Jaime Henrique Amorim Santos

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

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

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"Desenvolvimento de vacinas de subunidades contra a dengue baseadas no domínio III da proteína E e na proteína NS1 recombinantes"

Prezado Professor,

Informo que a sua licença para uso de animais em experimentação, constante no protocolo em epígrafe, **foi prorrogada até 23.04.2015.**

Reitero que havendo alteração de metodologia e inserção de novos alunos ao projeto de pesquisa vinculado à referida licença a CEUA/ICB deverá ser informada.

Cordialmente,

Prof. Dr. WOTHAN TAVARES DE LIMA
Coordenador - CEUA-ICB/ /USP

Ilmo.Sr.
Prof. Dr. LUIZ CARLOS DE SOUZA FERREIRA
Departamento de Microbiologia
Instituto de Ciências Biomédicas - USP

Aos heróis da minha vida, meus pais,
Jaime e Elza

Á minha riqueza, minha esposa, amiga, amante e cúmplice,
Paloma

Uma pequena dedicatória
às pessoas mais importantes da minha história.

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RESUMO

AMORIM-SANTOS, J. H. **Desenvolvimento de vacinas de subunidades contra a dengue baseadas no domínio III da proteína E e na proteína NS1 recombinantes**. 2012. 79 f. Tese (Doutorado em Biotecnologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2013.

O presente trabalho propõe o desenvolvimento e a caracterização de uma estratégia vacinal de caráter profilático contra o vírus da dengue (VD), baseada nas proteínas NS1 e domínio III da proteína E (EIII), empregando proteínas recombinantes em ensaios de imunização por via sub-cutânea em modelo murino. Estes antígenos foram obtidos pela clonagem e expressão de suas sequências de DNA codificadoras em sistema procarioto (*E. coli*). Além disso, formas atóxicas da toxina termo-lábil (LT_{G33D} e LTK63) de *E. coli* enterotoxigência (ETEC) foram obtidas e incorporadas como adjuvantes às formulações vacinais. As respostas celulares e humorais anti-NS1 e anti-EIII foram monitoradas por ELISA para anticorpos e citocinas, ICS (do inglês *intracellular citokine staining*) e atividade citotóxica *in vivo*. Observamos que animais imunizados com a NS1 recombinante adicionada da LT_{G33D} foram capazes de gerar respostas imunológicas com produção de anticorpos específicos e alta afinidade pelo antígeno. Em ensaios de desafio realizados para avaliar a proteção vacinal conferida à infecção por uma linhagem referência do o VD tipo 2 (NGC) observamos que essa formulação conferiu uma proteção de 50% aos animais imunizados. Paralelamente a esses resultados, demonstramos que a EIII não é um bom antígeno vacinal e que pode induzir anticorpos capazes de acentuar a infecção do VD. Descrevemos ainda a obtenção e a caracterização genética e patológica de um isolado clínico de VD tipo 2 naturalmente letal para camundongos Balb/C. A nova cepa viral (JHA1) demonstrou ser capaz de induzir perda de peso corporal, dano tecidual geral, e distúrbios hematológicos similares aos observados em humanos infectados pelo VD, podendo ser aplicada como modelo de infecção na avaliação de candidatos vacinais. Os resultados obtidos neste trabalho representam uma importante contribuição na área de desenvolvimento de estratégias vacinais contra a dengue e representam uma base importante para futuros estudos sobre a patologia da dengue.

Palavras-chave: Dengue. Vírus da dengue. Vacinas. Proteínas recombinantes.

ABSTRACT

AMORIM-SANTOS, J. H. **Subunit vaccine development against dengue fever based on the recombinant forms of the domain III of the E protein and the NS1 protein**. 2012. 79 p. Ph. D. thesis (Biotechnology) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2013.

The present study proposes the development and characterization of a strategy for prophylactic vaccination against dengue virus (VD) based on the NS1 protein and the domain III of the envelope glycoprotein (EIII), using recombinant proteins in subcutaneous immunization in a murine model. These antigens were obtained by cloning and expression of their DNA coding sequences in prokaryotic system (*E. coli*). In addition, the s non-toxic forms of the heat-labile toxin from enterotoxigenic *E. coli* (ETEC) (LTK63 and LTG33D) were obtained and incorporated as adjuvants to vaccine formulations. Anti-NS1 and anti-EIII cellular and humoral immune responses were monitored by antibody and cytokine ELISA, , intracellular citokine staining (ICS) and *in vivo* cytotoxic activity. We observed that animals immunized with the recombinant NS1 and LTG33D were capable to induce immune responses including specific antibodies with high affinity for the antigen. In challenge assays performed to evaluate the immunization protective efficacy such vaccine conferred protection of 50% against infection with a reference type 2 VD (VD2) strain(NGC). Alongside to these results, we demonstrated that EIII is not a good vaccine antigen and can induce the generation of antibodies that enhance DENV infection. We also described the isolation and the genetic and pathological characterization of a VD2 clinical isolate naturally lethal to immunocompetent Balb/c mice. The new strain was shown to cause weight loss, general tissue damage, and hematological disturbances similar to those observed in VDinfected humans, and therefore, may be applied as infection model to evaluate vaccine candidates. The results obtained in this study represent an important contribution to DENV vaccine development and established an important background for future studies of the dengue pathology.

Keywords: Dengue fever. Dengue virus. Vaccine. Recombinant proteins.

LISTA DE ABREVIATURAS E SIGLAS

µg	Micrograma (s)
µL	Microlitro (s)
AMP	Ampicilina
CFSE	*Carboxyfluorescein succinimidyl ester*
CTL	*Cytotoxic T lymphocyte* (linfócito T citotóxico)
DNA	*Desoxyribonucleic acid* (ácido desoxirribonucléico)
DO600	Densidade Optica no comprimento de onda de 600 nm
ELISPOT	*Enzyme-Linked Immunosorbent Spo*
FITC	Fluoresceína
IFN-γ	Interferon-γ
g	Força centrífuga relativa
Ig	Imunoglobulina
IL	Interleucina
KAN	Kanamicina
kDa	Quilodaltons
LB	Meio Luria-Bertani
M	Molar
mg	Miligrama (s)
mM	Milimolar
PBS	*Phosphatase buffered saline* (tampão salina fosfato)
PCR	*Polymeraso chain reaction* (reação de polimerase em cadeia)
PE	Ficoeritrina
RNA	*Ribonucleic acid* (ácido ribonucléico)
Th	T *helper*

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SUMÁRIO

1 REVISÃO DA LITERATURA

1.1 IMPACTO EPIDEMIOLÓGICO DA DOENÇA

A dengue é uma enfermidade causada pelo vírus da dengue (VD), um arbovírus da família *Flaviviridae*, gênero *Flavivirus*, com quatro tipos virais de relevância epidemiológica: VD-1, VD-2, VD-3 e VD-4. A doença acomete cerca de 100 milhões de pessoas por ano no mundo sendo que pelo menos 500 mil desenvolvem as formas mais graves da doença: a febre hemorrágica da dengue (FHD) e a síndrome de choque da dengue (SCD). A taxa de mortalidade da doença pode atingir 10% dos pacientes hospitalizados e 30% para pacientes não tratados (PONGSUMPUN et al., 2008).

No Brasil, os primeiros registros de epidemias de dengue ocorreram no Estado de São Paulo nos anos de 1851-1853 e 1916 e no Rio de Janeiro em 1923. Do começo do século XX aos anos 80, a doença foi praticamente eliminada do país, em virtude do combate ao vetor *Aedes aegypti*, durante campanha de erradicação da febre amarela. Na década de 80 foram registrados novos casos de dengue em função do ressurgimento do vetor a partir de países vizinhos. Em 1981-1982 os primeiros casos de dengue ocorreram em Boa Vista (RR). Em 1986 e 1987 surgiram registros da doença nos Estados do Rio de Janeiro, Alagoas, Ceará, Pernambuco, Bahia, Minas Gerais e São Paulo. No começo da década de 90 (1990 a 1992) a doença se espalha pelo interior do país com casos registrados em Mato Grosso do Sul, Mato Grosso, Tocantins e outros Estados brasileiros (AMORIM; ALVES; FERREIRA, 2009; BRASIL, 2012; WORLD HEALTH ORGANIZATION (WHO), 1997; PONGSUMPUN et al., 2008).

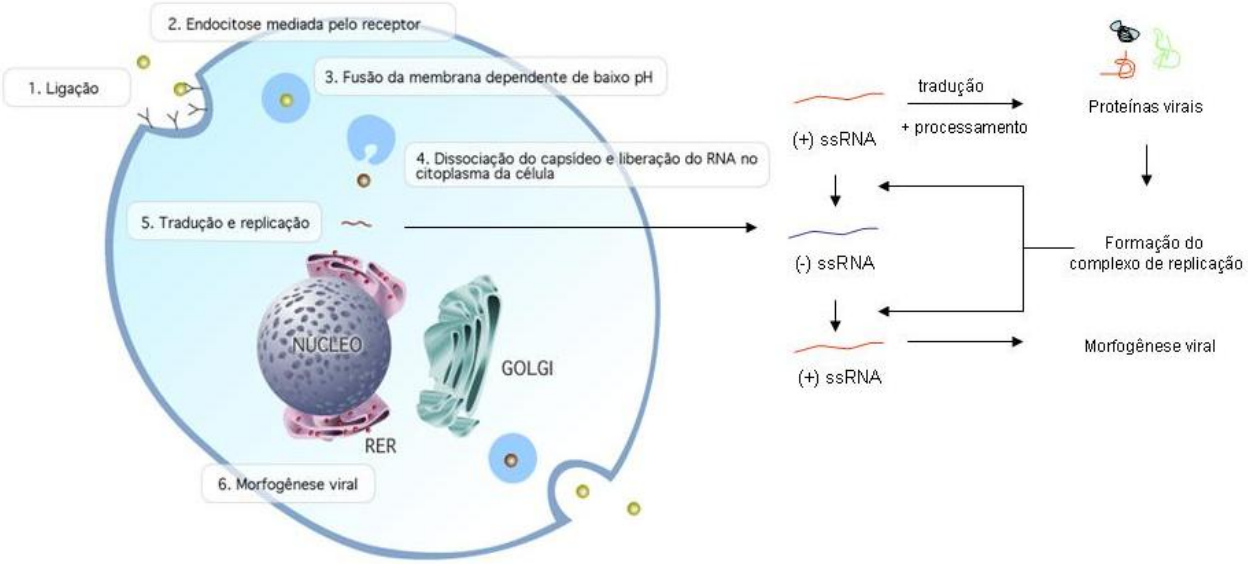
No período de 1986 a outubro de 1999 foram registrados no Brasil 1.104.996 casos de dengue em dezenove dos vinte e sete Estados da Federação. Observou-se grandes flutuações no número de casos notificados entre 1986 e 1993, seguido pelo aumento acentuado no número de notificações no período de 1994 a 1998. A partir do ano 2000, a doença assumiu caráter epidêmico com pico máximo de notificações no ano 2002 com grandes epidemias em 2002 e 2007 no Rio de Janeiro e número elevado de casos em 2008. Em 2009, temos o aumento acentuado da incidência da doença no Nordeste brasileiro, com destaque para a Bahia, onde no início de 2009 foram notificados 4.939 casos na cidade de Itabuna, 6.811 casos em Jequié e 1.533 em ilhéus (AMORIM; ALVES; FERREIRA, 2009; BRASIL, 2012).

1.2 O CICLO VIRAL E ASPECTOS MOLECULARES DA PATOGÊNESE

O VD infecta células alvo, preferencialmente células dendríticas, monócitos e hepatócitos, por meio da interação do domínio III da proteína do envelope viral (E), proteína majoritária presente na superfície da partícula viral, que interage com receptores presentes na membrana das células hospedeiras. A partícula viral penetra na célula após a formação de vesículas da membrana citoplasmática. No interior da vesícula, o pH ácido promove mudanças na estrutura da proteína E, que passa de um estado dimérico para uma forma trimérica, expondo uma região que promove a fusão das membranas do envelope viral e membrana vesicular, levando à dissociação do capsídeo e à liberação do material genético viral para o citoplasma da célula hospedeira (HENCHAL; PUNAK, 1990; WHITEHED et al., 2007).

Como o vírus possui RNA genômico com orientação positiva, a replicação inicia-se com a síntese, pela RNA polimerase, da fita de RNA com orientação negativa que serve de molde para a síntese de novas cópias do RNA viral (Figura 1). Durante um período de doze a dezesseis horas após a penetração na célula ocorre a formação da primeira progênie viral, com a replicação do RNA e a síntese das proteínas virais. O genoma viral é composto por uma fita simples de RNA com 10.173 bases, que codifica para uma poliproteína que depois de processada origina as três proteínas estruturais presentes na partícula viral: do capsídeo (C), de membrana (prM) e E, e sete proteínas não estruturais (NS), ausentes na partícula viral mas necessárias para a replicação nas células hospedeiras: NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5 (Figura 2) (AMORIM; ALVES; FERREIRA, 2009; DURBIN et al., 2008; WHITEHEAD et al., 2007).

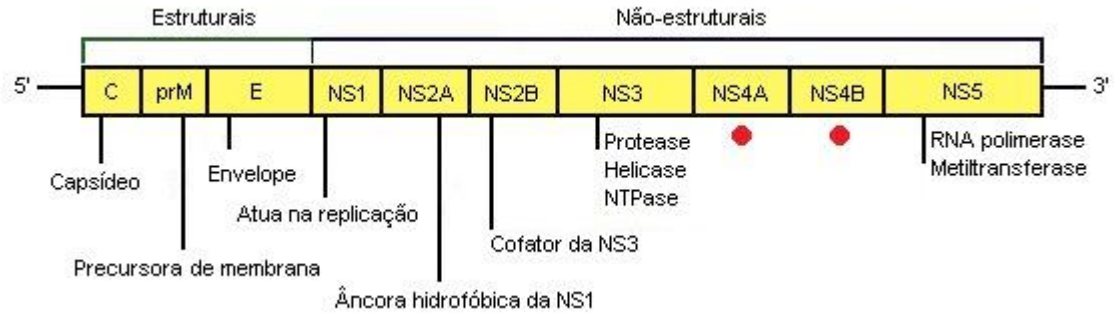
Figura 1- Etapas do processo infeccioso e da replicação do VD em células eucarióticas.



Após a infecção, a partícula viral se liga ao receptor celular (1) e há endocitose mediada pelo receptor (2). Ocorre a redução do pH do endossomo, o que promove a fusão da membrana viral à membrana do endossomo (3) e liberação do RNA no citoplasma celular (4). Em seguida, as proteínas virais são traduzidas e o vírus se replica (5), quando, então, formam-se novas partículas infectantes (6). RER: retículo endoplasmático rugoso; (+) ssRNA: RNA de fita simples com polaridade positiva; (-) ssRNA: RNA de fita simples com polaridade negativa. Fonte: Amorim, Alves e Ferreira. (2009).

Parte da imunidade protetora contra o VD surge após a geração de anticorpos neutralizantes contra a proteína E. A proteção imunológica é tipo-específica, ou seja, a infecção por um sorotipo viral, embora duradoura, não confere proteção aos outros três sorotipos virais. Uma particularidade da doença é o agravamento dos sintomas após infecções seqüenciais por diferentes sorotipos virais. Proposta inicialmente por Halstead em 1970, a "teoria da infecção seqüencial" defende a existência do fenômeno ADE, do inglês *antibody dependent enhancement* (HENCHAL; PUTNAK, 1990). De acordo com essa teoria, uma segunda infecção por um tipo diferente do vírus seria exacerbada pela ligação de anticorpos não neutralizantes, de anticorpos gerados durante a primeira infecção, às partículas virais do segundo sorotipo viral. Isso levaria a uma facilitação na entrada de vírions em células do hospedeiro, principalmente células dendríticas e monócitos, aumentando a carga viral, a intensidade da resposta inflamatória e danos em células endoteliais que causaria a forma hemorrágica da doença (DURBIN et al., 2008; ERAM et al., 1979; HALSTEAD, 1970; HALSTEAD, 1981; WHO, 1997).

Figura 2 - Esquema da organização das sequências de ácidos nucléicos codificadores das proteínas estruturais e não-estruturais do genoma do VD.



As funções das proteínas codificadas pelas sequências marcadas com círculos vermelhos ainda não estão totalmente elucidadas.
Fonte: Adaptada de Whitehead et al. (2007).

De fato, estudos recentes demonstraram que anticorpos não neutralizantes não interferem na infectividade da partícula viral, mas facilitam a entrada das partículas virais em células fagocíticas por meio da porção Fc dos anticorpos ligados aos vírus (VAN DER SCHAAR et al., 2009).

1.3 Estratégias vacinais voltadas para o controle do VD

Até o momento, não há uma vacina efetiva para o controle da dengue em seres humanos. Diversas estratégias vacinais contra a dengue mostram resultados positivos em condições experimentais e existe uma grande expectativa para que, em futuro, próximo uma vacina efetiva possa ser desenvolvida para o controle da dengue em seres humanos. As estratégias empregadas na pesquisa de uma vacina contra a dengue são diversas e se baseiam em diferentes tecnologias como o uso de partículas virais atenuadas ou inativas, vacinas de subunidades constituídas por proteínas virais purificadas, vacinas de DNA e vacinas vetorizadas constituídas por vetores vacinais quiméricos gerados por técnicas de engenharia genética.

1.4 Modelos experimentais para o estudo da dengue

A ausência de um modelo experimental capaz de simular a doença como observada em humanos, é uma das principais dificuldades encontradas no desenvolvimento de vacinas contra a dengue (YAUCH; SHERESTA, 2008). Diversos grupos de pesquisa no mundo buscam implementar um modelo experimental e alguns tipos de modelos já foram descritos. Os modelos diferem entre si quanto à adaptação ou não dos vírus e quanto à dose de vírus

administrada. Há modelos que analisam o efeito viral *in vitro*, (LIN et al., 2002) e há aqueles que utilizam animais para tal fim. Os modelos também variam de acordo com a via de inoculação estudada, sendo as mais utilizadas a intraperitoneal, subcutânea e intracraniana (FERREIRA et al., 2010; HALSTEAD et al., 1981). Há modelos que utilizam primatas não humanos para o estudo da patogenicidade do VD enquanto outros utilizam camundongos. Há modelos que utilizam camundongos imunocompetentes, que não possuem alterações genéticas, enquanto outros utilizam camundongos imunocomprometidos (TAN et al., 2009). Como a maioria dos modelos existentes ora utilizam camundongos imunocomprometidos de forma a diminuir sua capacidade de responder ao agente estranho, ora utilizam vírus adaptado ao modelo animal de forma a aumentar sua virulência, nenhum dos modelos atuais é capaz de simular a doença desenvolvida em humanos.

Seres humanos e mosquitos representam, até agora, os únicos hospedeiros da infecção natural pelo VD. Alguns primatas não humanos têm se mostrado permissivos à referida infecção, mas não reproduzem a doença observada em humanos (CLEMENTS et al., 2010). Por outro lado, eles desenvolvem viremia transitória e resposta de anticorpos, e tornaram-se úteis na avaliação da eficácia de candidatos vacinais e antivirais que precede os ensaios clínicos em seres humanos (BLANEY et al., 2005; SUN et al., 2006). No entanto, por razões éticas e econômicas, primatas não humanos não representam uma opção sustentável para a pesquisa com o VD.

Alternativamente, o modelo murino tem sido explorado (YAUCH; SHERESTA, 2008), mas uma grande dificuldade é que a maioria das cepas laboratoriais de VD não se replica eficientemente em camundongos e os pesquisadores precisam adaptá-las por passagens seriadas em cérebro de animais lactentes, o que insere um grande número de mutações e não permite a observação de um isolado clínico em estado selvagem em estudos patológicos (COLE et al., 1969; COSTA et al., 2007). Além disso, alguns grupos de pesquisa preferem estudar isolados clínicos em estado selvagem em modelos de camundongos geneticamente modificados para permitir a replicação do VD e o desenvolvimento de fenômenos patológicos (TAN et al., 2010). Apesar disso, o estudo da patogênese do VD com base nas cepas selvagens e linhagens não-modificadas em camundongos proporcionaria um melhor entendimento de como essa doença realmente ocorre, e facilitaria a execução de projetos no desenvolvimento de vacinas e medicamentos contra a dengue.

1.5 PERSPECTIVAS E PRINCIPAIS DESAFIOS PARA O DESENVOLVIMENTO DE UMA VACINA EFICAZ CONTRA A DENGUE

Ainda não existe uma vacina eficiente e segura para o controle da dengue. Embora várias formulações vacinais estejam em fase de teste clínico, as perspectivas para a disponibilidade de uma vacina eficiente e segura ainda são incertas e representam um desafio para pesquisadores e instituições que trabalham no problema. A vacina Chimerivax ™ (Sanofi-Aventis, França) com os estudos clínicos finalizados é uma esperança para que no futuro próximo uma vacina baseadas em vírus atenuados seja disponibilizada para uso em humanos. Essa vacina é basicamente uma quimera, resultante da inserção dos genes das proteínas prM e E do VD no genoma da cepa viral da febre amarela que é a base de uma vacina aprovada para uso em humanos (GUIRAKHOO et al., 2001). Outras formulações de vírus atenuados construídos com a inserção destes mesmos genes no genoma de cepas de VD com atenuações bem conhecidas também estão em fase de teste clínico (MEN et al., 1996). Uma vacina ideal contra a dengue deve conferir proteção contra os quatro tipos virais e impedir o agravamento da doença, como a febre hemorrágica e a síndrome do choque da dengue, nos indivíduos que tenham sido infectados previamente por pelo menos um tipo viral. No entanto, a falta de conhecimento mais sólido sobre a patogênese do vírus e dos mecanismos que geram as formas mais graves da doença lança preocupações sobre a segurança de vacinas baseadas em vírus atenuados (GUZMAN et al., 2010). A dificuldade em ativar respostas imunológicas equilibradas contra os quatro tipos virais representa também um grande desafio para o desenvolvimento de vacinas tetravalentes eficazes baseadas em vírus atenuados ou quiméricos.

As vacinas de subunidade surgem como uma alternativa bastante promissora, tendo em vista as limitações das vacinas vivas, onde a diferença em nível de virulência e/ou infectividade entre os sorotipos e cepas interfere diretamente na imunogenicidade de cada tipo viral (WHITEHEAD et al., 2007). Além disso, é possível combinar diferentes antígenos em uma mesma formulação vacinal, seja por co-administração simples ou fusão genética das proteínas antigênicas, permitindo ampliar as respostas imunológicas a diversos componentes do VD. Outros fatores dificultam o desenvolvimento de vacinas seguras e eficazes contra dengue. A ausência de modelos experimentais que permitam reproduzir de forma adequada os sintomas da doença em humanos representa uma dificuldade para teste de formulações vacinais promissoras antes dos testes clínicos, muito mais onerosos e demorados.

Finalmente, deve-se enfatizar que mesmo antes da disponibilidade de vacinas que permitam o controle profilático da dengue no Brasil e em outros países onde a doença é endêmica, medidas de controle de vetores artrópodos baseadas em ações governamentais e privadas são fundamentais para bloquear o caráter epidêmico da doença. A participação efetiva da comunidade, mobilizada por meio de campanhas educacionais, representa outro importante instrumento capaz de reduzir ou mesmo eliminar a disseminação da doença no país.

2 OBJETIVOS

Desenvolver novas vacinas de subunidade contra o vírus da dengue baseadas na proteína NS1 e domínio III da proteína E (EIII), em combinação a um derivado atóxico da toxina LT como adjuvante vacinal. As principais etapas experimentais para que esse objetivo possa ser alcançado são:

1. clonar e expressar as sequências de ácidos nucléicos codificadores das proteínas E (domínio III) e NS1 de vírus dengue sorotipo 2 (VD-2), cepa NGC, utilizando sistemas de expressão em bactérias (*Escherichia coli*);

2. purificar as proteínas do VD-2 por meio de cromatografia de afinidade em coluna niquelada;

3. expressar e purificar a forma atóxica da toxina termo lábil (LT) de *E. coli* enterotoxigênica (ETEC) com a mutação G33D na subunidade B (LT_{G33D});

4. realizar ensaios de imunização por via subcutânea com as proteínas NS1 ou EIII combinadas a uma forma atóxica de LT (LT_{G33D}), assim como outros adjuvantes vacinais, e determinação de respostas imunológicas, como produção de anticorpos séricos, ativação de complemento e indução de respostas celulares (linfócitos T $CD4^+$ e T $CD8^+$) específicas;

5. determinar o efeito protetor específico das diferentes formulações vacinais em ensaios de desafio intracerebral com o vírus DENV-2;

6. avaliar a indução de respostas auto-imunes em animais imunizados com as formulações vacinais baseadas nas proteínas NS1 e EIII do vírus DENV-2;

3 CAPÍTULO 1 - Expressão da proteína NS1 do VD-2 a partir de *Escherichia coli* com características estruturais e imunológicas preservadas em relação à proteína viral nativa

Nesta parte do trabalho, a sequência gênica codificadora da proteína NS1 (genoma do vírus da dengue sorotipo 2, cepa NGC, acessado no genebank cod. M29095, gi:323447) foi clonada em vetor de expresão pET28a, levando a super expressão protéica na linhagem de *E. coli* BL21*codonplus*(DE3)-RIL, com localização em corpúsculos de inclusão. Um método de *refolding* por diluição foi proposto para a NS1 ainda no extrato bacteriano, tornando possível a purificação desta como proteína solúvel. Uma completa caracterização da proteína NS1 incluindo análises de dicroísmo circular, *Western-blot*, *Dynamic Light Scattering* e a comparação de sua antigenicidade com a proteína nativa revelou formação de dímeros altamente termo-estáveis e com antigenicidade preservada.

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Refolded dengue virus type 2 NS1 protein expressed in *Escherichia coli* preserves structural and immunological properties of the native protein

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The dengue virus NS1 protein has been shown to be a protective antigen under different experimental conditions but the recombinant protein produced in bacterial expression systems is usually not soluble and loses structural and immunological features of the native viral protein. In the present study, experimental conditions leading to purification and refolding of the recombinant dengue virus type 2 (DENV-2) NS1 protein expressed in *Escherichia coli* are described. The refolded recombinant protein was recovered as heat-stable soluble dimers with preserved structural features, as demonstrated by spectroscopic methods. In addition, antibodies against epitopes of the NS1 protein expressed in eukaryotic cells recognized the refolded protein expressed in *E. coli* but not the denatured form or the same protein submitted to a different refolding condition. Collectively, the results demonstrate that the recombinant NS1 protein preserved important conformation and antigenic determinants of the native virus protein and represents a valuable reagent either for the development of vaccines or for diagnostic methods.

1. Introduction

Dengue fever is a major mosquito-born viral disease affecting people living in tropical and subtropical countries around the world. The disease is caused by the dengue virus, which belong to the *Flavivirus* genus of the *Flaviviridae* family, with four distinct serotypes: DENV-1, DENV-2, DENV-3 and DENV-4 (Lindenbach and Rice, 2001; Zhou et al., 2006). About 100 million cases of dengue fever (DF) are reported annually with at least 500 thousand cases with more severe symptoms, including dengue hemorrhagic fever (DHF). In these patients, mortality rates range from about 10% for patients admitted to hospital and up to 30% among patients not admitted to hospital (Gubler and Meltzer, 1999; Pongsumpun et al., 2008).

The NS1 protein is a 43–48 kDa glycoprotein expressed in infected mammalian cells as soluble monomers which form dimers in the lumen of the endoplasmic reticulum, which are transported subsequently to the cell surface where it remains either as a membrane-associated protein or released into extracellular milieu in the dimeric and hexameric forms (Winkler et al., 1988; Young et al., 2000). Although the function of the NS1 is not elucidated fully, available evidence suggests that this protein is involved in viral RNA replication (Lindenbach and Rice, 2001; Sampath and Padmanabhan, 2008). In addition, the high immunogenicity of the NS1 proteins of dengue and other flaviviruses has raised considerable interest both as an antigen for diagnostic methods (Chaiyaratana et al., 2009; Hang et al., 2009) and as component of subunit vaccine formulations (Schlesinger et al., 1987, 1993). Indeed, DNA vaccines encoding the DENV-2 NS1 protein conferred up to 100% protection to intracerebral virus challenge in murine model (Zhang et al., 1988; Costa et al., 2007).

Attempts to express the dengue virus NS1 protein in *Escherichia coli* strains have obtained limited success due mainly to the insolubility of the recombinant protein. In addition, the lack of post-translational modifications and altered secondary structure of the recombinant protein affects the formation of dimmers and results in decreased immunogenicity and antigenicity (Das et al., 2009; Zhou et al., 2006). In this study, the purification and refolding of the dengue virus type 2 (DENV-2) NS1 protein produced in *E. coli* are described. In contrast to published reports, the recombinant protein was purified as heat-stable dimers with preserved structural and antigenic determinants with regard to the protein expressed in eukaryotic cells.

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2. Materials and methods

2.1. Cloning of the dengue virus type 2 NS1 coding sequence

The pcENS1 plasmid (Costa et al., 2007) encoding the *ns1* gene from the DENV-2 New Guinea C (NGC) strain was used as template for PCR reactions. The cycling thermal parameters were followed: an initial denaturation step of 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 50.7 °C and 1 min at 72 °C, with a final extension step of 4 min at 72 °C in a Mastercycler Gradient (Eppendorf). The sense primer was 5′-ACATGCGAGGATCCGGAATGTCATACTCTAT-3′ (underlined sequence show the BamHI restriction site), and the anti-sense primer was 5′-GCCTTCTACTCGAGTTACGATAGAACTTCCTTTCTTA-3′ (underlined sequence shows the XhoI restriction site). After the amplification reaction, a 1076 pb of NGC DENV-2 NS1 gene sequence was obtained with flanking BamHI and XhoI restriction sites. The PCR product was purified with the IlustraTM GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences), digested with BamHI and XhoI, and then ligated into the corresponding BamHI and XhoI restriction sites of the linearized pET28a(+) expression vector (Novagen, Darmstadt, Germany), generating the recombinant plasmid pD2NS1, which was transformed subsequently in a chemically competent *E. coli* DH5α. Recombinant bacterial colonies were analyzed by digestion with BamHI and XhoI and PCR analysis (Sambrook et al., 1989). The inserted fragment was sequenced and compared with data reported for NGC sequence (GenBank Accession No. D00346). The cloned fragment (1076 pb) matched the NS1 gene sequence available at the GeneBank (data not shown). The expressed protein has a predicted molecular mass of 43.6 kDa corresponding to 356 amino acids of the NS1 protein and 32 amino acids encoded by the expression vector, including the N-terminal His-tag.

