear separation of immobilized pH ranging from 3 to 10 were rehydrated directly with the solubilized samples. The focusing protocol was 30 V for

180 Vh, 150 V for 300 Vh, 350 V for 350 Vh, 500 V for 500 Vh, 1000 V for 1,000 Vh, 3,000 V for 3,000 Vh, and 5,000 V for 40,000 Vh, with a 50 μ A/strip maximum-setting at 20 °C. The strips were equilibrated twice (reduced and alkylated) for 15 min in 15 mL equilibration solution (0.05 M Tris-HCl, pH 8.8, 6.0 M urea, 30 % [v/v] glycerol, and 2 % [w/v] SDS), first with the addition of 1 % DTT, and finally with 2.5 % iodoacetamide. After equilibration, the strips were attached to the 12 % SDS-PAGE. The gels were stained by Coomassie Blue R350 (PhastGel Blue R – GE Healthcare). The gels were analyzed with Image Master-2D Platinum version 6.0 software (GE Healthcare, USA).

Mass spectrometry and protein identification. The samples were analyzed by MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) mass spectrometry, using α -cyano-4-hydroxycinnamic acid as the matrix on an Ettan MALDI-TOF/Pro instrument (Amersham Biosciences, USA). The spots preparation, mass spectrometry and database search for protein identification were performed according to the protocol already described in Vieira et al (53). Prediction for protein localization was performed by LipoP program (49).

Human sera and microscopic agglutination test. Confirmed-leptospirosis serum samples were obtained from Instituto Adolfo Lutz collection, Sao Paulo, Brazil, as previously described (41). In brief, a laboratory-confirmed case of leptospirosis was defined by the demonstration of a four-fold microagglutination titer rise between paired serum samples. The probable predominant serovar was considered the titer that was the highest sample dilution with 50% of agglutination. MAT (microscopic agglutination test) was considered negative when the titer was below 100. In addition to the MAT-negative and MAT-positive paired samples of the same individuals with laboratory and clinic leptospirosis confirmations, we also employed sera from normal healthy donors (normal human sera - NHS), without a known history of leptospirosis, with confirmed negative MAT.

Human IgG and C3b deposition on leptospires. *L. interrogans* serovar Pomona strain LPF (2.5×10^7 cells/well) were diluted in 50 μ L IsPBS, coated onto microplates and allowed to stand at 37 °C for 1 h , followed by overnight at 4°C. The plates were washed twice with 200 μ L IsPBS and blocked with 5 % non-fat dry milk and 2.5 % BSA for 2 h at 37 °C. After addition of 50 μ L of pooled sera/well (NHS, MAT-negative or MAT-positive) as a source of IgG, the plates were allowed to incubate for 30 min on ice, and then washed three times. The coated leptospires were treated with 50

μL/well of 5 μg/mL uPA (urokinase, Sigma) and 40 μg/mL PLG (Calbiochem) for 2 h at 37°C, or only with uPA, only with PLG, or no additions, as negative controls. After three washings, the plates were incubated with anti-human IgG Fc region specific antibodies (Calbiochem) at a dilution of 1 : 5,000 for 1 h at 37°C, and then with secondary antibodies conjugated with peroxidase at a dilution of 1 : 10,000 for 1 h at 37°C. The IgG depositions on leptospires were evaluated by the peroxidase substrate OPD and readings were taken at 492 nm. When dilutions of NHS were used, they were performed with IsPBS.

The protocol of C3b deposition on leptospires was similar to the IgG deposition described before, with exception of the incubation with NHS, placed at 37°C for 50 min, and use of anti-human C3b primary antibodies (1 : 5,000) (Calbiochem), followed by secondary antibodies conjugated with peroxidase (1 : 5,000).

Human C3b degradation by leptospires. *L. interrogans* serovar Pomona strain LPF (10^8 leptospires/sample) were treated in 200 μL IsPBS with the addition of 10 μg PLG and 3U uPA (plasmin), 10 μg PLG or no additions (untreated). The cells were incubated for 1 h at 37°C with the PLG, and for one more hour after the addition of uPA. The cells were washed three times with IsPBS, and then resuspended in 100 μL IsPBS containing 15 μg/mL human purified C3b (Calbiochem), being incubated for 20 h at 37°C. The leptospires were removed by centrifugation, 20 μL of the supernatants were separated by 10 % SDS-PAGE and then transferred to nitrocellulose membranes in semi-dry equipment. The membranes were blocked by incubating overnight at 4°C with 5% non-fat dry milk and 1% BSA. The C3b detection was made by incubations with anti-human C3b antibodies and secondary antibodies conjugated with peroxidase, followed by ECL (GE Healthcare) development.

Serum susceptibility testing for treated leptospires. Complement-mediated killing of leptospires was evaluated after incubation with non-immune NHS, as described by Meri et al. (54), with some modifications. Approximately 5×10^8 *L. interrogans* serovar Pomona strain LPF per sample were treated with 40 μg PLG and 4U uPA in 200 μL IsPBS for 3 h at 37 °C. As a control, one aliquot of *L. interrogans* serovar Pomona strain LPF was incubated only with PBS. Then, the samples were divided into aliquots containing 1.0×10^8 cells, which were added by 80 μL NHS and 80 μL EMJH medium supplemented with 10% *Leptospira* enrichment. The bacteria suspensions were incubated at 37 °C for one hour, followed by a incubation of 5 min on ice for stopping complement activation. After, 20 μL of each sample (four replicates) were transferred to

microplates filled with 180 μL of EMJH medium/well. The plates were sealed with a sterile adhesive film and incubated at 30 °C for 4 days. Bacterial growth was determined by counting leptospires in a Petroff-Hausser chamber under dark-field microscopy.

Statistics. For the data that were tested for statistical significance, the Student's two-tailed test was applied, considering the minimum of $p < 0.05$.

RESULTS AND DISCUSSION

Binding of human plasminogen by *L. interrogans* cells. Live-immunofluorescence microscopy (L-IFA) was employed in order to evaluate the ability of *L. interrogans* cells to bind human PLG. L-IFA revealed that leptospires bind PLG in their outer surface along the entire cell, as seen when the bacteria were probed with antibodies anti-PLG (Fig. 1). PLG bound to leptospires does not seem to damage the bacterial membrane because no fluorescence of GroEL, a cytoplasmatic heat-shock protein (55), was achieved when serum anti-GroEL was employed (Fig. 1). The leptospiral recombinant proteins LipL32, an abundant outer membrane protein of *Leptospira* (56), used as positive control, showed an intense surface fluorescence. We also observed that the spirochete regular movement was conserved, suggesting that the flagella remained undamaged. Taken together, these data suggest that the PLG-binding does not interfere with the membrane structure or dynamics (36).

Activation and enzymatic activity of plasminogen-bound *Leptospira*. The binding of PLG to leptospiral surface was further analyzed by plasmin enzymatic activity. Leptospires were incubated with purified PLG, followed by plasmin activation after addition of uPA, conferring the bacteria a surface-associated plasmin activity (Fig. 2), as measured by the degradation of plasmin's chromogenic specific substrate (36).

The labeling with PLG alone did not trigger proteolytic activity suggesting that *L. interrogans* serovar Copenhageni is dependent on the host PLG activation system, probably not possessing an endogenous mechanism, as already reported for other pathogens (57-64). In contrast, the ability of PLG activation is shown for a number of pathogenic microorganisms (57-64).

Both the virulent and non-virulent strains of *L. interrogans* serovar Copenhageni tested were capable of capturing PLG, although the virulent strain seems to be more efficient in the PLG-binding than the non-virulent bacteria (~2.5 fold) (Fig. 2) (36). PLG is present in plasma at a concentration of approximately 20.8 ± 1.9 mg/100 mL (65). Our data shows that virulent *L. interrogans*, but not the attenuated strain, have the

ability to sequester PLG from human plasma in the conditions assayed. At any rate, once in the host, the infectious leptospires can acquire PLG when reach the blood circulation.

Inhibition by 6-aminocaproic acid (ACA) of proteolytic activity acquired by *L. interrogans*. It is well known that the lysine-binding sites of PLG kringle domains frequently mediate interactions with lysine residues of the cellular receptors (27). ACA, a lysine analogue, decreased the proteolytic activity of virulent *L. interrogans* serovar Copenhageni incubated with PLG, in a dose-dependent manner (Fig. 3). The total inhibition (100 %) of plasmin activity achieved with the higher ACA concentrations employed shows that the PLG interaction with these spirochetes occurs through the lysine-binding sites of the kringle domains (36).

Analysis of plasminogen-binding *L. interrogans* proteins on cell lysates. Assessment of leptospiral proteins binding to PLG was first visualized by affinity blotting using total whole-cell lysates (Fig. 4A). Our results are indicative that multiple leptospiral proteins are capable of binding PLG, although these affinity blotting experiments do not simulate the protein-PLG interaction *in vivo*. As expected, the data also demonstrate that there are similarities between the PLG-binding proteins of the virulent and non-virulent strains, but also revealed the presence of some reactive bands on the low-passage virulent strain that are absent in the non-virulent one, indicating that virulent strains have more PLG binding proteins. Thus, based on the data from enzymatic activity and affinity blotting presented here we cannot discriminate whether the increased bound activity of the virulent strain is due to only differential expression of a different set of membrane receptors, an increased expression of the same receptors, or both. Comparable to the results obtained with the intact leptospires, the affinity blotting was inhibited by the presence of the lysine analog, ACA, supporting the central role of lysine residues of the proteins in the PLG interactions (Fig. 4B) (36).

Plasmin-coated *Leptospira* degrades immobilized ECM molecules. Plasmin is a broad-spectrum serine protease that is capable to degrade directly laminin and fibronectin. To evaluate whether the plasmin associated to the leptospires surface can directly break these ECM components, purified soluble human fibronectin and laminin were adhered into 96-well plates, and the reaction was evaluated by exposing the ECM proteins with low-passage virulent *L. interrogans*, pre-treated with PLG/uPA. As shown in Fig. 5, the plasmin coated leptospires were able to significantly cleave down the immobilized ECM macromolecules, when compared to the ones exposed to untreated bacteria (36).

Given that leptospires are highly invasive pathogens that are thought to penetrate the skin or break in the skin to initiate infection, the ability of digesting ECM macromolecules via plasmin activity might be an important step for leptospiral pathogenesis. To date, this is the first proteolytic activity detected in *Leptospira* that can promote ECM degradation and it is a plausible mechanism that can contribute to leptospiral invasiveness (1, 66).

Recombinant leptospiral proteins bind human plasminogen. In order to investigate PLG binding proteins in *Leptospira* we benefitted from the availability of several recombinant proteins of *L. interrogans* available in our laboratory. We evaluated the ability of these proteins to interact with PLG *in vitro*. Table 1 summarizes the features of these proteins and gene conservation within the sequenced genomes (8-12). The binding of these proteins to PLG was quantified by ELISA and the results are shown in Fig. 6. Proteins LipL32, Lp29, Lp49, LipL40, MLP36, rLIC10494, rLIC12730, rLIC12238, Lsa66, Lp30, Lsa20 and Lsa33 presented significant binding to PLG while proteins, Lsa63, Lsa27, rLIC10509, MPL21, MPL17, rLIC12922, rLIC11030, Lsa25, BSA and gelatin did not show significant binding activity (Fig. 6) (37-40).

Activation of plasminogen-bound proteins. To evaluate whether purified PLG-binding proteins were independently capable to generate active plasmin in the presence of uPA PLG activator, we measured the degradation of plasmin specific chromogenic substrate in a modified ELISA. The PLG captured by the proteins could be converted into plasmin, as demonstrated indirectly by specific proteolytic activity (Fig. 7). The negative control BSA, that showed no PLG binding, produced no significant proteolytic activity, as well as the controls lacking PLG, uPA or the chromogenic substrate (37-40).

Characterization of the binding of recombinant proteins to PLG. The binding between each recombinant protein and PLG was assessed on a quantitative basis as illustrated in Fig. 8A, B and C. Dose-dependent and saturable binding was observed when increasing concentrations (0 to 1,000 nM) of the recombinant proteins MPL36, LipL40, LipL32, rLIC10494, rLIC12238, Lsa66, Lp30, Lsa33 and Lsa20 was allowed to individually adhere to a fixed amount of PLG (1 µg), indicating the specificity of the binding. For the proteins Lp29, Lp49, rLIC12730, Lsa25 and rLIC11030, the saturation level was not reached, even at the highest concentration tested (1,000 nM). Based on the ELISA data, the calculated dissociation equilibrium constants (K_D) for the recombinant proteins with PLG

are depicted in Fig. 8D; for the ones that reached equilibrium, the highest and the lowest K_D values were for Lsa20 (509.13 ± 77.47 nM) and MPL36 (3.52 ± 3.95 nM), respectively (37-40).

Proteomic approach to leptospiral PLG binding protein identification. In order to expand our knowledge on PLG binding proteins, we decided to employ two-dimensional gel electrophoresis followed by affinity immunoblotting, as an alternative strategy to identify novel leptospiral PLG-binding proteins. Total protein extracts of low passage *L. interrogans* were separated by isoelectric point and molecular mass (Fig. 9A), transferred to membranes, and incubated with PLG and anti-PLG antibodies for detection of reactive spots (Fig. 9B). The reactive spots were excised and prepared for MALDI-TOF mass spectrometry and protein identification. Under the conditions assayed, we could identify seven proteins capable of binding PLG, as depicted in Table 2. Two were annotated as membrane and five as cytoplasmic proteins (Table 2). Along with the membrane proteins, one is hypothetical and the other is a putative flagellar filament sheath protein (FlaA-1); the cytoplasmic proteins are citrate lyase, Tuf (elongation factor), acetyl-CoA acetyltransferase, glutamine synthetase and the heat shock protein DnaK. While the role of membrane proteins on the PLG sequestering within the host is expected, the meaning of cytoplasmic proteins remains to be investigated. Intriguingly, none of the PLG-binding recombinant proteins was detected in these experiments. One possible explanation is that the identified proteins, except LIC12816, are expressed in high amounts per cell, while the proteins assayed in our laboratory were below the detection limit, as reported by quantitative proteomics (67). However, LipL32 that was shown to have the highest expression level per leptospiral cell, was not detected in our proteomics studies. Although the reason for this is unknown, we might speculate that because LipL32 is always presented as isoforms in 2D gels (68) (Nally, 2007; Vieira, 2010), it is possible that these isoforms do not bind PLG. Several cytoplasmic proteins of other pathogenic microorganisms have been identified as PLG binding proteins, such as *Bifidobacterium animalis* DnaK (69), *Paracoccidioides brasiliensis*, *Schistosoma bovis* and *Streptococcus pneumoniae* enolases (70) and *Streptococcus pneumoniae* GAPDH (71). For some of these proteins, there is the possibility that at some moment they could be exposed in the bacterial outer membrane. Indeed, it has been recently reported that *B. burgdorferi* enolase, which is both a cytoplasmic and membrane associated protein, is also a PLG-binding protein (72). It is also possible that the PLG binding to these cytoplasmic proteins are not relevant for the bacteria.

Human IgG and C3b deposition on leptospires.

Plasmin-generation on surface of *L. interrogans* may facilitate the bacteria to degrade opsonizing IgG and C3b molecules, and therefore contribute to immune evasion. To test this hypothesis, microtiter plates coated with virulent *L. interrogans* serovar Pomona were incubated with NHS, as a source of C3b and nonspecific IgG molecules. Bacteria were then treated with PLG and uPA, and the resulting surface-associated opsonins were quantified by specific antibodies. The results show that the incubation of bacteria with PLG and uPA resulted in a decrease in IgG Fc region and C3b depositions at the leptospiral surface (Fig. 10A and 10B, respectively). No effect on IgG or C3b binding to *L. interrogans* was observed with only PLG or uPA (41).

IgG binding from human leptospirosis immune serum to *L. interrogans*.

To evaluate whether PLA activity observed against nonspecific human IgG bound to leptospires could also be functional to immune sera from patients diagnosed with leptospirosis, we performed the same experiment using MAT negative (pre-immune phase) and MAT positive (convalescent immune phase) pools of human sera. As expected, MAT positive sera resulted in significant higher IgG depositions on bacterial surface when compared to NHS and MAT negative sera (Fig. 11). When PLA was generated on leptospires, a noticeably decrease on the amount of IgG deposition from both negative and positive MAT sera was detected (41).

Opsonization obstruction by antibodies or complement system appears to be an interesting approach for the bacteria to evade the first line of human immune defense upon infection. In fact, the expression of proteins on the bacterial surface that sequester host complement regulators FH, FHL-1 or C4BP and therefore the inhibition of the complement cascade by the alternative or classical pathways has been employed by several pathogens (73-76). Moreover, the proteolytic cleavage of antibodies that prevents the activation of complement classical pathway or Fc receptor-mediated phagocytosis, is another well-known strategy employed by pathogenic bacteria (73-76).

Human C3b degradation by leptospires.

Meri and colleagues (54) reported the capability of *Leptospira*-bound factor H (FH) to act as a cofactor for the serum protease factor I in cleaving C3b. Thus, we decided to assess whether the decrease in C3b deposition by *Leptospira*-generated PLA was due to C3b cleavage. *L. interrogans* serovar Pomona were coated with PLA by treatment with PLG and uPA, and then incubated with human purified C3b in fluid-phase. The resulting supernatant together with anti-C3b specific antibodies was analyzed by Western blotting (Fig. 12). The data

revealed the appearance of two lower molecular mass bands of estimated 60 and 20 kDa, concurrently with the decrease in the masses of the alpha (105 kDa) and beta (75 kDa) C3b chains that took place only with PLA-coated leptospires. Untreated or leptospires only treated with PLG showed no gradation. The results unmistakably show that the human C3b is cleaved by leptospires-coated PLA, indicating that the reduction in deposition is most probably due to degradation (41).

Serum susceptibility testing for treated leptospires. It has been described that *L. interrogans* serovar Pomona is partially susceptible to complement killing upon incubation with normal human sera (54). To evaluate whether the PLG/PLA binding to leptospires confers protection on exposure to NHS, leptospires were treated with PLG+uPA (Pla) or with PBS, as negative control, and incubated for one hour with pooled NHS. The bacteria were harvested and counted in a dark-field microscopy using Petroff-Hausser chamber, at the fourth day of incubation. The number of untreated leptospires was almost four times less when compared to the plasmin-coated leptospires ($0.35 \times 10^8 \pm 0.1 \times 10^8$ versus $1.28 \times 10^8 \pm 0.3 \times 10^8$ leptospires/mL), suggesting that the leptospires endowed with active plasmin are less vulnerable to components present in NHS (41).

CONCLUSIONS

The interaction of the human PLG system has been suggested to be a feature that significantly contributes to the virulence of many bacterial pathogens by facilitating the initial anchoring to endothelium and penetration (27). Our group has been studying the interaction of *Leptospira* with PLG/plasmin generation system and the possible implications for pathogenesis. We demonstrated that leptospires interact with PLG and can acquire plasmin activity associated to the surface with no apparent damage to the surface, what occurs through multiple proteins. Although non-virulent leptospires can interact with PLG, there seems to be a correlation between the efficiency of PLG capturing and virulence, suggesting a role in virulence and infection. Moreover, the generation of enzymatically active PLA on the leptospiral surface decreased C3b and IgG depositions that may constitute a novel mechanism by which leptospires could evade the immune system until reach immunologically safe environments. This latter assertion will also have implication to the bacterial persistence within the host. We believe that the disclosure of this enzymatically system will shed light on the molecular mechanism of leptospiral pathogenesis.

Acknowledgments. We are deeply indebted to Alexsander Seixas de Souza (Departamento de Parasitologia, Instituto Butantan, Sao Paulo, Brazil) for use of the confocal microscope facilities and helpful discussion. This work was supported by FAPESP, CNPq and Fundação Butantan, Brazil; M.L.V.; M.V.A.; R.O.; R.S.M.; R.F.D. have scholarships from FAPESP (Brazil).

REFERENCES

1. Faine S, Adler B, Bolin C, Perolat P. *Leptospira* and *Leptospirosis*. Second ed. Melbourne, Australia: MediSci; 1999. p. 259.
2. Levett PN. *Leptospirosis*. *Clinical microbiology reviews*. 2001;14(2):296-326. Epub 2001/04/09.
3. Vinetz JM. *Leptospirosis*. *Current opinion in infectious diseases*. 2001;14(5):527-38.
4. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, et al. *Leptospirosis: a zoonotic disease of global importance*. *The Lancet infectious diseases*. 2003;3(12):757-71.
5. Ko AI, Galvao Reis M, Ribeiro Dourado CM, Johnson WD, Jr., Riley LW. *Urban epidemic of severe leptospirosis in Brazil*. *Salvador Leptospirosis Study Group*. *Lancet*. 1999;354(9181):820-5. Epub 1999/09/15.
6. Haake DA, Dundoo M, Cader R, Kubak BM, Hartskeerl RA, Sejvar JJ, et al. *Leptospirosis, water sports, and chemoprophylaxis*. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2002;34(9):e40-3. Epub 2002/04/10.
7. Plank R, Dean D. *Overview of the epidemiology, microbiology, and pathogenesis of Leptospira spp. in humans*. *Microbes and infection / Institut Pasteur*. 2000;2(10):1265-76.
8. Ren SX, Fu G, Jiang XG, Zeng R, Miao YG, Xu H, et al. *Unique physiological and pathogenic features of Leptospira interrogans revealed by whole-genome sequencing*. *Nature*. 2003;422(6934):888-93.
9. Nascimento AL, Verjovski-Almeida S, Van Sluys MA, Monteiro-Vitorello CB, Camargo LE, Digiampietri LA, et al. *Genome features of Leptospira interrogans serovar Copenhageni*. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica [et al]*. 2004;37(4):459-77.
10. Nascimento AL, Ko AI, Martins EA, Monteiro-Vitorello CB, Ho PL, Haake DA, et al. *Comparative genomics of two Leptospira interrogans serovars reveals novel insights into physiology and pathogenesis*. *Journal of bacteriology*. 2004;186(7):2164-72.
11. Bulach DM, Zuerner RL, Wilson P, Seemann T, McGrath A, Cullen PA, et al. *Genome reduction in Leptospira borgpetersenii reflects limited transmission potential*. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(39):14560-5.
12. Picardeau M, Bulach DM, Bouchier C, Zuerner RL, Zidane N, Wilson PJ, et al. *Genome sequence of the saprophyte Leptospira biflexa provides insights into the evolution of Leptospira and the pathogenesis of leptospirosis*. *PLoS ONE*. 2008;3(2):e1607.
13. Matsunaga J, Barocchi MA, Croda J, Young TA, Sanchez Y, Siqueira I, et al. *Pathogenic Leptospira species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily*. *Molecular microbiology*. 2003;49(4):929-45.
14. Ristow P, Bourhy P, da Cruz McBride FW, Figueira CP, Huerre M, Ave P, et al. *The OmpA-like protein Loa22 is*

- essential for leptospiral virulence. *PLoS pathogens*. 2007;3(7):e97. Epub 2007/07/17.
15. Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Moraes ZM, Lima DM, et al. Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC microbiology*. 2008;8:70. Epub 2008/05/01.
16. Luo Y, Liu Y, Sun D, Ojcius DM, Zhao J, Lin X, et al. InvA protein is a Nudix hydrolase required for infection by pathogenic *Leptospira* in cell lines and animals. *The Journal of biological chemistry*. 286(42):36852-63.
17. Boyle EC, Finlay BB. Bacterial pathogenesis: exploiting cellular adherence. *Current opinion in cell biology*. 2003;15(5):633-9. Epub 2003/10/02.
18. Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, Vieira ML, et al. A newly identified leptospiral adhesin mediates attachment to laminin. *Infection and immunity*. 2006;74(11):6356-64. Epub 2006/09/07.
19. Longhi MT, Oliveira TR, Romero EC, Goncalves AP, Moraes ZM, Vasconcellos SA, et al. A novel identified protein of *Leptospira interrogans* mediates binding to laminin. *Journal of medical microbiology*. 2009.
20. Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J, Haake DA. Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. *Infection and immunity*. 2007;75(5):2441-50.
21. Stevenson B, Choy HA, Pinne M, Rotondi ML, Miller MC, Demoll E, et al. *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. *PLoS ONE*. 2007;2(11):e1188.
22. Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Moraes ZM, Barbosa AS, et al. In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. *Infection and immunity*. 2008;76(6):2642-50. Epub 2008/04/09.
23. Hoke DE, Egan S, Cullen PA, Adler B. LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudomonas tunicata*. *Infection and immunity*. 2008;76(5):2063-9.
24. Atzingen MV, Gomez RM, Schattner M, Pretre G, Goncalves AP, de Moraes ZM, et al. Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells. *The Journal of infection*. 2009.
25. Carvalho E, Barbosa AS, Gomez RM, Cianciarullo AM, Hauk P, Abreu PA, et al. Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity. *FEBS letters*. 2009;583(8):1381-5. Epub 2009/03/31.
26. Vieira ML, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, Nascimento AL. Lsa63, a newly identified surface protein of *Leptospira interrogans* binds laminin and collagen IV. *The Journal of infection*. 60(1):52-64.
27. Lahteenmaki K, Kuusela P, Korhonen TK. Bacterial plasminogen activators and receptors. *FEMS microbiology reviews*. 2001;25(5):531-52.
28. Coleman JL, Sellati TJ, Testa JE, Kew RR, Furie MB, Benach JL. *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. *Infection and immunity*. 1995;63(7):2478-84.
29. Klempner MS, Noring R, Epstein MP, McCloud B, Hu R, Limentani SA, et al. Binding of human plasminogen and urokinase-type plasminogen activator to the Lyme disease spirochete, *Borrelia burgdorferi*. *The Journal of infectious diseases*. 1995;171(5):1258-65. Epub 1995/05/01.
30. Hu LT, Perides G, Noring R, Klempner MS. Binding of human plasminogen to *Borrelia burgdorferi*. *Infection and immunity*. 1995;63(9):3491-6.
31. Coleman JL, Gebbia JA, Piesman J, Degen JL, Bugge TH, Benach JL. Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice. *Cell*. 1997;89(7):1111-9. Epub 1997/06/27.
32. Coleman JL, Benach JL. Use of the plasminogen activation system by microorganisms. *The Journal of laboratory and clinical medicine*. 1999;134(6):567-76. Epub 1999/12/14.
33. Coleman JL, Benach JL. The generation of enzymatically active plasmin on the surface of spirochetes. *Methods (San Diego, Calif)*. 2000;21(2):133-41.
34. Nordstrand A, Shamaei-Tousi A, Ny A, Bergstrom S. Delayed invasion of the kidney and brain by *Borrelia crocidurae* in plasminogen-deficient mice. *Infection and immunity*. 2001;69(9):5832-9.
35. Haile WB, Coleman JL, Benach JL. Reciprocal upregulation of urokinase plasminogen activator and its inhibitor, PAI-2, by *Borrelia burgdorferi* affects bacterial penetration and host-inflammatory response. *Cellular microbiology*. 2006;8(8):1349-60. Epub 2006/08/03.
36. Vieira ML, Vasconcellos SA, Goncalves AP, de Moraes ZM, Nascimento AL. Plasminogen acquisition and activation at the surface of *leptospira* species lead to fibronectin degradation. *Infection and immunity*. 2009;77(9):4092-101.
37. Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, Vasconcellos SA, et al. In vitro identification of novel plasminogen-binding receptors of the pathogen *Leptospira interrogans*. *PLoS ONE*. 5(6):e11259.
38. Oliveira R, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, Nascimento AL. Characterization of novel OmpA-like protein of *Leptospira interrogans* that binds extracellular matrix molecules and plasminogen. *PLoS ONE*. 6(7):e21962.
39. Mendes RS, Von Atzingen M, de Moraes ZM, Goncalves AP, Serrano SM, Asega AF, et al. The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection. *Infection and immunity*. 79(11):4657-67.
40. Domingos RF, Vieira ML, Romero EC, Goncalves AP, Moraes ZM, Vasconcellos SA, et al. Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions. *BMC microbiology*. 2012;12(1):50. Epub 2012/04/03.
41. Vieira ML, de Moraes ZM, Vasconcellos SA, Romero EC, Nascimento AL. In vitro evidence for immune evasion activity by human plasmin associated to pathogenic *Leptospira interrogans*. *Microbial pathogenesis*. 51(5):360-5.
42. Turner LH. Leptospirosis. 3. Maintenance, isolation and demonstration of leptospirae. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 1970;64(4):623-46. Epub 1970/01/01.
43. Coleman JL, Roemer EJ, Benach JL. Plasmin-coated *Borrelia burgdorferi* degrades soluble and insoluble components of the mammalian extracellular matrix. *Infection and immunity*. 1999;67(8):3929-36. Epub 1999/07/23.
44. Nakai K, Horton P. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends in biochemical sciences*. 1999;24(1):34-6. Epub 1999/03/24.
45. Nakai K, Kanehisa M. Expert system for predicting protein localization sites in gram-negative bacteria. *Proteins*. 1991;11(2):95-110. Epub 1991/01/01.
46. Letunic I, Copley RR, Pils B, Pinkert S, Schultz J, Bork P. SMART 5: domains in the context of genomes and networks.

