Carina Buzzo de Lima

**Avaliação dos mecanismos moleculares
envolvidos na expressão de iNOS mediada pelo
eixo NAIP5/NLRC4-Caspase-1**

Tese apresentada ao Programa de Pós-Graduação em Imunologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, para a obtenção do Título de Doutor em Ciências.

São Paulo
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Área de concentração: Imunologia

Orientador: Profa. Dra. Karina Ramalho Bortoluci

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Candidato(a): Carina Buzzo de Lima.

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

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São Paulo, 27 de julho de 2010.

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"Papel dos receptores do tipo Toll e do tipo NOD-LRR na ativação metabólica e funcional de macrófagos"

Prezado Professor,

Informo que a sua licença para uso de animais em experimentação, constante no protocolo em epígrafe, **foi prorrogada até 06.12.2013.**

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. NIELS OLSEN SARAIVA CÂMARA
Vice-Coordenador da Comissão de Ética
no Uso de Animal - ICB /USP

Ilma.Sra.

Profa. Dra. KARINA RAMALHO BORTOLUCI

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CERTIFICADO

Certificamos que o protocolo registrado sob nº **109** nas fls. **51** do livro **2** para uso de animais em experimentação, sob a responsabilidade de **Karina Bastos** Coordenador(a) da Linha de pesquisa **"Papel dos receptores do tipo Toll e do tipo NOD-LRR na ativação metabólica e funcional de macrófagos"** do qual participou(aram) o(s) alunos **Carina Buzzo de Lima, Alexandra dos Anjos Cassado e o pesquisador Momtchilo Russo** está de acordo com os Princípios Éticos de Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela **COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL (CEEA)** em **06.12.2007**.

São Paulo, 11 de dezembro de 2007.

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

Profa. Dra. PATRÍCIA CASTELUCCI
Secretária
CEEA - ICB/USP

Validade: 11.12.2007 à 11.12.2010.

“Uma vida sem desafios não vale a pena ser vivida...”

Sócrates

Dedico esta tese aos meus pais, Luis Alberto e Marilda e as minhas irmãs e melhores amigas, Caren e Camila. Vocês são minha maior RIQUEZA, meu incentivo de aprendizado diário, obrigada por tudo!!!

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RESUMO

Buzzo CL. Avaliação dos mecanismos moleculares envolvidos na expressão de iNOS mediada pelo eixo NAIP5/NLRC4-Caspase-1. [tese (Doutorado em Imunologia)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2013.

A flagelina é um potente indutor da resposta imune, sendo seu reconhecimento compartilhado pelo receptor transmembrânico TLR5 e citosólico NAIP5/NLRC4. O TLR5 induz a ativação de genes inflamatórios de maneira dependente de *MyD88* enquanto NAIP5/NLRC4 formam um complexo multiproteico chamado inflamassoma, cuja ativação culmina no recrutamento e ativação da caspase-1 resultando na secreção de IL-1 β e IL-18 e piroptose. Embora ambos, TLR5 e NAIP5/NLRC4 cooperam para controlar infecções, pouco se sabe sobre os mecanismos efetores individuais induzidos a partir da ativação de cada um dos receptores. No intuito de avaliar as consequências do reconhecimento extra e intracelular da flagelina por macrófagos, nós demonstramos que somente a estimulação com a flagelina em sua forma livre (FLA-BS) foi capaz de induzir a produção de IL-6, como indicativo da ativação extracelular do TLR5. Por outro lado, a estimulação dos macrófagos com a flagelina inserida em vesículas lipídicas catiônicas (DOTAP) (FLA-BSDot) que permitem a entrega deste agonista no citosol celular, induziu apenas a produção de IL-1 β e morte celular, condizente com a ativação da caspase-1. Curiosamente, além da produção de IL-1 β , o estímulo com FLA-BSDot também foi capaz de induzir a expressão de iNOS, enzima responsável pela produção do óxido nítrico (NO), até então relacionada à cascata de ativação dos receptores TLRs. A expressão de iNOS em resposta à FLA-BSDot foi dependente do inflamassoma NAIP5/NLRC4 e da protease caspase-1 e independente das citocinas IL-1 β e IL-18 e da molécula adaptadora *MyD88*, descartando a via de ativação mediada pelos TLRs. Neste trabalho, investigamos os mecanismos moleculares envolvidos na expressão de iNOS mediada por caspase-1 em resposta à FLA-BSDot e verificamos o envolvimento do NF- κ B, mas não do IRF-1, nesta via, uma vez que a expressão de iNOS foi abolida na presença do inibidor da degradação do I κ B- α , PDTC. Inesperadamente, apesar da caspase-1 ser essencial na ativação de iNOS em resposta à FLA-BSDot, este estímulo induziu a ativação do NF- κ B em macrófagos caspase-1^{-/-}, mostrando que a flagelina citosólica induz a ativação deste fator de transcrição de maneira independente do inflamassoma. No entanto, verificamos o envolvimento da clivagem da enzima multifuncional associada à cromatina, poly(ADP-ribose)polymerase-1 (PARP-1), na expressão de iNOS mediada por caspase-1. Finalmente, esta via parece ter um papel importante no controle das infecções por *Legionella pneumophila* e *Salmonella Typhimurium*. Juntos, esses dados definem um mecanismo efetor adicional mediado por caspase-1 no controle de patógenos e fornecem um maior entendimento com relação às vias de sinalização induzidas a partir da ativação do inflamassoma NAIP5/NLRC4.

Palavras-chave: Inflamassomas. Caspase-1. iNOS. NF- κ B. PARP-1.

ABSTRACT

Buzzo CL. Evaluation of the molecular mechanisms involved in the iNOS expression by NAIP5/NLRC4-Caspase-1 axis. [Ph. D thesis (Immunology)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2013.

Flagellin is a potent inducer of the immune response, being its recognition shared by transmembrane TLR5 and cytosolic NAIP5/NLRC4 receptors. TLR5 induces the activation of inflammatory genes through *MyD88* pathway, whereas NAIP5/NLRC4 assembles multiprotein complexes called inflammasomes, culminating in caspase-1 activation, IL-1 β and IL-18 release and pyroptosis. Although both TLR5 and NAIP5/NLRC4 pathways cooperate to clear infections, little is known about the relative anti-pathogen effector mechanisms operating through each of them. In order to evaluate the consequences of the extra and intracellular recognition of flagellin by macrophages, we have demonstrated that only free flagellin stimulation (FLA-BS) was able to induce IL-6 production, as indicative of TLR5 extracellular activation. By the contrast, the stimulation of flagellin inserted in cationic lipid vesicle (DOTAP) (FLA-BSDot) that allows the delivery of flagellin to the cell cytosol induced only IL-1 β production and cell death, consistent with caspase-1 activation. Curiously, besides IL-1 β production, FLA-BSDot stimulation was also able to induce iNOS expression, an enzyme responsible to the nitric oxide (NO) production, previously associated with TLRs pathway. FLA-BSDot-induced iNOS expression was dependent of NAIP5/NLRC4 inflammasome and of the protease caspase-1 and independent of the IL-1 β and IL-18 cytokines and *MyD88* adaptor molecule, discarding the involvement of TLRs-induced signaling pathway. Extending our findings, here we have found that NF- κ B, but not IRF-1, was required for caspase-1-mediated iNOS activation in response to cytosolic flagellin, since iNOS activation was abrogated in the presence of a selective I κ B- α degradation inhibitor, PDTC. Unexpectedly, although the essential role of caspase-1 in the FLA-BSDot-induced iNOS activation, cytosolic flagellin also induced NF- κ B activation in caspase-1^{-/-} macrophages, indicating that its activation occurs in an inflammasome independent manner. However, we have found that caspase-1-mediated iNOS activation involves poly(ADP-ribose)polymerase-1 (PARP-1) cleavage, a nuclear chromatin-associated multifunctional enzyme. Finally, this pathway seems to be an important role in the control of *Legionella pneumophila* e *Salmonella Typhimurium*. Together, our data define an additional anti-pathogen effector mechanism mediated by caspase-1 in the control of pathogens and provide a better understanding regarding the signaling pathways induced upon NAIP5/NLRC4 inflammasome activation.

Keywords: Inflammasome. Caspase-1. iNOS. NF- κ B. PARP-1.

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1 INTRODUÇÃO

1.1 Os macrófagos e os Receptores da Imunidade Inata: TLRs e NLRs

As células da imunidade inata são fundamentais para o início e desenvolvimento de uma resposta imune a partir do reconhecimento de agentes possivelmente nocivos ao organismo. Nesse sentido, os macrófagos são considerados sentinelas imunológicas, pois se encontram distribuídos em diferentes tecidos, onde possuem a habilidade de reconhecer padrões moleculares que estão frequentemente associados à patógenos (*Pathogen associated molecular pattern* – PAMP) e que são distintos dos padrões apresentados por células próprias (1). Este reconhecimento ocorre através de uma variedade de receptores para reconhecimento de padrões (“*Pattern Recognition Receptors*” - PRR) que estão distribuídos em diferentes compartimentos celulares e são responsáveis por diversos mecanismos. Nesse contexto, a descoberta de duas grandes famílias de PRR, a família dos receptores do tipo *Toll* (“*Toll-like Receptor*”s- TLR) e, mais recentemente, os receptores do tipo NOD-LRR (“*Nucleotide-binding oligomerization domain – Leucin rich repeats*”) ou NLR, foi de fundamental importância para o entendimento dos mecanismos de reconhecimento imune inato e suas consequências para a resposta imune (Figura 1).

Figura 1 – Esquema dos receptores transmembrânicos TLR e citosólicos NLR.

A proteína Toll foi descrita em 1985 e foi relacionada à polarização dorso-ventral da mosca *Drosophila melanogaster*, durante sua embriogênese (2). Mais de dez anos depois, os

Tolls foram identificados como essenciais durante a resposta imune da *Drosophila*, uma vez que os insetos deficientes nesta proteína apresentavam alta incidência de infecções fúngicas (3). Em 1997, o homólogo de Toll em humanos, o TLR4, foi clonado por Dr. Ruslan Medzhitov e o falecido Dr. Charles Janeway, achado este que revolucionou o conhecimento dos mecanismos de reconhecimento do sistema imune inato (4). Um ano mais tarde, a descoberta do LPS, lipopolissacarídeos bacterianos presentes em bactérias Gram negativas, como ligante do TLR4, rendeu o prêmio Nobel em Medicina ao Dr. Bruce Beutler, em 2011 (5), o qual foi compartilhado com o Dr. Hoffman por seus achados a respeito do papel do Toll em *Drosophila*.

Os TLRs são glicoproteínas de membrana que possuem dois domínios; o domínio externo N-terminal, que consiste de, aproximadamente 16 - 18 seqüências ricas em leucina (LRR) ("*Leucine-rich repeat*"), sendo cada LRR composta de 20 - 30 aminoácidos; e o domínio intracelular C-terminal, chamado TIR, o qual mostra grande homologia com o domínio intracelular do receptor para IL-1 (IL-1R), (domínio Toll/IL-1R) (6).

Os TLRs são distribuídos diferencialmente, podendo estar acoplados à membrana plasmática, como TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 (expressos somente em humanos) e, TLR11, TLR12 e TLR13 (expressos somente em camundongos) ou associados às vesículas endossomais e retículo endoplasmático, como TLR3, TLR7, TLR8 e TLR9, e podem reconhecer diversos PAMPs provenientes de bactérias, vírus, protozoários e fungos.

Os ligantes melhor caracterizados são peptidoglicanas e lipoproteínas bacterianas, âncoras de GPI (glicofosfatidil inositol) presentes em protozoários e zimozan presente em fungos (ligantes de TLR2), RNA dupla-fita, comuns em vírus (ligante de TLR3), lipopolissacarídeos (LPS) presentes na parede de bactérias gram-negativas (ligante do TLR4), flagelina presente em bactérias móveis (ligante de TLR5) e seqüências de DNA ricas em CpG não metilados, presentes em bactérias e vírus (ligante de TLR9) (7).

A ativação dos TLRs por seus ligantes leva à dimerização do receptor e ao recrutamento de proteínas adaptadoras específicas como MyD88 ("*Myeloid differentiation primary-response protein 88*"), MAL/TIRAP ("*MyD88-adaptor like/TIR-associated protein*"), TRIF ("*Toll-receptor-associated activator of interferon*"), TRAM ("*Toll-receptor-associated molecule*") ou SARM ("*Sterile α - and armadillo-motif containing protein*") (8), que irão transduzir o sinal do TIR, ativando quinases e fatores de transcrição como NF- κ B ("*Nuclear factor kappa enhancer binding protein*") e IRFs ("*IFN-responsive factors*") (6). Além disso, os TLRs sinergizam com outros receptores da imunidade inata, sendo esta interação importante para a condução da resposta imune adaptativa (7).

A ativação do NF- κ B é rapidamente induzida em vários tipos celulares, em resposta à citocinas pró-inflamatórias e a produtos microbianos e infecções virais. Os mamíferos expressam cinco proteínas Rel (NF- κ B) que pertencem a duas classes. A primeira classe inclui RelA (p65), c-Rel (p50) e RelB, proteínas que são sintetizadas como produtos maduros e não requerem processamento proteolítico. O segundo grupo é codificado pelos genes *Nfkb1* e *Nfkb2*, cujos produtos são inicialmente sintetizados como grandes precursores, p100 e p105, respectivamente, e requerem processamento proteolítico, induzindo a forma madura das proteínas p50 e p52 (9).

Os dímeros contendo RelA ou c-Rel do NF- κ B estão no citoplasma através de interações com inibidores específicos, que formam o complexo dos I κ Bs. Esses inibidores sofrem rápida degradação, dependente de ubiquitina, após exposição a uma variedade de agonistas, os quais ativam o complexo dos IKKs. Esse complexo é composto por duas subunidades catalíticas, IKK α e IKK β , as quais podem fosforilar diretamente os I κ Bs, e uma subunidade reguladora, IKK γ (também chamado de NEMO), a qual a integridade é requerida para a ativação desta via (9).

A ativação do NF- κ B é representada por duas vias principais, sendo a via canônica, baseada na degradação de I κ B, principalmente por IKK β e, essencial para imunidade inata (10) e a via não canônica, a qual induz degradação de NF- κ B2 p100 por IKK α , e está envolvida no desenvolvimento de órgãos linfóides e na imunidade adaptativa (Figura 2) (9). Uma vez no núcleo, os dímeros são sujeitos à regulação, principalmente através da fosforilação de proteínas Rel, a qual é requerida para a indução completa de genes alvos de NF- κ B (11). Desta forma, a transcrição induzida por NF- κ B representa um evento central chave na defesa do hospedeiro e nas respostas inflamatórias, com geração de moléculas efetoras envolvidas na capacidade microbicida dessas células, na condução e resolução da resposta inflamatória e no desenvolvimento da resposta imune adaptativa (12). Essas moléculas são produtos de genes pró-inflamatórios (como citocinas inflamatórias e quimiocinas), ou provenientes do metabolismo lipídico (leucotrienos e prostaglandinas) e bioquímico dessas células (enzimas como NO-sintase induzida (iNOS), NADPH-oxidase, mieloperoxidase (MPO), hemeoxigenase-1 (HO-1) e indoleamina dioxigenase (IDO)).

Figura 2 – Esquema das vias de sinalização do NF-κB (Fonte: <http://oncosurg.com/?p=1518>).

Além dessas funções, o NF-κB também é conhecido por inibir o processo de morte celular programada, denominado apoptose (13-16). A atividade anti apoptótica do NF-κB depende da indução de genes, cujos produtos, tais como os inibidores celulares de apoptose (c-IAPs), caspase-8 - c-FLIP (“FLICE *inhibitory protein*”), A1 (também chamado de Bfl1), “TNFR-associated factor 1” (TRAF1) e TRAF2 são capazes de inibir a apoptose. As proteínas anti apoptóticas induzidas por NF-κB melhor caracterizadas são as IAPs, as quais podem se ligar e inibir diretamente caspases efetoras, tais como caspase-3 e caspase-7, tão bem quanto previnem ativação da pro-caspase-6 e pro-caspase-9 (17). Assim as proteínas IAPs podem inibir a apoptose induzida por ambas as vias, de receptores de morte e a via dependente da mitocôndria.

Além dos TLRs, outras duas grandes famílias de receptores recentemente descritas têm como característica peculiar a localização no citosol celular, a família dos NLRs (“*NOD-like receptors*”) e a família dos RLRs (“*RIG-like receptors*”). Enquanto a família dos RLRs possui apenas três membros, RIG-I, MDA5 e LGP2, os quais reconhecem RNA viral (18), acredita-se que a família dos NLRs possui mais de vinte membros, os quais reconhecem vários PAMPs e também estresse celular (19).

Os receptores NLRs possuem três domínios, sendo a região efetora variável N-terminal, podendo conter domínios do tipo CARD (“*Caspase activation and recruitment domain*”), PYD (“*PYD – pyrin domain*”) ou BIR (“*Baculovirus IAP repeat*”); um domínio

intermediário requerido para ligação de nucleotídeos e auto oligomerização chamado NACHT e a região C-terminal formada por um domínio LRR que parece ser responsável pelo reconhecimento dos PAMPs.

De acordo com os domínios N-terminais variáveis, os NLRs podem ser classificados como NLRBs (os quais contêm o domínio BIR), NLRCs (os quais contêm o domínio CARD) e NLRPs (os quais contêm o domínio PYD) (20) (Figura 3). Esses domínios são fundamentais para a indução de respostas imune específicas, as quais ocorrem através de interações proteína-proteína. O domínio CARD presente em alguns membros da família permite o recrutamento e ativação da caspase-1 e da serina-treonina quinase RIP2, também chamada de RICK (“*Receptor-interacting caspase-like apoptosis regulatory kinase*”) (21-23). No entanto, os NLRs que possuem domínios PYD ou BIR ao invés de domínios CARD, e, portanto, necessitam interagir com outras moléculas para induzir a ativação da caspase-1.

Figura 3: Esquema das proteínas da família dos receptores NLR (Fonte- <http://www.invivogen.com/review-nlr>).

A ativação dos NLRs ocorre a partir do reconhecimento de PAMPs, o qual induz a oligomerização da molécula, através do domínio NATCH, permitindo a aproximação das porções N-terminais e, então o recrutamento de moléculas adaptadoras, quando necessário, para a ativação (23, 24). Dentre os membros da família dos NLRs, o primeiro receptor identificado foi o NOD1 (também chamado de NLRC1), como uma proteína homóloga ao APAF, um conhecido regulador da apoptose, sendo o NOD1 responsável pela ativação de genes dependentes de NF- κ B e indução de apoptose (25). Dois anos depois, o mesmo grupo descreveu o NOD2 (ou NLRC2), outro membro desta família, mas cuja presença está restrita

a células de origem mielóides (26). Apesar da descoberta desses receptores, os primeiros ligantes de origem bacteriana foram descritos posteriormente. Sabe-se que NOD1 e NOD2 reconhecem muropeptídeos derivados do metabolismo de peptídeoglicana, um dos principais componentes da parede celular de bactérias Gram-positivas e Gram-negativas. Enquanto NOD2 reconhece muramildipeptídeo (MDP), presentes em todos os tipos de peptídeoglicanos (27), NOD1 detecta a presença de meso-DAP ou iE-DAP (“ γ -D-glutamyl-meso-diaminopimelic acid”), um aminoácido característico de muitas bactérias, tais como *Listeria monocytogenes* e *Bacillus SSP* (28). Mais recentemente, alguns trabalhos sugerem que NOD2 responde a RNA citosólico durante infecções virais (29-31). No entanto, a interação de NOD2 com MDP ou RNA ainda necessita ser elucidada.

Após ativação, NOD1 e NOD2 iniciam uma resposta pró-inflamatória dependente da ativação de NF- κ B (25), através do recrutamento de RIP2, a partir de interações CARD-CARD, essencial para a ativação deste fator de transcrição. Dessa maneira, após o reconhecimento de PAMPs no citosol, estes receptores iniciam uma cascata de sinalização intracelular que culmina na transcrição de inúmeros genes pró-inflamatórios de maneira semelhante à resposta dos TLRs.

Porém, outros membros da família dos NLRs estão envolvidos na ativação de caspases inflamatórias como a protease caspase-1 e a caspase-11. Esses membros dos NLRs formam plataformas complexas multiproteicas após o reconhecimento de PAMPs e ativam seus efetores através de auto-ativação induzida por aproximação. Em 2002, o primeiro complexo multiproteico que ativa caspases, formado por membros dos receptores NLRs foi descrito pelo grupo do falecido Dr. Jürg Tschopp e foi chamado de inflamassoma (32), o que se refere à inflamação, e é semelhante ao complexo que ativa a caspase-9 durante o processo de apoptose, conhecido como apoptossoma (Figura 4).

Figura 4: Representação esquemática do complexo inflamassoma (20).

1.2 Estrutura molecular dos Inflamassomas

Dentre os inflamassomas, podemos citar NLRP1, NLRP3 (também chamados de Nalp1 e Nalp3), NAIP5, NLRC4 (também chamado de Ipaf) e AIM2. AIM2, apesar de pertencer à família das proteínas PYHIN e não à família dos NLRs, também é capaz de formar o complexo inflamassoma e ativar a caspase-1 (33). Uma vez que os inflamassomas NLRP3 e AIM2 possuem o domínio N-terminal do tipo PYD, eles não conseguem ativar diretamente a caspase-1, sendo necessário recrutar a molécula adaptadora ASC, via seu domínio PYD. ASC, por sua vez, contém um domínio C-terminal recrutador e ativador de caspases (CARD) que se liga e recruta a pró-caspase-1, via interações CARD-CARD (34). Em contraste, os inflamassomas NLRP1 e NLRC4 possuem o domínio N-terminal do tipo CARD, podendo recrutar ASC ou interagir diretamente com a pró-caspase-1, via interações CARD-CARD (35). Desta forma, o complexo inflamassoma pode ou não conter ASC. Finalmente, o receptor NAIP5 difere dos demais, uma vez que sua porção N-terminal é composta por domínios do tipo BIR, ao invés de domínios CARD ou PYD. A interação de NAIP5 com outras moléculas na indução da caspase-1 será detalhada a seguir.

A via convencional de ativação dos inflamassomas culmina no recrutamento e na clivagem proteolítica da caspase-1. A caspase-1 é uma cisteíno-protease, expressa no citosol como um zimógeno inativo de 45 kDa e a associação da pró-caspase-1 com o complexo inflamassoma permite o seu processamento e ativação (34, 36). Assim, após o recrutamento para o interior da estrutura do inflamassoma, a pró-caspase-1 sofre auto-ativação por clivagem proteolítica tornando-se um heterodímero enzimaticamente ativo, composto por duas subunidades, p10 (10 kDa) e p20 (20 kDa). Uma vez ativa, a caspase-1 medeia o processamento e ativação das citocinas pró-inflamatórias IL-1 β e IL-18, que são geradas no citosol na forma de precursores inativos, (37) e foi inicialmente chamada de enzima conversora de IL-1 β (*IL-1-Converting Enzyme-ICE*) (38). Hoje, sabe-se que além das citocinas IL-1 β e IL-18, outros substratos da caspase-1 são conhecidos, como os precursores IL-33 e IL-1F7 (39).

Além do processamento e maturação destas citocinas, a ativação de caspase-1 e caspase-11 como resultado do engajamento da ativação dos inflamassomas tem sido relacionada com um processo de morte celular induzida por infecção, denominado piroptose (40, 41). O termo piroptose, do grego “pyro”, significando fogo ou febre, descreve um processo de morte celular altamente regulado e pró-inflamatório. Esta via pró-inflamatória de

morte celular é caracterizada por perda da integridade e posterior ruptura da membrana plasmática, levando à rápida lise celular com liberação do conteúdo intracelular pró-inflamatório, que, juntamente com a liberação das citocinas IL-1 β e IL-18 são responsáveis pela grande resposta inflamatória que acompanha este processo (40-42).

Como dito anteriormente, o complexo inflamassoma pode ou não conter ASC, uma vez que o NLRC4 possui domínios CARD, os quais podem recrutar diretamente a caspase-1 (35). Nesse sentido, Broz e colaboradores mostraram que a morte do macrófago e o processamento das citocinas IL-1 β e IL-18 em resposta a produtos microbianos, podem ser eventos separados, mediados por dois inflamassomas distintos. Os inflamassomas contendo NLRC4 que recrutam ASC induzem a clivagem proteolítica da caspase-1 com eficiente processamento de IL-1 β e IL-18. Ao contrário, os inflamassomas que não recrutam ASC, ativam a caspase-1, mas não induzem sua clivagem proteolítica, o que resulta em uma rápida morte celular por piroptose. De fato, a piroptose é acelerada na ausência de ASC, sugerindo um papel inibitório para este adaptador (43).

Sendo assim, a forma como a caspase-1 é processada determina os eventos celulares, e mostra como dois inflamassomas funcional e espacialmente distintos controlam infecções, apesar de os mecanismos envolvidos na ativação diferencial das duas vias não estarem completamente elucidados.

Apesar da piroptose se apresentar como uma forma de morte celular programada e molecularmente regulada como a apoptose, os mecanismos moleculares envolvidos em seu controle e, especialmente, suas consequências para a resposta imune, são distintos daquelas observadas para a apoptose, a qual inibe ativamente a inflamação. Enquanto as características de células apoptóticas são definidas por um grupo de caspases, tais como caspases-3, 6, 7, 8 e 9, as caspases-1 e 11 são responsáveis pelas mudanças morfológicas observadas durante a piroptose. Camundongos deficientes em caspase-1 sofrem apoptose e se desenvolvem normalmente, mostrando que a caspase-1 não participa da apoptose (44). Da mesma maneira, as caspases apoptóticas não estão envolvidas na piroptose e os seus substratos não sofrem proteólise durante a piroptose (40, 41).

1.3 Ativação dos Inflamassomas

A importância da ativação dos inflamassomas vem sendo cada vez mais estudada durante infecções bacterianas, fúngicas, virais e por protozoários, ainda que pouco se saiba a respeito dos seus agonistas e os mecanismos de ação.

Sabe-se que o NLRP1 reconhece a toxina letal anthrax (LT) (45), principal fator de virulência da *Bacillus anthracis*. Uma vez no citosol, a LT rapidamente cliva e inativa as vias das MAP kinases (MAPKKs), interferindo nas respostas do hospedeiro, incluindo quimiotaxia de neutrófilos, produção de citocinas e quimiocinas, apresentação de antígenos e co-estimulação (46). Além da interrupção da sinalização das MAPKKs, a LT também induz piroptose dos macrófagos (47, 48), sendo esta capacidade de resposta geneticamente mapeada ao gene do *Nlrp1*.

O mecanismo de ativação de NLRP1 não está completamente elucidado. A ativação de NLRP1 requer atividade proteolítica da LT e não apenas o reconhecimento da toxina sozinha, uma vez que a LT cataliticamente inativa não induz piroptose (48, 49). Contudo, não se sabe se a LT é capaz de clivar o NLRP1 diretamente e se esta clivagem é suficiente para sua ativação. Neste sentido, é possível que outros substratos da LT participem deste processo, como sugerido por alguns grupos, uma vez que a atividade do proteassoma parece ser requerida para a ativação de NLRP1 mas não de NLRP3 ou NLRC4 (48, 50, 51).

Diferente da maioria dos inflamassomas, o inflamassoma AIM2 requer um “priming” transcricional, induzido pela ativação do receptor de IFN-I. Esta via de ativação foi descrita a partir de estudos que mostraram a ativação da caspase-1 (52) em resposta à infecção por *Francisella tularensis*, a qual requereu a sinalização do receptor de IFN-I, mas não de outras vias conhecidas dos inflamassomas. Com base na ativação da caspase-1, os autores postularam a existência de um inflamassoma que poderia reconhecer DNA de dupla fita, liberado no citosol celular durante infecções (53). Após um ano, estas duas observações foram unificadas pela identificação do inflamassoma AIM2, que detecta DNA liberado no citosol celular após lise de vários patógenos, incluindo *F. tularensis* (33, 54-57).

A geração do camundongo AIM2^{-/-} confirmou que o AIM2 é ativado em resposta à *F. tularensis* (57-59), *L. monocitogenes* (58, 60-63) e *B. abortus* (64). Recentemente, foi sugerido que a *L. pneumophila*, deficiente nas proteínas do sistema de secreção também pode ativar o AIM2, via liberação de DNA no citosol de células hospedeiras (65). Importante, o AIM2 é o único inflamassoma pelo qual a interação direta do ligante ao receptor foi visualizada em células infectadas (57, 59), ou analisadas por cristalografia (66).

Assim como o AIM2, o inflamassoma NLRP3 também requer dois sinais: (a) indução transcricional do NLRP3 mediado pela via do NF-κB, induzida por ligantes de TLRs e

NODs ou citocinas inflamatórias e (b) um agonista para induzir sua oligomerização. Uma vez primado, o inflamassoma NLRP3 é ativado por estímulos, pelo menos *in vitro*, que incluem, mas não se limitam a materiais cristalinos ou particulados (cristais de ácido úrico, abestos, alum) (67, 68), sinalização de ATP através do receptor P2X₇ (69), infecções virais (70), produtos de fungos (71), RNA e DNA bacterianos (53, 72, 73), numerosas toxinas bacterianas e proteínas efetoras e protozoários (74, 75).