2.2. Expression of the recombinant NS1 protein

A chemically competent *E. coli* BL21-CodonPlus (DE3)-RIL strain was transformed with pD2NS1, to generate the BLNS1 lineage, or pET28a, to generate the BLempty strain. Both strains were cultivated in LB medium containing 50 μg/ml of kanamycin and 30 μg/ml of chloramphenicol at 37 °C until an OD_{600} of 0.5. An aliquot of bacterial of bacterial cells (t_0), collected for determination of colony forming units, was kept in ice and 0.5 mM isopropylthiogalactoside (IPTG) (Sigma) was added to the culture medium. After 4 h, another aliquot (t_4) was collected, cells were suspended in buffer A [100 mM Tris–HCl and 500 mM NaCl (pH 8.0)] and immediately lysed by sonication. After centrifugation and removal of unbroken cells, both soluble and insoluble fractions were recovered, 35 μg of total protein of each fraction were mixed with electrophoresis sample buffer and sorted by SDS-PAGE (Sambrook et al., 1989). Western blots were carried out with a reference mouse anti-DENV-2 ascitic fluid, supplied by ATCC, generated with DENV-2 proteins (1:1000 dilution in blocking buffer containing 5% skimmed milk in PBS–Tween 0.05%) and a goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma) (1:3000 dilution in blocking buffer) (Towbin et al., 1979). The reactive protein bands were developed with 5-bromo-4-chloro-3 indolyl phosphate toluidinium (BCIP) (Sigma) and nitroblue tetrazolium chloride (NBT) (Sigma).

2.3. Purification of the recombinant NS1 protein expressed in E. coli

The BLNS1 strain was cultivated in LB broth (containing 50 μg/ml kanamycin and 30 μg/ml of chloramphenicol) at 37 °C to an OD_{600} of 0.5. IPTG was added to a final concentration of 0.5 mM and cells harvested 4 h later. The pellet was suspended in buffer A and lysed by mechanical shearing using an APLAB-10 model homogenizer (ARTEPEÇAS, Brazil). After centrifugation, the inclusion body fraction was suspended in 20 ml of buffer B [100 mM Tris–HCl, 500 mM NaCl and 8 M urea (pH 8.0)]. By shaking gently at 4 °C overnight. The extract was centrifuged and the supernatant was filtered in a Sartorius Stedim apparatus with a 0.22 μm pore cellulose acetate filter (Biotech). Proteins were quantified in a GeneQuant spectrophotometer (GE Amershan Biosciences) and refolded by adding the volume into 2 l of buffer A in a 0.25 ml/min flow. After refolding, the sample was centrifuged, the supernatant filtered again in a cellulose acetate filter and 2-beta-mercaptoethanol added to a final concentration of 5 mM. The samples were submitted to nickel affinity chromatography using a Histrap™ FF column (GE Healthcare Life Sciences), previously equilibrated with buffer A, using a 1.8 ml/min flow in an Akta model FPLC (Amershan Pharmacia Biotech). The column was washed again with buffer A and then, with a linear gradient from buffer A to buffer C [100 mM Tris–HCl, 500 mM NaCl and 1 M imidazol (pH 8.0)]. The collected fractions containing the DENV-2 NS1 protein were pooled, treated with 10 U DNase (Promega) and dialyzed against sodium phosphate buffer (20 mM). The final protein yield was determined in a GeneQuant spectrophotometer (GE Amershan Biosciences). Aliquots of the refolded NS1 protein were suspended in electrophoresis sample buffer without reducing agent. To check for dimmer formation, aliquots containing 1 μg of the recombinant DENV-2 NS1 submitted or not to a heat-denaturing step, were subjected to SDS-PAGE and Western blot analyses. Thermal stability of NS1 dimers was determined after incubation at temperatures ranging from 4 °C to 100 °C for 10 min. Soluble NS1 protein was also obtained using a previously described refolding method based on slow dialyses to remove the denaturing reagent (Wu et al., 2003).

2.4. Circular dichroism (CD), dynamic light scattering (DLS) and size exclusion chromatography

Circular dichroism measurements were carried out with a JASCO J-810 spectropolarimeter equipped with a Peltier-type temperature controller and a thermostated cell holder, interfaced with a thermostatic bath. Spectra were recorded in 0.1 cm path length quartz cells at a protein concentration of 11 μM in 10 mM phosphate buffer at pH 8.0. Twenty consecutive scans were compiled and the average spectra stored. The data were corrected for the baseline contribution of the buffer and the observed ellipticities converted into the mean residue ellipticities [θ]. The secondary structure was estimated from fitted far-UV CD spectra using the Dichroweb server, method CDSSTR (Withmore and Wallace, 2004) according to data obtained from Jpred, PsiPred or Phyre servers (Bryson et al., 2005; Cole et al., 2008; Kelley and Sternberg, 2009). Thermal unfolding experiments were performed by increasing the temperature from 10 °C to 95 °C allowing temperature equilibration for 5 min before recording each spectrum. The far-UV CD spectra were recorded at the indicated temperatures using 11 μM of purified NS1 in 10 mM phosphate buffer at pH 8.0. DLS analysis was obtained at 20 °C using DLS Dynapro with 5 mM of protein at phosphate buffer 20 mM, pH 7.4, 150 acquisitions for 10 s and 60% laser.

Size exclusion chromatography was carried out in a HiLoad Superdex 75 prep grade (GE Healthcare) column equilibrated previously with buffer A using a flow of 1.0 ml/min in an Akta model FPLC (Amershan Pharmacia Biotech). Bovine serum albumin (BSA) (85 kDa), ovalbumin (OVA) (48 kDa) and lysozyme (19 kDa) (Pierce, Rockford) were used as standards. Eluted fractions containing the NS1 protein were sorted by SDS-PAGE followed by Western blots developed with anti-NS1 antibodies. Molecular masses were

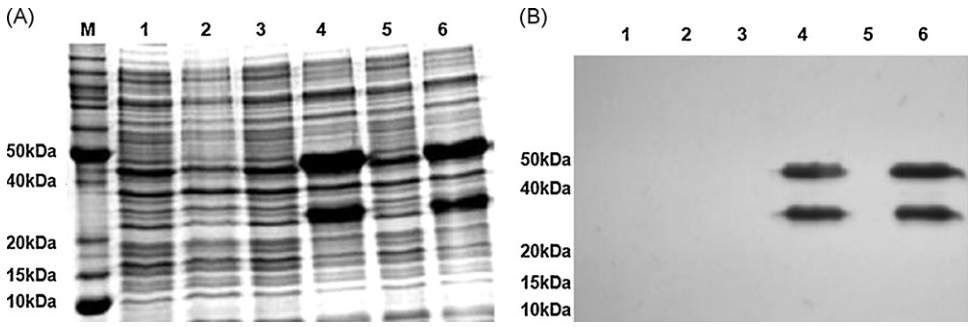

Fig. 1. Expression of the recombinant DENV-2 NS1 protein produced by the *E. coli* BLNS1 strain. (A) Coomassie blue-stained polyacrylamide gel of whole cell bacterial extracts. Samples: M, molecular mass marker; lane 1, whole cell extract of the non-induced BLempty strain; lane 2, whole cell extract of the BLempty strain after incubation with IPTG; lane 3: whole cell extract of the non-induced BLNS1 strain; lane 4, whole cell extract of the BLNS1 strain after induction with IPTG; lane 5, soluble protein fraction of the BLNS1 strain after induction with IPTG; lane 6, insoluble protein fraction of the BLNS1 strain after induction with IPTG. Each lane was loaded with 35 μg of total protein. (B) Western blot analysis of the whole cell extracts probed with mouse anti-DEN2 antibodies. Samples are the same described in (A). Molecular mass markers are indicated on the left sides of the figures.

inferred by linear regression using BSA, OVA and lysozyme retention times.

2.5. ELISA with the recombinant NS1 protein

Enzyme-linked immunosorbent assay (ELISA) with solid-phase bound NS1 protein was carried out with a mouse anti-NS1 serum generated in mice immunized with the plasmid pcTPANS1, a NS1-encoding DNA vaccine which allowed *in vivo* protein synthesis by transfected cells and conferred full protection to lethal virus challenge (Costa et al., 2007). MaxiSorp plates (Nunc) were coated for 1 h at 37 °C with different amounts (in 100 μl PBS) of the recombinant NS1 proteins generated in *E. coli* or the DENV-2 NS1 protein expressed in *Drosophila* cells (Hawaii Biotechnology Group Inc., USA). Plates were blocked overnight at 4 °C with 2% skimmed milk in 0.05% Tween 20–PBS (PBST). Serum samples were serially 2-fold diluted and added to wells washed previously with PBST. After 1 h at 37 °C, plates were washed with PBST and incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (Southern Biotechnology) for 1 h at 37 °C. Reactions were measured at A492 nm with ortho-phenylenediamine dihydrochloride (Sigma) and H_2O_2 as substrate and stopped with 9N H_2SO_4. Titers were established as the reciprocal of the serum dilution giving an absorbance above the value obtained with negative control sera (mice immunized with the pctPA vector) (Costa et al., 2007).

3. Results

3.1. Expression and purification of the recombinant DENV-2 NS1 protein

The DENV-2 NS1 coding sequence was cloned in the expression vector pET28a(+) and, after transformation of the *E. coli* BL21 (DE3) RIL strain, the expressed proteins, following 4 h incubation in the presence of IPTG, were monitored by SDS-PAGE and Western blot (Fig. 1). Protein bands with molecular masses of approximately 43 kDa and 32 kDa were detected in the insoluble protein extracts of the recombinant strain and the two protein bands reacted with the anti-NS1 antibodies. No cross-reacting protein was detected in cell extracts of the bacterial strain transformed with the pET28a vector. Expression of the recombinant NS1 protein reached an amount of approximately 135 mg of protein per liter of bacterial culture, as estimated by densitometry. Attempts to change both growth and inducing conditions of the bacterial culture did not increase the recovery yields (data not shown). Since all recombinant protein was detected in the insoluble fraction of the cell extract, different refolding methods were tested in order to generate a soluble recombinant protein. The best results were obtained after solubilization of the recombinant NS1 protein, following denaturation of inclusion bodies and refolding by a dilution method. Successful refolding of DENV-2 NS1 was achieved with a flow rate of 0.25 ml/min in 2 l of buffer A. After refolding, the cleared supernatant was applied to a Histrap™ FF (GE Healthcare Life Sciences) nickel affinity chromatography column and the bound proteins eluted with imidazol at concentrations ranging from 260 mM to 1000 mM. The purified protein was dialyzed finally with sodium phosphate buffer. Samples of the purified protein had two low molecular mass proteins with 43 kDa and 32 kDa, and two additional larger forms with approximately 50 kDa and 86 kDa (Fig. 2). The final protein yield following the refolding and purification steps reached 3.5 mg protein per liter of bacterial culture.

Incubation of the purified protein at different temperatures showed that the 86 kDa and 43 kDa bands were probably formed by dimmers and monomers of the recombinant NS1, respectively (Fig. 2). Heat-denatured samples contained only the 43 kDa and 32 kDa protein bands while non-heated samples contained mainly the 86 kDa and 50 kDa protein bands (Fig. 2). The NS1 dimers showed high thermal stability and the 86 kDa form was detected in polyacrylamide gels even after incubation at 80 °C for 10 min (Fig. 3). The same result was confirmed in the CD analyses in which loss of protein structure was detected only at temperatures above 75 °C (Fig. 3).

3.2. Spectroscopic and chromatographic analyses of the refolded NS1 protein

The spectroscopic analysis of NS1, based on the far-UV CD spectrum, showed two relative minima at 208 nm and 216 nm, characteristic of β-type proteins. In fact, prediction of the secondary structure content derived from the CD plot revealed that the recombinant protein has a total content of 41% β-sheets, 14% α-helixes and 45% loops (Fig. 4A), which is in accordance with data obtained from the Jpred, PsiPred or Phyre servers (data not shown). Interestingly, the DLS profile showed clearly that the purified NS1 protein is predominantly detected as a dimer with an apparent molecular mass of 92 kDa which fits within the estimated size of the 86 kDa protein detected in SDS-PAGE (Fig. 4B). Size exclusion chromatography confirmed that the recombinant NS1 protein generated after the refolding method had a molecular mass of approximately 88 kDa, the expected size of a protein dimer (Fig. 5). In order to determine the nature of the lower molecular mass proteins detected in SDS-PAGE of purified NS1 protein, fractions collected from the gel filtration analysis were

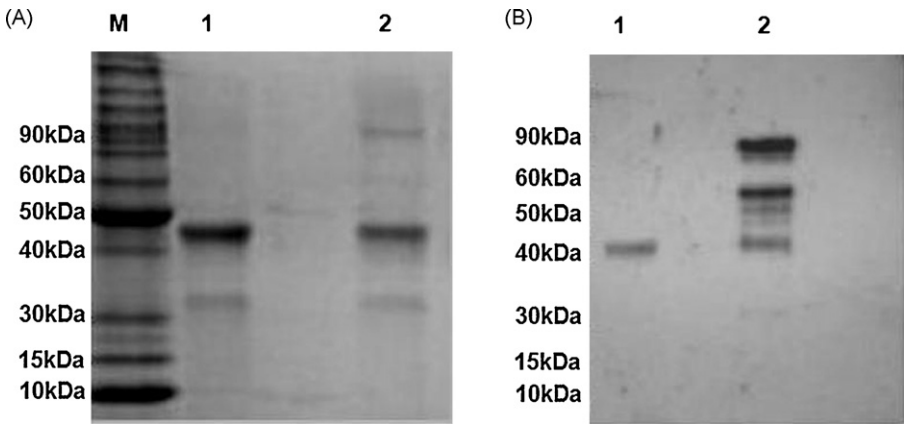

Fig. 2. The recombinant NS1 protein forms oligomers under non-denaturing conditions. Purified NS1 protein was denatured with urea and refolded according to the procedure described in Section 2. (A) Aliquots (1 μg/lane) were sorted into polyacrylamide gels after incubation at 100 °C for 10 min (lane 1) or kept at room temperature (lane 2). (B) Western blot of the samples probed with anti-DENV-2 NS1 specific antibodies. Sample numbers are the same shown in (A). Molecular mass markers are indicated as M and identified in the left sides of the figures.

sorted in polyacrylamide gels. The results indicated that extra bands detected by SDS-PAGE represent artifacts generated during the electrophoretic run (Fig. 5). As expected, heat-treated protein samples subjected to electrophoretic analysis under denaturing conditions showed a single band of approximately 43 kDa (Fig. 5).

3.3. Preserved antigenicity of the recombinant DENV-2 NS1 protein

The refolded NS1 protein was employed as solid-phase bound antigen in ELISA developed with antibodies raised in mice immunized with a protective NS1-encoding DNA vaccine (Costa et al.,

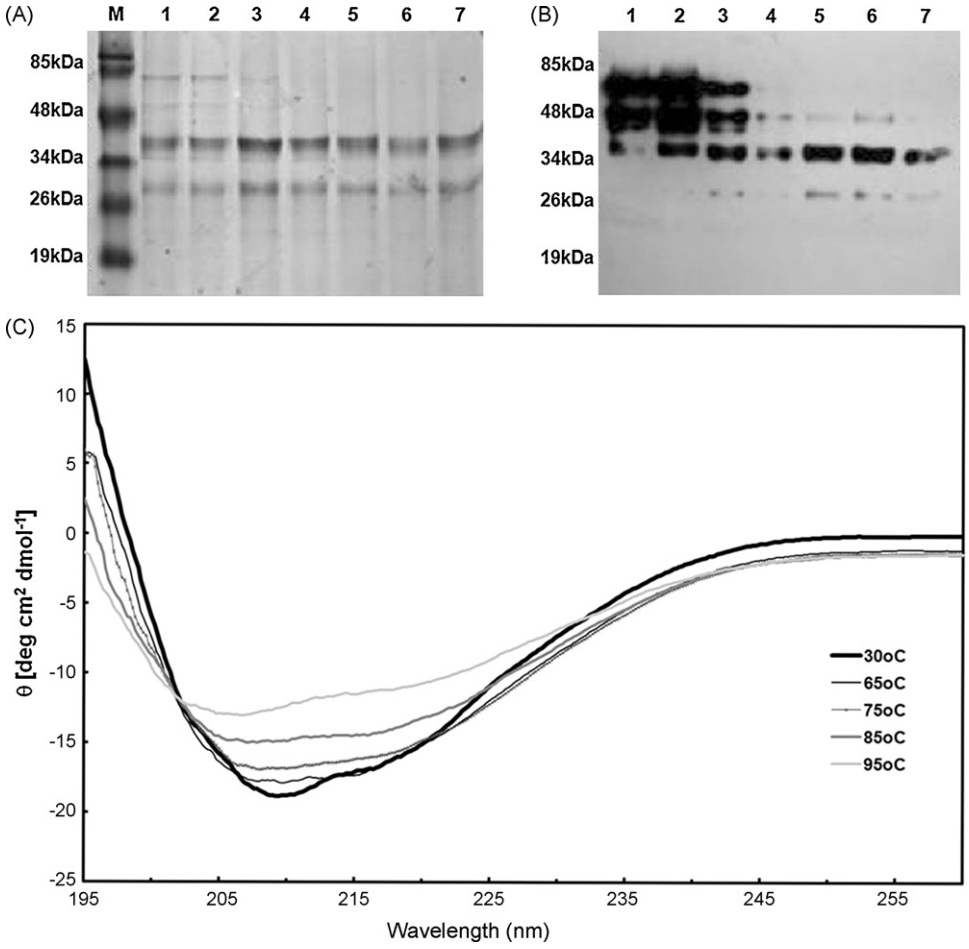

Fig. 3. Thermal stability of the NS1 oligomers. (A) Aliquots (1 μg/lane) of purified NS1 protein were incubated at different temperatures for 10 min in electrophoresis sample buffer before sorting on polyacrylamide gels and staining with Coomassie blue or (B) detected with anti-DENV-2 NS1 antibodies in a Western blot. Samples: M, molecular mass markers; lane 1, incubation at 30 °C; lane 2, incubation at 37 °C; lane 3, incubation at 80 °C; lane 4, incubation at 85 °C; lane 5, incubation at 90 °C; lane 6, incubation at 95 °C; lane 7, incubation at 100 °C. (C) Temperature-dependent denaturation of the recombinant NS1. The far-UV CD spectra of the NS1 samples incubated at different temperatures were recorded using 10 μM of purified protein aliquots in 10 mM phosphate buffer at pH 8.0.

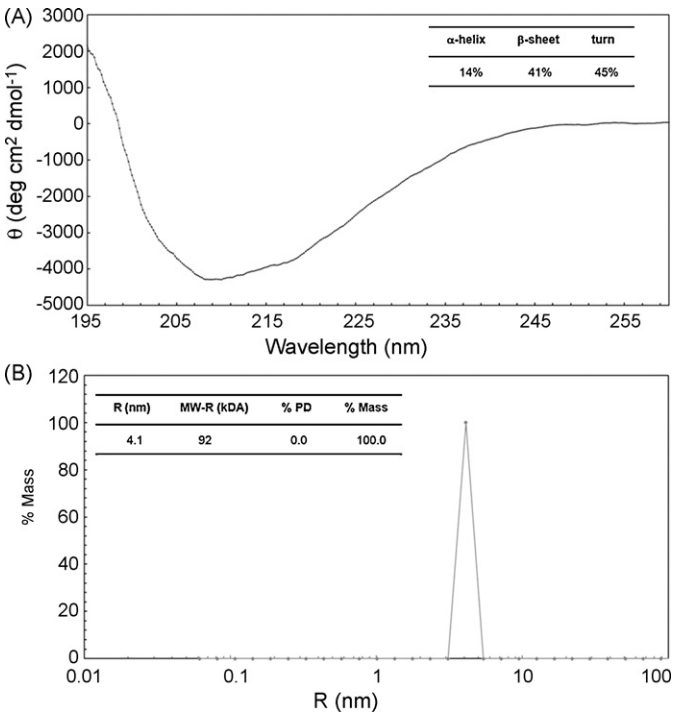

Fig. 4. Spectroscopic analysis of the refolded DENV2 NS1 protein. (A) Circular dichroism (CD) spectra and determination of the secondary structure features of the refolded NS1 protein. The amounts of β-sheets, α-helixes and turns are indicated on the right top of the figure. (B) Differential light scattering (DLS) analysis of the refolded NS1 protein. All protein obtained after the refold process has a molecular radius of 4.1 nm and a molecular mass of 92 kDa.

2007). In the first step the antigenicity of the recombinant NS1 protein prepared according to a previously described method (Wu et al., 2003) was compared with the protein prepared according to the conditions outlined above. As shown in Fig. 6A, the anti-NS1 serum reacted with at least 10-fold higher affinity with the recombinant NS1 protein prepared by the dilution refolding method. In a second step, the antigenicity of the refolded NS1 protein was compared with a recombinant protein generated in eukaryotic cells. As shown in Fig. 6B, both proteins reacted similarly with the anti-NS1 serum. Maximal titer values were achieved with 0.4 μg/well of antigen produced in *E. coli* while similar titer values were achieved with 0.1 μg/well of the NS1 protein produced in eukaryotic cells. The anti-NS1 serum did not react with both proteins after boiling the protein samples. As expected, no reaction was observed with the negative control serum collected from mice immunized with a DNA vaccine (empty vector, pcTPA) not encoding the NS1 protein (Fig. 6B).

4. Discussion

The dengue virus NS1 protein is a potential candidate for the design of subunit vaccines as well as diagnostic methods. Nonetheless, generation of recombinant NS1 protein from infected tissue culture insect cells is a laborious and costly, subjected to batch-to-batch variation making it difficult for routine large-scale production (Huang et al., 2006). Production of recombinant NS1 protein in *E. coli* strains is a much cheaper and a simpler procedure (Das et al., 2009; Hockney, 1994; Huang et al., 2006). Nonetheless, the recombinant protein purified from prokaryotic cells has been shown to be inadequate for immunological studies due to lack of conformational and antigenic determinants of the native protein (Georgiou and Valax, 1996; Hockney, 1994; Kolaj et al., 2009). In the present study, a recombinant DENV-2 NS1 protein produced in *E. coli* and treated by a refolding method showed preserved structural and antigenic determinants with regard to the protein produced in eukaryotic cells. The refolded protein remained fully soluble and formed dimers with enhanced thermal stability. More importantly, the recombinant protein was recognized efficiently by anti-NS1 antibodies generated in mice immunized with NS1-encoding DNA vaccine.

Initial screening of different *E. coli* BL21 strains showed that maximal protein yield was obtained with the BL21 (DE3) RIL strain that contains extra copies of tRNA encoding-genes (*argU*, *ileY*, and *leuW*) allowing recognition of AGA/AGG (R), AUA (I) and CUA (L) (Sahdev et al., 2008; Rosano and Ceccarelli, 2009). In spite of the enhanced expression, all the recombinant protein was recovered from inclusion bodies, which is in accordance with previous results reported by other groups (Das et al., 2009; Huang et al., 2001; Wu et al., 2003). The recombinant protein was expressed as inclusion bodies in *E. coli* and two major protein bands, with approximately 86 kDa and 43 kDa, were routinely detected in polyacrylamide gels indicating that the refolded protein assembled into dimers. Additional protein bands were observed routinely in SDS-PAGE analyses, but as showed by other analytical methods, such protein bands represent artifacts generated by the anomalous electrophoretic behavior of the NS1 protein, a finding reported previously by others (Das et al., 2009; Huang et al., 2006).

The recombinant NS1 was formed mainly by β-sheets and turns (41% β-sheets, 14% α-helixes and 45% loops). The generation of a soluble recombinant NS1 protein with preserved conformation is clearly an important tool for determination of the tertiary structure of the DENV-2 NS1 protein that still remains unsolved. Another interesting feature of the recombinant NS1 protein was the formation of heat-stable dimers. The NS1 dimers were detected even after incubation at 80 °C for 10 min. In contrast to the results obtained by other groups (Das et al., 2009; Wu et al., 2003) the present data show that the recombinant NS1 generated under the reported conditions does not require glycosylation or other post-translational

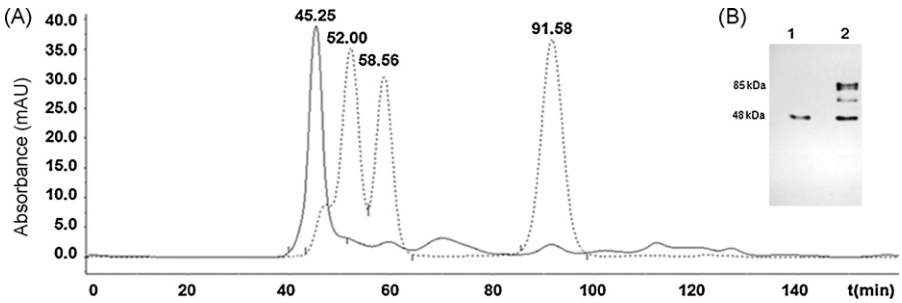

Fig. 5. Size exclusion chromatography of the refolded NS1 protein. (A) Retention times of purified proteins subjected to size exclusion chromatography. Dotted lines indicate retention times of BSA (52 min), OVA (58.56 min) and lysozyme (91.58 min). Solid line represents the retention profile detected with the refolded NS1 protein. (B) Western blot of pooled elution fractions containing the refolded NS1 protein. Samples: 1, protein heated for 10 min at 100 °C; 2, non-denatured NS1 protein.

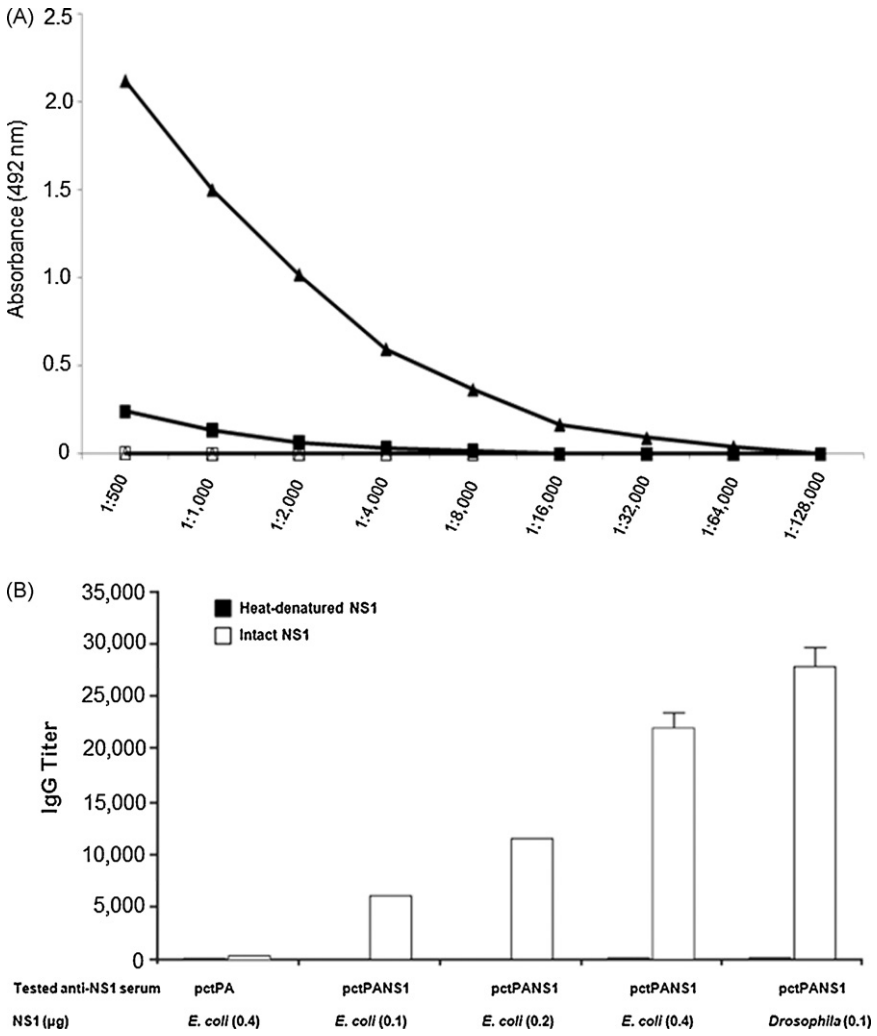

Fig. 6. Antigenicity of the refolded NS1 protein measured by reaction with the anti-NS1 serum generated in mice immunized with a DNA vaccine (pcTPANS1) encoding DENV-2 NS1. (A) Titration of the anti-NS1 serum with recombinant NS1 protein produced according to the method described in this study (black triangles) or a previous method (Wu et al., 2003) (black squares). The antigens were also incubated in the presence of a mouse serum harvested from mice immunized with plasmid pcTPA (without the DENV-2 *ns1* encoding gene) (open symbols). The amount of solid-phase bound antigen used was 0.2 μg/well. (B) Anti-NS1 antibodies titers of serum collected from mice immunized with pcTPANS1, as determined in ELISA plates treated with different amounts (0.1, 0.2, and 0.4 μg/well) of refolded (white columns) or heat-denatured (incubation at 100 °C for 10 min) NS1 protein (black columns, very low values). The same procedure was repeated with DENV-2 NS1 protein (0.1 μg/well) produced in *Drosophila* cells. As a negative control the purified recombinant NS1 was reacted with serum collected from mice immunized with pcTPA.

modifications to achieve the dimeric form. Such finding demonstrates further the usefulness of prokaryotic expression systems on the generation of heterologous proteins and the importance of adequate refolding methods in the generation of recombinant proteins with preserved structural and antigenic determinants.