- Nucleic acids research. 2006;34(Database issue):D257-60. Epub 2005/12/31.
- 47.Schultz J, Milpetz F, Bork P, Ponting CP. SMART, a simple modular architecture research tool: identification of signaling domains. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(11):5857-64. Epub 1998/05/30.
- 48.Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, Lassmann T, et al. Pfam: clans, web tools and services. Nucleic acids research. 2006;34(Database issue):D247-51. Epub 2005/12/31.
- 49.Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, Krogh A. Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein science : a publication of the Protein Society. 2003;12(8):1652-62. Epub 2003/07/24.
- 50.Brisette CA, Haupt K, Barthel D, Cooley AE, Bowman A, Skerka C, et al. *Borrelia burgdorferi* infection-associated surface proteins ErpP, ErpA, and ErpC bind human plasminogen. Infection and immunity. 2009;77(1):300-6. Epub 2008/11/13.
- 51.Pathirana RD, O'Brien-Simpson NM, Veith PD, Riley PF, Reynolds EC. Characterization of proteinase-adhesin complexes of *Porphyromonas gingivalis*. Microbiology. 2006;152(Pt 8):2381-94. Epub 2006/07/20.
- 52.Lin YP, Lee DW, McDonough SP, Nicholson LK, Sharma Y, Chang YF. Repeated domains of leptospira immunoglobulin-like proteins interact with elastin and tropoelastin. The Journal of biological chemistry. 2009;284(29):19380-91. Epub 2009/05/29.
- 53.Yun TH, Cott JE, Tapping RI, Slauch JM, Morrissey JH. Proteolytic inactivation of tissue factor pathway inhibitor by bacterial omptins. Blood. 2009;113(5):1139-48. Epub 2008/11/08.
- 54.Meri T, Murgia R, Stefanel P, Meri S, Cinco M. Regulation of complement activation at the C3-level by serum resistant leptospires. Microbial pathogenesis. 2005;39(4):139-47.
- 55.Haake DA, Matsunaga J. Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. Infection and immunity. 2002;70(9):4936-45. Epub 2002/08/17.
- 56.Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, et al. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infection and immunity. 2000;68(4):2276-85. Epub 2000/03/18.
- 57.Young KC, Shi GY, Wu DH, Chang LC, Chang BI, Ou CP, et al. Plasminogen activation by streptokinase via a unique mechanism. The Journal of biological chemistry. 1998;273(5):3110-6.
- 58.McCoy HE, Broder CC, Lottenberg R. Streptokinases produced by pathogenic group C streptococci demonstrate species-specific plasminogen activation. The Journal of infectious diseases. 1991;164(3):515-21.
- 59.Lijnen HR, De Cock F, Van Hoef B, Schlott B, Collen D. Characterization of the interaction between plasminogen and staphylokinase. European journal of biochemistry / FEBS. 1994;224(1):143-9. Epub 1994/08/15.
- 60.Schlott B, Guhrs KH, Hartmann M, Rocker A, Collen D. NH2-terminal structural motifs in staphylokinase required for plasminogen activation. The Journal of biological chemistry. 1998;273(35):22346-50.
- 61.Okada K, Ueshima S, Takaishi T, Yuasa H, Fukao H, Matsuo O. Effects of fibrin and alpha2-antiplasmin on plasminogen activation by staphylokinase. American journal of hematology. 1996;53(3):151-7.
- 62.Sodeinde OA, Subrahmanyam YV, Stark K, Quan T, Bao Y, Goguen JD. A surface protease and the invasive character of plague. Science. 1992;258(5084):1004-7. Epub 1992/11/06.
- 63.Leytus SP, Bowles LK, Konisky J, Mangel WF. Activation of plasminogen to plasmin by a protease associated with the outer membrane of *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America. 1981;78(3):1485-9. Epub 1981/03/01.
- 64.Li Z, Ploplis VA, French EL, Boyle MD. Interaction between group A streptococci and the plasmin(ogen) system promotes virulence in a mouse skin infection model. The Journal of infectious diseases. 1999;179(4):907-14. Epub 1999/03/09.
- 65.Collen D, Tytgat G, Claeys H, Verstraete M, Wallen P. Metabolism of plasminogen in healthy subjects: effect of tranexamic acid. The Journal of clinical investigation. 1972;51(6):1310-8.
- 66.Barocchi MA, Ko AI, Reis MG, McDonald KL, Riley LW. Rapid translocation of polarized MDCK cell monolayers by *Leptospira interrogans*, an invasive but nonintracellular pathogen. Infection and immunity. 2002;70(12):6926-32.
- 67.Malmstrom J, Beck M, Schmidt A, Lange V, Deutsch EW, Aebersold R. Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. Nature. 2009;460(7256):762-5. Epub 2009/07/17.
- 68.Cullen PA, Cordwell SJ, Bulach DM, Haake DA, Adler B. Global analysis of outer membrane proteins from *Leptospira interrogans* serovar Lai. Infection and immunity. 2002;70(5):2311-8. Epub 2002/04/16.
- 69.Candela M, Centanni M, Fiori J, Biagi E, Turroni S, Orrico C, et al. DnaK from *Bifidobacterium animalis* subsp. *lactis* is a surface-exposed human plasminogen receptor upregulated in response to bile salts. Microbiology (Reading, England). 156(Pt 6):1609-18.
- 70.Nogueira SV, Fonseca FL, Rodrigues ML, Mundodi V, Abi-Chacra EA, Winters MS, et al. Paracoccidioides brasiliensis Enolase Is a Surface Protein That Binds Plasminogen and Mediates Interaction of Yeast Forms with Host Cells. Infection and immunity. 78(9):4040-50.
- 71.Kunert A, Losse J, Gruszyn C, Huhn M, Kaendler K, Mikkat S, et al. Immune evasion of the human pathogen *Pseudomonas aeruginosa*: elongation factor Tuf is a factor H and plasminogen binding protein. J Immunol. 2007;179(5):2979-88.
- 72.Floden AM, Watt JA, Brissette CA. *Borrelia burgdorferi* enolase is a surface-exposed plasminogen binding protein. PLoS ONE. 6(11):e27502.
- 73.Blom AM, Hallstrom T, Riesbeck K. Complement evasion strategies of pathogens-acquisition of inhibitors and beyond. Molecular immunology. 2009;46(14):2808-17. Epub 2009/05/30.
- 74.Lambris JD, Ricklin D, Geisbrecht BV. Complement evasion by human pathogens. Nature reviews Microbiology. 2008;6(2):132-42. Epub 2008/01/17.
- 75.Zipfel PF, Wurzner R, Skerka C. Complement evasion of pathogens: common strategies are shared by diverse organisms. Molecular immunology. 2007;44(16):3850-7. Epub 2007/09/05.
- 76.Rooijackers SH, van Strijp JA. Bacterial complement evasion. Molecular immunology. 2007;44(1-3):23-32. Epub 2006/08/01.
- 77.Gamberini M, Gomez RM, Atzingen MV, Martins EA, Vasconcellos SA, Romero EC, et al. Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis. FEMS microbiology letters. 2005;244(2):305-13.
- 78.Gomez RM, Vieira ML, Schattner M, Malaver E, Watanabe MM, Barbosa AS, et al. Putative outer membrane proteins of *Leptospira interrogans* stimulate human umbilical vein

endothelial cells (HUVECS) and express during infection. Microbial pathogenesis. 2008;45(5-6):315-22.

79.Neves FO, Abreu PA, Vasconcellos SA, de Moraes ZM, Romero EC, Nascimento AL. Identification of a novel potential antigen for early-phase serodiagnosis of leptospirosis. Archives of microbiology. 2007;188(5):523-32.

80.Oliveira TR, Longhi MT, de Moraes ZM, Romero EC, Blanco RM, Kirchgatter K, et al. Evaluation of leptospiral recombinant antigens MPL17 and MPL21 for serological diagnosis of leptospirosis by enzyme-linked immunosorbent assays. Clin Vaccine Immunol. 2008;15(11):1715-22.

LEGENDS TO FIGURES

Figure 1. Recognition of PLG binding to *Leptospira* by L-IFA. Live virulent *L. interrogans* serovar Copenhageni isolates were treated with PLG, and the recognition was assessed through polyclonal anti-PLG antibodies under a confocal immunofluorescence microscope (PLG 1 to 3). PLG-treated leptospires stained for the proteins LipL32 (outer surface protein marker) or GroEL (a protoplasmic-cylinder marker) and untreated leptospires (PBS), are shown as controls. Panels A: DNA propidium iodide stained, B: FITC-stained and C: A+B composite images.

Figure 2. Cleavage of the plasmin-specific chromogenic substrate by plasmin-bound leptospires. Live low-passage virulent and high-passage non-virulent *L. interrogans* serovar Copenhageni cells received the following treatments: PBS only (PBS), uPA alone (uPA), 5 µg PLG alone (PLG), PLG in crescent quantities (0.5, 2 and 5 µg) together with uPA and 30 % human plasma together with uPA (30 % plasma). Bars represent mean absorbance as a measure of relative substrate degradation $\pm$ the standard deviation of three replicates for each experimental group and are representative of three independent experiments. * Virulent leptospires experiments: statistically significant ($P < 0.0001$) in comparison to the PBS control; ** non-virulent leptospires experiments: statistically significant ($P < 0.01$) in comparison to the PBS control. The 30% plasma, as PLG source, and all the PLG + uPA samples of the virulent leptospires were statistically significant ($P < 0.001$) in comparison to the same non-virulent bacteria tested samples.

Figure 3. Inhibition of PLG binding to *L. interrogans* by ACA. Low-passage virulent *L. interrogans* serovar Copenhageni cells were treated with the following: PLG together with uPA (PLG + uPA), PLG together with uPA with the addition of crescent concentrations of ACA (0.01 to 1,000 mM ACA), PLG alone (PLG), uPA alone (uPA), ACA alone (ACA) and no additions (PBS). The cleavage of the plasmin-specific substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride by the treated spirochetes was measured by absorbance

readings at 405 nm. Bars represent mean absorbance $\pm$ the standard deviation of three replicates for each experimental group and are representative of three independent experiments. Statistically significant substrate degradation inhibition results in comparison to the positive control (PLG + uPA) are shown: $P < 0.01$ (*) and $P < 0.0001$ (**).

Figure 4. Binding of human PLG to *L. interrogans* proteins by affinity blotting. Total protein extracts were resolved by 10% SDS-PAGE and electroblotted into nitrocellulose membranes. The membranes were incubated with PLG (A) or PLG + ACA (B), and the binding was detected by anti-PLG antibodies and peroxidase-conjugated antibodies. The reactivity was revealed by ECL (GE Healthcare) and exposure to X-Ray films. NV: high passage, non-virulent *L. interrogans* serovar Copenhageni strain M 20; Vir: low passage, virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. The arrows indicate reactivity protein regions seen in the virulent but absent in the non-virulent bacterial extract. Positions of protein molecular mass markers are shown on the left.

Figure 5. Fibronectin and laminin degradation by plasmin-coated leptospires. Spirochetes were incubated in IsPBS with no additions (UT) and with addition of PLG and uPA (plasmin). ELISA plate wells coated with 0.5 µg/well fibronectin (A) or laminin (B) were incubated for 20 h with the spirochetes from each experimental group to allow substrate digestion. Plates were then incubated with antibodies anti-laminin or anti-fibronectin. A reduction in absorbance was interpreted as substrate digestion, relative to controls lacking bacteria (0.5 µg/well purified laminin or cellular fibronectin, corresponding to 0% degradation). Percent degradation (a reduction in absorbance value relative to control lacking bacteria) was calculated as described in Materials and Methods. Bars represent mean percent substrate degradation relative to the positive control (0 % degradation) $\pm$ the standard deviation of three replicates. The experiment was independently performed three times with similar results. $P < 0.001$ (*) and $P < 0.01$ (**).

Figure 6. Binding of leptospiral recombinant proteins to human plasminogen. The recombinant proteins LipL32, Lsa63, rLIC10509, Lp29, Lp49, Lsa27, MPL21, MPL17, LipL40, MPL36, rLIC10494, rLIC12730, rLIC12922, rLIC12238, Lsa66, Lp30, Lsa20, rLIC11030, rLIC11834 and rLIC12253 were coated onto 96-well ELISA plates (10 µg/mL) and allowed to interact with purified human PLG (10 µg/mL). BSA and gelatin were used as a negative control for nonspecific binding. Binding was detected and quantified by specific antibodies. Bars represent the

mean absorbance values at 492 nm $\pm$ the standard deviation of four replicates for each protein and are representative of three independent experiments. Statistically significant binding in comparison to negative control BSA are shown by * ($P < 0.001$); ** ($P < 0.0001$); § ($P < 0.02$); $^{\#}$ ($P < 0.0005$). The binding of rLIC11834 and rLIC12253 was compared to its binding to gelatin $^{\times}$ ($P < 0.005$).

Figure 7. Activation of recombinant protein-PLG to enzymatically active plasmin. Cleavage of specific plasmin substrate by PLG bound to recombinant proteins was assayed by modified ELISA as immobilized proteins received the following treatment: PLG + uPA + specific plasmin substrate (PLG + uPA + S), or controls lacking one of the three components (PLG + uPA; PLG + S; uPA + S). BSA was employed as a negative control. Bars represent the mean absorbance values at 405 nm, as a measure of relative substrate cleavage, $\pm$ the standard deviation of four replicates for each experimental group and are representative of two independent experiments. Statistically significant differences are shown by * ($P < 0.00001$), ** ($P < 0.005$), *** ($P < 0.05$), § ($P < 0.001$), §§ ($P < 0.02$), §§§ ($P < 0.003$).

Figure 8. Characterization of recombinant proteins binding to PLG. In (A, B and C) PLG (10 $\mu\text{g/mL}$) was immobilized in 96-well ELISA plates, and each recombinant protein at 0 to 1,000 nM was added for interaction. The binding was detected using antiserum raised in mice against each protein at appropriate dilutions (1: 4,000 for LipL32; 1: 5,000 for rLIC12238, LipL40 and MPL36; 1:1,000 for Lp29, Lp49, Lsa20 and rLIC11030; 1:500 for rLIC12730, Lsa66 and Lp30; 1:750 for rLIC11834 and rLIC12253), followed by horseradish peroxidase-conjugated anti-mouse IgG. Data represent the mean absorbance values $\pm$ the standard deviation of six replicates for each experimental group. The results are representative of two independent experiments. In (D) The dissociation constant (K_D) was calculated based on ELISA data for the recombinant proteins that reached equilibrium up to a concentration of 1,000 nM.

Figure 9. Two-dimensional gel analysis of *L. interrogans* PLG binding proteins. Leptospiral total cell lysates were separated by isoelectric point (pH 3-10) and molecular mass on 12 % SDS-PAGE. Replicate gels were Coomassie Blue stained (A) or transferred to membranes and submitted to PLG affinity immunoblotting (B). The reactive spots are enumerated on A. The results are representative of three independent experiments performed in duplicates.

Figure 10. Human IgG and C3b binding to *L. interrogans*. Bacteria were coated onto microtiter plates (25×10^6 leptospires/well) and IgG (A) or C3b (B) were bound by incubating bacteria with 100% NHS. Bacteria were treated with PBS, 40 $\mu\text{g/mL}$ PLG (PLG), 5 $\mu\text{g/mL}$ uPA (uPA) or PLG + uPA (Pla). Presence of human IgG or C3b deposited on leptospires was determined by ELISA. Bars represent the mean absorbance values at 492 nm $\pm$ the standard deviation of three replicates for each experimental group and are representative of two independent experiments.

Figure 11. IgG binding from human leptospirosis immune serum to *L. interrogans*. Bacteria were coated onto microtiter plates (25×10^6 leptospires/well) and IgG was bound by incubating bacteria 50% non-immune human sera (NHS) or confirmed paired leptospirosis patients sera: MAT negative (MAT-) or MAT positive (MAT+). Bacteria were treated with PBS, 40 $\mu\text{g/mL}$ PLG (PLG), 5 $\mu\text{g/mL}$ uPA (uPA) or PLG + uPA (Pla). Presence of human IgG deposited on leptospires was determined by ELISA using Fc-specific anti-human IgG antibodies. Bars represent the mean absorbance values at 492 nm $\pm$ the standard deviation of three replicates for each experimental group and are representative of two independent experiments.

Figure 12. Detection of human C3b. Leptospires (10^8 /sample) were treated with 10 μg PLG and 3U uPA (Plasmin), only with PLG (PLG), or left untreated (UT). The cells were incubated with 15 $\mu\text{g/mL}$ human purified C3b for 20 h at 37°C, and the supernatants were resolved by SDS-PAGE and transferred to membranes. The C3b was detected by specific antibodies followed by ECL reagent development and exposition to X-Ray films. There are indicated the native human C3b **alpha chain** (105 KDa) and **beta-chain** (75 KDa), as well as the degradation products (**arrows**).

Table 1. Leptospiral selected proteins for characterization of the binding to PLG. Gene locus, protein name, NCBI reference sequence, features, gene conservation, sequence of the primers employed for DNA amplification, and molecular mass of expressed recombinant proteins.

Gene locus ¹	Recombinant protein given name	NCBI reference sequence number ²	Description/Function	Conservation (identity) ³	Sequence of primers for PCR amplification	Recombinant protein molecular mass (kDa)
LIC11352	LipL32 ^{a,h}	YP_001316	Major outer membrane protein (MOMP), LipL32 lipoprotein	Lai (100 %); LBH (98 %)	F: 5' CACCGGTGCTTTCGGTGGTCTG 3' R: 5' ATTACTTAGTCGCGTCAGAAGC 3'	30.2
LIC10314	Lsa63 ^{b,h}	YP_000304	Conserved hypothetical protein with Borrelia_P83 domain	Lai (98 %); LBH (87 %); LBP (39 %)	F: 5' GGATCCCTTATTTTCTCAGGAAAG 3' (BamH I) R: 5' GGTACCCTAAGGTTTAATTTTTT 3' (Kpn I)	63.0
LIC10509	rLIC10509 ^{c,h}	YP_000493	Putative lipoprotein	Lai (98%)	F: 5' CCGGGATCCAAAAAGAGCAAAGAAG 3' (BamH I) R: 5' GGTACCCTACTCGAGACAGCCAGGACCTTC 3' (Kpn I)	22.0
LIC12892	Lp29 ^{d,h}	YP_002808	Putative lipoprotein		F: 5' CTCGAGGCAGTACATTACAATCTTGCT 3' (Xho I) R: 5' CCATGGCTCTTAGGAGCCTGGAAA 3' (Nco I)	29.6
LIC10793	Lp49 ^{d,h}	YP_000772	Putative lipoprotein, Surface antigen	Lai (99%); LBH (86%)	F: 5' CTCGAGAGCGGAGACTTTTTCTTACTT 3' (Xho I) R: 5' CCATGGTTAAAAACCATCTCTACGATAAAC 3' (Nco I)	49.1
LIC12895	Lsa27 ^{e,h}	YP_002811	Putative lipoprotein	Lai (79%)	F: 5' GGATCCCTGAAATATACGAA 3' (EcoRI) R: 5' GAATTCCTACTGTTCTCCTTC 3' (BamHI)	27.0
LIC13131	MPL21 ^{a,f,h}	YP_003039	Hypothetical protein with Ycel domain	Lai (98%); LBP (45%)	F: 5' CACCACGTCTCAAAGTTACGCTTCAG 3' R: 5' TTCTACCATCCAGCTCGG 3'	21.9
LIC10765	MPL17 ^{a,f,h}	YP_000745	Conserved hypothetical protein	Lai (100%); LBH (80%); LBP (41%)	F: 5' CACCGAAAGTCCCCTAAGGTTCAAA 3' R: 5' TGCAGGAGTCCCACATTTTA 3'	15.4
LIC10091	LipL40 ^{a,h}	YP_000088	Putative lipoprotein	Lai (100%); LBH (84%)	F: 5' CCATGGGACTCGAGACGCCTCCTCTAAAGATCC 3' R: 5' CTCCATGGTCATTTCAAACTTCTACGGGGC 3'	39.0
LIC10054	MPL36 ^{a,h}	YP_000054	Putative lipoprotein with Rare lipoprotein A (RplA) like domain	Lai (100%); LBH (88%); LBP (50%)	F: 5' CACCACGTCTTGTCGTCGGTAGAG 3' R: 5' CCAAGTATTCTATTTATACGTCCGAG 3'	35.1
LIC10494	rLIC10494 ^{g,h}	YP_000478	Putative lipoprotein	Lai (99%)	F: 5' CACCACTGCTAGGGCTGCAGAAA 3' R: 5' ACTTTGAGAGCTTCGTCTCGT 3'	25.8
LIC12730	rLIC12730 ^{g,h}	YP_002650	Hypothetical protein with TPR domain and 4 NHL repetition	Lai (100%); LBH (90%); LBP (37%)	F: 5' CACCAGTTCTGACGGACTTCCAA 3' R: 5' TCTTGCGAATGAGTTGATCC 3'	77.4
LIC12922	rLIC12922 ^h	YP_002837	Conserved hypothetical protein	Lai (100%); LBH (89%); LBP (48%)	F: 5' CACCGAATCACTCAACAGAGTCATTGC 3' R: 5' ATCAATCTAAATGAAACGTCTCTTC 3'	40.0
LIC12238	rLIC12238 ^{h,i}	YP_002173	Hypothetical protein	Lai (99%); LBH (77%); LBP (39%)	F: 5' CTCGAGTGTTTTAAACCTACCGGAG 3' (Xho I) R: 5' AAGCTTCTACTTCATCGCTTTTCTATATC 3' (Hind III)	17.6
LIC10258	Lsa66 ⁱ	YP_000249	Hypothetical protein with ompA domain	Lai (99%); LBH (79%)	F: 5' GGATCCGAAGCCTTCTCACCCAATTG 3' (BamH I) R: 5' CCATGGTTAAAGTGAAAGATAAAAAATCGATTC 3' (Nco I)	65.7
LIC12880	Lp30 ⁱ	YP_002796	Putative lipoprotein	Lai (99%); LBH (76%)	F: 5' CTCGAGGAAGTTGTCCGAGTCTAT 3' (Xho I) R: 5' CCATGGTTATTGATTGTTAATTCAG 3' (Nco I)	30.7
LIC11469	Lsa20 ^j	YP_001430	Hypothetical protein	Lai (100%); LBH (82%); LBP (29%)	F: 5' CTCGAGCCAATTTCTTTTCGATCCAAATC 3' (Xho I) R: 5' AAGCTTCAATCCTCTACTGCAGCCC 3' (Hind III)	20.0
LIC11030	rLIC11030 ^{g,j}	YP_001000	Putati	Lai (100%)	F: 5' CTCGAGTGACAAACGAAAAAGAAGGT 3' (Xho I)	37.0

			ve lipoprotein with unknown domain (DUF1565)		R: 5' <u>AAGCTTTT</u> AGTTGCAAGGATTGGA 3' (Hind III)	
LIC11834	Lsa33 ^k	YP_001783	Putative lipoprotein	Lai (99%); LBH (87%); LBP (31%)	F: 5' <u>CTCGAGG</u> ATCTACAAGGTGGGGTTTTAC3' (Xho I) R: 5' <u>CCATGGT</u> TACTGAGGTTTACTTGGTCC3' (Nco I)	33.1
LIC12253	Lsa25 ^k	YP_002188	Conserved hypothetical protein	Lai (100%); LBH (77%); LBP (39%)	F: 5' <u>CTCGAGG</u> AGGAGAAACCGGACGATAC 3' (Xho I) R: 5' <u>CCATGGT</u> TAGGGAAGACTTCTAACACATC 3' (Nco I)	24.0

¹<http://aeg.lbi.ic.unicamp.br/world/lic/> [35]. ²<http://www.ncbi.nlm.nih.gov/protein/>. ³Protein BLAST - <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi> [71,72].^a Previously published by Gamberini et al. (77);^b Previously published by Vieira et al. (37);^c Previously published by Gómez et al. (78);^d Previously published by Neves et al. (79);^e Previously published by Longhi et al. (19);^f Previously published by Oliveira et al. (80);^g Previously published by Barbosa et al. (18);^h Previously published by Vieira et al. (37);ⁱ Previously published by Oliveira et al. (38);^j Previously published by Mendes et al. (39);
^k Previously published by Domingos et al. (40); Lai: *L. interrogans* serovar Lai (8); LBH: *L. borgpetersenii* serovar Hardjo-bovis (11); LBP: *L. biflexa* serovar Patoc (12).