Embora estruturalmente distintos, muitos produtos bacterianos que ativam o NLRP3 (nigericina, listeriolisina O, hemolisinas) compartilham a atividade de formação de poros na membrana, sugerindo que este “padrão de patogênese” (76) é, de alguma maneira detectado pelo inflamassoma NLRP3. Desta forma, dado a variedade de agonistas do NLRP3, postula-se que estes agonistas não ajam diretamente como ligantes deste receptor, mas ao invés disso eles induzem distúrbios nas células, os quais são sentidos pelo inflamassoma NLRP3.

Ainda neste contexto, processos como danos lisossomais, liberação de intermediários reativos do oxigênio (*ROS - Reactive oxygen species*) e efluxo de potássio tem sido relacionados à ativação do NLRP3. No entanto, uma vez que estas vias comuns podem resultar na perda da função do ribossomo por inibir a síntese protéica, a ativação do NLRP3 por estes estímulos tem sido discutida na literatura (77).

Há ainda, outros membros da família dos NLRs que são capazes de ativar a caspase-1, porém necessitam ser melhor caracterizados. O NLRP12 foi demonstrado ativar a caspase-1 antes mesmo do termo “inflamassoma” ter sido proposto (78) e polimorfismos associados ao NLRP12, como aqueles encontrados no NLRP3, estão associados com doenças inflamatórias hereditárias em humanos (79). Estudos utilizando camundongos *Nlrp12*^{-/-} mostraram o envolvimento do NLRP12 na imunidade e em doenças, como inflamação de cólon (80, 81). Ainda, o NLRP12 é requerido para migração de células mielóides em modelos de hipersensibilidade de contato (82) e, parece mediar um importante papel como regulador negativo da ativação do NF-κB (80). Além disso, recentes trabalhos mostraram um claro papel do NLRP12 na defesa imune contra a bactéria *Yersinia pestis* (83), por um mecanismo que parece envolver a produção de IL-18, a qual induz a produção de IFN-γ. Apesar destes achados, não se sabe se o NLRP12 detecta diretamente o patógeno ou se este receptor sente distúrbios na homeostase do hospedeiro.

É importante notar que diversas proteínas dos NLRs (como CIITA e NLRC5) parecem exercer funções não relacionadas ao inflamassoma, como por exemplo, na regulação da transcrição gênica. Um exemplo é o NLRP6, o qual tem sido sugerido regular as vias de

sinalização do NF- κ B e MAP kinases, após ativação dos TLRs, de maneira aparentemente independente de caspase-1 (84). Ao mesmo tempo, o papel do NLRP6 na regulação da inflamação intestinal parece ser dependente de ASC, caspase-1 e IL-18 (85, 86), sugerindo que o NLRP6 pode formar o complexo inflamassoma. Embora o NLRP6 possa exercer funções distintas não podemos assumir que estas proteínas agem via formação do inflamassoma.

Finalmente, outro inflamassoma que vem intrigando a comunidade científica, denominado “inflamassoma não canônico”, induz a ativação da caspase-11 por vias não convencionais. Após a confirmação de que camundongos deficientes em caspase-1 eram, na verdade deficientes em caspase-1 e caspase-11, os investigadores mostraram que a piroptose induzida após as infecções por *E. coli* e *Vibrio Cholerae* foi dependente da caspase-11, mas não da caspase-1 (87). Curiosamente, no entanto, o processamento e secreção de IL-1 β após estas infecções requereu o NLRP3 e a caspase-1, além da caspase-11 (87, 88), sugerindo que a caspase-11 pode induzir piroptose diretamente, mas somente induz a produção de IL-1 β via “downstream” NLRP3 e caspase-1 (89-91).

Recentes trabalhos mostram que a ativação da caspase-11 em resposta a bactérias Gram negativas requer as vias do TLR4, TRIF e do receptor do IFN do tipo I (89, 90), o que deu a idéia de que esta ativação poderia ser mediada pelo LPS. De fato, o LPS purificado (89, 90) (ou IFN-I) é capaz de induzir a ativação da caspase-11 e, além disso, camundongos caspase-11^{-/-} são resistentes ao choque tóxico induzido pelo LPS (87, 91). Uma vez que a piroptose induzida após a ativação dos inflamassomas NAIP/NLRC4/AIM2 e NLRP1B parece não requerer a caspase-11 (35, 87), é possível que um novo inflamassoma não canônico sirva como plataforma para ativá-la, no entanto, as moléculas envolvidas não são conhecidas (87).

1.4 Flagelina e o Inflamassoma NAIP5/NLRC4

O NLRC4 (também chamado de IPAF, CLAN e CARD12) foi identificado com base na sua similaridade com APAF-1 (*apoptotic protease-activating factor-1*), uma molécula relacionada a apoptose, e foi demonstrado por sua habilidade em recrutar e ativar a caspase-1 (92-94). Estudos iniciais com camundongos *Nlrc4*^{-/-} demonstraram sua importância na ativação da caspase-1 em resposta a *S. Typhimurium* (95, 96). Desde então, a ativação da caspase-1 por uma variedade de patógenos adicionais, incluindo, *L. pneumophila* (97), *P.*

aeruginosa (98, 99), *S. flexneri* (100), *Y. pestis* (101) e *A. veronii* (102) tem sido mostrada ser dependente do NLRC4.

O primeiro agonista descrito para NLRC4 foi à flagelina, apesar de a mesma não estar expressa em todas as bactérias acima citadas.

A flagelina extracelular pode ser reconhecida pelo TLR5 (103), presente na membrana plasmática, porém, quando levada ao citosol celular, através de sistemas de secreção do tipo III e IV, presentes em bactérias como *S. Typhimurium* e *L. pneumophila*, respectivamente, a flagelina é capaz de induzir a ativação do inflamassoma NAIP5/NLRC4 (104-106). A região da flagelina reconhecida pelos inflamassomas está na porção C-terminal presente no domínio D0 da molécula (104-108), diferente da região reconhecida pelo TLR5, a qual está presente no domínio D1 (109, 110) (Figura 5).

Figura 5 – Esquema da estrutura molecular da flagelina. (108).

A ativação do NLRC4 por bactérias flageladas também requer a expressão bacteriana de proteínas especializadas do sistema de secreção, tais como o sistema de secreção SPI-1 do tipo III (T3SS) de *S. Typhimurium* e o do tipo IV Dot/Icm (T4SS) de *L. pneumophila*. O papel fisiológico desses sistemas de secreção é translocar fatores virulentos bacterianos para dentro do citosol celular, como a flagelina (107, 111). No entanto, a função da flagelina no citosol das células hospedeiras era desconhecida. Nesse sentido, o hospedeiro natural da *L. pneumophila* são as amebas e não os mamíferos. Assim, a *Legionella* pode não ter evoluído

para evadir às respostas mediadas pelo NLRC4, enquanto outros patógenos adaptados aos mamíferos (como a *S. Typhimurium* e *Listeria monocytogenes*) parecem ter desenvolvido mecanismos regulatórios para limitar a expressão da flagelina em células infectadas.

De maneira interessante, bactérias deficientes em flagelina, como a *S. flexneri*, são capazes de ativar o NLRC4 (100, 105, 112), de maneira dependente da expressão do T3SS (98, 100, 105, 106, 112). Nesse sentido, Miao e colaboradores propuseram uma explicação para a ativação do NLRC4 independente de flagelina, demonstrando que proteínas do complexo T3SS secretório (proteínas *rod*) são capazes de ativar o NLRC4 (112). Assim como o domínio D0 da flagelina que ativa o NLRC4, acredita-se que as proteínas *rod* do T3SS possa adotar uma estrutura secundária α -hélice e oligomerizar para formar o canal de translocação de proteínas (112, 113). No entanto, apesar da analogia das propriedades funcionais e estruturais, a flagelina e as proteínas do T3SS compartilham pouca similaridade nas sequências de aminoácidos. A questão de como o NLRC4 poderia responder a diversos ligantes foi recentemente resolvida pela descoberta de que o reconhecimento da flagelina e das proteínas *rod* não é mediado diretamente pelo NLRC4 e sim pelo NAIP5 (105, 106). Utilizando técnicas como a de duplo-híbrido de levedura (“*yeast two-hybrid assay*”) ou imunoprecipitações, os autores dos trabalhos demonstraram uma interação física entre NAIP5 ou NAIP6 com NLRC4, após o reconhecimento da flagelina (105, 106), desvendando a estrutura do inflamassoma.

Por outro lado, enquanto a flagelina foi requerida para a ativação do NAIP5 e NAIP6, os mesmo autores demonstraram que o NAIP2 seria responsável pelo recrutamento de NLRC4 em resposta a proteínas do complexo secretório bacteriano como a PrgJ de *S. Typhimurium* e BsaK de *B. thailandensis*, sugerindo que os NAIPs 5 e 6 seriam os sensores universais da flagelina citosólica e o NAIP2 seria sensor de proteínas do complexo secretório, enquanto NLRC4 funcionaria como uma molécula adaptadora responsável pelo recrutamento e ativação da caspase-1. Tal observação parece fazer sentido pelo fato de que: (i) os NAIPs estão “*upstream*” do NLRC4; e (ii) o NLRC4 mas não os NAIPs possuem domínios CARD, os quais podem recrutar e interagir com a caspase-1 via interações CARD-CARD, após o reconhecimento da flagelina por NAIP.

Apesar destes achados, a região exata da flagelina para a ativação dos inflamassomas ainda necessita maiores elucidações. Neste sentido, em 2008, Lightfield e colaboradores inicialmente propuseram que os últimos 35 aminoácidos da porção C-terminal da molécula de flagelina seriam essenciais para a ativação de NAIP5, uma vez que a substituição do resíduo

de três leucinas por alaninas nesta região impediria a ativação de NAIP5 (107). No entanto, utilizando diferentes construções da molécula de flagelina, um trabalho recente demonstrou que as regiões presentes não apenas na porção C-terminal, mas também na porção N-terminal da flagelina são requeridas para a ativação do inflamassoma NAIP5/NLRC4 (108).

A análise feita por microscopia eletrônica do complexo inflamassoma após a ativação por flagelina permitiu a primeira predição de um modelo estrutural do inflamassoma NAIP5/NLRC4, o qual aparece em formato de disco com 11 a 12 moléculas, sendo apenas uma molécula de NAIP5 (Figura 6). No entanto, os mecanismos moleculares envolvidos na formação do complexo necessitam elucidações, uma vez que parece haver modificações pós-translacionais de seus componentes. A ativação da caspase-1 por NLRC4, por exemplo, parece requerer a fosforilação deste receptor na serina 533, a qual é mediada pela enzima PKC δ (114).

Figura 6: Modelo proposto para a formação e estrutura do inflamassoma NAIP5/NLRC4 (108).

Como dito anteriormente, o envolvimento do inflamassoma NAIP5/NLRC4 tem sido bastante estudado durante infecções com uma variedade de patógenos intracelulares. Os mecanismos efetores melhor caracterizados no controle das infecções mediado pela caspase-1 são o processamento das citocinas IL-1 β e IL-18 e a piroptose (115). De fato, a primeira evidência do papel da piroptose *in vivo* no controle de infecções foi demonstrada recentemente e envolve o inflamassoma NAIP5/NLRC4. Neste trabalho, os autores mostram que a piroptose do macrófago infectado *in vivo* com *S. Typhimurium*, resulta na liberação das bactérias para o meio extracelular, expondo-as à ação de neutrófilos recém-migrados, os quais eliminam as bactérias por um mecanismo que envolve a produção de intermediários reativos do oxigênio (ROS) (96).

Além da maturação de IL-1 β /IL-18 e piroptose, outros mecanismos efetores mediados pelo inflamassoma NAIP5/NLRC4 vem sendo estudados, como a secreção de mediadores

lipídios dependente de caspase-1, os quais induzem uma potente resposta inflamatória (116) e a maturação do fagossoma dependente da ativação de caspase-7, o que representa um mecanismo efetor no controle da *L. pneumophila* (117, 118). Nesse sentido, dados recentemente publicados por nosso grupo mostram outros mecanismos efetores não convencionais induzidos a partir do reconhecimento citosólico da flagelina. Estes mecanismos incluem uma via lisossomal que culmina na indução de uma morte celular inflamatória com liberação de IL-1 α e controle de respostas mediadas pelo inflamassoma NAIP5/NLRC4, como a secreção de IL-1 β e piroptose (119) (**APÊNDICE B**) e uma via de ativação de iNOS mediada pelo eixo NAIP5/NLRC4/Caspase-1 (120) (**APÊNDICE A**).

A ativação de iNOS pelo inflamassoma NAIP5/NLRC4 foi descrita durante o meu mestrado onde o objetivo inicial era avaliar as consequências do reconhecimento extra e intracelular da flagelina, mediado pela ativação de TLR5 e NAIP5/NLRC4, respectivamente. A grande maioria dos trabalhos da literatura envolvendo a ativação do inflamassoma NAIP5/NLRC4 e os mecanismos efetores consequentes desta ativação utiliza como ferramenta a infecção de macrófagos com bactérias inteiras, as quais contêm não só apenas a flagelina, mas outros muitos agonistas de TLRs e NLRs. Por este motivo, no intuito de avaliar o reconhecimento individual da flagelina por TLR5 e NAIP5/NLRC4, nós adotamos um modelo de ativação de macrófagos com flagelina purificada, a qual foi utilizada em duas formas: a estimulação com a flagelina livre (FLA-BS) ou inserida em vesículas catiônicas lipídicas, chamadas DOTAP (FLA-BSDot), as quais permitem a entrega da flagelina para o citosol celular, onde ativaria o inflamassoma NAIP5/NLRC4.

Vale ressaltar que a flagelina purificada utilizada neste trabalho é proveniente da bactéria Gram positiva, *Bacillus subtilis*, no intuito de eliminar o LPS como um possível contaminante. Além disso, como controle das nossas preparações, os macrófagos também foram transfectados com as vesículas DOTAP vazias, como descrito recentemente por nosso grupo (121).

Verificamos que somente a flagelina livre foi capaz de induzir a produção de IL-6, como indicativo da ativação extracelular do TLR5, enquanto que a flagelina citosólica induziu a produção de IL-1 β e morte celular, condizente com a ativação da caspase-1. A ausência de IL-6 em resposta à flagelina citosólica é um importante controle do experimento, pois descarta a possibilidade de que haja o reconhecimento do TLR5 por algum resquício de agonista livre durante as preparações de flagelina inserida no DOTAP.

Curiosamente, além da produção de IL-1 β , o estímulo com a flagelina citosólica também foi capaz de induzir a expressão de iNOS, enzima responsável pela produção do óxido nítrico (NO), até então relacionada à cascata de ativação dos receptores TLRs. Finalmente, verificamos que a expressão de iNOS induzida pela flagelina citosólica foi dependente não só do inflamassoma NAIP5/NLRC4, como também dependente da protease caspase-1 (**APÊNDICE A**).

Os estudos a respeito dos mecanismos moleculares envolvidos na ativação da iNOS por flagelina citosólica são o objeto do presente trabalho.

2 OBJETIVOS

- Avaliar o envolvimento da caspase-1 na regulação da transcrição da enzima iNOS em macrófagos estimulados com a flagelina citosólica.
- Avaliar a influência das citocinas pró-inflamatórias, IL-1 β e IL-18, induzidas a partir da ativação do inflamassoma NAIP5/NLRC4, na ativação da iNOS após o estímulo com flagelina citosólica.
- Avaliar o envolvimento dos fatores de transcrição NF- κ B e IRF-1 na ativação da iNOS em resposta à flagelina citosólica.
- Avaliar o envolvimento da enzima Poly(ADP-ribose)polymerase-1 (PARP-1) na regulação da transcrição do gene da iNOS por flagelina citosólica.
- Avaliar a influência da ativação da iNOS induzida por flagelina citosólica na capacidade microbicida dos macrófagos frente às infecções por *L. pneumophila* e *S. Typhimurium*.

3 MATERIAL E MÉTODOS

3.1 Animais

Camundongos da linhagem C57BL/6 e *knockouts* para caspase-1 e *MyD88* com 6-8 semanas de idade, mantidos em condições SPF (*Specific-pathogen free*), foram fornecidos pelo biotério de camundongos isogênicos da Universidade Federal de São Paulo - UNIFESP. Camundongos que apresentam a forma não clivável de PARP-1, induzido por uma mutação pontual, denominados D214N, e camundongos deficientes em caspase-7 com 6-8 semanas de idade, mantidos em condições SPF, foram fornecidos pelo biotério de camundongos isogênicos da Universidade de Zurique, na Suíça. Todos os camundongos foram mantidos em microisoladores com livre acesso à água e ração e toda a manipulação dos animais foi realizada em lugar apropriado no interior do biotério.

3.2 Linhagens bacterianas e flagelinas

As linhagens bacterianas *Salmonella enterica serovar Typhimurium* LT2 1412 (selvagem) e *S. Typhimurium* LT2 2157 (sem flagelina) (gentilmente cedidas pelo Prof^o Luís Carlos Ferreira, do Departamento de Microbiologia, ICB-USP) foram estocadas em meio Luria Bertani (LB) a - 80 °C. Para serem utilizadas, as bactérias primeiramente foram semeadas em meio LB ágar “over night” para crescimento das colônias. Então, uma das colônias foi recolhida e cultivada em meio LB líquido por 18h sob agitação (200rpm) a 37 °C. Posteriormente, este caldo LB foi diluído 10 vezes, e, então, cultivado sob agitação (1h30 - 3h) para obtenção das bactérias na fase exponencial, de acordo com uma curva de crescimento padrão. Ambas as flagelinas purificadas utilizadas, provenientes da bactéria *Salmonella Typhimurium* (FliCi) e da bactéria *Bacillus subtilis* (FLA-BS), foram obtidas da empresa InivoGen, reconstituídas com água livre de endotoxina e estocadas a -20°C na concentração de 100µg/mL.

3.3 Obtenção de macrófagos peritoneais (MPs)

Camundongos da linhagem C57BL/6 e deficientes em caspase-1 e *MyD88* foram inoculados intraperitonealmente (i.p) com amido de batata “*starch*” 1 - 1,5 % (Sigma) para o enriquecimento do peritônio por macrófagos. Após 4 dias, os camundongos foram

sacrificados em câmara fechada por inalação de Halotano (Cristália). Foram injetados 5 mL de PBS (1X) estéril e gelado na cavidade peritoneal para obtenção da suspensão celular. A suspensão de células foi centrifugada a 250 g por 5 minutos, e ressuspensa com 5 mL de meio RPMI 1640 completo, suplementado com 3 % de SBF (Sigma). Seguiu-se então, a contagem das células em câmara de Neubauer, após diluição da suspensão celular a 1:20 em azul de tripan. Após a contagem, onde verificamos a obtenção de, aproximadamente, 8×10^6 células por peritônio, as células foram então plaqueadas, em placas de 6, 12, 48 ou 96 poços (Costar), na concentração de 1×10^7 , 6×10^6 , 1×10^6 e 5×10^5 células por poço, respectivamente, em meio RPMI 1640 completo, suplementado com 3 % de SBF (Sigma), over night a 37 °C, em atmosfera contendo 5 % de CO₂. Após o período de aderência dos macrófagos, as células não aderentes foram retiradas através de vigorosas lavagens utilizando meio RPMI morno para prosseguirmos com os estímulos.

3.4 Obtenção de macrófagos derivados da medula óssea (MDMO)

MDMO foram preparados como previamente descrito (122). Os fêmures de camundongos selvagens ou deficientes em caspase-1, caspase-7 e D214N foram removidos e submetidos à lavagem com meio de cultura RPMI, contendo 10 % de soro fetal bovino para obtenção da suspensão de células. A suspensão celular foi centrifugada a 1000 rpm por 3 minutos, e ressuspensa com 10 mL de meio RPMI 1640 completo, suplementado com 10 % de SBF (Sigma). Seguiu-se então, a contagem das células em câmara de Neubauer, após diluição da suspensão celular a 1:20 em azul de tripan. Após a contagem, 2.5×10^7 células foram plaqueadas em placas petri não tratadas (10 cm) em meio RPMI 1640, suplementado com 10 % de SBF (Sigma), 1 % de glutamina, 1 % de penicilina/streptomicina e 20 % do sobrenadante de cultura da linhagem celular L929 (4 dias) , a qual contém o fator de crescimento para macrófago, M-CSF. As placas foram incubadas a 37 °C, em atmosfera contendo 5 % de CO₂. Após 4 dias, adicionou-se 10 mL de sobrenadante de L929 contendo M-CSF. Após 5 dias de incubação as placas foram lavadas com PBS morno para a retirada de células não aderentes. Os macrófagos derivados foram removidos com PBS gelado e plaqueados na concentração de 5×10^5 células por poço em placa de 24 poços over night contendo 5 % do sobrenadante de L929 para serem posteriormente estimuladas.

3.5 Transfecção da flagelina para o citosol celular

As flagelinas purificadas das bactérias *B. subtilis* e *S. Typhimurium* foram inseridas em vesículas lipídicas catiônicas, chamadas DOTAP (*dioleoyl trimethylammonium propane* - Roche), de acordo com as instruções do fabricante. Brevemente, a flagelina purificada foi adicionada ao DOTAP, na concentração de 1 µg de flagelina para cada 5 µl de DOTAP, na presença de HBS (HEPES-salina tamponada). Essa mistura foi homogeneizada gentilmente por 1 minuto e, então, mantida por 15 minutos em temperatura ambiente para a formação de complexos estáveis gerados espontaneamente. Após esse período, adicionou-se a quantidade apropriada de meio de cultura ao preparado flagelina/DOTAP (FLiCiDot ou FLA-BSDot) e este foi, então, diretamente adicionado às culturas. Desta maneira, a flagelina pôde ser levada ao citosol celular, como têm sido descrito na literatura (123).

3.6 Estimulação *in vitro*

Os macrófagos peritoneais ou derivados de medula óssea, obtidos como descrito anteriormente, foram estimulados *in vitro*, com flagelina purificada proveniente de *B. subtilis* ou *S. Typhimurium* em sua forma livre, FLA-BS e FLiCi, respectivamente, ou inseridas em vesículas lipídicas catiônicas (DOTAP (Roche)), FLA-BSDot ou FLiCiDOT, na concentração de 3 µg/mL em meio RPMI completo, suplementado com 3 % de SFB (Sigma), a 37 °C e a 5% de CO₂. Como controle, as células foram colocadas em meio de cultura sozinho ou juntamente com a vesícula DOTAP vazia.

3.7 Detecção das citocinas IL-1β e IL-6

A análise da produção das citocinas pró-inflamatórias IL-1β e IL-6 nos sobrenadantes das culturas foi determinada por ELISA *sandwich* de captura utilizando o kit comercial proveniente da *Pharmingen*, de acordo com as instruções do fabricante.

3.8 Ensaio de infecção *in vitro*

Para avaliar o envolvimento do óxido nítrico no controle de patógenos intracelulares, macrófagos peritoneais foram plaqueados em placa de 48 poços (3 x 10⁵ células/poço) com meio contendo 3 % de SFB, 1 % de glutamina, na ausência de antibiótico. Posteriormente, os macrófagos foram infectados com a bactéria *S. Typhimurium* WT (LT2 1412) ou mutante, sem flagelina (SL2157) (MOI 1:10 ou 0,1:1), por 1 hora na presença ou não do inibidor

seletivo da enzima iNOS (Aminoguanidina – AG) (SIGMA) [1 mM], responsável pela produção de óxido nítrico. Após esse período, a cultura foi lavada e então foi adicionado meio de cultura RPMI suplementado contendo 50 – 100 µg/mL de gentamicina por 90 minutos, para a retirada das bactérias extracelulares. Após esse período, as culturas foram mantidas com meio contendo 5 – 10 µg/mL ou de gentamicina, na presença ou não flagelina intracelular (FliDot) por 0h - 48h. Então, os macrófagos foram lisados com água estéril e plaquados em LB ágar, juntamente aos respectivos sobrenadantes, para a contagem das unidades formadoras de colônia (UFCs) após 12 - 18 horas em estufa sem CO₂ a 37 °C e, avaliados para a produção de NO pelo método de *Griess*.

3.9 Ensaio de morte celular por microscopia de fluorescência

Macrófagos peritoneais provenientes de camundongos C57BL/6 e deficientes em caspase-1 foram infectados com *Salmonella Typhimurium* como descrito no item anterior, e após 2 a 24h, adicionou-se uma solução de PBS contendo 25 µg/ml de Brometo de Etídio e 25 µg/ml de laranja de acridina. Todas as células foram coradas com Laranja de acridina enquanto apenas as células com poros e/ou rupturas na membrana permitiram a difusão de Brometo de Etídio para o interior do citosol. As imagens foram obtidas utilizando microscópio invertido de fluorescência nas objetivas de 20 e 40 X e a porcentagem de poros ou de células coradas com brometo de etídio foi obtida com o auxílio do *software* ImageJ.

3.10 Detecção de iNOS, IκB-α, PARP-1 clivado e phosphoNF-κB/p65 por *Western Blotting*

As células estimuladas com flagelina citosólica foram posteriormente lisadas com tampão SDS (*sodium dodecyl sulfate*) (50 mM Tris-HCl, Ph 6,8; 2 % SDS, 10 % glicerol e 2,5 % de β-mercaptoetanol). A amostra foi aquecida a 100 °C por 5 minutos e aplicada em gel de poliacrilamida (7,5 % - 15 %) contendo SDS e submetidas à eletroforese. As proteínas do gel foram então transferidas para uma membrana de PVDF e incubadas com anticorpos policlonais e específicos para iNOS (1:500) (Santa Cruz), IκB-α (1:250) (*Cell signalling*), PARP-1 clivado (Santa Cruz) (1:1000) e phosphoNF-κB/p65 (1:500) (*Cell signalling*). A detecção dos imunocomplexos foi realizada pelo método de quimioluminescência utilizando o kit ECL (*Amersham*), de acordo com as instruções do fabricante.

3.11 Detecção de phosphoNF-κB/p65 por imunofluorescência

MDMO (10^5 células/poço) foram plaqueados em placa de 24 well sob lamínulas redondas, com meio RPMI contendo 10 % de soro fetal bovino e mantidas por 16 horas em estufa à 37 °C e 5 % de CO₂. Após este período os macrófagos foram estimulados com LPS [100 ng/mL] ou FLA-BSDot [3 µg/mL] por 30 minutos em estufa à 37 °C e 5 % de CO₂. Como controle, os MDMO foram mantidos com meio de cultura ou com as vesículas DOTAP vazias. Então, o sobrenadante foi retirado e as células foram fixadas com 300 µL de paraformaldeído 4% diluído em PBS (1X) por 15 minutos à temperatura ambiente sob agitação. Após este período, as lamínulas foram bloqueadas com 300 µL de PBS contendo 2 % de BSA e 0.1 % de Triton X-100, por 45 minutos à temperatura ambiente. Após remoção da solução de bloqueio, as células foram incubadas com 200 µL do anticorpo primário (diluído em 2 % de BSA e 0.1 % de Triton X-100) rabbit anti-phosphop65 (1:250) por 1 hora sob agitação, lavadas 2 vezes com PBS para posterior incubação com 200 µL do anticorpo secundário red anti-rabbit (1:250) (diluído em 2 % de BSA e 0.1 % de Triton X-100), por 45 minutos sob agitação, no escuro. Após 1 lavagem com PBS, as células foram incubadas com 200 µL de PBS contendo DAPI (1:100) por 5 - 10 minutos sob agitação, lavadas 1 vez com PBS e as lamínulas foram preparadas para análise no microscópio de fluorescência (Leica).

3.12 Real time quantitativo- qPCR

Após estímulo com flagelina purificada ou infecção com *S. Typhimurium*, o RNA total dos macrófagos foi isolado pelo método de TRIzol ou por kit de separação por coluna (Qiagen). A concentração e purificação do RNA foi analisada por leitura em espectrofotômetro NanoDROP nd-1000. As amostras utilizadas foram avaliadas quanto à absorbância na razão de 260/280nm, indicando ausência de contaminantes de proteínas. A integridade do RNA foi avaliada em gel de agarose 1 % após incubação com brometo de etídio. O cDNA foi gerado a partir de 3µg do total do RNA utilizando o método Oligo(dT) e RNase H (Invitrogen) de acordo com as instruções do fabricante. A análise do Real Time PCR foi feita com sistema Taqman (*Applied Biosystems*) ou Syber Green. Como controle, foi

utilizado um gene endógeno de referência “*housekeeping*” (GAPDH) (catalogo nº 4352932) (*Applied Biosystems*).

3.13 Análises estatísticas

Todas as análises estatísticas foram realizadas com o auxílio de um *software* computacional chamado *Graphpad Prism*, da companhia *Graphpad Software Incorporation*, versão 3.02. O teste usado foi o ANOVA, seguido pelo teste Bonferroni. Valores de $P < 0,001$ (***), $P < 0,01$ (**) e $P < 0,05$ (*) foram considerados estatisticamente significativos.

4 RESULTADOS

4.1 Caspase-1 regula a transcrição do gene da iNOS em resposta à flagelina citosólica

Nosso modelo de ativação de macrófagos por flagelina purificada mostra que a expressão de iNOS, induzida após o estímulo com a flagelina citosólica (FLA-BSDot) (Figura 7 B), e não após o estímulo com a flagelina livre (FLA-BS) (Figura 7 A), é dependente da caspase-1 e independente de *MyD88*. Além disso, a caspase-1 foi dispensável para a expressão da iNOS após o estímulo com FLA-BS, sugerindo que as formas livre e citosólica da flagelina utilizam diferentes vias para ativar iNOS (Figura 7 A).

