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**A ativação de caspase-8 no inflamassoma de Naip5/NLRC4 em
resposta a infecção por *Legionella pneumophila***

Ribeirão Preto
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Danielle Pini Alves Mascarenhas

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Tese apresentada à Faculdade de
Medicina de Ribeirão Preto da
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obtenção do título de Doutor em
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Área de Concentração:
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Orientador: Prof. Dr. Dario Simões
Zamboni

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Resumo

RESUMO

Mascarenhas, D. P. A. **A ativação de caspase-8 no inflamassoma de Naip5/NLRC4 em resposta a infecção por *Legionella pneumophila***. 2018. Tese (Doutorado) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2018.

A bactéria *Legionella pneumophila* é um bacilo Gram-negativo, flagelado causador da doença dos legionários e febre de Pontiac. O inflamassoma mais importante no controle da replicação desta bactéria é o composto por Naip5/NLRC4, que é responsável pelo reconhecimento de flagelina. A ativação do inflamassoma de Naip5/NLRC4 pela flagelina induz a ativação de caspase-1, induzindo a formação de poros na membrana, piroptose e controle da replicação desta bactéria. A participação da proteína adaptadora ASC é essencial para a nucleação deste complexo e secreção de citocinas inflamatórias como IL-1 β e IL-18 por esta via. Além do controle da replicação de *L. pneumophila* pelo inflamassoma NLRC4 dependente de caspase-1, foi demonstrado que existe uma via induzida por NLRC4 independente de caspase-1/11. Dessa forma, camundongos e células *Nlrc4*^{-/-} são mais susceptíveis à infecção por esta bactéria do que as células *Casp1/11*^{-/-}. Neste trabalho, nós identificamos que a via independente de caspase-1/11 é composta por Naip5/NLRC4/ASC/Caspase-8 e é essencial para o controle da replicação de *Legionella* spp. flageladas em macrófagos e in vivo. Através da utilização de BMDMs *Casp1/11*^{-/-} e *Asc/Casp1/11*^{-/-} transduzidos com NLRC4-GFP ou ASC-GFP, identificamos que a formação de punctas de NLRC4 e ASC dependem do reconhecimento de flagelina e que ASC é essencial para a formação desses punctas. Também foi identificado que a infecção com *L. pneumophila* que expressa flagelina leva à ativação de caspase-8 de maneira dependente de ASC e Naip5, mas independente de caspase-1/11. De acordo com esses dados, o silenciamento de caspase-8 em macrófagos *Casp1/11*^{-/-} aumentou a susceptibilidade dessas células à infecção com *L. pneumophila* flagelada. Além disso, macrófagos e camundongos *Asc/Casp1/11*^{-/-} foram tão susceptíveis quanto os *Nlrc4*^{-/-} e mais susceptíveis que os *Casp1/11*^{-/-}. Nós observamos que o inflamassoma de NLRC4/ASC/Caspase-8 induz formação de poros e morte celular independente de gasdermina-D (GSDMD). Por meio da utilização de células de camundongos C57BL/6, foi observado que caspase-8 é recrutada para o inflamassoma de Naip5/NLRC4/ASC/Caspase-1. Entretanto, a ativação de caspase-8 só ocorre na

ausência de caspase-1 ou GSDMD. Nossos dados sugerem que a ativação de caspase-8 no inflamassoma composto por NLRC4/ASC/Caspase-8 representa uma via alternativa que opera para garantir o controle da replicação de bactérias flageladas em situações nas quais ou caspase-1 ou GSDMD estão inibidas.

Palavras-chave: Inflamassoma; NLRC4; Caspase-8; *Legionella pneumophila*; Controle da replicação.

Abstract

ABSTRACT

Mascarenhas, D. P. A. **The activation of caspase-8 by Naip5/NLRC4 inflammasome in response to *Legionella pneumophila* infection.** 2018. Tese (Doutorado) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2018.

Legionella pneumophila is a flagellated Gram-negative bacillus that is the causative agent of the legionnaire's disease and Pontiac fever. The most important inflammasome for the control of *L. pneumophila* replication is the Naip5/NLRC4, responsible for the flagellin recognition. The activation of the Naip5/NLRC4 inflammasome leads to caspase-1 activation, consequently pore formation, pyroptosis and control of bacterial replication. The participation of the adaptor molecule ASC is essential for this complex nucleation and the secretion of inflammatory cytokines like IL-1 β and IL-18 by this pathway. Besides the control of *L. pneumophila* replication by Naip5/NLRC4/Caspase-1 inflammasome, it was demonstrated there are NLRC4 responses independent of caspase-1/11. These explain why mice and macrophages *Nlrc4*^{-/-} are more susceptible than *Casp1/11*^{-/-}. In this work, we identified that the caspase-1/11-independent pathway is composed of Naip5/NLRC4/ASC/Caspase-8 and it is essential for the control of flagellated *Legionella* spp. replication in macrophages and *in vivo*. Infection of *Casp1/11*^{-/-} and *Asc/Casp1/11*^{-/-} macrophages, transduced with NLRC4-GFP or ASC-GFP, showed that flagellin-positive bacteria triggered puncta formation that is ASC-dependent. Accordingly, Naip5 and ASC, but not caspase-1/11, were required for caspase-8 activation in response to flagellated bacteria. Silencing caspase-8 in *Casp1/11*^{-/-} BMDMs increased the susceptibility to *L. pneumophila* infection. Furthermore, the macrophages and mice *Asc/Casp1/11*^{-/-} are as susceptible as *Nlrc4*^{-/-}, but more susceptible than *Casp1/11*^{-/-}. We also found that the NLRC4/ASC/Caspase-8 inflammasome induces GSDMD-independent pore formation and cell death. Using C57BL/6 cells, we observed that caspase-8 is recruited to Naip5/NLRC4/ASC/Caspase-1 inflammasome. However, caspase-8 is just activated in the absence of caspase-1 or GSDMD. Our data suggest that caspase-8 activation in the NLRC4/ASC/Caspase-8 inflammasome represents an alternative pathway that operates to ensure the control of flagellated bacteria replication in situations which either caspase-1 or GSDMD are inhibited.

Keywords: NLRC4; Inflammasome; Caspase-8; *Legionella pneumophila*; Control of replication.

Abreviaturas

ABREVIATURAS

AIM2: *Absent in melanoma 2*

ANOVA: Análise de variância

ASC: *Apoptosis-associated speck-like protein containing a caspase recruitment domain*

ATP: *Adenosine triphosphate*

BIR: *Baculovirus inhibitor of apoptosis repeat domain*

BMDMs: *Bone marrow-derived macrophages*

CARD: *Caspase activation and recruitment domain*

CT: *Cycle Threshold*

CYE: *Charcoal yeast extract agar* (Meio sólido para cultivo de *Legionella* spp)

DAMPs: *Damage-associated molecular pattern*

DNA: *Deoxyribonucleic acid*

cDNA: *Complementary DNA*

Dot: *Defective in organelle trafficking*

ECL: *Entry-level peroxidase substrate for enhanced chemiluminescence*

ELISA: *Enzyme-Linked Immunosorbent Assay*

***flaA*⁻:** *Legionella pneumophila* deficiente para o gene *flaA*, que codifica a flagelina.

***fliI*⁻:** *Legionella pneumophila* deficiente para o gene *fliI*, que codifica uma ATPase necessária para a secreção de flagelina na formação do flagelo.

FWD: *Primer forward*

g: Grama

GFP: *Green fluorescent protein*

GSDMA: Gasdermina-A

GSDMC: Gasdermina-C

GSDMD: Gasdermina-D

GSDME: Gasdermina-D

GSDMF: Gasdermina-F

h: Horas

HIN: *Hematopoietic IFN-inducible nuclear domain*

Icm: *Intracellular multiplication*

IFNs: Interferon

IL: Interleucina

IRAK: *IL-1R-associated kinases*

IRF: *Interferon Regulators Factors*

JNK: *JUN N-terminal kinase*

LCCM: *L929 cell conditioned media*

LCV: *Legionella-containing vacuole*

LDH: *Lactate dehydrogenase*

Lp: *Legionella pneumophila*

LPS: Lipopolissacarídeo

LRR: *Leucine-rich repeat*

Lvh: *L. pneumophila vir homologues*

MAL/TIRAP: *TIR domain containing adaptor protein MAL (Myd88 adaptor-like)*

MAMPs: *Microbe-associated molecular patterns*

MAPK: *Mitogen Activated Protein Kinases*

MCSF: *Macrophage colony-stimulating factor*

µg: Micrograma

µL: Microlitros

µM: Micromolar

mg: Miligrama

ml: Mililitro

mM: Milimolar

MOI: *Multiplicity of infection*

MyD88: *Myeloid differentiation primary response 88*

NAIP: *NLR family of apoptosis inhibitory protein*

NBD: *Nucleotide-binding and oligomerization domain*

ND: *Not detected* (Diferença estatística não detectada)

NF-κB: *Nuclear Factor- κB*

ng: Nanograma

NI: Não infectado

NLR: *Nucleotide-binding domain leucine-rich repeat-containing protein (Nod like receptor)*

NLRC4: *NLR family of CARD domain containing protein 4*

NLRP3: *NLR family of PYRIN domain containing protein 3*

nm: Nanômetro

Nod1: *Nucleotide binding and oligomerization domain containing 1*

Nod2: *Nucleotide binding and oligomerization domain containing 2*

NT shRNA: *Non-target shRNA*

O.D: *Optical density*

PBS: *Phosphate buffered saline*

PEI: *Polyethylenimine*

PI: *Propidium iodide*

PITs: *Pore-induced intracellular traps*

PRRs: *Pattern recognition receptors*

PYD: *Pyrin domain*

qPCR: *Quantitative polymerase chain reaction*

REV: *Primer reverse*

RIPA Buffer: *Radio immunoprecipitation assay buffer*

Ripk3: *Receptor-interacting protein (RIP) family of serine/threonine protein kinases*

RPMI: *Roswell Park Memorial Institute medium*

Rpsl: *30S ribosomal protein S12*

RNA: *Ribonucleic acid*

SBF: *Soro Bovino Fetal*

SDS: *Sodium dodecyl sulfate*

shRNA: *Small hairpin RNA*

T4ASS: *Type IVA secretion system*

T4BSS: *Type IVB secretion system*

TBS: *Tris buffered saline*

TIR: *Toll IL-1 receptors*

TLR: *Toll like receptors*

TNF: *Tumor necrosis factor*

TRAF: *TNF receptor-associated factors*

TRAM: *TRIF-related adaptor molecule*

TRIF: *TIR-domain-containing adapter-inducing interferon- β*

UFC: Unidades Formadoras de Colônia

WT: *Wild-type*

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Introdução

1 – INTRODUÇÃO

1.1- *Legionella pneumophila*

A bactéria *L. pneumophila* foi isolada pela primeira vez em 1976 na Filadélfia - EUA, após um surto entre os participantes da “Convenção Americana dos Legionários” que apresentavam sintomas característicos de uma pneumonia. Cerca de 200 pessoas foram infectadas e destas 29 não sobreviveram (Fraser *et al.*, 1977). Após este surto se iniciou uma corrida para a identificação do organismo causador da infecção. O desenvolvimento do meio ideal para o seu isolamento e a utilização das técnicas empregadas nos estudos com *Rickettsia*, outra bactéria intracelular, foram cruciais para a caracterização dessa bactéria. *L. pneumophila* foi então identificada como um bacilo Gram-negativo flagelado, e a doença causada por essa bactéria foi então denominada de “doença dos legionários” em referência aos legionários afetados (Mcdade *et al.*, 1977).

Hoje são conhecidas mais de 60 espécies de *Legionella*, sendo que todas já foram isoladas de amostras ambientais e pelo menos metade delas já foi reportada em infecção em humanos (Yu *et al.*, 2002). Dentre essas, as espécies de *Legionella* mais comumente isoladas de humanos são *L. pneumophila* e *L. longbeachae* seguidas de *L. bonzemanii*, *L. micdadei* e *L. dumoffii* (Yu *et al.*, 2002). Entretanto, mais de 90% dos casos de pneumonia relacionada à doença dos legionários, são resultados de infecções por *L. pneumophila*, dado que pode não ser correto devido à dificuldade de identificação de outras espécies de *Legionella* nos exames diagnósticos (Fraser *et al.*, 1977; Mcdade *et al.*, 1977; Khodr *et al.*, 2016).

As infecções por *Legionella* ocorrem principalmente em surtos, durante períodos mais quentes do ano (Phin *et al.*, 2014). Entretanto uma pequena parcela das pessoas exposta à *Legionella* desenvolve a doença dos legionários. Essa susceptibilidade está relacionada a alguns fatores como: fumo, idade avançada, doenças cardiovasculares crônicas, doenças respiratórias, abuso de álcool, diabetes e outras condições que possam levar a imunodeficiência (Phin *et al.*, 2014; Khodr *et al.*, 2016).