Table 2. *L. interrogans* PLG-binding proteins identified by MALDI-TOF mass spectrometry. The proteins are identified as the genomic nomenclature (LIC: *Leptospira interrogans* serovar Copenhageni), and are listed the respective NCBI reference sequence, predicted name/function and predicted cellular localization, as well as theoric molecular mass and isoelectric point (pI).

Gene locus ¹	NCBI reference sequence number ²	Gene/protein function	Localization ³	theoric molecular mass (kDa)	pI
LIC12816	YP_002733	Hypothetical protein	Membrane	14.7	9.5
LIC10788	YP_000767	flaA-1 (Flagellar filament sheath protein)	Membrane	35.0	7.6
LIC11194	YP_001164	Putative citrate lyase	Cytoplasm	37.2	6.1
LIC12875	YP_002791	tuf (Elongation factor Tu)	Cytoplasm	43.6	5.7
LIC12795	YP_002712	Acetyl-CoA acetyltransferase	Cytoplasm	47.4	6.0
LIC12407	YP_002339	Glutamine synthetase protein	Cytoplasm	55.4	5.9
LIC10524	YP_000508	dnaK (Heat shock protein)	Cytoplasm	69.1	5.1

¹<http://aeg.lbi.ic.unicamp.br/world/lic/> [35]. ²<http://www.ncbi.nlm.nih.gov/protein/>. ³Lipo P: Juncker et al., 200

Figure 1

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Microbial Pathogenesis

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Lsa30, a novel adhesin of *Leptospira interrogans* binds human plasminogen and the complement regulator C4bp

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ARTICLE INFO

Article history:

Received 5 April 2012

Received in revised form

13 June 2012

Accepted 14 June 2012

Available online xxx

Keywords:

Leptospira

Leptospirosis

Adhesion

Plasminogen

C4bp

ABSTRACT

Pathogenic *Leptospira* is the etiological agent of leptospirosis, a life-threatening disease that affects populations worldwide. Surface proteins have the potential to promote several activities, including adhesion. This work aimed to study the leptospiral coding sequence (CDS) LIC11087, genome annotated as hypothetical outer membrane protein. The LIC11087 gene was cloned and expressed in *Escherichia coli* BL21 (DE3) strain by using the expression vector pAE. The recombinant protein tagged with N-terminal 6XHis was purified by metal-charged chromatography and characterized by circular dichroism (CD) spectroscopy. The recombinant protein has the ability to mediate attachment to the extracellular matrix (ECM) components, laminin and plasma fibronectin, and was named Lsa30 (Leptospiral surface adhesin of 30 kDa). Lsa30 binds to laminin and to plasma fibronectin in a dose-dependent and saturable manner, with dissociation equilibrium constants (K_D) of 292 ± 24 nM and 157 ± 35 nM, respectively. Moreover, the Lsa30 is a plasminogen (PLG) receptor, capable of generating plasmin, in the presence of activator. This protein may interfere with the complement cascade by interacting with C4bp regulator. The Lsa30 is probably a new surface protein of *Leptospira* as revealed by immunofluorescence assays with living organisms and the reactivity with antibodies present in serum samples of experimentally infected hamsters. Thus, Lsa30 is a novel versatile protein that may play a role in mediating adhesion and may help pathogenic *Leptospira* to overcome tissue barriers and to escape the immune system.

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1. Introduction

Leptospirosis is a disease caused by spirochetes of the genus *Leptospira* that affects animals and humans worldwide. The transmission occurs by direct or indirect contact of humans with urine of infected animals [1]. In the urban surroundings, the disease is associated with the growing population and the lack of appropriate sanitation measures. Each year, more than 500,000 cases are reported with mortality rates that fluctuate between 10% and 40% [2]. However, these data are probably underestimated because of the non-specific clinical symptoms of leptospirosis that can vary from flu-like to fatal pulmonary hemorrhage and Weil's syndrome [3]. Effective control strategies are not very efficient since that pathogenesis of the disease is not well understood. Currently

veterinary vaccines against leptospirosis are composed by inactivated whole cells that have drawbacks such as not offering protection against serovars not included in composition. Only China [4] and Cuba [5] have human licensed vaccines with the same limitations as the veterinary ones.

Due to their location, surface proteins are in contact with the host and may be involved in leptospiral infectivity and protective immunity. We have characterized several of these proteins and identified novel leptospiral adhesins that might be involved in the first steps of host–pathogen interaction [6–13] (Fernandes et al., submitted to publication). After the adhesion, leptospires have to surmount tissue barriers in order to reach blood circulation and organs. Accordingly, we have reported the plasmin generation at the bacterial surface through the binding of the host PLG and the presence of an activator [14]. Moreover, our group has identified several leptospiral proteins as PLG – binding receptors [6,7,13,15]. Recently, we have described that plasmin generation on *Leptospira* decreased opsonization, a process that might help the bacteria to evade the immune system and increase survival [16].

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The present work intends to report a novel adhesin of *Leptospira* encoded by LIC11087 gene, named Lsa30, we have identified among the genome sequences of *Leptospira interrogans* serovar Copenhageni. The rationale to select this CDS was based on its cellular location by bioinformatics analysis. The gene was cloned into the expression vector pAE and the recombinant protein was expressed in *Escherichia coli* heterologous host system. Purified Lsa30 protein was assayed for its binding capacity to ECM components. We show that Lsa30 mediates the binding to laminin and to plasma fibronectin. Moreover, Lsa30 interacts with PLG and generates plasmin in the presence of activator. Lsa30 also binds C4bp complement regulator and may contribute to the bacterial immune evasion. The recognition of this protein by serum samples of infected hamsters suggested its expression during infection. It is likely that this novel protein could be involved in several steps of leptospiral pathogenesis.

2. Materials and methods

2.1. *Leptospira* strains and culture conditions

Leptospire were cultured at 28 °C under aerobic conditions in liquid Ellinghausen–McCullough–Johnson–Harris medium (EMJH, Difco) with 10% rabbit serum, enriched with L-asparagine (0.015%), sodium pyruvate (0.001%), calcium chloride (0.001%), magnesium chloride (0.001%), peptone (0.03%) and meat extract (0.02%) (all w/v). Low passage, virulent *L. interrogans* serovar Kennewicki strain Pomona Fromm was kept by iterative passages in Golden Syrian hamsters for maintenance. Recently weaned male Golden Syrian hamsters were intraperitoneally infected with 500 µL of approximately 1×10^4 virulent *L. interrogans* serovar Kennewicki strain Pomona Fromm. The animals were bled from the retro-orbital plexus after the appearance of symptoms, such as loss of weight and mobility (approximately 5 days post-infection).

2.2. In silico sequence analysis

Putative coding sequence (CDS) LIC11087 was identified on *L. interrogans* serovar Copenhageni genome sequences and selected based on cellular localization predicted by PSORT, <http://psort.nibb.ac.jp> [17] and CELLO, <http://cello.life.nctu.edu.tw/> [18,19] web servers. For prediction of function and conserved structural domains within the amino acid sequences, SMART (http://smart.embl-heidelberg.de/smart/show_motifs.pl [20,21], Pfam <http://www.sanger.ac.uk/Software/Pfam/> [22] and LipoP <http://www.cbs.dtu.dk/services/LipoP/> [23]) web servers were employed. Sequence analysis was performed by BLAST [24]. All sequences available in GeneBank were employed to study conservation and identity of LIC11087 using CLUSTAL 2.1 multiple sequence alignment at <http://www.ebi.ac.uk/Tools/msa/clustalw2/> [25].

2.3. Protein conservation among leptospiral strains

Bacterial cultures of *Leptospira* spp. were harvested by centrifugation, washed three times with PBS containing 5 mM MgCl₂ and resuspended in PBS. The purified recombinant protein, or the protein extracts from leptospire, were loaded into 12% SDS-PAGE, the proteins transferred to nitrocellulose membrane (Hybond ECL, GE Healthcare) in semidry equipment (GE Healthcare) and then analyzed by immunoblotting (see below).

2.4. Gene cloning, protein expression and purification

The gene was amplified from *L. interrogans* serovar Copenhageni strain Copenhageni, without signal peptide sequence tag. The PCR fragment was gel-purified and cloned into expression vector pAE

(Illustra GFX PCR DNA and Gel band purification kit; GE Healthcare). This vector has a 6XHis tag allowing purification of recombinant proteins by metal-chelating chromatography [26]. The cloning process was checked by DNA sequencing on an ABI Prism 3730_L sequencer (Houston, TX, USA) with appropriate T7 promoter-specific primers (Forward 5': TAATACGACTCACTATAGGG and reverse 5': CAGCAGCCAACTCAGTTCCT). Culture of *E. coli* BL21 (DE3) transformed with pAE-LIC11087 was grown in Luria-Bertani (LB) broth with 100 µg ampicillin ml⁻¹ at 37 °C until it reached O.D. 0.6–0.8. The expression of recombinant protein was induced with IPTG 0.25 mM (isopropyl-β-D-thiogalactopyranoside) overnight. The cells were harvested by centrifugation and the bacterial pellets were resuspended in lysis buffer (10 mM Tris–HCl [pH 8.0], 200 mM NaCl, 200 mg/ml lysozyme, 2 mM phenylmethylsulfonyl fluoride [PMSF], and 1% Triton X-100). The bacterial cell pellets were lysed on ice with the aid of a sonicator (Ultrasonic Processor; GE Healthcare). This protein was expressed in insoluble form, so it was solubilized in buffer containing 20 mM Tris–HCl (pH 12.0), 500 mM NaCl, 8 M urea. Lsa30 was purified through Ni²⁺ charged beads in chelating fast-flow chromatographic column (GE Healthcare). The refolding and washing process was performed on-column by gradually removing urea (6–0 M) and increasing concentrations of imidazole (5–60 mM), respectively. For removal of imidazole, the sample was dialyzed in buffer 20 mM Tris–HCl (pH 12.0), 500 mM NaCl and 0.1% glycine at 4 °C. The expression of recombinant protein was confirmed with detection of His-tag by anti-his tag antibodies (dilution 1:2000).

2.5. Circular dichroism (CD) spectroscopy

Purified recombinant Lsa30 protein was dialyzed against sodium phosphate buffer (pH 7.4). CD spectroscopy measurements were performed at 20 °C using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo) equipped with a Peltier unit for temperature control. Far-UV CD spectra were measured using a 1 mm-path-length cell at 0.5 nm intervals. The spectra were presented as an average of five scans recorded from 185 to 260 nm. The residual molar ellipticity is expressed in degree × centimeter per decimole. Spectra data was submitted to K2D2 software, <http://ogic.ca/projects/k2d2>, using the method that calculated the secondary structure content from the ellipticity experimental data [27].

2.6. Analysis of immunogenic potential of Lsa30 in animals

Female BALB/c mice (4–6 weeks old) were immunized subcutaneously twice with 10 µg of rLIC11087 adsorbed in 10% (vol/vol) of Alhydrogel [2% Al(OH)₃, Brenntag Biosector, Denmark]. Negative control mice were injected with PBS. Serum samples were collected before immunizations and 15 days after each immunization. The mice were bled from the retro orbital plexus and the sera of individual animals were analyzed by ELISA for determination of the antibody titers. All animal studies were approved by the Ethics Committee of the Instituto Butantan, São Paulo, SP, Brazil.

2.7. Immunoblotting assay

The purified Lsa30 was loaded into 12% SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare) in semi-dry equipment. Membrane was blocked with 5% non-fat dry milk and 2.5% BSA in PBS containing 0.05% Tween 20 (PBS-T) and then incubated with anti-His sequence tag monoclonal antibodies (1:2000) (GE Healthcare) or antibodies against Lsa30 (1:500) for 2 h at room temperature. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:5000; Sigma) in PBS-T for 1 h. The protein's reactivity was

developed by ECL reagent kit chemiluminescence substrate (GE Healthcare) with subsequent exposure to X-Ray film.

2.8. Liquid-phase immunofluorescence (L-IFA)

Localization of the protein encoded by LIC11087 on surface of *L. interrogans* was evaluated by Liquid-phase immunofluorescence (L-IFA) as described by Oliveira et al. [28]. The cells were grown and washed as described before. These cells were harvested at 10,000 rpm for 15 min and nuclei were stained with 50 µg/ml propidium iodide, for 1 h at 37 °C in the dark. The cells were washed and incubated for 30 min with polyclonal mouse antiserum against rLIC11087 at a 1:50 dilution. After washing, the samples were incubated for 30 min with secondary antibody at a dilution of 1:50, goat anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC; Sigma). Finally, the cells were washed twice and resuspended in PBS anti-fading solution (ProLong Gold; Molecular Probes). Immunofluorescence signal of labeled leptospire was detected using a confocal LSM 510 META microscope (Zeiss). The proteins LipL32 and DnaK were employed as outer membrane [29] and cytoplasmic [30,31] control, respectively.

2.9. Binding of Lsa30 to ECM and to serum components

The binding of Lsa30 to extracellular matrix and blood components was assessed by ELISA according to previously

published protocol [8]. Plates of 96 wells were coated with 1 µg of the components: laminin, collagen Type I, collagen Type IV, plasma fibronectin, cellular fibronectin, plasminogen, C4bp, complement, vitronectin, elastin, factor H, fibrinogen and ECM in 100 µL of PBS for 2 h at 37 °C. Gelatin and BSA were employed as negative controls. The wells were washed six times with PBS containing 0.05% Tween 20 (PBS-T) and then blocked with 200 µL of 10% (wt/vol) non-fat dry milk in PBS-T (overnight at 4 °C). One microgram of Lsa30 was added to each well and incubated for 3 h at 37 °C. The plates were washed three times and polyclonal antibody against Lsa30 was added in appropriate dilution for detection of protein binding. After the plates were washed, secondary antibody, HRP-conjugated goat anti-mouse IgG (Sigma), was added in dilution 1: 5000 and incubated for 1 h at 37 °C. The wells were washed three times, and o-phenylenediamine (OPD) (1 mg/ml) in citrate phosphate buffer (pH 5.0) plus 1 µL/ml H₂O₂ was added (100 µL per well). The reaction proceeded for 10 min and was interrupted by the addition of 50 µL of 4 N H₂SO₄. The absorbance at 492 nm was determined in a microplate reader (TP-reader; Thermo). Bindings were also confirmed by using HRP-conjugated anti-his tag monoclonal antibodies, previously titrated against the recombinant protein and used in a dilution that generates an OD_{492nm} value of approximately 1. For statistical analyses, the binding of recombinant protein to components was compared to its binding to negative control by Student's two-tailed *t* test.

Fig. 1. Analysis of LIC11087 coding sequence conservation among strains of *Leptospira* spp. by Clustal W2 sequence alignments. Blast analysis was performed among a collection of sequences in GeneBank database and leptospiral sequences were employed to perform Clustal W2 sequence alignments. In (A) are the alignments of LIC11087 with sequences identified in pathogenic strains: seqB to seqH are *L. interrogans* serovar Lai strains 56601 and IPAV, *L. noguchii* strain 2006001870, *L. santarosai* strain 2000030832, *L. weilii* strain 2006001855, *L. borgpetersenii* serovar Hardjo-bovis strains L550 and JB197, respectively. In (B) are the alignments of LIC11087 with sequences identified in saprophytic and intermediate strains: seqI to seqL are *L. biflexa* serovar Patoc Patoc 1 (Paris), *L. biflexa* serovar Patoc Patoc 1 (Ames), *L. licerasiae* serovar Varillal MMD08 (LlicsVM_010100009715) and *L. licerasiae* serovar Varillal VAR 010 (LEP1 GSC185_0037), respectively. (*) at the bottom of each alignment set denotes 100% of sequence identity. In (C) is the resulted phylogram of all sequence alignments showing the proximity of LIC11087 within the pathogenic strains of *Leptospira* while in a distant branches are the intermediate and saprophytic strains.

2.10. Dose-response curves

Microplate of 96-wells were coated overnight in PBS at 4 °C with 100 µL of 10 µg/ml PLG, plasma fibronectin, laminin or C4bp. Plates were then blocked and increasing concentrations of Lsa30 (0–1.75 µM) were added (100 µL/well in PBS). The assessment of bound proteins was performed by incubation for 1 h at 37 °C with the antiserum raised against each protein at the dilution of 1:1,500, followed by HRP-conjugated goat anti-mouse IgG (Sigma) (1:5000 in PBS). The reactions were detected with OPD as describe above. The ELISA data were used to calculate the dissociation constant (K_D) according to the method described by Pathirana et al. [32] and Lin et al. [33], based on the equation: $A = A_{\max} [\text{protein}] / (K_D + [\text{protein}])$, where A is the absorbance at a given protein concentration, $A_{\max}$ is the maximum absorbance for the ELISA plate reader (equilibrium), $[\text{protein}]$ is the protein concentration and K_D is the dissociation equilibrium constant for a given absorbance at a given protein concentration (ELISA data point).

2.11. Plasmin enzymatic activity assay

ELISA microplate was coated overnight with 10 µg/ml of Lsa30 in PBS at 4 °C. Gelatin was employed as negative control. Plate was washed once with PBS-T and blocked for 2 h at 37 °C with PBS with 10% (wt/vol) non-fat dry milk. The blocking solution was discarded and 100 µL/well of 10 µg/ml human PLG was added, followed by incubation for 2 h at 37 °C. Wells were washed three times with PBS-T, and then 4 ng/well of human uPA (Sigma–Aldrich) were added. Subsequently, 100 µL/well of plasmin-specific substrate D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride (Sigma–Aldrich) were added at a final concentration of 0.4 mM in PBS. Plate was incubated overnight at 37 °C and substrate degradation was measured by readings at 405 nm.

2.12. Reactivity of Lsa30 with serum samples from infected hamsters

The recognition of the protein Lsa30 by antibodies of serum samples from 42 infected hamsters was investigated by ELISA. The

Fig. 2. Analysis of Lsa30 expression by SDS-PAGE. (A) Analysis of Lsa30 protein expressed in *E. coli* BL21 (DE3), solubility and purification by SDS-PAGE 12%. M. molecular mass marker in kDa 1. LipL32, as protein molecular mass marker; 2. Bacterial cellular extract of untransformed *E. coli* BL21 SI (DE3) 3. Non-induced bacterial culture 4. Soluble fraction of IPTG-induced bacterial culture 5. Insoluble fraction of IPTG-induced culture 6. Purified recombinant protein. (B) Detection of Lsa30 by western blotting using monoclonal anti his-tag antibodies (1:2000). (C) Detection of Lsa30 by western blotting using homolog polyclonal antibodies (1:500). (D) Structural analysis of recombinant protein Lsa30 by circular dichroism. Lsa30 protein presents a predominant signal of α -helical with minimum ellipticity at 211 and 220 nm and a maximum at 192 nm. Far-UV CD spectra are presented as an average of five scans recorded from 183 to 260 nm.

detection of hamsters IgG against Lsa30 was performed with rabbit anti-hamster (Sigma, USA) at dilution 1:100, followed by secondary antibody goat anti-rabbit IgG (Sigma, USA) at dilution 1: 2000. The cutoff values were calculated at three standard deviations above the mean OD492 nm of sera from 8 healthy animals.

2.13. ECM and biological components

Macromolecules, including the control protein fetuin, were purchased from Sigma Chemical Co. (St. Louis, Mo.). Laminin and collagen Type IV were derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma, cellular fibronectin was

Fig. 3. Localization of Lsa30 in *Leptospira interrogans* by immunofluorescence. (A) DNA of live *Leptospira* stained by propidium iodide. (B) Antigens were recognized on bacteria surface by specific antibodies and secondary antibody conjugated with fluorescein isothiocyanate (FITC). (C) Co-localization showed in the merged images. Leptospire maintained in PBS or treated with antibodies against the cytoplasmic DnaK or against the outer membrane protein LipL32 were employed as negative and positive control, respectively.

derived from human foreskin fibroblasts, plasma fibronectin, human complement serum and fibrinogen were isolated from human plasma and collagen Type I was isolated from rat tail. Plasminogen native, purified from human plasma, was purchased from Merck. Human Factor H was from Calbiochem. C4bp was from Complement Technology, INC.

3. Results

3.1. Bioinformatic analysis of the LIC11087 sequence

The CDS LIC11087 was identified in the chromosome I of *L. interrogans* serovar Copenhageni genome sequences [34,35]. LIC11087 is predicted to be a membrane protein, based on the search for sequence motifs by the servers PSORT [17] and CELLO program [18,19]. No putative conserved domain within this sequence was detected according to SMART, PFAM and BLAST public web servers. BLAST analysis showed that CDS LIC11087 was identified in *L. interrogans* serovar Lai strains 56601 and IPAV (100% identity), *Leptospira borgpetersenii* serovar Hardjo-bovis JB197 and L550 strains (77% identity). LIC11087 CDS was also identified with high sequence similarity (79–98%) in *Leptospira noguchii* strain 2006001870, *Leptospira santarosai* strain 2000030832 and *Leptospira weilii* strain 2006001855. Sequence with partial similarity (29–30%) was identified in the saprophytic strains of *Leptospira biflexa*, Patoc 1 (Ames) and Patoc 1 (Paris), and in the strains of *Leptospira licerasiae* serovar Varillal MMD08 (LlicsVM_010100009715) and VAR 010 (LEP1GSC185_0037) that belongs to a group intermediate between pathogenic and saprophytic [36]. Multiple sequence alignment was performed with CLUSTAL 2.1 program comparing LIC11087 with the sequences from pathogenic strains (Fig. 1A) and the sequences from saprophytic and intermediate strains (Fig. 1B). The (*) at the bottom of sequences denotes high sequence identity. The phylogram of all sequences is depicted in Fig. 1C that clearly shows the similarity/proximity of LIC11087 with the sequence present in pathogenic strains while sequence present in saprophytic and intermediate strains have low similarity and are organized in a more distant branch.

3.2. Cloning of the LIC11087 gene and expression of Lsa30 recombinant protein

The amplified DNA fragment was cloned into the *E. coli* expression vector pAE. The pAE-LIC11087 positive clone was confirmed by DNA sequencing. The recombinant protein of 30 kDa was expressed as a full-length protein in *E. coli* BL21 (DE3) and purified by Ni²⁺ charged chromatography. Fig. 2A shows the SDS-PAGE analysis of protein expression and purification steps. The purified Lsa30 was recognized by monoclonal anti-his antibodies in immunoblotting (Fig. 2B) and by mice polyclonal antibodies against Lsa30 (Fig. 2C). The recombinant protein was expressed in insoluble form and was purified under denaturing conditions, followed by refolding process. The structural integrity of the purified protein was then assessed by CD spectroscopy, as depicted in Fig. 2D. The Lsa30 presents a predominant signal of α -helical with minimum ellipticity at near 210 and 220 nm and the maximum at 192 nm. The α -helical predominance was also confirmed by analysis of the spectrum data by K2D2 software, which showed 63% of alpha helix and 3.8% of β -sheets [27]. The results obtained showed that the recombinant protein is suitable for biological assays.

3.3. Detection of the protein corresponding to the LIC11087 CDS in the *Leptospira* by confocal microscopy

We used the liquid-phase immunofluorescence to demonstrate the expression of native LIC11087 protein in *L. interrogans*. The

genomic DNA of living leptospires was labeled by propidium iodide staining (Fig. 3, column A) followed by incubation with the anti-Lsa30 in the presence of anti-mouse IgG antibodies conjugated to FITC (Fig. 3 column B). In control experiments, a green fluorescence could be observed with antisera that recognizes LipL32 antigen (Fig. 3), an outer membrane protein but not with DnaK, a cytoplasmic protein marker (Fig. 3). The localization of the protein-green light on the leptospires was achieved by superimposing both fields and the results obtained are shown in Fig. 3, column C, for each protein, Lsa30, LipL32, DnaK. Lsa30 was weakly immunoreactive to intact spirochetes suggesting surface localization. No protein was detected with the control with PBS only.

3.4. Reactivity of Lsa30 with sera from infected hamsters

We have performed ELISA to analyze whether Lsa30 is recognized by antibodies present in experimentally infected hamsters. We have employed 42 serum samples of hamsters. Our data show that the recombinant protein was able to recognize IgG antibodies in 19 (45.2%) of infected hamster's tested sera (Fig. 4), suggesting that this protein is expressed during animal infection.

3.5. Protein conservation among leptospiral reference strains

Protein expression and conservation among *Leptospira* strains was assessed with total protein extracts from the same strains of *Leptospira* we have employed for DNA conservation analysis. Cell extracts were gel fractionated and western blotting analysis was performed by probing the membranes with polyclonal serum from mice immunized with Lsa30. However, no protein band was detected with the leptospiral strains (data not shown).

Fig. 4. Recognition of Lsa30 by IgG antibodies present in sera of infected hamsters. Positive sera (responders) were determined by ELISA with the recombinant protein and serum samples from experimentally infected hamsters. The reactivity was evaluated as total IgG antibodies. The cutoff values are defined as the mean plus 3 standard deviations obtained with sera from eight non-infected hamsters (horizontal bars).