A candidate vaccine antigen should preserve epitopes recognized by antibodies and T cells generated in animals infected with the virus pathogen. The antigenicity of the refolded NS1 was clearly higher than a protein purified according to a previous method (Wu et al., 2003). Under the testing conditions employed in the current study, the NS1 protein obtained by the conditions described above reacted at least 10 times better with antibodies generated in mice immunized with a protective DNA vaccine encoding the DENV2 NS1 protein (Costa et al., 2007). Similarly, comparison of the NS1 protein expressed in *E. coli* and the protein produced in eukaryotic cells showed that, on a molar basis, the bacterial protein has one forth of the antigenicity of the protein produced in insect cells. Such evidence indicates that the refolded NS1 protein produced in *E. coli* preserves important conformational epitopes of the native viral protein. The lower reactivity to the antibodies generated in mice immunized with the DNA vaccine certainly represents the lack of epitopes requiring glycosylation and other post-translational modifications that can only be reproduced in eukaryotic cell expression systems (Flamand et al., 1999; Pryor and Wright, 1994; Schlesinger et al., 1987).

The results of the present study represent the first successful attempt to obtain a recombinant NS1 protein in an *E. coli* host with preserved structural and antigenic determinants of the native viral protein. The present results indicate that the recombinant DENV-2 NS1 protein obtained according to the proposed refolding method may represent an important tool for the development of acellular vaccine formulations and diagnostic methods targeting dengue virus infection.

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4 CAPÍTULO 2 - Imunidade protetora ao VD-2 após imunização com a proteína NS1 recombinante e uso de uma forma não tóxica da toxina termolábil (LT) como adjuvante

Neste período, a proteína NS1 dimérica, descrita no capítulo anterior, foi aplicada como antígeno em diferentes formulações vacinais com o intuito de se eleger um adjuvante que modulasse a resposta imunológica contra NS1 mais protetora frente a desafio com o VD-2 pela via intracraniana em modelo murino. Nessa etapa, demonstrou-se que o adjuvante LT_{G33D}foi aquele que melhor modulou a resposta imunológica contra NS1 e conferiu maior proteção a desafio com o vírus. As respostas imunológicas humoral e celular foram monitoradas nos animais vacinados. Desta forma, demonstrou-se que as formulações utilizadas, baseadas em proteínas purificadas, induzem respostas imunológicas com pouca ativação de linfócitos T citotóxicos, os quais, por sua vez, parecem não contribuir significativamente para a proteção parcial vista nos ensaios de desafio.

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Protective immunity to DENV2 after immunization with a recombinant NS1 protein using a genetically detoxified heat-labile toxin as an adjuvant

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ABSTRACT

The dengue virus non-structural 1 (NS1) protein contributes to evasion of host immune defenses and represents a target for immune responses. Evidences generated in experimental models, as well as the immune responses elicited by infected individuals, showed that induction of anti-NS1 immunity correlates with protective immunity but may also result in the generation of cross-reactive antibodies that recognize platelets and proteins involved in the coagulation cascade. In the present work, we evaluated the immune responses, protection to type 2 dengue virus (DENV2) challenges and safety parameters in BALB/c mice vaccinated with a recombinant NS1 protein in combination with three different adjuvants: aluminum hydroxide (alum), Freund's adjuvant (FA) or a genetically detoxified derivative of the heat-labile toxin (LT_{G33D}), originally produced by some enterotoxigenic *Escherichia coli* (ETEC) strains. Mice were subcutaneously (s.c.) immunized with different vaccine formulations and the induced NS1-specific responses, including serum antibodies and T cell responses, were measured. Mice were also subjected to lethal challenges with the DENV2 NGC strain. The results showed that maximal protective immunity (50%) was achieved in mice vaccinated with NS1 in combination with LT_{G33D}. Analyses of the NS1-specific immune responses showed that the anti-virus protection correlated mainly with the serum anti-NS1 antibody responses including higher avidity to the target antigen. Mice immunized with LT_{G33D} elicited a prevailing IgG2a subclass response and generated antibodies with stronger affinity to the antigen than those generated in mice immunized with the other vaccine formulations. The vaccine formulations were also evaluated regarding induction of deleterious side effects and, in contrast to mice immunized with the FA-adjuvanted vaccine, no significant hepatic damage or enhanced C-reactive protein levels were detected in mice immunized with NS1 and LT_{G33D}. Similarly, no detectable alterations in bleeding time and hematological parameters were detected in mice vaccinated with NS1 and LT_{G33D}. Altogether, these results indicate that the combination of a purified recombinant NS1 and a nontoxic LT derivative is a promising alternative for the generation of safe and effective protein-based anti-dengue vaccine.

1. Introduction

Dengue fever is a common mosquito-borne viral disease that represents a major worldwide public health concern, particularly for those living in tropical countries and people traveling to these zones. Globally, more than 2.5 billion people are exposed to dengue virus (DENV) infection in endemic areas, and thousands of them die each year [1]. The spread of the virus observed over the last 25 years and the growing numbers of the more serious clinical cases, the dengue hemorrhagic fever (DHF) and the dengue shock syndrome (DSS), underscore the need for an effective anti-dengue vaccine [2,3]. Efforts to develop a DENV vaccine have mainly focused on attenuated or inactivated virus-based vaccine formulations. Despite the success of similar vaccine approaches in controlling other Flaviviruses, such as the yellow fever virus and the Japanese encephalitis virus, and several clinical trials conducted using most promising formulations, an effective dengue vaccine is still not available for human use [4–6]. Inefficient induction of protective immunity to the four viral types (DENV1, 2, 3 and 4),

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and safety concerns involving induction of antibody dependent enhancement (ADE), a mechanism believed to be involved in DHF and DSS occurrence, and deleterious cross-reactive reactions are the most relevant obstacles for the development of an effective dengue vaccine based on live virus particles [7].

DENV subunit vaccine formulation, based either on DNA or purified recombinant proteins represent safer alternatives to attenuated or recombinant viruses [3]. The most studied subunit vaccine approaches for dengue virus are based on either the complete envelope glycoprotein or fragments of this protein [1,8–11]. Immunization of mice with the DENV non-structural protein 1 (NS1), either as purified protein or encoded by DNA vaccines, have also shown promising results [12–16]. The DENV NS1 is a highly immunogenic 46–50 kDa glycoprotein expressed by infected cells both as a secreted oligomeric form and as a membrane-associated protein [17,18]. Although the precise functions of NS1 in the infection cycle remains unclear, it is accepted that this protein has an important role in the viral pathogenesis interfering with the complement activation cascade [19].

Mice immunized with NS1-based vaccines, particularly those encoded by DNA vaccines, develop protective immunity that involves both antibody and T cell responses [14–16]. In contrast, the protective immunity generated in mice immunized with purified NS1 protein alone seems to be based mainly on the generation of antigen-specific serum antibodies [12,13,20,21]. However, further studies have raised concern regarding the safety of NS1 as a vaccine antigen. Anti-NS1 antibodies detected in infected subjects or elicited in vaccinated mice may cross-react with proteins exposed on the surface of platelets, endothelial cells and proteins involved in the blood coagulation cascade, which may lead to vascular damages, thrombocytopenia and hemorrhage [22–27].

Adjuvants are key components of most vaccine formulations, particularly those based on purified proteins. Besides reducing the amount of antigen and number of doses required to achieve a specific immune response, adjuvants are modulators of the adaptive immunity but may lead to deleterious inflammatory reactions [28]. During decades aluminum hydroxide (alum) has been the only adjuvant alternative for human use. Nonetheless, new adjuvant alternatives became recently available for human use, such as MF59 and ASO4 [28,29]. Clearly, the identification of safe and effective adjuvants represents a key step on the development of new vaccine formulations.

The heat-labile enterotoxins (LT) are AB-type toxins produced by some enterotoxigenic *Escherichia coli* (ETEC) endowed with powerful adjuvant effects on both humoral and cellular immune responses to co-administered antigens [30,31]. Due to the intrinsic toxic effects of mucosal-delivered LT, attenuated or nontoxic LT mutants with preserved adjuvanticity have been generated by site-directed mutagenesis [31]. LT_{K63}, LT_{R72} and LT_{R192G}, with amino acid changes in the A subunit, and LT_{G33D} with a single point mutation at the B subunit, are the best characterized LT derivatives regarding both biological effects and immunological activities [32–35]. Replacing the glycine at position 33 of the B subunit with aspartate (G33D) abolishes LT binding to the GM1 ganglioside receptor and, consequently, reduces the toxin adjuvanticity following delivery via oral route [33]. Nonetheless, parenteral administration of LT_{G33D} has been shown to preserve the adjuvant properties of the protein for both B and T cell responses against co-administered antigens without induction of deleterious inflammatory reactions [35].

In this study, we evaluated the efficacy of anti-DENV vaccines based on a recombinant NS1 protein derived from type 2 DENV (DENV2) generated in a prokaryotic expression system with preserved structural and immunological features [36]. Vaccine formulations based on the recombinant NS1 protein admixed with three different adjuvants, alum, Freund's adjuvant [FA] and LT_{G33D}, were tested in mice trough parenteral administration. The results demonstrated that the adjuvant choice strongly affects both the immunogenicity and, more relevantly, the induction of protective immune responses in vaccinated mice. The results also indicate that the combination of recombinant NS1 and LT_{G33D} generates protective antibody responses without the induction of significant deleterious side effects.

2. Materials and methods

2.1. Ethics statement

All handling procedures and experiments involving mice were approved by the committee on the ethical use of laboratory animals from the Institute of Biomedical Sciences of São Paulo University, in accordance with the recommendations in the guidelines for the care and use of laboratory animals of the National Committee on the Ethics of Research (CONEP).

2.2. Virus and cell lines

The dengue 2 virus (DENV-2) strain New Guinea C (NGC) was used in the challenge assays [16,37,38]. DENV-2 NGC strain propagation was carried out in Vero cells cultured in medium 199 with Earle salts (E199) buffered with sodium bicarbonate (Sigma, USA), supplemented with 10% fetal bovine serum (FBS).

2.3. Generation of the mutated elt_{G33D} gene and purification of the recombinant NS1 and LT_{G33D}

The etl_{G33D} gene, mutated at amino acid position 33 of the B subunit, was generated by overlap extension splicing using the *elt* gene sequence of the ETEC H10407 strain [39,40]. The external primers used were 5′-CACGGTACCTCTTTCTTTATCG-3′ (*KpnI* restriction site underlined) and 5′-GGTTCTCTGCAGAGACATGC-3′ (*PstI* restriction site underlined). The internal primers responsible for introducing the mutation leading to the amino acid replacement G33D were 5′-GAATCGATGGCAGATAAAAG-3′ and 5′-CTCTTTTATCTGCCATCGAT-3′. The amplification reactions were performed as described previously [39]. The resulting fragment was purified using a gel purification kit (Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit, GE Healthcare), digested with restriction enzymes and then ligated into the corresponding *KpnI* and *PstI* sites of the linearized pBSPKS (−) vector [41], generating the recombinant plasmid $pKSLT_{G33D}$. The $pKSLT_{G33D}$ plasmid was subsequently introduced into chemically competent *E. coli* DH5α bacteria. One bacterial clone carrying the correct plasmid was named $LDVLT_{G33D}$. The correct sequence of the etx_{G33D} gene was confirmed by DNA sequencing. LT_{G33D} was purified by galactose-affinity chromatography following a standard LT purification procedure [40]. Briefly, the $LDVLT_{G33D}$ lineage was cultivated in Terrific Broth (TB) [42], containing 200 μg/ml of ampicillin, overnight at 37 °C in an orbital shaker set at 200 rpm. Cells were suspended at a 10% (w/v) concentration in TEAN buffer (50 mM Tris; 1 mM EDTA; 3 mM azide-Na and 200 mM NaCl; pH 7.5) and lysed by mechanical shearing in an APLAB-10 homogenizer (ARTEPEÇAS, Brazil). The soluble extract was applied into a XK 16/20 column (GE Amershan Biosciences) containing immobilized D-galactose gel (Pierce), extensively washed with TEAN buffer prepared with pyrogen-free water, and subsequently eluted with TEAN buffer containing 0.3 M galactose. The final amount of LT_{G33D} was determined in GeneQuant spectrophotometer (GE Amershan Biosciences). The purification of the DENV2 NS1 recombinant protein was achieved after denaturation/refolding steps of the protein expressed in bacterial cells and affinity chromatography, as previously reported

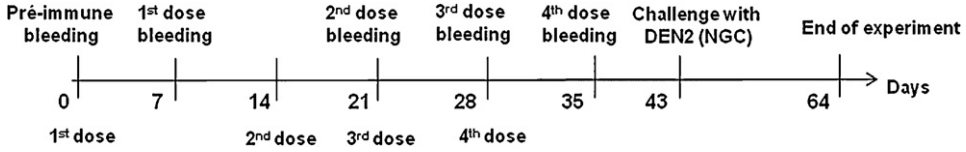

Fig. 1. Immunization regimen and virus challenge with the tested NS1-based vaccine formulations.

[36]. Endotoxin levels in LT_{G33D} and NS1 preparations were determined with the Chromogenic Limulus Amebocyte Lysate assay (Cambrex Bio Science) [43].

2.4. ELISA and immunoblot analyses of NS1 and LT_{G33D}

The recombinant NS1 and LT_{G33D} proteins were analyzed for purity and antigenicity by SDS-PAGE and Western blot. Protein aliquots (2 μg) were sorted in 15% polyacrylamide gels after heat treatment (100 °C for 10 min) or kept at room temperature with sample buffer [36,44]. Standard ELISA assays were performed as previously described [36,45]. The recombinant NS1 protein was tested in the non-heated or in heat-denatured state with serum samples collected from a DENV2-infected individual (kindly supplied by Dr. Bergman M. Ribeiro, Brasília University, FD, Brazil). A serum sample generated after immunization of mice with heat-denatured (100 °C for 10 min) NS1 in FA after the same immunization regimen described bellow (Fig. 1), was used in order to demonstrate that heat denaturation of the recombinant NS1 did not affect binding to ELISA plates. GM1-ELISAs using purified LT_{G33D} and parenteral LT derived from ETEC H10407 strain were carried out as reported previously [40].

2.5. Immunization regimens

BALB/c mice, 4–6 weeks old, were divided into groups ($n = 6$ for immune response monitoring and $n = 10$ for the virus challenges) and submitted to an immunization regimen comprising four doses of the tested vaccine formulations administered via the subcutaneous (s.c.) route on days 0, 14, 21 and 28 (Fig. 1). Mice were inoculated with 10 μg of NS1 alone or the same amount of NS1 combined with: 1.25 μg of alum (Rehydragel from Reheis), according to a standard procedure [46] that results in 99.7% binding of the protein to the solid matrix, Freund's adjuvant (50%, v/v), with the complete adjuvant in the first dose and the incomplete formulation in the subsequent injections; or 1 μg of LT_{G33D}. The amount of LT_{G33D} used in the vaccine formulations was based on previously reported results [36]. Sham-treated mice were injected with phosphate buffered saline (PBS). Mice were bled at the retro-orbital plexus before each vaccine dose and one week after the last administration. Serum samples were individually tested for reactivity to NS1, pooled and stored at −20 °C for subsequent analyses.

2.6. Determination of anti-NS1 serum antibody responses

Mouse sera were tested individually for the presence of NS1-specific antibodies by ELISA, as previously described [45]. Briefly, MaxiSorp plates (Nunc) were coated with 0.2 μg per well of the recombinant NS1 protein in 100 μL PBS and blocked for 1 h at 37 °C with 5% skim milk in 0.05% Tween-20–PBS (PBST). Serum samples were serially diluted and added to wells previously washed with PBST. After 1 h at room temperature, plates were washed with PBST and incubated with goat anti-mouse immunoglobulin (whole IgG isotype, IgG1 or IgG2a subclasses) conjugated with horseradish peroxidase (Southern Biotechnology) for 1 h at room temperature. Reactions were measured at $A_{490\,nm}$ with *ortho*-phenylenediamine dihydrochloride (Sigma) and H_2O_2 as substrate and with a 2 N H_2SO_4 stopping solution. Titers were established as the reciprocal of serum dilution which gave an absorbance two-fold higher than the SD values of the respective non-immunized samples.

2.7. Determination of cytokine-secretion patterns

One week after the last immunization, mice were euthanized and their spleens were harvested. Splenocytes were pooled and seeded (5×10^5 cells per well) in 12-well plates (Nunc) in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids, 10 mM HEPES buffer and 50 units/ml of penicillin–streptomycin. Cells were then incubated with purified NS1 at 37 °C with 5% CO_2 for 48 h. Culture supernatants were collected and tested individually for IFN-γ and IL-5 by ELISA, according to the manufacturer's instructions (BD Bioscience), as markers for activation of type 1 and type 2 Th responses, respectively.

2.8. Lethal challenges with DENV2

Two weeks after the final vaccine dose, mice were challenged with the DENV2 NGC strain, a mouse-adapted virus strain, as previously described [16,37,38]. Animals were anesthetized with a mixture of ketamine and xylazine [47] and intra cranially (i.c.) challenged with 30 μl of E199 medium supplemented with 5% FBS containing 4.32 $\log_{10}$ PFU of DENV-2, which corresponds to approximately 3.8 LD_{50}. Animals were monitored for 21 days, and mortality and morbidity rates were recorded.

2.9. ELISPOT

The IFN-γ ELISPOT assay was performed as previously described [40]. Two weeks after the immunization regimen, cells derived from spleens of vaccinated mice were placed (2×10^5 cells/well) in a 96-well micro titer plate (MultiScreen, Millipore) previously coated with 10 μg/ml of rat anti-mouse INF-γ monoclonal antibody (mAb) (BD Pharmingen). Cells were cultured at 37 °C with 5% CO_2 for 18 h in the presence or absence of 5 μg of the H-2d-restricted $CD8^+$ T cell-specific epitope AGPWHLGKL ($NS1_{265-273}$), a highly conserved epitope among the DENV serotypes [48]. As a positive control, cells from all groups were pooled and cultured in the presence of concanavalin A, as previously described [49]. After incubation, cells were washed away, and plates were incubated with a biotinylated anti-mouse INF-γ mAb (BD Pharmingen) at a final concentration of 2 μg/ml at 4 °C. After 16–18 h, the plates were incubated with diluted peroxidase-conjugated streptavidin (Sigma–Aldrich). The spots were developed using diaminobenzidine (DAB) substrate (Sigma–Aldrich) and counted with a stereo microscope (model SMZ645, Nikon).

2.10. In vivo evaluation of NS1-specific cytotoxic $CD8^+$ T lymphocytes

The in vivo assessment of the cytotoxic activity of $CD8^+$ T cells induced in the different immunization groups was carried out as previously described [40]. Splenocytes from naive mice were stained with 0.5 μM or 5 μM carboxyfluorescein diacetate

succinimidyl ester (CFSE) (Invitrogen) for 15 min at 37 °C. The cells labeled with 5 μM of CFSE were then pulsed with the $NS1_{265–273}$ oligopeptide (AGPWHLGKL) [48,50]. Both CFSE-labeled cell populations, $NS1_{265–273}$ pulsed or not, were transferred intravenously to vaccinated mice (2×10^7 cells of each population). One day later, the inoculated animals were euthanized and individual spleens were isolated to identify the two CFSE-labeled cell populations by multivariant FACScan analyses (FACSCalibur from BD Biosciences). The percentages of specific target cell killing were calculated for each individual by comparing the reduction of peptide-pulsed cells relative to that of the non-pulsed cells.

2.11. NS1-antibody affinity determination

The affinity of anti-NS1 antibodies was assessed by the ammonium thiocyanate elution-ELISA method, as previously described [51]. The procedure was similar to that of the standard ELISA with the inclusion of an extra step. After incubation with the pooled sera diluted according to titers obtained by ELISA, the plates were washed and ammonium thiocyanate, diluted in PBS, was added to the wells in concentrations ranging from 0 to 8 M. Plates were maintained at room temperature for 15 min. The percentage of antibody binding was calculated as the OD_{490} in the presence of ammonium thiocyanate × 100, divided by the OD_{490} in the absence of ammonium thiocyanate.

2.12. Monitoring tissue damages in vaccinated mice

Individual serum samples were used to determine glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and C-reactive protein (CRP) levels, using analytical kits as recommended by the supplier (Bioclin, Brazil).

2.13. Bleeding time and blood cell analyses

Bleeding time was measured at day seven following the fourth vaccine dose by creating a 3 mm incision at the tail tip. Blood droplets were collected on filter paper every 30 s for the first 3 min, and every 10 s thereafter. Bleeding was considered to be finished when the collected blood spot's diameter was less than 0.1 mm [22]. Complete blood cell counts were also taken at this time. Whole blood samples were collected in micro tubes containing 0.37 M EDTA. For hematocrit determination, micro capillaries were filled with blood samples, centrifuged at 5000 rpm for 5 min and properly positioned in a packed cell volume table for hematocrit scoring [52]. Red blood cell (RBC) and white blood cell (WBC) counts were carried out using a Neubauer chamber. Platelet numbers were determined according to the Fonio's method and neutrophil and lymphocyte differentiation was performed visually using a phase contrast microscope [52], (Eclipse E200 model, Nikon).

2.14. Statistical analyses

Statistical analyses were carried out using ANOVA and a subsequent Bonferroni's Multiple Comparison test. For survival and morbidity rates, Mantel–Cox and Gehan–Breslow–Wilcoxon tests were performed. Statistical significance was set as $p < 0.05$.

3. Results

3.1. Generation of purified NS1 and LT_{G33D}

Both NS1 and LT_{G33D} were produced by recombinant *E. coli* cells and tested for antigenicity and/or biological activity. The recombinant DENV2 NS1 protein was obtained mainly as dimers, as demonstrated after sorting in polyacrylamide gels (Fig. 2A). As demonstrated previously [36], the recombinant NS1 preserved, at least partially, some features of the native virus protein. In addition, the recombinant NS1 retained, at least in part, the antigenicity of the native protein as demonstrated by the reactivity of the recombinant protein with a serum sample collected from a DENV2 infected patient (Fig. 2B). The reactivity of the anti-NS1 serum sample was drastically reduced after heat denaturation of the recombinant protein, which indicates that conformational epitopes of the protein were lost. To demonstrate that the heat-denaturation treatment did not interfered with the binding of protein to the ELISA plates, the protein samples were reacted with a mouse serum raised in mice immunized with a heat-denatured NS1 (Fig. 2B). In contrast to antibodies raised in the DENV2 infected subject, this serum sample did not show any reduction in the recognition of the heat-denatured NS1 in ELISA, which indicated that denaturation of the recombinant protein did not affect the binding of the protein to the plate. The purified recombinant LT_{G33D} protein encompassed both the A and B subunits, as detected in polyacrylamide gels (Fig. 2C). As expected, the recombinant protein showed reduced binding to the GM1 ganglioside when compared to the parental toxin (Fig. 2D), as previously reported [35]. Both NS1 and LT_{G33D} preparations had low residual LPS concentrations (50 EU/mg and 82 EU/mg, respectively). The amount of endotoxin administered in each mice was 0.5 endotoxin units/dose and 0.582 endotoxin units/dose in samples containing NS1 alone or NS1 and LT_{G33D}, respectively, which did not interfere with the induced immune response of vaccinated mice (data not shown) [43].

3.2. Immune responses in mice immunized with NS1-containing vaccines

To determine the immunogenicity of the recombinant NS1 protein, BALB/c mice were s.c. immunized with the purified protein admixed with one of three different adjuvants (alum, FA or LT_{G33D}) using a four-dose vaccine regimen (Fig. 1). Under the testing conditions, 99.7% of the NS1 protein remained bound to the alum salts, while vaccines adjuvanted with FA or LT_{G33D} were prepared according to previously reported conditions [35,46]. Measurement of the serum anti-NS1 IgG responses showed that mice immunized with three or four doses of NS1 admixed with LT_{G33D} elicited stronger responses than those immunized with vaccines containing alum or FA ($p < 0.001$). In addition, assessment of the serum IgG subclass responses showed that mice immunized with NS1 and alum produced low IgG2a levels (IgG1/IgG2a ratios of 83) while those immunized with NS1 in combination with FA or LT_{G33D} elicited more balanced subclass responses with IgG1/IgG2a ratios of 4.3 and 1.8, respectively. A similar response profile was observed when assessing IFN-γ and IL-5 secretion in the culture supernatants of NS1-stimulated spleen cells collected from mice immunized with the three different vaccine formulations. As demonstrated in Fig. 3C, the IFN-γ/IL-5 ratio (5.74) detected in mice immunized with NS1 and LT_{G33D} was higher than the ratios detected in mice immunized with NS1 combined with alum or FA (0.32 and 3.52, respectively). Interestingly, mice immunized with LT_{G33D} and NS1 generated serum antibodies with enhanced avidity to the NS1 protein (Fig. 3D). The concentration of ammonium thiocyanate required to dissociate 50% of the antibodies bound to NS1 in sera collected from mice immunized with LT_{G33D} was approximately two and four-fold higher than the amounts of the reagent required to dissociate anti-NS1 antibodies generated in mice treated with FA and alum, respectively.

We also measured the induced T cell responses in mice immunized with the different NS1-based vaccine formulations. As shown in Fig. 3E and F, the tested vaccine formulations induced low anti-NS1 $CD8^+$ T cell responses in mice, as measured by the numbers of

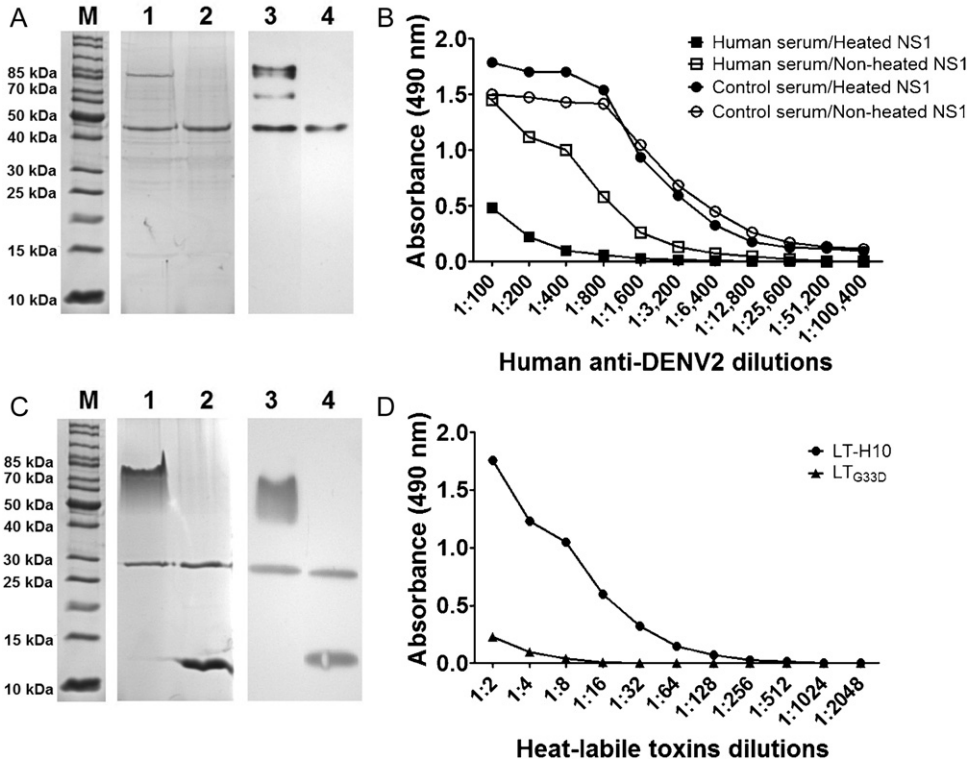

Fig. 2. Electrophoretic patterns and biological activities of the recombinant NS1 and LT$_{G33D}$. (A) Purified refolded NS1 protein detected in a SDS-polyacrylamide gel (lanes 1 and 2) and immunoblot (lanes 3 and 4). Aliquots of the purified protein (1 μg) were sorted in a 15% polyacrylamide gel stained with Coomassie brilliant blue (lanes 1 and 2) or transferred to a nitrocellulose membrane and reacted with serum collected from mice immunized with a DNA vaccine encoding the DENV2 NS1 protein (lanes 3 and 4). Heat-denatured samples (lanes 2 and 4) were obtained following protein incubation at 100 °C for 10 min M, molecular weight markers (band sizes indicated at the left side). (B) Reactivity of the intact recombinant NS1 protein with a serum sample collected from a DENV2-infected patient (open squares). Heat treatment (100 °C for 10 min) abolished the antigenicity of the protein as demonstrated by the lack of reactivity with the serum antibodies (closed squares). The control serum generated in mice against the heat-denatured form of the recombinant NS1 reacted in the same degree with the non-heated (closed circles) and the heated (open circles) forms of the antigen, showing that heat treatment did not affect NS1 binding to the plate. (C) Purification of the LT$_{G33D}$ by galactose-affinity chromatography. Protein samples (1 μg) were sorted in 15% polyacrylamide gels and stained with Coomassie brilliant blue (lanes 1 and 2) or reacted with an anti-LT mouse serum in a Western blot (lanes 3 and 4). Heat-denatured samples (lanes 2 and 4) were obtained following incubation of the protein at 100 °C for 10 min M, molecular weight markers (band sizes indicated at the left of the figure). (D) GM1-ELISA of the purified non-toxic LT$_{G33D}$ and the parental non-mutated LT1. GM1-binding mediated by the B subunit was ablated by the G33D B subunit mutation in LT$_{G33D}$.

NS1-specific IFN-γ secreting cells. In addition, experiments carried out with mouse splenocytes labeled with CFSE and pulsed with the MHC-I-restricted CD8$^+$ T cell specific AGPWHLGKL peptide showed that the induced antigen-specific cytotoxic responses induced in animals submitted to the different immunization regimen were low but with higher responses detected in mice immunized with NS1 admixed with FA or LT$_{G33D}$ regarding mice immunized with NS1 adjuvanted with alum.