As bactérias do gênero *Legionella* podem causar dois tipos principais de doença: a doença dos legionários e febre de Pontiac. A doença dos legionários é caracterizada por um período de incubação e 3 a 10 dias e por sintomas de uma broncopneumonia, podendo levar à morte (Kaufmann *et al.*, 1981; Doebbeling e

Wenzel, 1987). Já a febre de Pontiac é a manifestação mais frequente das infecções por *Legionella*, com tempo de incubação de 36 horas e sintomas muito parecidos com os da gripe, como por exemplo febre, calafrios, dores de cabeça e mialgia (Kaufmann *et al.*, 1981; Doebbeling e Wenzel, 1987). A febre de Pontiac, foi assim denominada após um surto de infecção em 1968 entre os funcionários do Departamento de Saúde de Pontiac, Michigan - EUA (Glick *et al.*, 1978). Entretanto, somente após o surto da doença dos legionários na Filadélfia, foi identificado que ambas as doenças eram causadas pelas mesma bactéria (Blaser, 1977).

O gênero *Legionella* apresenta bactérias intracelulares facultativas, que vivem em ambientes de água doce ou em solo (Fliermans *et al.*, 1981). Essas bactérias seguiram a sua evolução infectando protozoários unicelulares, incluindo diversas espécies de amebas de vida livre como *Acanthamoeba* e *Naegleria* (Rowbotham, 1980). A infecção em humanos ocorre pela inalação de gotículas de água contendo a bactéria. Essas gotículas de água são oriundas de sistemas de irrigação, fontes, torres de refrigeração ou de aparelhos de ar condicionado onde essa bactéria forma biofilmes (Fraser *et al.*, 1979; Morris *et al.*, 1979; Sethi e Brandis, 1983; Spitalny *et al.*, 1984; Newton *et al.*, 2010; Lucas *et al.*, 2018). Após a inalação das gotículas contaminadas as bactérias são fagocitadas pelos macrófagos alveolares onde apresentam um ciclo de vida muito similar ao que vivem no interior as amebas (Horwitz e Silverstein, 1980).

No meio intracelular, a *L. pneumophila* subverte as funções da célula hospedeira, inibindo a fusão do fagossomo com o lisossomo, impedindo a sua degradação (Horwitz, 1983). Em paralelo, essa bactéria recruta proteínas e organelas da célula hospedeira, como as mitocôndrias e vesículas do retículo endoplasmático, gerando um compartimento ideal para a sua replicação, com características de retículo endoplasmático rugoso, denominado de LCV (*Legionella-containing vacuole*) (Horwitz, 1983; Swanson e Isberg, 1995). A subversão das funções normais da célula hospedeira pela *L. pneumophila* é dependente do Sistema de Secreção do tipo IVB (T4BSS), denominado de Dot/Icm (*Defective in organelle trafficking / Intracellular multiplication*) (Marra *et al.*, 1992; Berger e Isberg, 1993; Berger *et al.*, 1994; Roy e Isberg, 1997; Roy *et al.*, 1998). Esse sistema de secreção é composto por 27 genes, importantes para translocar mais de 300 proteínas efetoras que podem agir em diversas vias da célula hospedeira (Coers *et al.*, 2000; Nagai *et al.*, 2002; Newton *et*

al., 2010; Khodr *et al.*, 2016). Mutações em genes que formam o Dot/Icm, levam a um defeito na formação do LCV e na replicação intracelular desta bactéria nas células hospedeiras (Newton *et al.*, 2010).

Além do T4BSS, também foi descrito que *L. pneumophila* expressa um T4ASS, denominado de *Lvh* (*L. pneumophila vir homologues*) importante para o translocamento de DNA e conjugação entre bactérias (Segal *et al.*, 1999). A transferência horizontal de genes entre as bactérias tem sido muito importante durante a história evolucionária desses organismos, gerando mecanismos para parasitar novos nichos, como ocorreu em relação aos humanos, considerados hospedeiros acidentais dessa bactéria (Cazalet *et al.*, 2004; Gomez-Valero e Buchrieser, 2013).

Infecções com *L. pneumophila* passaram a ser mais frequentes em humanos com as mudanças em nosso estilo de vida, como por exemplo o aumento do número de aparelhos de ar condicionado e torres de refrigeração em edifícios, o que possibilita o espalhamento do aerossol desta bactéria (Gomez-Valero e Buchrieser, 2013). Entretanto, a infecção por *L. pneumophila*, diferente do que ocorre nas amebas, é controlada em células de mamíferos (Yamamoto *et al.*, 1988; Cirillo *et al.*, 1999). Acredita-se que isso ocorra porque durante o seu processo evolutivo, a *L. pneumophila* sofreu pressões seletivas infectando protozoários unicelulares, entretanto o hospedeiro humano é considerado seu hospedeiro acidental (Lau e Ashbolt, 2009; Khodr *et al.*, 2016). Por essa razão *L. pneumophila* apresenta fatores de virulência para subverter certas vias de células eucarióticas comuns entre as amebas e os humanos (Gomez-Valero e Buchrieser, 2013). Entretanto, essa bactéria falha na inibição de outras vias mais específicas, como as de reconhecimento do sistema imune inato. Assim, em células de mamíferos, *L. pneumophila* é reconhecida por diversos receptores da imunidade inata, como os TLRs (*Toll-like Receptors*) e os NLRs (*Nod-like Receptors*), gerando respostas inflamatórias e sendo rapidamente controlada (Massis e Zamboni, 2011). Consequentemente, *L. pneumophila* é um excelente modelo para o estudo de novas vias de reconhecimento de MAMPs (*Microbe-associated molecular patterns*) e DAMPs (*Damage-associated molecular patterns*) por PRRs (*Pattern recognition receptors*) (Massis e Zamboni, 2011; Mascarenhas e Zamboni, 2017).

1.2- Reconhecimento de *Legionella pneumophila* por PRRs

Os receptores do tipo TLR foram identificados pela primeira vez em *Drosophila melanogaster* (Lemaitre et al., 1996). Em humanos e camundongos foram identificados 10 e 13 receptores do tipo TLR respectivamente (Rock et al., 1998). Esses receptores são proteínas presentes na membrana plasmática das células (TLR1, TLR2, TLR4, TLR5 e TLR6) ou em endossomos (TLR3, TLR4, TLR7, TLR8, TLR9 e TLR13) (O'Neill et al., 2013). Como estrutura geral os TLRs contêm um domínio N-terminal LRR (*Leucine-rich repeat*) extracelular de reconhecimento, um domínio transmembrana e um domínio intracelular C-terminal de sinalização (Medzhitov et al., 1997; Medzhitov, 2001). Já os TLRs endossomais apresentam o domínio N-terminal no interior do endossomo (Creagh e O'Neill, 2006; O'Neill et al., 2013). A sinalização por esses receptores ocorre por meio de proteínas adaptadoras contendo domínios TIR (*Toll IL-1 receptors*), também presentes na sinalização via IL-1 e IL-18, e são eles: TRAM (*TRIF-related adaptor molecule*), MAL/TIRAP {*TIR domain containing adaptor protein MAL (Myd88 adaptor-like)*}, MyD88 (*Myeloid differentiation primary response gene 88*) e TRIF (*TIR-domain-containing adapter-inducing interferon- β*) (Creagh e O'Neill, 2006).

Os TLRs podem ser ativados por diversas moléculas de vírus, bactérias, fungos e protozoários (Akira e Takeda, 2004; Kawai e Akira, 2010). Um exemplo é a ativação de TLR5 que ocorre pelo reconhecimento de flagelina, o principal componente do flagelo bacteriano. Já o receptor TLR4 é capaz de reconhecer LPS (lipopolissacarídeo), presente na parede celular de bactérias Gram-negativas. TLR3 é responsável por reconhecer dupla fita de RNA de infecções virais, enquanto TLR2 é capaz de reconhecer lipoproteínas e glicolipídios de diversos patógenos (Akira e Takeda, 2004).

A ativação desses receptores, desencadeia uma cascata sinalizadora com recrutamento de IRAK (*IL-1R-associated kinases*) e TRAF (*TNF receptor-associated factors*), levando à ativação das vias de MAPK (*Mitogen activated protein kinase*), JNK (*JUN N-terminal kinase*) e p38. Bem como a ativação de fatores de transcrição como NF- κ B (*Nuclear factor- κ B*) e IRF (*Interferon regulators factors*) (Kawai e Akira, 2010;

O'Neill *et al.*, 2013). De forma geral, a principal consequência da ativação dos TLRs é a indução de citocinas pró-inflamatórias, como TNF e IL-12, bem como de IFNs (Interferons). Além disso, o reconhecimento de moléculas pelos TLRs, bem como por outros PRRs, desencadeia na transcrição de certas proteínas como caspase-1, caspase-11 e NLRP3 (*NLR family of PYRIN domain containing protein 3*), bem como precursores de outras citocinas como pró-IL-1 β e pró-IL-18 em um processo denominado de “*priming*” (Bauernfeind *et al.*, 2009). Esse processo é essencial para a ativação de outras vias do sistema imune inato importantes de reconhecimento de patógenos, como a dos inflamassomas (Kawai e Akira, 2011; Latz *et al.*, 2013).

Muitos trabalhos têm demonstrados vias envolvendo o reconhecimento de MAMPs de *L. pneumophila* pelos TLRs. Um exemplo é o papel do receptor TLR2 no controle da infecção ou chamado “*clearance*” desta bactéria (Mascarenhas *et al.*, 2015). Trabalhos observaram que camundongos deficientes para *Tlr2* apresentam uma diminuição na secreção de citocinas como IL-12 e IL-10, e são mais susceptíveis à infecção que os camundongos C57BL/6 quando infectados com *L. pneumophila* (Hemmi *et al.*, 2000; Akamine *et al.*, 2005; Archer e Roy, 2006; Hawn *et al.*, 2006; Newton *et al.*, 2007; Archer *et al.*, 2009). A sinalização por TLR2 no reconhecimento de moléculas bacterianas como peptidoglicano e lipopeptídeos requer sua oligomerização com TLR1 ou TLR6, capazes de reconhecer lipoproteínas (Ahmadishoar e Kariminik, 2017). Um trabalho publicado em 2013 demonstrou que tanto TLR1 como TLR6 são essenciais para o reconhecimento de *L. pneumophila* por TLR2 e que polimorfismos em *Tlr6* estão ligados a casos de alto risco à doença dos legionários após a exposição a esta bactéria (Misch *et al.*, 2013).

Além do reconhecimento de *L. pneumophila* por TLR2, também foi demonstrado que a flagelina desta bactéria é reconhecida pelo receptor TLR5, regulando o recrutamento inicial de neutrófilos para o pulmão. Entretanto camundongos *Tlr5*^{-/-} não apresentam uma susceptibilidade significativa em relação aos C57BL/6 quando infectados com *L. pneumophila* (Hawn *et al.*, 2007; Archer *et al.*, 2009). Adicionalmente, assim como ocorre com outras bactérias Gram-negativas, o LPS de *L. pneumophila*, pode ser reconhecido por TLR4, entretanto o reconhecimento por esse receptor também não parece ser essencial o controle da infecção dessa bactéria (Lettinga *et al.*, 2002; Archer e Roy, 2006; Mascarenhas *et al.*, 2015).

Trabalhos tem demonstrado que camundongos deficientes para *Myd88* apresentam uma falha no controle da infecção de *L. pneumophila* (Archer e Roy, 2006; Hawn et al., 2006). Entretanto, não foi reportado nenhum camundongo deficiente para TLR tão susceptível quanto o *Myd88*^{-/-}. Mesmo camundongos duplo deficientes para os receptores *Tlr2/Tlr9* e *Tlr2/Tlr5* não são tão susceptíveis quanto os camundongos *Myd88*^{-/-} infectados com *L. pneumophila* (Archer et al., 2009). Recentemente demonstramos que além dos TLRs, a alta susceptibilidade do *Myd88*^{-/-} também ocorre pelo seu papel na sinalização via IL-1. Já que camundongos *Il-1r*^{-/-} são altamente susceptíveis à infecção por *L. pneumophila* e apresentam uma falha na *clearance* desta bactéria, bem como no recrutamento de neutrófilos para o pulmão (Mascarenhas et al., 2015). Desta maneira, existe uma grande cooperação entre as sinalizações que operam via MyD88, bem como uma grande redundância entre as sinalizações via TLR em resposta à *L. pneumophila*.

Além dos TLRs, uma outra família de PRRs citoplasmáticos foi descrita pela sua grande importância no reconhecimento de MAMPs e DAMPs, os NLRs (*Nucleotide-binding domain leucine-rich repeat-containing protein* ou *Nod-like receptors*). Homólogos desses receptores são encontrados em plantas, denominadas de “proteínas R”, importantes no reconhecimento de moléculas conservadas de bactérias patogênicas (Dangl e Jones, 2001). Os NLRs apresentam uma estrutura tripartida contendo: um domínio C-terminal LRR que atua como sensor de diferentes estímulos microbianos e de dano, um domínio NBD (*Nucleotide-binding and oligomerization domain*) responsável pela oligomerização do receptor após a ligação ao ATP e um domínio N-terminal variável, o qual determina a função efetora do receptor (Fig. 1). O domínio N-terminal pode ser um domínio CARD (*Caspase activation and recruitment domain*), um domínio PYD (*Pyrin domain*) ou um domínio BIR (*Baculovirus inhibitor of apoptosis repeat domain*) (Inohara e Nunez, 2003; Akira, 2009).