3.6. Adhesion of Lsa30 to ECM components

Laminin, collagen Type I, collagen Type IV, cellular fibronectin, plasma fibronectin, vitronectin, elastin and the control proteins, BSA and gelatin, were immobilized on microdilution wells and recombinant protein attachment was assessed by ELISA, using anti-Lsa30 polyclonal antiserum to probe the reactivity. Lsa30 protein exhibited efficient adhesiveness to laminin ($P < 0.001$) and plasma fibronectin ($P < 0.01$) compared to the control protein gelatin (Fig. 5A). The binding was also observed when monoclonal anti-his antibodies were used to probe the bound protein (Fig. 5B). No reaction was observed in the absence of Lsa30 when either anti-Lsa30 or anti-his were employed. No statistically significant adhesiveness was observed with Lsa30 when wells were coated with collagen Type I and IV and cellular fibronectin, vitronectin, elastin or the control proteins. The interaction between Lsa30 with laminin and plasma fibronectin was also assessed on a quantitative basis, as shown in Fig. 5C and D. A dose-dependent and saturable binding were observed when increasing concentrations of the recombinant protein (0–1.75 μM) were allowed to adhere to a fixed concentration of laminin or plasma fibronectin (1 μg each). In the case of laminin, saturation level was reached at a protein concentration of 1.5 μM (Fig. 5C)

and the calculated dissociation equilibrium constant (K_D) for Lsa30 with laminin is 292 ± 24 nM. Saturation level for plasma fibronectin was also reached at 1.5 μM of Lsa30 (Fig. 5D) with a calculated K_D of 157 ± 35 nM.

3.7. Interaction of Lsa30 to serum components

The ability of the protein Lsa30 to interact to serum components, human plasminogen, complement, factor H, C4bp *in vitro* was evaluated. The components and the control protein gelatin were individually immobilized onto 96-wells plates followed by incubation with Lsa30. The results obtained using polyclonal antibodies against the recombinant protein to probe the reactions showed that the protein interacts with C4bp and PLG (Fig. 6A). No reaction was observed with factor H, complement and the control proteins (Fig. 6A). Similar data were confirmed when binding was probed using monoclonal anti-his tag antibodies (Fig. 6B). These data were further confirmed when the reaction between Lsa30 with PLG and Lsa30 with C4bp were assessed on a quantitative basis as depicted in Fig. 6C and D, respectively. Dose-dependence was observed when increasing concentrations (0–1.75 μM) of the recombinant protein Lsa30 were allowed to adhere to a fixed amount (1 μg) of PLG or C4bp. In both cases, however, saturation

Fig. 5. Binding of Lsa30 to extracellular matrix components. (A) Evaluation of the binding of Lsa30 to laminin, collagen type I, collagen type IV, plasma fibronectin, cellular fibronectin, vitronectin, elastin and ECM gel. Gelatin and BSA were used as negative control. One microgram of each component was coated per well and after washes 1 μg of protein was added. The detection of bound protein was performed with antibodies against Lsa30 in dilution 1:1500. For statistical analyses, the attachment of recombinant protein to ECM components was compared to its binding to gelatin and BSA by two-tailed *t* test (*, $P < 0.00001$; **, $P < 0.001$). (B) Detection of Lsa30 bound to laminin or plasma fibronectin by monoclonal antibody anti-his tag at 1:1000 dilution (*, $P < 0.00001$; **, $P < 0.01$). (C) Dose response of the binding of Lsa30 to laminin (D) and to plasma fibronectin. For both experiments 1 μg of laminin and plasma fibronectin was coated per well and then protein, 0–1.75 μM , was added for ligand-binding characterization. The detection of Lsa30 bound to ECM was performed by using polyclonal antibodies against Lsa30 at 1:1500 dilutions. The equilibrium dissociation constant (K_D) was calculated based on ELISA data when the equilibrium concentration was reached. All experiments data represent the mean absorbance values standard deviation of three replicates for each experimental group.

Fig. 6. Interaction of Lsa30 to serum components evaluated by ELISA. (A) Binding of Lsa30 to human PLG, complement, human complement regulators, factor H and C4bp. Wells were coated with 1 μ g of each component and 1 μ g of protein was added for analysis. Gelatin and BSA were employed as negative controls. Polyclonal antibodies produced in mouse against Lsa30 were used at 1:1500 dilutions to detect the bound protein. For statistical analysis two-tailed *t* test was employed (*, $P < 0.005$; **, $P < 0.05$). (B) Detection of Lsa30 bound to PLG and to C4bp by monoclonal antibodies anti-his tag at 1:1000 dilution (*, $P < 0.05$). (C) Dose response curve of Lsa30 to PLG and (D) to C4bp. Characterization of ligand-binding interaction was performed by adding 0–1.75 μ M of Lsa30 protein to fixed ligand concentration. All experiments data represent the mean absorbance values standard deviation of three replicates for each experimental group. (E) Plasmin generation from PLG bound to Lsa30. Recombinant protein was coated into wells and PLG, plasmin substrate and uPA (PLG + S + uPA) were added. For control of the assay, one of the components was omitted. Gelatin was included as a negative control protein. The standard deviation of four replicates for each condition is shown and is representative of two independent experiments. Statistically significant signal is depicted (*, $P < 0.005$) and was calculated in comparison to the negative control (gelatin).

level was not reached within the protein concentration range used, suggesting low affinity of Lsa30 with the two ligands.

Leptospira and proteins within their surface can bind PLG and acquire proteolytic activity in the presence of an activator, as we have previously shown [7,13–15]. Therefore, we investigated whether Lsa30 bound to PLG could also generate the enzymatically

active plasmin. Microplates were coated with the recombinant protein, blocked, and then incubated with PLG. Unbound PLG was washed away and the urokinase-type PLG activator (uPA) was added together with a plasmin-specific chromogenic substrate. The reaction was carried out overnight and the plasmin activity was evaluated by measuring the cleavage of the substrate (absorbance

at 405 nm). The results show that the PLG captured by the Lsa30 protein could be converted into plasmin, as demonstrated indirectly by specific proteolytic activity (Fig. 6E). The negative control gelatin did not show any proteolytic activity, similar to the controls lacking one of the components, PLG, uPA or the chromogenic substrate.

4. Discussion

Since we have sequenced the whole genome of *L. interrogans* serovar Copenhageni [34,35] our group is committed to identify antigen candidates that would help fight leptospirosis. Bioinformatics have predicted 184 coding sequences that appeared to be exported to the leptospiral surface, and therefore with the potential to participate in the host–pathogen interactions [34]. The majority of these proteins are annotated as hypothetical with no assigned function. By data mining this pool of sequences, we have identified several novel leptospiral adhesins [7–13] and proteins that are capable to promote upregulation of cellular adhesion molecules (CAMs) in endothelial cells [37,38].

After cell adhesion, pathogens must penetrate tissues in order to reach blood circulation and target organs. Plasmin proteolytic activity can help the invasive bacteria to overcome these barriers. We have reported that similar to other pathogens, leptospires can generate plasmin on their surface by binding host PLG in the presence of activators [14]. This proteolytic activity can degrade fibronectin and laminin thus helping the leptospiral invasion [14] (Vieira et al., unpublished results).

We report in this work a novel *L. interrogans* protein encoded by the gene LIC11087, genome annotated as hypothetical. Due to the high degree of antigenic variation among leptospires, we examined the gene/protein conservation among a collection of sequences in GeneBank database. The LIC11087 coding sequence seems to be broadly spread and with high percentage of identity among pathogenic strains of *Leptospira*, while low homology was detected in leptospiral intermediate and saprophytic strains. However, we could not detect the presence of the protein encoded by LIC11087 in all these leptospiral extracts when probed with antibodies raised against the recombinant protein Lsa30. One possible explanation is the fact that this protein although identified by proteomics did not fall in protein abundance scale to have its number of copies calculated [39], indicating very low level of protein expression.

The LIC11087 CDS was expressed in *E. coli* as a 30 kDa full-length recombinant protein, named Lsa30. This protein was expressed in its insoluble from *E. coli* BL21 (DE3) expression host, purified under denaturing conditions followed by protein refolding through extensively dialysis. The secondary structure of the recombinant protein after the purification/refolding processes has been one of our concerns because some denatured proteins may lose their immunogenicity and immunoprotection activities [40]. CD spectroscopy showed a predominant signal of α -helical secondary structure content that makes the Lsa30 suitable for further studies.

The Lsa30 is probably a new surface protein of *Leptospira*, as shown by liquid-phase immunofluorescence assays with living organisms and the reactivity with antibodies present in serum samples of experimentally infected hamsters. The Lsa30 protein has extracellular matrix-binding activity and it is likely that it may have a role in the attachment to host. This protein exhibits a binding profile similar to the previously reported Lsa66 [7] and Lsa20 [13] that are laminin and plasma fibronectin interacting proteins, but distinct of other adhesins like Lsa24 and Lsa27 that are laminin-binding proteins [8]. Several adhesins have been reported to bind to different ECM macromolecules [41]. Indeed, the attachment of *Leptospira* to several ECM macromolecules has been previously shown [8]. The binding affinity dissociation constants estimated for

Lsa30 to laminin ($K_D = 292 \pm 24$ nm) showed similar value of that reported for OmpL37 [42], Lsa66 [7], Lsa33 and Lsa25 [6]. In the case of plasma fibronectin, the calculated K_D (157 ± 35 nm) is in the same order of magnitude when compared to the K_D values obtained with LenB [41], LigA [43], LipL32 [44,45], OmpL37 [42] and Lsa66 [7] and the same ECM component. Thus, it is possible that Lsa30 mediates the leptospiral adhesion process.

The spirochetes *Borrelia* spp. and *Treponema denticola* were shown to have PLG activation system with generation of plasmin on their surface with possible implications on the pathogenesis [46]. We have reported this PLG/PLA acquired proteolytic activity for the first time with *Leptospira* spp. [14]. Plasmin is a versatile serine protease with the ability to digest a broad spectrum of substrates, such as fibrin clots, connective tissue and components of extracellular matrices [47,48]. Verma and colleagues [49] have reported that LenA is a bacterial cell surface receptor for human PLG and that LenA-bound PLG could be converted to plasmin. Thus Lsa30, similarly to LenA [49] and other leptospiral proteins described [6,7,13,15], is a human PLG-binding receptor that generates plasmin in the presence of an activator. Thus, it is likely that Lsa30 may participate in the leptospiral invasion and dissemination.

C4b-binding protein (C4bp) controls the complement classical pathway by interfering with the formation and regeneration of C3 convertase and acting as a cofactor to the serine proteinase factor I in the proteolytic inactivation of C4b [50,51]. Leptospiral binding proteins to C4bp, factor H and factor H-like have been identified [41,52–54]. The interaction of C4bp and of factor H with other pathogens has been described, including the spirochetes *Borrelia* spp. [55,56]. Recently, we have identified two novel leptospiral adhesins, Lsa33 and Lsa25 that have the capacity of binding the human complement regulator C4bp [6]. Lsa30 also has the ability to interact with this complement regulator C4bp and may help the leptospires to evade the immune system.

5. Conclusions

We have identified among the sequences of *L. interrogans* serovar Copenhageni the LIC11087 CDS, genome annotated as hypothetical protein of unknown function. We have characterized this protein, named Lsa30, as a novel leptospiral adhesin. The Lsa30 protein acts as PLG–receptors and could confer proteolytic activity to the bacteria through the generation of plasmin. Moreover, this protein interferes with complement cascade by interacting with the regulator C4bp. Thus, Lsa30 is a multifunctional protein that may play a role on the *Leptospira*–host interactions.

References

- [1] Levett PN. Leptospirosis. *Clinical Microbiology Reviews* 2001;14:296–326.
- [2] Leptospirosis worldwide, 1999. Relevé épidémiologique hebdomadaire/Section d'hygiène du Secrétariat de la Société des Nations = Weekly epidemiological record/Health Section of the Secretariat of the League of Nations. 1999;74:237–42.
- [3] Faine S, Adler B, Bolin C, Perolat P. *Leptospira* and leptospirosis. Melbourne, Australia: MediSci; 1999.
- [4] Yan Y, Chen Y, Liou W, Ding J, Chen J, Zhang J, et al. An evaluation of the serological and epidemiological effects of the outer envelope vaccine to leptospirosis. *Journal of the Chinese Medical Association*: JCMS 2003;66:224–30.
- [5] Martinez R, Perez A, Quinones Mdel C, Cruz R, Alvarez A, Armesto M, et al. [Efficacy and safety of a vaccine against human leptospirosis in Cuba]. *Revista Panamericana De Salud Publica = Pan American Journal of Public Health* 2004; 15:249–55.
- [6] Domingos RF, Vieira ML, Romero EC, Goncalves AP, Morais ZM, Vasconcellos SA, et al. Features of two proteins of leptospirosis with potential role in host–pathogen interactions. *BMC Microbiology* 2012;12:50.
- [7] Oliveira R, de Morais ZM, Goncalves AP, Romero EC, Vasconcellos SA, Nascimento AL. Characterization of novel OmpA-like protein of leptospirosis that binds extracellular matrix molecules and plasminogen. *PLoS One* 2011;6:e21962.

- [8] Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, Vieira ML, et al. A newly identified leptospiral adhesin mediates attachment to laminin. *Infection and Immunity* 2006;74:6356–64.
- [9] Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Moraes ZM, Lima DM, et al. Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC Microbiology* 2008;8:70.
- [10] Atzingen MV, Gomez RM, Schattner M, Pretre G, Goncalves AP, de Moraes ZM, et al. Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells. *The Journal of Infection* 2009;59:264–76.
- [11] Longhi MT, Oliveira TR, Romero EC, Goncalves AP, de Moraes ZM, Vasconcellos SA, et al. A newly identified protein of leptospira interrogans mediates binding to laminin. *Journal of Medical Microbiology* 2009;58:1275–82.
- [12] Vieira ML, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, Nascimento AL. Lsa63, a newly identified surface protein of leptospira interrogans binds laminin and collagen IV. *The Journal of Infection* 2010;60:52–64.
- [13] Mendes RS, Atzingen MV, Moraes ZM, Goncalves AP, Serrano SM, Asega AS, et al. The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection. *Infection and Immunity* 2011;79:4657–67.
- [14] Vieira ML, Vasconcellos SA, Goncalves AP, de Moraes ZM, Nascimento AL. Plasminogen acquisition and activation at the surface of leptospira species lead to fibronectin degradation. *Infection and Immunity* 2009;77:4092–101.
- [15] Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, Vasconcellos SA, et al. In vitro identification of novel plasminogen-binding receptors of the pathogen Leptospira interrogans. *PLoS One* 2010;5:e11259.
- [16] Vieira ML, de Moraes ZM, Vasconcellos SA, Romero EC, Nascimento AL. In vitro evidence for immune evasion activity by human plasmin associated to pathogenic leptospira interrogans. *Microbial Pathogenesis* 2011;51:360–5.
- [17] Nakai K, Kanehisa M. Expert system for predicting protein localization sites in gram-negative bacteria. *Proteins* 1991;11:95–110.
- [18] Yu CS, Lin CJ, Hwang JK. Predicting subcellular localization of proteins for Gram-negative bacteria by support vector machines based on n-peptide compositions. *Protein Science: a Publication of the Protein Society* 2004;13:1402–6.
- [19] Yu CS, Chen YC, Lu CH, Hwang JK. Prediction of protein subcellular localization. *Proteins* 2006;64:643–51.
- [20] Schultz J, Milpetz F, Bork P, Ponting CP. SMART, a simple modular architecture research tool: identification of signaling domains. *Proceedings of the National Academy of Sciences of the United States of America* 1998;95:5857–64.
- [21] Letunic I, Copley RR, Pils B, Pinkert S, Schultz J, Bork P. SMART 5: domains in the context of genomes and networks. *Nucleic Acids Research* 2006;34:D257–60.
- [22] Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, Lassmann T, et al. Pfam: clans, web tools and services. *Nucleic Acids Research* 2006;34:D247–51.
- [23] Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, Krogh A. Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Science* 2003;12:1652–62.
- [24] Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 1997;25:3389–402.
- [25] Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. *Bioinformatics* 2007;23:2947–8.
- [26] Ramos CR, Abreu PA, Nascimento AL, Ho PL. A high-copy T7 Escherichia coli expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide [et al]. *Brazilian Journal of Medical and Biological Research = Revista Brasileira De Pesquisas Medicas E Biologicas/Sociedade Brasileira De Biofisica* 2004;37:1103–9.
- [27] Perez-Iratxeta C, Andrade-Navarro MA. K2D2: estimation of protein secondary structure from circular dichroism spectra. *BMC Structural Biology* 2008;8:25.
- [28] Oliveira TR, Longhi MT, de Moraes ZM, Romero EC, Blanco RM, Kirchgatter K, et al. Evaluation of leptospiral recombinant antigens MPL17 and MPL21 for serological diagnosis of leptospirosis by enzyme-linked immunosorbent assays. *Clinical and Vaccine Immunology* 2008;15:1715–22.
- [29] Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, et al. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infection and Immunity* 2000;68:2276–85.
- [30] Stamm LV, Gherardini FC, Parrish EA, Moomaw CR. Heat shock response of spirochetes. *Infection and Immunity* 1991;59:1572–5.
- [31] Guerreiro H, Croda J, Flannery B, Mazel M, Matsunaga J, Galvao Reis M, et al. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. *Infection and Immunity* 2001;69:4958–68.
- [32] Pathirana RD, O'Brien-Simpson NM, Veith PD, Riley PF, Reynolds EC. Characterization of proteinase-adhesin complexes of porphyromonas gingivalis. *Microbiology (Reading, England)* 2006;152:2381–94.
- [33] Lin YP, Lee DW, McDonough SP, Nicholson LK, Sharma Y, Chang YF. Repeated domains of leptospira immunoglobulin-like proteins interact with elastin and tropoelastin. *The Journal of Biological Chemistry* 2009;284:19380–91.
- [34] Nascimento AL, Ko AI, Martins EA, Monteiro-Vitorello CB, Ho PL, Haake DA, et al. Comparative genomics of two leptospira interrogans serovars reveals novel insights into physiology and pathogenesis. *Journal of Bacteriology* 2004;186:2164–72.
- [35] Nascimento AL, Verjovski-Almeida S, Van Sluys MA, Monteiro-Vitorello CB, Camargo LE, Digiampietri LA, et al. Genome features of leptospira interrogans serovar Copenhageni [et al]. *Brazilian Journal of Medical and Biological Research = Revista Brasileira De Pesquisas Medicas E Biologicas/Sociedade Brasileira De Biofisica* 2004;37:459–77.
- [36] Matthias MA, Ricaldi JN, Cespedes M, Diaz MM, Galloway RL, Saito M, et al. Human leptospirosis caused by a new, antigenically unique leptospira associated with a Rattus species reservoir in the Peruvian Amazon. *PLoS Neglected Tropical Diseases* 2008;2:e213.
- [37] Gomez RM, Vieira ML, Schattner M, Malaver E, Watanabe MM, Barbosa AS, et al. Putative outer membrane proteins of leptospira interrogans stimulate human umbilical vein endothelial cells (HUVECs) and express during infection. *Microbial Pathogenesis* 2008;45:315–22.
- [38] Vieira ML, D'Atri LP, Schattner M, Habarta AM, Barbosa AS, de Moraes ZM, et al. A novel leptospiral protein increases ICAM-1 and E-selectin expression in human umbilical vein endothelial cells. *FEMS Microbiology Letters* 2007;276:172–80.
- [39] Malmstrom J, Beck M, Schmidt A, Lange V, Deutsch EW, Aebersold R. Proteome-wide cellular protein concentrations of the human pathogen Leptospira interrogans. *Nature* 2009;460:762–5.
- [40] Pertinhez TA, Sforza ML, Alves AC, Ramos CR, Ho PL, Tendler M, et al. ¹H, ¹⁵N and ¹³C resonance assignments of the apo Sm14-M20(C62V) protein, a mutant of Schistosoma mansoni Sm14. *Journal of Biomolecular NMR* 2004;29:553–4.
- [41] Stevenson B, Choy HA, Pinne M, Rotondi ML, Miller MC, Demoll E, et al. Leptospira interrogans endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. *PLoS One* 2007;2:e1188.
- [42] Pinne M, Choy HA, Haake DA. The OmpL37 surface-exposed protein is expressed by pathogenic Leptospira during infection and binds skin and vascular elastin. *PLoS Neglected Tropical Diseases* 2010;4:e815.
- [43] Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J, Haake DA. Physiological osmotic induction of leptospira interrogans adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. *Infection and Immunity* 2007;75:2441–50.
- [44] Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Moraes ZM, Barbosa AS, et al. In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. *Infection and Immunity* 2008;76:2642–50.
- [45] Hoke DE, Egan S, Cullen PA, Adler B. LipL32 is an extracellular matrix-interacting protein of leptospira spp. and pseudomonas tunicata. *Infection and Immunity* 2008;76:2063–9.
- [46] Coleman JL, Benach JL. Use of the plasminogen activation system by microorganisms. *The Journal of Laboratory and Clinical Medicine* 1999;134:567–76.
- [47] Ponting CP, Marshall JM, Cederholm-Williams SA. Plasminogen: a structural review. *Blood Coagulation and Fibrinolysis* 1992;3:605–14.
- [48] Angles-Cano E, Rouy D, Lijnen HR. Plasminogen binding by alpha 2-antiplasmin and histidine-rich glycoprotein does not inhibit plasminogen activation at the surface of fibrin. *Biochimica Et Biophysica Acta* 1992;1156:34–42.
- [49] Verma A, Brissette CA, Bowman AA, Shah ST, Zipfel PF, Stevenson B. Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen. *Infection and Immunity* 2010;78:2053–9.
- [50] Blom AM. Structural and functional studies of complement inhibitor C4b-binding protein. *Biochemical Society Transactions* 2002;30:978–82.
- [51] Gigli I, Fujita T, Nussenzweig V. Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator. *Proceedings of the National Academy of Sciences of the United States of America* 1979;76:6596–600.
- [52] Barbosa AS, Monaris D, Silva LB, Moraes ZM, Vasconcellos SA, Cianciarullo AM, et al. Functional characterization of LcpA, a surface-exposed protein of Leptospira spp. that binds the human complement regulator C4BP. *Infection and Immunity* 2010;78:3207–16.
- [53] Castiblanco-Valencia MM, Fraga TR, Silva LB, Monaris D, Abreu PA, Strobel S, et al. Leptospiral immunoglobulin-like proteins interact with human complement regulators factor H, FHL-1, FHR-1, and C4BP. *The Journal of Infectious Diseases* 2012;205:995–1004.
- [54] Verma A, Hellwage J, Artiushin S, Zipfel PF, Kraiczky P, Timoney JF, et al. Lfha, a novel factor H-binding protein of Leptospira interrogans. *Infection and Immunity* 2006;74:2659–66.
- [55] Kraiczky P, Skerka C, Brade V, Zipfel PF. Further characterization of complement regulator-acquiring surface proteins of Borrelia burgdorferi. *Infection and Immunity* 2001;69:7800–9.
- [56] Hovis KM, McDowell JV, Griffin L, Marconi RT. Identification and characterization of a linear-plasmid-encoded factor H-binding protein (FhbA) of the relapsing fever spirochete Borrelia hermsii. *Journal of Bacteriology* 2004;186:2612–8.

The interaction of *Leptospira interrogans* with human proteolytic system enhances dissemination through endothelial cells and the levels of plasminogen activators and matrix metalloprotease-9

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We have recently reported the ability of *Leptospira* to capture plasminogen (PLG) and generate plasmin (PLA) bound on microbial surface in the presence of exogenous activators. In this work, we examined the effects of leptospiral PLG-binding for active penetration through endothelial cell barrier and activation. The results indicate that leptospires with PLG - associated or PLA- activated have enhanced migration activity through human umbilical vein endothelial cells (HUVECs) monolayers than untreated bacteria. *Leptospira*-coated PLG was capable to stimulate the release and expression PLG activators by HUVECs. Moreover, leptospires endowed with PLG or PLA promoted transcriptional upregulation and release of matrix metalloprotease-9 (MMP-9) to the culture media. Leptospirosis confirmed human serum samples showed higher levels of PLG activators and total MMP-9 than the normal serum samples. The highest level of PLG activators and total MMP-9 was detected with MAT negative serum samples, suggesting that this proteolytic activity stimulation occurs at the early stage of the disease. Furthermore, gelatin zymography profile obtained for MMP-9 with leptospirosis serum samples appears to be specific to leptospiral infection because serum samples of unrelated infectious diseases produced no degradation bands. Altogether, the data suggest that the *Leptospira*-associated PLG/PLA might represent a mechanism that contributes to bacterial penetration of endothelial cells through an activation cascade of events that enhances the proteolytic capability of the organism. To our knowledge, this is the first proteolytic activity associated with leptospiral pathogenesis described to date.

Leptospira interrogans is the causal agent of leptospirosis, a neglected zoonotic infectious disease of human and veterinary concern. The transmission of leptospirosis is associated with exposure of individuals in close proximity to wild or farm animals [1]. In the urban settings, leptospirosis became a major health problem due to the lack of adequate sanitation measurements and the large population of urban rodent reservoirs that contaminate the environment through their urine [2]. Although leptospiral genome, functional genomics and host-pathogen interactions are being actively investigated by many research groups, the molecular aspects of *Leptospira* virulence and pathogenesis or the origin of physiological leptospirosis symptoms and severity of disease remain to be understood [3-9].