3.3. Protective responses induced in mice immunized with NS1-based vaccines

Protective anti-DENV2 responses were measured in mice immunized with the different vaccination formulations following administration of a lethal i.c. challenge with the DENV2 NGC virus strain. As demonstrated in Fig. 4A, mice vaccinated with NS1 and LT$_{G33D}$ showed a 50% protection level. A lower but not statistically different result was observed in mice immunized with NS1 and FA (40% protection). In contrast, no protection was observed in mice immunized with NS1 combined with alum, non-adjuvanted NS1 or sham-treated animals. We also monitored the DENV2-associated morbidity and, as indicated in Fig. 4B, and mice immunized with NS1 combined with LT$_{G33D}$ or FA showed similar degree of partial limb paralysis (80% and 70% of the vaccinated mice, respectively). As expected, all mice immunized with NS1 and alum, NS1 or sham-treated animals showed severe limb paralysis before death by virus encephalitis.

3.4. Safety evaluation of the NS1-based vaccines

Previous studies indicated that anti-NS1 antibodies may recognize cross-reacting epitopes on platelets and endothelial cells, as well as proteins involved in the coagulation pathway, provoking hematological disturbances [22–26]. As a first step to investigate the safety of the NS1-based vaccine formulations, we measured biochemical markers of hepatic function and nonspecific tissue inflammatory reactions in vaccinated mice. As shown in Fig. 5A and B, GOT and GPT enzyme markers were significantly increased in mice immunized with NS1 admixed with FA but not in mice immunized with NS1 and LT$_{G33D}$. Similarly, C-reactive protein levels were, on average, higher in mice immunized with NS1 and FA than in mice immunized with NS1 and LT$_{G33D}$ or in sham-treated mice. These results indicate that incorporation of FA, but not LT$_{G33D}$, could induce mild inflammatory reactions among the vaccinated mice. In a second step, we determined hematological parameters that could indicate disturbances induced by the vaccine formulations adjuvanted with LT$_{G33D}$. For that purpose mice immunized with NS1 and LT$_{G33D}$ were monitored for hematocrit values, bleeding time,

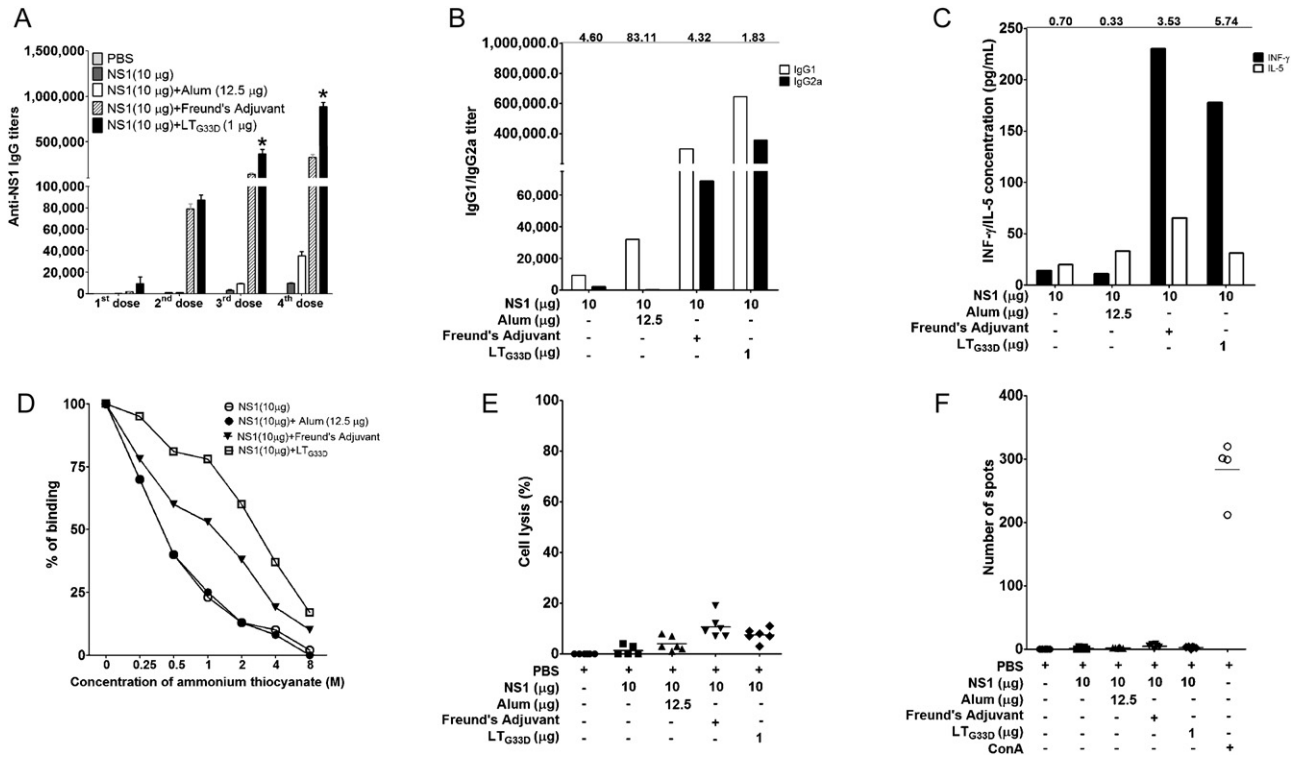

Fig. 3. Immune responses in mice immunized with NS-1-based vaccine formulations. (A) Serum IgG anti-NS1 responses detected in BALB/c mice immunized with purified NS1 in combination with different adjuvants. Mouse groups ($n = 6$) were s.c. immunized with four doses of 10 μg of purified NS1 admixed with alum (1.25 μg), FA (1:1, v/v), or LT$_{G33D}$ (1 μg). The anti-NS1 antibody responses were measured one week after each vaccine dose. Mouse groups immunized with non-adjuvanted NS1 and sham-treated mice were also included as controls to determine the adjuvant effects and non-specific reactions, respectively. Anti-NS1 titers were represented as reverse values of the maximal dilutions yielding $A_{490\,nm} > 0.1$. Values are based on individual responses and expressed as means ± SD. Mice immunized with three or four doses of NS1 admixed with LT$_{G33D}$ elicited stronger responses than those immunized with vaccines containing alum or FA ($^*p < 0.001$). (B) Anti-NS1 serum IgG subclasses responses in mice treated with the different vaccine regimens. Anti-NS1 IgG1 and IgG2a titers were represented as reverse values of the maximal dilutions yielding $A_{490\,nm} > 0.1$. The IgG1/IgG2a ratios of each vaccination group are indicated on the top of the figure. Values were determined using serum pools of each immunization groups. (C) Secreted cytokine responses measured in culture supernatants of spleen cells collected from mice vaccinated with the different immunization regimens. The INF-γ and IL-5 values were determined after stimulation with purified NS1. INF-γ/IL-5 ratios are indicated at the top of the figure. (D) Antigen affinity determination of anti-NS1 antibodies raised in mice treated with the different vaccination regimens. Antigen affinity was determined as the ammonium thiocyanate concentration (M) required to dissociate 50% of antibodies bound to NS1 on ELISA plates. (E) In vivo NS1-specific cytotoxic CD8$^+$ T cell activity in mice immunized with different vaccine formulations. Spleen cells collected from naïve mice were labeled with CFSE and pulsed with the H-2d-restricted CD8$^+$ specific NS1 epitope AGPWHLGKL (NS1$_{265-273}$) and subsequently i.v. inoculated in mice immunized with the vaccine regimens. Results are expressed as cell count reduction percentages with regard to cells labeled only with CFSE, 18 h after administration of the labeled cells in the tested mice. (F) Numbers of INF-γ-secreting CD8$^+$ T lymphocytes measured in ELISPOT following *in vitro* stimulation with the NS1$_{265-273}$ peptide. Concanavalin A was used as a stimulatory positive control. Data presented in this figure represent one of two independent experiments.

platelet counts and leukocyte counting, including neutrophils and lymphocytes. As indicated in Table 1, no evidence of hematological disturbance or hemorrhage was observed in mice immunized with NS1 and LT$_{G33D}$ up to seven days after immunization.

4. Discussion

In this study, we tested NS1-based vaccine formulations using a purified recombinant protein co-administered with different

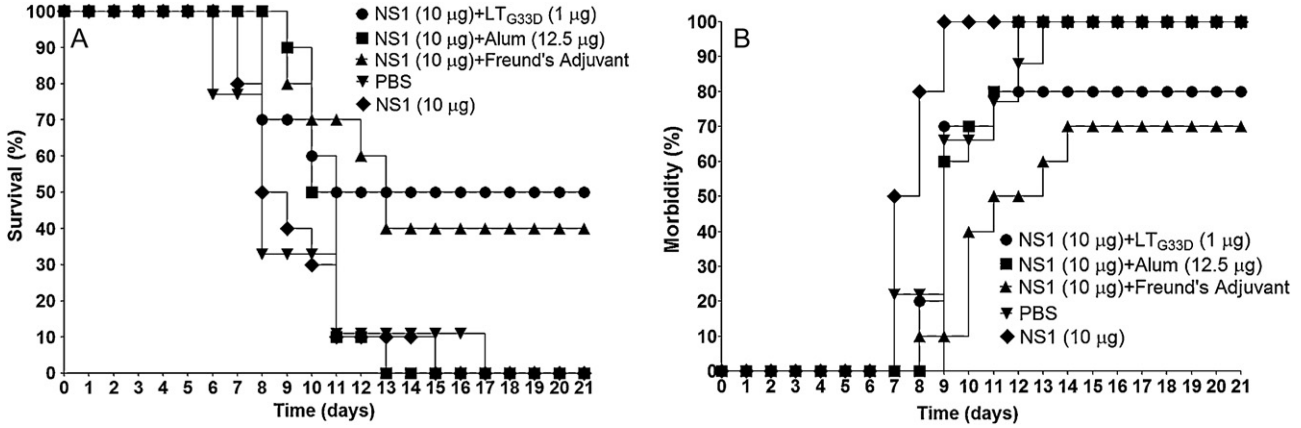

Fig. 4. Anti-DENV2 protection conferred to mice immunized with NS1-based vaccine formulations. Mice were i.c. challenged with 4.32 $\log_{10}$ PFU of the NGC DENV2 strain two weeks after the last vaccine dose. Survival (A) and morbidity (B) values were monitored for 30 days after the virus challenge.

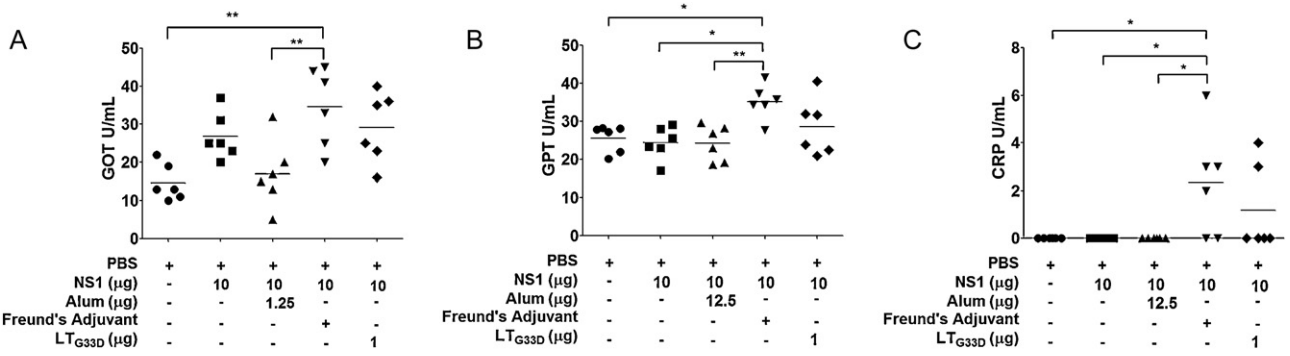

Fig. 5. Safety evaluation of the tested NS1-based vaccine formulations. (A) GOT, (B) GPT and (C) CRP levels were measured in serum samples collected from mice submitted to the different immunization regimens. Values are expressed as units/ml of blood. $^{*}p < 0.05$; $^{**}p < 0.01$. Data presented in this figure represent one representative result of two independently performed experiments.

adjuvants as an attempt to develop a safe and effective alternative for the control of dengue virus infection. The recombinant NS1 protein, despite production in bacterial cells, preserved important immunological features of the native protein, including specific reactivity with antibodies generated in a DENV-2 infected subject. In addition to alum and FA, we tested a nontoxic LT derivative, LT_{G33D}, as parenterally delivered adjuvants. Although devoid of the natural toxicity, the LT derivative showed strong adjuvant effects regarding induction of anti-NS1 serum antibodies. Mice immunized with vaccine formulations adjuvanted with LT_{G33D} showed partial protection to lethal encephalitis after challenge with a mouse-adapted DENV2 strain, similar to that achieved in mice immunized with NS1 adjuvanted with FA. However, in contrast to mice immunized with FA, mice immunized with NS1 and LT_{G33D} did not show any significant side effects regarding altered hepatic function and unspecific inflammatory reactions. In addition, mice immunized with NS1 and LT_{G33D} did not show any altered hematological parameters, such as neutropenia, and bleeding tendency. Altogether, these results demonstrated that the combination of NS1 and LT_{G33D} represents a promising alternative for the development of potentially safe and effective protein-based anti-dengue vaccines.

Parenteral administration of the recombinant NS1 protein admixed with one of three tested vaccine adjuvants (alum, FA and non-toxic LT derivative) had distinct effects regarding the induction of antigen-specific immune responses. Mice immunized with NS1 in combination with LT_{G33D} showed higher NS1-specific IgG titers compared to mice immunized with vaccines adjuvanted with alum or FA. These results were particularly relevant since alum still represents the first adjuvant choice for human vaccines. The rather low anti-NS1 antibody responses elicited in mice immunized with alum was not attributed to a defective binding of NS1 to the salt matrix and may reflect an inherent feature of the antigen. Although mice immunized with FA and NS1 elicited strong anti-NS1 antibody responses the use of this adjuvant is not acceptable for a potential human vaccine due to its reactogenicity. Thus, the demonstration that the administration of a non-toxic LT derivative induces elevated anti-NS1 IgG levels without exacerbated inflammatory reactions represents a relevant contribution for the development of new protein-based anti-dengue vaccines. Of particular interest was the observation that anti-NS1 antibodies elicited in mice immunized with LT_{G33D} have shown a clear increase in the avidity to the viral antigen. Previous studies based on immunization of rhesus monkeys with inactivated, live attenuated virus or DNA vaccines encoding the envelope protein showed that protective antibody responses correlated both with the serum antibody titers and avidity to the target antigen [10]. The finding that co-administration of LT_{G33D} may increase the affinity of the anti-NS1 antibodies to the target antigen may, therefore, represent an important feature of an adjuvant incorporated into a subunit-based anti-dengue vaccine.

Protection induced by NS1-encoding DNA vaccines to the DENV mouse encephalitis challenge model indicated that both antigen-specific B and T cells are important for the mounting of a protective immune response [14–16]. Under our experimental conditions, immunization with purified NS1 in combination with FA or LT_{G33D} resulted in the activation of B lymphocytes, as evaluated by the serum anti-NS1 levels, but less efficient activation of cytotoxic NS1-specific $CD8^+$ T cell-mediated responses. Activation of $CD4^+$ T helper lymphocytes was inferred indirectly both by the IgG subclass response as well as by the production of cytokines by NS1-stimulated splenocytes (IFN-γ for a Th1-biased pattern and IL5 for a Th2-biased response). Although IgG subclass response does not seem to be a particularly relevant parameter regarding DENV protection, INF-γ is known to interfere with viral replication and positively correlates with development of protective immunity [16,53]. In these two aspects both FA and LT_{G33D} showed similar behavior after s.c. administration to mice with a more balanced Th1/Th2 immune response pattern regarding animals immunized with NS1 and alum. It is conceivable that the partial protective immunity induced in mice immunized with FA or LT_{G33D} vaccine formulations is closely related to the circulating NS1-specific antibodies, in accordance to previous observations [12,13,20,21]. More proper evaluation of the protective role of anti-NS1 T cell responses, particularly those involving activation of cytotoxic responses, will

Table 1
Hematological analyses of mice immunized with purified NS1 and LT_{G33D}.

Hematological parameters[b]	Immunization groups[a]		
	PBS	NS1 (10 μg)	NS1 (10 μg) + LT_{G33D} (1 μg)
WBC	7.5 ± 1.28	6.1 ± 2.07	7.47 ± 2.92
NEU	1.5 ± 0.33	1.22 ± 0.37	1.42 ± 0.72
LYM	5.32 ± 0.99	4.52 ± 1.79	5.75 ± 2.24
RBC	6.53 ± 1.72	6.32 ± 2.13	5.48 ± 0.57
HCT[c]	36.83 ± 0.98	37.17 ± 0.98	37.5 ± 1.05
PLT	1.55 ± 0.35	1.57 ± 0.45	1.38 ± 0.32
BT[d]	188 ± 46	220 ± 151	195 ± 75

[a] Six Balb/c mice per immunization group were bled 7 days following the final dose.

[b] Blood samples were processed to determine white blood cells (WBC), neutrophils (NEU), lymphocytes (LYM), red blood cells (RBC), and platelets (PLT) numbers. WBC, NEU, LYM and PLT counts are given in 10^3 cells/μl. RBC count are expressed in 10^6 cells/μl.

[c] Hematocrit (HCT) values are given in percentages (%).

[d] The bleeding times (BT) are expressed in seconds. Data are expressed as the mean ± SD of individual measurements. Differences among groups were not seen after ANOVA and Bonferroni's Multiple Comparison test. Results contained in this table represent one of two independent experiments.

require the development of protein-based vaccines with improved effect on the induction of $CD8^+$ T cell-dependent responses or the testing of more complex vaccine regimens, such as those involving priming with NS1-encoding DNA vaccines.

The safety of the vaccine formulation is a major issue for those working on the development of anti-dengue vaccines. Although protein-based subunit vaccines tend to be safer than vaccines based on live attenuated or recombinant viruses [3], incorporation of an adjuvant required for induction of better immune response may result in undesirable side effects, including strong inflammatory reactions. In addition, previous studies showed that NS1-specific antibodies generated during DENV infection may cross-react with different host proteins including proteins exposed on the surface of platelets and endothelial cells [22–24,54]. In our experimental conditions, no hepatic damage, exacerbated inflammatory reactions and, more relevantly, altered hematological parameters have been detected in mice immunized with NS1 admixed with LT_{G33D}. These results further confirm that LT_{G33D} represents an effective and safe vaccine adjuvant, particularly following administrative via parenteral routes. Further experiments should address the question of deleterious effects induced in vaccinated mice following challenge with other DENV types.

Collectively the present results demonstrated that anti-DENV vaccines based on purified recombinant NS1 protein adjuvanted with a non-toxic LT derivative represent a new and promising alternative for the development of acellular-based dengue vaccines. The partial protection observed in the mouse lethal encephalitis model was achieved in a more restrictive condition if compared to previously reported studies [14–16], where the sham group had a significant survival rate, reflecting the protective potential of the tested vaccine formulation. Moreover, the incorporation of additional antigens to the vaccine preparation, such as the envelope protein or immunogenic domains derived from it, may improve the protective immunity induced in vaccinated subjects. Such ideas are presently under investigation and shall contribute for a better understanding of the immunological features of an effective protein-based anti-dengue vaccine.

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5 CAPÍTULO 3 - Estudo genético e patológico de um isolado clínico de VD-2 capaz de induzir encefalite e distúrbios hematológicos em camundongos imunocompetentes

Nessa parte do trabalho descrevemos um novo isolado de VD-2, obtido a partir do soro de um paciente hospitalizado, denominado de JHA1, que pode ser usado para estudar a doença em condições experimentais. A consolidação desse modelo experimental foi de grande importância para assegurar a autonomia do grupo em trabalhos envolvendo o VD2. A análise genética do isolado revelou que o JHA1 é um VD2, pertencente ao genótipo Americano. Uma análise de substituição de aminoácidos mostrou que essa cepa tem uma base genética para neurovirulência em camundongos. A neurovirulência dessa nova cepa foi confirmada por desafios intracranianos em modelo murino com camundongos Balb/c. A nova cepa mostrou-se naturalmente letal em camundongos imunocompetentes e causou perda de peso corporal, encefalite, danos teciduais e distúrbios hematológicos semelhantes aos observados em humanos infectados. O JHA-1 foi neutralizado por anticorpos gerados contra o domínio III da glicoproteína do envelope (EIII) da cepa de referência NGC, que é sabidamente neurovirulenta. Esses resultados indicam que esse novo isolado representa um modelo experimental promissor para avaliar candidatos vacinais e estudar a patogênese do VD em uma situação mais próxima da doença observada em humanos.

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A Genetic and Pathologic Study of a DENV2 Clinical Isolate Capable of Inducing Encephalitis and Hematological Disturbances in Immunocompetent Mice

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Abstract

Dengue virus (DENV) is the causative agent of dengue fever (DF), a mosquito-borne illness endemic to tropical and subtropical regions. There is currently no effective drug or vaccine formulation for the prevention of DF and its more severe forms, i.e., dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). There are two generally available experimental models for the study of DENV pathogenicity as well as the evaluation of potential vaccine candidates. The first model consists of non-human primates, which do not develop symptoms but rather a transient viremia. Second, mouse-adapted virus strains or immunocompromised mouse lineages are utilized, which display some of the pathological features of the infection observed in humans but may not be relevant to the results with regard to the wild-type original virus strains or mouse lineages. In this study, we describe a genetic and pathological study of a DENV2 clinical isolate, named JHA1, which is naturally capable of infecting and killing Balb/c mice and reproduces some of the symptoms observed in DENV-infected subjects. Sequence analyses demonstrated that the JHA1 isolate belongs to the American genotype group and carries genetic markers previously associated with neurovirulence in mouse-adapted virus strains. The JHA1 strain was lethal to immunocompetent mice following intracranial (i.c.) inoculation with a LD_{50} of approximately 50 PFU. Mice infected with the JHA1 strain lost weight and exhibited general tissue damage and hematological disturbances, with similarity to those symptoms observed in infected humans. In addition, it was demonstrated that the JHA1 strain shares immunological determinants with the DENV2 NGC reference strain, as evaluated by cross-reactivity of anti-envelope glycoprotein (domain III) antibodies. The present results indicate that the JHA1 isolate may be a useful tool in the study of DENV pathogenicity and will help in the evaluation of anti-DENV vaccine formulations as well as potential therapeutic approaches.

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Introduction

Infection with one of the four dengue virus (DENV) serotypes can be asymptomatic or can trigger a wide spectrum of clinical manifestations. The disease may yield symptoms ranging from a mild acute febrile illness, termed dengue fever (DF), to the more severe forms of the disease that include dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), characterized by fever, hemorrhage, thrombocytopenia, vascular leakage and viremia that is 10- to 100-fold greater than in DF [1]. The cellular and molecular mechanisms involved in DENV pathogenesis remain, at least in part, elusive. The current hypotheses regarding the mechanisms involved in dengue pathogenicity and the severity of disease symptoms range from dysfunction of the host immune system, with the generation of cross-reactive antibodies and T cells, to platelet depletion, endothelial cell apoptosis and complement activation with damage to host tissues [2–7].

The general health state and genetic profile of the host as well as the virulence variability among the DENV strains contribute to the severity of the disease symptoms and development of DHF/DSS [8–11]. However, the lack of a more suitable animal model for the study of the disease is a clear drawback to determining DENV pathogenesis and the immunological mechanisms involved in the disease progression or protection [12]. As a corollary, no effective anti-DENV drug or vaccine formulation is presently available for the treatment or prevention of the disease [13].

Humans and mosquitoes represent the only natural hosts for dengue viruses known to date. Some non-human primates are permissive to DENV and elicit specific immune responses, but they develop only transient viremia, without the specific symptoms observed in infected humans [14–17]. In addition, for ethical and economic reasons, non-human primates do not represent a sustainable option for the routine research of DENV pathogenicity. Different mouse-based models have been intensively explored as

experimental alternatives for the study of DENV pathogenesis and efficacy evaluation of anti-DENV vaccines [12].

Although representing a simple and straightforward tool for the study of DENV pathogenesis, mouse-based DENV infection models have several limitations. First and perhaps most importantly, wild-type DENV strains usually do not infect and kill mice. Thus, the DENV strains used in these studies must be adapted to this new host by serial passages in the brains of suckling mice [18,19]. During this selective process, different mutations in genes encoding structural and non-structural proteins are selected and confer the ability to replicate and kill the new host of the virus [19–23]. However, some of these mutations are expected to change different aspects of the virus physiology, such as cell and tissue tropism as well as the replication rate. Therefore, experimental models based on genetically modified virus strains are expected to have reduced relevance regarding the understanding of viral pathogenicity under natural conditions. Other DENV infection model approaches rely on immunocompromised mouse lineages that lack, for example, genes encoding IFN-γ and IFN-α/β receptors and thus allow the replication of non-adapted DENV strains [24,25]. Therefore, due to the complex regulatory roles played by these cytokines, the results based on immunodeficient lineages do not necessarily reproduce the conditions expected to be found in immunocompetent mouse lineages or humans [24,25].

Previous reports indicated that the ability of the mouse-adapted DENV strains to kill immunocompetent mice following administration via the intracranial (i.c.) route is a consequence of the selection of specific mutations during the adaptation process that allow them to replicate and cause encephalitis and death in these animals [18,20]. In contrast, a previous report of a non-adapted DENV strain capable of infecting and killing immunocompetent mice [26] indicates that the DENV natural genetic plasticity may represent the source of a more suitable DENV infection model for murine hosts. However, the characterization of such a DENV strain as an experimental model for the study of viral pathogenicity and/or the testing of prophylactic and therapeutic interventions has not been fully conducted.

In this study, we characterized a DENV strain recovered from a symptomatic subject that is naturally lethal to immunocompetent mice following i.c. administration. Genetic analysis of the isolate revealed that it is a DENV2 strain grouped within the American genotype. Amino acid sequence analyses demonstrated that the virus strain, named JHA1, has specific polymorphic markers of neurovirulence in mice. The infection with the DENV2 strain induced body weight loss, general tissue damage, altered hematological features indicative of plasma leakage, leucopenia, lymphocytopenia, neutropenia and hemorrhage in Balb/c mice. In addition, the JHA1 strain shares immunological determinants with the NGC strain, a reference DENV2 widely used in the testing of potential anti-dengue vaccine candidates. Collectively, these results indicate that the new DENV2 isolate exhibits several attributes of useful experimental models for studies aimed at understanding viral pathogenicity and vaccine testing.

Materials and Methods

Ethics Statement

All handling procedures and experiments involving mice were approved by the Committee for the Ethical Use of Laboratory Animals from the Institute of Biomedical Sciences of the University of São Paulo, in accordance with the recommendations in the guidelines for the care and use of laboratory animals of the National Committee on the Ethics of Research (CONEP). Serum sampling of human beings were approved by the Committee for Ethics in Research Involving Human Beings of the Tropical Medicine Institute of the University of São Paulo and all subjects provided written informed consent.

Serum Sampling and Viral Isolation

The human serum sample used in the present study was obtained from the Laboratory of Virology-LIM5 collection, at the Tropical Medicine Institute, University of São Paulo. The serum sample was originally collected at the city of Belém (Pará Federal State), in the northern region of Brazil, during the acute phase (day 4 after the symptoms onset) of the disease in a hospitalized patient with dengue fever. The virus was isolated after cultivation in the C6/36 cell lineage [27], as previously described [28,29].

Lethality and Propagation of Dengue Virus in Balb/c Mice

Once isolated, the DENV sample was propagated once in C6/36 cells. The cells were cultured in Leibovitz-15 medium (L-15) (Invitrogen, USA) supplemented with 5% fetal bovine serum (FBS) (Gibco, USA). The culture supernatant was collected, divided into aliquots and stored at $-80^{\circ}C$ or immediately titrated using a plaque assay on LLC-MK2 cells, as previously described [26]. Male Balb/c mice (9 weeks old; $n = 20$) were anesthetized with a mixture of ketamine and xylazine [30] and injected intracranially (i.c.) with 40 µL of L-15 medium supplemented with 5% FBS containing 4,000 plaque-forming units (PFU) or media only (mock group). Mice injected with virus ($n = 10$) were euthanized in the moribund state, 5 days post-infection (p.i.), and the brains were removed and individually macerated in 3 mL of DMEM medium (Gibco, USA) at $4^{\circ}C$. The brain macerates were combined in a 50-mL plastic tube and centrifuged at 405 g for 5 min. The supernatant was harvested, divided into aliquots and stored at $-80^{\circ}C$ for the virus seed stock. Five randomly chosen aliquots were subsequently titrated by plaque assay on LLC-MK2 cells.

Nucleotide Sequence and Phylogenetic Analysis

For DENV RNA extraction, 250 µL of an aliquot of the C6/36 cell culture supernatant, collected 4 days after infection with the DENV isolate, was admixed with 750 µL of TRIzol reagent (Invitrogen, USA) and incubated at room temperature for 5 min. A volume of 200 µL of chloroform was added to the initial mixture and incubated for 15 min at room temperature. The mixture was centrifuged at 20,000 g for 10 min at $8^{\circ}C$, and the aqueous phase was collected, added to 500 µL of isopropanol and incubated for 10 min at room temperature. The tube was again centrifuged at 20,000 g for 10 min at $8^{\circ}C$, the supernatant was removed, and the pellet was washed with 75% ethanol (v/v). The pellet was air dried and finally suspended in 20 µL of DEPC-treated water [31]. The extracted RNA was stored at $-80^{\circ}C$ or immediately subjected to reverse transcription-polymerase chain reaction (RT-PCR) using a specific primer set, according to the manufacturer's instructions (SuperScript III First-Strand Synthesis SuperMix, Invitrogen, USA). For the amplification reaction, the sense primer used was 5′-GGAATGTCATACTCTAT-3′, and the anti-sense primer was 5′-TTACGATA-GAACTTCCTTTCTTA-3′. The amplified sequence encompassed nucleotides 1822 to 3477 of the virus genome, which includes the beginning of domain III of the envelope glycoprotein (EIII) coding sequence to the end of the NS1 coding sequence. The amplified band was purified with the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, USA) and was directly used in the sequencing reactions. The sample was sequenced four times in both orientations (MegaBACE Sequencer, GE Healthcare, USA). The nucleotide sequence obtained was submitted to GenBank (accession number

JQ686088) and aligned with other DENV- and yellow fever virus-equivalent nucleotide sequences using the ClustalW tool. The alignments were used to construct a midpoint-rooted phylogenetic tree by the neighbor-joining method using the Tamura Nei model, implemented by the software MEGA 5.05, with 1,000 bootstrap replicates [32,33]. Amino acid polymorphism analyses were performed via alignment of the inferred amino acid sequence with sequences from strains of the same genotype, i.e., the India/1957 and Indonesia/1977 strains (GenBank accession numbers FJ538927 and GQ398257, respectively), and the New Guinea C (NGC) strain, which is a reference virus strain selected for neurovirulence in mice (GenBank accession number M29095). The markers for mouse neurovirulence were searched according to a previous report [20]. The comparisons were performed with the amino acid sequences corresponding to part of the envelope glycoprotein (domain III, the adjacent stem-anchor region and the NS1 signal peptide) of the JHA1, India/1957 and Indonesia/1977 strains and with the entire envelope glycoprotein sequence of the NGC strain. Similar analyses were performed with the complete amino acid sequence of the mature NS1 protein of the four DENV2 strains.