Figura 1: Estrutura geral dos receptores NLRs. De maneira geral os NLRs apresentam uma estrutura tripartida contendo: um domínio C-terminal LRR (*Leucine-rich repeat*), um domínio NBD (*Nucleotide-binding and oligomerization domain*) e um domínio N-terminal variável, podendo ser um domínio CARD (*Caspase activation and recruitment domain*), um domínio PYD (*Pyrin domain*) ou um domínio BIR (*Baculovirus inhibitor of apoptosis repeat domain*). Tanto NLRP3 quanto NLRP1 apresentam PYD como domínios N-terminais. Já NLRC4 apresenta um domínio de recrutamento de caspase CARD, e Naip5 apresenta um domínio BIR. Adaptado de: TING, Jenny PY et al. *Nature Reviews Immunology*, 2008.

Os NLRs são ativados por diversos MAMPs e DAMPs como flagelina, cristais de ácido úrico, peptidoglicano e toxinas bacterianas, sendo de grande importância no controle da infecção de bactérias intracelulares como *L. pneumophila* (Kufer *et al.*, 2005; Ting *et al.*, 2008; Kim *et al.*, 2016). Como exemplo Naip5 (*NLR family of apoptosis inhibitory protein 5*), um NLR capaz de reconhecer flagelina bacteriana de *L. pneumophila* gerando a formação de um complexo intracelular que induz o controle da replicação desta bactéria (Zamboni *et al.*, 2006). Ou também os receptores Nod1 (*Nucleotide binding and oligomerization domain containing 1*) e Nod2 (*Nucleotide binding and oligomerization domain containing 2*), que são capazes de sinalizar em resposta à infecção por *L. pneumophila*, sendo importantes para o *clearance* e

recrutamento de células para o pulmão em resposta a esta bactéria (Shin *et al.*, 2008; Berrington *et al.*, 2010; Frutuoso *et al.*, 2010).

Além destes, diversos receptores NLRs são ativados em resposta à infecção por *L. pneumophila* (Massis e Zamboni, 2011; Mascarenhas e Zamboni, 2017). Após ativados esses NLRs se oligomerizam, levando à ativação de diversas vias como de MAPK, indução de fatores de transcrição como NF- κ B, e também a formação de um complexo proteico denominado de inflamassoma com a ativação de caspases (Ting *et al.*, 2008; Guo *et al.*, 2015).

1.3- Ativação dos inflamassomas por *Legionella pneumophila*

Os inflamassomas são caracterizados por um agregado de proteínas que são formados no citoplasma da célula e induzem a ativação e clivagem de caspases inflamatórias como caspase-1 e caspase-11 (Fig. 2) (Martinon *et al.*, 2002). As caspases ativas induzem a formação de poros na membrana celular e uma morte celular característica denominada de piroptose, além da secreção de citocinas pró-inflamatórias como IL-1 β e IL-18 (Cookson e Brennan, 2001; Fink e Cookson, 2006).

Tem sido amplamente estudada a ativação dos inflamassomas pela *L. pneumophila*, sendo essa bactéria um excelente modelo para o estudo destas vias. Um dos mais importantes inflamassomas ativados por esta bactéria é o de Naip5/NLRC4, responsável pelo reconhecimento de flagelina bacteriana e ativação de caspase-1 (Fig. 2) (Ren *et al.*, 2006; Zamboni *et al.*, 2006; Kofoed e Vance, 2011; Zhao *et al.*, 2011). Outro inflamassoma também muito estudado, principalmente pela ampla gama de agonistas pelo qual é ativado é o de NLRP3 (Latz *et al.*, 2013). Esse inflamassoma é ativado por diversos fatores, entretanto todos parecem convergir para efluxo de potássio, que parece ser o principal ativador desta via (Rivers-Auty e Brough, 2015; Ruhl e Broz, 2015). Além dos inflamassomas dependentes dos receptores NLRs, *L. pneumophila* também ativa vias independentes destes receptores, como o inflamassoma de AIM2 (*Absent in melanoma 2*) e de caspase-11, responsáveis pelo reconhecimento de dupla fita de DNA e LPS de bactérias Gram-negativas, respectivamente (Mascarenhas e Zamboni, 2017).

A formação da maioria destes inflamassomas depende da proteína adaptadora ASC (*Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain*). Essa proteína apresenta dois domínios de interação proteína-

proteína, um domínio PYD que se liga aos receptores NLRs, e um domínio CARD que se liga às caspases, funcionando assim como uma ponte entre elas (Fig. 2) (Masumoto *et al.*, 1999; Motani *et al.*, 2011; Proell *et al.*, 2013). Trabalhos demonstram que ASC facilita a nucleação dos inflamassomas, sendo essencial para a formação de “*punctas*” ou “*specks*” (Masumoto *et al.*, 1999; Proell *et al.*, 2013). Essas estruturas são características da ativação dos inflamassomas e podem ser observadas por meio de microscopia óptica. A aproximação das moléculas de caspase nas estruturas dos punctas possibilita o autoprocessamento dessas proteínas, e posteriormente a secreção de citocinas pró-inflamatórias (Boucher *et al.*, 2018). Em 2010 um trabalho utilizando formas mutantes de caspase-1, demonstrou que ASC é importante para a formação dos punctas e autoproteólise das moléculas de caspase-1 (Broz *et al.*, 2010). Entretanto, a forma de caspase-1 ativa e não clivada é capaz de levar à indução de piroptose de maneira independente de ASC. Este estudo sugere que a formação dos inflamassomas ocorrem em duas etapas, a primeira sem a presença de ASC, onde ocorre a ativação das caspases e indução de piroptose. Em um segundo momento ocorre o recrutamento de ASC e formação dos punctas, importante para o autoprocessamento das caspases, gerando assim a clivagem e secreção de citocinas pró-inflamatórias como IL-1 β e IL-18.

Figura 2: Estrutura dos inflamassomas ativados em resposta à *Legionella pneumophila*. Nesta figura estão demonstradas as estruturas dos componentes que formam os inflamassomas em resposta à *L. pneumophila*. O inflamassoma de Naip5/NLRC4/Caspase-1 é ativado em resposta à flagelina bacteriana. O domínio C-terminal da flagelina é reconhecido pelo domínio NBD do Naip5 que se oligomeriza e recruta NLRC4. A ligação entre Naip5 e NLRC4 também ocorre pelo domínio NBD. Após essa interação ocorre o recrutamento e ativação de caspase-1, que se liga à NLRC4 pelo seu domínio CARD. O reconhecimento de flagelina também ocorre pelo receptor Naip6 e pelo único receptor do tipo Naip em humanos. Já o inflamassoma de caspase-11 reconhece LPS de bactérias Gram-negativas, pela ligação direta entre o domínio CARD de caspase-11 e o lipídio A do LPS. A via de AIM2 é ativada pelo reconhecimento de fita dupla de DNA de *L. pneumophila* pelo domínio HIN do receptor AIM2. Esse inflamassoma é dependente da proteína adaptadora ASC, que se liga ao AIM2 pelo seu domínio PYD, levando ao recrutamento de caspase-1. Por fim o inflamassoma de NLRP3 reconhece reduzidas concentrações de K⁺ na célula levando à ativação do inflamassoma e secreção de citocinas pró-inflamatórias como IL-18 e IL-1β. Esse inflamassoma também necessita da proteína adaptadora ASC. A ligação entre ASC e NLRP3 ocorre pelos seus domínios PYD. Estão indicados os seguintes domínios: HIN, PYD, NBD, LRR, BIR, CARD e caspase. De: MASCARENHAS, Danielle PA; ZAMBONI, Dario S. *Journal of leukocyte biology*, 2017.

Caspases são proteases baseadas em cisteína que clivam suas proteínas-alvo em resíduos de ácido aspártico (Creagh, 2014). Elas são classificadas como caspases inflamatórias (caspase-1 e caspase-11 em camundongos e caspase-4 e caspase-5 em humanos) e caspases apoptóticas (iniciadoras: caspase-2, caspase-8, caspase-9 e caspase-10 e efetoras: caspase-3, caspase-6 e caspase-7) de acordo com as vias em que estão envolvidas. Além das caspases inflamatórias, trabalhos têm demonstrado a participação de outras caspases na formação dos inflamassomas, como caspase-3, caspase-7 e principalmente caspase-8 (Masumoto *et al.*, 2003; Hasegawa *et al.*, 2005; Akhter *et al.*, 2009; Kumar *et al.*, 2010; Abdelaziz, Gavrilin, Akhter, Caution, Kotrange, Khweek, Abdulrahman, Hassan, *et al.*, 2011; Motani *et al.*, 2011; Pierini *et al.*, 2012; Man *et al.*, 2013; Sagulenko *et al.*, 2013; Man *et al.*, 2014; Vajjhala *et al.*, 2015; Kawamoto *et al.*, 2017; Rauch *et al.*, 2017; Schneider *et al.*, 2017). A participação dessas caspases parece ser de grande importância na indução de respostas independentes de caspase-1 induzidas pelos inflamassomas, como a secreção de citocinas pró-inflamatórias. Além disso também tem sido demonstrado o papel das vias dependentes de caspase-8 no *priming* dos inflamassomas, como o de NLRP3 (Gurung *et al.*, 2014). Entretanto, ainda se sabe muito pouco sobre o papel dessas caspases não inflamatórias nos inflamassomas, principalmente no que se refere ao controle de infecção à patógenos como a *L. pneumophila*.

Grande parte das informações e descobertas acerca dos inflamassomas ocorreu com a utilização de *L. pneumophila* como modelo (Mascarenhas e Zamboni, 2017). A principal delas foi pela observação de que macrófagos de camundongos A/J são mais susceptíveis a infecção por *L. pneumophila* quando comparados a outras linhagens de camundongos como C57BL/6 e BALB/c (Yamamoto *et al.*, 1988). Por meio da utilização de genética clássica essa resistência foi mapeada em um locus autossômico nomeado de *Lgn1* no cromossomo 13, e foi identificado que esses genes correspondiam aos receptores Naip1, Naip3, Naip6, Naip5 e Naip2 expressos em macrófagos (Beckers *et al.*, 1995; Dietrich *et al.*, 1995; Diez *et al.*, 2000). A partir dessas descobertas, foi verificado que Naip5 era o responsável pela resistência contra *L. pneumophila* em macrófagos, levando à ativação de caspase-1 de maneira dependente de Dot/Icm (Zamboni *et al.*, 2006). Em camundongos A/J o alelo de Naip5 não é funcional, levando à alta susceptibilidade desses camundongos à infecção por *L. pneumophila* (Diez *et al.*, 2003; Wright *et al.*, 2003). Também foi demonstrando que

Naip5, interage com NLRC4, após o reconhecimento de flagelina, induzindo a ativação do inflamassoma (Amer *et al.*, 2006; Molofsky *et al.*, 2006; Ren *et al.*, 2006). Entretanto somente após o desenvolvimento do camundongo *Naip5*^{-/-}, foi confirmado que esse receptor interage com o domínio C-terminal conservado da flagelina de *L. pneumophila*, recruta NLRC4, levando à ativação de caspase-1 (Fig. 3) (Lightfield *et al.*, 2008; Kofoed e Vance, 2011; Zhao *et al.*, 2011). Adicionalmente também foi descrita a participação de Naip6 no reconhecimento de flagelina e interação com NLRC4, entretanto Naip5 é mais efetivo nessa função (Kofoed e Vance, 2011; Zhao *et al.*, 2011; Rauch *et al.*, 2016; Zhao *et al.*, 2016).

Além da interação com Naip5, NLRC4 também interage com os receptores Naip1 e Naip2. O inflamassoma Naip1/NLRC4 reconhece a proteína PrgI (*needle protein*), já o de Naip2/NLRC4 reconhece a proteína PrgJ (*rod protein*), ambas do Sistema de Secreção do Tipo III de bactérias (Kofoed e Vance, 2011; Zhao *et al.*, 2011; Rayamajhi *et al.*, 2013; Rauch *et al.*, 2016; Zhao *et al.*, 2016).

Figura 3: Ativação dos inflamassomas por *Legionella pneumophila* e suas consequências. *L. pneumophila* é reconhecida por diversos inflamassomas. O reconhecimento de flagelina pela via de Naip5/NLRC4 é dependente de Dot/Icm e induz a formação de dois complexos. O primeiro é independente de ASC e leva à ativação, mas não clivagem de caspase-1, com consequente formação de poros e controle da replicação bacteriana. O segundo complexo é dependente de ASC e induz a formação dos punctas, clivagem de caspase-1 e secreção de citocinas pró-inflamatórias. *L. pneumophila* também é reconhecida pelo inflamassoma de AIM2, pela ligação deste receptor com dupla fita de DNA. AIM2 juntamente com ASC, leva ao recrutamento e ativação de caspase-1, induzindo piroptose e secreção de citocinas pró-inflamatórias. Já caspase-11 reconhece o LPS de *L. pneumophila*, assim como de outras Gram-negativas, levando à clivagem de GSDMD. Após essa clivagem, o domínio N-terminal de GSDMD é transportado para a membrana plasmática interna onde se oligomeriza e forma a estrutura do poro, levando à indução de piroptose. Os poros formados pelos inflamassomas de Naip5/NLRC4, AIM2 e caspase-11 levam a um efluxo de potássio e com isso a ativação do inflamassoma de NLRP3, gerando a secreção de citocinas pró-inflamatórias como IL-18 e IL-1 β . Adaptado de: MASCARENHAS, Danielle PA; ZAMBONI, Dario S. *Journal of leukocyte biology*, 2017.