L. interrogans has the capacity to rapidly disseminate causing systemic infection after skin penetration. Indeed, the rapid translocation of polarized MDCK cell monolayers by virulent leptospires has been reported [10]. Yet, the mechanisms involved in such translocation and dissemination were not understood [10]. Bacterial interaction with the hosts' fibrinolytic system by surface PLG capture and/or PLG activation to PLA have been suggested to be a feature that significantly contributes to the virulence of several pathogens by facilitating penetration and invasion [11]. It has been shown that PLA-coated *Borrelia burgdorferi* resulted

in an enhanced penetration of endothelial cell monolayers [12]. We have firstly described the interaction of *Leptospira* with PLG/PLA system and some possible implications for pathogenesis [13]. We demonstrated that leptospires interact with PLG and can acquire PLA activity associated to the surface without interfering in cell growth and viability, which occurs through multiple receptors [13-17]. Although non-virulent leptospires can interact with PLG, there seems to be a correlation between the efficiency of PLG capturing and virulence, suggesting a role in virulence and infection. In fact, PLG receptors seem to be differentially expressed in virulent strains. Moreover, the generation of enzymatically active PLA on the leptospiral surface lead to extracellular matrix (ECM) components degradation, decreased C3b levels and IgG opsonization that may permit leptospires to reach the bloodstream and disseminate to multiple organs [10,13,18].

In the present study we focused to elucidate possible mechanisms involved when *Leptospira* associated with proteolytic activity are in contact with human cells. We show that PLA generation on the surface of leptospires facilitates the bacterial transposition across human endothelial cell barrier when compared with leptospires lacking this proteolytic activity. We also show that the interaction with mammalian cells promoted an upregulation of PLG activators, thus increasing PLA generation on

the bacterial surface. Furthermore, we describe that the interaction of PLA-associated *Leptospira* with human monolayers cells results in an upregulation of MMPs and stimulates the activation and release of these enzymes from HUVECs. Moreover, we demonstrate that confirmed leptospirosis serum samples present different circulating amounts of PLG activators and MMPs, when compared to normal individuals. Our data provide the first evidence of mechanisms involved during *Leptospira* - mammalian cells interactions that would contribute to tissue penetration and rapid bacterial dissemination.

MATERIALS AND METHODS

Bacteria isolates and culture conditions. Virulent *L. interrogans* serovar Pomona strain LPF is routinely cultured by iterative passages in Golden Syrian hamsters for maintenance of virulence. Recent-weaned hamsters were intraperitoneally infected with 500 µL containing approximately 1.0×10^4 virulent leptospires. The animals were sacrificed after appearance of symptoms such as loss of weight and mobility (approximately 5 days post-infection). Kidney and liver were removed and macerated, and the organs-derived leptospires were cultured at 28°C in modified Elinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with 10% *Leptospira* enrichment EMJH (BD, Difco).

Human serum samples collection. Serum samples from patients with confirmed leptospirosis were obtained from the collection of Instituto Adolfo Lutz, São Paulo, Brazil; serum samples from patients with unrelated infectious diseases were obtained from the collections of the Laboratório de Imunoepidemiologia, SUCEN, São Paulo, Brazil; Laboratório de Protozoologia, IMT/USP, São Paulo, Brazil (sera from patients with Chagas' disease) Laboratório de Virologia, IMT/USP, São Paulo, Brazil (sera from patients with human immunodeficiency virus [HIV] infection and dengue); and the Núcleo de Estudos em Malária, SUCEN/IMT/USP, São Paulo, Brazil (sera from patients with malaria). Human sera were donated for use for research purposes. Normal human sera were obtained from healthy donors, with no reported leptospirosis history.

Microscopic agglutination test (MAT). The MAT of the serum samples was performed according to Faine et al. [2]. In brief, an array of serovars of *Leptospira* spp. as antigens were employed: Australis, Autumnalis, Bataviae, Canicola, Castellonis, Celledoni, Copenhageni, Cynopteri, Djasiman, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc, Pomona, Pyrogenes, Sejroe, Shermani, Tarassovi and Wolffi. All the strains were maintained in EMJH liquid medium (Difco, USA) supplemented with 10%

rabbit sera, at 28°C. A laboratory-confirmed case of leptospirosis was defined by the demonstration of a four-fold microagglutination titer rise between paired serum samples. The probable predominant serovar was considered the titer that was the highest sample dilution with 50% of agglutination. MAT was considered negative when the titer was below 1:100. In addition of the MAT-negative and MAT-positive samples from individuals with laboratory and clinic leptospirosis confirmations, we also employed sera from normal healthy donors, without a known history of leptospirosis and confirmed negative MAT.

Endothelial cells extraction and cultivation. The HUVECs (Human Umbilical Vein Endothelial Cells) were obtained by collagenase digestion of umbilical veins as detailed in Jaffe et al. [19]. The cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 media (Cultilab) supplemented with 10% fetal bovine sera (FBS) (Cultilab), 45 µg/mL heparin (Roche), 1 mM sodium pyruvate (Spectrum Chemical), 2 mM L-glutamin (Sigma), 100 IU penicilin (Cultilab), 100 µg/mL streptomycin (Cultilab), 50 µM 2-mercaptoetanol (Pharmacia Biotech), 25 µg/mL endothelial growth factor (Sigma) and 0.75% mice brain extract. Cells were maintained at 37°C in a humidified 5% CO₂ incubator. Confluent HUVECs were passaged with 0.25% trypsin and 0.02% EDTA (Cultilab) and seeded in appropriate culture plates. First passage confluent HUVECs were used in all experiments. The use of HUVECs for experimentation is approved by the Ethics Committee of Universidade Federal de São Paulo (UNIFESP), under the protocol number 0664/05.

Leptospiral transmigration across HUVECs monolayers. Polycarbonate membrane (5 µm) transwell chambers of 6.5 mm (Corning) were prepared with 100 µL/well 4% gelatin. First passage HUVECs (15,000/well) were seeded over the gelatin layer, and incubated with 1.5 mL RPMI complete culture media until total confluence of the monolayer (approximately 24h). *L. interrogans* serovar Pomona (5×10^8 /sample) were treated with 50 µg PLG (Calbiochem) in 200 µL IsPBS (low salt Phosphate Buffered Saline – PBS with 50 mM NaCl) for 1.5h, followed by addition of 5 U uPA (urokinase) (Sigma) for 1h at 37°C. As controls, samples were treated only with PLG or uPA, or with no additions (PBS). After three washes with IsPBS, each sample was resuspended in 200 µL RPMI complete culture media lacking FBS, and 100 µL of the bacterial suspension were added over the HUVECs together with 100 µL culture media in the upper chamber. 400 µL of culture media were added to the lower chamber. After 4h incubation at 37°C in 5% CO₂ atmosphere, the lower chamber contents were collected, centrifuged at 10,000 x g, and the leptospires were counted in Petroff-Hausser chamber by dark field microscopy.

Leptospiral HUVECs activation. HUVECs were seeded (15,000 cells/well) onto cell culture microplates (96 wells), and incubated until total adherence for 16h at 37°C in 5% CO₂ atmosphere. *L. interrogans* serovar Pomona strain LPF (5 x 10⁸/sample) were treated with 50 µg PLG (in 200 µL IsPBS) for 1.5h, followed by addition of 5U uPA and incubation for 1h at 37°C. Controls were treated lacking uPA, PLG or both PLG and uPA. After three washings with IsPBS, each sample was resuspended in 500 µL RPMI medium. The bacterial suspensions (100 µL/well + 100 µL/well RPMI) were transferred to the microplates containing the HUVECs previously washed with PBS for the removal of remnant FBS. As controls, HUVECs were incubated only with RPMI medium, lacking the bacteria. After 6h or 24h incubation times at 37°C, the media were collected, centrifuged for cell and bacteria removal, and frozen in aliquots at -80°C until use.

Quantification of PLG activators. Human serum samples were quantified as uPA and tPA (tissue-type plasminogen activator) presence by commercial kits based on capture ELISA (AssayMax Human Urokinase and AssayMax Human Tissue-Type Plasminogen Activator from Assaypro). The protocols were performed as recommended by the manufacturer, with the dilution of the samples by 25%.

Urokinase chromogenic activity assay. Human serum samples were subjected to uPA chromogenic activity direct assay (Assaypro) according to the instructions provided by the manufacturer. The samples were diluted 5 times prior the analysis.

Zymography. 8% SDS-PAGE (sodium dodecyl sulphate-polyacrilamide gel electrophoresis) were co-polymerized with 0.1% (m/v) gelatin (Difco) or type I human collagen (Sigma). Non-denaturing five times concentrated gel application solution (250 mM Tris-HCl pH 6.8; 2% [wt/v] SDS; 0.1 g/mL bromophenol blue and 10% [v/v] glycerol) was added to the samples without heating step. The samples were loaded, and after running, the gels were washed twice for 15 min with 2.5% (v/v) Triton X-100 in 50 mM Tris-HCl pH 8.4, followed by two washes of 5 min in 50 mM Tris-HCl pH 8.4. The gels were incubated overnight (~18h) at 37°C in activation solution (50 mM Tris-HCl pH 8.4, 5 mM CaCl₂, 1 µM ZnCl₂), and then coomassie blue stained (0.25% coomassie blue R, 45% [v/v] methanol, 10% [v/v] glacial acetic acid). The stain excess was removed by de-staining solution (45% [v/v] methanol, 10% [v/v] glacial acetic acid), until adequate visualization of the degradation regions. Control experiments were performed with the activation solution lacking CaCl₂ and ZnCl₂, or with the addition of 25 mM EDTA to the activation solution.

For experiments with human sera, samples were diluted 20 times, and 10 µL were applied per well. The sera were selected randomly from our collection, independently of the MAT titer and serovar(s) involved. For the cell culture supernatants zymography, 20 µL of the samples were applied onto each gel well. Culture supernatants from HT1080 cells (human fibrosarcoma cell line) were employed as positive control for pro-MMP-2 and pro-MMP-9 degradation activity.

Metalloprotease activity test. MMP-2 and MMP-9 were quantified by specific activity assay systems (GE Healthcare), as recommended by the manufacturer. When total (pro and active) enzymes were quantified, the p-aminophenylmercuric acetate (APMA) was employed to the samples. Serum samples were diluted 20 and 50 times for MMP-9 and MMP-2 determinations, respectively. Cell culture supernatants were analyzed without dilutions.

Real-time PCR. Total RNA from the HUVECs after activation by leptospiral contact was extracted using the RNeasy mini kit (GE Healthcare), as recommended by the manufacturer. The RNA was quantitated by spectrophotometry using a NanoDrop (Thermo). The complementary DNA (cDNA) was synthesized using 1,000 ng total RNA, the SuperScript III Reverse Transcriptase kit (Invitrogen) and oligo dT primers, according to standard protocols. The resulting cDNA was used for quantitative PCR using TaqMan Gene Expression Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) in a 7300 Real-Time PCR System (Applied Biosystems). The genes evaluated were human MMP-9, uPA, tPA and ACTB (Beta Actin) (Applied Biosystems) (catalog numbers hs00957555_m1, hs01547050, hs00263492 and 4333762T, respectively). ACTB was employed as constitutive control. Reaction conditions were as described in the TaqMan Gene Expression Master Mix with 50 ng of cDNA in a total volume of 20 µL, and the cycling protocol was as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 60 s. Each reaction was performed in triplicate and the cycle threshold (CT) values were calculated using 7300 System SDS Relative Quantification Study software (Applied Biosystems). The relative expression ratio was calculated by the comparative 2^{-ΔΔCt} method [20] using ACTB as constitutive control, and 6 h incubation time of RPMI sample (HUVECs) incubated without leptospires, as calibrator. The range for target genes relative expression (maximum and minimum) was calculated by: 2^{-ΔΔCt} with ΔΔCt + s and ΔΔCt - s, where s is the standard deviation of the ΔΔCt value.

RESULTS

Leptospiral transmigration through HUVECs monolayers is enhanced by PLA.

To evaluate whether PLA generation on the surface of leptospires affects bacterial tissue penetration, virulent low-passage *L. interrogans* serovar Pomona strain LPF were used to infect primary cultures of HUVECs monolayers in transwell chambers. The results show that when the organisms were treated with PLG or PLG+uPA (PLA), the leptospires migrated to the lower chamber across the cell monolayers more efficiently than the control leptospires (PBS) (Fig. 1). The fastest migration of leptospires through the monolayer was achieved when the bacteria generate PLA on their surface compared to the ones treated only with PLG. Interestingly, migration was enhanced when leptospires were treated only with PLG when compared to the PBS-treated control cells, suggesting the release of PLG activators from the endothelial cells.

HUVECs activation by leptospires. In our previous work, we have shown that leptospires do not have the capacity to convert PLG to active PLA, and are dependent on the host's fibrinolytic activation system [13]. As leptospires treated with PLG but lacking its activator showed higher migration activity across the cellular barrier when compared to PBS-treated control bacteria (Fig. 1), we speculated whether leptospires could also promote an upregulation of PLG activators when in contact with HUVECs. Endothelial cells were incubated with leptospires and the total RNA of the cells were extracted, followed by cDNA synthesis and real-time PCR evaluation. HUVECs were tested for uPA and tPA PLG activators transcriptions; the ACTB gene was used as endogenous constitutive control for the normalization of both activators levels. The data obtained indicate that leptospires in contact with HUVECs promoted on these cells an upregulation at the transcriptional level of both PLG activators when compared to cells non-exposed to the bacteria (RPMI-treated only) (Fig. 2A and 2B). The stimulation of the uPA transcription was clearly observed after 24 h of HUVECs-leptospires incubation; the transcription stimulation was higher when PLG was present, but the strongest induction of uPA and tPA transcriptional levels was achieved with PLA-associated leptospires (more than 10 times for uPA and 2 times for tPA) (Fig. 2A and 2B). A negligible increase on the transcription was observed upon 6 h of HUVECs-organism incubation for both activators. The control, endothelial cells incubated with RPMI only, produced insignificant differences in the levels of both PLG activators.

MMPs secretion by HUVECs incubated with leptospires. It has been proposed that PLA generated from PLG is an activator of pro-MMPs [21]. The PLA generated in the spirochete *B. burgdorferi* was shown

to activate pro-MMP-9 in human cells [22]. We thus set up to investigate whether the PLA-generated activity on leptospires was capable to promote the activation or release of MMPs by HUVECs co-incubated *in vitro* with leptospires plus the PLG/PLA proteolytic system. Gelatin zymography assays of the cell culture supernatants were analyzed after incubation of HUVECs-PLA associated leptospires and controls for 6 and 24h. HT1080 cell culture media was employed as a control for constitutive secretion of pro-MMP-2 (~72 kDa) and pro-MMP-9 (~92 kDa). As shown in Figure 3, protein degradation bands relative to pro-MMP-2 activity upon gelatin substrate were visible on all samples, including both controls lacking leptospires or samples incubated with treated or untreated leptospires for 6 or 24h (Fig. 3). Supernatants from 24h incubation show protein degradation bands relative to pro-MMP-9 and active MMP-2 (~66 kDa) in on all samples, although apparently in a higher level on samples incubated with leptospires in comparison with the control lacking the bacteria (HUVECs). The band corresponding to active MMP-9 (~78 kDa) became visible, though weak, in the samples from HUVECs incubated 24h with leptospires, treated or untreated, being absent in the control without bacteria (Fig. 3). Thus, we decided to evaluate MMPs activity on the cell culture supernatants, from the same systems described above, by using an ELISA based on capture. The results show that there was no increase either of active or total MMP-9 after 6 h incubation. However, there was a statistically significant increase of total MMP-9 (Fig. 4A) with all the supernatants from HUVECs incubated with leptospires, PLG treated for 24 h or not, confirming the data obtained with gelatin zymography analysis (Fig. 3). Active MMP-9 remained unaffected in the same supernatants after 24 h incubation (Fig. 4A). Total or active MMP2 showed no significant differences after 6 or 24 h incubation in the presence or absence of leptospires, either PLG treated or not (Fig. 4B). We further assess whether the higher release of total MMP-9 by HUVECs exposed to the bacteria was due to an upregulation of this enzyme at the transcriptional level by performing real-time PCR using total HUVECs cDNA, as template, and specific primers to MMP-9. The results indicate that PLA-associated *Leptospira*, or bacteria plus PLG or bacteria plus uPA promoted an upregulation of MMP-9 from HUVECs, being the highest transcriptional level observed with PLA-coated leptospires incubated for 24 h (Fig. 4C).

Detection of PLG activators on confirmed leptospirosis serum samples. *L. interrogans* can incorporate PLG onto their surface and PLA is generated in the presence of exogenous PLG activators [13]. Therefore we decided to investigate human serum samples from confirmed leptospirosis cases for the presence of uPA and tPA PLG activators using commercial kits based on capture ELISA.

Serum samples at the beginning (MAT negative) or convalescent (MAT positive) phase of leptospirosis were employed. Samples from healthy donors were employed as comparative purpose. The results reveal that both uPA and tPA activators are present in higher quantity in serum samples of confirmed leptospirosis when compared to samples from healthy donors (Fig. 5A and 5B). The highest level for both activators was detected at the early phase of disease (MAT negative), suggesting that the bacteria induce the release of both uPa and tPA activators from the hosts during the first days of the illness. Very low levels of activators were detected from healthy donors, particularly in the case of uPA (Fig. 5A). To investigate whether the increase of activators on these samples was not only at concentration level but also on the activity, we measured uPA specific activity of each serum sample employed. The results show that an increase on uPA activity was observed with leptospirosis serum samples when compared to normal healthy donors (Fig. 5C). The uPA activity was higher at the beginning of the disease, MAT negative samples, reinforcing previous data on uPA concentration at this phase of the illness.

Zymography profile analysis of normal and leptospirosis human serum samples. The interaction of leptospires with the PLG activation system, PLG or PLG+uPA (PLA), has the capacity to promote an upregulation of MMP-9 from human endothelial cells (shown in Fig. 4). These results prompted us to investigate whether the MMPs activity and expression is altered during natural human leptospirosis by performing zymography analysis. Human serum sample (0.5 μ L) was applied onto SDS-PAGE co-polymerized with gelatin as a substrate for gelatinase (MMP)-activity proteases. After incubation in activation solution, the gels were coomassie blue stained, and the colorless bands represent regions where the substrate was degraded. The gelatin zymogram profile obtained for MAT negative and MAT positive of confirmed leptospirosis serum samples and of the healthy individuals (normal) is presented in Fig. 6A. The arrows indicate the visual intensity discrepancy between the healthy and leptospirosis serum samples, corresponding to the molecular mass of approximately 90 kDa (Fig. 6A), most probably of pro-MMP-9, reflecting higher degradation activity during the disease. We further confirmed these activity data by performing the same experiment but with collagen type I zymography. The results depicted in Fig. 6B revealed elevated levels of degradation in the same region (~90 kDa) with the leptospirosis serum samples compared with the serum from normal healthy donors. Additional control experiments were performed using the activation solution lacking CaCl_2 and ZnCl_2 , or with the addition of 25 mM EDTA. Under these conditions, no degradation bands were observed (data not shown),

indicating that the proteolytic activity was due to metal-dependent activity of the MMPs.

To further validate the data obtained by zymography experiments, we also performed MMP activity assays and analyzed total and active MMP-9 and MMP-2 in all serum samples. The results reveal an elevated level of total MMP-9 at the early stage of the disease, MAT negative serum samples, while this level tends to reach normal values during the convalescent phase (MAT positive) (Fig. 7A). In contrast, the amount of active MMP-9 was very low, and similar values were detected in normal and leptospirosis serum samples. The total MMP-9 concentration level observed among MAT negative samples corresponds to approximately 2.5 times the normal samples mean concentration (Fig. 7A). The total MMP-2 quantification analysis indicates that the activity of this protease is drastically reduced in both phases of leptospirosis, MAT negative and positive, when compared to normal healthy serum samples (Fig. 7B). The active MMP-2 levels during both phases of the disease were low and similar to the ones of human normal healthy serum (Fig. 7B).

Zymography analysis of leptospirosis and non-related infectious diseases serum samples. In view of the presence of gelatin/collagen enhanced degradation activity in leptospirosis serum samples (Fig. 6) in concert with the elevated levels total MMP-9 detected in MAT negative serum samples (Fig. 7A), we decided to investigate whether this elevated activity was restricted to leptospirosis or common to other infectious disease. We performed gelatin zymography and compared both phases of leptospirosis with dengue, malaria, HIV and Chagas disease human serum samples. Our data show a strong gelatinase activity present in serum samples of both phases of leptospirosis, pro-MMP-9 (indicated by the arrows), contrasting with the absence of degradation bands in all serum samples of other infectious diseases tested. The profile of the zymography obtained for dengue, malaria and Chagas are similar to the ones observed with the normal human serum samples used as controls (Fig. 6). HIV samples resulted in a different profile where some other degradation bands of lower mass than the pro-MMP-9 were evident.

DISCUSSION

L. interrogans is a highly invasive pathogen that promotes a rapid systemic infection after tissue penetration. The ability of *Leptospira* to penetrate through the monolayer appears to be associated with pathogenicity because pathogenic strain revealed a faster translocation than the nonpathogenic one [10]. Previous work in our laboratory has described the ability of *Leptospira* to capture PLG on the surface and to generate PLA in the presence of exogenous uPA activator [13]. Several outer membrane

leptospiral proteins with expected role in pathogenesis and virulence have been identified as PLG-binding receptors, suggesting that the interaction with the fibrinolytic system might be important during leptospirosis [14-17,23]. Indeed, the PLA-associated *Leptospira* renders the bacteria with proteolytic activity capable of degrading ECM components [13] that in turn, may help bacterial penetration and dissemination. Moreover, PLA-coated leptospires have also shown the ability to degrade IgG and C3b that could facilitate the bacterial immune evasion [18].

We focused the present study on the analysis of the effects of leptospiral PLG-binding and PLA-generation on the dissemination and activation of endothelial cells. We show that *Leptospira* with surface-associated PLG or active PLA-coated have an enhanced migration activity through endothelial cell barriers when compared to leptospires without the proteolytic system. It has been shown that the Lyme disease *B. burgdorferi* coated with PLA migrate faster thorough cell monolayers than the bacteria without the proteolytic activity [12]. As mentioned before, PLA-coated *Leptospira* have the capacity to degrade ECM components *in vitro* [13]. Moreover, there is no leptospiral secreted protease with ECM proteolytic activity reported to date. Therefore, the faster transposition of cellular barrier observed with *Leptospira* endowed with the PLG/PLA system is most probably due to the PLA proteolytic activity conferred to the bacteria. In fact, the PLG/PLA binding have been described to facilitate the keratinocyte invasion by *Streptococcus pyogenes* [24]. Furthermore, it has been reported that the fungal pathogen *Cryptococcus neoformans* coated with PLG promotes extracellular matrix invasion [25]. Surprisingly, the cellular transmigration efficiency of leptospires treated only with PLG and lacking uPA was higher than the untreated control. Previous studies in our laboratory have indicated that *Leptospira* do not have an endogenous mechanism for PLG activation [13]. We therefore hypothesized that the bacterial contact could stimulate the expression and/or secretion of PLG activators by the HUVECs, transforming the bound PLG into enzymatically active PLA. The increased level of uPA and tPA transcripts revealed that the expression of both activators is, in fact, upregulated when the HUVECs are co-incubated with the leptospires. Intriguingly, the upregulation of the activators was negligible after 6 h incubation (see Fig. 2), while the enhanced penetration was already detected after 4 h treatment (see Fig. 1). Thus, we assume that *Leptospira*-associated PLG was most probably activated in the early hours of incubation by release of tPA from the endothelial cells. Indeed, it is well documented that endothelial cells contain several populations of regulated secretory organelles, including the tPA organelle [26], and that the secretion of this activator is induced during injury or inflammation process [27].

Dengue virus was also reported to stimulate the release of tPA from human endothelial cells [28]. Accordingly, the higher availability of PLG activators could elicit the activation of the leptospiral-bound PLG to active enzyme, triggering PLA-mediated ECM proteolysis and promoting an enhanced migration activity.

Interestingly, serum samples of confirmed leptospirosis cases have higher amounts of both PLG activators available in circulation compared to normal serum samples. We were able to confirm that in the case of uPA, the activity in leptospirosis serum samples is also elevated, suggesting that there is no concomitant increase in uPA inhibitors, like PLG activator inhibitors PAI-1 and PAI-2. The fact that higher PLG activators levels were detected in MAT negative serum samples suggests that this activation is elicited at the beginning of the invasion process, thus increasing the PLA proteolytic activity generation that would be important during the leptospiral invasion.

MMPs are a family of metalloendopeptidase that degrade the protein components of ECM and therefore are essential for tissue remodeling during various physiological processes [29]. An imbalance of MMPs expression and activity regulation can lead to numerous pathologic events, such as microbial invasion, cancer progression, inflammation and tissue damage [30,31]. Indeed, it has been demonstrated that activated MMP-9 enhances the invasive phenotype of cultured cells by increasing their ability to degrade ECM [32]. MMPs are well-known to be direct and actively involved in the origin of pathology in some infections, as MMPs expression can be stimulated by a number of pathogens [22,30,33]. Furthermore, PLA can induce direct or indirectly MMPs expression, and is able to activate MMPs by the proteolytic cleavage of the pro-peptide domains that confer latency to the secreted pro-MMPs [22,34-36]. It has been suggested that the spirochete *B. burgdorferi* promoted an upregulation of MMPs mediating an activation cascade that is started by PLA-bound to the bacterial surface [22]. The incubation of leptospires with HUVECs stimulates an upregulation and secretion of MMP-9 by endothelial cells, an activity that could contribute to the disruption of ECM and endothelial tissues, favoring the penetration and invasion of the bacteria. It is possible that this cascade of events occurs during human leptospirosis infection because elevated levels of circulating total MMP-9, but not MMP-2, were detected in serum samples of confirmed leptospirosis cases. Interestingly, similar to the levels detected of PLG activators, MMP-9 concentration was higher with MAT negative serum samples, suggesting that the cascade of proteolytic activity generation takes place at the early stage of the illness. PLA-generated on the surface of bacteria seems to be associated to spirochete infection such as, Lyme disease, relapsing fever and leptospirosis because non-related infectious diseases appear to lack this activity. Further studies are needed to understand

the effects of the high levels of MMP-9 during human leptospirosis in addition to the facilitation of penetration and invasion of tissues.

CONCLUSIONS

The present work shows that the interaction of *L. interrogans* with the PLG/PLA fibrinolytic systems enhances the migration of the bacteria through endothelial monolayers cells. Moreover, *Leptospira*-bound PLG has the capacity to stimulate the release and expression of PLG activators from endothelial cells, thus promoting an increase generation of PLA-coated leptospires. Furthermore, this proteolytic system is capable to elicit an upregulation of MMP-9 from HUVECs. Altogether, our results suggest that leptospires associated with the fibrinolytic system can trigger a cascade of proteolytic activity initiated by PLA generation after the binding of PLG to the leptospiral surface. MMP-9 together with PLA-associated *Leptospira* may represent one possible mechanism that contributes to the invasion and the rapid dissemination of this organism.