Determination of the LD_{50} in Mice and Pathological Symptoms Induced by the JHA1 Isolate

Male Balb/c mice (9 weeks old) were divided into seven groups ($n = 10$), which were administered i.c. viral loads containing 25, 50, 100, 150, 200 or 300 PFU diluted to a final volume of 40 µL with DMEM. A mock group was injected with DMEM alone. The animals were monitored daily for mortality and changes in body weight, which were recorded over a period of 21 days. The body weight loss was daily calculated for each animal until the day of death or the conclusion of the monitoring period and represented as the percentage of the final weight compared to the initial weight. Seven days post injection (p.i.), the animals were bled via the retro-orbital plexus for the individual determination of hematocrit and lactate dehydrogenase (LDH) levels. For hematocrit determination, heparinized microcapillary tubes (Precision Glass Line, CRAL, Brazil) were filled with blood samples, centrifuged at 4,000 g for 5 min and properly positioned in a packed cell volume table for hematocrit scoring [34,35]. LDH levels were determined with an analytical kit, as recommended by the supplier (Laborclin, Brazil).

Lethal Infection with the JHA1 Isolate and Histopathological Analyses

To evaluate the histopathological effects elicited by the JHA1 isolate, 9-week-old male Balb/c mice ($n = 12$) were injected i.c. with 300 PFU of the viral stock. Mice were bled via the retro-orbital plexus ($n = 6$) on days 2, 4, 6 and 7 p.i. for individual serum sampling. The samples were used to test the reactivity of antibodies to recombinant NS1 and EIII proteins (days 2, 4, 6 and 7 p.i.) and INF-γ production (day 7 p.i.). For blood cell analyses, the animals were bled on days 4, 7 and 8 p.i. ($n = 6$), and the blood samples were transferred to microtubes containing EDTA (0.375 M). On the day 8 p.i. (moribund state), the animals were euthanized, and the brains, spleens, lungs, kidneys, liver, colon and bone marrow were removed and individually macerated in DMEM at 4°C (3 mL for each brain, liver, couple of lungs or kidneys, and individual colon; 2 mL for each spleen; and 0.5 mL for each bone marrow). The macerates were transferred to 15-mL plastic tubes and centrifuged for 5 min at 405 g. The virus concentrations in the supernatants were determined using a plaque assay on LLC-MK2 cells. For histological studies, the mice were euthanized ($n = 3$), and the brains were fixed with 10% formaldehyde and processed and stained with hematoxylin and eosin.

IgG and Cytokine ELISA

Mice sera were tested individually for the presence of NS1 and EIII-specific antibodies by ELISA, as previously described [36]. Briefly, MaxiSorp plates (Nunc, Denmark) were coated with 0.2 µg per well of the recombinant NS1 or EIII proteins (based on the genome sequence of the NGC virus strain) in 100 µL PBS and blocked for 1 h at 37°C with 5% skim milk in 0.05% Tween-20/PBS (PBST). The serum samples were serially diluted and added to wells previously washed with PBST. After 1 h at room temperature, the plates were washed with PBST and incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (Southern Biotechnology, USA) for 1 h at room temperature. The reactions were measured at A_{492nm} with ortho-phenylenediamine dihydrochloride (Sigma-Aldrich, USA) and H_2O_2 as a substrate after the addition of a 2 N H_2SO_4 stop solution. The titers were established as the reciprocal of serum dilutions that yielded an absorbance two-fold higher than the SD values of the respective mock-infected animals. The serum samples collected on day 7 p.i. were tested individually using an INF-γ, IL-1β or TNF-α ELISA, according to the manufacturer's instructions (BD Bioscience, USA).

Blood Cell Analyses

Whole blood samples were used to evaluate six hematological parameters: red blood cell (RBC) and white blood cell (WBC) counts, hematocrit (HTC), platelet number (PTL) and lymphocyte (LYM) and neutrophil (NEU) differentiation. RBC and WBC counts were performed using a Neubauer chamber, HCT was determined as described above, PLT numbers were determined according to Fonio's method, and NEU and LYM differentiation was performed visually using a phase-contrast microscope [34,35] (Eclipse E200 model, Nikon, Japan). At the day 7 p.i., mouse plasma were used to perform the prothrombin time test according to the kit manufacturer's instructions (Bioclin, Brazil).

Determination of Cross-reacting Epitopes Shared by the Envelope Proteins of the JHA1 and NGC Strains

The immunological relationship between the JHA1 and NGC strains was demonstrated by the labeling of infected cells and virus-neutralization assays performed with antibodies raised in mice immunized with domain III of the recombinant envelope (EIII) protein derived from the NGC strain. Anti-EIII antibodies were obtained in Balb/c mice subjected to an immunization regimen of three doses administered subcutaneously (s.c.) (days 0, 14 and 28) and containing 10 µg of the recombinant EIII combined with complete Freund's adjuvant (50% v/v) (first dose) and incomplete Freund's adjuvant in the subsequent injections. The serum samples were pooled, titered with the recombinant protein (final reverse titer of 900,000) and used in JHA1-infected mammalian cells. Sterile microscope cover slips were positioned in wells of Nunc 6-well plates containing 10^5 cells/well (LLC-MK2) in DMEM supplemented with 5% FBS. After incubation at 37°C with 5% CO_2 for 12 h, the cells were gently washed with sterile PBS (pH 7), infected with JHA1 and suspended in DMEM at a multiplicity of infection of 1 (MOI = 1) for 1 h at 37°C with 5% CO_2. The cells were incubated under the same conditions for 96 h and then fixed with 4% paraformaldehyde for 20 min at 4°C, permeabilized with saponin and treated with the anti-EIII serum or serum from non-immune mice for 1 h at room temperature. Subsequently, the cells were treated with goat anti-mouse IgG

conjugated to FITC (Invitrogen, USA) and DAPI (Thermo Scientific, USA). The samples were analyzed using an immunofluorescence microscope (Asiovert S100, Zeiss, Germany).

The serum samples were also tested for virus neutralization activity using the JHA1 isolate in plaque assays with the LLC-MK2 cell line, as previously described [37]. In brief, viral stock aliquots were diluted to yield 40 plaques per well (in 6-well plates). The serum pools from immunized or non-immune mice were inactivated (56°C for 30 min), and serial two-fold dilutions beginning at a 1:2 dilution were added to the virus suspension in a final volume of 400 µL and incubated at 37°C for 1 h. The incubation mixtures were seeded with 2×10^5 LLC-MK2 cells and incubated for 1 h at 37°C with 5% CO_2. After incubation, the cells were covered with a mixture containing E199 medium, 1% carboxymethyl cellulose and 2% FBS and incubated at 37°C with 5% CO_2 for 7 days for viral plaque formation.

The examination of cross-reactive epitopes shared by the NGC and JHA1 strains was also performed with the $PRED^{BALB/C}$ program that predicted the specific class I and II MHC epitopes for Balb/c mice [38]. The epitopes with high scores were compared in both DENV2 strains. The experimentally determined MHC-I-restricted $CD8^+$ T cell-specific NS1 epitope (AGPWHLGKL) was also compared in both virus strains [39,40].

Statistical Analyses

Statistical analyses were performed using a Student's *t*-paired test or ANOVA and a subsequent Bonferroni's multiple comparison test. Statistical significance was set as $p<0.05$.

Results

Genetic Study of the DENV JHA1 Strain

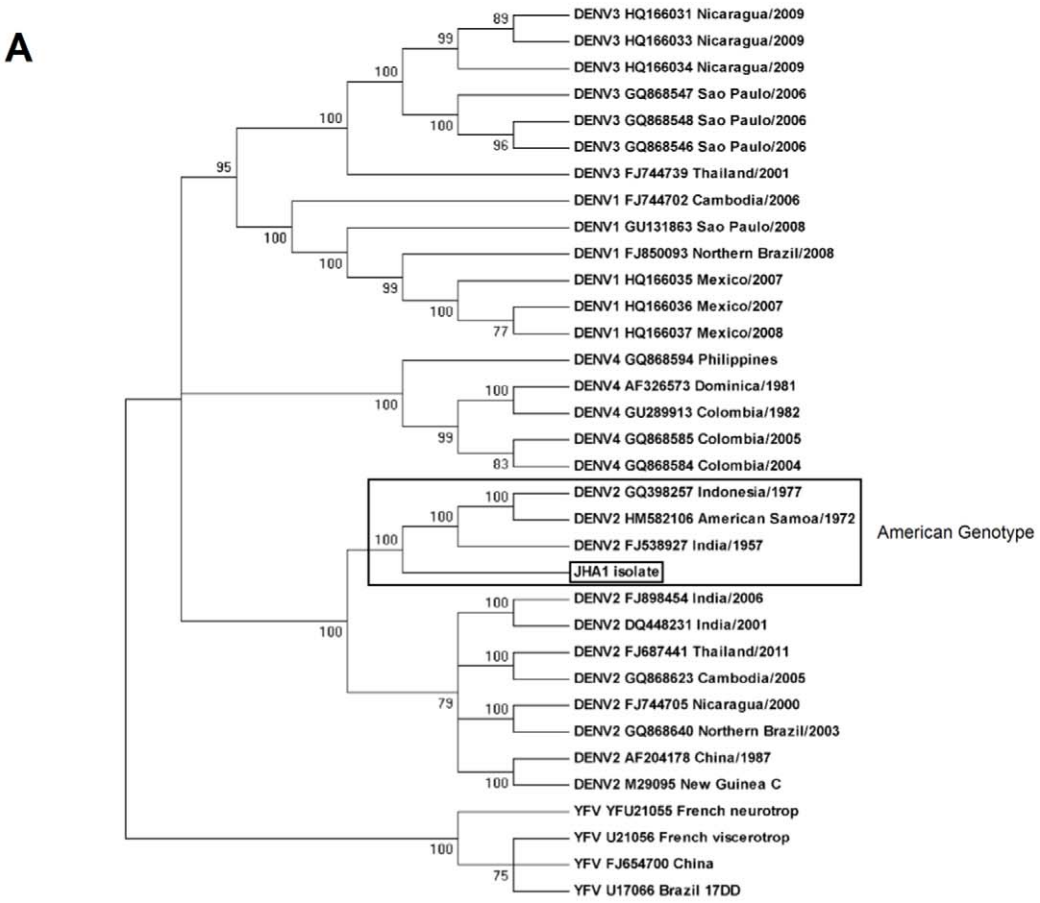
Sequencing of the genes encoding the E (domain III, stem-anchor region and NS1 signal peptide) and NS1 proteins of the JHA1 strain, ranging from nucleotides 1822 to 3477 of the virus genome, and phylogenetic analyses with orthologous nucleotide sequences of different DENV and yellow fever virus (YFV) strains available at the Genbank demonstrated that the strain was a type 2 dengue virus that lies within the American genotype (Figure 1A). Comparison of the amino acid sequences of the JHA1 strain with other DENV2 strains, including the mouse-adapted neurovirulent NGC strain, revealed 23 polymorphic sites (Figure S1). Among these, three polymorphic sites were located on sites previously shown to be involved in neurovirulence in mice [20]. The replacements of an aspartic acid (D) with an asparagine (N) and a phenylalanine (F) with a leucin (L) at positions 390 and 402 of the envelope glycoprotein (E protein), respectively, as well as the replacement of an arginine (R) with a glutamine (Q) at position 105 of the NS1 protein, were ascribed to neurovirulence in mice. These replacements were present in the NGC strain but absent in the other two DENV2 strains isolated from human subjects (Figures 1B, 1C and Table S1). Additional unique polymorphic sites in the E protein sequence of the JHA1 strain were detected, including the replacement of a methyonine (M) with an alanine (A) at position 301, the replacement of a threonine (T) with a glycine (G) at position 303 (see Figure S1) and the replacement of a serine (S) with an arginine (R) at position 363 (see Fig. 1B). Collectively, the sequence analyses indicated that the JHA1 strain would naturally possess features of the NGC strain, particularly regarding neurovirulence in mice.

The DENV2 JHA1 Strain is Lethal in Immunocompetent Adult Mice

After isolation, the JHA1 strain was propagated in the C3/36 cell line and tested for lethality in adult Balb/c mice following i.c. administration. Under these conditions, all animals inoculated with 4,000 PFU of the JHA1 died 5 days later. Subsequent experiments performed to determine the minimum lethal dose showed that the JHA1 strain had an estimated LD_{50} of 50 PFU, while the smallest tested dose (25 PFU) caused a final lethality of 20% under the test conditions (Fig. 2A). Mice infected with 100, 150, 200 or 300 PFU died between 8 and 15 days after challenge and exhibited statistically significant body weight loss compared with the mock-treated animals (Figure 2B). Mice infected with lethal virus loads had morbidity signs (hind limb paralysis and distorted spinal cords) within 24 h before dying; however, morbidity signals were not detected among survivors (data not shown). In addition, the hematocrit values of mice infected with 300 PFU showed a significant increase seven days after challenge when compared with the control group and mice infected with lower viral loads (Figure 2C). Moreover, the serum LDH levels were increased in mice infected with 200 and 300 PFU in comparison to mock-infected animals, suggestive of general tissue damage (Figure 2D). Virus obtained from infected mice were further submitted to sequencing of the genes encoding the EIII, stem-anchor region and NS1 protein and no acquired mutations could be detected with regard to the parental strain (data not shown). Together, these results indicate that the JHA1 strain can kill adult Balb/c mice infected via the i.c. route by causing general tissue injury, including hematological disturbances indicative of plasma leakage.

Hematological Alterations and Brain Damage in Mice Infected with the JHA1 Strain

To investigate the hematological disturbances detected in mice infected with the JHA1 isolate, the animals were inoculated i.c. with 300 PFU ($6\times LD_{50}$), and the number of red cells, white cells, and platelets, as well as the hematocrit values, were monitored. As shown in Table 1, no significant cellular parameter was altered on day 4 p.i. However, a clear increase in the hematocrit values and a sharp decrease in the number of white blood cells were observed in JHA1-infected mice 7 days after the challenge. One day later, there was a sharp decrease in the hematocrit values and red blood cell count in virus-infected animals. At this time point, most of the animals were in a moribund state and had begun to die (Table 1). The numbers of lymphocytes and neutrophils were also reduced on days 7 and 8 p.i., but no alteration in the platelet numbers could be detected in mice infected with the JHA1 strain. In contrast, a significant alteration in the coagulation pattern, measured by the prothrombin-dependent coagulation time, was detected in JHA1-infected mice. The prothrombin time was increased by approximately ten-times in mice infected with the JHA1 strain with regard to mock-treated mice, an indication that the coagulation function was drastically impaired among infected mice (Table 1). In addition to the hematological disturbances, the JHA1 strain resulted in brain tissue damage. As shown in Figure 3A, extensive gliosis scars were observed in brain tissue samples collected from mice infected with the JHA1 strain. Moreover, mononuclear cells were detected in the blood vessels and the surrounding brain parenchyma but were not observed in samples collected from the mock-treated animals (Figures 3D and 3F). No apparent spleen or liver damage could be detected in mice infected i.c. with the JHA1 strain (data not shown). These results



B

301th and 303th amino acids of the E protein

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DENV2_FJ538927_India/1957          MCTGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGR 350
DENV2_GQ398257_Indonesia/1977      MCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGR 350
JHA1_isolate                       ACGGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGR 350
DENV2_M29095_New_Guinea_C          MCTGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGR 350
                                    * ****:**************************************************

DENV2_FJ538927_India/1957          LITVNPIVTEKDSPVNIEAEPPFGDSYIIIGVEPGQLKLDWFKKGSSIGQ 400
DENV2_GQ398257_Indonesia/1977      LITVNPIVTEKDSPVNIEAEPPFGDSYIIIGVEPGQLKLDWFKKGSSIGQ 400
JHA1_isolate                       LITVNPIVTEKDRPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQ 400
DENV2_M29095_New_Guinea_C          LITVNPIVTEKDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQ 400
                                   ************ ***************************:***********
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363th amino acid and 390th amino acid of the E protein

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DENV2_FJ538927_India/1957          MFETTMRGAKRMAILGDTAWDFRSLGGVFTSIGKALHQVFGAIYGAAFSG 450
DENV2_GQ398257_Indonesia/1977      MFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSG 450
JHA1_isolate                       MLETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSG 450
DENV2_M29095_New_Guinea_C          MIETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSG 450
                                   *:******************** ****************************
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402th amino acid of the E protein

C

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DENV2_FJ538927_India/1957          EEGICGIRSVTRLENLMWKQITPELNHILSENEVKLTIMTGDIKGIMQVG 100
DENV2_GQ398257_Indonesia/1977      EEGICGIRSVTRLENLMWKQITSELNHILSENEVKLTIMTGDIKGIMQVG 100
DENV2_M29095_New_Guinea_C          EEGICGIRSVTRLENLMWKQITPELNHILSENEVKLTIMTGDIKGIMQAG 100
JHA1_isolate                       EEGICGIRSVTRLENLMWKQITPELNHILSENEVKLTIMTGDIKGIMQVG 100
                                   ********************.************************.*

DENV2_FJ538927_India/1957          KRSLRPQPTELRYSWKTWGKAKMLSTELHNQTFLIDGPETAECPNTNRAW 150
DENV2_GQ398257_Indonesia/1977      KRSLRPQPTELRYSWKTWGKAKMLSTELHNQTFLIDGPETAECPNTNRAW 150
DENV2_M29095_New_Guinea_C          KRSLQPQPTELKYSWKTWGKAKMLSTESHNQTFLIDGPETAECPNTNRAW 150
JHA1_isolate                       KRSLQPQPTELRYSWKTWGKAKMLSTELHNQTFLIDGPETAECPNTNRAW 150
                                   ****:******:*************** **********************
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105th amino acid of the NS1 protein

Figure 1. Genetic study of the JHA1 DENV2 strain recovered from a symptomatic subject. (A) Phylogenetic tree showing that the JHA1 strain (indicated by the rectangle) is grouped within the type 2 dengue viruses based on nucleotide sequences encompassing the envelope protein (domain III of the E protein) and the NS1 protein. The JHA1 strain was clustered in the American genotype (India/1957, American Samoa/1972 and Indonesia/1977) strains. Numbers at the nodes are bootstrap values (1000 replicates). DENV sequences were retrieved from GenBank as identified on the right side of the figure. In most cases, the sequences were also identified by the virus isolation area and the year of isolation. (B) Genetic analyses of polymorphic sites within the main envelope (E) protein sequence of the JHA1 strain were performed with the NGC strain, which served as the model for neurovirulence in mice, and the India/1957 and Indonesia/1977 strains, which are unable to infect mice. One amino acid substitution in the JHA1 strain was identical (position 390) and another was similar (position 402) to those observed at the same positions of the NGC strain but not in the other DENV2 strains. Other mutations at positions 301, 303 and 363 of the JHA1 E protein are unique to the JHA1 strain. (C) Genetic analysis of polymorphic sites of the deduced NS1 amino acid sequence. The amino acid replacement at position 105 was found in the NGC strain but not in the other DENV2 strains.
doi:10.1371/journal.pone.0044984.g001

indicate that the JHA1 strain kills mice mainly via encephalitis with extensive tissue damage.

Replication and Induction of Inflammatory and Adaptive Immune Responses in JHA1-infected Mice

To determine whether the JHA1 strain remained viable in the tissues of infected mice, organ (brain, spleen and liver) and blood samples were collected 7 days after the challenge with 300 PFU. Viable virus particles were detected in the brains of infected mice (mean number of 70,832±33,228 PFU/brain). The virus was also detected in the spleens of infected mice, albeit at lower levels (333±376 PFU/spleen). No viable virus particles could be recovered from the blood, liver, lungs, kidneys, colon and bone marrow samples. The production of IFN-γ in the serum of virus-infected animals was also determined to be an indication of an acute inflammatory reaction. As indicated in Figure 4B, the IFN-γ serum concentration was significantly enhanced in mice infected

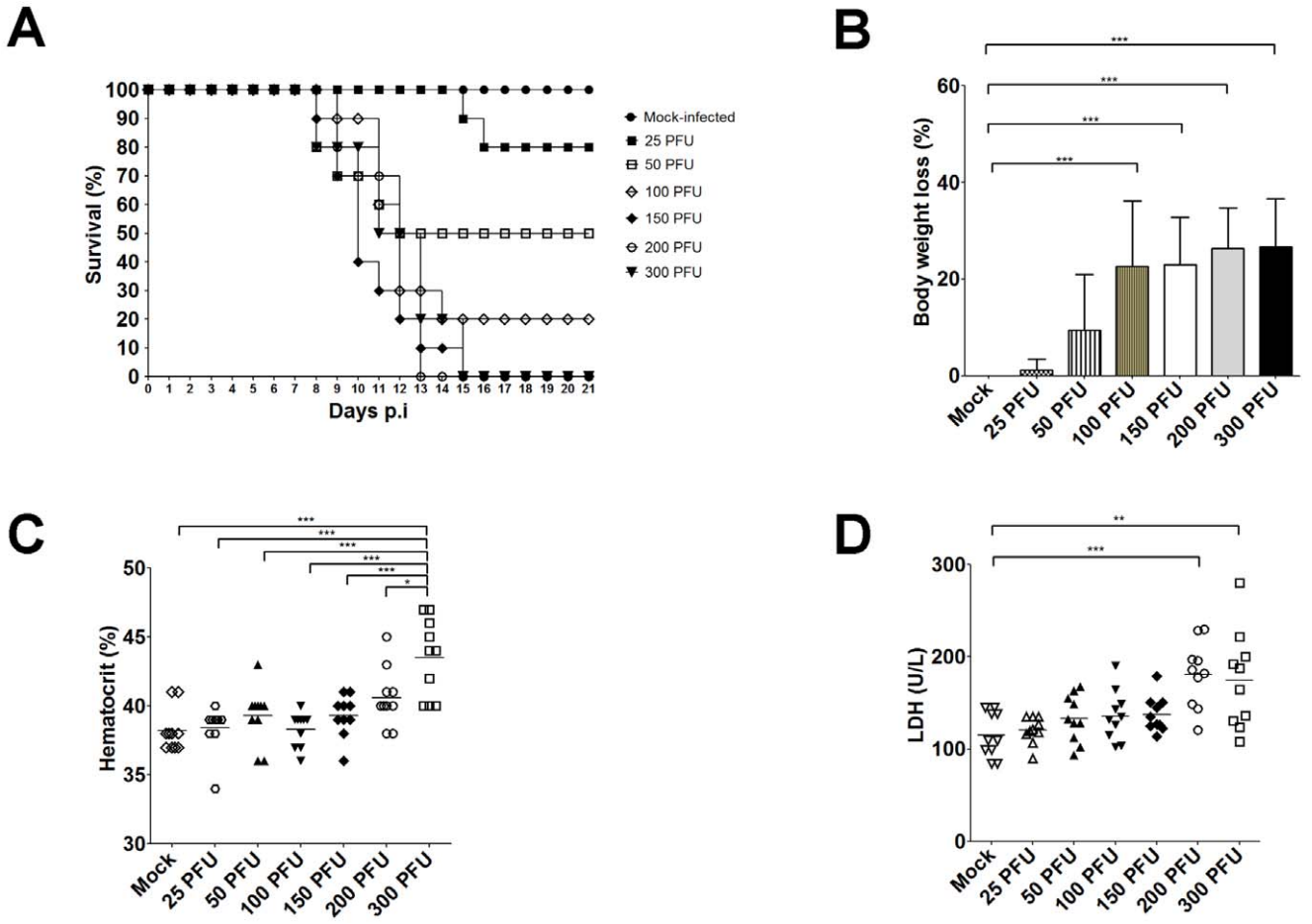

Figure 2. The JHA1 isolate is lethal to immunocompetent mice and causes tissue damage. (A) Survival curves of male Balb/c mice infected i.c. with different viral loads (25 to 300 PFU) in a final volume of 40 µL. Mice were monitored for 21 days after the challenge. (B) Body weight loss in mice infected with the JHA1 strain. The body weights of infected and mock-infected animals were monitored daily, and the differences between the initial and final measurements were calculated and are presented as percentages. (C) Hematocrit determination in mice infected with the JHA1 strain. The animals were bled on the seventh day after the challenge. (D) Serum LDH levels in JHA1-infected mice. The serum samples were collected 7 days after the challenge. Statistically significant differences were determined with the ANOVA test and a subsequent Bonferroni's multiple comparison test. Statistically significant differences are indicated with asterisks: *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$. Data are representative of three independent experiments.
doi:10.1371/journal.pone.0044984.g002

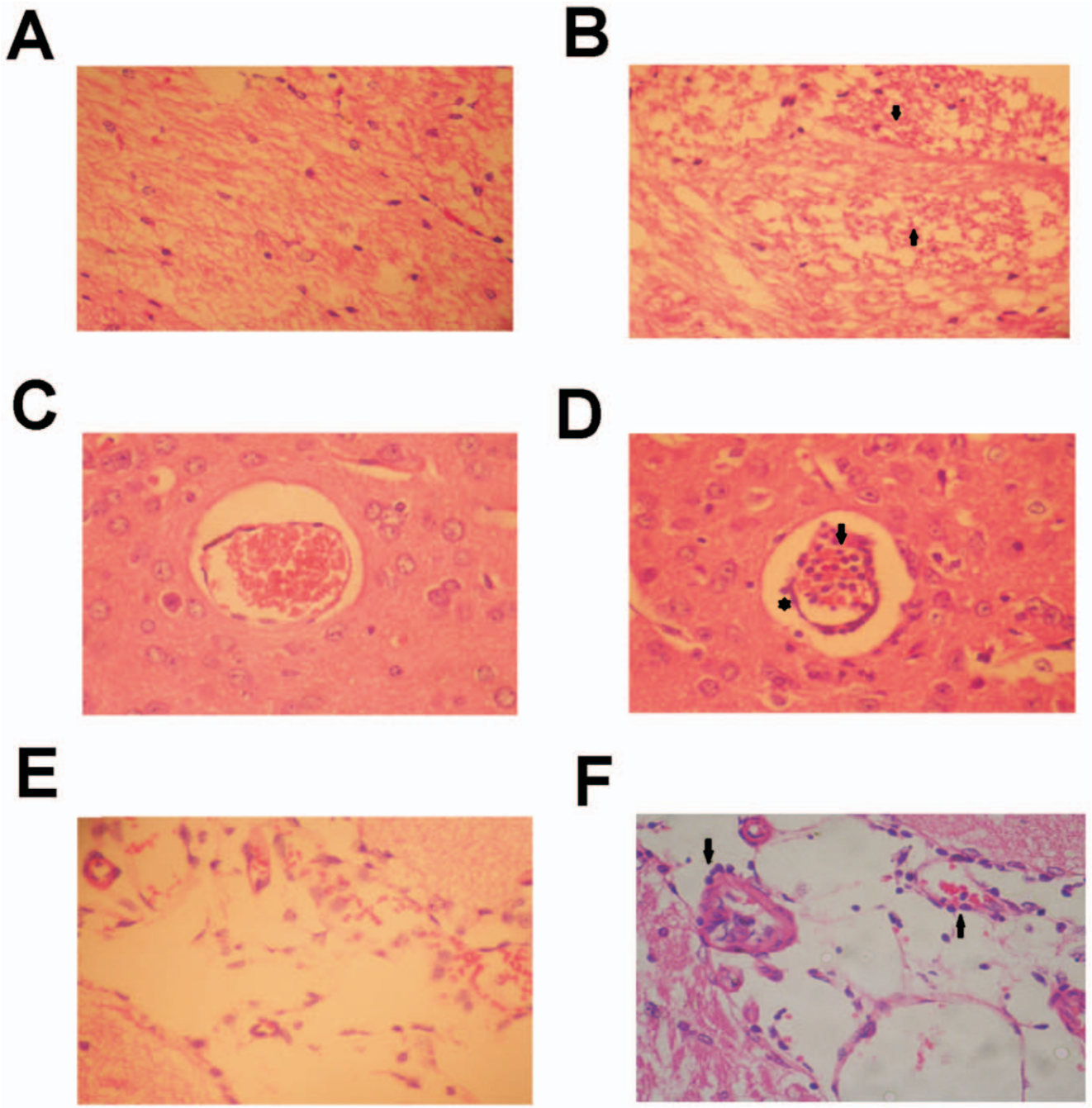

Figure 3. Pathological brain tissue alterations in mice infected with the JHA1 strain. (A) Mock-treated mice with preserved brain parenchyma with typical cellular components. (B) Brain tissue of JHA1-infected mice showing a gliosis scar, in which dead neurons were replaced by astrocytes. The gliosis scar is indicated by the arrows. (C) Brain blood vessel (transverse view) in mock-treated mice. (D) Brain blood vessel in JHA1-infected mice filled with mononuclear cells (arrow). Some of the cells are observed outside the endothelial epithelium (asterisk) and are present in the brain parenchyma (arrowheads). (E) Brain blood vessel (longitudinal view) in mock-treated mice. (F) Brain blood vessel of JHA1-infected mice with infiltration of mononuclear cells (arrowheads). Infected and mock-infected mice were euthanized on day 8 p.i. Brain tissue samples were fixed with 1% formaldehyde, processed and stained with hematoxylin and eosin. Magnification: 400x. Images are representative of three independent experiments.
doi:10.1371/journal.pone.0044984.g003

with the JHA1 strain compared with the mock-treated animals. But IL-1β and TNF-α were not detected in mice sera of both groups (data not shown). In addition, a dramatic increase in the anti-NS1 serum IgG titers and, to a lesser degree, the anti-EIII IgG levels was detected in the serum of JHA1-infected mice (Figure 4C). Neither NS1 nor EIII-specific antibody responses were detected in mock-infected mice, as evaluated both by ELISA and Western blot (Figure 4C and data not shown). Collectively, these results indicate that the JHA1 strain replicates and induces acute inflammatory reactions and early adaptive immune responses in adult mice infected via the i.c. route.