L. pneumophila como modelo possibilitou um grande desenvolvimento na elucidação dessas vias inflamatórias. Além da ativação dos inflamassomas por essa espécie, já foi relatado a ativação do inflamassoma de Naip5/NLRC4/Caspase-1 também por outras espécies de *Legionella* flageladas como: *L. bonzemani*, *L. gratiana*, *L. micdadei* e *L. jordanis*, bem como outras espécies de bactérias contendo flagelina como *Salmonella* Typhimurium e *Pseudomonas aeruginosa* (Franchi *et al.*, 2006; Miao *et al.*, 2006; Molofsky *et al.*, 2006; Franchi *et al.*, 2007; Sutterwala *et al.*, 2007; Suzuki *et al.*, 2007; Pereira, Marques, *et al.*, 2011; Pereira, Morgantetti, *et al.*, 2011).

A ativação do inflamassoma de Naip5/NLRC4/ Caspase-1 é uma das vias mais importantes no controle da replicação de *Legionella* spp., entretanto os mecanismos responsáveis por esse controle ainda não são completamente conhecidos (Zamboni *et al.*, 2006; Kofoed e Vance, 2011; Pereira, Morgantetti, *et al.*, 2011; Zhao *et al.*, 2011; Cerqueira *et al.*, 2015; Rauch *et al.*, 2016; Zhao *et al.*, 2016). Acredita-se que a alta indução de poros na membrana e consequente morte por piroptose pode ser um dos mecanismos pelos quais as bactérias intracelulares são controladas após a ativação desse inflamassoma (Miao *et al.*, 2010; Silveira e Zamboni, 2010). Já que a morte celular por piroptose lisa as células onde essas bactérias estão se multiplicando e também leva à uma propagação da inflamação, por meio do extravasamento de citocinas e DAMPs, recrutamento assim novas células para o sítio de infecção. Trabalhos recentes demonstraram que a indução de piroptose não leva diretamente à morte das bactérias intracelulares. Entretanto, essas bactérias ficam presas nos restos das células mortas após a piroptose, em uma estrutura denominada de PITs (*Pore-induced intracellular traps*) (Jorgensen, Lopez, *et al.*, 2016; Jorgensen, Zhang,

et al., 2016). Os PITs mantêm as bactérias presas no lugar, impedindo que infectem novas células. A indução de piroptose leva à liberação de DAMPs, citocinas pró-inflamatórias e eicosanoides recrutando novas células para o sítio de infecção como neutrófilos (Barry *et al.*, 2013; Casson *et al.*, 2013; Mascarenhas *et al.*, 2015). Essas células recrutadas fagocitam os restos celulares com as bactérias presas, em um processo denominado de eferocitose (Jorgensen, Lopez, *et al.*, 2016; Jorgensen, Zhang, *et al.*, 2016). Foi demonstrado que essas bactérias após passarem por células que sofreram piroptose, ficam mais susceptíveis à morte por espécies reativas de oxigênio (ROS), antibióticos e apresentam uma menor capacidade de infectar outras células. Desta maneira a piroptose parece ser um mecanismo eficiente de controle da replicação de bactérias intracelulares.

A morte celular por piroptose é caracterizada pela formação inicial de poros na membrana, por onde ocorre o influxo e efluxo de íons, gerando uma lise osmótica da célula com liberação do seu conteúdo no meio extracelular, juntamente com citocinas pró-inflamatórias (Cookson e Brennan, 2001; Fink e Cookson, 2006; Larock e Cookson, 2013; Evavold *et al.*, 2018). Até 2015 não se conhecia os mecanismos pelo qual a ativação do inflamassomas e as caspases induziam a formação de poros nas membranas e morte celular por piroptose. Contudo, através da utilização de “*high throughput screening*” com *CRISPR/Cas9*, foi identificada a proteína responsável pela formação dos poros na membrana, denominada de gasdermina-D (GSDMD) (Fig. 4) (Kayagaki *et al.*, 2015; Shi *et al.*, 2015). Tanto a caspase-1 quanto a caspase-11 clivam essa proteína, liberando um fragmento C-terminal e um N-terminal. O fragmento N-terminal da GSDMD migra para a membrana plasmática onde se oligomeriza e se liga à lipídios específicos da membrana plasmática interna, formando a estrutura do poro e desencadeando assim a piroptose (Fig. 4) (Kayagaki *et al.*, 2015; Shi *et al.*, 2015; Aglietti *et al.*, 2016; Ding *et al.*, 2016; Liu *et al.*, 2016; Sborgi *et al.*, 2016). Recentemente, foi demonstrado que a secreção de IL-1 β e IL-18, que não são liberadas por uma via secretória convencional, ocorre pelos poros de GSDMD (Keller *et al.*, 2008; Evavold *et al.*, 2018). Além do papel de GSDMD na indução de piroptose, também foi demonstrada a participação de outras gasderminas na indução de morte celular, como GSDME, também conhecida como Dfna5 (*Deafness Autosomal Dominant 5*), após sua clivagem por caspase-3 (Rogers *et al.*, 2017; Wang *et al.*, 2017).

Figura 4: Estrutura do poro de membrana formado por gasdermina-D. Após a ativação das caspases inflamatórias (caspase-1 ou caspase-11 em camundongos e os homólogos de caspase-11, caspase-4 e caspase-5 em humanos) GSDMD é clivada em um ácido aspártico liberando um domínio C-terminal e um N-terminal. O domínio N-terminal de GSDMD migra para a membrana plasmática, onde se liga à lipídios específicos da membrana plasmática interna. A formação do poro ocorre com a oligomerização de 16 monômeros de GSDMD. O poro formado tem cerca de 10-15nm. Um desbalanço iônico pela formação desse poro causa a entrada de água na célula induzindo a lise celular. Essa lise celular leva à liberação do conteúdo citoplasmático em meio extracelular ativando novas células pelo reconhecimento de DAMPs. Acredita-se que por esse poro são secretadas algumas citocinas com secreção não convencional como IL-1 β e IL-18. Também são capazes de passar por esse poro outros compostos como iodeto de propídio (PI). Adaptado de: KOVACS, Stephen B; MIAO, Edward A. *Trends in Cell Biology*, 2017.

Além da piroptose, em resposta a infecção por *L. pneumophila*, o inflamassoma de Naip5/NLRC4/Caspase-1 também induz a formação de punctas e secreção de citocinas pró-inflamatórias como IL-1 β e IL-18, de maneira dependente da molécula adaptadora ASC (Coers *et al.*, 2007; Case *et al.*, 2009; Case e Roy, 2011). Entretanto o papel de ASC no controle da infecção desta bactéria tem sido controverso. Trabalhos utilizando células de humanos demonstraram que ASC é importante no controle da replicação de *L. pneumophila* (Abdelaziz, Gavrilin, Akhter, Caution, Kotrange, Khweek, Abdulrahman, Grandhi, *et al.*, 2011). Já trabalhos utilizando macrófagos de camundongo demonstraram que ASC não é importante para a indução de piroptose e controle da replicação dessa bactéria. Desta maneira, ainda existe um desacordo em relação ao papel de ASC e o controle da replicação de *L. pneumophila*.

Outros inflamassomas além de Naip5/NLRC4/Caspase-1 também são ativados em resposta à *L. pneumophila*. Um deles é o inflamassoma de AIM2, responsável pelo reconhecimento de fita dupla de DNA (Fig. 2 e 3) (Burckstummer *et al.*, 2009). Esse receptor apresenta dois domínios, um deles denominado de domínio HIN (*Hematopoietic IFN-inducible Nuclear Domain*) que se oligomeriza após a interação com fita dupla de DNA e um domínio PYD que interage com ASC (Fig. 2) (Fernandes-Alnemri *et al.*, 2009; Hornung *et al.*, 2009; Roberts *et al.*, 2009). A ativação do inflamassoma de AIM2 por *L. pneumophila* foi identificado pela observação de que bactérias deficientes para o efector *SdhA* geravam uma maior morte celular em macrófagos (Laguna *et al.*, 2006). Alguns anos depois foi demonstrado que *SdhA* regula a integridade de vacúolo de *L. pneumophila* (Creasey e Isberg, 2012). A deleção desse gene leva à uma desestabilidade de vacúolo e sua consequente lise, induzindo a liberação do DNA da bactéria no meio citoplasmático onde é reconhecido pelo AIM2. O reconhecimento por esse receptor desencadeia a ativação de caspase-1, secreção de IL-1 β e piroptose em células humanas e de camundongos, processos que são independentes de flagelina e da degradação da bactéria no citoplasma (Fig. 3) (Ge *et al.*, 2012).

L. pneumophila, assim como outras bactérias Gram-negativas, também é reconhecida por caspase-11 (Fig. 2 e 3) (Kayagaki *et al.*, 2011). Essa molécula é capaz de reconhecer o LPS (lipopolissacarídeo) dessas bactérias (Shi *et al.*, 2014). A ligação entre essas moléculas ocorre pelo domínio CARD da caspase-11 e o lipídio A do LPS (Shi *et al.*, 2014; Zanoni *et al.*, 2016). Após essa ligação ocorre uma

oligomerização de caspase-11 e ativação do inflamassoma, desencadeando respostas como piroptose pela clivagem de GSDMD e secreção de IL-1 α (Fig. 3) (Case *et al.*, 2013; He *et al.*, 2015; Kayagaki *et al.*, 2015; Shi *et al.*, 2015; Aglietti *et al.*, 2016; Liu *et al.*, 2016; Sborgi *et al.*, 2016). A ativação desse inflamassoma é independente de flagelina e da via de Naip5/NLRC4/Caspase-1, que gera a maior e mais rápida indução de piroptose em resposta à *L. pneumophila* (Case *et al.*, 2013; Casson *et al.*, 2013; Cunha *et al.*, 2015).

O papel do inflamassoma de caspase-11 no controle da replicação de *L. pneumophila* é controverso. Entretanto nosso grupo demonstrou através da utilização dos camundongos *Casp1^{-/-}Casp11^{tg}* e *Casp11^{-/-}* que somente caspase-1, mas não caspase-11 é importante para o controle da replicação de *L. pneumophila* flagelada (Cerqueira *et al.*, 2015). Esse trabalho foi essencial para diferenciar as respostas dependentes de caspase-1 e caspase-11, já que por muito tempo o camundongo *Casp1/11^{-/-}* foi utilizado pelos pesquisadores como sendo deficiente apenas para caspase-1.

Em 2017, um trabalho do nosso grupo demonstrou que camundongos deficientes para *Aim2/Casp11* são mais susceptíveis à infecção por *L. pneumophila* deficiente para flagelina (Cunha *et al.*, 2017). Foi verificado que a ativação dos inflamassomas de AIM2 e caspase-11 induz a formação de poro. Esse dano na membrana gerado pelo poro leva a um efluxo de potássio, e com isso a ativação do inflamassoma de NLRP3 em resposta à infecção por *L. pneumophila* (Fig. 3).

A ativação de NLRP3, uma molécula que também é membro da família dos NLRs, pode ocorrer por duas vias principais. A via canônica de ativação, que é ativada por diversos componentes como ATP, RNA bacteriano, toxinas que levam à formação de poro (como nigericina) e cristais de urato monossódico (Kanneganti *et al.*, 2006; Mariathasan *et al.*, 2006; Martinon *et al.*, 2006; Sutterwala, Ogura, Szczepanik, *et al.*, 2006; Sutterwala, Ogura, Zamboni, *et al.*, 2006). Já a ativação não canônica depende da ativação prévia de outros inflamassomas, como por exemplo a ativação de caspase-11 por bactérias Gram-negativas (Kayagaki *et al.*, 2011; Case *et al.*, 2013; Casson *et al.*, 2015; Schmid-Burgk *et al.*, 2015). Entretanto, tanto a via canônica quanto a não canônica de ativação do inflamassoma de NLRP3 parecem resultar de um processo comum, que é o efluxo de potássio (Rivers-Auty e Brough, 2015; Ruhl e Broz, 2015; Cunha *et al.*, 2017).

O inflamassoma de NLRP3 não é importante no controle da infecção de *L. pneumophila* (Case *et al.*, 2009). Entretanto, esse inflamassoma em cooperação com o inflamassoma de Naip5/NLRC4/Caspase-1 são essenciais para a clivagem de caspase-1 e secreção de citocinas pró-inflamatórias como IL-1 β e IL-18 ambos de maneira dependente de ASC (Zamboni *et al.*, 2006; Case *et al.*, 2013).