Vale ressaltar que os macrófagos *MyD88*^{-/-} não induziram a expressão de iNOS seguido do estímulo com FLA-BS, uma vez que esta molécula é essencial na via de sinalização mediada por TLR5 após o reconhecimento da flagelina, confirmando o seu genótipo (Figura 7 A). Além disso, o fato do *MyD88* ser dispensável para a expressão de iNOS em resposta à flagelina citosólica reforça os dados obtidos anteriormente sobre a independência da sinalização mediada pelos TLRs na expressão de iNOS dependente de caspase-1 (APÊNDICE A).

Após observarmos a expressão de iNOS a partir da ativação dos inflamassomas, demos continuidade na avaliação dos mecanismos moleculares envolvidos neste achado. Para avaliar se a caspase-1 regula a transcrição do gene da iNOS, os macrófagos foram estimulados com a flagelina citosólica e o mRNA para o gene da iNOS foi avaliado por qPCR. Verificamos que a alta expressão de iNOS induzida nos MDMO selvagens em resposta à FLA-BSDot, foi perdida nos MDMO caspase-1^{-/-} (Figura 7 C). Este dado demonstra que a caspase-1 regula a expressão da iNOS em resposta a flagelina também a nível transcripcional.

Figura 7: Regulação da iNOS mediada por caspase-1 em resposta à flagelina citosólica: MPs (A e B) (3×10^5 células/poço) ou MDMO (C) (5×10^5 células/poço) provenientes de camundongos C57BL/6, caspase-1^{-/-} ou MyD88^{-/-} foram estimulados com flagelina purificada em sua forma livre (FLA-BS) (A) ou inserida em vesículas lipídicas catiônicas (DOTAP) para inserção no citosol celular (FLA-BS-Dot) (B e C) [3 µg/ml]. Como controle os macrófagos foram estimulados com as vesículas DOTAP vazias (Dot). (A e B) Análise da expressão de iNOS por *Western Blotting* (24h). (C) Análise do mRNA para iNOS por qPCR (3-4h). ***P≤0,001. Os números representam a média +/- desvio padrão de triplicatas. Os experimentos foram repetidos três vezes mostrando o mesmo perfil.

4.2 A expressão de iNOS dependente de caspase-1 não requer IL-1β e IL-18

Sabe-se que a caspase-1 desempenha um importante papel na inflamação por promover o processamento das citocinas pró-inflamatórias IL-1β e IL-18, as quais podem estar envolvidas na expressão de iNOS (39, 124). Sendo assim, o próximo passo foi avaliar o envolvimento destas citocinas na regulação da expressão de iNOS em resposta à flagelina citosólica, adicionando ou não, anticorpos neutralizantes para ambas, na cultura.

O estímulo com a flagelina citosólica induziu a produção de IL-1 β , como indicativo da ativação da caspase-1. Como esperado, esta produção foi abolida na presença de anti-IL-1 β (Figura 8 A).

Ao contrário, ambas as citocinas, IL-1 β e IL-18 foram dispensáveis para a expressão de iNOS em resposta a flagelina, uma vez que a adição dos anticorpos neutralizantes na cultura não alterou a expressão de iNOS nos MPs (Figura 8 B). Pelo contrário, a expressão de iNOS parece aumentar na presença de anti-IL-1 β e anti-IL-18, o que sugere um mecanismo compensatório mediado pelos macrófagos. A independência de IL-1 β e IL-18 na expressão de iNOS em resposta a flagelina citosólica foi suportada pelos dados obtidos com MDMO, os quais também regularam a expressão desta enzima (Figura 8 C) mesmo na presença de anti-IL-1 β e anti-IL-18.

Sabe-se que os receptores para IL-1 β e IL-18, assim como a sinalização do receptor TLR5, dependem da molécula adaptadora *MyD88* (39, 103, 125). Dessa forma, a independência de ambas as citocinas na expressão de iNOS por flagelina citosólica foi reforçada pelo fato de que macrófagos *MyD88*^{-/-} também induziram a expressão de iNOS nas mesmas condições (Figura 7 A).

Vale ressaltar que a regulação da iNOS em resposta a flagelina citosólica não pode ser atribuída à ativação inespecífica da utilização dos anticorpos neutralizantes, uma vez que ambos não induziram a expressão de iNOS na ausência de flagelina (Figura 8 C). Além disso, nenhum efeito dos anticorpos anti-IL-1 β e anti-IL-18 foram observados na produção de IL-6 em resposta a flagelina citosólica (Figura 8 D).

Figura 8: Avaliação do envolvimento de IL-1β e IL-18 na expressão de iNOS em resposta à flagelina citosólica: MPs (3×10^5 células/poço) (A, B e D) ou MDMO (C) (5×10^5 células/poço) provenientes de camundongos C57BL/6 foram estimulados com flagelina purificada em sua forma livre (FLA-BS) (A e D) ou inserida em vesículas lipídicas catiônicas (DOTAP) para inserção no citosol celular (FLABS-Dot) [$3 \mu\text{g}/\text{ml}$] por 24 horas, na presença ou não dos anticorpos neutralizantes para as citocinas IL-1β e IL-18. Como controle os macrófagos foram estimulados com as vesículas DOTAP vazias (Dot). A produção de IL-1β (A) e IL-6 (D) foi avaliada por ELISA. (B e C) Análise da expressão de iNOS por *Western Blotting*. *** $P \leq 0,001$ em relação ao grupo controle; ### $P \leq 0,001$ em relação ao grupo “FLA-BSDot + anti-IL-1β”. Os números representam a média $\pm$ desvio padrão de triplicatas. Os experimentos foram repetidos três vezes mostrando o mesmo perfil.

4.3 A flagelina citosólica ativa NF-κB, mas não IRF-1

Sabe-se que o promotor do gene da iNOS contém sítios de ligação para os fatores de transcrição NF-κB e IRF-1 (126, 127). Sendo assim, o fato da expressão de iNOS ser dependente da caspase-1 e independente das citocinas IL-1β e IL-18 levantou a questão de como a caspase-1 poderia interagir com o NF-κB para ativar iNOS.

Assim, tornou-se necessária a investigação do envolvimento do NF- κ B e IRF-1 na ativação de iNOS em resposta a flagelina citosólica. No entanto, um estudo mostrou que a restrição da infecção por *L. pneumophila* depende da ativação dos fatores de transcrição IRF-1 e IRF-8, mediada pelo inflamassoma NAIP5/NLRC4, importante para a regulação de iNOS e IL-12 (128). Por esta razão, inicialmente avaliamos a modulação de IRF-1 e IRF-8 em MPs provenientes de camundongos C57BL/6 infectados com *S. Typhimurium* para análise da expressão do mRNA de IRF-1 e IRF-8 por qPCR. Observamos uma regulação positiva de IRF-1 após 90 minutos da infecção com *S. Typhimurium* (Figura 9 A), porém até o período de 6 horas pós-infecção, não foi observada modulação da expressão de IRF-8 nos MPs (Figura 9 B).

Figura 9: Avaliação da expressão de IRF-1 e IRF-8 em MPs infectados com *S. Typhimurium*. MPs (3×10^5 células/poço) provenientes de camundongos C57BL/6 foram infectados com *S. Typhimurium* (MOI 1:10) por 1 hora. Após a infecção, as células foram tratadas com gentamicina [100 μ g/ml] por 1.5h, para eliminar as bactérias extracelulares. Então, as culturas foram lavadas e mantidas com gentamicina [10 μ g/ml] por 1.5, 3 e 6 horas pós infecção. Após esse período os MPs infectados foram lisados com água estéril e o mRNA foi isolado pelo método de TRIzol para a análise da expressão de IRF-1 (A) e IRF-8 (B) por qPCR. O gene GAPDH foi utilizado com controle. Os números representam a média $\pm$ desvio padrão de triplicatas. ** $P \leq 0,01$ em relação ao grupo controle. Os experimentos foram repetidos duas vezes mostrando o mesmo perfil.

Apesar da indução de IRF-1 durante a infecção por *S. Typhimurium*, a flagelina parece não exercer papel importante nesta regulação. Observamos que, ao contrário do rIFN- γ (Figura 10A), conhecido como indutor da ativação de IRF-1, a flagelina citosólica purificada não foi capaz de induzir a ativação deste fator de transcrição (Figura 10 B).

No entanto, assim como o LPS, potente ativador da via do NF- κ B, utilizado como controle (Figura 10 D), o estímulo com a FLA-BSDot também foi capaz de induzir a ativação deste fator de transcrição, observada por *Western Blotting* (Figura 10 C), através da fosforilação da subunidade p65, e por imunofluorescência (Figura 10 D), o que poderia

sugerir seu envolvimento na expressão de iNOS por inflamassomas. Vale ressaltar que as vesículas DOTAP vazias não induziram ativação de NF- κ B (Figura 10 D).

Figura 10: Avaliação da ativação de IRF-1 e NF- κ B em macrófagos estimulados com flagelina citosólica: MPs (A, B, C) (3×10^5 células/poço) ou MDMO (D) (5×10^5 células/poço) provenientes de camundongos C57BL/6 foram estimulados com rIFN- γ [5 ng/mL] (A), LPS [100 ng/mL] (D) ou com flagelina purificada inserida em vesículas lipídicas catiônicas (DOTAP) para inserção no citosol celular (FLA-BSDot) (B, C e D) [3 μ g/ml]. Como controle os macrófagos foram estimulados com a vesícula DOTAP vazia (Dot). (A e B) Análise da

expressão do mRNA do gene IRF-1 por qPCR. A ativação do NF- κ B foi avaliada por *Western Blotting* (C) ou por imunofluorescência (D). Os números representam a média $\pm$ desvio padrão de triplicatas. *** $P \leq 0,001$ em relação ao grupo controle. Os experimentos (A, B e C) foram repetidos duas vezes mostrando o mesmo perfil.

4.4 A ativação de iNOS dependente de caspase-1 parece requerer a ativação do NF- κ B

Para avaliar o envolvimento do NF- κ B na expressão de iNOS por flagelina citosólica, MPs selvagens e deficientes em *MyD88* e caspase-1, foram estimulados na presença ou não do inibidor de NF- κ B, “Pyrrolidine Dithiocarbamate” (PDTC), o qual impede a degradação de I κ B- α e conseqüente translocação do dímero p65/p50 para o núcleo. Como controle da atividade do inibidor, observamos que os MPs estimulados com a flagelina em sua forma livre induziram a ativação do NF- κ B, através da degradação de I κ B- α , a qual foi inibida pela adição do PDTC, de maneira dose dependente (Figura 11 A).

Da mesma maneira observada para o estímulo com a flagelina livre, a expressão de iNOS induzida pela flagelina citosólica nos MPs selvagens e deficientes em *MyD88*, também foi inibida na presença do PDTC (Figura 11 B). Como esperado, a flagelina citosólica não induziu a expressão de iNOS na ausência de caspase-1 e, portanto, não observamos nenhum efeito da adição do PDTC na cultura (Figura 11 B). Estes dados em conjunto, mostram que a flagelina citosólica induz a expressão de iNOS de maneira dependente de caspase-1 e NF- κ B.

Figura 11: Avaliação do envolvimento do NF- κ B na expressão de iNOS por flagelina citosólica: MPs (3×10^5 células/poço) provenientes de camundongos C57BL/6 (A e B), *MyD88*^{-/-} (B) ou caspase-1^{-/-} (B) foram pré-tratados com PDTC por 1 hora, seguido do estímulo com flagelina purificada em sua forma livre (FLA-BS)

(A) ou inserida em vesículas lipídicas catiônicas (DOTAP) para inserção no citosol celular (FLA-BSDot) [3 µg/ml] (B). Como controle os macrófagos foram estimulados com as vesículas DOTAP vazias (Dot). (A) Curva dose resposta da atividade do PDTC [30 µM, 60 µM ou 100 µM] para análise da ativação de IκB-α (10 minutos) por *Western Blotting*. (B) Análise da influência do PDTC [60 µM] na expressão de iNOS (24h) por *Western Blotting*. Os experimentos foram repetidos duas vezes mostrando o mesmo perfil.

No entanto, apesar de não observarmos expressão de iNOS nos macrófagos deficientes em caspase-1, o estímulo com a flagelina citosólica foi capaz de induzir a ativação de NF-κB nessas células, observada através da degradação de IκB-α por *Western Blotting* (Figura 12), abrindo a questão de como a caspase-1 e o NF-κB poderiam interagir para induzir a expressão de iNOS.

Figura 12: Avaliação do envolvimento da caspase-1 na ativação de NF-κB induzida flagelina citosólica: MPs (3x10⁵ células/poço) provenientes de camundongos C57BL/6 ou caspase-1^{-/-} foram estimulados com flagelina purificada inserida em vesículas lipídicas catiônicas (DOTAP) para inserção no citosol celular (FLA-BSDot) [3 µg/ml] por 10 minutos para análise da expressão de NF-κB através da degradação de IκB-α por *Western Blotting*. Os experimentos foram repetidos duas vezes mostrando o mesmo perfil.

4.5 A clivagem de PARP-1 está envolvida na expressão de iNOS mas não de IκB-α em resposta a flagelina citosólica

Os dados anteriores desafiam a hipótese de uma interação direta entre a caspase-1 e o NF-κB na expressão de iNOS. Nesse contexto, o grupo do Dr. Michael Hottiger, da Universidade de Zurique, na Suíça, recentemente demonstrou que os inflamassomas também são capazes de utilizar a enzima responsável por reparar danos no DNA, PARP-1 (Poly(ADP-ribose) polymerase-1), para ativar genes alvos dependentes de NF-κB, após ativação da caspase-1. Diante da similaridade dos mecanismos envolvidos na ativação de genes dependentes de NF-κB, envolvendo caspases, estabelecemos uma colaboração com o grupo mencionado acima para avaliar o envolvimento de PARP-1 na ativação de iNOS pelo eixo NAIP5/NLRC4-Caspase-1/NF-κB.

Para melhor entender sobre o envolvimento da caspase-1 na regulação da iNOS mas não na ativação de NF- κ B, ambos induzidos em resposta a flagelina citosólica, inicialmente confirmamos o potencial da flagelina citosólica em induzir a ativação da caspase-1. Para isso, MPs selvagens foram estimulados com a flagelina citosólica e a ativação da caspase-1 foi avaliada por *Western Blotting*. De fato, o estímulo com a FLA-BSDot foi capaz de induzir a clivagem e ativação direta da caspase-1, observada através da subunidade p20 desta protease (Figura 13). Esses dados confirmam que a flagelina citosólica induz a ativação da caspase-1, necessária para a regulação da transcrição do gene da iNOS.

Figura 13: Avaliação da ativação da caspase-1 por flagelina citosólica: MDMO (5×10^5 células/poço) provenientes de camundongos C57BL/6 foram estimulados com a flagelina purificada inserida em vesículas lipídicas catiônicas (DOTAP) para inserção no citosol celular (FLA-BSDot) [3 μ g/ml] por 1.5h para análise da ativação da caspase-1 através de sua clivagem proteolítica por *Western Blotting*. Como controle experimental os macrófagos foram estimulados com as vesículas DOTAP vazias (Dot).

Além da caspase-1, a caspase-7 também foi demonstrada estar envolvida na regulação da transcrição de genes dependentes de NF- κ B, após clivagem de PARP-1 (129). Desta forma, para avaliar o envolvimento da caspase-7 na expressão de iNOS, MDMO selvagens ou deficientes em caspase-7 foram estimulados com a flagelina citosólica e a expressão de iNOS foi avaliada por qPCR. Os animais deficientes em caspase-1 foram utilizados como controle. Observamos que na ausência da caspase-7 houve uma redução significativa na expressão de iNOS, induzida pela flagelina citosólica, ainda que esta redução tenha sido em menor escala quando comparada aos MDMO deficientes em caspase-1 (Figura 14 D). Curiosamente, na ausência da caspase-1 e caspase-7, a análise da expressão de outro gene dependente de NF- κ B, o I κ B- α , utilizado como controle de um gene não relacionado, mostrou níveis elevados em sua expressão em resposta à FLA-BSDot, quando comparados aos MDMO selvagens

(Figura 14 E). Juntos, esses dados mostram o envolvimento das caspases-1 e 7 na regulação da expressão de iNOS em resposta a flagelina citosólica.

Vale ressaltar que, diferente dos animais deficientes em caspase-1 utilizados nos experimentos anteriores, que também são deficientes em caspase-11, os animais deficientes em caspase-1 utilizados no grupo do Dr. Hottiger, não apresentam expressão de caspase-1 (Figura 14 A), mas expressam caspase-11 (Figura 14 B). O genótipo dos macrófagos deficientes em caspase-7 foi confirmado por *Western Blotting* (Figura 14 C), avaliado em MDMO estimulados com LPS, um conhecido ativador da caspase-7 (129).

Figura 14: Avaliação do envolvimento da caspase-7 na expressão de iNOS por flagelina citosólica. MDMO provenientes de camundongos C57BL/6 (5×10^5 células/poço) foram estimulados com flagelina purificada inserida em vesículas lipídicas catiônicas (DOTAP) para inserção no citosol celular (FLA-BSDot) [3 µg/ml] para análise da expressão das caspases-1 e 11 (A, B), iNOS (D) e IκB-α (E) (3-4 horas) por qPCR. A expressão da caspase-7 (C) foi analisada por *Western Blotting*. Como controle experimental os macrófagos foram estimulados com as vesículas DOTAP vazias (Dot). Os números representam a média $\pm$ desvio padrão de triplicatas. *** $P \leq 0,001$ e ** $P \leq 0,01$ em relação ao grupo “Wild type + FLA-BSDot” ## $P \leq 0,01$ em relação ao grupo controle. Os experimentos (A, D e E) foram repetidos duas vezes mostrando o mesmo perfil.

O envolvimento das caspases na expressão de iNOS induzida por flagelina citosólica foi confirmado após o tratamento dos MDMO selvagens com o inibidor de caspases, pan-z-

VAD, seguido do estímulo com FLA-BSDot, o que resultou em uma redução significativa na expressão de iNOS (Figura 15 A). Além disso, a expressão de I κ B- α induzida pela flagelina citosólica não foi alterada na presença de z-VAD nos macrófagos selvagens, atribuindo especificidade ao gene da iNOS, dependente de estímulo (Figura 15 B).

Figura 15: Avaliação da influência do z-VAD na expressão de iNOS em resposta à flagelina citosólica. MDMO provenientes de camundongos C57BL/6 (5×10^5 células/poço) foram ou não pré-tratados com o inibidor de caspases (pan-z-VAD) 1 hora antes do estímulo com a flagelina purificada inserida em vesículas lipídicas catiônicas (DOTAP) para inserção no citosol celular (FLABS-Dot) [3 μ g/ml] por 3-4 horas para análise da expressão de iNOS (A), ou I κ B- α (B) por qPCR. Como controle os macrófagos foram estimulados com as vesículas DOTAP vazias (Dot). Os números representam a média $\pm$ desvio padrão de triplicatas. **P $\leq$ 0,01 em relação ao grupo controle. Os experimentos foram repetidos duas vezes mostrando o mesmo perfil.

Uma vez que a clivagem de PARP-1 induzida por caspases resulta na descondensação da cromatina, o que facilita a expressão de genes alvos dependentes de NF- κ B (129), estes dados sugerem o envolvimento das caspases-1 e 7 na regulação do gene da iNOS, através da clivagem de PARP-1. Para avaliar a importância da clivagem de PARP-1 na expressão de iNOS em resposta à flagelina citosólica, utilizamos MDMO provenientes de camundongos denominados D214N. Nesta linhagem de camundongo foi introduzido um ponto de mutação no sítio de clivagem de caspases presente no gene de PARP-1 pela estratégia de “*knockin*” (KI) na qual a conversão do aspartato para uma asparagina no códon 214 do gene resulta em uma proteína PARP-1 resistente à clivagem por caspases, denominada forma não clivável. Estes macrófagos foram estimulados com flagelina citosólica para a análise da expressão dos genes dependentes de NF- κ B, iNOS e I κ B- α por qPCR.

A figura 16 A mostra que a alta expressão de iNOS induzida pela flagelina citosólica foi comprometida nos MDMO D214N, sugerindo o envolvimento da clivagem de PARP-1 na ativação de iNOS mediada pelo inflamassoma NAIP5/NLRC4. Ainda, mais uma vez a clivagem de PARP-1 mostrou ser específica para ativação do gene da iNOS, uma vez que a expressão de I κ B- α induzida pela flagelina citosólica não foi alterada nos MDMO D214N (Figura 16 B).

Figura 16: Avaliação da clivagem de PARP-1 na expressão de iNOS em resposta à flagelina citosólica. MDMO provenientes de camundongos C57BL/6 ou D214N (5×10^5 células/poço) foram estimulados com flagelina purificada inserida em vesículas lipídicas catiônicas (DOTAP) para inserção no citosol celular (FLABS-Dot) [$3 \mu\text{g/ml}$] por 3-4 horas para análise da expressão de iNOS (A) ou I κ B- α (B) por qPCR. Como controle os macrófagos foram estimulados com as vesículas DOTAP vazias (Dot). Os números representam a média $\pm$ desvio padrão de triplicatas. *** $P \leq 0,001$ em relação ao grupo “C57BL/6+FLA-BSDot”. Os experimentos foram repetidos três vezes ou mais mostrando o mesmo perfil.

Os dados em conjunto nos permitem propor um modelo para a regulação molecular da iNOS dependente do inflamassoma NAIP5/NLRC4, onde a flagelina citosólica induz a ativação da caspase-1 e da caspase-7, as quais poderiam contribuir para induzir a clivagem de PARP1, resultando na descondensação da cromatina, o que facilitaria a expressão de iNOS.

4.6 Relevância biológica da via de ativação de iNOS por inflamassomas

4.6.1 A ativação de iNOS independente de MyD88 contribui para o controle da infecção por *L. pneumophila*

Uma vez que o inflamassoma NAIP5/NLRC4 participa do controle da infecção por *L. pneumophila* (97), o próximo passo foi avaliar o papel biológico dessa via de ativação de iNOS por inflamassomas no controle da infecção por *L. pneumophila*. Para isso, MPs foram infectados na presença ou não do inibidor seletivo de iNOS, a aminoguanidina (AG). Para avaliar a dependência da flagelina na ativação de iNOS e no controle da infecção, os macrófagos também foram infectados com *L. pneumophila* mutante, sem flagelina (*FlaA*). Finalmente, para descartar a via convencional de ativação de iNOS por TLRs, MPs provenientes de camundongos deficientes em *MyD88* também foram avaliados.

A inibição de iNOS durante a infecção pela *L. pneumophila* WT resultou no aumento da susceptibilidade dos MPs selvagens (Figura 17 A), sugerindo um papel da iNOS no controle da infecção. No entanto, quando esses MPs foram infectados com *L. pneumophila FlaA*, nenhum efeito da adição de AG foi observado, sugerindo que o controle mediado pela iNOS depende de flagelina (Figura 17 B). Interessante, o tratamento com AG também tornou os MPs *MyD88*^{-/-} mais susceptíveis à infecção com a *L. pneumophila* WT mas não com a *L. pneumophila FlaA*, sugerindo que a iNOS exerce seu papel protetor no controle da infecção de maneira dependente de flagelina e independente de *MyD88*.

Figura 17: Avaliação do envolvimento da iNOS no controle da infecção por *L. pneumophila*: MDMOs provenientes de camundongos C57BL/6 ou *MyD88*^{-/-} foram infectados com *L. pneumophila* WT (A) ou mutante,

deficiente em flagelina (*FlaA*) (**B**) por 72 horas (MOI de 0,015), na presença ou não do inibidor seletivo de iNOS, aminoguanidina (AG) [0,1 mM]. As unidades formadoras de colônias (UFCs) foram contadas após plaqueamento em meio LB ágar após 96 horas de incubação à 37 °C. As barras representam media e desvio * $P \leq 0,05$ quando comparado com o grupo controle. Os experimentos foram repetidos três vezes mostrando o mesmo perfil.

4.6.2 A ativação de iNOS dependente de caspase-1 contribui para o controle da infecção por S. Typhimurium

Sabe-se que o inflamassoma NAIP5/NLRC4 desempenha um papel importante no controle da infecção por patógenos intracelulares como *P. aeruginosas*, *S. flexineri*, *L. pneumophila* e *S. Typhimurium*. Uma vez demonstrado o envolvimento da iNOS, no controle da *L. pneumophila* de maneira independente de *MyD88*, em resposta à flagelina citosólica, o próximo passo foi avaliar a relevância biológica desta via frente a infecção por *S. Typhimurium*. Para iniciarmos nossos estudos, padronizamos uma curva de crescimento de *S. Typhimurium* para posterior análise dos mecanismos efetores induzidos a partir da infecção, nos MPs. Para isso, o crescimento bacteriano foi monitorado a cada hora, por contagem das unidades formadoras de colônia (UFC) em meio LB ágar, e em paralelo, a leitura de densidade óptica (D.O), foi realizada em espectrofotômetro a 600 nm, como descrito em material e métodos. Através dessa curva de crescimento padrão, sabemos o tempo exato em que, a cepa de *S. Typhimurium* WT ou mutante, sem flagelina (*ΔfliC*), atinge sua fase exponencial (virulência), observado pelo pico máximo de crescimento, que ocorre em torno de 2 - 3 horas, antes de retornar a fase estacionária. Como mostra a figura 18, ambas as cepas, *S. Typhimurium* WT e *ΔfliC*, alcançam a fase exponencial ao mesmo tempo, entre 2 - 3 horas, sendo possível utilizá-las paralelamente durante a infecção dos MPs.

Figura 18: Padronização da curva de crescimento de *S. Typhimurium* WT e $\Delta fliC$ (sem flagelina). As cepas de *S. Typhimurium* WT e $\Delta fliC$ foram semeadas em meio LB ágar e incubadas over night em estufa sem CO₂ a 37 °C, para o isolamento de unidades formadoras de colônia (UFC). Após este período, uma colônia isolada foi cultivada em meio LB líquido por 18h em shaker sob agitação (200 rpm) a 37 °C. Este concentrado de bactéria foi diluído 10 vezes, e então mantido sob agitação por um período de 36 horas. As UFCs e a leitura da densidade óptica (D.O) da turbidez bacteriana foram realizadas a cada hora de incubação, de acordo com os tempos indicados. Os números representam a média +/- desvio padrão de triplicatas. Os experimentos foram repetidos três vezes mostrando o mesmo perfil.

4.6.3 Papel da piroptose no controle da infecção por *S. Typhimurium*

Trabalhos recentes apontam a indução de piroptose, induzida por flagelina citosólica, dependente de caspase-1 como um dos principais mecanismos efetores para controlar a infecção por *S. Typhimurium* (40, 96, 130-133).

De fato, utilizando a marcação com o corante vital laranja de acridina e o brometo de etídio, o qual é incorporado em células que perdem a integridade da membrana plasmática, observamos uma alta frequência de morte celular nos MPs selvagens quando comparados com MPs caspase-1^{-/-} em tempos precoces à infecção (1 hora) por *S. Typhimurium* (Figura 19 A). Ainda, a baixa frequência de morte celular apresentada por MPs caspase-1^{-/-} foi correlacionada com a alta susceptibilidade destas células, observada pelo aumento de 10 vezes na carga bacteriana quando comparados aos MPs selvagens (Figura 19 B), confirmando o papel da piroptose dependente de caspase-1 no controle da infecção por *S. Typhimurium*.

Além disso, dados de nosso grupo mostram que esta rápida morte celular mediada pela caspase-1 é dependente da presença da flagelina, uma vez que a infecção pela *S. Typhimurium* WT induz uma frequência de morte celular muito maior nos MPs do que a infecção pela *S. Typhimurium* $\Delta fliC$, sem flagelina (119).

Apesar de a piroptose representar um mecanismo efetor importante no controle da infecção por *S. Typhimurium*, observamos que ao menos metade das células selvagens e deficientes em caspase-1 sobrevive ao longo da infecção (6 horas) (Figura 19 A). Além disso, ao observarmos os macrófagos remanescentes, notamos uma alta carga bacteriana no interior dos MPs deficientes em caspase-1 quando comparados aos MPs selvagens (Figura 19 C), sugerindo que mecanismos efetores dependentes de caspase-1 poderiam operar de maneira independente da morte celular para o controle da infecção.

Figura 19: Papel da piroptose no controle da infecção por *S. Typhimurium*. MPs provenientes de camundongos C57BL/6 ou deficientes em caspase-1 (3×10^5 células/poço) foram infectados com *S. Typhimurium* (MOI 1:10) por 1 hora. Após a infecção, as células foram tratadas com gentamicina [100 µg/ml] por 1.5h, para eliminar as bactérias extracelulares. Então, as culturas foram lavadas e mantidas com gentamicina [10 µg/ml] por 6 horas. A morte celular foi avaliada por microscopia de fluorescência após marcação com brometo de etídio (vermelho) e laranja de acridina (verde) (A e C). A contagem das unidades formadoras de colônias (UFCs) foi realizada após lise dos MPs com água estéril e plaqueamento em meio LB ágar, 12 – 18 horas após incubação a 37 °C (B). Os números representam a média +/- desvio padrão de triplicatas. *** $P \leq 0,001$ em relação ao grupo controle (C57BL/6). Os experimentos foram repetidos três vezes mostrando o mesmo perfil

4.6.4 Papel não redundante da iNOS dependente de caspase-1 no controle da infecção por *S. Typhimurium*.

Apesar de a morte celular desempenhar um importante papel no controle de infecções, acredita-se que este não seja o único mecanismo efetor suficiente para a eliminação do patógeno. A importância dos intermediários reativos do oxigênio e do nitrogênio no controle de patógenos intracelulares por macrófagos é bem descrita na literatura (134).