Table 1. Hematological alterations detected in mice infected with the JHA1 strain[a].

Hematological	Day 4 p.i.		Day 7 p.i.		Day 8 p.i.[c]	
Parameters[b]	Mock-infected	Infected	Mock-infected	Infected	Mock-infected	Infected
HCT[d]	40.3±1.6	39.3±1.2	40.8±1.9	44.5* ±2.0	40.6±1.7	30.0*** ±2.0
RBC	7.6±3.0	7.9±1.2	8.7±0.7	11.1±3.3	8.0±0.6	5.8*** ±1
WBC	9.0±1.9	5.8±3.2	11.0±0.5	2.4*** ±0.6	8.4±1.1	2.5*** ±0.6
LYM	5.8±2.4	4.6±2.9	8.3±0.8	1.7*** ±0.4	7.9±0.8	1.5*** ±0.5
NEU	2.0±0.5	1.0±0.8	1.7±0.2	0.6*** ±0.2	1.2±0.1	0.5*** ±0.2
PLT	1.2±0.1	1.2±0.1	1.3±0.2	1.3±0.2	1.2±0.1	1.2±0.2
PT[e]			19.5±5.4	217.2±115**		

[a]Male Balb/c mice (n = 12) were inoculated i.c. with 300 PFU of the JHA1 strain or mock-treated. Mice were bled via the retro-orbital plexus on days 4, 7 and 8 p.i. for hematological analyses.
[b]Blood samples were processed to determine the concentration of red blood cells (RBCs), white blood cells (WBCs), lymphocytes (LYMs), neutrophils (NEUs), and platelets (PLTs). WBC, NEU, LYM and PLT counts are expressed as 10^3 cells/μL. RBC counts are expressed as 10^6 cells/μl.
[c]On day 8 p.i., the animals had morbidity signals, such as hind limb paralysis and spine cord curvature, which was indicative of a moribund state.
[d]Hematocrit (HCT) values are given as percentages (%) of packed cell volume. Data are expressed as the mean ± SD of individual measurements. Comparisons between mock-treated and virus-infected mice were performed by t-paired tests on days 4, 7 and 8 p.i., with statistical significance set as $p<0.05$. (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. The results are based on one representative experiment of three independently performed experiments yielding similar results.
[e]The prothrombin times (PT) were individually measured on day 7 p.i. both in mock-treated and virus infected mice, in order to access coagulation mechanism integrity. Values are given in seconds. A t-paired test was performed, with statistical significance set as $p<0.05$. (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$ regarding mock-treated and infected groups.
doi:10.1371/journal.pone.0044984.t001

The JHA1 Strain Shares Immunological Determinants with the NGC Strain

In contrast to the DENV2 NGC strain, a viral strain adapted for neurovirulence in mice [41], our results demonstrated that the JHA1 strain was capable of replicating and killing adult Balb/c mice infected via the i.c. route without the need of adaptation steps for the new host. As the NGC strain represents a reference strain both in studies focusing on DENV pathogenesis and the evaluation of anti-dengue vaccine efficacy, we determined the extent of common immunological determinants between the NGC and JHA1 DENV2 strains. Computational analysis performed with the $PRED^{BALB/C}$ program indicated that the class I and II MHC-restricted epitopes of the E glycoprotein (domain III and stem-anchor region) and the NS1 proteins were identical in both virus strains (Table S2). In addition, sera collected from mice immunized with a recombinant EIII protein derived from the NGC were capable to recognize the protein found in JHA1 (Figure 5). As indicated in Figures 5A and 5B, sera of sham-treated mice did not recognize the viral particles inside infected cells, while anti-EIII antibodies, raised in mice immunized with a recombinant

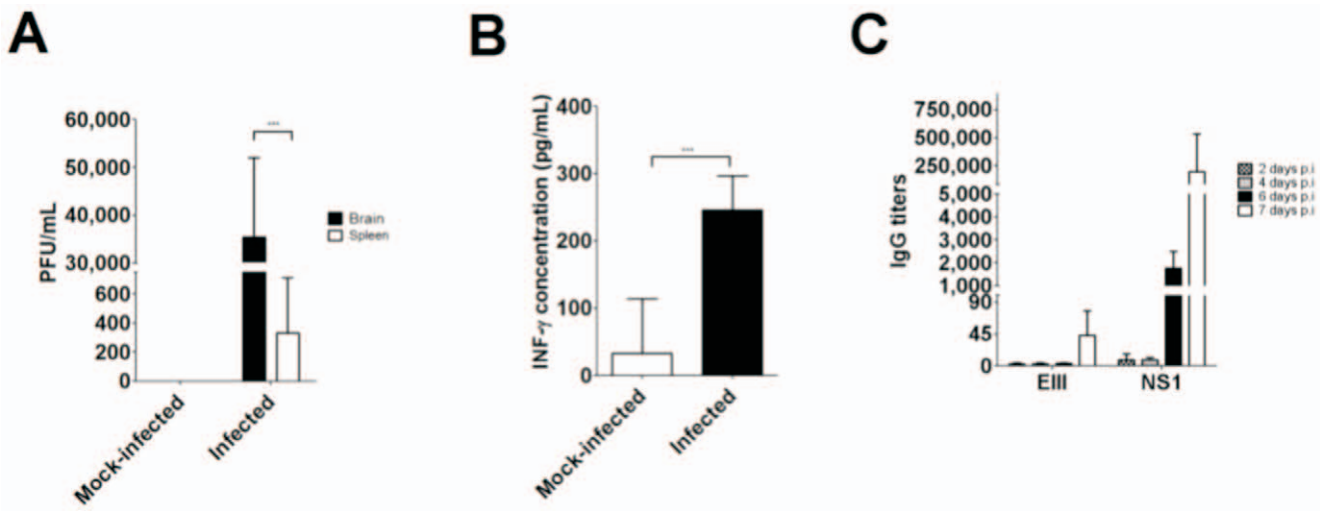

Figure 4. Determination of viable virus particles and induction of inflammatory and adaptive immune responses in mice infected with the JHA1 strain. (A) Replication of the JHA1 strain in brain and spleen tissues. Mice infected i.c. with 300 PFU were euthanized on day 7 p.i., and both the brains and spleens were removed and processed as described in the Methods section. (B) Quantification of INF-γ levels in the serum samples of mice infected with the JHA1 strain. The serum samples were collected seven days after the challenge. (C) Virus-specific serum IgG responses induced in mice infected with the JHA1 strain. The serum samples collected on days 2, 4, 6 and 7 p.i. were tested individually for the presence of NS1- or EIII-specific antibodies by ELISA. Background values detected in serum samples collected from mock-treated animals were reduced from those measured among virus-infected mice.
doi:10.1371/journal.pone.0044984.g004

protein derived from the NGC strain, reacted with the JHA1 particles (Figures 5C and 5D). Moreover, the same anti-EIII serum was shown to neutralize the JHA1 strain, as demonstrated in virus-neutralization assays (Figure 5E). Considering similar cross-reactivity of the NS1-specific antibodies (Figure 4C), these evidences indicate that the JHA1 and NGC strains share important immunological determinants both in the main envelope glycoprotein and the NS1 protein.

Discussion

The incidence of DF and DHF/DSS cases has dramatically increased during the last decade in different parts of the tropical world and particularly among Latin America countries, in which the disease has assumed a seasonal epidemic character with remarkable numbers of infected subjects and fatalities [42,43]. This scenario is worsened by the fact that the viral pathogenicity, particularly regarding aspects dictating the infection course and the onset of more serious symptoms, has not been elucidated. This knowledge gap is attributed at least in part to the lack of adequate experimental models that reproduce the pathological and immunological events either leading to the disease or controlling it. In the present study, we described the characterization of a DENV2 strain, named JHA1, which is naturally lethal to mice. After isolation from a symptomatic patient, a virus seed stock was obtained after a minimal number of replication rounds and

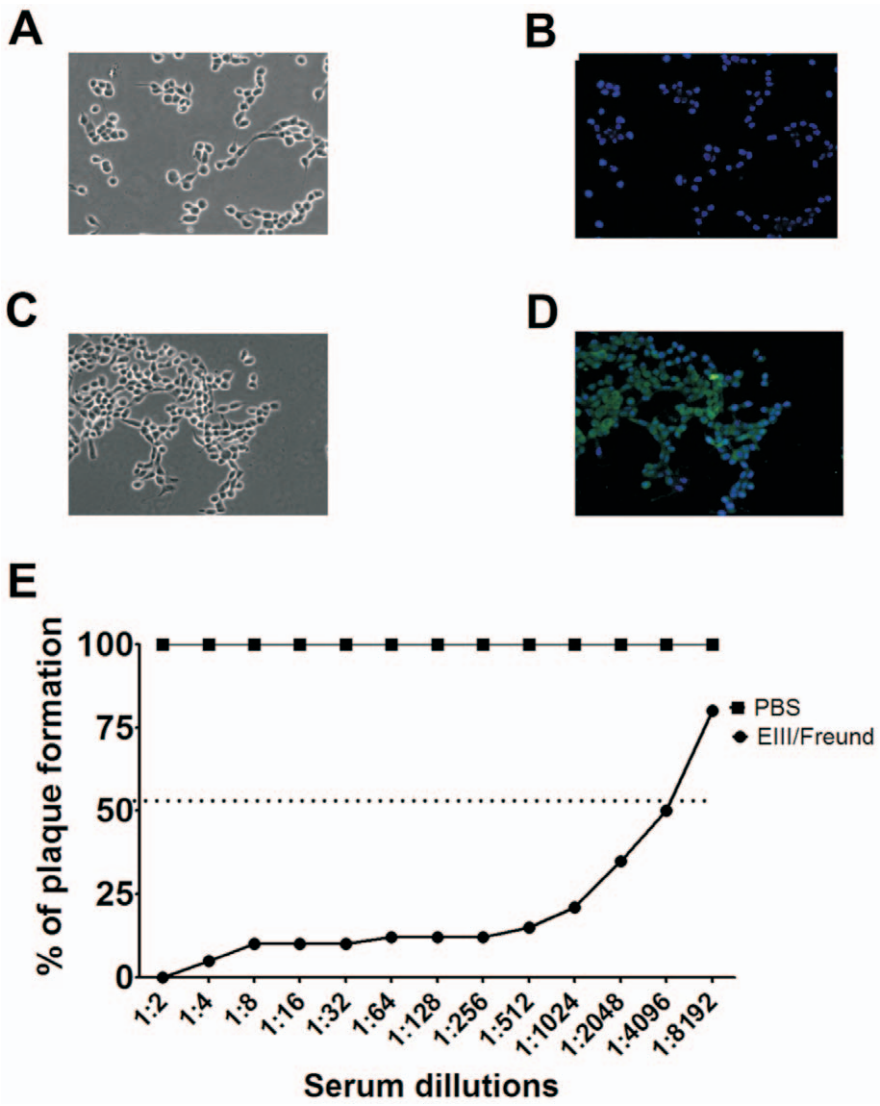

Figure 5. The JHA1 and NGC strains share immunological determinants within the major envelope protein. LLC-MK2 cells were infected with the JHA1 strain and probed with a serum pool raised in mice immunized with EIII derived from the NGC strain or in non-immune animals. (A) Phase-contrast microscopy of the infected cells probed with serum from sham-treated mice. (B) Immunofluorescence microscopy of the infected cells probed with serum from sham-treated mice. The photograph was merged with the same field observed using phase-contrast microscopy. (C) Phase-contrast microscopy of infected cells probed with the anti-EIII serum pool. (D) Immunofluorescence microscopy of infected cells probed with the serum pool of mice immunized with the EIII protein derived from the NGC strain JHA1. The picture was merged with the same field observed in phase contrast. (E) Virus neutralization assay performed with the serum from JHA1-infected mice immunized with the EIII protein derived from the NGC strain. Aliquots containing 40 PFU were incubated with different dilutions of the anti-EIII serum pool (ranging from 1:2 to 1:8,192) for 30 min and subsequently transferred to wells with LLC-MK2 cells. One week later, the number of virus plaques was counted. Magnification: 400x. Images are representative of three independent experiments.
doi:10.1371/journal.pone.0044984.g005

demonstrated to replicate, induce symptoms and kill Balb/c mice infected via the i.c. administration route. The exacerbate murine virulence of the JHA1 strain probably lies in the natural genetic polymorphism of the virus, in which at least three amino acid replacements were previously ascribed to DENV2 neurovirulence in mice. JHA1-infected mice showed altered hematological parameters similar to those observed in severe dengue cases, such as plasma leakage and hemorrhage. The virus showed rather low infective doses (LD_{50} of 50 PFU) after administration via the i.c. route and, in addition, induced encephalitis, acute inflammatory responses and early antibody responses to the NS1 and, to a lesser extent, the envelope glycoprotein, in agreement with the pathological signs detected among infected subjects. Together, the present findings indicate that the JHA1 strain represents a new and useful tool for the study of DENV pathogenicity and yields promising perspectives regarding the development of a more rational and effective screening program for drugs and vaccine candidates.

A phylogenetic analysis grouped the JHA1 strain as a type 2 DENV (DENV2) strain clustered within strains of the American genotype. Interestingly, representative viruses of this group were isolated in India, American Samoa and Indonesia, which is explained by the transmission of DENV2 strains between Asia and the Americas sometime around 1948 [44,45]. The DENV2 JHA1 strain was also shown to carry important genetic markers associated with neurovirulence in mice when compared with strains of the same genotype. Two amino acid replacements, D390N and F402L, in the E protein were previously found to be related to neurovirulence in mice [20]. In addition, the replacement at position R105Q of the NS1 protein was also found to be related to neurotropism in mice and is identical or equivalent to those replacements observed in the DENV2 NGC strain [20]. Other amino acid replacements, specifically located at the EIII (positions 301, 303 and 363), were found only in the JHA1 strain. Although not yet ascribed to murine neurovirulence, mutations in this domain that are directly involved in binding host cell receptors may affect host-virus interactions [46–48]. The higher virulence of the JHA1 strain compared with the NGC strain, as inferred both by the lower LD_{50} and the lack of morbidity signs among animals who survived a sublethal dose [22,35,49,50], may also be attributed to the unique amino acid replacement detected in the E/NS1 protein sequence as well as in other protein sequences of the virus.

The JHA1 strain shares immunological determinants with the reference DENV2 NGC strain, as demonstrated by the specific reactivity of anti-EIII and anti-NS1 antibodies. To date, the NGC strain has been frequently used in studies employing immunocompetent mice and as the genetic background for the construction of chimeric-, DNA- and protein-based DENV2 vaccine candidates [4,16,22,35,50,51]. The present data demonstrated that the JHA1 strain may represent a complementary tool for the evaluation of NGC-based vaccine candidates. In addition, our results indicate that the JHA1 strain may also be used in experimental models without the need to determine how to handle a virus strain in which different mutations have been selected during adaptation to the new host, which is therefore different from the original virus strains.

Pathological damage caused by the JHA1 strain was mainly restricted to the brain and local endothelial cells. In contrast to DHS/DHF cases [43,52], no significant pathological damage was detected in the liver or spleen of the infected animals. This indicates that the cellular targets of the JHA1 strain are mainly restricted to the cerebral tissue, leading to death by encephalitis. Such pathological features may restrict the dissemination of the virus to other tissues, as supported by the unsuccessful attempts to infect mice with the JHA1 strain using other administration routes (unpublished observations). Nevertheless, the detection of viable virus particles in the spleen and the induction of rather early virus-specific antibody responses indicate that at least some virus particles leak from the cerebral tissue and may reach distant organs. The presence of viable virus particles in the spleen may also be a consequence of the high concentration of mononuclear cells in this organ, known to be permissive to DENV replication [1,2,14]. Some of the features detected in mice lethally infected with the JHA1 strain have also been observed in infected subjects with unusual DENV infection manifestations, who suffer from pain ascribed to transient encephalitis and weight loss [53–56]. Other DENV experimental models based on immunocompromised mice could also reproduce some of these symptoms (encephalitis and weight loss) but in a different time period [25]. The fact that the JHA1 virus could replicate and kill immunocompetent mice without accumulation of selective mutations allowing adaptation to the new host emphasizes the fact that the natural genetic variability of the virus may hinder relevant information regarding host specificity and tissue tropism among immunocompetent hosts.

Specific hematological alterations observed in JHA1-infected mice were similar to those recorded in severe DENV infection cases, such as the transient increase in hematocrit values followed by a sharp decrease indicative of hemorrhage [25,54,57,58]. In addition, the observed reduction in the number of lymphocytes and neutrophils has also been recorded in DHF and DSS cases in which the depletion of progenitor cells occurs due to medullar virus replication [34,59–62]. In contrast, no alteration was observed in the platelet number during the infection course, which is a hallmark DHF characteristic. A similar feature was also reported for a DENV3 strain capable of infecting immunocompetent mice [26]. However, the coagulation function in mice infected with the JHA1 strain was drastically altered, as measured by the prothrombin time test. This result has a close relationship with the hemorrhage process observed in the JHA1-infected animals and may also be related to the high levels of anti-NS1 antibodies detected in these mice, once that such antibodies were previously shown to interfere with coagulation mechanisms [4,6,7]. Nevertheless, the conclusion that the JHA1 strain reproduces, under experimental conditions, most of the hematological disturbances observed in severe forms of DENV infection cases must be considered in future studies that are designed to provide a better understanding of viral pathogenicity.

The JHA1 strain was also shown to induce acute inflammatory reactions and early adaptive immune responses in infected mice. Infected animals had increased serum levels of INF-γ, an important antiviral cytokine that is lacking in AG129 mice (lacking interferon-α/β and -γ receptors), which are frequently used as a DENV infection model [24,25] Thus, the lack of detectable virus particles in blood samples and histological injuries in organs other than the brain of mice infected with the JHA1 strain may be attributed to the early virus replication control triggered by the rapid increase in INF-γ in immunocompetent Balb/c mice. Similar to the response detected in humans [63], early antibody responses detected in mice infected with the JHA1 strain were mainly associated with the NS1 protein. These results are indicative of the higher immunogenicity of the NS1 protein compared with the E protein and reflect the early synthesis and secretion of the NS1 protein during the infection course [43,63]. Together, these findings indicate that in mice, the JHA1 strain can reproduce some of the inflammatory reactions and early antibody responses that are detected in DENV-infected subjects without the

need to use genetically modified mice. Such features open the possibility of using the JHA1 strain to evaluate immunization regimens that induce protective innate and adaptive immune responses leading to protection from lethal virus challenges.

It is important to highlight that the results presented in this study were obtained by using an inbred immunocompetent mouse lineage, which differ from conditions naturally found among human populations. Probably some of the reported symptoms and features observed in JHA1-infected mouse may differ from those observed in outbred animals. Nonetheless, all experimental murine models aiming to the study of dengue pathogenesis are based on isogenic lineages [6,26,64]. In addition, the infection route presented in this study is clearly far from that observed in the infection natural course, with disease symptoms based mainly on encephalitic manifestations, which are accepted as unusual manifestations in infected humans [53–56], but in contrast, were also reported in other mouse models with DENV inoculation through the intracranial route [65–67]. Thus, the infection model presented here is not able to reproduce the complete set of symptoms seen in DF or its more severe forms, DHF and DSS. Nonetheless, we report here, for the first time, the contribution of reproducing most of the hematological disturbances seen in infected humans by using non-modified virus and mice. Despite the important differences regarding the natural infection, the present model represents a step forward in the study of different aspects of DENV pathogenesis and may help of the screening of different anti-viral approaches.

In conclusion, the present study provides important contributions to the study of DENV2 pathogenicity that may aid the development of antivirus prophylactic and therapeutic approaches. As previously observed [26], our results demonstrate that the endogenous DENV2 genetic plasticity represents an important source of information that may contribute to a better understanding of viral pathogenicity, including the ability of viruses to replicate in a murine host and the severity of the induced symptoms. These results also indicate that a similar approach may be applied to other DENV types that could lead to the identification of virus strains with more adequate features for the development of alternative infection models.

Supporting Information

Figure S1 Multiple sequence alignment of the amino acid sequences corresponding to the EIII/NS1 region of the JHA1, India/1957, Indonesia 1977 and NGC strains. Polymorphic sites are indicated and placed into three different regions of the analyzed sequences: domain III (red), stem-anchor and NS1 signal peptide (blue) of the E protein (inside the black rectangle) and the entire NS1 protein (outside of the black rectangle).
(TIF)

Table S1 Comparison of amino acid sequences of the envelope glycoprotein and nonstructural NS1 protein regions possibly involved with neurovirulence in mice of the NGC and JHA1 DEN2 strains.
(DOC)

Table S2 Predicted T-cell epitopes shared by the JHA1 and NGC DENV2 strains found at the sequences encoding the EIII/NS1 proteins. [a] Inferred amino acid sequences of NGC and JHA1 strains were submitted to the computational system $PRED^{BALB/C}$ to predict specific epitopes for class I ($H2\text{-}K^d$, $H2\text{-}L^d$ and $H2\text{-}D^d$) and class II ($H2\text{-}IE^d$ and $H2\text{-}IA^d$) MHC molecules of Balb/c mice (Zhang et al., 2005). The predicted epitopes with higher scores were compared between the two strains to infer the conservation of these immunological determinants. This comparison was also applied to the experimentally determined $CD8^+$ T cell-restricted epitope of the DENV2 NS1 protein, AGPWHLGKL (Gao et al., 2008). [b] Predicted epitopes with higher scores within the EIII/NS1 region of the JHA1 isolate and the $CD8^+$ T cell-restricted epitope of the DENV2 NS1 protein, all of which were fully conserved between the JHA1 and NGC strains. [c] Location of the conserved epitope within the EIII/NS1 region of the strains subjected to the analysis. [d] Epitope located in the NS1 protein previously demonstrated to be specific for $CD8^+$ T lymphocytes and widely conserved among several DENV2 strains.
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: JHA MESA MLC LCSF. Performed the experiments: JHA RSPB RPSA JEL. Analyzed the data: JHA RSPB LCSF. Wrote the paper: JHA LCSF.

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6 CAPÍTULO 4 - Uma resposta imunológica estritamente humoral contra o domínio III da glicoproteína de envelope do vírus dengue induz ADE homotípico

Para investigar se o correlato de proteção na dengue é, de fato, a geração de anticorpos neutralizantes, obtivemos a EIII do VD-2 na forma de proteína recombinante e a utilizamos como antígeno vacinal, coadministrado ou não com diferentes adjuvantes em camundongos da linhagem Balb/c. As respostas imunológicas induzidas nos animais atingiram altos níveis de anticorpos, capazes de neutralizar o vírus em ensaios *in vitro* conduzidos com células desprovidas de receptores Fc. Entretanto, ao avaliarmos a eficácia protetora dessas formulações em desafios *in vivo*, constatamos que os animais imunizados com a EIII morreram e desenvolveram sinais de morbidade de forma precoce em relação ao grupo controle não imunizado. Constatamos ainda que os mesmos anticorpos, quando testados em ensaios de neutralização *in vitro* conduzidos com células expressando receptores Fc, induziram aumento nos níveis de infecção nessas células. Os resultados obtidos neste trabalho indicam que uma resposta estritamente humoral direcionada contra o EIII do vírus dengue aumenta os níveis de infecção e acelera o aparecimento de danos. As contribuições trazidas neste trabalho são as primeiras evidências de que o processo de desenvolvimento de vacinas contra a dengue deve ser revisto, começando pela reconsideração do principal correlato de proteção para a doença. Os resultados indicam ainda que a(s) resposta(s) imunológica(s) protetora(s) contra a doença são complexa(s) e ainda mal conhecidas.

6.1 INTRODUÇÃO

O vírus dengue (VD) é o causador da dengue, a arbovirose mais comum que atinge seres humanos. Somente estes apresentam manifestações clínicas da infecção, produzindo um período de viremia de aproximadamente sete dias. Nos demais primatas, a viremia é baixa, de curta duração e assintomática (HOLMES et al.,1998). A dengue é endêmica em países de clima tropical e subtropical, pois estas regiões apresentam condições adequadas para o desenvolvimento de seus artrópodes vetores, os mosquitos *Aedes albopictus* e *Aedes aegypti* (GUZMAN et al., 2010). As fêmeas infectadas destas espécies transferem o vírus para o homem ao realizarem o repasto sanguíneo, necessário à maturação de seus ovos. Assim que o mosquito infectado transfere o vírus para o homem, ocorrem as etapas do processo infeccioso e replicação viral, sendo as células dendríticas, monócitos e macrófagos as principais células-

alvo (WHITEHEAD et al., 2007). Dentro do hospedeiro humano, o VD se liga ao receptor de membrana da célula alvo pela interação deste com o domínio III (EIII) da glicoproteína de envelope (E). Em seguida, acontece a endocitose mediada pelo receptor, redução do pH no interior do endossomo e consequente mudança conformacional da proteína E com exposição do peptídeo de fusão localizado no domínio II da proteína E, o que promove a fusão do envelope viral com a membrana do endossomo e liberação do capsídeo no citoplasma da célula hospedeira (SAMPATH; PADMANABHAN, 2009). Se essas etapas forem cumpridas, o vírus libera seu material genético no citoplasma para síntese de novos componentes (material genético e proteínas) que serão destinados à sua replicação.

As principais estratégias vacinais em desenvolvimento para o controle da dengue são baseadas na ideia de impedimento da infecção. Admite-se que o correlato de proteção é a indução de uma resposta imunológica com altos níveis de anticorpos neutralizantes, capazes se ligarem ao domínio EIII e impedirem a interação com os receptores da célula hospedeira e, consequentemente, a infecção (WHITEHEAD et al., 2007). Além disso, preconiza-se que uma vacina ideal para o controle da dengue deve ser capaz de induzir anticorpos neutralizantes contra os quatro sorotipos virais, para que não haja risco de ADE (do inglês *antibody dependent enhanceement*-ADE) (WHITEHEAD et al., 2007). Por esta razão, acredita-se que uma vacina que não seja capaz de gerar imunidade neutralizante contra os quatro sorotipos virais pode induzir o desenvolvimento de formas graves da dengue.

Diversas formulações vacinais foram propostas para o controle da dengue baseadas em tal raciocínio. O curioso é que as formulações baseadas no EIII, na forma de proteína purificada, costumam induzir anticorpos considerados neutralizantes em ensaios *in vitro*, mas nenhum grupo de pesquisa demonstrou a capacidade protetora dessas vacinas em ensaios de desafio *in vivo* (CHIANG et al., 2012; ETEMAD et al., 2008; LENG et al., 2009; ZHANG et al., 2007). Por outro lado, quando a proteína E ou seu domínio III são utilizados como antígenos vacinais na forma de vacina de DNA os níveis de proteção podem chegar a 100% (AZEVEDO et al., 2011; DE PAULA et al., 2008; RAVIPRAKASH et al., 2003). Além disso, sabe-se que vacinas baseadas em vírus inativados induzem proteção parcial e que vacinas baseadas em vírus atenuados induzem altos níveis de proteção em suas versões monovalentes (WHITEHEAD et al., 2007). O que essas últimas versões de vacinas têm de diferente em relação à primeira deve estar relacionado ao direcionamento de anticorpos contra outros domínios da proteína E também importantes no processo infeccioso. Além disto, vacinas baseadas em vírus atenuados ou DNA promovem a expressão do antígeno dentro da

célula hospedeira, o que pode modular de forma completamente diferente a resposta imunológica. Assim, cabe fazer a pergunta: o correlato de proteção na dengue é mesmo a geração de anticorpos ditos neutralizantes contra o EIII? Ou melhor: a neutralização *in vitro* corresponde à neutralização *in vivo*? A resposta parece ser não.

Para investigar essa questão, obtivemos a porção EIII na forma de proteína recombinante purificada e a utilizamos como antígeno vacinal, coadministrado ou não com diferentes adjuvantes em camundongos da linhagem Balb/c. As respostas imunológicas induzidas nos animais contiveram altos níveis de anticorpos, capazes de neutralizar o vírus em ensaios *in vitro* conduzidos com células desprovidas de receptores Fc. Entretanto, ao avaliarmos a eficácia protetora dessas formulações em desafios *in vivo*, constatamos que os animais imunizados com a EIII morreram mais rapidamente e desenvolveram sinais precoces de morbidade, queo grupo controle não imunizado. Constatamos ainda que os mesmos anticorpos, quando testados em ensaios de neutralização *in vitro* conduzidos com células expressando receptores Fc, induziram aumento nos níveis de infecção nessas células. Essas são as primeiras evidências de que uma resposta estritamente humoral direcionada contra o EIII do vírus dengue aumenta os níveis de infecção e acelera o aparecimento de danos. O conhecimento produzido nesse trabalho deve servir de base para uma busca racional de uma vacina efetiva contra a dengue.

6.2 MATERIAIS E MÉTODOS

6.2.1 CLONAGEM DA SEQUÊNCIA CODIFICADORA DA EIII DO VD2

O plasmídeo pE2, um derivado do pcDNA3, que alberga o gene codificador da EIII da cepa NGC (gentilmente cedido pela Dra. Ada Maria de B. Alves, Fundação Oswaldo Cruz, RJ, Brasil) foi utilizado como modelo para reações de PCR. O conjunto de parâmetros térmicos foi: uma etapa de desnaturação inicial de 5 minutos a 94 ºC seguida por 30 ciclos de 30 segundos a 94 ºC, 1 minuto a 50,7 ºC e 1 minuto a 72 ºC, com uma etapa final de extensão de 4 minutos a 72 ºC no termociclador Mastercycler Gradient (Eppendorf). O oligonucleotídeo iniciador senso utilizado foi 5'-ACATGCGAGGATCCGGAATGTCATACTCTAT-3' (a sequência sublinhada mostra o sítio de restrição *BamHI*), e anti-senso foi 5'-

GCCTTCTACTCGAGTTAGATAGAACTTCCCTTTCTTA - 3' (a sequência sublinhada mostra o sítio de restrição *XhoI*).