Durante muito tempo acreditou-se que o único inflamassoma realmente efetivo no controle da replicação de *L. pneumophila* era o de Naip5/NLRC4/Caspase-1 (Yamamoto *et al.*, 1988; Zamboni *et al.*, 2006). Contudo, Pereira, Morgantetti, *et al.* (2011) demonstraram que além dessa via dependente de NLRC4, flagelina e caspase-1, existe uma via independente de caspase-1/11 que age no controle da replicação de espécies de *Legionella* flageladas. Foi demonstrado que camundongos *Nlrc4*^{-/-} são mais susceptíveis à infecção por *L. pneumophila* flagelada do que camundongos *Casp1/11*^{-/-}. Ainda, quando foram utilizadas bactérias deficientes para flagelina (*flaA*⁻) em macrófagos *Casp1/11*^{-/-}, foi observado uma maior multiplicação bacteriana quando comparado à infecção por bactérias selvagem (WT Lp). Indicando que existe uma via dependente de flagelina e NLRC4 e independente de caspase-1/11 que atua no controle da replicação de *L. pneumophila*. Uma hipótese para esse novo inflamassoma encontrado é de que NLRC4 poderia utilizar uma caspase apoptótica para atuar nesta via. Essa hipótese é corroborada por alguns trabalhos que já detectaram a ativação de caspases não inflamatórias em resposta à infecção por *L. pneumophila*, como caspase-3 e caspase-7 (Gao e Abu Kwaik, 1999; Akhter *et al.*, 2009; Amer, 2010; Abdelaziz, Gavrilin, Akhter, Caution, Kotrange, Khweek, Abdulrahman, Hassan, *et al.*, 2011; Zhu *et al.*, 2013). Entretanto, os mecanismos e os componentes dessa via independente de caspase-1/11 ainda são obscuros, bem como o papel de ASC e dessas caspases não inflamatórias no controle da replicação de *L. pneumophila*.

Objetivo

2 – OBJETIVO

Determinar e caracterizar a via de sinalização dependente de flagelina/NLRC4 e independente de caspase-1/11 que leva ao controle da infecção de *L. pneumophila*.

2.1 – Objetivos específicos

- Avaliar o papel de ASC no controle da replicação de *L. pneumophila*.
- Identificação das proteínas que compõem o inflamassoma dependente de NLRC4 e independente de caspase-1/11 em resposta à *L. pneumophila* flagelada.
- Avaliar o papel de caspases apoptóticas na sinalização induzida por *L. pneumophila*.
- Analisar o papel do reconhecimento de flagelina por Naip5 para a ativação do inflamassoma dependente de NLRC4 e independente de caspase-1/11.
- Avaliar a formação de poro e secreção de citocinas pela via independente de caspase-1/11.
- Caracterizar as respostas independentes de caspase-1/11 no controle da replicação de *L. pneumophila*.

Materiais e Métodos

3 – MATERIAIS E MÉTODOS

3.1 – Bactérias

Neste estudo foram utilizadas as bactérias *L. pneumophila* da cepa selvagem JR32 (WT Lp) e seus mutantes isogênicos deficientes para motilidade (*flil*⁻) e para flagelina (*flaA*⁻) (Ren *et al.*, 2006; Pereira, Morgantetti, *et al.*, 2011). As bactérias *L. micdadei* (ATCC 33218) e *L. gratiana* (ATCC 49413) foram utilizadas para gerar cepas resistentes à estreptomicina. Mutantes para *RpsI* de *L. micdadei* e *L. gratiana* foram isoladas após plaqueadas em placas contendo 100 µg/ml de estreptomicina (Zhu *et al.*, 2015). Todas as bactérias foram cultivadas em placas com meio CYE {1% de extrato de levedura (Oxoid), 1% MOPS (Sigma-Aldrich), pH 6.9, 3.3 mM de L-cisteína (Sigma-Aldrich), 0.33 mM Fe(NO)₃ (Sigma-Aldrich), 1.5% ágar (Oxoid) e 0.2% de carvão ativado (Sigma-Aldrich)} (Feeley *et al.*, 1979). Para os experimentos de infecção *in vitro* e *in vivo*, todas as bactérias cresceram a 36°C por 48 horas. Para a realização das infecções, as bactérias foram ressuspensas em água estéril e diluídas de acordo com a densidade óptica (O.D) a 600nm.

3.2 – Cultura de macrófagos derivados de medula óssea

Para a cultura de macrófagos derivados de medula óssea (BMDMs – *Bone marrow-derived macrophages*), foram retirados fêmures e tíbias de animais eutanasiados por dose excessiva de anestésico. As células precursoras de macrófagos obtidas da medula óssea de camundongos C57BL/6, *Nlrc4*^{-/-}, *Asc*^{-/-}, *Casp1*^{11/-}, *Asc/Casp1*^{11/-}, *Aim2/Casp1*^{11/-}, *Casp1*^{-/-}*Casp1*^{tg}, *Casp1*^{11/-} e *Gsdmd*^{-/-} foram diferenciadas *in vitro* durante 7 dias, em estufa a 37°C e 5% de CO₂, em meio RPMI 1640 +HEPES (Gibco - Thermo Fisher Scientific) suplementado com 20% de soro bovino fetal (SBF) (Gibco - Thermo Fisher Scientific), 30% de meio condicionado por células L929 (LCCM: fonte de M-CSF, importante para diferenciação de células progenitoras em macrófagos), 2 mM L-Glutamina (Sigma-Aldrich), 100 U/mL de penicilina/estreptomicina (Sigma-Aldrich) (Marim *et al.*, 2010). Após a diferenciação, os macrófagos foram coletados pela lavagem das monocamadas com PBS (*phosphate-buffered saline*) pH 7.4 gelado (Gibco - Thermo Fisher Scientific), posteriormente, distribuídos em placas de poços tratadas e cultivados em meio RPMI 1640 +HEPES contendo 10% de SBF e 2 mM L-Glutamina para serem posteriormente infectados.

3.3 – Infecções in vitro e determinação de UFC

Nos experimentos com infecções in vitro as culturas foram infectadas em uma multiplicidade de infecção (MOI) de 0.015, 5 ou 10 bactérias/célula, seguidas de uma centrifugação de 300 X g por 5 minutos a temperatura ambiente e posteriormente incubadas a 37°C e 5% CO₂. Para a determinação de UFC (unidades formadoras de colônias), 2 X 10⁵ células foram plaqueadas em placa de 24 poços. Após a adesão das células, a cultura foi infectada com MOI 0.015 ou 10, seguidas de uma centrifugação de 300 X g por 5 minutos a temperatura ambiente. No caso de experimentos com MOI 10, após 1 hora de infecção os poços foram lavados duas vezes com PBS morno e 1 ml de meio RPMI 1640 +HEPES com 10% de SBF e 2 mM L-Glutamina foi adicionado a cada poço e as placas foram então incubadas a 37°C e 5% CO₂ durante os tempos indicados. Para a determinação de UFC, o sobrenadante de cada poço foi coletado e foi adicionado 1 ml/poço de água estéril. O lisado celular foi combinado ao sobrenadante do respectivo poço. As combinações de lisado mais sobrenadante de cada poço foram diluídas em água estéril, plaqueadas em meio CYE e incubadas por 4 dias a 36°C (Pereira, Morgantetti, *et al.*, 2011; Cerqueira *et al.*, 2015; Mascarenhas *et al.*, 2015).

3.4 – Transdução retroviral e quantificação de NLRC4-GFP e ASC-GFP

NLRC4 e ASC murinos foram clonados em vetor pEGFP (N2) (Clontech) usando os sítios de restrição XhoI e BamHI (Case e Roy, 2011). NLRC4-GFP e ASC-GFP foram clonados em vetor retroviral específico de murino pMSCV2.2 (Clontech). O sistema de vetor pCL foi utilizado para o empacotamento dos retrovírus das células Hek Peak transfectadas e posteriormente mantidas em meio contendo 10% de SBF e 2 mM L-Glutamina. O sobrenadante das células Hek Peak contendo os retrovírus foi coletado após 2 dias da transfecção, filtrado utilizando filtro de 0.45 µm e utilizado para a transdução dos BMDMs. BMDMs foram obtidos de camundongos *Casp1/11^{-/-}*, *Asc/Casp1/11^{-/-}* e *Aim2/Casp1/11^{-/-}* e plaqueados em meio para diferenciação. Após 3 dias de diferenciação o sobrenadante contendo os retrovírus foram adicionados aos BMDMs com 20% de SBF, 25% de LCCM e 2 mM L-Glutamina. Após a diferenciação 2 X 10⁵ células/poço foram plaqueadas em placa de 24 poços contendo lamínulas de vidro de 12 mm e cultivadas em meio suplementado com 10% de SBF e 2 mM L-Glutamina. Para os experimentos de colocalização com caspases, as culturas foram

tratadas com 20 μ M de Z-VAD (*Promega*) por 1 hora e infectados com MOI 1, 3 e 10. Após a infecção as placas foram centrifugadas a 300 X g a temperatura ambiente e incubadas a 37°C e 5% de CO₂. Após 1, 3 ou 10 horas de infecção as células foram fixadas com paraformaldeído 4%, permeabilizadas e bloqueadas com 0.05% de saponina mais 10% de soro de cabra (tampão de bloqueio), as lamínulas foram então montadas em lâminas com *Prolong Gold Antifade Reagent* contendo DAPI (*Molecular probes – Thermo Fisher Scientific*). Para os experimentos de colocalização, após a permeabilização e bloqueio as células foram incubadas com anti-caspase-8 (*Enzo*, diluição de 1:50), anti-caspase-8 clivada (*Cell signaling*, diluição de 1:800), anti-caspase-3 clivada (*Cell signaling*, diluição de 1:400), anti-caspase-7 clivada (*Cell signaling*, diluição de 1:400), anti-ASC (*Adipogen*, diluição de 1:250), anti-ASC (*Santa Cruz*, 1:50), anti-caspase-1 (*Santa Cruz*, diluição de 1:500) ou anti-AIM2 (*Cell Signaling*, diluição de 1:400) diluídos em tampão de bloqueio, por 1 hora. Após incubados com o anticorpo primário, as células foram lavadas três vezes com PBS e então incubadas com os anticorpos secundários conjugados com o fluoróforo Alexa Fluor 594 ou 647 (*Molecular probes – Thermo Fisher Scientific*, diluição 1:3000), DAPI e as lamínulas foram montadas com *Prolong Gold Antifade Reagent*. O número de punctas de NLRC4-GFP ou ASC-GFP em relação as células transduzidas e o número de colocalizações foi quantificado em microscópio invertido Leica DMI 4000B com objetiva de 100X (*Leica Microsystems*). As imagens de microscopia de multifóton foram adquiridas utilizando o sistema *LSM 780 Zeiss AxioObserver* (*Zeiss*) com lente de óleo de imersão de aumento de 63X e analisadas e utilizando o software *ImageJ*.

3.5- Ativação de caspase-8

Para investigar a ativação de caspase-8, 1 X 10⁵ células/poço foram plaqueadas em placas brancas de 96 poços. Após a sua adesão, as células foram infectadas com WT Lp, *flii*⁻ e *flaA*⁻ (MOI 10) por 8 horas. A ativação de caspase-8 foi medida utilizando o kit de *Caspase-8 Glo Assay* (*Promega*) de acordo com o recomendado pelo fabricante. Para a avaliação da ativação de caspase-8 por análise de *Western Blotting*, foram plaqueados 1 X 10⁷ células/poço em placa de 6 poços e infectados com *flii*⁻ e *flaA*⁻ (MOI 10). Após 8 horas de infecção as células foram lisadas com tampão RIPA {25 mM Tris-HCl (pH 7.6) (*J.T.Baker*), 150 mM NaCl (*J.T.Baker*), 1% NP-40 (*Fluka Biochemika*), 1% deoxicolato de sódio (*Sigma- Aldrich*), 0.1% SDS}

com inibidor de protease (*Roche*). Os lisados foram centrifugados a 20.500 X g por 15 minutos a 4°C e posteriormente os sobrenadantes foram ressuspensos em tampão de amostra {50 mM Tris (pH 6.8), 2% SDS (*USB Corporation*), 0.1% azul de bromofenol (*Synth*), 10% glicerol (*Sigma- Aldrich*), 2.5% β-mercaptoetanol (*J.T.Baker*)} e fervidos por 5 minutos. As amostras foram então separadas em gel de SDS-PAGE 15% e posteriormente transferidas para uma membrana de nitrocelulose de 0.22 μm (*GE Healthcare*), utilizando o sistema de transferência *Semidry Transfer Cell* (*Bio-Rad*). Após a transferência, as membranas foram bloqueadas por 1 hora a 25°C em tampão TBS (*Tris buffered saline*) {60.5 g/L de Tris (*J.T.Baker*) e 87.5 g/L de NaCl (*J.T.Baker*) com pH ajustado para 8.0 com HCl} com 0.01% de Tween-20 (*Sigma-Aldrich*) e 5% de leite em pó desnatado. O anticorpo primário anti-caspase-8 (*Enzo*, diluição 1:500) foi diluído em tampão de bloqueio e incubado na membrana por cerca de 12-14 horas a temperatura de 4°C. As membranas foram lavadas três vezes com TBS-Tween e então incubadas com anticorpo secundário anti-rato conjugado a peroxidase (*KPL*, diluição 1:3000) diluído em tampão de bloqueio por 1 hora a temperatura ambiente. Após a incubação do anticorpo secundário, as membranas foram lavadas novamente três vezes com TBS-Tween e foi utilizado *ECL Prime Western Blotting Detection Reagent* (*GE Healthcare*) para a detecção dos anticorpos marcados observados no aparelho *Amersham Imager 600* (*GE Healthcare*).