Acknowledgements. This work has benefited from Grants from FAPESP, CNPq and Fundação Butantan. MLV has a Ph.D. scholarship from FAPESP (Brazil)

REFERENCES

- Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, et al. (2003) Leptospirosis: a zoonotic disease of global importance. *Lancet Infect Dis* 3: 757-771.
- Faine S, Adler B, Bolin C, Perolat P (1999) *Leptospira* and Leptospirosis. Second ed. Melbourne, Australia: MediSci. pp. 259.
- Ren SX, Fu G, Jiang XG, Zeng R, Miao YG, et al. (2003) Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. *Nature* 422: 888-893.
- Nascimento AL, Verjovski-Almeida S, Van Sluys MA, Monteiro-Vitorello CB, Camargo LE, et al. (2004) Genome features of *Leptospira interrogans* serovar Copenhageni. *Braz J Med Biol Res* 37: 459-477.
- Bulach DM, Zuerner RL, Wilson P, Seemann T, McGrath A, et al. (2006) Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proc Natl Acad Sci U S A* 103: 14560-14565.
- Picardau M, Bulach DM, Bouchier C, Zuerner RL, Zidane N, et al. (2008) Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PLoS One* 3: e1607.
- Adler B, Lo M, Seemann T, Murray GL (2011) Pathogenesis of leptospirosis: the influence of genomics. *Vet Microbiol* 153: 73-81.
- Cinco M (2010) New insights into the pathogenicity of leptospires: evasion of host defences. *New Microbiol* 33: 283-292.
- Evangelista KV, Coburn J (2010) *Leptospira* as an emerging pathogen: a review of its biology, pathogenesis and host immune responses. *Future Microbiol* 5: 1413-1425.
- Barocchi MA, Ko AI, Reis MG, McDonald KL, Riley LW (2002) Rapid translocation of polarized MDCK cell monolayers by *Leptospira interrogans*, an invasive but nonintracellular pathogen. *Infect Immun* 70: 6926-6932.
- Lahteenmaki K, Kuusela P, Korhonen TK (2001) Bacterial plasminogen activators and receptors. *FEMS Microbiol Rev* 25: 531-552.
- Coleman JL, Sellati TJ, Testa JE, Kew RR, Furie MB, et al. (1995) *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. *Infect Immun* 63: 2478-2484.
- Vieira ML, Vasconcellos SA, Goncalves AP, de Moraes ZM, Nascimento AL (2009) Plasminogen acquisition and activation at the surface of *Leptospira* species lead to fibronectin degradation. *Infect Immun* 77: 4092-4101.
- Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, et al. (2010) In vitro identification of novel plasminogen-binding receptors of the pathogen *Leptospira interrogans*. *PLoS One* 5: e11259.
- Oliveira R, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, et al. (2011) Characterization of novel OmpA-like protein of *Leptospira interrogans* that binds extracellular matrix molecules and plasminogen. *PLoS One* 6: e21962.
- Mendes RS, Von Atzingen M, de Moraes ZM, Goncalves AP, Serrano SM, et al. (2011) The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection. *Infect Immun* 79: 4657-4667.
- Domingos RF, Vieira ML, Romero EC, Goncalves AP, Moraes ZM, et al. (2012) Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions. *BMC Microbiol* 12: 50.
- Vieira ML, de Moraes ZM, Vasconcellos SA, Romero EC, Nascimento AL (2011) In vitro evidence for immune evasion activity by human plasmin associated to pathogenic *Leptospira interrogans*. *Microb Pathog* 51: 360-365.
- Jaffe EA, Nachman RL, Becker CG, Minick CR (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 52: 2745-2756.
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408.
- Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, et al. (1997) Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet* 17: 439-444.
- Gebbia JA, Coleman JL, Benach JL (2001) *Borrelia* spirochetes upregulate release and activation of matrix metalloproteinase gelatinase B (MMP-9) and collagenase 1 (MMP-1) in human cells. *Infect Immun* 69: 456-462.
- Verma A, Brissette CA, Bowman AA, Shah ST, Zipfel PF, et al. (2010) Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen. *Infect Immun* 78: 2053-2059.
- Siemens N, Patenge N, Otto J, Fiedler T, Kreikemeyer B (2011) *Streptococcus pyogenes* M49 plasminogen/plasmin binding facilitates keratinocyte invasion via integrin-integrin-linked kinase (ILK) pathways and protects from macrophage killing. *J Biol Chem* 286: 21612-21622.
- Stie J, Bruni G, Fox D (2009) Surface-associated plasminogen binding of *Cryptococcus neoformans*

- promotes extracellular matrix invasion. PLoS One 4: e5780.
26. Knipe L, Meli A, Hewlett L, Bierings R, Dempster J, et al. (2010) A revised model for the secretion of tPA and cytokines from cultured endothelial cells. Blood 116: 2183-2191.
 27. Oliver JJ, Webb DJ, Newby DE (2005) Stimulated tissue plasminogen activator release as a marker of endothelial function in humans. Arterioscler Thromb Vasc Biol 25: 2470-2479.
 28. Huang YH, Lei HY, Liu HS, Lin YS, Chen SH, et al. (2003) Tissue plasminogen activator induced by dengue virus infection of human endothelial cells. J Med Virol 70: 610-616.
 29. Stamenkovic I (2003) Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol 200: 448-464.
 30. Elkington PT, O'Kane CM, Friedland JS (2005) The paradox of matrix metalloproteinases in infectious disease. Clin Exp Immunol 142: 12-20.
 31. Sbardella D, Fasciglione GF, Gioia M, Ciaccio C, Tundo GR, et al. (2012) Human matrix metalloproteinases: an ubiquitous class of enzymes involved in several pathological processes. Mol Aspects Med 33: 119-208.
 32. Ramos-DeSimone N, Hahn-Dantona E, Sipley J, Nagase H, French DL, et al. (1999) Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. J Biol Chem 274: 13066-13076.
 33. Vanlaere I, Libert C (2009) Matrix metalloproteinases as drug targets in infections caused by gram-negative bacteria and in septic shock. Clin Microbiol Rev 22: 224-239, Table of Contents.
 34. Liotta LA, Goldfarb RH, Brundage R, Siegal GP, Terranova V, et al. (1981) Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane. Cancer Res 41: 4629-4636.
 35. Liotta LA, Goldfarb RH, Terranova VP (1981) Cleavage of laminin by thrombin and plasmin: alpha thrombin selectively cleaves the beta chain of laminin. Thromb Res 21: 663-673.
 36. Lahtenmaki K, Edelman S, Korhonen TK (2005) Bacterial metastasis: the host plasminogen system in bacterial invasion. Trends Microbiol 13: 79-85.

LEGENDS TO FIGURES

Figure 1. Penetration of HUVECs monolayers by leptospires. *L. interrogans* serovar Pomona strain LP was treated with **PLG**, **PLG+uPA (PLA)** or only with **PBS**. The HUVECs monolayers previously seeded above the transwell chamber polycarbonate membrane were inoculated with the leptospires (2.5×10^8). After 4h, the leptospires present in the lower chamber were recovered and counted by dark field microscopy. The bars represent the media $\pm$ standard deviation of two independent counts of each experimental duplicate. The data are representative of three independent experiments. * = $P < 0.0025$ and ** = $P < 0.03$.

Figure 2. *L. interrogans* upregulates the transcription of uPA (A) and tPA (B) from HUVECs. Primary cultures of HUVECs were incubated for 6 or 24 h with *L. interrogans* serovar Pomona without treatment (**PBS**), or treated with **PLG**, **uPA** or **PLG+uPA (PLA)**. As negative control, HUVECs were incubated only with the culture medium, without bacteria (**RPMI**). The HUVECs' total RNA was extracted, and with the cDNAs as template, real time PCR was employed for CT analysis and fold change calculation relative to the HUVECs incubated in the absence of bacteria, for 6 h. The bars represent the range of the relative fold change from triplicates, calculated as described in M&M. The data are representative of two independent experiments.

Figure 3. Gelatin zymography analysis of MMPs released into the culture supernatants of HUVECs in response to incubation with *Leptospira*. Primary cultures of HUVECs were incubated for 6 or 24h with *L. interrogans* serovar Pomona without treatment (**PBS**), or treated with **PLG**, **uPA** or **PLG+uPA (PLA)**. As negative control, HUVECs were incubated only with the culture medium, without bacteria (**HUVECs**). **HT1080** supernatants were employed as positive controls for pro-MMP-2 (~72 KDa) and pro-MMP-9 (~92 KDa) constitutive release. The samples were separated by 8% SDS-PAGE co-polymerized with 0.1% gelatin. After overnight incubation in activation solution, the gels were stained with coomassie blue. The clear bands represent the regions where the gelatin substrate was degraded. The degradation bands of **pro-MMP-2**, **intermediate MMP-2**, **active MMP-2**, **pro-MMP-9** and **active MMP-9** are indicated.

Figure 4. Analysis of MMP-9 and MMP-2 levels released into the culture supernatants of HUVECs in response to incubation with *Leptospira*. Primary cultures of HUVECs were incubated for 6 or 24h with *L. interrogans* serovar Pomona without treatment (**PBS**), or treated with **PLG**, **uPA** or **PLG+uPA (PLA)**. As negative control, HUVECs were incubated only with the culture medium, without bacteria (**RPMI**). The cleared cell culture supernatants were evaluated for MMP-9 (**A**) or MMP-2 (**B**) activity by commercial ELISA kit. Total MMP-9 and MMP-2 were achieved by APMA (M&M) activation of the pro-MMPs. The bars represent the media of duplicates from two independent experiments $\pm$ the standard deviation. (**C**) The cDNA obtained by extraction of the HUVECs' total RNA was subjected to real time evaluation for MMP-9 gene transcription. The data were normalized according to ACTB constitutive control. The bars represent the range of the relative fold change from triplicates, calculated as described in M&M. The data are representative of two independent experiments.

Figure 5. Measurement of PLG activators concentration and uPA activity in normal and confirmed leptospirosis serum samples. Paired serum samples of confirmed leptospirosis individuals, in the initial (MAT negative) or convalescent (MAT positive) phase, or serum samples from healthy donors (Normal), were quantified for the presence of uPA (A) or tPA (B) by specific capture ELISA kits. The quantification was based on uPA and tPA standard curves. The symbols represent the individual dosage of each sample and the horizontal bars, the medium. In (C) are the measurements of uPA activity by specific activity test. Plotted in the graphic is the activity determinate of each serum sample. The horizontal bars represent the mean of the serum samples for each group.

Figure 6. Zymography profile of healthy and leptospirosis serum samples. Human serum samples of healthy donors (Normal) and leptospirosis paired serum samples at early (MAT negative) and convalescent (MAT positive) phase of the disease were separated by 8% SDS-PAGE co-polymerized with 0.1% gelatin (A) or type I collagen (B). After incubation in activation solution, the gels were coomassie blue stained. The clear bands represent the regions where the substrate was degraded. The arrows indicate the regions with most pronounced difference in degradation between patients and normal human sera samples. M: molecular mass marker, 1-9: individual serum samples.

Figure 7. Evaluation of total amount and enzymatic activity of MMP-9 and MMP-2 from human serum samples. Human serum samples from healthy donors (Normal), leptospirosis serum samples at the initial (MAT -) and convalescent phase (MAT +) phases were quantified for total contents and enzymatic activity of MMP-9 and MMP-2. Total or enzymatic activity of MMP-9 or MMP-2 was achieved by APMA activation of the pro-MMPs. Bars represent the means of the samples (n=8) quantified in duplicates $\pm$ the standard deviation. * $P<0,02$.

Figure 8. Comparison of zymography profile from confirmed leptospirosis and other infectious diseases serum samples. In (A) Leptospirosis serum samples at the initial (lanes 1 and 2) or convalescent phase (lanes 3 and 4), and unrelated infectious diseases sera: Dengue (lanes 5-7), Malaria (lanes 8-10); In (B), HIV (Human Immunodeficiency Virus) (anes 5-7) and Chagas' disease (lanes 8-10). The samples were separated by 8% SDS-PAGE co-polymerized with 0.1% gelatin, incubated in activation solution and stained by coomassie blue. The clear bands represent the regions where the substrate was digested. The arrows indicate the regions with most pronounced difference in degradation between Leptospirosis samples in comparison to the other diseases.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

OmpL1 is an extracellular matrix- and plasminogen- interacting protein of *Leptospira* spp.

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Leptospirosis is a zoonosis of multisystem involvement caused by pathogenic strains of the genus *Leptospira*. Surface proteins that are capable of mediating host-pathogen interactions are important for understanding the bacterial pathogenesis as well as to identify protective antigens. In this work we describe that the protein OmpL1 is a novel leptospiral adhesin and a plasminogen (PLG) receptor. The recombinant protein was expressed in *E. coli* BL21 (DE3) Star pLyS, as inclusion bodies, refolded and purified in metal chelating chromatography. The protein presented a typical β -strands secondary structure as evaluated by circular dichroism spectroscopy. The recombinant protein reacted with antibodies in serum samples of convalescent leptospirosis patients with high specificity when compared to serum samples from individuals with unrelated diseases. These data strengthen the usefulness of OmpL1 as a diagnostic marker of leptospirosis. Characterization of recombinant OmpL1 in immunized BALB/c mice showed that the protein has the capacity to elicit humoral and cellular immune responses, as denoted by high antibody titers and proliferation of lymphocytes. We demonstrate that OmpL1 has the ability to mediate the attachment to laminin and plasma fibronectin, with a K_D value of 2099.93 ± 871.03 nM and 1239.23 ± 506.85 nM, respectively. OmpL1 is also a PLG- receptor, with a K_D of 368.63 ± 121.23 nM, capable of generating enzymatically active plasmin. This is the first report that shows and characterizes OmpL1 as an adhesin and PLG-binding protein of *Leptospira* spp. that being expressed during infection may play a role in the bacterial pathogenesis.

Leptospirosis is been considered an important re-emerging infectious disease. It is a zoonosis caused by pathogenic *Leptospira* spp. that is transmitted from reservoir hosts to humans through water and soil contaminated with their urine [1]. Symptoms of the disease include fever, vomiting, headache, diarrhea, abdominal and generalized muscle pain. Progression to multi-organ system complications, known as Weil's syndrome, occurs in 5-15% of cases, with mortality rates of 5-40% [1,2,3,4].

At present, available vaccines are based on inactivated whole cell or membrane preparations of pathogenic leptospires. They confer protective responses mostly through the induction of antibodies against leptospiral lipopolysaccharide [5,6]. These vaccines, however, do not induce long-term protection against infection and do not provide cross-protective immunity against leptospiral serovars not included in the vaccine preparation [6]. Due to the large number of leptospiral serovars [7], conserved and protective antigens are being pursued.

Surface exposed proteins are potential targets for inducing immune responses during infection and may also mediate the initial adhesion process to host cells. Indeed, several of these proteins have been reported as leptospiral adhesins [8,9,10,11,12,13]. Furthermore, well conserved outer membrane proteins

(OMPs) are promising vaccine targets, because they would have the advantage in inducing cross-protective immunity [14]. After adherence, pathogens have to overcome host tissue barriers to reach blood circulation and organs. We have reported that leptospires bind PLG at their surface and that proteolytic activity is achieved due to plasmin generation [15].

OmpL1 is a 31 kDa leptospiral transmembrane OMP, containing 320 amino acid residues, characterized by Haake and colleagues [16]. Several studies have pointed out the importance of OmpL1 as a serological marker for the diagnosis of human [17,18,19,20,21,22] and canine leptospirosis [23,24].

This work describes the expression and characterization of recombinant OmpL1, the immune response induced in BALB/c mice and the reactivity of this protein with leptospirosis positive serum samples. We also describe the ability of OmpL1 to mediate the attachment to various extracellular matrix (ECM) and serum components. We report that OmpL1 is a novel surface adhesin that binds to laminin and plasma fibronectin, interacts with PLG, is expressed during infection [25] and may participate in the leptospiral pathogenesis.

MATERIAL AND METHODS

Bacteria strains and serum samples. The pathogenic high passage non-virulent *Leptospira* strains used were *L. interrogans* serovar Canicola strain Hound Utrech IV, *L. interrogans* serovar Copenhageni strain M 20, *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. interrogans* serovar Pomona strain Pomona, *L. borgpetersenii* serovar Castellonis strain Castellon 3, *L. borgpetersenii* serovar Whitcombi strain Whitcomb, *L. kirshneri* serovar Grippotyphosa strain Moskva V, *L. kirshneri* serovar Cynoptery strain 3522C, *L. santarosai* serovar Shermani strain 1342 K, *L. noguchii* serovar Panama strain CZ 214 and the non-pathogenic *L. biflexa* serovar Patoc strain Patoc. The leptospire were cultured at 28°C under aerobic conditions in liquid EMJH medium (Difco®) with 10% rabbit serum, enriched with L-asparagine (wt/vol: 0.015%), sodium pyruvate (wt/vol: 0.001%), calcium chloride (wt/vol:0.001%), magnesium chloride (wt/vol: 0.001%), peptone (wt/vol:0.03%) and meat extract (wt/vol: 0.02%) [26]. Low passage, virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 and *L. interrogans* serovar Pomona strain LPF were also used. *Leptospira* cultures are maintained in Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil. Serum samples of confirmed leptospirosis hamsters were obtained by experimental infection with virulent leptospire. Confirmed- leptospirosis human serum samples were from Instituto Adolfo Lutz collection, São Paulo, Brazil. Serum samples from patients with unrelated infectious diseases were obtained from serum collection of Laboratório Imunoepidemiologia, SUCEN, SP, Brazil; Laboratório Protozoologia, IMT/USP, Brazil, Laboratório Virologia, IMT/USP, Brazil and Nucleo Estudos em Malária, SUCEN/IMT/USP, Brazil. *E. coli* DH5α was used as the cloning host strain and *E. coli* BL21 (DE3) Star pLyS [27] was used as the host strain for the expression of the recombinant protein.

Cloning, expression and purification of recombinant OmpL1. Amplification of the OmpL1 gene was performed by PCR from *L. interrogans* serovar Copenhageni strain M20 genomic DNA using the primer pairs F: 5'GGATCCAAAACATATGCAATTGTAGG and R: 5'GGTACCTTAGAGTTCGTGTTTATAACC. Gene sequence was amplified without the signal peptide tag, which was determined by software SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>). PCR fragment was cloned into pGEM-T easy vector (Promega) and subcloned into the *E. coli* expression vector pAE [28] at *Bam*HI and *Kpn*I restriction sites. The plasmid pAE-OmpL1, containing the correct DNA sequence, was used to transform *E. coli* BL21 (DE3)

Star pLyS. Protein expression was obtained by inoculating 10 mL of a culture grown overnight in 800 mL of Luria-Bertani medium containing 50µg/mL ampicillin and 34µg/mL chloranfenicol. The culture was grown with continuous shaking at 37 °C to an optical density 0.6 at 600 nm and then induced for 3 h under constant agitation at 37 °C in the presence of 0.1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). The cells were harvested by centrifugation, the bacterial pellet was resuspended in sonication buffer (20mM Tris-HCl, pH 8.0, 200mM NaCl, 200 mg/mL lysozyme, 2mM PMSF and 1% Triton X-100) and lysed on ice with the aid of a sonicator tip (Ultrasonic Processor, GE Healthcare). The insoluble fraction was resuspended in a buffer containing 20mM Tris-HCl, pH 8.0, 500 mM NaCl, 8M urea and 1mM β-mercaptoethanol. The protein was purified through Ni²⁺-charged beads of chelating fast-flow chromatographic column (GE Healthcare), extensively washed with buffer containing 20mM Tris-HCl, pH 8.0, 500mM NaCl and increasing imidazole concentrations (5-60mM). Bound protein was eluted with buffer containing 1M imidazole. The efficiency of the purification was evaluated by 12% SDS-PAGE. Fractions containing the recombinant protein were extensively dialyzed against PBS (phosphate-buffered saline pH 7.4) containing 0.1% glycine for 24 h.

Circular Dichroism spectroscopy. Purified recombinant protein was dialyzed against sodium phosphate buffer (pH 7.4). Circular dichroism (CD) spectroscopy measurements were performed at 20°C using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo) equipped with a Peltier unit for temperature control. Far-UV CD spectra were measured using a 1 mm-path-length cell at 0.5 nm intervals. The spectra were presented as an average of five scans recorded from 185 to 260 nm. The residual molar ellipticity is expressed in degree X centimeter per decimole. Spectra data was submitted to K2D2 software, <http://ogic.ca/projects/k2d2>, using the method that calculated the secondary structure content from the ellipticity experimental data [29].

Microscopic agglutination test (MAT). The microscopic agglutination test was performed according to Faine et al. [30]. In brief, an array of 22 serovars of *Leptospira* spp. as antigens were employed: Australis, Autumnalis, Bataviae, Canicola, Castellonis, Celledoni, Copenhageni, Cynopteri, Djasiman, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc, Pomona, Pyrogenes, Sejroe, Shermani, Tarassovi and Wolffi. All the strains were maintained in EMJH liquid medium (Difco, USA) at 29 °C. A laboratory-confirmed case of leptospirosis was defined by the demonstration of a four-fold microagglutination

titer rise between paired serum samples. The probable predominant serovar was considered to be the one with the highest dilution that could cause 50 % of agglutination. MAT was considered negative when the titer was below 100.

Antiserum production against recombinant protein and isotype determination. The recombinant protein was mixed with 10 % (vol/vol) of Alhydrogel (2 % Al(OH)₃, Brenntag Biosector, Denmark), used as adjuvant. Ten micrograms of OmpL1 were subcutaneously administered in five female BALB/c mice (4-6 weeks old). Two subsequent booster injections were given at two-week intervals with the same protein preparation. Negative control mice were injected with PBS. Two weeks after each immunization, the mice were bled from the retro-orbital plexus and the resulting sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for determination of antibody titers. Prior to experiments, anti-recombinant protein sera were adsorbed to a suspension of *E. coli* to avoid the reactivity with anti-*E. coli* antibodies [31]. For isotype determination, total IgG (immunoglobulin G), IgG1 and IgG2a were measured by incubation of the pooled mice sera with recombinant protein followed by incubation with horse radish peroxidase (HRP)-conjugated anti-mouse total IgG or goat anti-mouse IgG1 or IgG2a (1:2,000) followed by incubation with HRP-conjugated anti-goat IgG (1:10,000). The OD492 nm values exhibited by different dilution of mice sera were compared to a curve generated by coating the plates with different concentrations of mice total IgG, IgG1 or IgG2a. All animal studies were approved by the Ethics Committee of the Instituto Butantan, São Paulo, SP, Brazil.

Immunoblotting assay. The purified recombinant protein was loaded into 12% SDS-PAGE and fractioned proteins were transferred into nitrocellulose membranes (Hybond ECL; GE Healthcare) in semi-dry equipment. Membranes were blocked with 10% non-fat dry milk, 1% BSA in PBS containing 0.05% Tween 20 (PBS-T) and then incubated with anti-OmpL1 (1:1,600) mouse polyclonal serum or monoclonal anti-his tag antibodies (1:1,000) (GE Healthcare) for 2 h at room temperature. After washing, the membranes were incubated with HRP-conjugated anti-mouse IgG (1:5,000; Sigma) in PBS for 1 h. The protein reactivity was revealed by ECL reagent kit chemiluminescence substrate (GE Healthcare); the luminescence generated by the reaction was detected with the aid of Carestream Molecular Imaging (Carestream Health, Inc.) connected to Gel Logic 2200PRO.

OmpL1 genomic DNA and protein conservation among leptospiral strains. Leptospiral genomic DNAs

were extract as previously described [12] and sample integrity was verified by amplification of 16S ribosomal DNA. Cloning primers of OmpL1 were used for DNA amplification of *Leptospira* spp. Fragments were visualized in 1% agarose gel. For protein conservation, cultures of *Leptospira* spp. were harvested by centrifugation, washed three times with PBS low-salt containing 5mM MgCl₂ and resuspended in PBS. A sample of each strain was applied onto a 12% SDS-PAGE and after separation, proteins were transferred into nitrocellulose membrane on a semidry equipment (GE Healthcare). The membrane was blocked overnight at 4 °C with 10% nonfat dry milk, 1% BSA in PBS-T. After three washings with PBS-T, the membrane was incubated for 3 h at room temperature with the antiserum against OmpL1 (1:500) produced in mice, followed by washings and incubation with HRP-conjugated goat anti-mouse IgG (1:5,000) for 1 h at room temperature. The membrane was then washed and the reactivity was revealed as described above;

ELISA for detection of human and hamsters antibodies. Human and hamster IgG antibodies against OmpL1 were detected by ELISA as previously described [32]. In brief, negative and positive MAT serum samples from 32 confirmed-leptospirosis patients and 44 experimentally infected hamsters, were diluted (1:100) and evaluated for total IgG using HRP-conjugated anti-human IgG antibodies, 1:5,000 (Sigma, USA), and total IgG using HRP-conjugated anti-hamster IgG antibody, 1:5,000. Cut-off values were set at three standard deviations above the mean OD492 nm of sera from 10 healthy individuals from the city of São Paulo, Brazil, or 5 non-infected hamsters. Reaction specificity was evaluated with serum samples from patients diagnosed with unrelated infection diseases, as follows: dengue (n=13), malaria (n=12), HIV infection (n=15) and Chagas' disease (n=20). The sera were used at 1:100 dilutions and the values for specificity were determined by the method of Galen and Gambino [33]

Lymphoproliferation assay. At the end of the immunization protocols, BALB/c mice were sacrificed their spleens were aseptically removed and suspended in RPMI (Roswell Park Memorial Institute Medium- RPMI-1640 medium containing 2mM L-glutamine, 100 IU/mL of Penicillin, 100 µg/mL of Streptomycin and 50 µg/mL of Gentamycin). Spleen were macerated and after erythrocytes lysis splenocytes were resuspended in 1 mL of RPMI containing 10% fetal bovine serum and counted after staining with 0.4% trypan blue for viability. Splenocytes were plated in triplicate at 5x10⁵ cells/well of 96-well flat bottom cell culture plates (Costar, Corning). Spleen cells were stimulated with 5 µg/mL of ConA (Sigma), employed as positive control, 5µg/mL of OmpL1 or medium alone, used as negative

control. Cells were cultured for 48 h at 37 °C and 5% CO₂ in a humidified atmosphere and proliferative rates were determined as a function of DNA synthesis, measured by the incorporation of bromodeoxyuridine (BrdU) by BrdU ELISA colorimetric kit (Roche Diagnostic, Indianapolis, IN). Cells were labeled for 2 h with 20 µL of BrdU labeling solution. Subsequently, anti-BrdU conjugated with HRP (1:100) was added and incubated for 90 min. The enzyme substrate solution was added and incubation was carried at room temperature for 15 min. The enzymatic reaction was stopped by adding 1M H₂SO₄ and the optical density was measured at 450 nm in a microplate reader. Stimulation Index (S.I.) was calculated as the ratio between the mean OD of cells cultured with the antigen and the mean OD of cells cultured in medium only, in the immunized group.