Após a reação de amplificação, a sequência gênica da EIII, composta por 309 pares de bases (pb), foi obtida flanqueada pelos sítios de restrição *BamHI* e *XhoI*. O produto da PCR foi purificado com o kit *IlustraTM GFXTM PCR DNA and Gel Band Purification* (GE Healthcare Life Sciences), digerido com *BamHI* e *XhoI* e, então, ligado nos sítios correspondentes de *BamHI* e *XhoI* no vetor de expressão pET28a(+) (Novagen, Darmstadt, Alemanha), gerando o plasmídeo recombinante pD2EIII, que, posteriormente, foi transformado em *E. coli* DH5α quimicamente competente. O DNA plasmidial de colônias bacterianas transformadas foram analisadas por digestão com *BamHI* e *XhoI* e PCR (SAMBROOK e RUSSEL, 2001). O clone selecionado para a etapa de expressão foi sequenciado.

6.2.2 EXPRESSÃO DA PROTEÍNA EIII RECOMBINANTE

A cepa BL21 (DE3) de *E.coli* quimicamente competente foi transformada com o pD2EIII, para gerar a linhagem BLEIII. Uma linhagem "vazia" foi gerada pela transformação com o pET28a para ser utilizada como controle. As duas linhagens foram cultivadas individualmente em 50 mL de meio LB (Luria Bertani) contendo 50 µg/mL de canamicina e 30 µg/mL de cloranfenicol a 37 ºC até uma densidade ótica de 0,5 a 600nm. Uma alíquota de células bacterianas (t_0), coletada após ajuste das unidades formadoras de colônia (UFC), foi mantida em gelo e 0,5 mM do indutor de expressão IPTG (do inglês *isopropyl β-D-1-thiogalactopyranoside*) (Sigma) foi adicionado ao meio de cultura. Após 4 horas de indução, outra alíquota (t_4) foi coletada com ajuste das UFC em relação à t_0, as células foram suspensas em tampão A [fosfato de sódio a 100 mM e NaCl a 500 mM pH 6,5] e imediatamente lisadas por sonicação (ciclo de 1,5 minuto em sonicação, dividido em etapas de 15 segundos, com 10 segundos de intervalo entre as etapas, em amplitude de 30%). Após centrifugação (15557 x g por 30 minutos), ambas as frações solúvel e insolúvel foram recuperadas, 35 µg de proteína total de cada fração foram misturadas com tampão de amostra de eletroforese [Tris-HCL a 200 mM pH 6,8; SDS a 8% (massa/volume); Glicerol a 40% (volume/volume), Azul de Bromofenol a 0,4% (massa/volume); 2-β-mercaptoetanol a 200 mM] e submetidas à eletroforese em gel de poliacrilamida SDS-PAGE (do inglês *sodium dodecyl sulfate polyacrylamide gel electrophoresis*) (SAMBROOK; RUSSEL, 2001). Ensaios de *Western*

blot foram realizados com 3H5-1, um IgG1 monoclonal de camundongo anti-VD2 de referência (Millipore), numa diluição 1:1000 em tampão de bloqueio contendo 5% de leite desnatado em PBS (tampão fosfato salino, do inglês *Phophate Buffered Saline* [NaCl a 137 mM, KCl a 2,7 mM, Na_2HPO_4 a 10 mM, KH_2PO_4 a 2 mM]) acrescido de 0,05% de Tween 20 (PBST) e anticorpos anti-IgG de camundongos produzidos em cabra conjugados com peroxidase (Sigma) (diluição 1:3000 em tampão de bloqueio) (AMORIM et al., 2010; SAMBROOK; RUSSEL, 2001). As bandas reativas de proteínas foram identificadas com a solução de revelação apropriada (SAMBROOK; RUSSEL, 2001).

6.2.3 PURIFICAÇÃO DA EIII RECOMBINANTE

A linhagem BLEIII foi cultivada em 1L de meio LB contendo 50 µg/mL de canamicina e 30 µg/mL de cloranfenicol a 37 ºC até uma densidade ótica de 0,5 a 600 nm. O IPTG foi adicionado para uma concentração final de 0,5 mM e as células foram colhidas 4 horas mais tarde após centrifugação (12857 x g por 10 minutos). As células sedimentadas foram suspensas em tampão A e submetidas à lise mecânica (600 psi por 5 minutos) em um homogeneizador modelo APLAB-10 (ARTEPEÇAS, Brasil). Após a centrifugação (20670 x g por 60 minutos), a fração contendo os corpos de inclusão foi suspensa em 20 mL de tampão B [100 mM de fosfato de sódio, 50 0mM de NaCl e 8M de uréia (pH 6,5)], através de leve agitação a 4 ºC por toda a noite. O extrato foi centrifugado e o sobrenadante foi filtrado em um aparato *Stedim Sartorius* com um filtro de acetato de celulose com poros de até 0,22 µM (Biotech). As proteínas foram quantificadas em espectrofotômetro GeneQuant (GE Amershan Biosciences) e submetidas a um processo de *refolding* por diluição em 2 L de tampão A em um fluxo de 0,25 mL/minuto. Depois do *refolding*, a amostra foi clarificada por centrifugação (20670 x g por 60 minutos), com posterior filtração do sobrenadante, e foi adicionado 2-beta-mercaptoetanol para uma concentração final de 5mM. As amostras foram submetidas à cromatografia de afinidade ao níquel usando uma coluna *Histrap*TM FF (GE Healthcare Life Sciences), previamente equilibrada com tampão A, num fluxo de 1,8 mL/minuto no cromatógrafo ÄKTA FLP (Amershan Pharmacia Biotech). A coluna foi lavada novamente com tampão A e, em seguida, foi aplicado um gradiente linear de tampão A para o tampão C [100 mM de fosfato de sódio, 500 mM de NaCl e 1 M de imidazol (pH 6,5)] para eluição da amostra que foi coletada de forma fracionada, de acordo com o gradiente estabelecido dividido em 30 partes iguais. As frações coletadas contendo a EIII do VD2 foram agrupadas,

tratadas com 10U DNAse (Promega) e dialisadas contra PBS 1x a um pH de 6,5. A produção final de proteína foi determinada em espectrofotômetro GeneQuant (GE Amershan Biosciences).

6.2.4 ANTIGENICIDADE E PROVAS DE FUNÇÃO BIOLÓGICA DA EIII RECOMBINANTE

Para a prova de antigenicidade, a EIII recombinante foi usada para sensibilizar placas de ELISA após ou não o tratamento térmico (incubação a 100 ºC por 10 minutos). Em resumo, o anticorpo monoclonal 3H5-1 foi usado para testar a antigenicidade e o ensaio foi realizado conforme descrito anteriormente (AMORIM et al., 2010).

Para avaliação da função biológica, lamínulas estéreis foram posicionadas em placas de seis poços (Nunc), onde $1x10^5$ células LLC-MK2/poço (ATCC) foram plaqueadas em DMEM suplementado com 5% de SFB, e incubadas à 37 ºC com 5% de CO_2 por 48 horas. As células foram gentilmente lavadas com PBS estéril (pH 7,0) e imediatamente incubadas com 10 µg de EIII recombinante com ou sem tratamento térmico por 30 minutos a 37 ºC com 5% de CO_2. Após a incubação, as células foram fixadas com paraformaldeído a 4% por 20 minutos a 4ºC e tratadas com o anticorpo monoclonal 3H5-1 por 1 hora em TA. Posteriormente, as células foram tratadas com anti-IgG de camundongo produzido em cabra conjugado com FITC (isotiocianato de fluoresceína) (Invitrogen) e DAPI (4,6-diamidino-2-fenilindol) (Thermo Scientific). As amostras foram analisadas em microscópio de imunofluorescência (Asiovert S100, Zeiss). Alternativamente, após tratamento com o anti-IgG conjugado ao FITC, as células foram suspensas em PBS e passadas em citometria de fluxo, num citômetro modelo FACS-Calibur (BD).

6.2.5 REGIME DE IMUNIZAÇÃO

Camundongos da linhagem Balb/c, com seis semanas de idade, foram agrupados de acordo com a formulação vacinal utilizadae com *n*=15. Os animais foram injetados pela via intramuscular (i.m.) com as seguintes formulações vacinais, dissolvidas em tampão fosfato salino (PBS): PBS apenas como controle, 10 µg de EIII apenas, 10 µg de EIII e 12,5 µg de $Al(OH)_3$, (Rehydragel, Reheis) como adjuvante, 10 µg de EIII e 1 µg de LTK63 como adjuvante (obtida de acordo com protocolo estabelecido em nosso laboratório em AMORIM et al., 2012b) ou o equivalente a 1000 unidades formadoras de placa (UFP) da cepa JHA1 de

VD2 (AMORIM et al., 2012a) previamente inativadas por calor e coadministradas com 12,5 µg de $Al(OH)_3$. A formulação vacinal contendo vírus inativado foi retitulada imediatamente após injeção nos animais, para assegurar sua inativação. O regime vacinal consistiu na aplicação de quatro doses com intervalo de 15 dias entre as duas primeiras doses e 7 dias entre as duas últimas. Coletas de sangue para obtenção de soro foram feitas nos camundongos pelo plexo retro-orbital antes da primeira dose (soro pré-imune) e sete dias após cada imunização. Inicialmente, as amostras de soro foram testadas individualmente para reatividade contra EIII e depois, misturadas respeitando-se a organização dos grupos e estocadas a – 20 ºC para análises subseqüentes

6.2.6 AVALIAÇÃO DA GERAÇÃO DE ANTICORPOS ESPECÍFICOS PARA A EIII

O soro de cada animal foi testado quanto à presença de anticorpos específicos para a EIII por ELISA. Placas Maxisorp (Nunc) foram sensibilizadas por 12 h a 4 ºC com 0,2 µg de EIII (em 100 µL de PBS) por poço. O bloqueio foi feito por 1 h a 37 ºC com PBS acrescido de Tween-20, a 20% (v/v) e leite em pó desnatado a 5% (m/v) (PBST). As amostras de soro foram serialmente diluídas na placa previamente bloqueada. Após 1 h à temperatura ambiente, as placas foram lavadas com PBST e incubadas com Imunoglobulinas conjugadas à peroxidase produzidas em cabra contra IgG, IgG1 ou IgG2a de camundongo (Southern Biotechnology) por mais 1 h à temperatura ambiente. As reações foram mensuradas à $A_{492\ nm}$ com dihidrocloreto de orto-fenilenediamina (Sigma) e H_2O_2 como substrato e paradas com H_2SO_4 a 2 N. Os títulos foram calculados como previamente descrito pelo nosso grupo (AMORIM et al., 2012b).

6.2.7 DESAFIO COM INFECÇÃO DE DENGUE 2 EM CAMUNDONGOS BALB/C

Duas semanas após a última dose vacinal, os camundongos foram desafiados com $2xDL_{50}$ (100 UFP) da cepa JHA1 de dengue 2, pela via intracraniana (AMORIM et al., 2012a). Os animais foram anestesiados com um mistura de quetamina e xilazina (ERHARDT et al., 1984) e inoculados com 30 µL da suspensão viral. As partículas virais foram diluídas em DMEM puro. Imediatamente após o procedimento de inoculação, o restante da suspensão foi novamente titulado em células LLCMK2 (AMORIM et al., 2012b). Os animais foram monitorados por 21 dias e os percentuais de mortalidade e morbidade foram registrados.

6.2.8 MARCAÇÃO INTRACELULAR DE CITOCINAS

A marcação intracelular de IFN-γ foi realizada em amostras de sangue retiradas dos animais imunizados 5 dias após o desafio com o vírus dengue (*n*=5 por grupo). As amostras foram tratadas com tampão ACK (BioSource International; Camarillo, Estados Unidos) para lise específica de eritrócitos e, em seguida, centrifugadas a 100 *g* por 5 min. Os leucócitos periféricos (PBMCs) foram novamente tratados com ACK, centrifugados e ressuspensos em DMEM. Os PBMCs foram cultivados numa concentração de $1x10^6$ células/poço em placas apropriadas de 96 poços (BD Bioscience) por 5 h a 37 ^oC com os seguintes aditivos: 10% de soro fetal bovino (SFB), β-mercaptoethanol 10^{-6} M, brefeldina A (GolgiPlug; BD Bioscience) a 1 µg/mL, aminoácidos, vitaminas e a proteína EIII como estímulo numa concentração de 5 µg/mL (exceto nos poços de controle negativo). A marcação extracelular foi feita com anticorpos monoclonais anti-CD4 e anti-CD8a de camundongo conjugado ao fluoróforo Cy e FITC, respectivamente (BD Bioscience). E a marcação intracelular foi feita com um anticorpo monoclonal anti- IFN-γ (BD Bioscience). Após esse procedimento, as amostras foram lidas em um citômetro de fluxo modelo FACS Calibur (BD Bioscience).

6.2.9 ENSAIOS BIOQUÍMICOS PARA AVALIAÇÃO DE INTEGRIDADE TECIDUAL

Amostras individuais de soro coletadas 7 dias após a última imunização ou 5 dias após o desafio (*n*=5) foram utilizadas para determinar os níveis de transaminase glutâmico-oxalacética (TGO), transaminase glutâmico-pirúvica (TGP) e desidrogenase láctica (LDH), como indicado pelo fabricante dos *kits* de quantificação de TGO, TGP e LDH (Bioclin, Brasil).

6.2.10 AVALIAÇÃO DA INTEGRIDADE HEMATOLÓGICA

Amostras individuais de sangue total coletadas 7 dias após a última imunização ou 5 dias após o desafio (*n*=5) foram utilizadas para estudar o hematócrito e avaliar o perfil de coagulação dos animais nessas duas situações, conforme procedimentos previamente descritos (AMORIM et al., 2012a).

6.2.11 TESTE DE NEUTRALIZAÇÃO VIRAL IN VITRO

Amostras de soro foram agrupadas de acordo com a formulação vacinal e testadas para a atividade de neutralização contra o JHA1 em ensaio de placa realizado com células LLC-MK2, como descrito anteriormente (AMORIM et al., 2012a). Alternativamente, um ensaio para avaliar a capacidade de neutralização das mesmas amostras em células U937 (ATCC), que expressam receptores do tipo Fc, foi implementado. Neste ensaio, a detecção de células infectadas foi feita pela marcação extracelular da proteína NS1, com anticorpos de camundongos anti-NS1 previamente descritos (AMORIM et al., 2012b), por citometria de fluxo, com uma marcação final utilizando um anticorpo monoclonal anti-IgG de camundongo conjugado ao FITC (BD Bioscience). A infecção das células utilizadas em cada poço ($1x10^6$ células) foi padronizada para 50%. Diluições seriadas dos soros referentes a cada grupo de imunização a partir de 1:10 (relação vírus:soro) foram realizadas em alíquotas do vírus JHA1 com UFPs suficientes para infectar 50% das células e incubadas à 37 ºC por 1 hora. As misturas foram incubadas juntamente com as células por 1 h, as quais, em seguida, foram centrifugadas a 100 g por 5 min e lavadas com RPMI duas vezes. As células foram incubadas em meio RPMI com suplemento de 10% de SFB, 2 mM de glutamina e 5% de CO_2 a 37 ^oC por 48 h. Após esse período, as células foram marcadas extracelularmente com anticorpos anti-NS1 e em seguida, anticorpos anti-IgG de camundongo conjugado ao FITC. As amostras foram lidas no citômetro modelo FACS Calibur (BD Bioscience).

6.2.12 TESTE DE NEUTRALIZAÇÃO VIRAL IN VIVO

O protocolo de incubação da mistura vírus-soro foi repetido para cada grupo, apenas na diluição de 1:10. As misturas, padronizadas quanto ao volume, foram injetadas pela via intracraniana em camundongos machos Balb/C *naive* com 6 semanas de vida (n=5 por grupo). Os animais foram monitorados por 21 dias e os percentuais de mortalidade foram registrados.

6.2.13 ANÁLISES ESTATÍSTICAS

Análises de variância (ANOVA) foram realizadas com subsequente teste de Dunnet ou teste de comparaçao múltipla de Bonferroni. A significância estatística foi considerada quando $p < 0,05$. Para as análises de sobrevivência e morbidade, foram aplicados os métodos

estatísticos de Mantel-Cox e Gehan-Breslow-Wilcoxon, sendo admitida significância estatística quando $p < 0,05$.

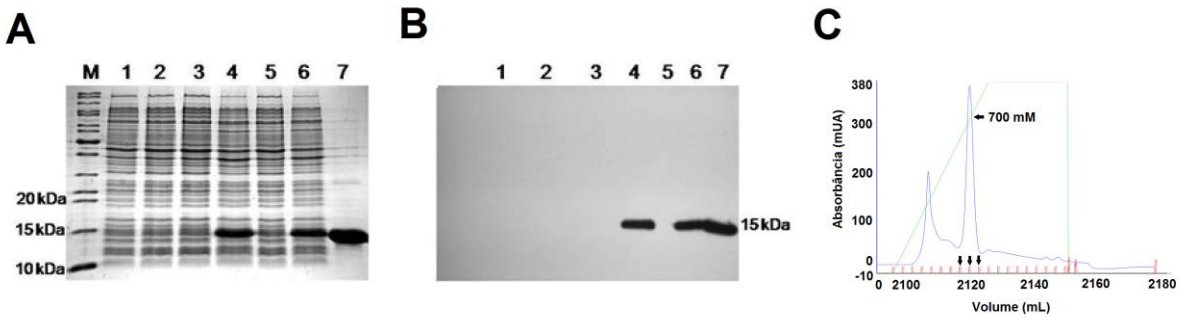
6.3 RESULTADOS

6.3.1 OBTENÇÃO E CARACTERIZAÇÃO DA FORMA RECOMBINANTE DO EIII

Para investigar se uma resposta imunológica de anticorpos dirigidos contra o EIII do VD pode conferir proteção em ensaios de desafio, clonamos e expressamos em *E. coli* a forma recombinante deste antígeno. Uma banda com massa molecular de 15 kDa foi detectada no extrato insolúvel de proteínas da BLEIII (Figura 3A) e reagiu com os anticorpos monoclonais anti-VD (Figura 3B). Uma vez que toda a proteína recombinante foi detectada na fração insolúvel do extrato celular, foi testado um método de *refolding* com uma taxa de fluxo de 0,25 mL/minuto em 2 L de tampão A. Após o *refolding*, o sobrenadante foi aplicado a uma coluna cromatográfica de afinidade por níquel *Histrap*TM FF (GE Healthcare Life Sciences) para cromatografia de afinidade e as proteínas foram eluídas com imidazol em concentrações que variavam de 420 mM a 1000 mM (Figura 3C). A proteína purificada foi dialisada com PBS. A produção final de proteína após *refolding* e a purificação atingiu 42 mg de proteína por litro de cultura bacteriana.

A função biológica da EIII recombinante foi testada por meio da observação da sua capacidade de se ligar aos receptores celulares de mamíferos. As células foram incubadas com 10 µg da EIII recombinante que sofreu *refolding* após ou não a desnaturação por calor (100 ºC por 10 minutos), por 30 minutos e devidamente marcadas para análise em microscópio de fluorescência ou em citometria de fluxo, usando anticorpos monoclonais anti-VD 3H5-1. Nas figuras de 4A a 4E é possível observar que a EIII submetida ao *refolding* foi capaz de se ligar aos receptores celulares de mamíferos, o que foi abolido quando a proteína foi desnaturada pelo calor. Além disso, foi observado que a EIII recombinante produzida preserva epítopos conformacionais que reagem com o anticorpo específico para o VD. Mas esta reatividade é diminuída em aproximadamente 3 vezes quando a proteína é desnaturada por calor (Figura 4D). Esses resultados mostram que a proteína obtida preserva estrutura, antigenicidade e atividade biológica em relação ao EIII nativo, sendo, portanto, um antígeno adequado para o estudo em questão.

Figura 3 - Expressão e purificação da EIII recombinante do VD2.

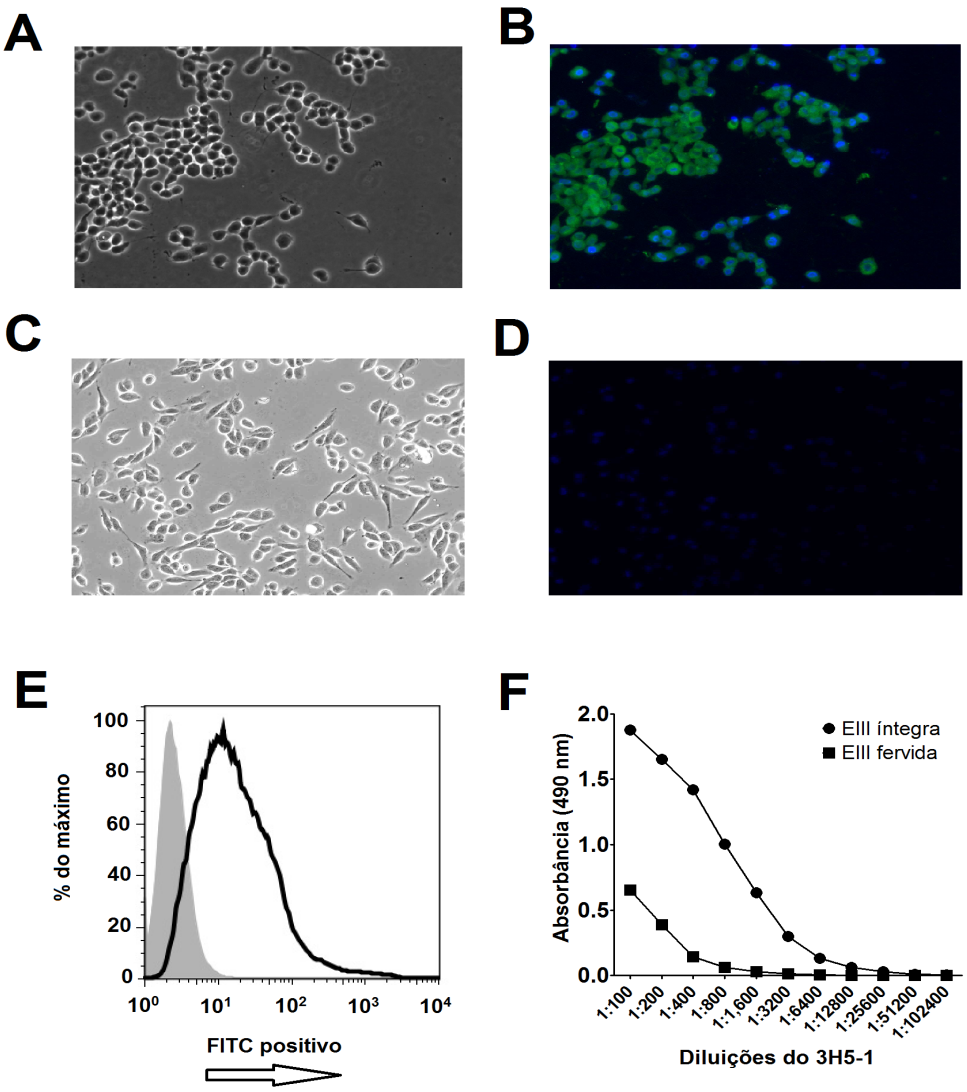


(A) Extrato total da célula bacteriana em gel de poliacrilamida corado com *coomassie blue* (SAMBROOK; RUSSEL, 2001). Amostras: M, marcador de massa molecular; 1, extrato celular total da cepa vazia não-induzida; 2, extrato celular total da cepa vazia após a incubação com IPTG; 3: extrato celular total da cepa BLEIII não-induzida; 4, extrato celular total da cepa BLEIII após indução com IPTG; 5, fração de proteína solúvel da cepa BLEIII após indução com IPTG; 6, fração de proteína insolúvel da cepa BLEIII após indução com IPTG e 7, EIII recombinante purificada. Nos poços de extrato total e das frações solúvel e insolúvel foram carregados 35 µg de proteína total. No poço da EIII purificada foram carregados 5 µg de proteína total. (B) Análise por *Western blot* dos extratos celulares totais incubados com o anticorpo de camundongo anti-VD2. As amostras são as mesmas descritas em (A). Os marcadores de massa molecular são indicados nos lados esquerdo e direito da figura. (C) Cromatograma da purificação por afinidade ao níquel da EIII recombinante. As frações contendo a proteína pura estão indicadas pela seta e foram eluídas com 700 mM de imidazol.

6.3.2 ESTUDO DAS RESPOSTAS IMUNOLÓGICAS GERADAS NOS ANIMAIS IMUNIZADOS COM AS FORMULAÇÕES VACINAIS CONTENTO EIII

A Figura 5A mostra que a combinação de 10 µg de EIII e 1 µg da LTK63 induziu a produção da maior quantidade de IgG anti-EIII específica entre todas as formulações testadas. Entretanto, a formulação que promoveu a geração de respostas imunológicas contra EIII com perfil mais pronunciadamente deslocado para o braço Th1, visto pela predominância de IgG2a em relação à IgG1 foi a mistura de vírus inativados e Alum, com razão IgG1/IgG2a de 0,03 (Figura 5B). O uso do antígeno apenas resultou numa relação IgG1/IgG2a de 0,1, seguido de 0,64 para EIII e LTK63, e 3,69 para EIII e Alum, sendo esta última a formulação que induziu a resposta humoral com perfil mais Th2 (Figura 5B).

Figura 4 - A EIII recombinante obtida retém função biológica e antigenicidade em relação à proteína nativa.

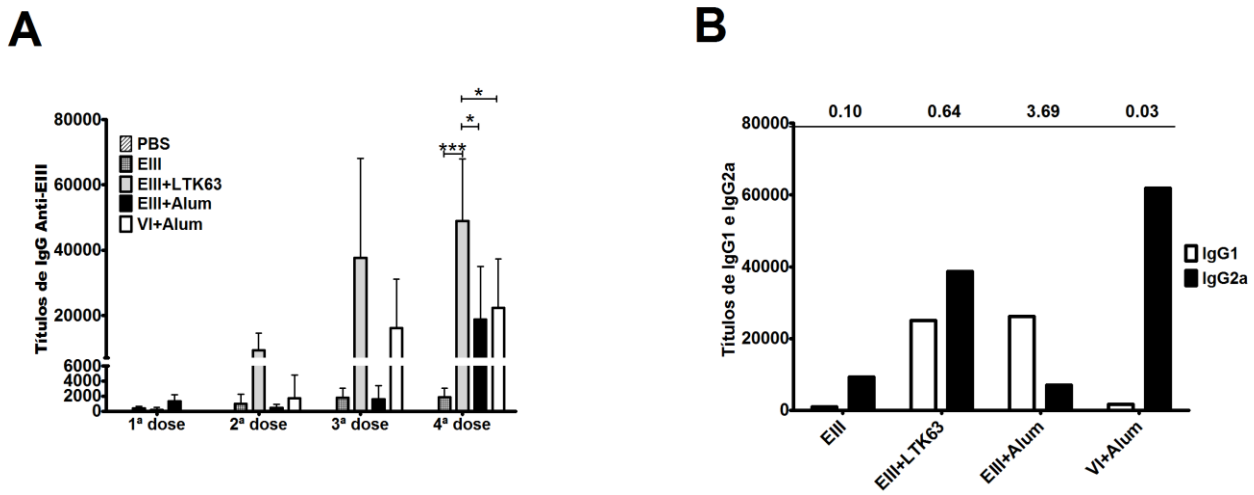


Para a prova de função biológica, células LLC-MK2 foram incubadas com EIII após ou não a desnaturação pelo calor, marcadas com anticorpos 3H5-1 por 1 hora e, em seguida, marcadas com DAPI (azul) e anti-IgG de camundongo produzido em cabra conjugado com FITC (verde) por mais 1 hora. Células incubadas com a EIII não-desnaturada foram observadas em microscópio de imunofluorescência apenas em contraste de fase (A) ou com fluorescência (B). O mesmo foi aplicado para as células incubadas com a EIII desnaturada pelo calor (C e D). Aumento de 100x. A capacidade de ligação da EIII obtida a receptores celulares da LLMCK2 também foi avaliada por citometria de fluxo (histograma cinza para proteína desnaturada e histograma não preenchido para proteína íntegra) (E). A antigenicidade da EIII recombinate também foi avaliada nas condições desnaturada ou intacta por ELISA, realizado com o anticorpo monoclonal anti-VD 3H5-1 (F).

Em relação às respostas imunológicas celulares, medidas em ensaios de marcação intracelular de INFγ, não foi observada nenhuma indução significativa de proliferação de linfócitos T produtores dessa citocina (Figura 6). Os estímulos *in vitro* foram feitos tanto com o VD JHA1 (Figuras 6A e 6C) quanto com a proteína recombinante EIII (Figuras 6B e 6D),

por 72 h, mas nenhuma detecção significativa de INFγ, tanto para células TCD4 ou TCD8 foi observada. Em conjunto, esses resultados indicam que as respostas imunológicas induzidas pelas formulações testadas foram predominantemente humorais, com os anticorpos exercendo papel principal na resposta aos antígenos EIII e vírus inativados.

Figura 5 - Perfil das respostas anti-EIII induzidas pelas diferentes formulações de vacinas.



(A) Título de IgG anti-EIII nas diferentes doses. Um nível elevado de produção de IgG anti-EIII por camundongos imunizados com a formulação contendo 10 µg de EIII e 1 µg de LTK63 foi observada em comparação com camundongos imunizados com as outras formulações na quarta dose. (B) Em comparação com a formulação contendo apenas 10 µg de EIII, formulações contendo EIII ou vírus inativados adicionados a adjuvantes mostraram diferentes proporções de IgG1/IgG2a.