3.6 – Silenciamento retroviral de caspase-8, Naip5 e GSDMD

Para o silenciamento retroviral de caspase-8, Naip5 e gasdermina-D (GSDMD), células Hek Peak foram transfectadas com vetores lentivirais codificando shRNA (*small hairpin RNA*) para caspase-8 (*Sigma-Aldrich* - Seq1: TRCN0000231279; Seq2: TRCN0000231281), Naip5 (*Sigma-Aldrich* - TRCN0000114742) e GSDMD (*Sigma-Aldrich* - TRCN0000219619) e o como controle um vetor com uma sequência não relacionada (NT – *non-target shRNA*) (Franco *et al.*, 2017). A transfecção das células Hek Peak foi realizada com o auxílio de PEI (*Polyethylenimine*) (*Sigma-Aldrich*). As células transfectadas foram mantidas em meio RPMI 1640 +Hepes suplementado com 10% de SBF e 2 mM L-Glutamina a 37°C e 5% CO₂. Após 2 dias de transfecção o sobrenadante das células Hek Peak contendo os retrovírus foi coletado, filtrado utilizando filtro de 0.45 μm e utilizado para a transdução dos BMDMs. BMDMs foram obtidos de camundongos C57BL/6, *Casp1/11^{-/-}* e *Asc/Casp1/11^{-/-}* e plaqueados em

meio para diferenciação. Após 3 dias de diferenciação o sobrenadante contendo os retrovírus codificando os shRNAs e o NT shRNA foram adicionados aos BMDMs com 20% de SBF e 30% de LCCM. Após um dia de transdução as células foram selecionadas com 3 µg/ml de puromicina por 2 dias e então plaqueadas e utilizadas de acordo com cada experimento. Para a confirmação do silenciamento das células foi realizado *Western Blotting* para observação da pro-caspase-8 (como descrito anteriormente), bem como qPCR em tempo real para caspase-8, Naip5 e GSDMD como descreveremos a seguir.

3.7 – qPCR em Tempo Real

RNA total foi extraído de 2×10^6 células utilizando o kit de isolamento de RNA *Illustra RNAspin (Ge Healthcare)* de acordo com o recomendado pelo fabricante. Após a extração, alíquotas de 2 µl foram utilizadas para determinar a concentração de RNA total em equipamento de *NanoDrop (Thermo Fisher Scientific)* e 1 µg do RNA extraído foi utilizado para a conversão em cDNA utilizando o kit *iScript™ cDNA Synthesis (Bio-Rad)* e o auxílio do termociclador *Mastercycler gradient (Eppendorf)* de acordo com o recomendado pelo fabricante. O cDNA (10 µg) foi utilizado para a quantificação da expressão de caspase-8 (*TaqMan Assay: Casp8 – Mn01255716_m1 (Thermo Fisher Scientific)*) por PCR em tempo real utilizando *TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific)* de acordo com o recomendado pelo fabricante. O gene de β -actina (*TaqMan Assay: Actb – Mn00607939_s1 (Thermo Fisher Scientific)*) foi utilizado com controle para normalização da expressão das amostras. A quantificação de Naip5 e GSDMD foi realizada utilizando 25 ng de cDNA e 10 µM de cada primer 1X *SYBR Green* (Naip5: FWD: GTTGAGATTGGAGAAGACCTCG, REV-CACACGTGAAAGCAACCA TGG; GSDMD: FWD: TCATGTGTCAACCTGTCAATCAAGGACAT, REV-CATCGACGACATCAGAGACTTTGAAGGA). A normalização da expressão de Naip5 e GSDMD foi realizada pela expressão do gene *Gapdh (glyceraldehyde-3-phosphate dehydrogenase)* (Gapdh: FWD- AGGTCGGTGTGAACGGATTTG, REV-TGTAGACCATGTAGTTGAGGTCA). A reação de PCR em tempo real foi realizada no equipamento *Vii7 Real-Time PCR System (Thermo Fisher Scientific)*. Os resultados foram analisados utilizando o método $2^{-\Delta\Delta CT}$ e foram expressos em relação ao grupo

de referência. A porcentagem de silenciamento foi estimada utilizando a equação: $\{1 - (2^{-\Delta\Delta CT}) \times 100\}$ (Milstein *et al.*, 2013).

3.8 – Camundongos e infecções in vivo

Os camundongos utilizados neste estudo foram criados e mantidos nos Biotérios da Universidade de São Paulo no Campus de Ribeirão Preto (Biotério Central, Centro de Criação de Camundongos Especiais, Biotério do Departamento de Biologia Celular e Molecular e Biotério do Departamento de Imunologia e Bioquímica). Foram utilizados os camundongos C57BL/6 (Jax 000664), *Nlrc4*^{-/-}, *Casp1*¹¹¹^{-/-}, *Asc*^{-/-}, *Casp1*¹¹¹^{-/-} e *Casp1*^{-/-}*Casp1*¹¹¹^{tg} (Kuida *et al.*, 1995; Lara-Tejero *et al.*, 2006; Sutterwala, Ogura, Szczepanik, *et al.*, 2006; Kayagaki *et al.*, 2011). Camundongos duplo-deficientes foram gerados pelo intercruzamento da progênie F1 dos camundongos parentais. Todos os camundongos foram pareados por sexo e idade (todas as infecções foram feitas com camundongos de pelo menos 8 semanas) e todos os camundongos são do *background* genético de C57BL/6. Para os experimentos com infecções in vivo, foram utilizados cerca de 5-7 animais/grupo como indicado em cada figura. Anteriormente às infecções, os camundongos foram anestesiados com cetamina e xilazina (50 mg/kg e 10 mg/kg, respectivamente) por via intraperitoneal. Após anestesiados os camundongos foram infectados com 40 µl de PBS contendo 1×10^5 bactérias/camundongo por via intranasal. Para a determinação do número de bactérias no pulmão, os camundongos foram sacrificados e seu pulmão retirado, triturado e homogeneizado em 5 ml de água estéril por 30 segundos com homogeneizador de tecidos *Power Gen 125* (Thermo Fisher Scientific). Os pulmões homogeneizados foram então diluídos em água e plaqueados em meio CYE para a determinação de UFC/pulmão após 4 dias a 36°C (Pereira, Marques, *et al.*, 2011; Pereira, Morgantetti, *et al.*, 2011; Mascarenhas *et al.*, 2015).

3.9 – Ensaio de formação de poro

A formação de poro nos BMDMs foi quantificada baseada na incorporação de iodeto de propídio (PI), um corante fluorescente capaz de entrar apenas em células com a membrana danificada (Case *et al.*, 2013; Cerqueira *et al.*, 2015). Para isso, 1×10^5 células/poço foram plaqueados em placa preta com o fundo transparente de 96

poços. Antes da infecção o meio foi substituído por RPMI sem vermelho de fenol (*Sigma-Aldrich*) contendo 15 mM de Hepes (*Sigma-Aldrich*), apenas 0.038 g/ml de NaHCO₃ (*J.T.Baker*), suplementado com 2% de SBF, 2 mM de L-Glutamina, e posteriormente adicionado 6 µl de PI e anticorpo anti-*L. pneumophila* (diluição 1:1000). Os macrófagos foram infectados com MOI 10 e posteriormente a placa foi centrifugada a 300 X g por 5 minutos a temperatura ambiente. Os BMDMs infectados foram mantidos a 37°C, com o PI sendo excitado a 535 nm. A fluorescência de emissão foi lida a 617 nm a cada 5 minutos utilizando um fluorímetro de placa *Spectra Max i3x* (*Molecular Devices*). A formação total de poro foi determinada pela lise das células com a adição de 34 µl/poço (contendo 200 µl de meio) de Triton X-100 (*Sigma-Aldrich*) 9%.

3.10 – Ensaio de liberação de Lactato desidrogenase (LDH) e ELISA

Nos ensaios de liberação de Lactato desidrogenase (LDH) e ELISA, 5 X 10⁵ células/poço foram plaqueadas em placa de 24 poços. No ensaio de LDH, antes da infecção o meio foi substituído por RPMI sem vermelho de fenol contendo 15 mM de Hepes, 2 g/L de NaHCO₃, suplementado com 2% de SBF e 2 mM de L-Glutamina. As células foram infectadas com MOI 10 seguidas de uma centrifugação de 300 X g por 5 minutos a temperatura ambiente. Após 8 horas de infecção o sobrenadante das células foi coletado para a dosagem de liberação de LDH com o kit *CytoTox 96 LDH-release* (*Promega*) de acordo com as recomendações do fabricante. O valor do LDH total foi determinado pela lise das células com Triton X-100 9%. Para a determinação da secreção de citocinas pelo método de ELISA (*Enzyme-linked immunosorbent assay*) após aderidos os BMDMs foram infectados com MOI 10. Após 1 hora de infecção as culturas foram lavadas duas vezes com PBS morno e o meio foi substituído por um novo meio RPMI 1640 suplementado com 10% de SBF e 2 mM de L-Glutamina. As células foram incubadas por 24 horas a 37°C e 5% de CO₂, e então seus sobrenadantes foram coletados para a dosagem de citocinas utilizando os kits de ELISA de acordo com as recomendações do fabricante (*BD Biosciences*).

3.11 – Declaração ética

Os cuidados com os animais neste trabalho estão de acordo as diretrizes institucionais de ética em experimentação animal e foram aprovadas pela Comissão de Ética em Experimentação Animal da Faculdade de Medicina de Ribeirão Preto (CETEA – Número de protocolo: 218/2014). Para a eutanásia, os camundongos foram tratados com cetamina e xilazina (50 mg/kg e 10 mg/kg, respectivamente) por via intravenosa.

3.12- Análise Estatística

Na realização das análises estatísticas do presente estudo, foi utilizado o programa *GraphPad Prism 5.0* Para comparar dados entre diferentes grupos experimentais, foi empregado o *teste t não paramétrico* ou teste de análise de variância ANOVA seguido de análise pós-teste *Bonferroni*. O teste não-paramétrico *Mann-Whitney* foi utilizado nas análises dos experimentos in vivo. As diferenças foram consideradas estatisticamente diferentes quando $p < 0.05$ e foram indicadas com asteriscos nas figuras.

Resultados

4 – RESULTADOS

4.1 – O controle da replicação de *L. pneumophila* em BMDMs é dependente do reconhecimento de flagelina e de NLRC4, independente de ASC e parcialmente dependente de caspase-1

Foi demonstrado anteriormente que o controle da replicação de *L. pneumophila* ocorre pela via já descrita de Naip5/NLRC4/Caspase-1 e por uma via dependente de NLRC4, mas independente de caspase-1/11 (Zamboni *et al.*, 2006; Pereira, Morgantetti, *et al.*, 2011). Entretanto os mecanismos pelos quais essa via atua eram desconhecidos. Com o objetivo de caracterizar as respostas independentes de caspase-1/11 no controle da replicação de *L. pneumophila*, foi utilizada a cepa JR32 WT Lp de *L. pneumophila* (Wild-type), e seus mutantes isogênicos *fliH*⁻ e *flaA*⁻. A bactéria *fliH*⁻ é deficiente para uma ATPase importante para a secreção de flagelina pelo aparato flagelar; conforme foi demonstrado anteriormente, bactérias *fliH*⁻ induzem ativação do inflamassoma de Naip5/NLRC4 normalmente (Merriam *et al.*, 1997; Pereira, Morgantetti, *et al.*, 2011). Por outro lado, a bactéria *flaA*⁻ é deficiente para flagelina, e, portanto, não é capaz de ativar o inflamassoma de Naip5/NLRC4 (Amer *et al.*, 2006; Molofsky *et al.*, 2006; Ren *et al.*, 2006). Deste modo, o melhor controle para a bactéria *flaA*⁻ é a bactéria *fliH*⁻, já que ambas são deficientes para motilidade, mas somente a *fliH*⁻ expressa flagelina. Para avaliar o crescimento dessas bactérias na via independente de caspase-1/11, macrófagos derivados de medula óssea (BMDMs – *Bone marrow-derived macrophages*) de camundongos C57BL/6, *Nlrc4*^{-/-}, *Casp1/11*^{-/-} e *Asc*^{-/-} foram infectados com WT Lp, *fliH*⁻, e *flaA*⁻ com baixo (0.015) e alto (10) MOI e lisados após 0, 24, 48, 72 e 96 horas para a determinação das unidades formadoras de colônias (UFCs). Em ambos os MOIs os macrófagos C57BL/6 e *Asc*^{-/-} foram altamente restritivos à replicação de *L. pneumophila* WT Lp e *fliH*⁻ (Fig. 5A-D). Confirmando dados anteriores de que ASC não é importante no controle da replicação de *L. pneumophila* na presença de caspase-1/11 (Zamboni *et al.*, 2006; Case *et al.*, 2009). Por outro lado, macrófagos derivados de camundongos *Casp1/11*^{-/-} infectados com WT Lp e *fliH*⁻ apresentaram um controle da replicação intermediário nos dois MOIs utilizados. Já os macrófagos derivados de camundongos *Nlrc4*^{-/-} infectados com todas as bactérias foram altamente susceptíveis à infecção (Fig. 5A-F), assim como as células infectadas com a bactéria mutante para flagelina (*flaA*⁻), por não ser

reconhecida pela via de NLRC4 (Fig. 5E-F). Esses dados corroboram com os achados de que além do inflamassoma de Naip5/NLRC4/Caspase-1 existe uma nova via dependente do reconhecimento de flagelina e de NLRC4 e independente de caspase-1/11 (Pereira, Morgantetti, *et al.*, 2011). A partir desses dados, nós utilizamos dos macrófagos derivados de camundongos *Casp1/11*^{-/-} para a caracterização dessa via de controle da replicação de *L. pneumophila*, na ausência de caspase-1/11.