Como demonstrado previamente, a via de ativação de iNOS, mediada pelo eixo NAIP5/NLRC4-caspase-1, contribui para o controle da infecção por *L. pneumophila*. Além disso, uma vez que demonstramos que a expressão de iNOS em resposta à flagelina citosólica depende da caspase-1, a iNOS foi a primeira hipótese de mecanismo efetor operante nos macrófagos remanescentes da morte celular.

Para avaliarmos o efeito da iNOS no controle da infecção por *S. Typhimurium*, MPs selvagens foram infectados na presença ou não de AG. Ainda, a dependência da flagelina na ativação da iNOS durante a infecção foi avaliada em MPs infectados com *S. Typhimurium AfliC*, mutante, deficiente em flagelina.

A infecção com a *S. Typhimurium WT* induziu a produção de NO em MPs selvagens (Figura 20 A). A presença de AG aboliu a produção de NO e, paralelamente tornou os MPs selvagens susceptíveis à infecção, uma vez que essas células apresentaram carga bacteriana elevada na presença do inibidor. Estes dados mostram o envolvimento da iNOS no controle da infecção por *S. Typhimurium* (Figura 20 B).

A infecção com a *S. Typhimurium AfliC* não induziu níveis significativos de NO pelos MPs, demonstrando o papel da flagelina na ativação da iNOS (Figura 20 C). Além disso, a presença de AG não alterou a susceptibilidade dos MPs infectados com a *S. Typhimurium AfliC*, sugerindo que a produção de NO induzida pela flagelina está envolvida no controle da *Salmonella* (Figura 20 D).

O papel da iNOS dependente de flagelina no controle da *S. Typhimurium* foi confirmado pela adição de flagelina citosólica exógena nas culturas dos MPs infectados com a *S. Typhimurium* deficiente neste agonista. A reposição da flagelina induziu a produção de NO nos MPs, chegando a níveis semelhantes àqueles obtidos pela infecção com a *S. Typhimurium WT* (Figura 20E e ver Figura 20 A). Além disso, a adição da flagelina citosólica exógena tornou os MPs resistentes à infecção, observada pela redução significativa de UFCs em comparação com MPs infectados com a *S. Typhimurium AfliC*, que não receberam a flagelina exógena (Figura 21 F). Por fim, a presença de AG aboliu novamente a produção de NO induzida pela flagelina exógena em MPs infectados com a *S. Typhimurium AfliC* (Figura 20 E), o que se correlacionou com a perda da resistência dos macrófagos (Figura 20 F). Esses dados mostram o papel da flagelina na indução de NO e controle da infecção.

Figura 20: Envolvimento da iNOS e da flagelina no controle da infecção por *S. Typhimurium*. MPs provenientes de camundongos C57BL/6 (3×10^5 células/poço) foram infectados com *S. Typhimurium* selvagem (WT) ou *S. Typhimurium* mutante, sem flagelina (ΔfliC) (MOI 0,1:1) por 1 hora, na presença ou não do inibidor de iNOS, aminoguanidina (AG) [1 mM]. Após a infecção, as células foram tratadas com gentamicina [50 $\mu\text{g}/\text{mL}$] por 1 hora para eliminar as bactérias extracelulares. Após, as culturas foram lavadas e mantidas com gentamicina [5 $\mu\text{g}/\text{mL}$] por mais 24 horas, na presença ou não de flagelina purificada inserida em vesículas lipídicas catiônicas (DOTAP) para inserção no citosol celular (FliDot) [1 $\mu\text{g}/\text{mL}$] proveniente da bactéria *S. Typhimurium*. Os MPs foram lisados com água estéril e a contagem das UFCs foi feita após plaqueamento em LB ágar, 12-18 horas após incubação a 37 °C. Os números representam a média $\pm$ desvio padrão de triplicatas. *** $P \leq 0,001$ em relação ao grupo controle; ## $P \leq 0,01$ em relação ao grupo “FliDot”.

Diferente dos MPs selvagens, na ausência de caspase-1 não houve produção de NO em resposta à infecção pela *S. Typhimurium* WT e Δ fliC, nem após a estimulação com flagelina exógena (Figura 21). Esses dados comprovam que a produção de NO dependente de flagelina requer a presença de caspase-1 e sugerem que a susceptibilidade apresentada pelos MPs deficientes em caspase-1 durante a infecção por *S. Typhimurium* possa ser explicada, em parte, pela deficiência na ativação da iNOS e conseqüentemente, na produção de NO.

Figura 21: Avaliação do papel da caspase-1 na produção de NO para o controle da infecção por *S. Typhimurium*. MPs provenientes de camundongos C57BL/6 ou caspase-1^{-/-} (3×10^5 células/poço) foram infectados com *S. Typhimurium* selvagem (WT) ou mutante, sem flagelina (Δ fliC) (MOI 0,1:1) por 1 hora na presença ou não do inibidor de iNOS, aminoguanidina (AG) [1 mM]. Após a infecção, as células foram tratadas com gentamicina [50 μg/ml] por 1 hora para eliminar as bactérias extracelulares. Após, as culturas foram lavadas e mantidas com gentamicina [5 μg/ml] por mais 24 horas na presença ou não de flagelina purificada inserida em vesículas lipídicas catiônicas (DOTAP) para inserção no citosol celular (FliDot) [1 μg/mL] proveniente da bactéria *S. Typhimurium*. Os sobrenadantes das culturas foram retirados para a dosagem da produção de NO pelo método de Griess. Os números representam a média $\pm$ desvio padrão de triplicatas. *** $P \leq 0,001$ em relação ao grupo controle; ### $P \leq 0,01$ em relação ao grupo “*S. Typhimurium* Δ fliC”.

5 DISCUSSÃO

A flagelina é capaz de induzir uma potente resposta imune a partir de seu reconhecimento extra ou intracelular mediado pelos receptores TLR5 e NAIP5/NLRC4, respectivamente, sendo este último capaz de recrutar e ativar a caspase-1. Os mecanismos efetores após a ativação da caspase-1 melhor caracterizados são o processamento das citocinas pró-inflamatórias IL-1 β e IL-18 e a morte celular por piroptose.

Neste trabalho, demonstramos que a caspase-1 também foi capaz de regular a expressão da enzima iNOS, em resposta à flagelina citosólica, tanto a nível transcripcional (Figura 7C) como traducional (Figura 7B) (**APÊNDICE A**) (120). Esta regulação ocorreu de maneira independente da molécula adaptadora *MyD88*, assim como das citocinas pró-inflamatórias IL-1 β e IL-18 e do fator de transcrição IRF-1, porém requereu a ativação do NF- κ B. Por fim, a regulação da iNOS pela caspase-1 parece envolver mecanismos epigenéticos, uma vez que este fenômeno envolve a clivagem de PARP-1, uma enzima associada ao DNA que regula a estrutura da cromatina, deixando-a mais compacta ou descondensada, o que dificulta ou facilita a ação dos fatores de transcrição (129).

O gene da iNOS contém sítios de ligação para os fatores de transcrição NF- κ B e IRF-1 (126, 127), os quais são conhecidamente ativados por estímulos induzidos por TLR ou IFN- γ , respectivamente. A flagelina citosólica, ao contrário do estímulo com IFN- γ , não foi capaz de induzir a ativação de IRF-1, no entanto, a ativação de NF- κ B por este estímulo foi similar ao encontrado em resposta a ligantes de TLR, como o LPS (ligante de TLR4) e a flagelina livre (ligante de TLR5). Ainda, a inibição da via do NF- κ B por adição de PDTC aboliu a expressão de iNOS induzida tanto por flagelina livre como por flagelina citosólica. Esses dados demonstram que ambas as vias de sinalização induzidas por flagelina podem levar à expressão da iNOS de maneira dependente de NF- κ B, no entanto, enquanto a flagelina livre utiliza a molécula adaptadora *MyD88*, os efeitos da flagelina citosólica requerem a protease caspase-1.

Curiosamente, a caspase-1 é necessária para a ativação da iNOS dependente de NF- κ B em resposta à flagelina citosólica mas é dispensável para a ativação desse fator de transcrição, descartando uma interação direta entre a caspase-1 e o NF- κ B. Sendo assim, o mecanismo mais provável para explicar o envolvimento da caspase-1 na ativação da iNOS pela flagelina citosólica seria o “*crosstalk*” de vias de sinalização simultâneas induzidas a partir da ativação da caspase-1 que poderiam culminar na expressão de iNOS.

Uma das possibilidades seria a influência das citocinas IL-1 β e IL-18, substratos da caspase-1 e conhecidos ativadores de iNOS (39). A influência da IL-1 β na ativação da iNOS foi demonstrada em células endoteliais do cérebro, em astrócitos em resposta ao IFN- γ e TNF-

α (124) e em macrófagos em resposta à ativação do inflamassoma NLRP3 por *Leishmania amazonensis*, *Leishmania braziliensis* e *Leishmania infantum chagasi* (74).

No entanto, o fato de macrófagos *MyD88*^{-/-} serem capazes de expressar iNOS em resposta a flagelina citosólica descarta a interferência de IL-1 β e IL-18, uma vez que a sinalização pelos receptores de IL-1 β e IL-18 (IL-1R e IL-18R) requerem a molécula adaptadora *MyD88*. Além disso, a neutralização de IL-1 β e IL-18 não foi capaz de inibir a expressão da iNOS em resposta à flagelina citosólica, confirmando a independência dessas citocinas na ativação de NF- κ B.

A transcrição gênica induzida por NF- κ B pode ser regulada por modificação pós traducional reversível do próprio NF- κ B (p.e acetilação), através do recrutamento de cofatores (CBP/p300), ou por modificações na estrutura da cromatina (135-137). Neste sentido, a enzima nuclear associada à cromatina, PARP-1, responsável por reparar danos no DNA, usa NAD⁺ (“*nicotinamide adenine dinucleotide*”) para sintetizar poly(ADP-ribose) (PAR) (138). A formação de polímeros PAR ocorre em resposta a estímulos mitogênicos e estresse genotóxico (139-142), e tem sido implicada em uma variedade de processos, tais como manutenção da estabilidade genômica, regulação da transcrição, metabolismo e morte celular (143).

No entanto, PARP-1 parece regular a transcrição de genes-alvo do NF- κ B de maneira independente de sua atividade enzimática. Hassa e colaboradores mostraram evidências de que PARP-1 age como um co-ativador do NF- κ B, através de sua interação direta com ambas as subunidades do NF- κ B, p50 e p65 (144, 145). Além da interação direta com fatores de transcrição, outros estudos mostram o papel de PARP-1 como regulador transcricional através de modificações na estrutura da cromatina.

Em condições fisiológicas PARP-1 regula negativamente a transcrição gênica por promover a compactação da cromatina, independente de sua atividade enzimática (146, 147). Em resposta a estímulos inflamatórios, PARP-1 sofre clivagem por caspases, e esta clivagem promove a sua liberação da cromatina. Isto resulta na descondensação da cromatina, tornando o DNA mais acessível para a transcrição gênica (129, 148). Entretanto, pouco se sabe a respeito dos mecanismos envolvidos na clivagem de PARP-1. Sabe-se que PARP-1 é clivada e inativada durante a apoptose por caspases executoras (148).

Neste trabalho nós verificamos que a caspase-1 foi indispensável para a expressão da iNOS em resposta à flagelina citosólica. Além disso, a clivagem de PARP-1 também parece estar envolvida na expressão desta enzima, uma vez que MDMO D214N apresentaram

expressão de iNOS comprometida quando comparados aos MDMO selvagens. Isto sugere um envolvimento da caspase-1 na clivagem de PARP-1 em resposta à flagelina citosólica. Ainda, a caspase-7, mas não caspase-11 também parece estar envolvida na expressão da iNOS. Juntos estes dados sugerem uma regulação da expressão da iNOS por caspase-1 e caspase-7, o que parece ser específico para este gene, uma vez que a expressão de I κ B- α não foi alterada na ausência de PARP-1 clivável, caspase-1 ou caspase-7.

Nesse sentido, Malireddi e colaboradores demonstraram que a clivagem de PARP-1 durante a piroptose induzida por LPS, Pam3 ou manana em combinação com ATP ou nigericina foi dependente da ativação do inflamassoma NLRP3 e do processamento das caspases-1 e 7 (149). Erenér e colaboradores descreveram de maneira mais detalhada a clivagem de PARP-1 a partir da ativação do inflamassoma NLRP3 (129). Os autores mostraram que a estimulação dos macrófagos com LPS induz a ativação da caspase-1 dependente do inflamassoma NLRP3/ASC. A caspase-1 ativada cliva a caspase-7, a qual é translocada para o núcleo e recrutada para o sítio inicial de transcrição de alguns genes alvo. Nesse sítio, a caspase-7 cliva PARP-1, e esta clivagem resulta na liberação de PARP-1 da cromatina, permitindo sua descondensação e, induzindo assim, a expressão gênica de alguns genes alvo dependentes de NF- κ B como *il-6*, *csf2* e *lif*, mas não *ip10*, (129).

Ainda que nossos dados não comprovem a clivagem de PARP-1, o fato de a flagelina citosólica induzir a ativação de caspase-1 e caspase-7 via o inflamassoma NAIP5/NLRC4 sugere a possibilidade da ação das mesmas na clivagem de PARP-1 e seu envolvimento na indução de iNOS dependente de NF- κ B.

A ativação de iNOS mediada por clivagem de PARP-1 foi previamente demonstrada, onde macrófagos D214N falharam em induzir a expressão desta enzima após o estímulo com LPS sozinho ou em combinação com IFN- γ (148). Esses dados em conjunto, sugerem um papel da caspase-1 em modificações epigenéticas, as quais podem estar envolvidas na regulação de genes dependentes de NF- κ B em resposta a estímulos distintos. No entanto, o envolvimento da caspase-1 a partir da ativação de inflamassomas na clivagem de PARP-1, necessita maiores elucidações.

Finalmente, verificamos que a ativação de iNOS induzida por inflamassomas está envolvida no controle das infecções por *Legionella pneumophila*, *Salmonella Typhimurium* e *T. cruzi* (**APÊNDICE C**) (75). A influência da ativação da iNOS dependente de flagelina e independente de *MyD88* durante a infecção por *L. pneumophila* foi verificada pelo fato de que a adição de AG induziu um aumento da carga bacteriana em macrófagos. A *S. Typhimurium*

WT induziu a produção de NO de maneira dependente de flagelina e caspase-1. Mais uma vez, a inibição de iNOS pela adição de aminoguanidina reverteu o efeito do NO no controle da infecção por macrófagos.

A piroptose vem sendo descrita como um importante mecanismo efetor no controle de *S. Typhimurium* por macrófagos (96). Nesse sentido, Miao e colaboradores confirmaram o papel da piroptose como mecanismo efetor no controle da infecção por *Salmonella* demonstrando pela primeira vez o papel da piroptose *in vivo* no controle de patógenos intracelulares. A piroptose do macrófago dependente de NLRC4 e da caspase-1 resultou na liberação das bactérias para o meio extracelular, deixando-as expostas à fagocitose por neutrófilos, que realizam o “*killing*” das mesmas, por um mecanismo dependente de espécies reativas do oxigênio.

Apesar do importante papel da piroptose, a ativação de iNOS no controle da infecção por *S. Typhimurium* parece ser aditivo à piroptose do macrófago, uma vez que esta via parece controlar a replicação bacteriana pelos macrófagos remanescentes da rápida morte celular. Interessante, a ativação de iNOS também parece um mecanismo efetor mediado pelo inflamassoma NLRP3 (75). Demonstramos que o *T. cruzi* ativa NLRP3, o qual induz a produção de NO de maneira dependente de caspase-1 e independente de IL-1R (75). Vale ressaltar que animais deficientes em NLRP3 e caspase-1, mas não animais deficientes em IL-1R, apresentam altos níveis de parasitemia na fase aguda da infecção pelo *T. cruzi* e alto índice de infecção em macrófagos, demonstrando que a via de indução de iNOS por inflamassomas também está envolvida no controle de protozoários.

Pouco se sabe sobre o envolvimento da clivagem de PARP-1 durante infecções bacterianas. Nesse contexto, existem evidências da clivagem de PARP-1 durante a infecção com *S. Typhimurium*, a qual foi dependente da ativação do NLRC4, caspase-1 e caspase-7 (149). Estes achados, juntamente com o envolvimento de PARP-1 na regulação de genes dependentes de NF- κ B (129), como iNOS (148, 150), corroboram com o envolvimento de PARP-1 na regulação da iNOS induzida por flagelina citosólica nos modelo de infecção acima citados, ainda que a sua ação direta necessite ser comprovada.

A descrição da ativação da iNOS como um mecanismo efetor adicional no controle das infecções intracelulares mediado por inflamassomas, assim como a sugestão do envolvimento dessas plataformas em alterações epigenéticas são as principais contribuições deste trabalho, as quais se somam a outros mecanismos não convencionais que vêm sendo descritos para o inflamassoma NAIP5/NLRC4, tais como a liberação de mediadores lipídicos

(116) e indução da maturação do fagossoma dependente de caspase-7, com impacto no controle da infecção por *L. pneumophila* (117, 118).

6 CONCLUSÃO

Nossos dados, em conjunto, nos permitem propor um modelo de uma nova via para a ativação da iNOS (Figura 22), onde (1) a flagelina citosólica induz ativação do inflamassoma NAIP5/NLRC4 e, paralelamente, a ativação de NF- κ B (por uma via ainda desconhecida), (2) o que resulta na ativação da caspase-1 e caspase-7, as quais seriam responsáveis por induzir a clivagem de PARP-1 e (3) esta clivagem resultaria na liberação de PARP-1 da cromatina, tornando-a mais acessível para a transcrição do gene da iNOS, dependente de NF- κ B. Esses dados estão no manuscrito em preparação *"Role of Poly(ADP-ribose)polymerase-1 cleavage in the iNOS activation by inflammasomes."*

Figura 22: Modelo proposto para regulação molecular da via de ativação da iNOS pelo inflamassoma NAIP5/NLRC4 em resposta à flagelina citosólica.

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APÊNDICES

APÊNDICE A

A Novel Pathway for Inducible Nitric-oxide Synthase Activation through Inflammasomes^{*S}

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Innate immune recognition of flagellin is shared by transmembrane TLR5 and cytosolic Nlrc4 (NOD-like receptor family CARD (caspase activation recruitment domain) domain containing 4)/Naip5 (neuronal apoptosis inhibitory protein 5). TLR5 activates inflammatory genes through MYD88 pathway, whereas Nlrc4 and Naip5 assemble multiprotein complexes called inflammasomes, culminating in caspase-1 activation, IL-1 β /IL-18 secretion, and pyroptosis. Although both TLR5 and Naip5/Nlrc4 pathways cooperate to clear infections, little is known about the relative anti-pathogen effector mechanisms operating through each of them. Here we show that the cytosolic flagellin (FLA-BSDot) was able to activate iNOS, an enzyme previously associated with TLR5 pathway. Using *Nlrc4*- or *Naip5*-deficient macrophages, we found that both receptors are involved in iNOS activation by FLA-BSDot. Moreover, distinct from extracellular flagellin (FLA-BS), iNOS activation by intracellular flagellin is completely abrogated in the absence of caspase-1. Interestingly, IL-1 β and IL-18 do not seem to be important for FLA-BSDot-mediated iNOS production. Together, our data defined an additional anti-pathogen effector mechanism operated through Naip5 and Nlrc4 inflammasomes and illustrated a novel signaling transduction pathway that activates iNOS.

Innate immune system employs intracellular and extracellular pattern recognition receptors (PRRs)² to detect microbial products through the identification of pathogen-associated molecular patterns (PAMPs). Among many families of PRRs,

Toll-like receptors (TLRs) and NOD-like receptors (NLRs) have been described as central elements in triggering innate immune responses (1, 2).

TLRs and NLRs have been shown to activate distinct biochemical pathways, resulting in diverse mechanisms of resistance to pathogens. These mechanisms may act independently or may cooperate to the resolution of infections. TLRs primarily activate transcriptional factors such as NF- κ B and IFN-responsive factors (IRF) (3, 4), culminating in the production of effector molecules such as inflammatory cytokines and type I interferons. The contribution of these pathways to resistance to infection is manifold: recruitment of other inflammatory cells, production of cytotoxic and cytostatic molecules, and activation of adaptive immune response. NLRs, on the other hand, although also capable of activating NF- κ B, have a peculiar ability of initiating an inflammatory response through caspase-1 activation via the assembly of a multiprotein complex called inflammasome (5–7). Besides the well known role of caspase-1 on IL-1 β and IL-18 maturation and secretion, this protease has also been described to induce macrophages death by a process known as pyroptosis (6, 7). Importantly, pyroptosis has been ascribed as a mechanism of host immune response against certain pathogens, such as *Salmonella*, *Legionella*, *Pseudomonas*, and *Shigella*, although it is still not clear how pyroptosis effectively contributes for pathogen clearance (7).

The recognition of PAMPs by TLRs and NLRs occur independently. However, there are cases where a certain PAMP can be recognized by both TLRs and NLRs. Flagellin, the monomeric subunit of flagella present in several Gram-negative and Gram-positive bacteria is a good example of this redundancy. Extracellular flagellin is recognized by transmembrane TLR5 (8), whereas cytosolic sensing of flagellin requires Naip5 (neuronal apoptosis inhibitory protein 5) and Nlrc4 (NLR family CARD (caspase activation recruitment domain) domain-containing 4), also called Ipaf (ICE-(IL-1-converting enzyme) protease activating factor), both members of the NLR family (9–13). It was recently demonstrated that both Nlrc4 and Naip5 are required for signaling in response to the 35-amino acid of a peptide located on the C-terminal D0 domains of *Legionella pneumophila* and *Salmonella typhimurium* flagellins and that this region of flagel-

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² The abbreviations used are: PRR, pattern recognition receptor; NLR, NOD-like receptor; TLR, Toll-like receptor; IRF, IFN-responsive factors; Naip5, neuronal apoptosis inhibitory protein 5; CARD, caspase activation recruitment domain; iNOS, inducible nitric-oxide synthase; BMDM, bone marrow-derived macrophage; PM, peritoneal macrophage; z-VVAD-fmk, benzylloxycarbonyl-VVAD-fluoromethyl ketone; PAMP, pathogen-associated molecular pattern; DOT, DOTAP (lipidic vesicles).

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lin is distinct from the D1 domain sensed by TLR5 (11). Importantly, TLR5 engagement by flagellin leads to NF- κ B activation, whereas the recognition of flagellin by Naip5 and Nlrc4 induces the assembly of inflammasomes with the recruitment and activation of pro-caspase-1 (7).

Regardless the particularities between intracellular and extracellular receptor pathways, the relative contribution of TLR5 and Naip5/Nlrc4 inflammasomes to host resistance to infection is still not clear. Here, by using a purified flagellin, we defined an additional anti-pathogen effector mechanism operating through Naip5 and Nlrc4 inflammasomes that was previously associated with TLR pathway (14, 15), namely the activation of inducible nitric-oxide synthase (iNOS), the enzyme responsible for nitric oxide (NO) production (16). Interestingly, distinct from extracellular flagellin, iNOS activation by intracellular flagellin is completely abrogated in the absence of caspase-1 but not with neutralization of IL-1 β and/or IL-18. Importantly, this effect is preserved in Myd88-deficient macrophage, ruling out the participation of TLR5. Together, our data indicated that by using distinct pathways, TLR and NLR systems can operate with a certain level of redundancy, which might improve the host resistance against infections.

EXPERIMENTAL PROCEDURES

Mice—6–8-Week-old wild-type (WT) C57BL/6 and Myd88-deficient female mice were bred in our animal facilities at the University of São Paulo. Nlrc4- and caspase-1-deficient mice were previously described (17) and backcrossed for six generations with C57BL/6. Mice with 99.2% A/J genome harboring the wild-type *Lgn1* allele derived from C57BL/6 were constructed by backcrossing a progeny of C57BL/6 $\times$ A/J with A/J for 6 generations as described in supplemental Fig. 1. Briefly, each generation was screened by PCR using 2 pairs primers that amplify fragments within microsatellite regions flanking the *Lgn1* locus, thus, differentiating C57BL/6 from A/J regions of chromosome 13 (13). The resultant mice (F7 AJxB6 *Lgn1*^{B6/AJ}) are heterozygous for the dominant *Lgn1* locus and show full Naip5 functions. In contrast, littermate controls homozygous for the A/J allele (F7 AJxB6 *Lgn1*^{AJ/AJ}) harbors two copies of the defective Naip5 allele from A/J mice (13, 18). Naip5 functions of the F7 mice were monitored by measuring restriction of *L. pneumophila* multiplication in macrophages (supplemental Fig. 1). These mice were bred and constructed in the animal facilities at the University of São Paulo-Ribeirão Preto. Animal studies used protocols approved by the University of São Paulo Committee on Use and Care of Animals.

Preparation of Mouse Macrophages—Bone marrow-derived macrophages (BMDM) were prepared as described (19). Bone marrow cells were cultured for 7 days at 37 °C in 5% CO₂ in RPMI 1640 medium supplemented with 30% L929 supernatant containing macrophage-stimulating factor, 10% heat-inactivated fetal calf serum (FCS), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 units/ml streptomycin and penicillin. All supplements were purchased from Invitrogen. Peritoneal macrophages (PM) were obtained as described (20). Briefly, peritoneal lavage was collected 4 days after injection of mice with 1% starch solution (Sigma). Adherent macrophages

were prepared by overnight culture in RPMI supplemented with 10% heat inactivated FCS and antibiotics, resulting in a population that comprises 97–99% of F4/80⁺ cells (data not shown).

Stimulation of Macrophages with Flagellin—PM and BMDM (3×10^5 cells seeded in 96-well plates) were cultured for 5–24 h at 37 °C in 5% CO₂ in RPMI 1640 medium supplemented with 3% FCS, amino acids, and antibiotics in the presence of purified flagellin from *Bacillus subtilis* (FLA-BS) or *S. typhimurium* (FliC) (Invivogen) (1–6 μ g/ml) in its free form or inserted into DOTAP (Roche Diagnostics), a cationic lipid formulation that permits its delivery to cell cytosol (21). DOTAP was used accordingly to the manufacturer's instructions. Briefly, DOTAP (50 μ l) was incubated for 15 min in serum-free media with 9 μ g of purified flagellin. After incubation, 2.95 ml of RPMI 1640 medium was added, and an aliquot of 200 μ l was added to 3×10^5 macrophages (for 3 μ g/ml final concentration). In some cultures endogenous caspase-1 was blocked with specific inhibitor z-YVAD-fmk (Calbiochem) at a final concentration of 2–50 μ M, and IL-1 β and IL-18 were neutralized by adding anti-mouse IL-1 β or IL-18 (AbD Serotec) at final concentration of 1 and 5 μ g/ml, respectively.

Measurement of Cytokines and Nitric Oxide—IL-1 β and IL-18 were measured in culture supernatants by enzyme-linked immunoabsorbent assay (ELISA) (kits from BD Biosciences) following the manufacturer's instructions. Culture supernatants were also assayed for NO by the Griess reaction. Briefly, 50 μ l of supernatant was incubated with 50 μ l of Griess reagent for 5 min at ambient temperature, and the NO₂ concentration was determined by measuring the optical density at 550 nm in reference to a standard NaNO₂ solution.

Western Blot—Western blot was performed as previously described (22). Cells were harvested, washed once in ice-cold PBS, lysed directly in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 2.5% β -mercaptoethanol), and boiled for 5 min. Samples were resolved under reducing conditions for 2 h at 15 V in SDS-polyacrylamide gels. Proteins were then transferred onto PVDF membranes for 1 h in a semi-dry system. Blots were blocked for 3 h in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween) containing 0.1% sodium azide and 5% nonfat dried milk and then probed with polyclonal antibodies to iNOS (1:1000) and β -actin (1:20,000) overnight (Santa Cruz Biotechnology). Reactions were detected with suitable secondary antibody (1:2000) conjugated to horseradish peroxidase (The Jackson Laboratory and Amersham Biosciences) using enhanced chemiluminescence solution (Pierce).

Real-time Quantitative PCR—Total RNA was isolated using TRIzol RNA isolation. Concentration and purity of the RNA were verified by spectrophotometer NanoDROP nd-1000. The absorbance ratio at 260/280 nm of all samples ranged from 1.8 to 2.0, indicating they all were contaminant-free. The RNA integrity has been assayed on a 1% agarose gel stained with ethidium bromide. cDNA was generated from 3 μ g of total RNA using oligo(dT) and the Moloney murine leukemia virus reverse transcriptase RNase H (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR analysis

was performed with the Taqman System (Applied Biosystems) (catalog no. Mm0130989_m1; Nos2). To standardize all the samples, the housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (catalog no. 4352932) was used as an endogenous reference gene (TaqMan Endogenous Control concentration-limited primer, Applied Biosystems).