Evaluation of cytokine production. For analysis of secreted cytokines, spleen cells were isolated and cultured as described above, except that the culture was made in 24 well tissue culture plates, each well containing 5x10⁶ cells. After 48 h, cell-free culture supernatants were collected and stored for short-term at -20 °C. IL-4, IL-10, IFN-gamma and TNF-alpha were measured by ELISA (PreproTech) according to the manufacturer's instructions. Briefly, capture antibody was added to each well and the plates were incubated overnight at 4 °C, followed by incubation with blocking buffer for 1h. Standard cytokines were diluted to create a concentration curve. Then, 100 µL of each supernatant sample were added and plates were incubated for at least 2h. After that, antibody detection solution was added (1:2,000) followed by 2h of incubation; substrate was added to each well and incubation proceeded at room temperature for reaction development; readings were taken at 405 nm with wavelength correction set at 650 nm in an ELISA plate reader. Results are expressed in pg/mL.

Binding of OmpL1 to ECM and to serum components. Macromolecules, including the control protein fetuin, were purchased from Sigma Chemical Co. (St. Louis, Mo.). Laminin-1 and collagen Type IV were derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma, cellular fibronectin was derived from human foreskin fibroblasts, elastin was derived from human aorta, plasma fibronectin, human complement serum and fibrinogen were isolated from human plasma and collagen Type I was isolated from rat tail. ECM gel is composed primarily of laminin, collagen type IV, heparan sulfate proteoglycan and entactin. PLG native, purified from human plasma, was purchased from Calbiochem. Human Factor H was from Calbiochem, complement mix was from Sigma and C4bp from

Complement technology. Protein attachment to individual macromolecules of the extracellular matrix and serum components was analyzed according to a previously published protocol [8]. Briefly, ELISA plates (Costar High Binding, Corning) were coated with 1 µg of component or bovine serum albumin (BSA, negative control), gelatin and fetuin (highly glycosylated attachment-negative control protein) in 100 µL of PBS for 3 h at 37 °C, and then blocked overnight at 4° C. One µg of OmpL1 was added per well in 100 µL of PBS, and protein was allowed to attach to the different substrates for 2 h at 37 °C. After washing six times with PBS-T, bound proteins were detected by adding an appropriate dilution of mouse antiserum that gives an OD_{492 nm} value of 1 determined by titration in 100 µL of PBS (1:1,600). Incubation proceeded for 1 h at 37 °C, and after three washings with PBS-T, 100 µL of a 1:5,000 dilution of HRP-conjugated goat anti-mouse IgG in PBS was added per well and incubated for 1 h at 37 °C. The wells were washed three times, and o-phenylenediamine (1 mg/mL) in citrate phosphate buffer (pH 5.0) plus 1 µL/mL H₂O₂ was added (100 µL per well). The reaction was allowed to proceed for 10 min and interrupted by the addition of 50 µL of 8 M H₂SO₄. Readings were taken at 492 nm in a microplate reader (Multiskan EX; Labsystems Uniscience). For statistical analyses, the binding of recombinant proteins to ECM macromolecules was compared to its binding to all three negative controls used by Student's two-tailed t test, and *P* value was given related to comparison to gelatin, used as negative control for the following experiments. Bindings were also confirmed by using HRP-conjugated anti-his tag monoclonal antibodies, previously titrated against the recombinant protein and used in a dilution that generates an OD_{492 nm} value of approximately 1.

Dissociation equilibrium constant (*K_D*) for the OmpL1 binding to ECM and to serum components. First, ELISA plates were coated with 100µl of 10 µg/mL ECM or serum components and allow to adhere for 3h at 37 °C. Plates were then blocked overnight and increasing concentrations of the purified OmpL1 were added (100 µl/well in PBS) and incubated for 2 h at 37 °C. The assessment of bound protein was performed by incubation for 1 h at 37 °C with the antiserum raised against the protein at the dilution of 1:1,600, followed by HRP-conjugated goat anti-mouse IgG (Sigma) (1:5,000 in PBS). The ELISA data were used to calculate the dissociation constant (*K_D*) according to the method previously described [34] based on the equation: $A = A_{max} [\text{protein}] / (K_D + [\text{protein}])$, where *A* is the absorbance at a given protein concentration, *A_{max}* is the maximum absorbance for the ELISA plate reader (equilibrium), *[protein]* is the protein concentration and *K_D* is the dissociation equilibrium

constant for a given absorbance at a given protein concentration (ELISA data point).

Characterization of the binding of OmpL1 to laminin and to PLG. In order to evaluate sugar moiety contribution to the laminin-OmpL1 interaction, microplates were coated with 1 µg of laminin in 50 mM sodium acetate buffer, pH 5.0, and incubated for 16 h at 4°C. Wells were washed three times with the same buffer, and immobilized laminin was treated with different sodium metaperiodate concentrations (5 to 100 mM) for 15 min at 4°C in the dark. After three washes with 50 mM sodium acetate buffer, wells were blocked with 200 µl of PBS-T 10% (w/v) non-fat dry milk for 2 h at 37°C. Binding of OmpL1 (1 µg in PBS per well) to periodate-treated laminin was assessed as described above. To determine the role of lysines in PLG-recombinant protein interactions, the lysine analog 6-aminocaproic acid (ACA) (Sigma-Aldrich) was added together with the recombinant protein at a final concentration of 2 mM or 20 mM to the PLG-coated wells. The detection of bound protein was performed as previously described.

Plasmin enzymatic activity assay. 96-well ELISA plates were coated overnight with 10 µg/mL recombinant protein, or BSA and Lsa63 [35], for negative control, in PBS at 4°C. Plates were washed once with PBS-T and blocked for 2 h at 37°C with PBS with 10% (wt/vol) non-fat dry milk. The blocking solution was discarded and 100 µl/well of 10 µg/mL human PLG was added followed by incubation for 2 h at 37°C. Wells were washed three times with PBS-T, and then 4 ng/well of human uPA (Sigma-Aldrich) was added. Subsequently, 100 µl/well of plasmin-specific substrate D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride (Sigma-Aldrich) was added at a final concentration of 0.4 mM in PBS. Plates were incubated overnight at 37°C and substrate degradation was measured by taken the readings at 405 nm.

Binding interference of OmpL1 with ECM and PLG. 96-well plates were coated overnight at 4°C with 100 µl of 10 µg/mL laminin, plasma fibronectin or PLG in PBS, washed three times and blocked with 10% (w/v) non-fat dry milk for 2 h at 37 °C. Concomitantly, OmpL1 (1 µg in 100µL of PBS) was incubated with increasing concentrations (0-1 µg in 100µL of PBS) of the other components separately, and then transferred to the coated plates, which was then incubated for 2 h at 37 °C. After four washings, recombinant protein binding was quantified by specific antibodies as described above.

Inhibition of live leptospires binding to laminin, plasma fibronectin and PLG by OmpL1. ELISA

plates were coated with laminin, plasma fibronectin or PLG (1 µg/well). The plates were washed and blocked with 10% non-fat dry milk in PBS-T for 2 h at 37°C. The blocking solution was discarded, and the wells were incubated for 2 h at 37°C with increasing concentrations of recombinant protein (0 to 1.5µM). After three washings, 100 µL/well of 4×10^7 live *L. interrogans* serovar Copenhageni strain M20 were added for 90 min at 37°C. The unbound leptospires were washed and the quantification of bound leptospires was performed indirectly by anti-LipL32 antibodies produced in mice (1:4,000), due to the fact that LipL32 is a major outer membrane leptospiral protein [36] the procedure was followed by HRP-conjugated anti-mouse IgG antibodies, essentially as described in Barbosa et al.[8]. The detection was performed by OPD as previously described.

RESULTS

Cloning, expression and purification. Oligonucleotides for PCR amplification (see Materials and Methods) were designed based on genome sequences of *L. interrogans* serovar Copenhageni, excluding the signal peptide. The amplified coding sequence was cloned into an *E. coli* pAE vector [28] and the protein was expressed with 6XHis tag at the N-terminal. Recombinant protein was expressed in the bacterial pellet, in its insoluble form, as inclusion bodies (Fig. 1A, lane 4). Protein was recovered from inclusion bodies after solubilization with 8M urea. The purification was performed by metal chelating chromatography after refolding for removal of urea and was evaluated by SDS-PAGE, as shown in Fig. 1A, lane 6. The recombinant protein bands were confirmed by western blotting probed with polyclonal antibodies raised in mice against OmpL1 (Fig. 1B, lane 1) and with anti-His tag monoclonal antibodies (Fig.1B, lane 3). The calculated 32.4 kDa molecular mass of the recombinant protein OmpL1 comprise the vector fusion plus the encoded amino acids sequence. Structural integrity of the purified protein was assessed by circular dichroism (CD) spectroscopy. As depicted in Fig. 1C, CD spectrum shows the minima at 215-220 nm, and the maximum at approximately 196 nm, typical of β-strands secondary structure content in the recombinant protein. The OmpL1 β-strands predominance was also confirmed by analysis of the generated spectrum by K2D2 software, which showed 34% of β-strand [29].

Genomic DNA and protein conservation among leptospiral strains. Genomic DNA integrity was confirmed by amplification of 16S DNA. A single band corresponding to OmpL1 gene was observed in all pathogenic strains tested, while no band was found in the saprophytic *L. biflexa* genomic DNA (Fig. 2A).

Protein expression and conservation among *Leptospira* strains was assessed with total protein extracts from the same pathogenic, high passage, non-virulent strains of *Leptospira*, used for genomic DNA conservation, the saprophytic strain *L. biflexa* serovar Patoc and the virulent, low-passage strains, *L. interrogans* serovars Copenhageni and Pomona. Cell extracts were gel fractionated, proteins membrane transferred and western blotting analysis was performed by probing the membranes with polyclonal serum from mice immunized with OmpL1. The serological reactivity showed conservation of OmpL1 coding sequences in the main serovars of *L. interrogans* (Fig. 2B). However, with two serovars of *L. borgpetersenii* only weak protein bands were detected (Fig. 2B). No protein band was detected with *L. santarosai* serovar Shermani and with the saprophytic *L. biflexa* strain. Reactivity was also detected with both *L. interrogans* virulent strains employed.

Evaluation of immunological response elicited in mice by OmpL1. The antibody immune response induced by OmpL1 in mice was analyzed by ELISA in a quantitative basis. As depicted in Fig. 3A, antibodies IgG1 and IgG2a against OmpL1 were found, with a predominance of IgG1 isotype. Statistically significant value for lymphocyte proliferation was achieved with animals immunized with the OmpL1, stimulated with the recombinant protein ($P<0.05$) (Fig. 3B), having an average stimulation index of 2.8. High proliferation level was obtained when cells were treated with ConA, employed as positive control of the experiment (not shown). Addition of OmpL1 to lymphocytes from animals that have not been primed with the recombinant protein produced non-significant levels of proliferation (data not shown). Supernatants of cultured spleen cells from OmpL1 immunized mice were analyzed for the presence of cytokines, IL-10, IFN- γ TNF- α and IL-4. The cytokine profiles obtained showed a predominance of IL-10 and IFN- γ , both occurring in high levels (Fig. 3C and 3D), similar to the ones of the ConA positive control (not shown). A statistically significant cytokine level was obtained in the OmpL1-primed group when cells were stimulated with OmpL1 ($P<0.001$ for IL-10 and $P<0.0001$ for IFN- γ); lower value, although statistically significant, was observed with TNF- α level ($P<0.05$) when the cells from OmpL1-primed group were induced or not with the recombinant protein (Fig. 3E). In contrast, no IL-4 stimulation was achieved when comparing spleen cells from animal groups under the same experimental conditions (Fig. 3F). Measurements of the same parameters with spleen cells from control animals immunized with PBS either stimulated or not with the recombinant protein produced negligible results (not shown).

Reactivity of OmpL1 with human and hamster leptospirosis serum samples. We have performed ELISA to analyze whether the OmpL1 is recognized by antibodies present in confirmed leptospirosis cases from humans and experimentally infected hamsters. We have employed 32 paired human samples of early (MAT negative) and convalescent (MAT positive) phases of the disease and 44 samples of MAT-confirmed serum from hamsters. Our data show that the recombinant protein was able to recognize specific IgG antibodies in 90% of infected hamster's tested sera (Fig. 4A). High level of responders (75%) was obtained for IgG in human MAT positive sera while a total of 9% of serum samples presented IgG antibodies against OmpL1 in the early phase of the disease (MAT negative) (Fig. 4B). The cutoff value calculated with healthy human sera was 0.374. Due to the non-specific clinical symptoms of leptospirosis, we analyzed the reactivity of the recombinant protein OmpL1 with serum samples from patients with other febrile illnesses, including dengue (n=13), malaria (n=12), Chagas' disease (n=20), and HIV infection (n=15). The reactivity obtained with OmpL1 and these serum samples was similar to the ones obtained with serum samples from healthy donors (Fig. 4B). The specificity of OmpL1 was calculated to be 100% for all unrelated diseases tested, except for dengue, to which the specificity was calculated to be 92.3%

Adhesion of OmpL1 to ECM components. As native OmpL1 has surface-exposed domains [16,37], we decided to evaluate whether this protein could mediate host colonization by adhering to extracellular matrix proteins. Thus, laminin, collagen Type I, collagen Type IV, cellular fibronectin, plasma fibronectin, ECM gel, elastin and the control proteins gelatin, BSA and fetuin were immobilized on 96-wells microdilution plates and recombinant protein attachment was assessed by ELISA, as previously described [8]. As shown in Fig. 5A, OmpL1 protein exhibited statistically significant adhesiveness to laminin and plasma fibronectin ($P<0.01$). The adherence of OmpL1 to ECM gel was expected, as laminin is one of the components (see M&M). No statistically significant adhesiveness was observed with OmpL1 protein when wells were coated with collagen Type I and IV, cellular fibronectin, elastin or with control proteins. The bindings were confirmed by performing similar assay but using anti-his tag monoclonal antibodies (Fig. 5B). The interaction between OmpL1 to laminin and to plasma fibronectin was also assessed on a quantitative basis by fixing the ECM components and varying protein concentration, as depicted in Fig. 5C and 5D, respectively. Binding saturation level was achieved with protein concentration of $\sim 5 \mu\text{M}$ for laminin and plasma fibronectin, with K_D of $2099.93 \pm 871.03 \text{ nM}$ and $1239.23 \pm 506.85 \text{ nM}$,

respectively. The role of sugar moiety in laminin was also investigated by incubating this component with increasing concentrations of sodium metaperiodate. A reduction of 20% on the binding could be noticed only with the highest concentration tested, suggesting that the carbohydrate moieties do not have a major role in the binding of OmpL1 to laminin (Fig. 5E).

Recombinant leptospiral OmpL1 binds to human PLG. In our previous work we have shown that leptospires bind PLG on their surface [15] and we have also described several proteins that are probably PLG receptors [35]. Based on this assumption, we decided to evaluate if OmpL1 can also adhere to human PLG *in vitro*. In addition, we investigated whether the recombinant protein can also bind factor H, human complement and C4bp, as previously reported for other recombinant proteins [38,39,40] (Domingos et al., manuscript submitted to publication). Our data show that OmpL1 binds to human PLG ($P < 0.01$), while no or very low reactivity was detected with the other components and the control proteins (Fig. 6A). This binding was also confirmed using anti-his monoclonal antibodies (Fig. 6B). The interaction between OmpL1 and PLG was also assessed on a quantitative basis, keeping PLG and varying the protein concentration, as shown in Fig. 6C. Binding saturation level was reached with 2 μ M of OmpL1 with a K_D of 368.63 ± 121.23 nM.

It is well known that PLG kringle domains frequently mediate interactions with lysine residues of the bacterial receptors [41]. These domains were shown to participate in the binding of PLG with intact live *L. interrogans* serovar Copenhageni strain L1–130, since the derivative and analogue of lysine, ACA, almost totally inhibited binding [15]. Based on these findings, the participation of lysine residues in the binding of the recombinant protein was evaluated by the addition of ACA to the assay. As depicted in Fig. 6D, when 2 mM of ACA was added to the reaction, the binding of the proteins to PLG was almost completely abolished ($P < 0.01$), strongly suggesting the participation of these domains in OmpL1 interaction with PLG.

Plasmin generation from PLG-bound. It has been demonstrated that enzymatically active plasmin is generated by PLG bound to the surface of *L. interrogans* when its activator is present [15]. To assess whether the PLG bound to OmpL1 generates proteolytic activity, as previously reported with other recombinant proteins [11,12,35,38,42], a microplate was coated with OmpL1, blocked, and then incubated with PLG. Unbound PLG was washed away, uPA (urokinase)-type PLG activator together with a plasmin-specific chromogenic substrate were added. The reaction was carried out overnight and the plasmin activity was evaluated by measuring the cleavage of the plasmin-

specific substrate D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride at 405 nm. The PLG bound to OmpL1 protein could be converted into plasmin, as indirectly demonstrated by specific proteolytic activity (Fig. 6E). Reaction controls lacking PLG, uPA or the chromogenic substrate showed no significant enzymatic activity. The adhesin Lsa63 that is not a PLG-binding protein and therefore does not generate plasmin [35] was also included as a negative control (Fig. 6E).

Inhibition of *L. interrogans* attachment to laminin, PLG or to plasma fibronectin by OmpL1. It has been reported that several recombinant proteins [8,11,12,43] exhibit an inhibitory effect on the binding of leptospires to PLG and ECM macromolecules. We thus carried out similar experiments to evaluate whether OmpL1 has similar effect on leptospiral adherence to laminin, PLG and plasma fibronectin. The results show that the addition of increasing concentration of OmpL1 reduced the leptospiral binding to laminin, PLG and to plasma fibronectin. Binding reduction in the number of leptospires adhering to PLG was statistically significant with 0.1 μ M of OmpL1 ($P < 0.05$) while to laminin significance was reached with 1 μ M of protein ($P < 0.05$) and to plasma fibronectin with 1.5 μ M ($P < 0.05$) (Fig. 7A). These results suggest that OmpL1 compete with the native leptospiral protein for the component binding sites. The experiment was performed in triplicate and this figure shows one representative data of two independent experiments.

Binding competition of OmpL1 to ECM and PLG by different ligands. As OmpL1 showed significant binding to PLG, laminin and plasma fibronectin, we decided to evaluate if these macromolecules compete for the same protein binding sites. Thus, we evaluated the competition among the components in a combination of two each time. The increasing concentration of laminin or PLG had no effect on the binding of OmpL1 to plasma fibronectin (Fig. 7B). Similarly, the addition of laminin or plasma fibronectin caused no changes on the binding of recombinant protein to PLG (Fig. 7C). The binding of laminin to OmpL1 was not affected by the addition of plasma fibronectin (Fig. 7D) contrasting with the effect produced by the addition of PLG, which unexpectedly, caused an increased on the interaction (Fig. 7D).

DISCUSSION

Leptospiral outer membrane proteins are the main focus of our research. Because of their location, these proteins may be involved in mechanisms of leptospiral pathogenesis and protective immunity. We have reported that leptospires have ability to adhere to laminin, cellular fibronectin, plasma fibronectin,

collagen I and IV [8], and to date, several leptospiral adhesins ECM-binding proteins have been identified. These include 36-kDa fibronectin-binding protein[44], LfhA/Lsa24 [8,38], LigA and LigB proteins [10,45], Len-family proteins [46], Lsa21 [9], LipL32 [13,47], Lsa27 [48], Lp95 [49], TlyC [50], LipL53 [51], Lsa63 [52], OmpL37 [43], Lsa66 [12], Lsa20 [11], Lsa25 and Lsa33 (Domingos et al., manuscript submitted to publication). After adherence, leptospires rapidly disseminates to several organs that is thought to be mediated by rapid translocation across host cell monolayers [53]. Besides motility and chemotaxis, *Leptospira* invasion may be mediated by proteolytic activity capable of cleaving host cell membranes. Indeed, we have described that leptospires acquire host-derived plasmin by receptor-bound PLG and can degrade fibronectin and laminin (Vieira et al., manuscript submitted to publication) that could help bacterial dissemination [15,54]

OmpL1 is a transmembrane outer-membrane protein of *Leptospira* firstly described by Haake and colleagues [16]. This protein has been identified in pathogenic *Leptospira* spp. by proteomics [55,56,57,58], exhibited synergistic immunoprotective capacity in combination with LipL41 in the Golden Syrian hamster model of leptospirosis [59] and has been studied by several groups for its human and veterinary diagnostic capability [17,18,19,20,21,22,23,24]. In this work, we report that OmpL1, encoded by the gene LIC10973, is an adhesin of *Leptospira* that interacts with laminin and plasma fibronectin, is a PLG-binding receptor capable of generating plasmin and is specifically recognized by antibodies in serum samples of human confirmed leptospirosis.

The DNA coding sequence of OmpL1 is conserved in all serovars of *L. interrogans* and other pathogenic species tested, but is absent in the saprophytic *L. biflexa*. These results corroborate previous findings that this protein is expressed in high passage pathogenic and low passage, virulent strains of *Leptospira* [16,59,60,61]. The LIC10973 gene was cloned and expressed in *E. coli* as a 32.4 kDa full-length recombinant protein, which comprises the native sequence of OmpL1 plus a 6X His tag at N-terminal. The purified protein exhibited a single major band in SDS-PAGE and was recognized by monoclonal anti-His tag antibodies and its homolog antibodies from mice immunized with the recombinant protein. Assessment of secondary structure of the recombinant protein after the purification process has been performed by CD spectroscopy that showed a typical of β -strands secondary structure content in the recombinant protein, which is in agreement with the native structure of OmpL1, characterized as an outer membrane-spanning protein, whose topological model contains ten amphipathic transmembrane β -strands [16,62].

Several researchers have suggested the importance of OmpL1 as a serological antigen for the diagnosis of human [17,18,19,20,21,22] and canine leptospirosis [23,24]. Our data showed high percentage of anti-OmpL1 IgG responders when experimentally infected-hamsters or MAT+ human serum samples were used, which is in agreement with previous published studies. Moreover, the results corroborate previous findings that this protein is expressed during infection [25]. Further evaluation of OmpL1 with human serum samples of confirmed unrelated infectious diseases revealed high specificity of this antigen, which reinforces the importance of OmpL1 as an antigen for the development of leptospirosis diagnostic kit.

Lin and colleagues [63] evaluated combined B and T cell epitopes of OmpL1 and of LipL41 conserved regions for their capacity to promote immune responses in BALB/c mice, and reported that the selected epitopes can induce a Th1-response. It has been already shown that OmpL1 is highly immunogenic in animal models, but these antibodies are probably not protective because OmpL1 alone did not protect hamsters against the challenge with virulent leptospires [59]. High levels of OmpL1 IgG antibodies detected in the sera from immunized BALB/c mice were mainly from IgG1 subclass indicating Th2-type response. Our data show that the recombinant OmpL1 was also capable of activating cell-mediated immune response indicated by lymphocyte proliferation from OmpL1 immunized mice. Moreover, OmpL1 was capable to induce both cellular and humoral immune response as demonstrated by the levels of IFN- γ and TNF- α (Th1) and IL-10 (possibly Th2) cytokines. In fact, Vernel-Pauillac and Merien [64] have demonstrated that pathogenic leptospires can elicit Th1 response together with anti-lipopolysaccharide antibodies in animal model. Moreover, the protective immune mechanism of LigA DNA vaccine was evaluated in immunized animals and shown to be conferred by both Th1 and Th2 immune responses [65]. It is worth mentioning that the recombinant protein LipL32, a major leptospiral antigen, has failed to confer protection against virulent bacteria in several challenge studies [66,67]. However, protection of this antigen was achieved when hamsters were immunized with recombinant BCG (rBCG) expressing LipL32 [68]. Recombinant BCG has the ability to elicit strong cellular as well as humoral immune responses [69]. Thus, it is possible that not only humoral but cellular immune responses are important in the immunoprotection against leptospirosis. Understanding the immune response elicited by leptospiral recombinant antigens may provide new insights on the mechanisms of anti-*Leptospira* immunity and may facilitate the design for an improved recombinant-based vaccine.

OmpL1 is a surface exposed protein that is expressed during infection. It is therefore possible that it may play a role in the attachment to the host. Indeed, OmpL1 protein exhibits extracellular matrix-binding properties. This protein binds laminin and plasma fibronectin contrasting to the previously reported Lsa24 [8], Lsa27 [48] and Lsa20 [11], that are laminin-binding adhesins, but comparable to other reported adhesins, namely Len family protein [46], LigA and LigB proteins [10], Lsa21 [9], LipL32 [13,47], TlyC [50], OmpL37 [43] and Lsa66 [12] that showed a broader spectrum binding profile to ECM. The calculated K_D for the binding of OmpL1 to laminin and to plasma fibronectin (2099.93 ± 871.04 and 1239.23 ± 506.85 nM, respectively) is, however, lower when compared to the K_D values obtained with Lsa66 and the same ECM components (55.4 ± 15.9 nM and 290.8 ± 11.8 nM) [12].