Figura 5: O controle da replicação de *L. pneumophila* é dependente de NLRC4 e flagelina, parcialmente dependente de caspase-1 e independente de ASC. Macrófagos derivados de medula óssea (BMDMs) de C57BL/6 (círculo aberto), *Nlrc4*^{-/-} (quadrado fechado), *Casp1/11*^{-/-} (triângulo fechado) e *Asc*^{-/-} (triângulo invertido aberto) foram infectados com *L. pneumophila* WT Lp (A-B), *flit* (C-D) e *flaA*⁻ (E-F) com MOI de 0.015 (A, C e E) ou 10 (B, D e F). As células foram lisadas após 0, 24, 48, 72 ou 96 horas para a determinação das unidades formadoras de colônias (UFCs). Dados demonstram a média ± o desvio padrão das triplicatas. *, $P < 0.05$ comparado com as células *Casp1/11*^{-/-}, ANOVA. Os dados apresentam um experimento representativo de dois experimentos com resultados similares. De: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

4.2- A formação de punctas de NLRC4 por *L. pneumophila* contendo flagelina é dependente de ASC

A formação e ativação dos inflamassomas gera um agregado citoplasmático muito característico, capaz de ser visto em microscópio óptico, denominado de “*puncta*” ou “*speck*” (Proell *et al.*, 2013). A formação dos punctas leva à auto proteólise das caspases, com consequente secreção de citocinas pró-inflamatórias (Martinon *et al.*, 2002). Desta maneira, nosso próximo objetivo foi avaliar como ocorre a formação do inflamassoma dependente de NLRC4 e independente de caspase-1/11 pela observação dos punctas de NLRC4. Para isso utilizamos um sistema retroviral expressando NLRC4 fusionado à GFP já padronizado anteriormente (Case e Roy, 2011). BMDMs derivados de camundongos *Casp1/11^{-/-}* foram transduzidos com NLRC4-GFP e então infectados com WT Lp, *flii*⁻ e *flaA⁻* (MOI 1, 3 e 10) por 2, 4 e 8 horas. Nós observamos que a quantidade de punctas de NLRC4-GFP aumenta conforme o tempo e a quantidade de bactérias/macrófagos (Fig. 6A). As bactérias WT Lp e *flii*⁻ induziram uma maior formação de punctas em comparação à bactéria *flaA⁻*, que apresentou baixas porcentagens de formação de punctas de NLRC4-GFP. Esse dado indica que há formação de puncta de NLRC4 em resposta à flagelina de *L. pneumophila* mesmo na ausência de caspase-1/11.

Diversos trabalhos têm demonstrado o papel essencial da molécula adaptadora ASC na nucleação dos inflamassomas, incluindo o inflamassoma de NLRC4, AIM2 e NLRP3 (Masumoto *et al.*, 1999; Fernandes-Alnemri *et al.*, 2007; Case e Roy, 2011; Pierini *et al.*, 2012; Man *et al.*, 2013; Stutz *et al.*, 2013). Dessa maneira nosso próximo passo foi avaliar o papel de ASC na formação do puncta de NLRC4-GFP em resposta à *L. pneumophila* na ausência de caspase-1/11. Para isso foram cruzados animais *Asc^{-/-}* e *Casp1/11^{-/-}* gerando uma prole F1 que foi posteriormente cruzada gerando camundongos triplo nocautes para *Asc/Casp1/11^{-/-}* (Cunha *et al.*, 2017). BMDMs desses camundongos, assim como de *Casp1/11^{-/-}* foram transduzidos com NLRC4-GFP e posteriormente infectados com *flii*⁻ por 8 horas para a quantificação de punctas de NLRC4-GFP. Não foi possível detectar nenhum puncta de NLRC4-GFP nos macrófagos *Asc/Casp1/11^{-/-}* infectados com *flii*⁻ (Fig. 6B). Entretanto a ausência de caspase-1/11 não afetou a formação dos punctas como demonstrado anteriormente (Fig. 6A). Esses dados confirmam que ASC é essencial para a nucleação do

inflamassoma de NLRC4 em resposta à infecção por *L. pneumophila* contendo flagelina, mesmo na ausência de caspase-1/11.

A partir desses resultados nosso próximo objetivo foi identificar novos componentes do inflamassoma dependente de NLRC4 e independente de caspase-1/11, como por exemplo outras caspases. Tem sido demonstrado que caspases apoptóticas, como a caspase-3, caspase-7 e caspase-8 podem participar da formação dos inflamassomas por meio da interação com ASC (Masumoto *et al.*, 2003; Hasegawa *et al.*, 2005; Akhter *et al.*, 2009; Kumar *et al.*, 2010; Abdelaziz, Gavrillin, Akhter, Caution, Kotrange, Khweek, Abdulrahman, Hassan, *et al.*, 2011; Motani *et al.*, 2011; Pierini *et al.*, 2012; Man *et al.*, 2013; Sagulenko *et al.*, 2013; Man *et al.*, 2014; Vajjhala *et al.*, 2015; Kawamoto *et al.*, 2017; Rauch *et al.*, 2017). Desta maneira, macrófagos *Casp1/11*^{-/-} foram transduzidos com NLRC4-GFP e infectados com *flii* por 8 horas. Nesse experimento antes da infecção as células foram tratadas por 1 hora com inibidor de pan-caspase Z-VAD para bloquear a ativação das caspases e facilitar a visualização dos punctas. Após a infecção as células foram fixadas e coradas com anticorpo anti-caspase-3, anti-caspase-7 ou anti-caspase-8. Nós não detectamos um número significativo de punctas de NLRC4-GFP colocalizados com caspase-3 e caspase-7 (Fig. 7A). Entretanto, foi encontrado uma alta taxa de colocalização entre os punctas de NLRC4-GFP e os punctas de caspase-8, indicando que a caspase-8 pode participar da formação do inflamassoma de NLRC4 na ausência de caspase-1/11. Para confirmar esses dados, nós avaliamos a colocalização de caspase-8 e ASC nos punctas de NLRC4-GFP em macrófagos *Casp1/11*^{-/-} infectados com *flii*. Tanto os punctas de caspase-8 quanto de ASC apresentaram mais de 80% de colocalização com os punctas de NLRC4-GFP (Fig. 7B). Confirmando esses dados, imagens adquiridas por microscopia de multifóton revelaram que NLRC4, ASC e caspase-8 estão no mesmo puncta, indicando que o inflamassoma independente de caspase-1/11 é composto por NLRC4/ASC/Caspase-8 (Fig. 7C).

A fim de corroborar os dados anteriores foi utilizado um sistema retroviral expressando ASC fusionado a GFP, também já padronizado anteriormente (Case e Roy, 2011). BMDMs derivados de camundongos *Casp1/11*^{-/-} foram transduzidos com ASC-GFP e infectados com WT Lp, *flii* e *flaA*⁻ (MOI 10) por 1 e 8 horas de infecção. Nós observamos que a formação dos punctas de ASC-GFP ocorre logo após a infecção com WT Lp ou *flii*, mas não com *flaA*⁻ (Fig. 8A). Após 8 horas de infecção

ocorre um aumento na formação de punctas de ASC-GFP, inclusive nas células infectadas com *flaA*⁻, entretanto essas células ainda apresentam uma formação de punctas inferior se comparadas às células infectadas com WT Lp e *fliI*⁻, sendo a formação de punctas em tempos mais tardios parcialmente dependente de flagelina. Para validar a participação de caspase-8 na via independente de caspase-1/11, macrófagos *Casp1/11*^{-/-} transduzidos com ASC-GFP foram infectadas com *fliI*⁻ e incubadas com anticorpo anti-caspase-3, anti-caspase-7 ou anti-caspase-8. Como observado com NLRC4, nas células transduzidas para ASC-GFP a porcentagem de colocalização entre ASC, caspase-3 e caspase-7 não foi significativa (Fig. 8B). Entretanto, foi encontrado uma alta taxa de colocalização entre ASC-GFP e caspase-8 (Fig. 8B-C). Em conjunto esses dados indicam que a flagelina de *L. pneumophila* leva à formação de um inflamassoma independente de caspase-1/11 composto por NLRC4/ASC/Caspase-8.

Figura 6: *L. pneumophila* leva à formação de puncta de NLRC4-GFP de maneira dependente de flagelina e ASC e independente de caspase-1/11. Macrófagos derivados de medula óssea (BMDMs) de *Casp1/11^{-/-}* (A-B) e *Asc/Casp1/11^{-/-}* (B) foram transduzidos com retrovírus expressando NLRC4-GFP e infectados com *L. pneumophila* WT Lp, *fliI*⁻ ou *flaA*⁻ (MOI 1, 3 ou 10). As células foram fixadas após 2 (A), 4 (A) ou 8 (A-B) horas de infecção e a porcentagem de células transduzidas contendo puncta de NLRC4-GFP foi quantificada por meio de microscopia de epifluorescência. Dados demonstram a média $\pm$ o desvio padrão das triplicatas. *, $P < 0.05$ comparado com as células infectadas com *flaA*⁻ ou como indicado, teste *t* não paramétrico. Os dados apresentam um experimento representativo de cinco (A) e dois (B) experimentos com resultados similares. Adaptado de: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

Figura 7: ASC e caspase-8, mas não caspase-3 e caspase-7 colocalizam o puncta de NLRC4-GFP em resposta à *L. pneumophila* contendo flagelina. Macrófagos derivados de medula óssea (BMDMs) de *Casp1/11^{-/-}* foram transduzidos com retrovírus expressando NLRC4-GFP e infectados com *L. pneumophila flii* (MOI 10). Após 8 horas de infecção as células foram fixadas e incubadas com anti-caspase-3 (A), anti-caspase-7 (A), anti-caspase-8 (A-C) ou anti-ASC (B-C). (A-B) A porcentagem de colocalização entre os punctas de NLRC4-GFP, caspase-3, caspase-7, caspase-8 e ASC foi quantificada por meio de microscopia de epifluorescência. (C) Imagens representativas da colocalização entre NLRC4-GFP (verde), caspase-8 (vermelho) e ASC (magenta) em BMDMs *Casp1/11^{-/-}* transduzidos com NLRC4-GFP e infectados com *flii* (MOI 10) por 8 horas. As células foram fixadas e marcadas para anti-caspase-8, anti-ASC e o núcleo foi corado com DAPI (ciano). As imagens foram adquiridas por microscopia de multifóton utilizando objetiva de óleo de imersão de aumento de 63X. As imagens foram analisadas utilizando o software *ImageJ*. Barra de escala, 10 µm. Dados demonstram a média ± o desvio padrão das triplicatas. *, $P < 0.05$, teste *t* não paramétrico. NI, não infectado. Os dados apresentam um experimento representativo de dois experimentos com resultados similares. Adaptado de: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

Figura 8: Puncta de ASC-GFP é induzido em resposta à flagelina de *L. pneumophila* e colocaliza com caspase-8. Macrófagos derivados de medula óssea (BMDMs) de *Casp1/11^{-/-}* foram transduzidos com retrovírus expressando ASC-GFP e infectados com *L. pneumophila* WT Lp (A), *flii*⁻ (A-C) ou *flaA*⁻ (A) (MOI 10). (A) As células foram fixadas após 1 ou 8 horas de infecção e a porcentagem de células transduzidas contendo puncta de ASC-GFP foi quantificada por meio de microscopia de epifluorescência. (B) Após 8 horas de infecção com *flii*⁻ (MOI 10), as células foram fixadas e incubadas com anti-caspase-3, anti-caspase-7 ou anti-caspase-8. A porcentagem de colocalização entre os puncta de ASC-GFP, caspase-3, caspase-7 ou caspase-8 foi quantificada por meio de microscópio de epifluorescência. (C) Imagens representativas da colocalização entre ASC-GFP (verde) e caspase-8 (vermelho) em BMDMs *Casp1/11^{-/-}* transduzidos com ASC-GFP e infectados com *flii*⁻ (MOI 10) por 8 horas. As células foram fixadas e marcadas para anti-caspase-8 e o núcleo foi corado com DAPI (ciano). As imagens foram adquiridas por microscopia de multifóton utilizando objetiva de óleo de imersão de aumento de 63X. As imagens foram analisadas utilizando o software *ImageJ*. Barra de escala, 10 μ m. Dados demonstram a média $\pm$ o desvio padrão das triplicatas. *, $P < 0.05$, teste *t* não paramétrico. NI, não infectado. Os dados apresentam um experimento representativo de três (A) e dois (B-C) experimentos com resultados similares. Adaptado de: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

4.3- O inflamassoma de NLRC4/ASC/Caspase-8 leva ao controle da replicação de *Legionella* spp. flageladas na ausência de caspase-1/11 em macrófagos

Anteriormente observamos que NLRC4, ASC e caspase-8 estão no mesmo puncta, sugerindo que caspase-8 participa do inflamassoma independente de caspase-1/11 com NLRC4 e ASC. A partir desses dados, nós avaliamos se caspase-8 é ativada em resposta à infecção por *L. pneumophila*. Com esse objetivo, BMDMs de camundongos *Casp1/11^{-/-}* e *Asc/Casp1/11^{-/-}* foram infectados com *L. pneumophila flit⁻* e *flaA⁻* (MOI 10). Após 8 horas foi avaliada a ativação de caspase-8 por meio da adição de um substrato para caspase-8 ativa que quando clivado resulta em um sinal luminescente (*Caspase-8 Glo Assay*) (Zwolaneck *et al.*, 2014). Nós detectamos uma alta ativação de caspase-8 em células *Casp1/11^{-/-}* infectadas com *flit⁻*, mas não com *flaA⁻* (Fig. 9A). Já nas células deficientes para *Asc/Casp1/11* a ativação de caspase-8 não foi significativa, indicando que ASC é essencial para a ativação de caspase-8, o que vai de acordo com os dados anteriores indicando que ASC é essencial para a formação deste inflamassoma. Para confirmar esses dados, nós avaliamos a ativação de caspase-8 por *Western Blot* observando a clivagem da pro-caspase-8 (p55) para a forma clivada (p18). Nós verificamos uma alta clivagem de caspase-8 em p18 nos macrófagos *Casp1/11^{-/-}* infectados com *flit⁻*, o que também fica evidente pela redução da forma p55 (Fig. 9B). Entretanto, os macrófagos *Casp1/11^{-/-}* infectados com *flaA⁻* ou *Asc/Casp1/11^{-/-}* apresentaram uma baixa clivagem de caspase-8, confirmando que a ativação e clivagem de caspase-8 é dependente de flagelina e ASC.