Macrophage Infection with *L. pneumophila*—BMDMs from C57BL/6- and *Myd88*-deficient mice (2×10^5 cells seeded in 24-well plates) were infected with wild-type or flagellin-deficient mutant FlaA *L. pneumophila* at a multiplicity of infection of 0.015 for 24, 48, and 72 h in the presence or absence of 0.1 mM aminoguanidine (selective inhibitor of iNOS (23); SIGMA). Macrophages were lysed in sterile H₂O, and the cell lysates were combined with the tissue culture supernatant from that well, ensuring that all bacteria in the well would be counted. Serial dilutions from each well were plated on charcoal yeast extract agar plates. Colony-forming units were assessed by counting of bacterial colonies present after 96 h of incubation at 37 °C.

Statistical Analysis—Experiments were performed always in duplicate or triplicate at least three times. Data are presented as the mean values $\pm$ S.D. Statistical analysis of the data were carried out using one-way analysis of variance and Tukey as a post-test. Differences between experimental groups were considered significant for $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

RESULTS

Recognition of Extracellular or Cytosolic *B. subtilis* Flagellin Differentially Stimulates Macrophages—We first characterized the macrophage responses to extracellular (FLA-BS) or intracellular (FLA-BSDot) flagellin. PMs were stimulated with LPS, FLA-BS, or FLA-BSDot (1–6 μ g/ml), and the secretion of IL-6, IL-1 β , and macrophage lysis (monitored by LDH release) were determined respectively as surrogate markers of NF- κ B and caspase-1 activation. As expected, only FLA-BSDot induced IL-1 β maturation and secretion and caspase-1-dependent macrophage lysis in a dose-dependent manner (Fig. 1, A and B, and supplemental Fig. 2). Conversely, high levels of IL-6 were only observed in the presence of FLA-BS and LPS (Fig. 1C). DOTAP by itself did not induce either IL-1 β or IL-6 secretion (Fig. 1). Also, as expected, LPS alone was not able to induce IL-1 β secretion (Fig. 1). Moreover, as expected, the level of IL-1 β secreted in response to FLA-BSDot stimulation alone is markedly lower than that observed after LPS priming (data not shown). IL-18 was not detected in these conditions (data not shown). Our results are in agreement with the literature showing that TLRs and NLRs drive macrophage activation in different ways.

Cytosolic Flagellin Induces iNOS Expression by Macrophages—FLA-BS stimulation of PMs resulted in iNOS expression, which correlated with NO secretion (Fig. 2A). However, unexpectedly, iNOS expression and NO production were also observed when PMs were stimulated with FLA-BSDot. Similar results were also observed using adherent BMDM (Fig. 2B), indicating that FLA-BSDot up-regulates iNOS activity in different macrophage populations. Therefore, our data show for the first time that both extracellular and cytosolic flagellins are able to activate iNOS.

FIGURE 1. Differential activation of macrophages by extracellular and cytosolic *B. subtilis*. PM from C57BL/6 mice ($3 \times 10^5/200 \mu$ l) were stimulated with 1, 3, and 6 μ g/ml LPS or purified flagellin from *B. subtilis* in its free form (FLA-BS) or inserted into DOTAP, a cationic lipid vesicles that permits its delivery to cell cytosol (FLA-BSDot). The release of IL-1 β (A), IL-6 (C), and macrophage lysis (B) were determined in culture supernatants at 24 h by ELISA and at 6 h by LHD release, respectively. Numbers represent the mean $\pm$ S.D. of triplicate samples. Data are representative of three independent experiments.

Nlrc4 and Naip5 Participate in FLA-BSDot-induced iNOS Expression—Because cytosolic flagellin has been shown to be sensed by Nlrc4 and Naip5 inflammasomes (9–13), we next

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FIGURE 2. Cytosolic flagellin induces iNOS activation. PM (A) or BMDM (B) from C57BL/6 mice ($3 \times 10^5/200 \mu\text{l}$) were stimulated with $3 \mu\text{g/ml}$ purified flagellin from *B. subtilis* in its free form (FLA-BS) or inserted into DOTAP, a cationic lipid vesicle that permits its delivery to cell cytosol (FLA-BSDot). After 24 h iNOS expression was evaluated in cell extracts by Western blot, and nitrite concentrations in culture supernatants were determined by Griess method. Numbers represent the mean $\pm$ S.D. of triplicate samples. *, $p < 0.05$; **, $p < 0.01$ when compared with control group. Data are representative of three independent experiments.

determined their contribution to FLA-BSDot-induced iNOS activation by using BMDM from mice deficient in each of these molecules. As shown in Fig. 3, FLA-BSDot-induced iNOS expression in *Nlr4*^{-/-} BMDM was significantly lower than observed in WT C57BL/6 cells, but not absent, suggesting that *Nlr4* is important but not essential for iNOS expression in

FIGURE 3. *Nlr4* and *Naip5* are required for cytosolic flagellin-induced iNOS expression. BMDM from wild-type and *Nlr4*^{-/-} C57BL/6 mice (A and C) or *Naip5*-competent (*Lgn1*^{B6/AJ}) and *Naip5*-deficient (*Lgn1*^{AJ/AJ}) mice (B and D) were stimulated with $3 \mu\text{g/ml}$ purified flagellin from *B. subtilis* inserted into DOTAP, a cationic lipid vesicle that permits its delivery to cell cytosol (FLA-BSDot). After 24 or 5 h, iNOS expression was evaluated in cell extracts by Western blot (A and B) or at transcriptional levels by real-time RT-PCR (C and D), respectively. Data are representative of three independent experiments. Numbers represent the mean $\pm$ S.D. of triplicate samples. #, $p < 0.05$; ###, $p < 0.001$ when compared with control group. *, $p < 0.05$; **, $p < 0.01$ when compared with FLA-BSDot group from deficient mice. Ctrl, control.

response to cytosolic flagellin (Fig. 3A). A similar pattern of results was observed at transcriptional levels by real-time RT-PCR, where a significant decrease in the iNOS expression was observed in *Nlr4*^{-/-} BMDM when compared with WT C57BL/6 cells (Fig. 3C). The *Naip5* allele from A/J mouse strain is partially defective due to a series of amino acid substitutions (18, 24). Thus, we took advantage of these mutations and generated a pair of mice with a similar genetic background (A/J) that differentially expresses functional (*Lgn1*^{B6/AJ}) or mutant (*Lgn1*^{AJ/AJ}) *Naip5* allele (see “Experimental Procedures” and supplemental Fig. 1). FLA-BSDot stimulates *Naip5*-competent (*Lgn1*^{B6/AJ}) but not *Naip5*-deficient (*Lgn1*^{AJ/AJ}) BMDM to up-regulate iNOS protein levels (Fig. 3B). Notwithstanding the absence of iNOS protein in *Naip5*-deficient (*Naip5*^{AJ/AJ})

FIGURE 4. Caspase-1 is required for cytosolic flagellin-induced iNOS expression. PMs or BMDM from C57BL/6 or caspase-1 $^{-/-}$ mice were stimulated with 3 μ g/ml purified free (FLA-BS) or cytosolic (FLA-BSDot) flagellin from *B. subtilis* (A, B, and E) or flagellin from *S. typhimurium* (FliC or FliCDot, respectively) (C and D) in the presence or absence of z-YVAD-fmk (2 μ M). After 24 or 5 h, iNOS expression was evaluated in cell extracts by Western blot (B, C, and D) or at transcriptional levels by real-time RT-PCR (E), respectively. IL-1 β secretion was determined in culture supernatants by ELISA (A). Numbers represent the mean $\pm$ S.D. of triplicate samples. ***, $p < 0.001$ when compared with control group. ###, $p < 0.001$ when compared with FLA-BSDot group from caspase-1 $^{-/-}$ BMDM. Data are representative of three independent experiments.

BMDM, low levels of iNOS transcripts were still observed by RT-PCR (Fig. 3D). These data indicate that functional Naip5 plays a major role for FLA-BSDot-induced iNOS. Similar results were obtained with flagellin purified from *S. typhimurium* (data not shown), a well characterized Naip5 and Nlrc4 agonist (11). Taken together, our results established that flagellin from *B. subtilis* is also a Naip5 and Nlrc4 agonist and that both receptors are involved in the up-regulation of iNOS.

FLA-BSDot-induced iNOS Expression Is Dependent on Caspase-1—Because cytosolic flagellin recognition by inflammasomes results in caspase-1 activation (9–13), we asked whether caspase-1 was involved in FLA-BSDot-induced iNOS. Therefore, macrophages were cultured with FLA-BS or FLA-BSDot in the presence or absence of z-YVAD-fmk, a synthetic peptide that irreversibly inhibits caspase-1. As expected, z-YVAD-fmk inhibited IL-1 β secretion in response to FLA-BSDot (Fig. 4A). Importantly, it also abrogated the effect of

FLA-BSDot on iNOS expression (Fig. 4B). However, no effect of z-YVAD-fmk was observed in FLA-BS-induced iNOS expression (Fig. 4B). The same results were obtained with FliC and FliCDot from the Gram-negative *S. typhimurium*, showing that the effect was not restricted to flagellin from the Gram-positive *B. subtilis* (Fig. 4C). The requirement of caspase-1 for iNOS induction in response to cytosolic flagellin was confirmed at protein (Fig. 4D) and transcriptional levels (Fig. 4E) in caspase-1 $^{-/-}$ BMDM, demonstrating that extracellular and intracellular flagellin use different pathways to activate iNOS.

FLA-BSDot-induced iNOS Expression Does Not Depend on IL-1 β and IL-18—Because caspase-1 activation induced by cytosolic flagellin was shown to result in the secretion of IL-1 β and IL-18 (6), we next determined whether these cytokines participate in iNOS up-regulation. Macrophages were cultured with FLA-BSDot in the presence or absence of neutralizing anti-IL-1 β and anti-IL-18 antibodies. Anti-IL-1 β antibodies sequestered IL-1 β present in FLA-BSDot-stimulated PM culture supernatants (Fig. 5A) but did not inhibit FLA-BSDot-induced iNOS expression (Fig. 5B). On the contrary, if anything, anti-IL-1 β antibodies seem to slightly increase iNOS expression. The independence of IL-1 β for iNOS activation in response to FLA-BSDot was supported by data obtained with

BMDM, which up-regulated iNOS expression (Fig. 5C) without any detectable IL-1 β production (data not shown). Similar results were obtained with neutralizing anti-IL-18 or the combination of both anti-IL-1 β and anti-IL-18 antibodies (Fig. 5, B and C). It is important to emphasize that IL-18 was not found in the supernatants from FLA-BSDot-stimulated PMs or BMDMs. Together, our data indicate that cytosolic flagellin induces iNOS activation by a mechanism dependent on Nlrc4/Naip5-caspase-1 axis but independent of IL-1 β and IL-18.

FLA-BSDot-induced iNOS Expression Is Independent of MYD88—Because IL-1 and IL-18 receptors as well as TLR5 signaling depend on MYD88 adaptor molecule (3, 8, 25), PMs from *myd88* $^{-/-}$ mice were stimulated with FLA-BSDot to evaluate the influence of this pathway on cytosolic flagellin-induced iNOS activation. As shown in Fig. 6, FLA-BSDot-mediated iNOS expression and LDH release are also observed in the absence of MYD88, supporting our claim that these responses are indepen-

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FIGURE 5. Induction of iNOS expression by cytosolic flagellin is independent of IL-1 β and IL-18. PMs (A, B) and BMDM (C) from C57BL/6 were stimulated with 3 μ g/ml purified free (FLA-BS) or cytosolic (FLA-BSDot) flagellin from *B. subtilis* in the presence or absence of neutralizing anti-IL-1 β (1 μ g/ml) or anti-IL-18 (5 μ g/ml). After 24 h, iNOS expression was evaluated in cell extracts by Western blot, and IL-1 β secretion was determined in culture supernatants by ELISA. Numbers represent the mean $\pm$ S.D. of triplicate samples. ***, $p < 0.001$ when compared with control group. ###, $p < 0.001$ when compared with FLA-BSDot in the presence of neutralizing anti-IL-1 β . Data are representative of three independent experiments.

dent of IL-1 and IL-18 receptors and TLR5 signaling. Moreover, the flagellin-induced TLR-independent iNOS-activation seems to participate in the restriction of *L. pneumophila* infection. Selective inhibition of iNOS by aminoguanidine increased at the same extent the growth of wild-type *L. pneumophila* in both WT and *myd88*^{-/-} C57BL/6 macrophages (supplemental Fig. 4A). However, aminoguanidine had no effect on the growth of the flagellin-deficient mutant FlaA *L. pneumophila* in either macrophage (supplemental Fig. 4B). Together, these results

FIGURE 6. MYD88 is not required for cytosolic flagellin-induced macrophage responses. PMs from *Myd88*^{-/-} mice were stimulated with 3 μ g/ml purified cytosolic flagellin from *B. subtilis* (FLA-BSDot). After 6 and 24 h macrophage lysis was monitored by LDH release in culture supernatants (B), and iNOS expression was evaluated in cell extracts by Western blot (A), respectively. Data are representative of three independent experiments.

indicate that NO induced by flagellin through a TLR-independent pathway participate in the control of *L. pneumophila* infection by macrophages.

DISCUSSION

Flagellin is one of the rare protein structures sensed by the innate immune system and can activate both TLR and NLR receptors. Extracellular flagellin is recognized by transmembrane TLR5 (8), whereas cytosolic flagellin is sensed by Naip5 and Nlr4 (9–13, 26, 27). Interestingly, TLR5 seems to broadly recognize flagellated bacteria, whereas sensing of Nlr4 and Naip5 depends on specialized transport systems present in virulent bacteria, such as the type III secretion systems of *Salmonella* (10, 27) and the type IV (T4SS) of *Legionella* (9, 13). These secretion systems allow the delivery of bacterial products directly into the host cell cytoplasm. In experimental models, Nlr4- and Naip5-induced caspase-1 activation can be achieved by delivering purified flagellin into the host cell cytosol by means of cationic liposomes or by transducing cells with appropriated gene-expression systems (10, 27, 28). Consistent with this view, TLR5 stimulation primarily leads to the activation of microbicidal effector mechanisms that are supposedly effective in controlling less

aggressive, non-pathogenic bacterial infection. In contrast, a more dramatic effector mechanism consisting of a Nlrc4/Naip5-caspase-1-induce inflammatory cell death is observed only in infections with virulent bacteria (7).

Therefore, the existence of different PRRs capable of sensing the same agonist or different agonists present on the same pathogen is obviously beneficial to the host. The combined action of distinct, non-redundant, specialized responses should lead to a synergistic, more robust immunity against potential pathogens. Extracellular and intracellular recognition of flagellin induces such distinct macrophage responses. High levels of IL-6 were observed only with FLA-BS, whereas IL-1 β secretion and pyroptosis were detected only with FLA-BSDot (Fig. 1 and supplemental Fig. 2). However, as the expression of PRRs is not ubiquitous and, therefore, not all host cells are equally prepared to properly recognize and handle potential pathogens, it should be interesting for the host immunity to have different PRR family sharing at least some microbicidal mechanisms.

iNOS activation by TLRs can be considered one of the major effector mechanism for controlling intracellular pathogens (29). TLRs are able to recruit NF- κ B and IRF transcription factors, both necessary to induce iNOS expression and activation (30, 31). In contrast, Nod1 and Nod2 are the only NLR members known to activate NF- κ B (32), resulting in the activation of a large repertoire of genes, including iNOS (33). Nlrps, Naip5, and Nlrc4 inflammasomes, on the other hand, mediate caspase-1 activation and the consequent IL-1 β and IL-18 secretion and/or a programmed cell death called pyroptosis (1, 7, 34). As macrophage activation by cytosolic flagellin occurs independently of TLR5 (10, 27), our data demonstrating iNOS up-regulation by FLA-BSDot (Fig. 2) highlights the plasticity of innate immunity recognition strategies, adding a role for NO on inflammasome-mediated microbicidal pathways.

Despite the fact that both Nlrc4 and Naip5 can induce caspase-1 activation, IL-1 β secretion, and pyroptosis in response to *L. pneumophila* and *S. typhimurium* flagellin, it is still obscure how exactly Nlrc4 and Naip5 individually participate in the recognition of flagellin and in the assembly of the inflammasomes. Nlrc4 can bind directly to pro-caspase-1 via a CARD-CARD homotypic interaction, but the molecular partner(s) of Naip5 remains to be solved. Interestingly, Naip5 do not participate in every function of the Nlrc4-inflammasome. Nlrc4 has been shown to be crucial in host defense for a number of pathogens such as *Salmonella*, *Legionella*, *Pseudomonas*, and *Shigella*, and its genetic defect cannot be substituted by a functional Naip5 (9–12, 17, 35–37). On the other hand, Naip5 is indispensable for the resistance to *L. pneumophila* infection (13). Interestingly, FLA-BSDot-induced iNOS expression was significantly reduced in Nlrc4-deficient macrophages and completely abolished in the absence of a functional Naip5 (Fig. 3). By using highly sensitive techniques such as real-time RT-PCR, we were able to detect a slight transcriptional induction of iNOS in the mice harboring a partially mutant A/J Naip5 allele and still significantly less when compared with Nlrc4-deficient macrophages. The partial functionality of Naip5 from A/J mice (11) could

account for the observed flagellin-dependent iNOS mRNA induction, an effect that possibly would not be observed in Naip5^{−/−} mice. Another possible explanation for these results is that Naip5 plays an additional Nlrc4-independent role in the post-transcriptional regulation of iNOS.

Importantly, we show here for the first time that, distinct from free flagellin, the activation of iNOS by cytosolic flagellin via Nlrc4/Naip5 is dependent on caspase-1 (Fig. 4). The genetic or pharmacological inactivation of caspase-1 resulted in the abrogation of iNOS protein and mRNA expression in response to FLA-BSDot. Caspase-1 promotes the release of IL-1 β and IL-18, which in turn can activate iNOS (25). In fact, it was recently shown that IFN- γ - and TNF- α -induced iNOS activation in astrocytes and cerebral endothelial cells is dependent on caspase-1-mediated IL-1 β secretion (38). However, our data show that neutralization of IL-1 β and/or IL-18 with specific antibodies resulted in a rather increased iNOS expression by FLA-BSDot, ruling out the requirement for these cytokines in flagellin-mediated iNOS up-regulation (Fig. 5). These observations are reinforced by the fact that *myd88*^{−/−} macrophages are able to express iNOS in response to cytosolic flagellin (Fig. 6) besides their deficiency in both IL-1 and IL-18 receptor, signaling pathways. Also, the observed up-regulation of iNOS cannot be attributed to unspecific macrophage activation as anti-IL-1 β - and anti-IL-18-neutralizing antibodies were not able to activate iNOS in the absence of flagellin (Fig. 5C). Moreover, anti-IL-1 β and anti-IL-18 have no effect on IL-6 produced in response to FLA-BSDot, showing their particular involvement on iNOS modulation (supplemental Fig. 3). These data indicate that IL-1 β and IL-18 are not required for iNOS activation in response to FLA-BSDot but do not discard their participation in some aspects of iNOS regulation.

Because the iNOS promoter contains binding sites for NF- κ B and IRF-1 (30, 31), additional investigation is required to elucidate whether the up-regulation of iNOS by caspase-1 involves the activation of either or both transcription factors. Recently, it was demonstrated that IRF-1 and IRF-8 transcription factors seem to be regulated during Naip5 and Nlrc4-mediated sensing of *L. pneumophila* (39). Moreover, these authors observed that, in the absence of flagellin, there is a defective activation of *IL-12p40* and *iNOS*, the IRF-1 and IRF-8 transcriptional targets. Despite the clear evidences of Nlrc4 and Naip5-mediated caspase-1 and IRF-1/IRF-8 activation, it is not clear whether these events are correlated.

Additionally, although Nlrc4 can directly interact and activate caspase-1 via its N-terminal CARD domain (40, 41), the activation of caspase-1 by Naip5 possibly requires interaction with other proteins since Naip5 possesses a BIR domains, which for other inhibitor of apoptosis proteins family members are known to interact with different proteins (42). Regardless of the possible interaction between Naip5 and Nlrc4 (13), these NLR do not share all caspase-1-mediated functions (11), opening the possibility of interactions among Naip5 and other unknown proteins. Because multiple complexes containing more than one NLR member can be found within the same cell (34, 43), the sensing of cytosolic flagellin could lead to multiprotein complexes formation including Nlrc4, Naip5, caspase-1, IRF-1,

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IRF-8, or alternatively, to other NLR members and proteins involved in the activation of iNOS.

Although there are still missing pieces in the Nlr4 and Naip5 inflammasome complexes, their role in macrophage clearance of bacterial pathogens is clear. Defects on Nlr4 or caspase-1 render macrophages susceptible to infections such as *S. typhimurium* (10, 27), *Pseudomonas aeruginosa* (35, 36) and *Shigella flexneri* (37), whereas Naip5 deficiency lead to susceptibility against *L. pneumophila* infection (13). Because the same defects also lead to resistance to caspase-1-mediated cell death, pyroptosis seems to be the major effector mechanism to control these pathogens. However, the requirement of macrophage death during an infection and the relevance of pyroptosis *in vivo* are still matters of debate (7). In this regard, a Naip5/Nlr4-caspase-1-dependent caspase-7 activation and phagosome maturation responsible for the inhibition of bacteria replication (44) was recently described.

Here, we are describing a novel pathway for iNOS activation orchestrated by the Naip5 and Nlr4 and operated through caspase-1, adding a new effector mechanism mediated by inflammasomes. Importantly, this pathway cooperate with TLR-induced NO production for the control of *L. pneumophila* since pharmacological inhibition of iNOS renders higher susceptibility of both WT C57BL/6 and *myd88*^{-/-} macrophages to flagellated *L. pneumophila* but not to flagellin-mutant bacteria (supplemental Fig. 4). The role of iNOS to the resistance against *L. pneumophila* was previously demonstrated (45, 46). However, iNOS seem not to be involved in the control of *L. pneumophila* by macrophages from Naip5-deficient A/J mice (47), corroborating our data showing that the TLR-independent iNOS activation is observed only in the presence of functional flagellin. It remains to be determined how caspase-1-mediated cellular events, such as pyroptosis, pro-inflammatory cytokines secretion, caspase-7, and iNOS activation cooperate to the control of infections.

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APÊNDICE B

Cytosolic flagellin-induced lysosomal pathway regulates inflammasome-dependent and -independent macrophage responses

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NAIP5/NLRC4 (neuronal apoptosis inhibitory protein 5/nucleotide oligomerization domain-like receptor family, caspase activation recruitment domain domain-containing 4) inflammasome activation by cytosolic flagellin results in caspase-1-mediated processing and secretion of IL-1 β /IL-18 and pyroptosis, an inflammatory cell death pathway. Here, we found that although NLRC4, ASC, and caspase-1 are required for IL-1 β secretion in response to cytosolic flagellin, cell death, nevertheless, occurs in the absence of these molecules. Cytosolic flagellin-induced inflammasome-independent cell death is accompanied by IL-1 α secretion and is temporally correlated with the restriction of *Salmonella* Typhimurium infection. Despite displaying some apoptotic features, this peculiar form of cell death do not require caspase activation but is regulated by a lysosomal pathway, in which cathepsin B and cathepsin D play redundant roles. Moreover, cathepsin B contributes to NAIP5/NLRC4 inflammasome-induced pyroptosis and IL-1 α and IL-1 β production in response to cytosolic flagellin. Together, our data describe a pathway induced by cytosolic flagellin that induces a peculiar form of cell death and regulates inflammasome-mediated effector mechanisms of macrophages.

Flagellin, the monomeric subunit of flagella present in Gram-negative and Gram-positive bacteria, is one of the few protein structures that can activate both transmembrane and cytosolic pattern recognition receptors of the innate immune system. Extracellular flagellin is recognized by the transmembrane Toll-like receptor (TLR)5 (1). On the other hand, flagellin can be directly delivered into the cytosol by transport systems, such as the type III secretion system (T3SS) of *Salmonella* (2) and the type IV secretion system (T4SS) of *Legionella* (3). Once in the cytosol, flagellin is sensed by the inflammasome complex comprised of the NOD-like receptor (NLR) proteins neuronal apoptosis inhibitory protein (NAIP)5 and NLRC4 [NLR family, caspase activation recruitment domain (CARD) domain-containing 4] (2–5).

Both TLR5 and NAIP5/NLRC4 receptors recognize conserved regions of flagellin. TLR5 is thought to detect a region of flagellin located in the D1 domain (6), whereas a sequence of three leucine residues that is present in the C-terminal D0 domain of flagellin is required to activate the NAIP5/NLRC4 inflammasome (7). Despite some redundant roles that are attributed to NLRC4 and NAIP5 in flagellin-mediated macrophage activation (7), a new model for NAIP5/NLRC4 inflammasome activation in response to flagellin was recently proposed (8, 9). In this model, NAIP5 acts as an immune sensor protein that specifically binds to flagellin (9). The interaction between NAIP5 and flagellin promotes the recruitment of NLRC4 through the NOD domain. The formation of this protein complex leads to the association of NLRC4 with procaspase-1 via CARD-CARD interactions. Additionally, NLRC4 can recruit the adaptor protein ASC (apoptosis-associated speck-like protein containing a

caspase recruitment domain), which also contains a CARD domain and is able to recruit and process procaspase-1.

Caspase-1 activation results in the cleavage and secretion of biologically active forms of the inflammatory cytokines interleukin (IL)-1 β and IL-18 (10) and the induction of a form of cell death named pyroptosis (11). The activation of caspase-1 in response to cytosolic flagellin by the NAIP5/NLRC4 inflammasome complex can also induce other effector mechanisms to restrict infections, such as caspase-7-dependent phagosome maturation (4, 12) and the activation of inducible nitric oxide synthase (iNOS) by macrophages (13). Both of these effector mechanisms lead to the inhibition of *Legionella pneumophila* replication. Importantly, caspase-1-induced IL-1 β and IL-18 are not involved in phagosome maturation (4, 12), induction of pyroptosis (14), or iNOS activation (13), suggesting that caspase-1 mediates independent effects that cooperate to clear infections.

Although the NAIP5/NLRC4 inflammasome complex is involved in the control of many bacterial infections, such as infection with *Salmonella* Typhimurium (2, 5), *Shigella flexneri* (15), *Pseudomonas aeruginosa* (16, 17), *L. pneumophila* (3, 4), and *Listeria monocytogenes* (18), the precise effector mechanism mediated by these receptors is not completely understood. Among the NAIP5/NLRC4 inflammasome-mediated effector

Significance

This study describes a unique form of cell death induced by a cytosolic flagellin that does not require NLRC4 (nucleotide oligomerization domain-like receptor family, caspase activation recruitment domain domain-containing 4), ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), or caspase-1/11 but is regulated by lysosomal cathepsins B and D. Inflammasome-independent cell death is characterized by loss of membrane integrity and IL-1 α secretion and participates in the clearance of *Salmonella* Typhimurium by macrophages. Lysosomal pathway activated by flagellin also regulates inflammasome-dependent responses such as IL-1 β and IL-1 α secretion and pyroptosis. Together, our data identify a pathway induced by cytosolic flagellin that cooperates with inflammasomes to the clearance of infections.

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mechanisms that have been implicated with intracellular bacterial replication control, pyroptosis has received great attention.

Pyroptosis has been described as a programmed cell death pathway that uniquely depends on caspase-1 (19). Recently, it was demonstrated that the enteric pathogenic bacteria *Escherichia coli*, *Citrobacter rodentium*, and *Vibrio cholerae* and the cholera toxin B subunit can trigger the activation of a noncanonical inflammasome that targets caspase-11 (also known as caspase-4 in humans and related to caspase-1) (20). These stimuli induce cell death in a caspase-11-dependent fashion, but the process is not dependent on ASC, NLRC4, or caspase-1. Interestingly, this process of cell death (also named pyroptosis) is accompanied by the secretion of IL-1 α but not by the secretion of IL-1 β (which requires caspase-1). Importantly, the 129 mouse strain that was used to generate the first caspase-1^{-/-} mutants (21, 22) harbors a mutation in the caspase-11 locus that impairs caspase-11 function. Because of the close proximity in the genome between the caspase-1 and caspase-11 genes, the two proteins cannot be segregated by recombination. Therefore, these caspase-1^{-/-} mice are also defective for caspase-11 (20).

Importantly, although pyroptosis is regulated by caspase activation, similarly to apoptosis, inhibition of or genetic deficiency in apoptotic caspase does not rescue cells from pyroptosis (11, 23). In addition, pyroptosis and apoptosis provide distinct outcomes for the immune response, which may be explained by the different morphological and biochemical changes that are observed in cells undergoing these forms of cell death (24, 25). Activation of caspase-1/11 results in the rapid formation of pores in the plasma membrane that dissipate cellular ionic gradients. This process allows the influx of water into the cells, resulting in cell swelling, osmotic lysis, and the release of intracellular contents (25, 26). The loss of plasma membrane integrity and the secretion of inflammatory mediators during pyroptosis, including IL-1 β and IL-18, results in the induction of a strong inflammatory response (27). The inflammatory milieu produced by pyroptosis could result in the recruitment of effector cells to the site of infection as a mechanism of pathogen clearance. Recently, it was demonstrated that the ectopic expression of the *Salmonella* flagellin protein FliC during the intracellular phase of infection triggers pyroptosis of infected cells in vivo (14). The bacteria released by the pyroptotic macrophages were controlled by infiltrating neutrophils through a reactive oxygen species-dependent mechanism.