Tabela 1 - Título de neutralização viral para os soros dos grupos de imunização.

Grupos[a]	Título neutralizante[b]
PBS	0
EIII	20
EIII+Alum	40
EIII+LTK63	160
VI+Alum	640
Protegidos	1280

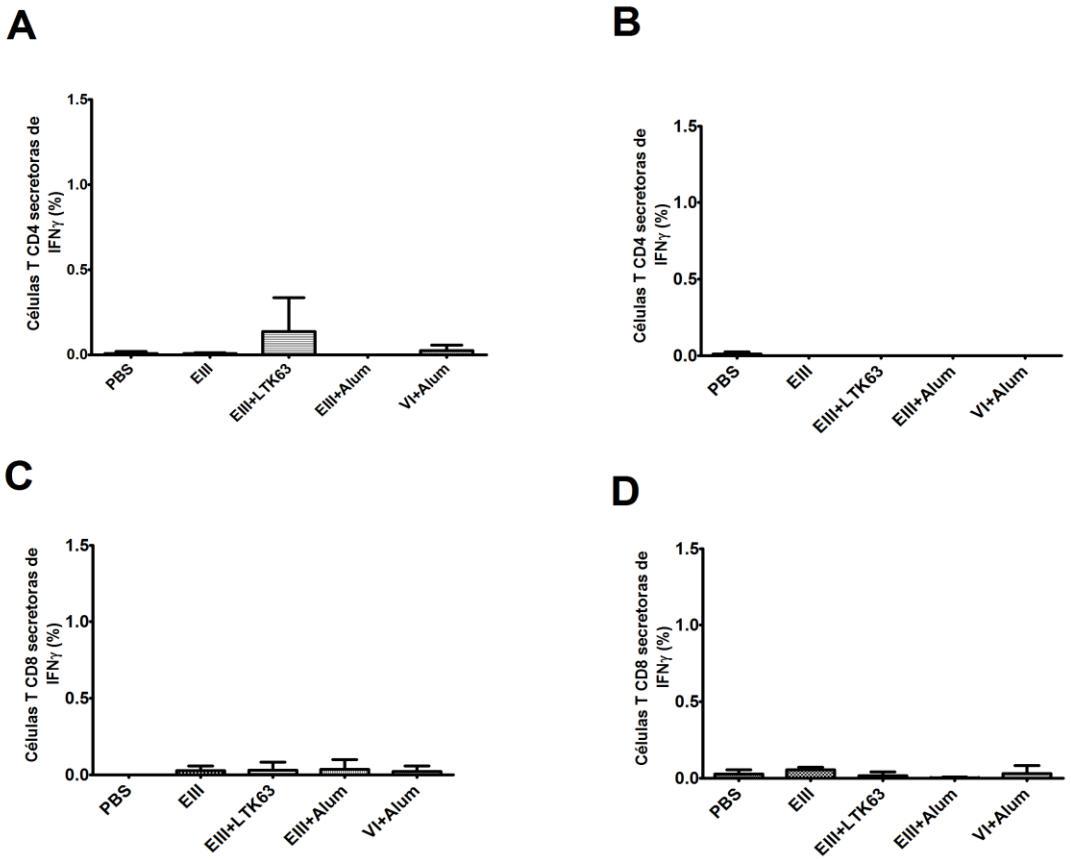
a - Os animais foram imunizados com as formulações descritas na seção de materiais e métodos e os soros referentes à última dose vacinal foram agrupados de acordo com a formulação vacinal.
b - Ensaios de neutralização viral foram realizados em células LLCMK2, desprovidas de receptores fc. O título neutralizante representa a última diluição do soro capaz de reduzir a formação de placas em pelo menos 50% em relação ao controle.

6.3.3 AVALIAÇÃO DE SEGURANÇA E DA EFICIÊNCIA PROTETORA DAS FORMULAÇÕES VACINAIS

Para verificar se alguma das formulações vacinais testadas induzia algum dano tecidual nos animais imunizados, provas bioquímicas para medição de enzimas marcadoras de

dano tecidual geral (LDH) ou localizados no fígado (TGO e TGP) foram realizadas. Não houve alteração estatisticamente significativa na quantidade da enzima LDH presente no soro dos animais (Figura 7A) indicando que as imunizações não induziram nenhum dano tecidual ou hemólise até o sétimo dia após a última dose vacinal. Também não houve alteração significativa nas enzimas TGO e TGP (Figuras 7B e 7C, respectivamente), indicando que nenhum dano hepático foi induzido com o regime de imunização. Além disso, foi demonstrado que os soros dos animais imunizados foram capazes de neutralizar o VD JHA1 *in vitro* quando o ensaio foi conduzido em células LLCMK2, sendo que o soro do grupo imunizado com vírus inativado e Alum foi significativamente mais neutralizante (Tabela 1). Esses resultados indicam, em conjunto, que as formulações vacinais testadas induzem respostas imunológicas com anticorpos considerados neutralizantes *in vitro* quando a célula testada não apresenta receptores do tipo Fc. Além disso, essas formulações não induzem, por si, danos teciduais nos animais imunizados.

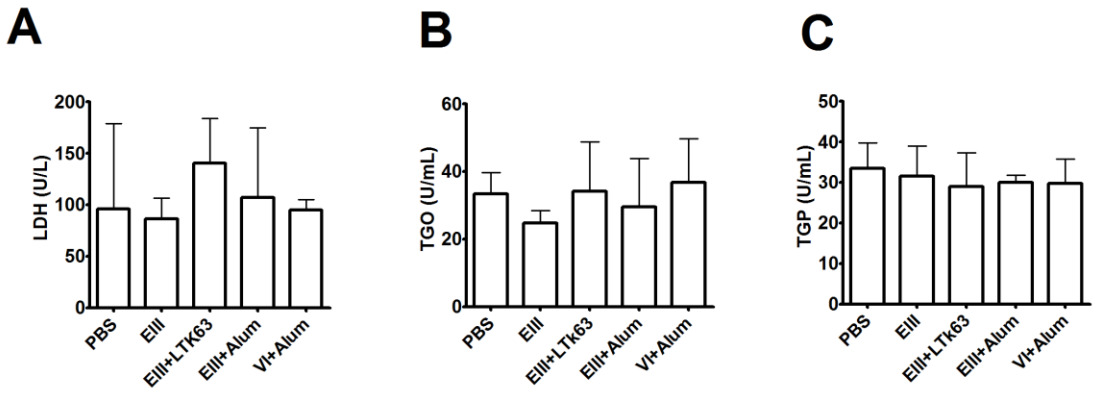
Figura 6 - Imunidade celular induzida pelas formulações vacinais testadas em camundongos BALB/c vacinados.



Não foi detectada resposta intracelular significativa de IFNγ em linfócitos T $CD4^+$ tanto após estimulação com o JHA1 (A) quanto com a EIII recombinante (B). Do mesmo modo, nenhuma detecção intracelular significativa de IFNγ em linfócitos T $CD8^+$ foi observada, tanto após estimulação com o JHA1 (C) quanto com a EIII recombinante (D).

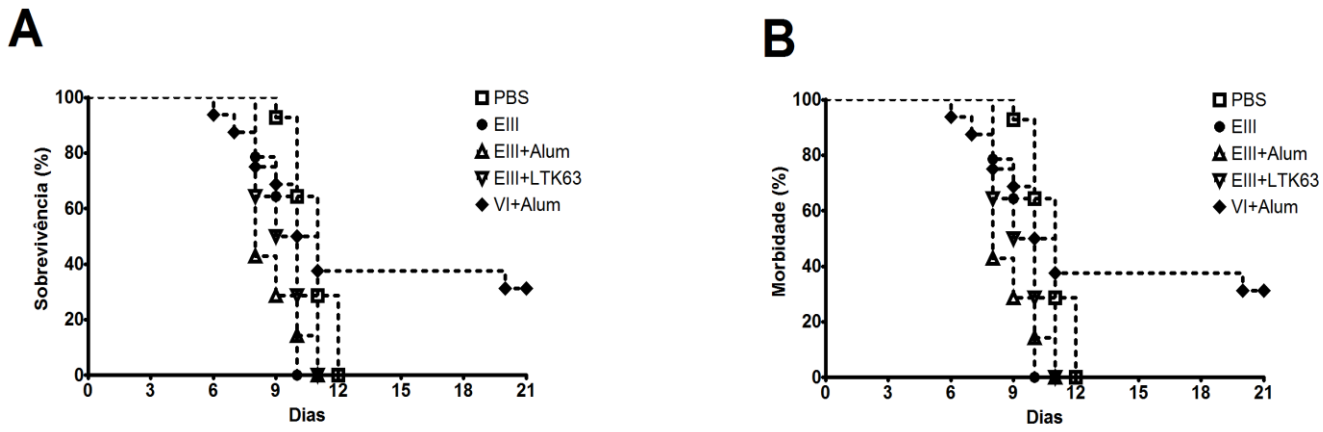
Apesar de as formulações vacinais testadas não induzirem danos nos animais imunizados, em condições de desafio com o JHA1 foi observado que essa condição não se manteve. Animais imunizados com a EIII, contendo ou não adjuvantes, morreram mais rapidamente (a partir do quinto dia pós-infecção) do que os animais injetados com PBS, os quais começaram a morrer a partir do nono dia pós-infecção ($p < 0,05$) (Figura 8A). De forma semelhante, esses mesmos animais apresentaram sinais de morbidade, como paralisia de membros inferiores e perda de peso antes dos animais do grupo controle injetado com PBS ($p < 0,05$) (Figura 8B). O único grupo onde foi observada alguma proteção ao desafio com o vírus foi aquele imunizado com vírus inativado e Alum com 30% de sobrevivência e 30% de proteção quanto à morbidade. Esses resultados indicam que formulações vacinais contendo EIII na forma de proteína não conferem proteção einduzem a aceleração do processo infeccioso nos animais imunizados.

Figura 7 - Marcação bioquímica de enzimas associadas a danos teciduais nos soros dos animais imunizados.



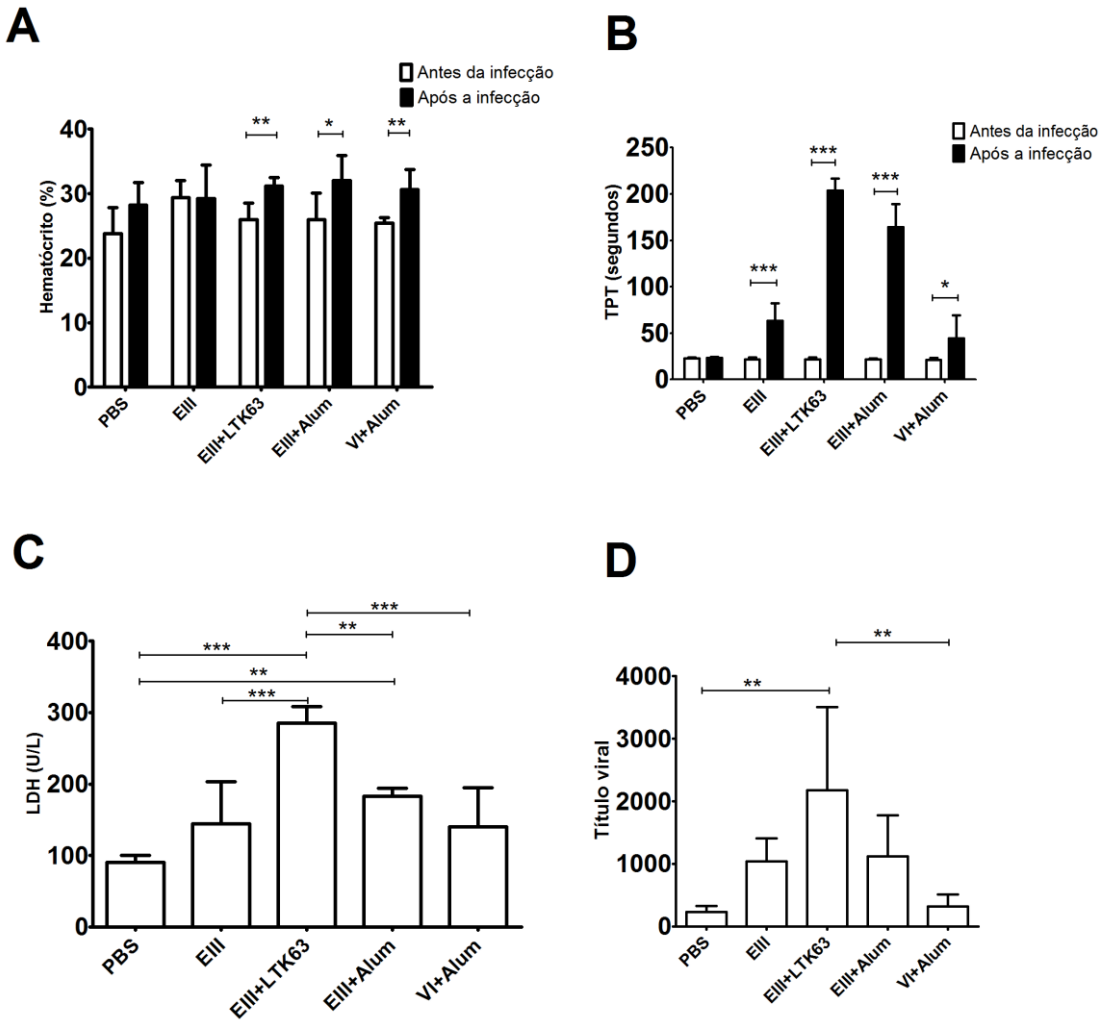
Os níveis de LDH (A), TGO (B) e TGP (C) não foram alterados pela imunização dos animais com as formulações testadas.

Figura 8 - Avaliação da capacidade protetora das formulações vacinais contendo EIII ou vírus inativados.



Após o esquema vacinal, os camundongos foram desafiados pela via i.c. com VD JHA1. Os animais foram monitorados por 21 dias e os dados referentes à sobrevivência (A) e à morbidade (B) foram anotados.

Figura 9 - Avaliação dos danos gerados nos animais imunizados após o desafio com o VD JHA1.



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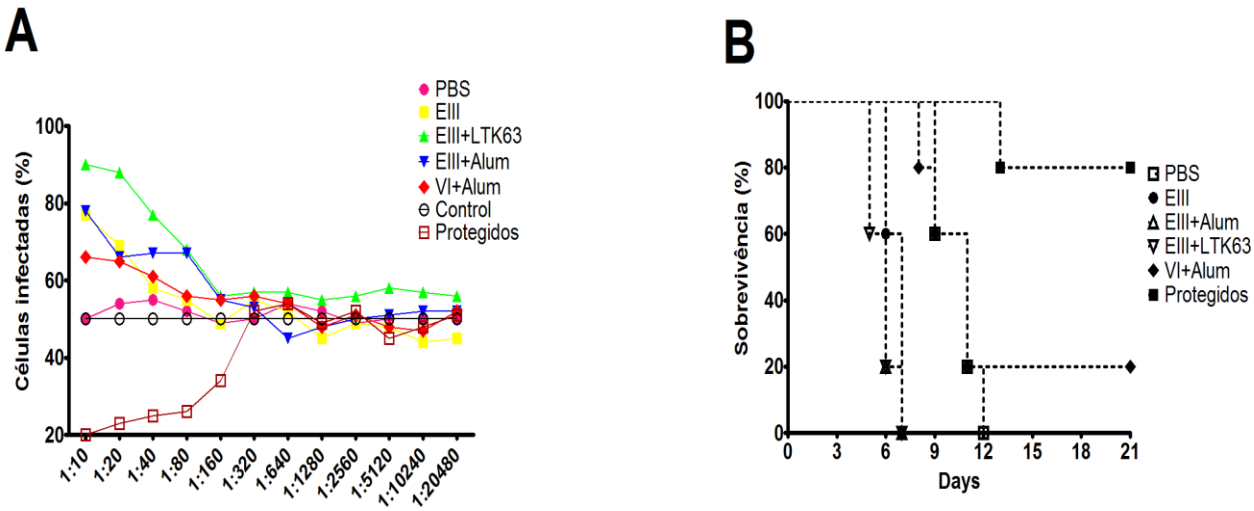
(A) Medidas de hematócrito nos animais 7 dias após a última dose vacinal (antes da infecção) ou 5 dias após o desafio (após a infecção). (B) Medidas do tempo de protrombina (TPT) no plasma dos animais 7 dias após a última dose vacinal (antes da infecção) ou 5 dias após o desafio (após a infecção). (C) Medidas da enzima desidrogenase láctica (LDH) no soro dos animais 5 dias após o desafio. (D) Determinação do título viral no cérebro dos animais imunizados 5 dias após o desafio. As diferenças entre os grupos de imunização para os parâmetros avaliados foram determinadas por ANOVA e posterior teste de comparações múltiplas de Bonferroni, onde * indica p <0,05; **, p <0,01 e ***, p <0,001.

6.3.4 Detecção de danos nos animais imunizados após o desafio com o VD JHA1

A detecção do aparecimento de danos foi realizada nos animais imunizados após o desafio para que a relação entre resposta imunológica e controle ou acentuação de danos fosse estabelecida. Nenhum grupo experimental apresentou alteração no hematócrito após o regime vacinal, mas essa situação foi modificada para aumento significativo nos grupos imunizados com EIII coadministrada com LTK63 e Alum e também para o grupo de animais imunizados com vírus inativado e Alum (Figura 9A). De forma semelhante, o tempo de pró-trombina

também não foi alterado nos animais imunizados 7 dias após a última dose vacinal, mas foi significativamente alterado nos grupos que receberam formulações vacinais contendo EIII ou vírus inativado (Figura 9B). Após o desafio, os níveis de LDH nos animais imunizados com LTK63 também foram alterados significativamente em relação a todos os outros grupos de imunização, enquanto que o grupo imunizado com EIII e Alum apresentou níveis de LDH alterados em relação ao grupo injetado com PBS apenas (Figura 9C). Em concordância com esses resultados, os títulos do VD JHA1 no cérebro dos animais imunizados e desafiados foi significativamente maior no grupo imunizado com EIII e LTK63 em relação ao grupo imunizado com vírus inativado e Alum e também em relação ao grupo injetado com PBS. Coletivamente, esses resultados indicam que a morte precoce após o desafio dos animais imunizados em relação aos animais apenas injetados com PBS foi devida ao aumento na infecção viral, que acelerou o aparecimento de distúrbios hematológicos e danos teciduais.

Figura 10 - Uma resposta estritamente humoral contra a EIII induz um ADE homotípico *in vitro* e *in vivo*.



(A) Teste de neutralização viral *in vitro*, com o soro coletado dos animais imunizados ou desafiados e protegidos em células U937. (B) Teste de neutralização viral *in vivo*, onde animais *naive* foram desafiados com um mistura de soro e vírus previamente incubados a 37 ^{o}C por 1 h e monitorados por 21 dias.

6.3.5 *O AUMENTO DA INFECÇÃO É DEVIDO AO DIRECIONAMENTO DE PARTÍCULAS VIRAIS OPSONIZADAS COM ANTICORPOS ANTI-EIII PARA CÉLULAS EXPRESSANDO RECEPTORES DO TIPO FC*

Para entender porque o processo infeccioso foi acelerado nos animais imunizados, resolvemos realizar ensaios de neutralização *in vitro* com células U937, as quais expressam

receptores Fc. Além disto, realizamos um ensaio de neutralização *in vivo* onde uma mistura de vírus e soro foi injetada em animais para avaliação da capacidade neutralizante desses anticorpos frente à variedade de células existente no animal vivo. Os soros coletados de animais imunizados com a proteína EIII ou com vírus inativados induziram aumento na capacidade de infecção do VD JHA1 nas células U937 (Figura 10A). A exceção foi o soro coletado de animais imunizados com vírus inativado e Alum que sobreviveram ao desafio, o qual foi capaz de neutralizar o vírus até altas diluições (Figura 10A). Esse mesmo soro foi o único capaz de induzir um nível significativo de proteção nos animais desafiados com a mistura soro-vírus (p= 0,002). Esses resultados indicam que, embora uma resposta estritamente humoral contra o EIII induza a geração de anticorpos capazes de neutralizar o VD em ensaios *in vitro* convencionais, esses mesmos anticorpos podem acentuar a infecção de forma semelhante a uma ADE *in vivo* ao direcionar o vírus para receptores Fc presentes em células alvo para a replicação do vírus.

6.4 DISCUSSÃO

Neste trabalho uma questão central no desenvolvimento de vacinas contra a dengue foi estudada: anticorpos contra o domínio III (EIII) da proteína de envelope do vírus dengue (VD) desempenham um papel central na imunidade protetora? Para estudar essa questão, o EIII foi obtido na forma recombinante com estrutura, função biológica e antigenicidade preservadas. A proteína recombinante foi utilizada como antígeno vacinal em diferentes formulações em comparação com uma formulação vacinal contendo o VD inativado coadministrado com Alum como adjuvante. Demonstramos que as formulações vacinais testadas induzem a geração de respostas imunológicas com perfis estritamente humorais, com anticorpos capazes de neutralizar o VD em ensaios *in vitro* conduzidos com células desprovidas de receptores do tipo Fc. Entretanto, demonstramos também que estes mesmos anticorpos são capazes de acentuar a infecção quando os ensaios de neutralização são conduzidos com células que expressam esse tipo de receptor e que, além disso, estes anticorpos acentuam a infecção também na condição *in vivo*.

No estudo desta questão, algumas características importantes que direcionam melhor o estudo devem ser destacadas. Primeiro, o antígeno recombinante obtido preserva estrutura, função e antigenicidade em relação ao antígeno nativo. Isso foi demonstrado inclusive pelo reconhecimento da EIII recombinante em ELISA por anticorpos de animais imunizados com

o vírus inativado. Essa característica garante uma correlação direta entre a resposta gerada contra o antígeno recombinante e a resposta gerada contra o antígeno nativo. Segundo, foi demonstrado que as respostas imunológicas geradas foram predominantemente humorais, com os anticorpos desempenhando papel central na resposta imunológica gerada. Isso permite um estudo focado na pergunta levantada, sem a interferência de uma resposta celular significativa, o que dificultaria as conclusões frente aos testes realizados para avaliar as hipóteses propostas acerca da pergunta central. Entretanto, a utilização de um modelo experimental baseado em uma infecção intracraniana em camundongos para avaliar as respostas imunológicas geradas dificulta o estabelecimento de uma relação mais direta entre o que foi observado neste estudo e o que acontece no hospedeiro humano. Ainda assim, a cepa viral utilizada como modelo de infecção neste estudo é um isolado clínico previamente descrito pelo nosso grupo (AMORIM et al., 2012a), naturalmente capaz de infectar e matar camundongos imunocompetentes, o que traz algumas vantagens no estabelecimento dessa relação frente aos modelos experimentais que utilizam cepas virais atenuadas ou camundongos com receptores de interferon geneticamente anulados.

A principal evidência apresentada nesse estudo, em resposta à pergunta levantada inicialmente, é a de que uma resposta estritamente humoral contra o EIII do VD desencadeia um ADE homotípico quando o animal imunizado entra em contato com o vírus. As respostas imunológica geradas com as formulações vacinais testadas não induzem, por elas mesmas, lesão tecidual nos animais. No entanto, quando esses animais são desafiados, a infecção deixa de seguir seu curso normal e passa a se desenvolver mais rapidamente e com maior gravidade, com distúrbios hematológicos e danos teciduais graves nos animais imunizados. Em concordância com estes achados, a carga viral nos animais imunizados com a EIII e desafiados também é aumentada. Estes dados parecem ter uma relação direta com o título de anticorpos induzidos pela formulação vacinal, pois o grupo de imunização onde os maiores títulos de IgG anti-EIII foram computados (EIII e LTK63) é justamente o grupo onde são observados danos hematológicos e teciduais mais pronunciados, bem como a carga viral mais aumentada.

Foi demonstrado aqui que a explicação para este fenômeno está no direcionamento das partículas virais para células com receptores Fc, as quais, em geral, são os principais alvos para replicação do VD (HALSTEAD, 1981; WHITEHEAD et al., 2007). Até aqui fica claro que anticorpos considerados neutralizantes em ensaios *in vitro* convencionais não são necessariamente protetores *in vivo* e este conceito precisa ser revisto na avaliação de futuros

candidatos vacinais para controle da dengue. Obviamente, a neutralização *in vivo* por anticorpos dirigidos contra o EIII pode requerer "potência" suficiente para saturar todos os domínios EIII disponíveis nas partículas virais circulantes (VAN DER SCHAAR et al., 2009; WILLIAMS et al., 2012), situação que não foi induzida pelas formulações vacinais contendo a EIII como antígeno vacinal testadas neste trabalho. Entretanto, a formulação contendo o vírus inativado como antígeno vacinal conferiu proteção parcial no grupo de animais imunizados. Além disto, o soro dos animais protegidos foi capaz de neutralizar o VD *in vitro* tanto em ensaios conduzidos com células desprovidas de receptores Fc (LLCMK2) quanto em células (U937) que expressam esse tipo de receptor. Esses soros também foram capazes de neutralizar o vírus *in vivo* conferindo 80% de proteção no desafio realizado com a mistura de soro e vírus em animais *naive*. Por terem sido imunizados com o vírus inteiro, embora inativado, a resposta humoral induzida parece ter tido um repertório mais amplo de anticorpos, direcionados contra diferentes epitopos de proteínas vírais importantes no processo infeccioso. O bloqueio do domínio EIII pode impedir o contato do vírus com a célula hospedeira pela via clássica de infecção, dependente de clatrinas, mas se o vírus entrar na célula por uma via alternativa, como no caso da ligação de vírus opsonizados a receptores Fc, outros mecanismos podem ser necessários para que a infecção não seja completada (DA SILVA VOORHAM et al., 2012). Neste caso, se o peptídeo de fusão, contido no domínio II também não for bloqueado, o vírus pode fundir seu envelope à membrana do endossomo e completar seu ciclo infeccioso (VAN DER SCHAAR et al., 2009). Sendo assim, um repertório de anticorpos mais abrangente capaz de se ligar a diferentes domínios estruturais envolvidos nas etapas de ligação e penetração do ciclo viral parece ser mais efetiva na neutralização propriamente dita do VD. A partir deste raciocínio é possível concluir que uma resposta humoral focada no EIII parece não ser a melhor alternativa para uma proteção *in vivo* contra o VD.

Pelas evidências apresentadas e discutidas até aqui, a geração de uma resposta imunológica com anticorpos considerados neutralizantes não é um correlato de proteção confiável. No entanto, para as condições experimentais apresentadas neste trabalho, como explicar uma maior carga viral e maiores danos hematológicos causados por uma mesma dose viral inoculada como desafio? A resposta pode estar novamente relacionada ao título de anticorpos induzido pela imunização. Mesmo com a quantidade de partículas virais viáveis normalizada, quantidades diferentes de anticorpos anti-EIII, os quais demonstramos aqui que

não são necessariamente neutralizantes, podem ter efeitos diferentes sobre partículas virais imaturas ou inativas, as quais compõem a maior parte das populações de vírus de animais (VAN DER SCHAAR et al., 2009). Essas partículas virais não contribuem de forma significativa numa infecção primária, justamente por não serem capazes de se replicar. Entretanto, numa infecção secundária ou no caso de indivíduos imunizados, onde estão circulantes anticorpos anti-VD, essas partículas virais podem se tornar infectantes caso o repertório de anticorpos presentes não seja realmente capaz de neutralizá-las (DA SILVA VOORHAM et al., 2012; VAN DER SCHAAR et al., 2009). Esses anticorpos podem direcionar partículas virais de VD para infectar células pela via alternativa utilizando receptores Fc. Entende-se assim que quanto mais anticorpos não neutralizantes circulantes, mais partículas virais consideradas imaturas serão capazes de completar o ciclo infeccioso, resultando numa maior carga viral, assim como foi observado neste trabalho.

Em conclusão, o presente estudo demonstra, pela primeira vez, que o principal correlato de proteção para VD mais amplamente aceito e citado na literatura atual não é confiável. O conceito de neutralização para VD aceito até hoje é limitado ao contemplar apenas uma alternativa de ligação viral à célula hospedeira, deixando de fora vias alternativas de ligação e, mais ainda, outras etapas do ciclo de replicação, como a penetração, que também podem ser bloqueadas por anticorpos e que parecem ser tão ou mais importantes do que a ligação sob o ponto de vista de impedimento da infecção. Além disso, outras formas de neutralização viral dependentes de anticorpos, incluindo a virólise causada pela ação conjunta de anticorpos e sistema complemento também não são geralmente consideradas como correlato de proteção. As contribuições trazidas neste trabalho são as primeiras evidências de que o processo de desenvolvimento de vacinas contra a dengue deve ser revisto, começando pela reconsideração do principal correlato de proteção para a doença, para a qual a resposta imunológica protetora parece ser bastante dinâmica e complexa.

7 CONSIDERAÇÕES FINAIS

Como conclusões dos resultados obtidos até o momento podemos destacar os seguintes pontos: (i) obtivemos a proteína NS1 do vírus dengue produzida em sistema de expressão procarioto com a maior conservação de antigenicidade já descrita, o que torna esse reagente um instrumento valioso não somente em estudos vacinais, mas também em estudos de diagnóstico e sobre a patogênese viral; (ii) o antígeno NS1 obtido mostra-se como um promissor candidato vacinal e poderá compor uma vacina eficiente e segura contra a dengue baseada em proteínas recombinantes; (iii) um modelo de infecção capaz de reproduzir parte dos sintomas obervados em humanos com formas graves da doença foi desenvolvido a partir do uso de camundongos selvagens e de um isolado clínico do vírus dengue. Isto traz à tona a ideia de que a própria variabilidade genética natural deste vírus pode fornecer à ciência cepas capazes de reproduzir, em camundongos, pelo menos parte dos sintomas observados em humanos, sem a necessidade de qualquer adaptação do vírus; (iv)a proteína EIII não se mostrou um bom candidato vacinal apesar de preservar todos os atributos da proteína viral nativa; (v) os resultados obtidos indicam que o principal correlato de proteção na dengue, o qual é baseado na neutralização do domínio EIII por anticorpos, precisa ser revisto e corrigido.

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