The interaction with the host PLG, a key component of the host fibrinolytic system, has been shown for several invasive gram-positive and gram-negative bacteria [70,71]. We have reported that *Leptospira* species were also capable to bind PLG and generate active plasmin on their surface [15]. Moreover, we have demonstrated that the generation of this proteolytic activity makes virulent *L. interrogans* capable of degrading purified extracellular matrix fibronectin [15] and laminin (Vieira et al., manuscript submitted to publication) a step that may contribute to leptospiral invasiveness [58]. In addition, we have identified proteins that act as leptospiral PLG-receptors [35]. OmpL1 also shows PLG-binding activity that seems to occur via PLG kringle domains with a K_D of 368.63 ± 121.23 nM. This K_D value is in the same order of magnitude as the one reported for the adhesin Lsa20 [11] but lower when compared to the ones reported with several recombinant proteins in our laboratory [35]. As previously shown with other protein-PLG-receptors [11,12,35,42], plasmin is actively generated in the presence of urokinase activator by PLG-bound OmpL1. Thus, it is possible that this protein may contribute to leptospiral infectiveness.

The low binding affinity of OmpL1 to ECM and to PLG could be compensated by the high copy number reported for this protein in *L. interrogans* [56] that was shown to increase after 7 days of serum treatment [57]. No interference on the binding of OmpL1 with these ligands was observed except the increased binding effect produced by PLG increased concentration on the binding of recombinant protein to laminin suggesting that somehow the binding is benefited by the presence of PLG. OmpL1 exhibited an inhibitory effect on the binding of intact *L. interrogans* to immobilized laminin, plasma fibronectin or PLG, as was previously observed with the ECM- and PLG- interacting proteins Lsa24 [8],

Lsa63 [52], LigA/LigB [10], OmpL37 [43] and Lsa20 [11].

In conclusion, we report in this work for the first time that OmpL1 is an adhesin and a PLG-binding protein of *Leptospira* spp. The interaction of OmpL1 with PLG in the presence of host activator can generate plasmin, rendering the bacteria with proteolytic activity capable of degrading host cell membranes. Thus, as an ECM- and PLG binding protein, OmpL1 may promote the attachment of the bacteria to mammalian hosts and may help the leptospires to disseminate during the infection process. The high number of human leptospirosis (MAT +) responders associated with the specificity of the IgG antibody response to OmpL among serum samples from patients with febrile diseases such as dengue, malaria, HIV, and Chagas disease, strengthens the potential of OmpL1 as a diagnostic marker for leptospirosis. OmpL1 promotes the proliferation of lymphocytes and activates both Th1 and Th2 cytokines from immunized BALB/c mice. It is conceivable that OmpL1 administered with adequate immunomodulator might exhibit protective activity against lethal challenges.

REFERENCES

1. Faine S, Adler B, Bolin C, Perolat P (1999) *Leptospira* and *Leptospirosis*. Second ed. Melbourne, Australia: MediSci. pp. 259.
2. Levett PN (2001) *Leptospirosis*. *Clin Microbiol Rev* 14: 296-326.
3. Ko AI, Galvao Reis M, Ribeiro Dourado CM, Johnson WD, Jr., Riley LW (1999) Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group. *Lancet* 354: 820-825.
4. Plank R, Dean D (2000) Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. *Microbes Infect* 2: 1265-1276.
5. de la Pena-Moctezuma A, Bulach DM, Kalambaheti T, Adler B (1999) Comparative analysis of the LPS biosynthetic loci of the genetic subtypes of serovar Hardjo: *Leptospira interrogans* subtype Hardjoprajitno and *Leptospira borgpetersenii* subtype Hardjobovis. *FEMS Microbiol Lett* 177: 319-326.
6. Adler B, de la Pena Moctezuma A (2009) *Leptospira* and leptospirosis. *Vet Microbiol*.
7. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, et al. (2003) *Leptospirosis*: a zoonotic disease of global importance. *Lancet Infect Dis* 3: 757-771.
8. Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, et al. (2006) A newly identified leptospiral adhesin mediates attachment to laminin. *Infect Immun* 74: 6356-6364.
9. Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Moraes ZM, et al. (2008) Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC Microbiol* 8: 70.
10. Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J, et al. (2007) Physiological osmotic induction of

- Leptospira interrogans adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. *Infect Immun* 75: 2441-2450.
11. Mendes RS, Von Atzingen M, de Moraes ZM, Goncalves AP, Serrano SM, et al. (2011) The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection. *Infect Immun* 79: 4657-4667.
 12. Oliveira R, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, et al. (2011) Characterization of novel OmpA-like protein of Leptospira interrogans that binds extracellular matrix molecules and plasminogen. *PLoS One* 6: e21962.
 13. Hoke DE, Egan S, Cullen PA, Adler B (2008) LipL32 is an extracellular matrix-interacting protein of Leptospira spp. and Pseudoalteromonas tunicata. *Infect Immun* 76: 2063-2069.
 14. Koizumi N, Watanabe H (2005) Leptospirosis vaccines: past, present, and future. *J Postgrad Med* 51: 210-214.
 15. Vieira ML, Vasconcellos SA, Goncalves AP, de Moraes ZM, Nascimento AL (2009) Plasminogen acquisition and activation at the surface of leptospira species lead to fibronectin degradation. *Infect Immun* 77: 4092-4101.
 16. Haake DA, Champion CI, Martinich C, Shang ES, Blanco DR, et al. (1993) Molecular cloning and sequence analysis of the gene encoding OmpL1, a transmembrane outer membrane protein of pathogenic Leptospira spp. *J Bacteriol* 175: 4225-4234.
 17. Dong H, Hu Y, Xue F, Sun D, Ojcius DM, et al. (2008) Characterization of the ompL1 gene of pathogenic Leptospira species in China and cross-immunogenicity of the OmpL1 protein. *BMC Microbiol* 8: 223.
 18. Flannery B, Costa D, Carvalho FP, Guerreiro H, Matsunaga J, et al. (2001) Evaluation of recombinant Leptospira antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. *J Clin Microbiol* 39: 3303-3310.
 19. Sun A, Wang Y, Du P, Wu S, Yan J A (2011) sensitive and specific IgM-ELISA for the serological diagnosis of human leptospirosis using a rLipL32/1-LipL21-OmpL1/2 fusion protein. *Biomed Environ Sci* 24: 291-299.
 20. Zhang XY, Yu Y, He P, Zhang YX, Hu BY, et al. (2005) Expression and comparative analysis of genes encoding outer membrane proteins LipL21, LipL32 and OmpL1 in epidemic leptospires. *Acta Biochim Biophys Sin (Shanghai)* 37: 649-656.
 21. Qiu XF, Xu HF, Guo ZQ, Wang J, Yan J (2008) [Establishment and application of ELISAs based on rLipL32/1-LipL21-OmpL1/2 fusion antigen of Leptospira interrogans]. *Zhejiang Da Xue Xue Bao Yi Xue Ban* 37: 592-598.
 22. Natarajaseenivasan K, Vijayachari P, Sharma S, Sugunan AP, Selvin J, et al. (2008) Serodiagnosis of severe leptospirosis: evaluation of ELISA based on the recombinant OmpL1 or LipL41 antigens of Leptospira interrogans serovar autumnalis. *Ann Trop Med Parasitol* 102: 699-708.
 23. Okuda M, Sakai Y, Matsuchi M, Oikawa T, Watanabe M, et al. (2005) Enzyme-linked immunosorbent assay for the detection of canine Leptospira antibodies using recombinant OmpL1 protein. *J Vet Med Sci* 67: 249-254.
 24. Iwamoto E, Wada Y, Fujisaki Y, Umeki S, Jones MY, et al. (2009) Nationwide survey of leptospira antibodies in dogs in Japan: results from microscopic agglutination test and enzyme-linked immunosorbent assay. *J Vet Med Sci* 71: 1191-1199.
 25. Barnett JK, Barnett D, Bolin CA, Summers TA, Wagar EA, et al. (1999) Expression and distribution of leptospiral outer membrane components during renal infection of hamsters. *Infect Immun* 67: 853-861.
 26. Turner LH (1970) Leptospirosis. 3. Maintenance, isolation and demonstration of leptospires. *Trans R Soc Trop Med Hyg* 64: 623-646.
 27. Studier FW (1991) Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J Mol Biol* 219: 37-44.
 28. Ramos CR, Abreu PA, Nascimento AL, Ho PL (2004) A high-copy T7 Escherichia coli expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. *Braz J Med Biol Res* 37: 1103-1109.
 29. Perez-Iratxeta C, Andrade-Navarro MA (2008) K2D2: estimation of protein secondary structure from circular dichroism spectra. *BMC Struct Biol* 8: 25.
 30. Faine S, Adler B, Bolin C, Perolat P (1999) Leptospira and Leptospirosis. Melbourne, Australia MediSci.
 31. Gruber A, Zingales B (1995) Alternative method to remove antibacterial antibodies from antisera used for screening of expression libraries. *Biotechniques* 19: 28, 30.
 32. Oliveira TR, Longhi MT, de Moraes ZM, Romero EC, Blanco RM, et al. (2008) Evaluation of leptospiral recombinant antigens MPL17 and MPL21 for serological diagnosis of leptospirosis by enzyme-linked immunosorbent assays. *Clin Vaccine Immunol* 15: 1715-1722.
 33. Galen RS (1975) The enzyme diagnosis of myocardial infarction. *Hum Pathol* 6: 141-155.
 34. Lin YP, Lee DW, McDonough SP, Nicholson LK, Sharma Y, et al. (2009) Repeated domains of leptospira immunoglobulin-like proteins interact with elastin and tropoelastin. *J Biol Chem* 284: 19380-19391.
 35. Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, et al. (2010) In vitro identification of novel plasminogen-binding receptors of the pathogen Leptospira interrogans. *PLoS One* 5: e11259.
 36. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, et al. (2000) The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect Immun* 68: 2276-2285.
 37. Pinne M, Haake DA (2009) A comprehensive approach to identification of surface-exposed, outer membrane-spanning proteins of Leptospira interrogans. *PLoS One* 4: e6071.
 38. Verma A, Hellwage J, Artiushin S, Zipfel PF, Kraiczky P, et al. (2006) LfhA, a novel factor H-binding protein of Leptospira interrogans. *Infect Immun* 74: 2659-2666.
 39. Castiblanco-Valencia MM, Fraga TR, Silva LB, Monaris D, Abreu PA, et al. (2012) Leptospiral Immunoglobulin-like Proteins Interact With Human Complement Regulators Factor H, FHL-1, FHR-1, and C4BP. *J Infect Dis* 205: 995-1004.

40. Barbosa AS, Monaris D, Silva LB, Morais ZM, Vasconcellos SA, et al. (2010) Functional characterization of LcpA, a surface-exposed protein of *Leptospira* spp. that binds the human complement regulator C4BP. *Infect Immun* 78: 3207-3216.
41. Lahteenmaki K, Kuusela P, Korhonen TK (2001) Bacterial plasminogen activators and receptors. *FEMS Microbiol Rev* 25: 531-552.
42. Verma A, Brissette CA, Bowman AA, Shah ST, Zipfel PF, et al. (2010) Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen. *Infect Immun* 78: 2053-2059.
43. Pinne M, Choy HA, Haake DA (2010) The OmpL37 surface-exposed protein is expressed by pathogenic *Leptospira* during infection and binds skin and vascular elastin. *PLoS Negl Trop Dis* 4: e815.
44. Merien F, Truccolo J, Baranton G, Perolat P (2000) Identification of a 36-kDa fibronectin-binding protein expressed by a virulent variant of *Leptospira interrogans* serovar icterohaemorrhagiae. *FEMS Microbiol Lett* 185: 17-22.
45. Lin YP, Chang YF (2007) A domain of the *Leptospira* LigB contributes to high affinity binding of fibronectin. *Biochem Biophys Res Commun* 362: 443-448.
46. Stevenson B, Choy HA, Pinne M, Rotondi ML, Miller MC, et al. (2007) *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. *PLoS ONE* 2: e1188.
47. Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Morais ZM, et al. (2008) In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. *Infect Immun* 76: 2642-2650.
48. Longhi MT, Oliveira TR, Romero EC, Goncalves AP, de Morais ZM, et al. (2009) A newly identified protein of *Leptospira interrogans* mediates binding to laminin. *J Med Microbiol* 58: 1275-1282.
49. Atzingen MV, Gomez RM, Schattner M, Pretre G, Goncalves AP, et al. (2009) Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells. *J Infect*.
50. Carvalho E, Barbosa AS, Gomez RM, Cianciarullo AM, Hauk P, et al. (2009) Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity. *FEBS Lett*.
51. Oliveira TR, Longhi MT, Goncalves AP, de Morais ZM, Vasconcellos SA, et al. (2010) LipL53, a temperature regulated protein from *Leptospira interrogans* that binds to extracellular matrix molecules. *Microbes Infect* 12: 207-217.
52. Vieira ML, de Morais ZM, Goncalves AP, Romero EC, Vasconcellos SA, et al. (2010) Lsa63, a newly identified surface protein of *Leptospira interrogans* binds laminin and collagen IV. *J Infect* 60: 52-64.
53. Barocchi MA, Ko AI, Reis MG, McDonald KL, Riley LW (2002) Rapid translocation of polarized MDCK cell monolayers by *Leptospira interrogans*, an invasive but nonintracellular pathogen. *Infect Immun* 70: 6926-6932.
54. Vieira ML, de Morais ZM, Vasconcellos SA, Romero EC, Nascimento AL (2011) In vitro evidence for immune evasion activity by human plasmin associated to pathogenic *Leptospira interrogans*. *Microb Pathog* 51: 360-365.
55. Nally JE, Whitelegge JP, Aguilera R, Pereira MM, Blanco DR, et al. (2005) Purification and proteomic analysis of outer membrane vesicles from a clinical isolate of *Leptospira interrogans* serovar Copenhageni. *Proteomics* 5: 144-152.
56. Malmstrom J, Beck M, Schmidt A, Lange V, Deutsch EW, et al. (2009) Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. *Nature* 460: 762-765.
57. Schmidt A, Beck M, Malmstrom J, Lam H, Claassen M, et al. (2011) Absolute quantification of microbial proteomes at different states by directed mass spectrometry. *Mol Syst Biol* 7: 510.
58. Vieira ML, Pimenta DC, de Morais ZM, Vasconcellos SA, Nascimento AL (2009) Proteome Analysis of *Leptospira interrogans* Virulent Strain. *Open Microbiol J* 3: 69-74.
59. Haake DA, Mazel MK, McCoy AM, Milward F, Chao G, et al. (1999) Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. *Infect Immun* 67: 6572-6582.
60. Haake DA, Suchard MA, Kelley MM, Dundoo M, Alt DP, et al. (2004) Molecular evolution and mosaicism of leptospiral outer membrane proteins involves horizontal DNA transfer. *J Bacteriol* 186: 2818-2828.
61. Zuerner R, Haake D, Adler B, Segers R (2000) Technological advances in the molecular biology of *Leptospira*. *J Mol Microbiol Biotechnol* 2: 455-462.
62. Shang ES, Exner MM, Summers TA, Martinich C, Champion CI, et al. (1995) The rare outer membrane protein, OmpL1, of pathogenic *Leptospira* species is a heat-modifiable porin. *Infect Immun* 63: 3174-3181.
63. Lin X, Sun A, Ruan P, Zhang Z, Yan J (2011) Characterization of conserved combined T and B cell epitopes in *Leptospira interrogans* major outer membrane proteins OmpL1 and LipL41. *BMC Microbiol* 11: 21.
64. Vernel-Pauillac F, Merien F (2006) Proinflammatory and immunomodulatory cytokine mRNA time course profiles in hamsters infected with a virulent variant of *Leptospira interrogans*. *Infect Immun* 74: 4172-4179.
65. Faisal SM, Yan W, Chen CS, Palaniappan RU, McDonough SP, et al. (2008) Evaluation of protective immunity of *Leptospira* immunoglobulin like protein A (LigA) DNA vaccine against challenge in hamsters. *Vaccine* 26: 277-287.
66. Lucas DS, Cullen PA, Lo M, Srikram A, Sermswan RW, et al. (2011) Recombinant LipL32 and LigA from *Leptospira* are unable to stimulate protective immunity against leptospirosis in the hamster model. *Vaccine* 29: 3413-3418.
67. Branger C, Chatrenet B, Gauthier A, Aviat F, Aubert A, et al. (2005) Protection against *Leptospira interrogans* sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. *Infect Immun* 73: 4062-4069.
68. Seixas FK, da Silva EF, Hartwig DD, Cerqueira GM, Amaral M, et al. (2007) Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. *Vaccine* 26: 88-95.

69. Dennehy M, Williamson AL (2005) Factors influencing the immune response to foreign antigen expressed in recombinant BCG vaccines. *Vaccine* 23: 1209-1224.
70. Coleman JL, Benach JL (1999) Use of the plasminogen activation system by microorganisms. *J Lab Clin Med* 134: 567-576.
71. Sun H (2006) The interaction between pathogens and the host coagulation system. *Physiology (Bethesda)* 21: 281-288.

LEGENDS TO FIGURES

Figure 1. Expression, purification and Western blotting analysis of purified recombinant OmpL1.

(A) Recombinant OmpL1 expression analysis on SDS-PAGE; M: molecular mass protein marker; 1: non-induced total bacterial extract; 2: total bacterial cell lysates after induction; 3 and 4: soluble and insoluble (pellet) fractions of the induced culture, respectively; 5: flow-through fraction; 6: purified recombinant protein in Coomassie blue stained gel. (B) Western blotting analysis of the recombinant protein probed with pooled polyclonal antiserum raised in mice immunized with OmpL1 (1) or PBS (2), or with anti-His tag monoclonal antibodies (3). (C) CD spectrum of OmpL1 protein depicting a predominance of β -sheets in its secondary structure composition. Far-UV CD spectrum is presented as an average of five scans recorded from 185 to 260 nm.

Figure 2: DNA and OmpL1 protein conservation among *Leptospira* strains.

(A) genomic DNA of several species of *Leptospira* were used as template for PCR amplification of 16S ribosomal DNA (constitutive control) and OmpL1 genes. The negative control (12) denotes a reaction without DNA template. (B) *Leptospira* whole cell lysates and recombinant OmpL1 (+, used as a positive control) were separated by SDS-PAGE, transferred into membranes and probed with antiserum against the recombinant protein followed by anti-mouse IgG peroxidase conjugated. Reactivity was detected by ECL kit. *L. interrogans* serovars: Copenhageni (1), Canicola (2), Icterohaemorrhagiae (3) Pomona (4), *L. borgpetersenii* serovars: Castellonis (5), Whitcombi (6), *L. kirshnery* serovars Cynoptery (7) and Grippotyphosa (8), *L. santarosai* serovar Shermani (9), *L. noguchii* serovar Panama (10), and *L. biflexa* serovar Patoc (11). For protein conservation virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (12) and serovar Pomona strain LPF (13) were also included.

Figure 3: Immune response evaluation in BALB/c mice immunized with OmpL1.

(A) Total IgG antibodies and subclasses were determined in sera from BALB/c mice immunized with OmpL1. OD values were from different pooled sera dilution and compared

against a concentration curve of total IgG, IgG1 and IgG2a. (B) Lymphocyte proliferation response was analyzed from spleens of immunized mice with OmpL1; cells were cultured followed by a pulse with medium alone, ConA (5 μ g/mL) or OmpL1 (5 μ g/mL). Lymphocytes stimulated with the medium alone were used as a negative control and those stimulated with con A were used as positive control. The proliferative response was measured by ELISA BrdU colorimetric assay. The data represent the mean OD values of three determinations $\pm$ S.D. of two independent experiments. For statistical analysis, OD values of OmpL1 immunized group treated with the recombinant protein were compared against OmpL1 immunized group treated with medium alone by the two-tailed *t* test (**P*<0.05). Spleen cells were isolated and cultured in 24 wells tissue culture plates, each well containing 5 $\times$ 10⁶ cells. After 48 h, cell-free culture supernatants were collected and IL-10 (C), IFN- γ (D), TNF- α and IL-4 (F) cytokines were measured by a sandwich ELISA according to the manufacturer's instructions. For statistical analysis, concentration values from OmpL1 immunized group pulsed with the recombinant protein were compared against OmpL1 immunized group treated with medium alone by the two-tailed *t* test (**P*<0.05, ****P*<0.001 and *****P*<0.0001).

Figure 4: Recognition of OmpL1 by IgG antibodies of hamsters, of individuals diagnosed with leptospirosis and of individuals diagnosed with unrelated diseases.

Positive sera (responders) were determined by ELISA with the recombinant protein and (A) serum samples from experimentally infected hamsters; (B) serum samples from leptospirosis patients in both phases of the disease, MAT+ and MAT-, and serum samples from patients diagnosed with unrelated febrile diseases (B). The reactivity was evaluated as total IgG antibodies. The cutoff values are defined as the mean plus 3 standard deviations obtained with sera from ten healthy individuals or 5 non-infected hamsters (horizontal bars).

Figure 5: Binding of OmpL1 to ECM components.

(A) Wells were coated with 1 μ g of laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, ECM gel, elastin and the control proteins gelatin, BSA and fetuin. 1 μ g of the recombinant protein was added per well and the binding was measured by ELISA. Data represent the mean $\pm$ the standard deviation from three independent experiments. For statistical analyses, the attachment of recombinant protein to the ECM components was compared to its binding to all negative controls by the two-tailed *t* test, although the *P* value given here refers to comparison against gelatin (**P* < 0.05 and ***P*<0.01). (B) Significant binding activity was confirmed by ELISA

employing anti-his tag monoclonal antibodies. Data represent the mean $\pm$ the standard deviation from three independent experiments. For statistical analyses, the attachment of recombinant protein was compared to its binding to gelatin by the two-tailed *t* test ($*P < 0.05$, $**P < 0.01$ and $***P < 0.001$). OmpL1 dose-dependent binding experiments to laminin (C) and to plasma fibronectin (D): each point was performed in triplicate and expressed as the mean absorbance value at 492 nm $\pm$ standard error for each point. Gelatin was included as a negative control. The equilibrium dissociation constant (K_D) value is depicted for each ligand in C and D, and was calculated based on ELISA data for the OmpL1 recombinant protein that reached equilibrium at a given concentration. (E) Immobilized laminin was treated with sodium metaperiodate (5 to 100 mM) for 15 min at 4°C in the dark followed by OmpL1 interaction. The mean absorbance values at 492 nm ($\pm$ the standard deviations of three independent experiments) were compared to those obtained with untreated laminin (0 mM).

Figure 6. OmpL1 binds to PLG and generates active plasmin. (A) Wells were coated with 1 μ g of PLG, factor H, complement mix, C4bp and the control proteins gelatin, BSA and fetuin. 1 μ g of the recombinant protein was added per well and the binding was measured by ELISA. Data represent the mean $\pm$ the standard deviation from three independent experiments. For statistical analyses, the attachment of recombinant protein to the serum components was compared to its binding to all negative controls by the two-tailed *t* test, although the *P* value given here is relative to comparison against gelatin ($**P < 0.01$). (B) Significant binding activity was confirmed by ELISA employing anti-his monoclonal antibodies. Data represent the mean $\pm$ the standard deviation from three independent experiments. For statistical analyses, the attachment of recombinant protein was compared to its binding to gelatin by the two-tailed *t* test ($**P < 0.01$ and $***P < 0.001$). (C) OmpL1 dose-dependent binding experiments: each point was performed in triplicate and expressed as the mean absorbance value at 492 nm $\pm$ standard error for each point. Gelatin was included as negative control. The equilibrium dissociation constant (K_D) is depicted and was calculated based on ELISA data for the OmpL1 recombinant protein that reached equilibrium at a given concentration. (D) Binding of OmpL1 to PLG was carried out in the presence or absence (no inhibition) of the lysine analogue 6-aminocaproic acid (ACA). The bound PLG was detected and quantified by specific antibodies. Bars represent the mean absorbance at 492 nm $\pm$ the standard deviation of triplicate and are representative of two independent experiments. For statistical analyses, the attachment of recombinant protein in the presence of

ACA was compared to its binding to PLG without ACA (no inhibition) by the two-tailed *t* test ($**P < 0.01$). (E) Plasmin generation by PLG bound to recombinant protein, indirectly measured by cleavage of specific plasmin substrate, was assayed by modified ELISA; Immobilized recombinant OmpL1 protein received the following treatment: PLG+uPA+specific plasmin substrate (PLG+uPA+S), or controls lacking one of the three components (PLG+uPA; PLG+S; uPA+S). BSA and Lsa63 were employed as negative controls. Bars represent the mean absorbance values at 405 nm, as a measure of relative substrate cleavage, $\pm$ the standard deviation of three replicate for each experimental group and are representative of two independent experiments. Statistically significant differences are shown in comparison to BSA ($*P < 0.05$).

Figure 7. Binding inhibition of leptospires to ECM or to PLG by OmpL1 and competition assays of OmpL1-binding to ECM and to PLG. (A) Laminin, plasma fibronectin or PLG (1 μ g/well) were adsorbed to microtiter plates followed by incubation with increasing concentrations of OmpL1 (0 to 1.5 μ M) for 90 min at 37°C. After washings, live leptospires (100 μ L/well of 4×10^7) were added and the quantification of bound leptospires was performed indirectly by anti-LipL32 antibodies produced in mice (1: 4,000 dilution), followed by HRP-conjugated anti-mouse IgG antibodies. Each point represents the mean absorbance value at 492 nm $\pm$ the standard deviation of three replicates. Data are representative of 2 independent experiments ($*P < 0.05$). The effect of the component on each other interaction to OmpL1 was performed by competition assays with the addition of increasing masses of different components (0 to 1.00 μ g in 100 μ L PBS); (B) The effect of PLG or laminin on the binding of plasma fibronectin (10 μ g/mL) to immobilized OmpL1 (10 μ g/mL) was performed with the addition of increasing PLG or laminin masses (0 to 1.00 μ g in 100 μ L PBS); (C) The effect of laminin and plasma fibronectin on the binding of PLG to immobilized OmpL1 and (D) the effect of PLG and plasma fibronectin on the binding of laminin to immobilized OmpL1. Statistically significant interference was determined by the two-tailed *t* test ($**P < 0.01$).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7