Despite the evidence implicating pyroptosis as an important host defense mechanism to clear intracellular pathogens, the molecular regulation of pyroptosis is poorly understood. Here, we analyzed the regulation of macrophage death using purified flagellin as a single, death-inducing stimulus. Our data demonstrate that cytosolic flagellin is able to induce cell death in the absence of caspase-1/11. Although displaying some apoptotic features, such as cell shrinkage and the formation of membrane blebs, cytosolic flagellin-induced caspase-1/11-independent cell death does not require apoptotic caspases but depends on lysosomal events. Similar to pyroptosis, cytosolic flagellin-induced caspase-1/11-independent cell death results in the release of intracellular inflammatory contents. Caspase-1/11-independent cell death also contributes to the control of *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) infection by macrophages, supporting the existence of an effector mechanism important to restrict bacterial infection. Finally, our data provide evidences that lysosomal cathepsins also regulate IL-1 β secretion and pyroptosis in response to cytosolic flagellin. Taken together, our results suggests lysosome events as a central regulator of both inflammasome-dependent and inflammasome-independent macrophage responses induced by cytosolic flagellin.

Results

Purified Flagellin Protects both Wild-Type and TLR5-Deficient Mice from Flagellin-Deficient *Salmonella* Typhimurium Infection. Recent studies have suggested that flagellin-induced pyroptosis controls the infection of virulent bacteria, such as *L. pneumophila* and *Salmonella* Typhimurium (14). To investigate this phenomenon in more detail, we inoculated the *Salmonella* Typhimurium flagellin-expressing 1412 strain (ST) and flagellin-deficient 2157 strain (ST Δ Fli) in wild-type (WT) and TLR5^{-/-} mice and evaluated the number of colony-forming units (CFUs) recovered after 6 h from the peritoneal cavity (PEC). Recognition of flagellin was involved in the protection, since a higher bacterial load was found when mice were infected with ST Δ Fli strain (Fig. 1A, black boxes) in comparison with ST (Fig. 1A, white boxes). Surprisingly, the i.p. inoculation of purified flagellin from *Salmonella* Typhimurium, 3 h after infection with ST Δ Fli strain, significantly reduced the bacterial load in PECs of both WT and TLR5^{-/-} mice, suggesting that a mechanism dependent on flagellin but independent of TLR5 can operate to clear *Salmonella* Typhimurium infection in mice (Fig. 1A, gray boxes).

Because inoculation of mice with purified flagellin potentially stimulates both TLR5 and NLRC4-dependent responses (28), we decided to investigate the relative contribution of these receptors to macrophage responses to flagellin. Peritoneal macrophages (PMs) were stimulated with 1, 3, or 6 μ g/mL (correspondent to 0.3, 1, or 2 $\times 10^{-7}$ M) of purified flagellin from *Bacillus subtilis* (FLA-BS) or with FLA-BS inserted into cationic lipid vesicles, which permits the delivery of the flagellin into the cellular cytosol (FLA-BSDot). Free flagellin is thought to activate TLR5, whereas cytosolic flagellin is believed to activate NAIP5/NLRC4 receptors (2, 13). As expected, only treatment with cytosolic flagellin induced the secretion of IL-1 β by macrophages (Fig. S1A), whereas IL-6 was only observed when macrophages were treated with free flagellin (Fig. S1B). Additionally, death in PM was also only observed when cells were treated with cytosolic flagellin, as assessed by LDH release (Fig. S1C), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (Fig. S1D), and loss of vital acridine orange (AO) dye in combination with ethidium bromide (EtBr) incorporation (Fig. S1E). All of these responses occurred in a dose-dependent manner. Being derived from a Gram-positive bacterial species devoid of lipopolysaccharide (LPS) in the cell envelope, flagellin from *B. subtilis* was used to prevent any interference of LPS in treated cells. Moreover, similar levels of IL-1 β were found in WT and TLR2^{-/-} PMs culture after stimulation with cytosolic flagellin, discarding the interference of any contamination with TLR2 ligands in flagellin preparations (Fig. S2A). Cell death (Fig. S2B) and IL-1 β production (Fig. S2C) were also observed when cells were treated with purified *Salmonella* Typhimurium FliC flagellin (FliCdot) but not when cells were treated with the peptide of *Plasmodium vivax* VK210 circumsporozoite protein (pepVK210Dot) trapped into lipid vesicles. Similarly to purified native *Salmonella* Typhimurium FliC flagellin, a recombinant flagellin produced in an *E. coli* strain (BL21DE) (rFliCdot) also induced macrophage death (Fig. S2B) and IL-1 β production (Fig. S2C). In contrast, these responses were not obtained with a recombinant p24 protein from HIV (rp24Dot), produced in the same *E. coli* strain and purified by the same nickel-affinity chromatography, discarding the influence of nonspecific cytotoxic effects of flagellin preparations. Of note, the purity of flagellin preparations is similar (Fig. S2D), and all proteins were inserted into N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) and used in similar concentrations. These results indicate that only intracellular flagellin is capable of inducing macrophage responses, suggesting a role for inflammasome activation in cell viability. Finally, MyD88^{-/-} and WT PMs displayed similar morphology (Fig. S2E) and similar levels

Fig. 1. Cell death induced by cytosolic flagellin occurs in the absence of the NLRC4/ASC-caspase-1/11 axis. (A) CFU numbers recovered from the peritoneal cavity of C57BL/6 and TLR5-deficient mice infected with the flagellin-proficient *Salmonella* Typhimurium 1412 strain (ST WTfli) or the flagellin deficient *Salmonella* Typhimurium 2157 strain (ST Δfli) (5×10^3 bacteria per mouse) or the ST Δfli strain plus purified FliC flagellin (5 μ g per mouse) (ST Δfli + FliC). Numbers represent the means $\pm$ SEM ($n = 3$ per group). * $P < 0.05$ compared with ST Δfli-infected group. (B and C) IL-1 β levels from supernatants of PMs stimulated with purified *B. subtilis* flagellin trapped into DOTAP (FLA-BSDot) (3 μ g/mL) for 6 h were determined by ELISA (C). Numbers represent the means $\pm$ SEM ($n = 2$). *** $P < 0.001$ compared with knockout groups. (D) LDH levels quantified in culture supernatants after 6 h using a LDH kit. Numbers represent the means $\pm$ SEM ($n = 2$). $^1P < 0.05$; $^{22}P < 0.01$ compared with WT group. (E) Cytotoxicity assessed as the percentage of EtBr single-positive cells in fluorescence micrographs according to AO/EtBr staining. Numbers represent the means $\pm$ SEM of at least seven images per treatment. $^{111}P < 0.001$ compared with control group; *** $P < 0.001$ compared with WT group. (F) Representative micrographs of FLA-BSDot-stimulated PMs at 6 h. AO-stained PMs (green) show viable cells in each field, and EtBr (red) indicates permeabilized PMs. At right are merged images. Micrographs represent an original magnification of 400 $\times$. All data are representative of four (C57BL/6:00 PMs vs. caspase-1/11 $^{-/-}$ PMs) or two independent experiments.

of LDH liberation (Fig. S2F) when stimulated with cytosolic flagellin. These data eliminate the possibility that the MYD88 pathway participates in cytosolic flagellin-induced cell death.

Caspase-1/11, NLRC4, and ASC Are Not Required for Cytosolic Flagellin-Induced Cell Death. Currently, it is thought that after exposure to cytosolic flagellin, caspase-1 is recruited to the NAIIP5/NLRC4 inflammasome complex, where it is activated. The ASC adaptor molecule may or may not participate in this process (5, 7, 29). IL-1 β production by PM from C57BL/6 WT mice in response to cytosolic flagellin increased over time in culture, peaking at 6 h (Fig. 1B). Using caspase-1/11-deficient (Fig. 1B), NLRC4-deficient, and ASC-deficient (Fig. 1C) cells, we observed that the cytosolic flagellin-mediated secretion of IL-1 β was dramatically reduced in the absence of these molecules, thus confirming their requirement for the secretion of IL-1 β in response to cytosolic flagellin. Low levels of LDH were released by NLRC4 $^{-/-}$ and caspase-1/11 $^{-/-}$ macrophages in comparison with WT and ASC $^{-/-}$ macrophages 1–3 h after cytosolic flagellin stimulation (Fig. 1D). However, the levels of LDH released from cytosolic flagellin-stimulated WT and caspase-1/11 $^{-/-}$ PM was similar 6 h after stimulation (Fig. 1D). Surprisingly, although less pronounced compared with the cell death observed in WT and ASC $^{-/-}$ cultures, a high frequency of macrophage death after cytosolic flagellin stimulation was also observed in the absence of NLRC4 and caspase-1/11, as assessed by loss of vital AO dye in combination with EtBr incorporation (Fig. 1E and F). Similar results were observed when 6 μ g/mL of cytosolic flagellin was used for stimulation (Fig. S3A–C) and with

bone marrow-derived macrophages (BMDMs) (Fig. S3D and E). It is noteworthy that BMDM are not able to release IL-1 β in response to cytosolic flagellin in the absence of priming with TLR ligands, in contrast to observed with starch-elicited PMs (Fig. 1B and C). Elicited PMs are composed mainly by newly recruited macrophages originated from inflammatory monocytes, which could present basal levels of pro-IL-1 β because they hold a constitutively activated form of caspase-1 (30, 31). Taken together, these data indicate that whereas IL-1 β secretion is dependent on NLRC4, ASC, and caspase-1/11, cytosolic flagellin-induced cell death occurs in the absence of these molecules.

Flagellin Induces Inflammasome-Dependent Pyroptosis and Inflammasome-Independent Cell Death, both Contributing to the Clearance of *Salmonella* Typhimurium. We then evaluated cell death in WT and caspase-1/11 $^{-/-}$ PMs that were infected with the ST and ST Δfli. Of note, the plates were centrifuged at 290 $\times g$ for 10 min to enable the same rate of ingestion of the two *Salmonella* Typhimurium strains by the macrophages, overcoming the absence of flagellum in ST Δfli, as described previously (2). At 1 h postinfection, a higher frequency of cell death was observed in WT PMs compared with caspase-1/11 $^{-/-}$ PM (Fig. 2A). The early caspase-1/11-dependent cell death appeared to be dependent on the presence of flagellin because infection of WT macrophages with the ST induced a higher frequency of PMs death compared with infection with ST Δfli strain (Fig. 2B and C). Flagellin-dependent death was reduced and displayed slower kinetics after infection ST in caspase-1/11 $^{-/-}$ PMs (Fig. 2A–C), as demonstrated

previously by cytosolic flagellin stimulation. Early caspase-1/11-dependent cell death was correlated with a reduction in the number of CFUs observed in WT PMs compared with caspase-1/11^{-/-} PMs (Fig. 2D). These data confirm the role of early caspase-1/11-dependent pyroptosis in the control of *Salmonella* Typhimurium infection by macrophages. Importantly, however, recognition of flagellin also conferred protection in a caspase-1/11-independent manner, because significantly higher numbers of CFUs were found in caspase-1/11^{-/-} macrophages infected with the ST Δ Fli strain with regard to PMs infected with the ST strain (Fig. 2D). Moreover, adding cytosolic flagellin during infection with ST Δ Fli strain resulted in a marked increase in cell death (Fig. 2E) and a decrease in the numbers of CFUs (Fig. 2F) found in macrophages

from caspase-1/11^{-/-}, as well as WT mice, corroborating our previous in vivo data (Fig. 1A). Altogether, these data indicate that both flagellin-induced caspase-1/11-dependent pyroptosis and caspase-1/11-independent cell death participate in the clearance of *Salmonella* Typhimurium by macrophages.

Flagellin-Induced, Caspase-1/11-Independent Cell Death Displays Apoptotic Features but Does Not Rely on Caspase Activity.

To evaluate the morphological and biochemical features of caspase-1/11-independent macrophage death, cell morphology after stimulation with cytosolic flagellin was assessed. One hour after stimulation, WT PMs were permeable to EtBr before the loss of vital AO staining (Fig. S4A). These data are indicative of the formation of pores in the plasma membrane (26, 32). Double staining with AO/EtBr was not observed in caspase-1/11^{-/-} macrophages (Fig. S4A). Giemsa-stained slides indicated that the majority of WT PM appeared to be swelled compared with caspase-1/11^{-/-} PMs that seemed to have a reduction in volume (Fig. S4B). Moreover, time-lapse, phase-contrast images indicated a higher frequency of swollen cells in WT PM that increase in size over time, resulting in the accumulation of cellular debris (Fig. 3A). Despite the presence of cells with a similar morphology compared with WT PMs, we found that the majority of caspase-1/11^{-/-} PMs died with apoptotic characteristics, which included the loss of cell volume and the formation of membrane blebs (Fig. 3A). The flagellin-induced caspase-1/11-independent cell death closely resembled apoptotic cells (Fig. S4C). To determine whether macrophages stimulated with cytosolic flagellin are biased toward apoptotic cell death in the absence of NLRC4 or caspase-1/11, we compared the flagellin-induced caspase-1/11-independent cell death to actinomycin D (ActD)-induced apoptosis. Of note, ActD treatment induced the same frequency of cell death (Fig. S4D, black bars) and hypodiploid nuclei (Fig. S4E, center graphs, and Fig. S4F, black bars) in both WT and caspase-1/11^{-/-} PMs. These data demonstrate that the lack of caspase-1/11 did not interfere with the apoptotic program of cell death. The frequency of hypodiploid nuclei observed 3 h after stimulation with cytosolic flagellin is strongly reduced in the absence of caspase-1/11 (Fig. 3B), demonstrating the involvement of caspase-1/11 in DNA fragmentation that occurs in response to cytosolic flagellin. However, a decrease in full-length caspase-3, which is associated with the appearance of the active, cleaved 17/19-kDa caspase-3, was observed only in ActD-treated WT, caspase-1/11^{-/-}, and NLRC4^{-/-} macrophages but not in any of cytosolic flagellin-treated WT, caspase-1/11^{-/-}, and NLRC4^{-/-} cells (Fig. 3C). The involvement of cytosolic flagellin in the activation of apoptotic caspase-7 and caspase-9 were also investigated using a fluorimetric assay. ActD-treated WT or caspase-1/11^{-/-} macrophages displayed caspase-7 (Fig. 3D) and caspase-9 (Fig. 3E) activation that was abrogated by the addition of z-VAD-fmk and LEHD-fmk (pan-caspase and caspase-9 inhibitors, respectively). In contrast, activation of caspase-7 or caspase-9 was not observed in response to cytosolic flagellin stimulation (Fig. 3D and E), suggesting that the apoptotic machinery is not involved in cytosolic flagellin-induced cell death. According to these data both, AO loss (Fig. S4B, white bars) and the formation of hypodiploid nuclei (Fig. S4C, right graphs, and Fig. S4D, white bars) induced by ActD were strongly inhibited by Q-VD-fmk, a pan-caspase inhibitor. In contrast, Q-VD-fmk did not interfere with cell death in cytosolic flagellin-stimulated caspase-1/11^{-/-} (Fig. 3F) and NLRC4^{-/-} macrophages (Fig. 3G), as measured by AO/EtBr staining. Thus, the absence of apoptotic caspase activation and the lack of caspase interference in cytosolic flagellin-induced cell death indicate that caspase-1/11^{-/-} PMs did not have a bias toward the classical apoptotic program of cell death.

Fig. 2. Flagellin-induced caspase-1/11-independent cell death contributes to the control of *Salmonella* Typhimurium infection. (A) Cytotoxicity assessed in WT and caspase-1/11-deficient PMs infected with *Salmonella* Typhimurium 1412 strain (St WTfli) or *Salmonella* Typhimurium 2157 strain (St Δ Fli) [multiplicities of infection (MOI), 5:1], as the percentage of EtBr single-positive cells in fluorescence micrographs according to AO/EtBr staining. Numbers represent the means $\pm$ SEM ($n = 3$). *** $P < 0.001$ compared with the caspase-1/11^{-/-} group. Data are representative of three independent experiments. (B) Representative micrographs of stimulated PMs at 3 h. AO-stained PMs (green) show viable cells in each field, and EtBr (red) indicates permeabilized PMs. Micrographs represent an original magnification of 400 $\times$. (C) Cytotoxicity assessed as the percentage of EtBr single-positive cells in fluorescence micrographs according to AO/EtBr staining of St WTfli-stimulated PMs at 3 h. *** $P < 0.001$ compared with the St Δ Fli strain-infected group. Data are representative of three independent experiments. (D) CFUs recovered from PMs infected with St WTfli or St Δ Fli (MOI, 5:1) at 3 h. *** $P < 0.001$ compared with the ST Δ FliC-infected group. Data are representative of three independent experiments. (E) Cytotoxicity assessed as the percentage of EtBr single-positive cells in fluorescence micrographs according to AO/EtBr staining of PMs infected with the St Δ FliC strain (MOI, 5:1) concomitantly treated (open symbols) or not with FliCdot (closed symbols). Data are representative of two independent experiments. (F) The number of CFUs recovered from the peritoneal cavity of mice infected with Δ FliC strain (MOI, 5:1) in the presence or absence of FliCdot. Numbers represent the means $\pm$ SEM ($n = 3$). Data are representative of two independent experiments.

Fig. 3. Cell death induced by cytosolic flagellin is independent of caspase activity and culminates in IL-1 α release. (A) The cell morphology of FLA-BSDot-stimulated PMs (3 μ g/mL) accompanied in phase contrast microscopy for 3.5 h. Micrographs represent an original magnification of 400 $\times$. Data are representative of three independent experiments. (B) Percentage of hypodiploid nuclei evaluated by flow cytometry according to PI incorporation in FLA-BSDot- or empty DOTAP-treated PMs at 3 h. Numbers represent the means $\pm$ SEM ($n = 3$). *** $P < 0.001$ compared with the WT group. (C) Western blot analysis for caspase-3 activation in BMDMs stimulated with FLA-BSDot (6 μ g/mL) or ActD (5 μ g/mL) at 6 h. Activation of caspase-3 was determined by decrease in the full-length caspase-3 (35 kDa) associated with the appearance of the cleaved 17/19-kDa caspase-3. (D and E) Fluorimetric assay for caspase-7 (D) and caspase-9 (E) activity in FLA-BSDot or ActD-treated PMs at 4 h. Cell-free extracts were incubated with caspase-7 or caspase-9 fluorimetric substrates in the presence or absence of specific caspase inhibitors. Numbers represent the means $\pm$ SEM ($n = 3$). Results represent relative fluorescence units (RFU). (F and G) Cytotoxicity assessed as the percentage of EtBr single-positive cells in fluorescence micrographs according to AO/EtBr staining in PMs stimulated with FLA-BSDot (3 μ g/mL). Cells were treated or not with Q-VD-fmk (20 μ M) 1 h prior to stimulation. Numbers represent the means $\pm$ SEM ($n = 3$). (H) Cytotoxicity in PMs stimulated with FLA-BSDot (6 μ g/mL) or ActD (5 μ g/mL). Numbers represent

Caspase-1/11-Independent Cell Death Induced by Cytosolic Flagellin Retains an Inflammatory Outcome Characterized by the Release of IL-1 α . Despite displaying some apoptotic features, almost 40% of caspase-1/11^{-/-} PMs that were stimulated with cytosolic flagellin were permeable to EtBr at 3 h, whereas almost all ActD-treated cells remained impermeable (Fig. 3H). In comparison, up to 12 h after ActD treatment, a high frequency of ActD-treated PM from both WT and caspase-1/11^{-/-} mice lost vital AO but remained impermeable to EtBr, indicating that these cells died but did not lose plasma membrane integrity. Importantly, even less than WT cells, cytosolic flagellin-stimulated caspase-1/11^{-/-} PMs released large amounts of IL-1 α (Fig. 3I), which is a known damage signal released by necrotic cells (33). In contrast, apoptotic cell death induced by ActD did not result in IL-1 α release, even after 24 h of stimulation (Fig. 3I), when almost all cells were already dead, consistent with the idea that apoptotic cells retain these molecules within apoptotic bodies. These data demonstrate that cytosolic flagellin-induced caspase-1/11-independent cell death retains the same inflammatory outcome as the classical caspase-1/11-dependent pyroptosis, both characterized by the loss of cell integrity and release of intracellular proinflammatory contents, corroborating with the idea that cytosolic flagellin does not activate the classic apoptotic machinery in the absence of caspase-1/11.

Lysosomal Cathepsins Regulate Caspase-1/11-Dependent and -Independent Macrophage Responses to Cytosolic Flagellin. Given the peculiar features displayed by caspase-1/11-deficient dying cells after cytosolic flagellin stimulation, we investigated the participation of alternative cell death mechanisms. Lysosomal membrane permeabilization (LMP) induced by a variety of stimuli activates a programmed cell death process independent of the classical apoptotic machinery (34–36). This unusual form of cell death induces a mixed necrotic or subapoptotic semblance (37), similar to what we observed in caspase-1/11-independent cell death induced by cytosolic flagellin. To investigate the ability of cytosolic flagellin to induce loss of lysosomal acidity, a hallmark of lysosomal damage, we analyzed macrophages in accordance to AO staining upon flagellin stimulation. AO is a cell permeable dye with lysosomotropic properties that accumulate within acidic lysosomes. When elicited by blue light, the fluorescence emitted by AO varies from red (at high concentrations into the intact acidic lysosomes) to green (under low concentrations in the cytosol or nucleus) (37–39). A large number of spots with bright green fluorescence were observed in both WT and caspase-1/11^{-/-} PMs a few minutes after stimulation with cytosolic flagellin (Fig. 4A) but not with empty DOTAP or medium (Fig. S5A). Because the appearance of a high green AO fluorescence indicates the disruption of lysosomes filled with AO, which then disperse diffusely through the cell cytosol (37–39), our data suggest that cytosolic flagellin can induce loss of lysosomal membrane integrity and leakage of the lysosomal contents into the cell cytosol. The lysosomal damage was likely to be a result of the transfection with cytosolic flagellin but not the transfection of any protein, because a similar staining pattern was observed with FliC flagellin from *Salmonella* Typhimurium inserted into DOTAP but not with other proteins such as VK210 and rp24 also inserted into DOTAP (Fig. S5A). These results indicate that different preparations of flagellin induced the same effect on macrophages death, in contrast to that observed with nonrelated proteins.

the means $\pm$ SEM of at least five images per treatment. *** $P < 0.001$ compared with the Act-D group. (I) IL-1 α levels from supernatants of PMs stimulated with FLA-BSDot (3 μ g/mL) or ActD (5 μ g/mL) were determined by ELISA *** $P < 0.001$ compared with the knockout group. Data are representative of two independent experiments.

Fig. 4. Major role for lysosomal permeability in flagellin-induced macrophage death. (A) Representative fluorescence micrographs of PMs stimulated with FLA-BSDot (3 µg/mL) and stained with AO/EtBr. (Left) Cells with high cytoplasmic green fluorescence. (Center and Right) AO-stained PMs with decreased intensity in cytoplasmic green fluorescence. Micrographs represent an original magnification of 400×. (B–E) Cytotoxicity assessed as the percentage of EtBr single-positive cells in fluorescence micrographs in PMs stimulated with FLA-BSDot (3 µg/mL). Cells were pretreated or untreated with the lysosomal acidification inhibitor bafilomycin A1 (20 nM) (B), cathepsin B inhibitor CA074-Me (25 µM) (C), cathepsin D inhibitor pepstatin A (25 µM) (D) or both CA074-Me and pepstatin A (E), 1.5 h prior stimulation. Numbers represent the means ± SEM of at least 15 images per treatment. ****P* < 0.001 compared with the pretreated group. Data are representative of two independent experiments.

The presence of bafilomycin A1, which specifically inhibits a vacuolar H⁺-ATPase (V-ATPase), preventing lysosomal acidification, abolished cytosolic flagellin-induced cell death in caspase-1/11^{-/-} macrophages (Fig. 4B). Moreover, the presence of bafilomycin also had a significant inhibitory effect on cell death in WT macrophages stimulated with cytosolic flagellin, which suggests the involvement of lysosomal pathways in both pyroptosis and caspase-1/11-independent cell death induced by cytosolic flagellin. To further examine the role of lysosomal pathways in the regulation of cell death induced by cytosolic flagellin, the involvement of lysosomal cathepsins B and D was evaluated by the addition of the selective inhibitors, N-(L-3-*trans*-propylcarbonyl-oxirane-2-carbonyl)-L-isoleucyl-L-proline methyl ester (CA074-Me) and pepstatin A, respectively. No significant effect on the levels of cell death induced by cytosolic flagellin in both WT and caspase-1/11^{-/-} macrophages was observed in the presence of CA074-Me (Fig. 4C) or pepstatin A alone (Fig. 4D). However, when added together, cell death was halved in both WT and caspase-1/11^{-/-} macrophages (Fig. 4E), indicating a redundant role for cathepsins B and D during cytosolic flagellin-induced cell death. Despite lysosomal disruption and cathepsin B release are associated with NLRP3 inflammasome activation (40, 41), cytosolic flagellin-stimulated NLRP3^{-/-} macrophages displayed similar frequencies of dead cells (Fig. S5D) and IL-1α release (Fig. S5E) compared with WT macrophages, ruling out the participation of NLRP3 in lysosomal-mediated inflammatory cell death induced by cytosolic flagellin.

Interestingly, the presence of CA074-Me during cytosolic flagellin stimulation significantly inhibited caspase-1-dependent IL-1β production (Fig. 5A). The inhibition of cathepsin B also reduced IL-1α production by WT macrophages, at levels similar to those released by caspase-1/11^{-/-} cells (Fig. 5B), suggesting that cathepsin B might regulate caspase-1/11 activities in response to cytosolic flagellin. In contrast to the effect of CA074-Me, the presence of pepstatin A did not affect IL-1β (Fig. 5C) or IL-1α (Fig. 5D) production in response to the stimulation with cytosolic flagellin, which suggests that cathepsin B may have a major role in the regulation of caspase-1-dependent IL-1β production and partially caspase-1/11-dependent IL-1α secretion in response to cytosolic flagellin. It is noteworthy that CA074-Me inhibitor is broadly accepted as a "specific" inhibitor of cathepsin B (40, 42, 43). However, some findings obtained with CA074-Me are not reproduced in cathepsin B knockout cells (41). In addition, because it has been demonstrated that caspase inhibitors can interact with cathepsin B, thus inhibiting its activity (44), we tested the possibility of the cross-reactivity between CA074-Me and recombinant caspase-1. As shown in Fig. 5E, CA074-Me was not able to directly inhibit caspase-1 activity, whereas inhibited cathepsin B activity in a dose-dependent manner,

Fig. 5. Lysosomal cathepsins regulate cytokine production in response to cytosolic flagellin. IL-1β (A and C) or IL-1α (B and D) levels from supernatants of PMs stimulated with 3 µg/mL cytosolic flagellin from *B. subtilis* (FLA-BSDot) were determined by ELISA. Cells were pretreated or not with the cathepsin B inhibitor CA074-Me (A and B), cathepsin D inhibitor pepstatin A (C and D), with the indicated concentrations, 1.5 h prior to stimulation with FLA-BSDot. Data obtained in A and B are representative of two independent experiments. Numbers represent the means ± SEM (*n* = 2). Data obtained in C and D are shows as the mean of two independent experiments. Numbers represent the means ± SEM (*n* = 4). **P* < 0.05; ***P* < 0.01; ****P* < 0.001 compared with the untreated group. (E) Recombinant cathepsin B and caspase-1 were pretreated with CA074-Me and their residual activity measured and represented as means ± SEM (*n* = 3).

Fig. 6. The relationship between inflammasome and lysosomal pathway for cytokine production and cell death. Bacterial flagellin inserted into macrophage cytosol through T3SS/T4SS secretion systems or by lipid vesicles can induce the activation of both NAIP5/NLRC4 inflammasome complex (labeled A) and lysosomal pathway (labeled B). Cytosolic flagellin could induce lysosome rupture or LMP that permits the release of active cathepsins into cell cytosol. Inflammasome activation results in caspase-1/11-mediated pyroptosis and IL-1 β production and IL-1 α secretion. However, the inflammasome-mediated macrophages responses seem to be a result of the cross-talk between inflammasome complex and lysosomal pathway, where cathepsin B could regulate the activation of NAIP5/NLRC4 inflammasome or caspase-1 activities, because it is involved with IL-1 β production (labeled C). On the other hand, the lysosomal pathway orchestrates the cytosolic flagellin induced caspase-1/11-independent inflammatory cell death.

discarding the nonspecific effect of CA074-Me in the inhibition of caspase-1 and suggesting that the inhibition of caspase-1-dependent macrophages responses to cytosolic flagellin was a result of cross-talk between cathepsin B and caspase-1 and not an off-target effect of CA074-Me. Together, these data indicate that lysosomal events, particularly the activated cathepsins, participate in the regulation of caspase-1/11-dependent and -independent responses after cytosolic flagellin stimulation.

Discussion

Caspase-1-mediated cellular signaling induced by flagellin stimulation seems to be strictly coordinated. Activation of caspase-1 by the NAIP5/NLRC4 inflammasome complex promotes rapid formation of pore in the plasma membrane that culminates in cell swelling, osmotic lysis, and the release of the proinflammatory cytokines IL-1 β and IL-18 (11, 25). Taken together, these caspase-1-dependent events characterize the pyroptosis process. However, we show here that cytosolic flagellin also induces cell death in the absence of caspase-1/11. Cytosolic flagellin-induced caspase-1/11-independent cell death occurs with slower kinetics in comparison with pyroptosis, and dying cells have some apoptotic-like features, including loss of cell volume and the formation of structures that resemble membrane blebs. Despite the initial events that distinguish it from pyroptosis, cytosolic flagellin-induced caspase-1/11-independent cell death seems to result in the same inflammatory outcome, culminating in cell lysis with the release of intracellular contents including the proinflammatory cytokine IL-1 α . Moreover, cytosolic flagellin-induced caspase-1/11-independent cell death strongly correlated with the restriction of *Salmonella*

Typhimurium infection by macrophages and could explain the reduction of in vivo infection found in caspase-1/11 $^{-/-}$ mice after the inoculation of flagellin. Importantly, the inhibition of cathepsin B and cathepsin D reduced cytosolic flagellin-induced cell death by half, in both WT and caspase-1/11 $^{-/-}$ macrophages. In addition, IL-1 β production in response to cytosolic flagellin was abrogated in the absence of active cathepsin B. Taken together, our data indicate that cytosolic flagellin activates the lysosomal pathway that, in its turn, regulate both caspase-1/11-dependent and caspase-1/11-independent macrophages responses.

Despite the fact that flagellin may be recognized by both TLR5 and NAIP5/NLRC4 inflammasome, their individual activation results in nonredundant cellular responses. Caspase-1 activation, cytotoxicity, and release of IL-1 β have been demonstrated to proceed normally in TLR5-deficient BMDM during *Salmonella* Typhimurium infection (5). In agreement with this study, our results demonstrated that the introduction of purified flagellin from *B. subtilis* directly into the macrophage cytosol using transfection lipid vesicles (DOTAP) was sufficient to induce caspase-1-dependent IL-1 β secretion, the formation of membrane pores and the early LDH release by macrophages. These same effects were not observed when macrophages were stimulated with purified flagellin in its free form. Stimulation with free flagellin only resulted in IL-6 production because of its extracellular recognition by TLR5. Moreover, the LDH release and cell death that was induced by cytosolic flagellin were still observed when MyD88 was absent, suggesting that the TLR5, IL-1R, and IL-18R signaling pathways are not required for macrophage death.

Because flagellin-induced pyroptosis is accompanied by cell permeabilization, most studies have assessed this peculiar program of cell death by analyzing early liberation of LDH, which is indicative of a loss of cell integrity. In contrast to C57BL/6 WT PMs, LDH was not identified in NLRC4^{-/-} and caspase-1/11^{-/-} macrophage cultures early after stimulation with cytosolic flagellin. Using distinct approaches to characterize cell death, we observed that LDH liberation did not necessarily correlate with cell death. Stimulation of macrophages with a low dose of cytosolic flagellin was sufficient to induce LDH liberation but was not sufficient to induce cell death. Moreover, caspase-1/11^{-/-} macrophages lost AO dye and incorporated EtBr at 3 h after stimulation with cytosolic flagellin, but no LDH release was identified in these cultures. These data suggest that the release of cellular inflammatory contents and cell death, which are two important consequences of the recognition of cytosolic flagellin, possibly represent two independent events. The disagreement observed between the cytotoxicity assays can be explained by the nature of each assay (45). LDH release is predicted on the release and accumulation of the enzyme into the culture supernatant after a loss in plasma membrane integrity. Conversely, the EtBr/AO assay is based on the loss of acidic compartments, specifically the acidity of nuclei, in combination with an increased cell membrane permeability that permits the EtBr diffusion. Therefore, it is possible that some temporary membrane damage could lead to the release of LDH without inducing cell death. Conversely, other intracellular events could culminate in a loss of AO staining before any cell membrane damage that would allow the liberation of LDH. Therefore, the techniques that are used to characterize cell death can be misinterpreted when used alone. Here, we determined that caspase-1/11 is not required for cell death in response to cytosolic flagellin stimulation as proposed previously (19, 24).

Caspase-1/11-dependent pyroptosis has been described as an important effector mechanism to clear bacterial infections (19, 46). It is believed that the death of infected macrophages by this pathway results in the loss of a replication niche for the infecting bacteria and exposes the bacteria to an inflammatory microenvironment that is optimized for the control of bacterial replication. Miao and his collaborators (14) provided the first in vivo evidence to suggest that pyroptosis could be a host defense mechanism to clear intracellular pathogens. They demonstrated that the induction of flagellin expression during *Salmonella* Typhimurium infection led to NLRC4-dependent pyroptosis, which resulted in the exposure of the released bacteria to infiltrating neutrophils. The bacteria were then killed by the production of reactive oxygen species. Although there was not a direct correlation between cell death and neutrophils-mediated killing in caspase-1/11^{-/-} mice, the authors showed that caspase-1/11^{-/-} mice were more permissive to *Salmonella* Typhimurium infection compared with WT mice (14). Our experiments demonstrated that the i.p. inoculation of flagellin significantly reduces the bacterial load in WT and TLR5^{-/-} mice, indicating that flagellin induces the control of in vivo infection by a mechanism independent of TLR5. Then, we hypothesize that the flagellin-dependent control of *Salmonella* infection by caspase-1/11^{-/-} macrophages is correlated with caspase-1/11-independent cell death. In fact, caspase-1/11^{-/-} macrophages underwent flagellin-dependent cell death in vitro during infection with *Salmonella* Typhimurium. The slow kinetics of caspase-1/11-independent cell death was likely related to the higher number of CFUs observed in caspase-1/11^{-/-} macrophages compared with WT cells. However, caspase-1/11^{-/-} macrophages were more susceptible to flagellin-deficient *Salmonella* Typhimurium compared with flagellin-sufficient *Salmonella* Typhimurium. Finally, the stimulation of both WT and caspase-1/11^{-/-} macrophages with cytosolic flagellin significantly reduces the infection of flagellin-deficient *Salmonella* Typhimurium in both macrophages. Most importantly, this

reduction is temporally correlated with the induction of cell death, which corroborates our hypothesis.

In contrast to proIL-1 β , proIL-1 α is not a caspase-1 substrate and does not require processing to allow receptor binding (47). However, the role of caspase-1 in the regulation of IL-1 α release is still a matter of debate (21, 33, 48). Recently, it was demonstrated that for most NLRP3 soluble activators, including ATP and nigericin, the release of IL-1 α was dependent on NLRP3, ASC, and caspase-1 (49). However, the ability of caspase-1 in controlling IL-1 α secretion occurs independently of its protease activity. In contrast, some particles or crystals such as silica and MSU induced processing and secretion of IL-1 α in a manner independently of inflammasome. In our hands, the secretion of IL-1 α in response to cytosolic flagellin seems to be partially dependent on caspase-1 because caspase-1/11^{-/-} macrophages is able to release IL-1 α , but with levels significantly lower in comparison with that found in WT macrophages cultures. The secretion of IL-1 α , in vivo, was equally or more important than the secretion of IL-1 β to induce IL-6 production and neutrophil recruitment during model of peritonitis induced by MSU (49). Therefore, cell injury that is concomitant to the release of IL-1 α could be an important consequence of flagellin-induced caspase-1/11-independent cell death that contributes to the inflammatory milieu at the site of bacterial infection.

Interestingly, cytosolic flagellin-stimulated macrophages displayed some apoptotic characteristics in the absence of caspase-1/11 and NLRC4, such as loss in cell volume and the membrane blebbing. These results suggest the possibility that, in the absence of inflammasome activation, macrophages could have a bias toward apoptotic program of cell death. In fact, it was demonstrated that in the absence of caspase-1, dying *Salmonella*-infected macrophages could exhibit apoptotic or even autophagic features (50, 51). Chromatin condensation and DNA breakage observed in the absence of caspase-1 is mediated by caspase-2 and requires the SipB protein from *Salmonella*. However, cytosolic flagellin-induced caspase-1/11-independent cell death seems to be regulated by a pathway distinct from classical apoptosis. Conversely to ActD-induced apoptosis, caspase-1/11-independent cell death induced by cytosolic flagellin resulted in the loss of cell integrity, observed by the entry of EtBr and in the release of intracellular contents few hours after stimulation, thus maintaining a necrotic outcome (33). Moreover, cytosolic flagellin did not activate apoptotic caspases 3, 7, and 9 and the broad pharmacological inhibition of caspases did not modify the kinetics or outcome of cytosolic flagellin-induced cell death in the absence of caspase-1/11. Despite the independency of caspases, cytosolic flagellin-induced caspase-1/11-independent cell death is abrogated in the presence of bafilomycin, which suggests the involvement of the lysosomal pathway. Concomitant inhibition of cathepsin B and cathepsin D have a significant negative effect on cytosolic flagellin-induced cell death, which indicates a redundant role of these two proteins and supports the involvement of lysosomal damage-mediated program of cell death. Thus, our work adds a flagellin-mediated mechanism by which *Salmonella* Typhimurium can kill the infected macrophage in the absence of caspase-1 and caspase-11.

It is well established that moderate lysosomal damage with slow liberation of lysosomal hydrolases into the cell cytosol can culminate in controlled lysosome-mediated apoptotic cell death (37). Once in cytosol, cathepsins B and D can induce apoptosis in a synergistic manner by simultaneously cleavage of the proapoptotic protein Bid, and the antiapoptotic members of Bcl-2 family, like Bcl-2, Bcl-xL, Ncl-1e, and XIAP (52–56). According to this, the early cell shrinkage and formation of membrane blebs observed in cytosolic flagellin-stimulated caspase-1/11^{-/-} macrophages and that resembles apoptosis could be a result of the activation of the apoptotic machinery by lysosomal cathepsins into cell cytosol. However, different from apoptotic cells,

cytosolic flagellin-stimulated caspase-1/11^{-/-} macrophages undergo rapid loss of membrane integrity, which is consistent with a massive induction of lysosomal membrane permeability and consequent loss of organelle membrane integrity (37). This lysosomal damage-mediated cell death program commonly results in uncontrolled necrotic cell death without apoptotic caspase activation where macrophages exhibit promiscuous features of apoptosis and necrosis, consistent with our observations. The mechanism by which flagellin induces massive loss of lysosome membrane integrity has yet to be elucidated. Therefore, the fact of distinct flagellin preparations but not other nonrelated proteins were able to induce lysosomal cell death, discard nonspecific effects of possible contaminants in preparations, and point out the unique lysosomotropic propriety of flagellin.

In our model, the lysosomal pathway has a central role in flagellin-induced cell death because the inhibition of either lysosome acidification or cathepsin B and D activation, but not the absence of caspase-1/11, suppresses cell death. In addition, because cell death occurs less prominently and with distinct characteristics in the absence of caspase-1/11, it is possible that these two proinflammatory caspases act as accessory/catalytic factors rather than being the central effector mechanism of cytosolic flagellin-induced cell death. This is reminiscent to what occurs during apoptosis, particularly after insults that triggers the intrinsic pathway, where the apoptotic effector caspases -3, -6, and -7 are also not required for cell death, but they are essential for imprinting apoptotic characteristics, such as the appearance of “eat me” or “find me” signals, DNA degradation, and inactivation of DAMPs, in cells that are already destined to die (57–59). Likewise, we propose that pyroptosis, as defined as a caspase-1/11-dependent cell death, is rather a program of cell demise capable of imprinting particular characteristics to the cells committed to die, such as the secretion of IL-1 β /IL-18.

There are few and conflicting data about the relationship between inflammasomes and lysosomal damage-mediated cell death. In some instances, LMP, a hallmark characteristic of lysosomal-mediated cell death, can occur upstream or downstream of inflammasome activation (42, 60). In other circumstances, LMP is an independent event that occurs simultaneously with caspase-1 activation, thus contributing to cell death (38). It has been described that the inhibition of cathepsin B activation abolishes caspase-1-dependent IL-1 β and IL-18 production in addition to caspase-1-independent pyroptosis that are mediated by NLRP3 inflammasome (42). Despite the similarities of the cathepsin B effects, we clearly show here that cytosolic flagellin-induced lysosomal cell death is not mediated by NLRP3. However, it is important to note that it is still possible that NLRP3-dependent responses may compensate for the lack of Nlr4 in Nlr4 knockout mice and, similarly, Nlr4-dependent responses may be sufficient in the absence of NLRP3. Future studies could address this question using Nlr4/Asc and Nlr4/NLRP3 double knockouts. One of the few studies that have attempted to investigate the regulation of this pathway suggested that phosphorylated tyrosine kinase Syk is physically associated with ASC, and Syk is required to activate both the NLRP3 inflammasome and cathepsin B in macrophages that were stimulated with hemozoin from *Plasmodium*, but not with uric acid or

silica (60). Because IL-1 β production is abolished in the absence of active cathepsin B or Syk, these data suggest that the phosphorylation of Syk and the activation of cathepsin B are upstream of caspase-1 activation. However, the mechanisms involved in the activation of cathepsin B and NLRP3 by phosphorylated Syk remain to be elucidated. Recently, cathepsin B was demonstrated to be involved in pyroptosis induced by *Bacillus anthracis* lethal toxin (LT) (38). Interestingly, it was observed that LMP could be upstream or downstream of the NLRP1b inflammasome activity. LMP required a NLRP1b LT-responsive allele of NLRP1b and caspase-1 activation, but LT-induced mitochondrial outer membrane permeabilization seems to be sufficient to induce LMP and can function as a positive regulator of NLRP1b inflammasome activities. Here, we demonstrated evidences of the regulation of NAIP5/NLRC4 inflammasome activities by lysosomal pathway, because inhibition of cathepsin B impacts cytosolic flagellin-induced inflammasome-mediated effects.

Taken together, our results indicate that NAIP5/NLRC4 inflammasome (labeled “A” in Fig. 6) and lysosomal pathway (labeled “B” in Fig. 6) are both activated after cytosolic flagellin stimulation. Both NAIP5/NLRC4 inflammasome and lysosomal pathway seem to synergistically contribute to cell death/pyroptosis, IL-1 β production and IL-1 α secretion (labeled “C” in Fig. 6). Moreover, lysosomal cathepsins regulate flagellin-induced cell death in the absence of NAIP5/NLRC4 inflammasome. Given that this peculiar form of cell death is also involved in the clearance of bacterial infection, our results provide further understanding of the molecular mechanisms that coordinate the activation and death of macrophages in response to flagellin and its biological significance to host resistance against infections with flagellated bacteria.

Methods

Animal studies were all performed using protocols that were approved by the Federal University of São Paulo Committees on the Use and Care of Animals (CEP 0159/11). Details on methods and any associated references are available in *SI Methods*. Briefly, PMs were obtained by peritoneal lavage 4 d after i.p. treatment with starch solution (1%) (Sigma Aldrich). Purified flagellins from *B. subtilis* (FLA-BS) and *Salmonella* Typhimurium (FliC) were purchased from Invivogen. The recombinant *Salmonella* Typhimurium FliC flagellin and the HIV p24 protein were expressed in the *E. coli* BL21 DE strain, and the recombinant proteins were purified by nickel affinity chromatography, as described previously (61, 62). VK210 peptide of *P. vivax* protein was purchased from GenScript. PMs were cultured in the presence of purified flagellin from *B. subtilis* or *Salmonella* Typhimurium in its free form (FLA-BS or FliC) or inserted into DOTAP (FLA-BSDot or FliCDot) (Roche Diagnostics), a cationic lipid formulation that permits its delivery to cell cytosol (63). DOTAP was used accordingly to the manufacturer's instructions, and protein concentration was calculated to maintain the same molarity. Cytokines were measured in culture supernatant with ELISA kits from BD (OptEIA), following the manufacturer's instructions. Pore formation and cytotoxicity was assessed using EtBr incorporation in combination with AO (Sigma) staining as described previously (64).

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APÊNDICE C

NLRP3 Controls *Trypanosoma cruzi* Infection through a Caspase-1-Dependent IL-1R-Independent NO Production

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Abstract

Trypanosoma cruzi (*T. cruzi*) is an intracellular protozoan parasite and the etiological agent of Chagas disease, a chronic infectious illness that affects millions of people worldwide. Although the role of TLR and Nod1 in the control of *T. cruzi* infection is well-established, the involvement of inflammasomes remains to be elucidated. Herein, we demonstrate for the first time that *T. cruzi* infection induces IL-1 β production in an NLRP3- and caspase-1-dependent manner. Cathepsin B appears to be required for NLRP3 activation in response to infection with *T. cruzi*, as pharmacological inhibition of cathepsin B abrogates IL-1 β secretion. NLRP3^{-/-} and caspase1^{-/-} mice exhibited high numbers of *T. cruzi* parasites, with a magnitude of peak parasitemia comparable to MyD88^{-/-} and iNOS^{-/-} mice (which are susceptible models for *T. cruzi* infection), indicating the involvement of NLRP3 inflammasome in the control of the acute phase of *T. cruzi* infection. Although the inflammatory cytokines IL-6 and IFN- γ were found in spleen cells from NLRP3^{-/-} and caspase1^{-/-} mice infected with *T. cruzi*, these mice exhibited severe defects in nitric oxide (NO) production and an impairment in macrophage-mediated parasite killing. Interestingly, neutralization of IL-1 β and IL-18, and IL-1R genetic deficiency demonstrate that these cytokines have a minor effect on NO secretion and the capacity of macrophages to control *T. cruzi* infection. In contrast, inhibition of caspase-1 with z-YVAD-fmk abrogated NO production by WT and MyD88^{-/-} macrophages and rendered them as susceptible to *T. cruzi* infection as NLRP3^{-/-} and caspase-1^{-/-} macrophages. Taken together, our results demonstrate a role for the NLRP3 inflammasome in the control of *T. cruzi* infection and identify NLRP3-mediated, caspase-1-dependent and IL-1R-independent NO production as a novel effector mechanism for these innate receptors.

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Introduction

Trypanosoma cruzi is an intracellular trypanosomatid protozoan that is transmitted to the human host by blood-feeding *Reduviidae* bugs from the subfamily Triatominae. *T. cruzi* is the causative agent of Chagas disease and American trypanosomiasis, a chronic infectious disease. While Chagas disease is endemic in Latin America, a significant increase in confirmed cases of Chagas disease has recently been reported in the USA, Canada, Japan, Australia and Europe, indicating that it is an emerging disease [1] [2] [3]. Due to increasing immigration from endemic countries and a lack of regular screening in blood banks and hospitals (with a few exceptions), *T. cruzi* infection is a potential public health issue in the USA and Europe.

The control of *T. cruzi* by the immune system depends on both innate and adaptive responses. Innate immune cells are responsible for the initial recognition of the parasite, as well as the initiation and coordination of adaptive responses [4]. The transmembrane Toll-like Receptor (TLR) family of pattern recognition receptors (PRRs) plays a central role in the recognition of *T. cruzi* by the immune system [5]. TLR4 [6], TLR2 [7] [8] [9],

TLR9 [10] and TLR7 [11] are responsible for sensing glycoinositolphospholipid-containing ceramides (GPIs), glycosylphosphatidylinositol (GPI) anchors from the trypomastigote form of the parasite (t-GPI mucin), *T. cruzi* DNA and *T. cruzi* RNA, respectively. These receptors initiate a signaling cascade that is dependent on the adaptor molecule MyD88 and culminates in the activation of pro-inflammatory genes that are crucial for resistance to *T. cruzi* infection, including IL-12 [12] [13] [14] [15], IFN- γ [16] and the microbicidal molecule nitric oxide (NO) [17] [18]. MyD88^{-/-} mice are highly susceptible to *T. cruzi* infection, possibly because of defects in the production of pro-inflammatory cytokines [19]. In addition to TLR, NOD1, a member of the cytosolic NOD-like receptor (NLR) family, plays a role in controlling *T. cruzi* infection [20]. NOD1^{-/-} macrophages exhibit impaired production of pro-inflammatory cytokines and NO, and NOD1^{-/-} mice succumb to the acute phase of *T. cruzi* infection. Despite evidence for the critical role of NOD1 in controlling *T. cruzi*, the agonist responsible for NOD1 activation remains unknown.

In addition to NOD1 and NOD2, the cytosolic PRR family includes inflammasomes, which are multiprotein complexes that

Author Summary

Inflammasomes are cytosolic innate receptors that are emerging as central effectors in the control of infections and inflammatory pathologies. NLRP3 is the most studied member of inflammasomes with established role in the control of bacterial and viral infections. This manuscript describes original studies on the involvement of NLRP3 inflammasome in the control of *Trypanosoma cruzi*, the etiological agent of Chagas disease, a chronic infectious illness that affects millions of people in the world. *T. cruzi* activates NLRP3 inflammasome by a mechanism involving cathepsin B. NLRP3^{-/-} and caspase1^{-/-} mice display high parasitemia during acute phase of *T. cruzi* infection, which could be explained by a severe defect in the production of nitric oxide (NO) and in the impairment of their macrophages to control intracellular parasites. Interestingly, inhibition of caspase-1, but not the neutralization of IL-1 β and IL-18, the best-studied caspase-1 substrates, abrogated NO production by WT and MyD88^{-/-} macrophages and rendered them as susceptible to *T. cruzi* infection as NLRP3^{-/-} macrophages. Together, our results indicate a caspase-1-dependent and IL-1 β and IL-18-independent pathway for NO production as a new effector mechanism played by NLRP3 to control *T. cruzi* infection.

contain a sensor protein from the NLR or pyrin and HIN domain-containing protein (PYHIN) families. The signaling cascade initiated by inflammasomes includes the recruitment of the apoptosis-associated speck-like (ASC) protein (which contains a caspase activating and recruitment domain (CARD) and pro-caspase-1 or pro-caspase-11 (pro-caspase-4 in humans), culminating in the activation of these proteases (reviewed in [21] [22] [23]). Active caspase-1 mediates the maturation and secretion of the IL-1 β and IL-18 pro-inflammatory cytokines and, along with caspase-11, induces the pyroptosis cell death program.

NLRP3, NLRC4 and AIM2 are the best-characterized inflammasome complexes. NLRC4 is activated in response to bacterial proteins such as flagellin [24] or the inner rod component of the bacterial type III secretion system (T3SS) [25], whereas AIM2 senses cytosolic bacterial [26] and viral DNA [27]. NLRP3 is activated by several different classes of molecules, including pathogen-associated molecular patterns (PAMPs) from viruses [28], bacteria [29] [30] [31], fungi [32] and protozoa [33]; signals from endogenous damage-associated molecular patterns (DAMPs) such as uric acid and ATP [34] [35]; and crystalline or particulate structures such as alum [36] [37] and silica [38]. NLRP3 activation by these molecules, which are not structurally related, appears to be influenced by ROS production, lysosomal rupture or potassium efflux, which are believed to result in distinct activation signals [39] [40] [41].

Inflammasomes have been implicated in the host response to several pathogens. NLRP3 has been identified as an effector in the control of *Mycobacterium* [42], *Cytrobacterium* [43], *Salmonella* [44], *Brucella* [45], Influenza A virus [46] [47] [48], *Candida* [32] and other pathogens. However, the *in vivo* role of the NLRP3 inflammasome in the host defense against *T. cruzi* has not been elucidated. Here, we demonstrate that the NLRP3 inflammasome controls *T. cruzi* parasitemia by inducing NO production via a caspase-1-dependent, IL-1R-independent pathway.

Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (<http://www.cobea.org.br/>). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institutional Animal Care and Use Committee at the Federal University of Sao Paulo (Id # CEP 0162/11).

Animals

C57BL/6 wild type (WT), caspase-1^{-/-}, NLRP3^{-/-}, MyD88^{-/-}, IL-1R^{-/-}, iNOS^{-/-} and IFN- γ ^{-/-} mice were bred in our animal facilities at the Federal University of São Paulo. The caspase-1^{-/-} mice were kindly provided by Dr. Richard Flavell (Yale University, USA), and the NLRP3^{-/-} mice were kindly provided by Dr. Vishva Dixit (Genentech, USA). Eight-week-old female mice were infected subcutaneously (s.c.) with 10³ blood-derived trypomastigotes from the Y strain of *T. cruzi*, which was obtained from the Dante Pazzanese Institute. Parasitemia was monitored by counting the number of bloodstream trypomastigotes in 5 μ L of fresh blood collected from the tail vein. Spleens were removed 10 days after infection. All animal procedures were developed with all efforts to minimize suffering and were approved by the Ethics Committee for Animal Care of the Federal University of São Paulo UNIFESP (Id # CEP 0162/11).

Preparation of mouse macrophages

Peritoneal macrophages (PMs) were obtained by peritoneal lavage 4 days after intraperitoneal injection of a 1% starch solution (Sigma Aldrich). Peritoneal cells were cultured overnight in RPMI 1640 medium supplemented with 3% heat-inactivated FCS and antibiotics. Non-adherent cells were removed by washing with warm RPMI medium.

In vitro infection with *T. cruzi*

PMs (1.5 $\times$ 10⁶ cells/300 μ L) cultured in RPMI medium were infected with trypomastigotes from *T. cruzi* strain Y (3 parasites/cell) purified from a monkey epithelial cells (LLCMK₂). The cells were treated with a caspase-1 inhibitor (z-YVAD-fmk) (10 μ M) (MBL International Corporation), an iNOS inhibitor (1 mM) (Aminoguanidine, AG), a cathepsin B inhibitor (Ca-074-Me) (Sigma Aldrich), a K⁺ channel inhibitor (Glybenclamide, GLB) (InVivoGen), an ROS inhibitor (Apocynin, APO) (25 μ M to 100 μ M) (Sigma Aldrich) or neutralizing antibodies against IL-1 β and IL-18 (0.1, 1 and 10 ng/mL) (BD Pharmingen). Supernatants were collected 24 h after infection to detect IL-1 β , IFN- γ and IL-6, and 48 h after infection to measure the NO concentration.

Microbicidal activity of macrophages

PMs (3 $\times$ 10⁵) from C57BL/6 wild type (WT), caspase-1^{-/-}, NLRP3^{-/-}, MyD88^{-/-}, iNOS^{-/-} and IL-1R^{-/-} mice were infected with trypomastigotes from *T. cruzi* Y (1:5) for 2–4 h. Extracellular parasites were removed by washing, and after 48 hours the chambers were fixed with methanol and stained with DAPI (4', 6-Diamidino-2-phenylindole) (Sigma Aldrich). The frequency of infected macrophages and the number of amastigotes inside the macrophages were evaluated by fluorescence microscopy (600 $\times$). At least 500 macrophages were counted for each representative experiment.

Measurement of cytokines and NO

Supernatants from *in vitro*-infected PMs (1.5×10^6 cells/300 μ l) or cultured spleen cells from infected mice (3×10^6 cells/300 μ l) were collected after 24 h. IL-1 β and IL-6 were measured using enzyme-linked immunosorbent assay (ELISA) kits from BD Pharmingen (OptEIA) according to the manufacturer's instructions. The levels of IL-1 β were determined using a standard curve generated with the 17 KDa mature form of recombinant mouse IL-1 β , as previously described [49]. IFN- γ was detected by sandwich ELISA using a purified anti-IFN- γ capture antibody (BD Pharmingen) and a biotin rat anti-mouse IFN- γ antibody (BD Pharmingen). IFN- γ levels were calculated based on a standard curve generated using recombinant cytokine (BD Pharmingen). The nitrite concentration was determined by the Griess reaction. Briefly, 50 μ l of Griess reagent solution (1% sulfanilamide, 0.1% naphthylene diamine dihydrochloride, 2% H_3PO_4) was added to 50 μ l samples, and the absorbances were measured at 540 nm.

Statistical analysis

Experiments were performed in duplicate or triplicate, and at least three independent experiments were performed. Data are presented as the mean $\pm$ S.D. Statistical analysis of the data was carried out using one-way or two-way ANOVA and Tukey's post-test. Differences between the experimental groups were considered significant as follows: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Results

T. cruzi-induced NLRP3 activation is dependent on cathepsin B

The NLRP3 inflammasome is activated by a variety of stimuli, including viral, bacterial, fungal and protozoan pathogens. However, the activation of NLRP3 by *T. cruzi* has not been previously reported. To determine the ability of *T. cruzi* to activate NLRP3, we evaluated the secretion of IL-1 β by *T. cruzi*-infected peritoneal macrophages (PMs). *T. cruzi* infection induced the secretion of IL-1 β by PMs isolated from WT mice, but not from PMs isolated from NLRP3 $^{-/-}$ and caspase-1 $^{-/-}$ mice (Figure 1A). Lysosomal cathepsins, ROS and/or potassium efflux have been reported to influence NLRP3 activation in response to diverse stimuli [35] [36] [37]. To investigate the role of these molecules in *T. cruzi*-induced NLRP3 activation, PMs from WT mice were infected with *T. cruzi* in the presence of pharmacological inhibitors of cathepsin B (Ca-074ME), NADPH oxidase (apocynin, APO) and potassium channels (glybenclamide, GLB). IL-1 β secretion by infected macrophages was inhibited by Ca-074ME in a dose-dependent manner, but was not inhibited by APO or GLB (Figure 1B). Taken together, these results indicate that *T. cruzi* is able to induce NLRP3 activation by a mechanism that involves cathepsin B.

NLRP3- and caspase-1-deficient mice exhibit high levels of parasitemia and are defective in NO secretion during the acute phase of *T. cruzi* infection

Next, we evaluated the role of NLRP3 in host resistance to *T. cruzi* infection by assessing parasitemia and mortality in NLRP3 $^{-/-}$ and caspase-1 $^{-/-}$ mice infected with *T. cruzi* Y. A significantly greater number of *T. cruzi* parasites were found in the blood of both NLRP3 $^{-/-}$ and caspase-1 $^{-/-}$ mice compared to WT mice (Figure 2A). Interestingly, the degree of parasitemia exhibited by the NLRP3 $^{-/-}$ and caspase-1 $^{-/-}$ mice was comparable to that exhibited by the MyD88 $^{-/-}$ and iNOS $^{-/-}$ mice, which are considered susceptible models for *T. cruzi* infection [18] [19]

Figure 1. *T. cruzi*-induced NLRP3 activation. PMs from WT, NLRP3 $^{-/-}$ and caspase-1 $^{-/-}$ mice (1.5×10^6 cells/300 μ l) were infected with *T. cruzi* Y trypomastigotes (3 parasites per cell). The supernatants were collected after 24 h for the evaluation of IL-1 β levels by ELISA. Bars represent the mean $\pm$ S.D. of triplicate samples, where ** $p > 0.01$ compared to the WT group (A). *T. cruzi*-infected PMs (3 parasites per cell) (1.5×10^6 cells/300 μ l) were treated with Ca-074ME, APO or GLB in doses of 25, 50 and 100 μ M, or left untreated (-). After 24 h, the supernatants were collected and IL-1 β production was evaluated by ELISA. Bars represent the mean $\pm$ S.D. of triplicate samples, where *** $p > 0.001$ compared to the untreated group. The experiments were repeated three times, and representative results are shown (B). doi:10.1371/journal.pntd.0002469.g001

(Figure 2A and Table S1). The greatest parasite burden was found in IFN- γ $^{-/-}$ mice (Figure 2A and Table S1). To evaluate the pro-inflammatory responses of NLRP3 $^{-/-}$ and caspase-1 $^{-/-}$ mice during the acute phase of *T. cruzi* infection, we assessed the level of cytokines and NO in the supernatants of cultured spleen cells isolated 10 days after infection. MyD88 $^{-/-}$ mice were used as a positive control for susceptibility to infection. Unlike MyD88 $^{-/-}$ spleen cells, the spleen cells isolated from NLRP3 $^{-/-}$ and caspase-1 $^{-/-}$ mice produced considerable levels of IL-6 (Figure 2B) and IFN- γ (Figure 2C), although they did not produce as much IFN- γ as the WT cells. As expected, IL-1 β was produced by splenocytes isolated from WT mice, but not by splenocytes isolated from NLRP3 $^{-/-}$, caspase-1 $^{-/-}$ and MyD88 $^{-/-}$ mice (Figure 2D). In agreement with a previous report [19], the production of NO by spleen cells from infected MyD88 $^{-/-}$ mice was significantly reduced compared to spleen cells isolated from WT mice (Figure 2E). Surprisingly, however, the levels of NO produced by splenocytes from NLRP3 $^{-/-}$ and especially caspase-1 $^{-/-}$ mice were even lower than the levels of NO produced by splenocytes

Figure 2. NLRP3 and caspase-1 are involved in the control of *T. cruzi* infection. WT, MyD88^{-/-}, NLRP3^{-/-}, caspase-1^{-/-}, IFN- γ ^{-/-} and iNOS^{-/-} mice were subcutaneously infected with 10³ *T. cruzi* blood trypomastigotes. Parasitemia was quantified by counting the parasites in 5 μ L of tail blood obtained on days 4 to 20 after infection (A). Supernatants from cultured spleen cells (3 $\times$ 10⁶ cells/300 μ L) isolated from naive (-) or infected (10 days) mice were collected after 24 h. The levels of IL-6 (B), IFN- γ (C) and IL-1 β (D) were assessed by ELISA, and NO secretion was assessed by the Griess reaction (E). Numbers represent the mean $\pm$ S.D. (n=5). *** p>0,001, ** p>0,01 and * p>0,05 compared to the WT group. Mortality was assessed by daily inspection of the cages (F). Experiments were repeated two times with IFN- γ ^{-/-} and iNOS^{-/-} mice (n=6) and five times with MyD88^{-/-}, NLRP3^{-/-}, caspase-1^{-/-} mice (n=6) showing similar results. doi:10.1371/journal.pntd.0002469.g002

isolated from MyD88^{-/-} mice (Figure 2E). The defect in NO production by spleen cells isolated from the NLRP3^{-/-} and caspase-1^{-/-} mice could explain the high levels of parasitemia observed in the iNOS^{-/-}, NLRP3^{-/-} and caspase-1^{-/-} mice (Figure 2A). However, unlike the MyD88^{-/-} and IFN- γ ^{-/-} mice, which succumbed to infection, the NLRP3^{-/-} and caspase-1^{-/-} mice survived infection (Figure 2F). These data suggest that NLRP3 and caspase-1 are required for NO production and parasite control during the acute phase of *T. cruzi* infection.

NLRP3-dependent NO secretion is critical for *T. cruzi* killing by macrophages

Because macrophages are the major source of NO during *T. cruzi* infection, the deficiency in NO production exhibited by the caspase-1^{-/-} and NLRP3^{-/-} mice could reflect an impairment in macrophage function. To test this hypothesis, PMs from WT, MyD88^{-/-}, NLRP3^{-/-}, and caspase-1^{-/-} mice were infected *in vitro* with *T. cruzi* trypomastigotes. After 48 h, the DAPI-stained slides were analyzed by fluorescence microscopy. The NLRP3^{-/-} and caspase-1^{-/-} macrophages demonstrated a marked deficiency in the control of infection, as the number of amastigotes (Figure 3A) and the frequency of infected cells (Figure 3B) were even higher than in the susceptible MyD88^{-/-} macrophages. Notably, the difference in the amastigotes numbers of the macrophages derived from the *knockouts* and the WT mice was not related to the invasion rate of *T. cruzi*, as similar numbers of amastigotes were observed in the macrophages from WT, NLRP3^{-/-} and caspase-1^{-/-} mice after 2 h of infection (Figure S1A). In contrast, 48 h after infection, the number of amastigotes present in the WT macrophages only was significantly reduced (Figure S1A). Therefore, in the absence of NLRP3 and caspase-1, macrophages were more permissive to parasite replicative or less efficient to kill them, or both. Importantly, pyroptosis (macrophage death induced by inflammasome activation) was not observed during *T. cruzi* infection. The majority of the *T. cruzi*-infected macrophages isolated from WT mice stained with the vital dye acridine orange and did not incorporate ethidium bromide, in contrast to macrophages stimulated with cytosolic flagellin, a potent pyroptotic stimulus [49] (Text S1 and Figure S1B). As observed in the spleen cells from infected mice (Figure 2), unlike MyD88^{-/-} macrophages, macrophages isolated from NLRP3^{-/-} and caspase-1^{-/-} mice secreted the pro-inflammatory cytokine IL-6 (Figure 3C) but did not produce NO (Figure 3D). Furthermore, NLRP3-dependent NO production in response to *T. cruzi* infection appeared to be regulated by cathepsin B, as Ca-074ME, but not APO or GLB, significantly reduced the levels of NO produced by macrophages from WT mice (Figure 3E). These results correlate well with our observations of IL-1 β production (Figure 1B). Moreover, treatment with Ca-074ME significantly increased the frequency of infection in macrophages isolated from WT and MyD88^{-/-} mice (Figure 3F), as well as the number of amastigotes found inside these cells (data not shown). In contrast, Ca-074ME treatment had no effect in PMs from NLRP3^{-/-} and even resulted in a small increase in levels of infection in PMs from caspase-1^{-/-} mice (Figure 3F). Taken together, these results

suggest that the NLRP3 inflammasome mediates NO production in response to *T. cruzi* infection by a mechanism that involves cathepsin B.

NLRP3-dependent NO secretion in response to *T. cruzi* infection is mediated by caspase-1 and does not require IL-1R

To confirm the role of NLRP3-dependent NO secretion in the control of *T. cruzi* infection, macrophages were infected in the presence of AG, a selective iNOS inhibitor. Treatment with AG increased the number of intracellular parasites found in WT and MyD88^{-/-} macrophages by 100% and 25%, respectively (Figure 4A), indicating that the NO produced by these macrophages contributes to the control of *T. cruzi* infection. Conversely, no significant effect was observed in NLRP3^{-/-} macrophages treated with AG (Figure 4A), supporting the idea that NLRP3-mediated NO secretion is involved in the control of parasite replication. Because NLRP3 activation leads to caspase-1 activation and consequent IL-1 β secretion, we next asked whether caspase-1 and IL-1 β are required for NLRP3-dependent NO production in response to *T. cruzi* infection, and what role these molecules play in the control of infection by macrophages. In contrast to the caspase-1^{-/-} macrophages, we observed only a minor increase in the number of amastigotes in macrophages isolated from IL-1R^{-/-} mice compared to macrophages isolated from WT mice (Figure 4B). These results correlate with the ability of the IL-1R^{-/-} macrophages to produce NO (Figure 4C; white bars). Moreover, in the presence of the caspase-1 inhibitor (z-YVAD-fmk) or AG, the NO production (Figure 4C) and the capacity to control of *T. cruzi* infection (Figure 4D) of the WT and IL-1R^{-/-} macrophages were abrogated. Importantly, treatment with z-YVAD-fmk or AG had no significant effect on NO production (Figure 4C) and amastigote numbers (Figure 4D) in macrophages from caspase-1^{-/-} and iNOS^{-/-} mice. This finding indicates that caspase-1 plays a major role in the induction of NLRP3-dependent NO production and the control of *T. cruzi* infection by macrophages.

NLRP3-mediated NO production acts synergistically with MyD88-mediated pathways to control *T. cruzi* infection

As demonstrated in Figure 3D, MyD88^{-/-} macrophages secreted NO, although at reduced levels compared to WT macrophages. Because MyD88 participates in the IL-1R and IL-18R signaling pathways, these results confirm that NO production in response to *T. cruzi* infection is mediated by a signaling pathway that involves NLRP3/caspase-1 and is independent of IL-1 β and IL-18. In fact, the neutralization of IL-18 or IL-1 β plus IL-18 reduced NO production by *T. cruzi*-infected macrophages from WT mice by only 15–20% (Figure 5A). Notably, treatment with z-YVAD-fmk abrogated the secretion of NO by MyD88^{-/-} macrophages (Figure 5B) and rendered them highly susceptible to *T. cruzi* replication (Figure 5C). Together, these results suggest that MyD88 and caspase-1, as well as IL-1 β and IL-18 to a lesser extent, cooperate to clear *T. cruzi* infection by inducing NO production.

Figure 3. Macrophages isolated from NLRP3^{-/-} and caspase-1^{-/-} mice are impaired in NO secretion and the control of *T. cruzi* infection. PMs (3×10^5 /well of chamber slides) from WT, MyD88^{-/-}, NLRP3^{-/-} and caspase-1^{-/-} mice were infected with *T. cruzi* Y trypomastigotes (1:5) for 2–4 h. Extracellular parasites were removed by washing. After 48 hours, the chambers were fixed with methanol, stained with DAPI and evaluated by fluorescence microscopy (600×). The number of amastigotes (A) and the frequency of the infected cells (B) were counted in 1000 macrophages from a representative experiment. Experiments were repeated four times with similar results. Supernatants from non-infected (-) and *T. cruzi*-infected PMs (3 parasites per cell) (1.5×10^6 cells/300 μl) were collected after 24 h to evaluate the IL-6 levels by ELISA (C), and after 48 h to measure NO secretion by the Griess reaction (D). Numbers represent the mean $\pm$ S.D. (n = 5). *** p > 0.001 and ** p > 0.01 compared to the WT group. *T. cruzi*-infected PMs (3 parasites per cell) (1.5×10^6 cells/300 μl) from WT mice were treated with Ca-074ME, GLB or APO in doses of 25, 50 and 100 μM, or left untreated. After 48 h, supernatants were collected to evaluate the level of NO production by the Griess reaction (D). Numbers represent the mean $\pm$ S.D. of triplicate samples. *** p > 0.001 compared to the untreated group. (E). PMs (3×10^5 /well of chamber slides) from WT mice were infected with *T. cruzi* Y trypomastigotes (5 parasites/cell) for 2–4 h. After removing extracellular parasites, Ca-074ME (50 μM) was added to the cultures. After 48 h, the chambers were fixed with methanol, stained with DAPI and evaluated by fluorescence microscopy (600×) to determine the frequency of infected cells. Bars represent the mean $\pm$ S.D. of triplicate samples, and ** p > 0.001 and * p > 0.01 compared to the untreated group (F). doi:10.1371/journal.pntd.0002469.g003

Discussion

Cells from the innate immune system play a key role in sensing and controlling the acute phase of *T. cruzi* infection. While TLR2, TLR4, TLR7, TLR9 and NOD1 play well-established roles in host resistance to *T. cruzi* infection, it is unknown whether inflammasomes, multiprotein platforms that are emerging as central regulators in many infections and inflammatory pathologies, are involved. Herein, we show that *T. cruzi* activates NLRP3 inflammasomes by a mechanism that involves lysosomal cathepsins. NLRP3 and the effector protease caspase-1 appear to participate in controlling the acute phase of *T. cruzi* infection, as NLRP3^{-/-} and caspase-1^{-/-} mice exhibit a very prominent peak parasitemia, despite their ability to secrete IL-6 and IFN-γ. However, in the absence of NLRP3 and caspase-1, NO production is significantly impaired, indicating the involvement of NLRP3 inflammasomes in inducing NO secretion. The

deficiency in NO secretion reflects in the inability of macrophages to control intracellular amastigotes. Interestingly, NLRP3-dependent NO secretion is completely abrogated in the absence of caspase-1, but not in the absence of IL-1R, which could explain the ability of IL-1R^{-/-} macrophages, but not caspase-1^{-/-} macrophages, to prevent *T. cruzi* replication. In fact, macrophages isolated from MyD88^{-/-} mice, which are unable to respond to TLRs agonists (except TLR3), IL-1 and IL-18, secrete NO, although at low levels compared to WT cells. Moreover, the inhibition of caspase-1 in MyD88^{-/-} macrophages renders them susceptible to *T. cruzi* infection as NLRP3^{-/-} and caspase-1^{-/-} macrophages. Collectively, our data demonstrate the existence of a novel NO induction pathway that involves NLRP3 inflammasomes (Figure 6).

Recently, it has been reported that common pathways involving the production of ROS, disruption of lysosomes and/or potassium efflux influence NLRP3 activation, as they are activated by several

Figure 4. Caspase-1 but not IL-1R mediates *T. cruzi*-induced NO secretion. PMs (3×10^5 /well of chamber slides) from WT, MyD88^{-/-} and NLRP3^{-/-} mice were infected with *T. cruzi* Y trypomastigotes (5 parasites/cell) for 2–4 h. After the extracellular parasites were removed, AG (1 mM) was added to the cultures. After 48 h, the chambers were fixed with methanol, stained with DAPI and evaluated by fluorescence microscopy (600 $\times$). The numbers represent the increase in the number of amastigotes compared to untreated cells, and 1000 macrophages were counted from a representative experiment (A). PMs (3×10^5 /well of chamber slides) from WT, caspase-1^{-/-} and IL-1R^{-/-} mice (3 parasites/cell) were infected with *T. cruzi* Y trypomastigotes (5 parasites/cell) for 2–4 h. Chambers were obtained as described and evaluated by fluorescence microscopy (600 $\times$). The number of amastigotes was counted in 1000 macrophages from a representative experiment. The experiments were repeated two times with similar results (B). *T. cruzi*-infected PMs (3 parasites per cell) ($1,5 \times 10^6$ cells/300 μ l) from WT, caspase-1^{-/-}, iNOS^{-/-} and IL-1R^{-/-} mice were treated or not with z-YVAD-fmk (10 μ M) or AG (1 mM). After 48 h, nitrite accumulation was measured in the culture supernatants by the Griess reaction. Bars represent the mean $\pm$ S.D. of triplicate samples. *** $p > 0,001$ compared to the untreated group (C). *T. cruzi*-infected PMs (3×10^5 /well of chamber slides) from WT, caspase-1^{-/-}, iNOS^{-/-} and IL-1R^{-/-} mice (3 parasites/cell) were treated or not with z-YVAD-fmk (10 μ M) or AG (1 mM). Chambers were obtained as described above and evaluated by fluorescence microscopy (600 $\times$). The frequency of infected macrophages was counted in 1000 macrophages from a representative experiment. Bars represent the rate of increase compared to untreated cells, where *** $p > 0,001$ compared to the untreated group. The experiments were repeated two times with similar results (D).
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PAMPs and DAMPs that are not structurally related [39] [40] [41]. *T. cruzi* infection induced IL-1 β secretion by spleen cells as well as by macrophages isolated from WT mice, but not by cells isolated from NLRP3^{-/-} and caspase-1^{-/-} mice, which suggests that *T. cruzi* activates NLRP3 inflammasomes. Importantly, IL-1 β production was also reduced in MyD88^{-/-} cells, which is expected since pro-IL-1 β is transcriptionally regulated by signals dependent on TLR [50]. Interestingly, the pharmacological inhibition of cathepsin B abrogated *T. cruzi*-induced IL-1 β secretion, suggesting that lysosomal pathways are involved in NLRP3 activation in response to *T. cruzi* infection. In fact, *T. cruzi* invades a variety of cell types by recruiting lysosomes to the plasma membrane, which they fuse with to form a steady parasitophorous vacuole [51]. *T. cruzi* requires the acidic environment of the lysosome to exit the parasitophorous vacuole and replicate in cell cytosol. We suggest that the lysosomes are disrupted by this

process, leading to liberation of cathepsins which then mediate NLRP3 activation by an as yet unknown mechanism.

NLRP3 inflammasomes appear to control *T. cruzi* replication during the acute phase of infection, as NLRP3^{-/-} and caspase-1^{-/-} mice exhibit a very prominent peak parasitemia 11 d after infection. While NLRP3 is involved in many inflammatory disorders, little is known about the role of NLRP3 in controlling infections. Recently, we demonstrated that NLRP3 and AIM2 inflammasomes are involved in the control of *Brucella abortus* infection through an IL-1 β - and IL-18-dependent mechanism [45]. NLRP3-induced IL-1 β secretion also mediates host resistance to *C. rodentium*, [43], *M. kansasii* [42], *S. typhimurium* [44], Influenza A virus [46] and *C. albicans* [32] [52]. However, NLRP3 does not appear to play a role in the control of the protozoan parasite *Plasmodium* [33] [53]. Conversely, the absence of NLRP3 or IL-1 β resulted in increased survival of the malaria strain *P.*

Figure 5. NLRP3-mediated NO production acts synergistically with MyD88-mediated pathways to control *T. cruzi* infection. PMs from WT mice were infected with *T. cruzi* Y (3 parasites per cell) (1.5×10^6 cells/300 μ l) in the presence of neutralizing antibodies against IL-1 β , IL-18 or both cytokines in doses of 0,1, 1 and 10 μ g/mL, or left untreated. After 48 h, nitrite accumulation was measured in the culture supernatants by the Griess reaction. Bars represent the mean $\pm$ S.D. of triplicate samples where ** $p > 0,01$ and * $p > 0,05$ when compared to untreated group (A). *T. cruzi*-infected PMs from WT and MyD88^{-/-} mice (3 parasites/cell) were treated or not with z-YVAD-fmk (10 μ M). After 48 h, nitrite accumulation was measured in culture supernatants by Griess Reaction. Bars represent the mean $\pm$ S.D. of triplicate samples where *** $p > 0,001$ and * $p > 0,01$ when compared to untreated group (B). PMs (3×10^5 /well of chamber slides) from MyD88^{-/-} mice were infected with tripomastigotes from *T. cruzi* Y (5 parasites/cell) for 2–4 h. After removal of extracellular parasites, z-YVAD-fmk (10 μ M) was added to cultures. After 48 h chambers were prepared and analyzed as described above to evaluate the numbers of amastigotes. Results were obtained in 1000 counted macrophages from a representative experiment. Experiments were repeated three times with the same profile of results (C). doi:10.1371/journal.pntd.0002469.g005

chabaudi adami DS, indicating that NLRP3 promotes malaria pathology. The first demonstration of the involvement of inflammasomes in the control of a protozoan infection was recently described in collaboration with our group [54]. In this article, we demonstrated that the NLRP3 inflammasome is involved in the control of *L. amazonensis*, *L. braziliensis* and *L. infantum chagasi* through a mechanism that involves IL-1R-dependent NO production.

Interestingly, deficiencies in NLRP3 and caspase-1 result in high levels of parasites in the blood and inside macrophages, which is similar to results from MyD88^{-/-} and iNOS^{-/-} mice. However, iNOS^{-/-}, NLRP3^{-/-} and caspase-1^{-/-} mice do not succumb to infection, unlike MyD88^{-/-} mice. The pronounced deficiency in IFN- γ production exhibited by MyD88^{-/-} cells, but not by NLRP3^{-/-} and caspase-1^{-/-} cells, could explain this difference, as IFN- γ ^{-/-} mice also succumb early to infection. It is well-established that the deficiency in IFN- γ secretion by MyD88^{-/-} mice infected with *T. cruzi* reflects an impairment, not only in the microbicidal capacity of the macrophages but also in DC and TCD8 cell functions, as well an antibody switch to IgG2a and FC γ R induction [55]. This could also explain why the highest number of parasites was observed in IFN- γ ^{-/-} mice

compared to all the other immunodeficient mouse strains studied here.

Despite the ability to secrete pro-inflammatory cytokines, spleen cells and macrophages from NLRP3^{-/-} and caspase-1^{-/-} mice were not able to secrete NO in response to *T. cruzi* infection. The role of NO in the control of *T. cruzi* infection is well established [56]. Mice that are deficient in iNOS [18] or have been treated with pharmacological iNOS inhibitors [57] [58] exhibit high levels of parasites in the blood and can succumb to the acute phase of infection, depending on the parasite and mouse strain. In our model, the NLRP3^{-/-} and caspase-1^{-/-} mice behaved in a manner identical to the iNOS^{-/-} mice, with a higher magnitude of peak parasitemia compared to WT mice; however, most of the NLRP3^{-/-}, caspase-1^{-/-} and iNOS^{-/-} mice survived infection. Macrophages isolated from the NLRP3^{-/-} and caspase-1^{-/-} mice failed to kill amastigotes and exhibited an infection index that was even higher than that observed in MyD88^{-/-} macrophages. The incapacity to control *T. cruzi* infection demonstrated by NLRP3^{-/-} and caspase-1^{-/-} macrophages correlated with a deficiency in NO secretion. Moreover, adding AG to *T. cruzi*-infected macrophages increased the parasite burden in WT and MyD88^{-/-} macrophages. Conversely, no effect of AG was

Figure 6. The proposed model for NLRP3-mediated control of *T. cruzi*. TLR2, TLR4, TLR7 and TLR9 recognize *T. cruzi* PAMPs and control infection by MyD88 adaptor molecule-mediated effector mechanisms. *T. cruzi* infection leads to lysosomal disruption, which may result in cathepsin B release into the cytosol and NLRP3 inflammasome activation. The activation of the NLRP3 inflammasome results in caspase-1 activation, which induces NO secretion in an IL-1R-independent manner. Caspase-1-dependent NO secretion appears to cooperate with MyD88-dependent pathways to control *T. cruzi* infection.

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observed in NLRP3^{-/-} cells, indicating that this inflammasome is required for NO production in response to *T. cruzi* infection.

The maturation and secretion of the pro-inflammatory cytokines IL-1β and IL-18 and the induction of pyroptosis, a specialized form of inflammatory cell death, are the major effector mechanisms induced by activated caspase-1 [21] [23]. While pyroptosis is known to play a role in controlling bacterial infections [59], this specialized cell death process did not appear to occur during *T. cruzi* infection, as the majority of the macrophages were viable 48 hs after infection. Recently, we described a caspase-1-dependent pathway for iNOS activation in response to cytosolic flagellin that is mediated by NAIP5/NLRC4-inflammasomes [60]. Caspase-1-dependent iNOS activation is involved in the control of *Legionella pneumophila* [60] and *Salmonella typhimurium* (unpublished data from our group) by macrophages. Interestingly, MyD88, IL-1β and IL-18 are dispensable for iNOS activation in response to cytosolic flagellin. IL-1β and IL-18 also appear to play a minor role in the induction of NO production and the capacity of macrophages to control *T. cruzi* infection, as a genetic defect in IL-1R, as well as the neutralization of IL-1β and IL-18, have little influence on these activities. Conversely, NO secretion was not observed in caspase-1^{-/-} macrophages or in macrophages treated with z-YVAD-fmk, confirming the existence of a pathway of NLRP3-mediated NO secretion in response to *T. cruzi* infection

that is dependent of caspase-1 but independent of IL-1β and IL-18, the best studied caspase-1 substrates. However, the mechanism by which caspase-1 mediates NO secretion remains unclear. Recently, it was demonstrated that a chromatin-associated multifunctional enzyme PARP1 (also called ARTD1) negatively regulates the transcription of NF-κB-dependent target genes by a pathway that requires NLRP3/caspase-1/caspase-7 activation [61]. Mutating the PARP1 cleavage site D214 rendered PARP1 uncleavable and inhibited PARP1 release from chromatin and chromatin decondensation, thereby inhibiting the expression of cleavage-dependent NF-κB target genes such as *il-6*, *cfs2* and *lif*, but not *ip-10* [61]. Earlier studies showed that PARP1 cleavage is essential for stimulus-dependent transcriptional activation of transiently transfected reporter plasmids containing the *inos* and *mip2* NF-κB sites in mouse lung fibroblasts (MLFs) [62] [63]. Additionally, *inos* expression is significantly reduced in the presence of noncleavable PARP1 (D214N), compared to cleavable PARP1 (WT or enzymatically inactive) [64]. It was recently demonstrated that treatment with Olaparib (a pharmacological inhibitor of PARP-1 enzymatic activity) reduced *T. cruzi* replication in VERO cells [65]. However, the enzymatic activity of PARP-1 is not related to the role of cleaved PARP-1 in the regulation of chromatin condensation and access to transcription factors [60]. Therefore, further studies are required to understand

the molecular machinery involved in the regulation of iNOS activation by the caspase-1/IL-1 axis.

TLR pathways [66], IFN- γ [67] and IFN- γ -related cytokines such as IL-12 [15] [68] are known to be the major inducers of NO production in response to *T. cruzi* infection. However, we demonstrate here that pharmacological inhibition of caspase-1 abrogates NO secretion by spleen cells and macrophages from WT and MyD88^{-/-} mice and renders their macrophages as susceptible to *T. cruzi* infection as NLRP3^{-/-}, caspase-1^{-/-} and iNOS^{-/-} cells. This indicates that the requirement of NLRP3 inflammasomes for NO secretion bypasses the need for MyD88, and identifies a novel NLRP3-dependent pathway that stimulates NO production in response to *T. cruzi* infection. Despite the minor effect of IL-1R and IL-18 on NO secretion in response to *T. cruzi* infection, the amount of NO produced by macrophages and cultured spleen cells from MyD88^{-/-} mice is significantly lower than that produced by WT cultures. The fact that caspase-1 inhibition in MyD88^{-/-} cells abrogates NO production and renders macrophages more susceptible to *T. cruzi* infection suggests that the NLRP3 and MyD88 pathways cooperate to induce NO secretion and promote host resistance to *T. cruzi*. Taken together, our data describe a novel effector mechanism mediated by NLRP3 inflammasomes that contributes to the control of *T. cruzi* infection.

Supporting Information

Figure S1 Inflammatory cell death is not involved in the control of *T. cruzi* by macrophages. PMs (3×10⁵/well of chamber slides) from WT mice were infected with *T. cruzi* Y trypomastigotes (1:5). After 2 h or 48 h, the chambers were fixed with methanol, stained with DAPI and evaluated by fluorescence microscopy (600×). Bars represent the mean ± S.D. of triplicate samples of the number of amastigotes found in 100 macrophages (MOs). * p>0,01 compared to the 2 h group. The number of amastigotes was counted in 1000 PMs from a representative of two experiments (A). PMs (3×10⁵/well of chamber slides) from WT

mice were stimulated with 6 µg/ml of purified flagellin from *Bacillus subtilis* inserted into DOTAP (Fladot) for 6 h, or were infected with *T. cruzi* Y (5:1) for 6–48 h. Cytotoxicity was assessed by fluorescence microscopy according to the loss of vital acridine orange staining and incorporation of ethidium bromide (B). (TIF)

Table S1 Effect of NLRP3 and caspase-1 in the control of the acute phase of *T. cruzi* infection. WT, MyD88^{-/-}, NLRP3^{-/-}, Caspase-1^{-/-}, IFN- γ ^{-/-} and iNOS^{-/-} mice were subcutaneously infected with 10³ *T. cruzi* blood trypomastigotes. Parasitemia was quantified by counting the parasites in 5 µL of tail blood obtained on days 4 to 20 after infection. Global Parasitemia (GP) represents the mean of sum of total blood parasites found in each mouse strain and S.D. (n = 6). *** p>0,001 compared to the WT group and #p>0,001 compared to IFN- γ ^{-/-} group. No significant difference was observed among MyD88^{-/-}, iNOS^{-/-}, Caspase-1^{-/-} and NLRP3^{-/-} groups. AUC – Area under Curve. Experiments were repeated two times with IFN- γ ^{-/-} and iNOS^{-/-} mice and five times with MyD88^{-/-}, NLRP3^{-/-}, caspase-1^{-/-} mice showing similar results. (DOCX)

Text S1 Ethidium bromide and acridine orange stain. (DOCX)

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Author Contributions

Conceived and designed the experiments: VMG KCM CLB ACT KRB. Performed the experiments: VMG KCM BHM DF. Analyzed the data: VMG KCM CLB ACT MMR JMA KRB. Contributed reagents/materials/analysis tools: MMR KRB. Wrote the paper: VMG KRB. Critically revised the manuscript: CLB ACT MMR JMA.

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