A partir dos dados de que NLRC4/ASC/Caspase-8 formam um inflamassoma e que caspase-8 é ativada em resposta à flagelina de *L. pneumophila*, nosso próximo passo foi avaliar o papel de caspase-8 no controle da replicação de *L. pneumophila* independente de caspase-1/11 e mediado por NLRC4 em macrófagos. Com esse objetivo foram utilizados retrovírus expressando duas sequencias independentes de shRNA para o silenciamento de caspase-8 em BMDMs *Casp1/11^{-/-}*. Como controle foram adicionados aos experimentos retrovírus expressando uma sequência não relacionadas (NT – *Non-target*). Por meio de análises de *Western Blot* e Real Time qPCR, nós detectamos uma diminuição na expressão de caspase-8 nas células silenciadas com a Seq1 e Seq2 em relação às células não silenciadas (NT) (Fig. 10A-B). Também foi possível identificar que a Seq1 apresenta um melhor silenciamento de caspase-8 em comparação com a Seq2. É importante ressaltar que o silenciamento

dos macrófagos não pode ser completo em relação à caspase-8, já que essa proteína é importante para a sobrevivência das células (Varfolomeev *et al.*, 1998; Kaiser *et al.*, 2011). Para confirmar o silenciamento de caspase-8 por meio de ensaios funcionais, BMDMs silenciados com as duas sequências de shRNA para caspase-8 ou com a sequência NT foram infectados com *flit* por 8 horas e então foi avaliado o número de punctas e ativação de caspase-8 nessas células. Nós detectamos uma redução significativa tanto na formação de puncta quanto na ativação de caspase-8 nas células silenciadas em comparação às não silenciadas para caspase-8, confirmando assim que nosso sistema de silenciamento funciona corretamente (Fig. 10C-D). A partir desses dados macrófagos silenciados com as duas sequências de shRNA para caspase-8 e a sequência NT, foram infectados com *flit* e *flaA*⁻. Após 0, 24, 48 ou 72 horas as células foram lisadas e plaqueadas em meio CYE para a determinação das UFCs. Nós observamos que o silenciamento de caspase-8 leva a um aumento da replicação nos macrófagos *Casp1/11*^{-/-} infectados com *flit*, mas não com *flaA*⁻, indicando que caspase-8 é importante para o controle da replicação de *L. pneumophila* dependente de flagelina, na ausência de caspase-1/11 (Fig. 10E-F). Esse dado suporta a hipótese de que caspase-8 compõe o inflamassoma de NLRC4, juntamente com ASC e que esse inflamassoma leva ao controle da replicação de *L. pneumophila* de maneira independente de caspase-1/11.

Em seguida nós avaliamos o papel de ASC no controle da infecção mediado por NLRC4, já que nossos dados anteriores indicam que essa proteína adaptadora é essencial para a formação do inflamassoma de NLRC4/ASC/Caspase-8. Para isso BMDMs de camundongos C57BL/6, *Nlrc4*^{-/-}, *Casp1/11*^{-/-} e *Asc/Casp1/11*^{-/-} foram infectados com *L. pneumophila flit* e *flaA*⁻ (MOI 10) e lisados após 0, 24, 48 ou 72 horas para a determinação das UFCs. Nós confirmamos que os macrófagos C57BL/6 são altamente restritivos, os *Casp1/11*^{-/-} parcialmente restritivos e os *Nlrc4*^{-/-} susceptíveis em resposta à infecção por *L. pneumophila* contendo flagelina (*flit*) (Fig. 11A). Em paralelo, macrófagos *Asc/Casp1/11*^{-/-} foram altamente susceptíveis à infecção por *flit*, apresentando um fenótipo muito parecido com o das células *Nlrc4*^{-/-}. Como observado anteriormente, todos os macrófagos infectados com a bactéria *flaA*⁻ apresentaram um alto crescimento bacteriano, já que essa bactéria não possui flagelina e não é reconhecida pela via de NLRC4 (Fig. 11B). Para analisar se o inflamassoma de NLRC4/ASC/Caspase-8 seria uma via específica de

reconhecimento de flagelina de *L. pneumophila*, nós realizamos infecções com outras espécies flageladas de *Legionella*, como *L. gratiana* e *L. micdadei* (Silveira e Zamboni, 2010). Assim como nas infecções com *flii*⁻, com *L. gratiana* e *L. micdadei*, os macrófagos *Asc/Casp1/11*⁻ foram tão susceptíveis quanto os *Nlrc4*⁻, já os *Casp1/11*⁻ foram parcialmente restritivos e os C57BL/6 altamente restritivos (Fig. 11C-D). Esses dados indicam que as células *Casp1/11*⁻ controlam mais a infecção por apresentarem a via de reconhecimento de flagelina NLRC4/ASC/Caspase-8 funcional.

Para confirmar essa hipótese foi realizado o silenciamento de caspase-8 com as suas sequências de shRNA e a sequência não específica (NT) em macrófagos *Asc/Casp1/11*⁻. O silenciamento foi validado por *Western Blot*, confirmando uma diminuição da expressão de caspase-8 nas células silenciadas (Fig. 11E). BMDMs transduzidos com as duas sequências de silenciamento de caspase-8 e a sequência NT, foram infectados com *flii*⁻ e *flaA*⁻, e lisados após 0, 24, 48 ou 72 horas para a determinação das UFCs. Como observado na figura 10E, o silenciamento de caspase-8 em macrófagos *Casp1/11*⁻ infectados com *flii*⁻, leva a um aumento do número de bactérias nessas células (Fig. 11F). Entretanto, o silenciamento de caspase-8 não alterou o crescimento das bactérias *flii*⁻ nas células *Asc/Casp1/11*⁻, ou das bactérias *flaA*⁻ tanto em macrófagos *Asc/Casp1/11*⁻ quanto em *Casp1/11*⁻, o que indica que ASC e caspase-8 estão na mesma via (Fig. 11G-I). Em conjunto esses dados confirmam que o inflamassoma de NLRC4/ASC/Caspase-8 leva ao controle da replicação de *Legionella* spp. em macrófagos, pelo reconhecimento de flagelina e independente das vias de caspase-1/11.

Figura 9: Caspase-8 é ativada em resposta à flagelina de *L. pneumophila* de maneira dependente de ASC e independente de caspase-1/11. Macrófagos derivados de medula óssea (BMDMs) de *Casp1/11*^{-/-} e *Asc/Casp1/11*^{-/-} foram infectados com *L. pneumophila flii*⁻ e *flaA*⁻ com MOI 10. Após 8 horas de infecção a ativação de caspase-8 foi avaliada utilizando um substrato luminescente de caspase-8 (*Caspase-8 Glo Assay*) (A) ou por *Western Blot* (B). (B) Após 8 horas de infecção as células foram lisadas com tampão RIPA. Os lisados celulares foram separados em gel SDS-PAGE, transferidos para membrana de nitrocelulose e incubados com anti-caspase-8. A pro-caspase-8 p55 e a forma clivada p18 estão indicadas. Dados demonstram a média ± o desvio padrão das triplicatas. *, $P < 0.05$, teste *t* não paramétrico. NI, não infectado. URL, unidades relativas de luminescência. Os dados apresentam um experimento representativo de três (A) e dois (B) experimentos com resultados similares. De: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

Figura 10: Caspase-8 é importante para o controle da replicação de *L. pneumophila* mediado por NLRC4 e independente de caspase-1/11. Macrófagos derivados de medula óssea (BMDMs) de *Casp1/11^{-/-}* foram transduzidos com retrovírus expressando sequências de shRNA para o silenciamento de caspase-8 (Seq1 e Seq2) e uma sequência não relacionada (NT- *non-target*). O silenciamento foi confirmado por análises de *Western Blot* (A) e qPCR em Tempo Real (B). (A) Células não infectadas foram lisadas com tampão RIPA. Os lisados das células foram separados em gel SDS-PAGE, transferidos para membrana de nitrocelulose e incubados com anti-caspase-8 (pro-caspase-8 p55) e anti-α-actina. (B) Quantificação da expressão de caspase-8 por RT-qPCR em macrófagos silenciados para duas sequências de shRNA para caspase-8 e sequência não relacionada. O gene *β-Actina* foi utilizado como controle para normalização dos níveis de expressão. Os números acima das barras indicam a porcentagem de silenciamento comparado com a sequência NT pela equação: $\{1 - (2^{-\Delta\Delta C_t}) \times 100\}$. (C-D) BMDMs *Casp1/11^{-/-}* transduzidos foram infectados com *L. pneumophila flit* (MOI 10). Após 8 horas de infecção as células foram fixadas e o número de punctas de caspase-8 foram quantificados com auxílio de microscópio de epifluorescência (C), e a ativação de caspase-8 foi avaliada pela adição um substrato luminescente de caspase-8 (*Caspase-8 Glo Assay*) (D). (E-F) As células transduzidas com a sequência NT (triângulo fechado), shRNA Casp8 Seq1 (quadrado aberto) ou Seq2 (losango fechado) foram infectadas com *flit*⁻ e *flaA*⁻ (MOI 10) e lisadas após 0, 24, 48 ou 72 horas para a

determinação das unidades formadoras de colônias (UFCs). Dados demonstram a média $\pm$ o desvio padrão das triplicatas. *, $P < 0.05$ comparado com as células transduzidas com a sequência NT ou como indicado, teste t não paramétrico (C-D) e ANOVA (E-F). NI, não infectado. NT, não relacionado (*non-target*). Os dados apresentam um experimento representativo de três experimentos com resultados similares. De: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

Figura 11: ASC é importante para o controle da replicação de *L. pneumophila* mediado pelo inflamassoma de NLRC4/Caspase-8 e independente de caspase-11. (A-D) Macrófagos derivados de medula óssea (BMDMs) de camundongos C57BL/6 (círculo aberto), *Nlrc4*^{-/-} (quadrado fechado), *Casp1/11*^{-/-} (triângulo fechado) e *Asc/Casp1/11*^{-/-} (triângulo aberto) foram infectados com *L. pneumophila flit* (A), *flaA* (B), *L. gratiana* (C) e *L. micdadei* (D) (MOI 10). As células foram lisadas após

0, 24, 48 ou 72 horas para a determinação das unidades formadoras de colônias (UFCs). (E-I) BMDMs de camundongos *Casp1/11^{-/-}* e *Asc/Casp1/11^{-/-}* foram transduzidos com retrovírus expressando sequências de shRNA para o silenciamento de caspase-8 (Seq1 e Seq2) e uma sequência não relacionada (NT- *non-target*). (E) O silenciamento foi confirmado por análises de *Western Blot*. Células não infectadas foram lisadas com tampão RIPA. Os lisados celulares foram separados em gel SDS-PAGE, transferidos para membrana de nitrocelulose e incubados com anti-caspase-8 (pro-caspase-8 p55) e anti- α -actina. (F-I) As células transduzidas com a sequência NT (triângulo fechado), shRNA Casp8 Seq1 (quadrado aberto) ou Seq2 (losango fechado) foram infectadas com *flii⁻* e *flaA⁻* (MOI 10) e lisadas após 0, 24, 48 ou 72 horas para a determinação das UFCs. Dados demonstram a média $\pm$ o desvio padrão das triplicatas. *, $P < 0.05$ comparado com as células transduzidas com a sequência NT, ANOVA. NT, não relacionado (*non-target*). Os dados apresentam um experimento representativo de quatro (A), dois (B-D) ou um (E-I) experimentos com resultados similares. De: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

4.4 – ASC é essencial para o controle da replicação de *Legionella* flagelada mediado pelo inflamassoma de NLRC4/ASC/Caspase-8 in vivo

Nos dados anteriores nós observamos que a via de NLRC4/ASC/Caspase-8 é importante para o controle da replicação de *Legionella* spp. em macrófagos. A partir disso, nosso próximo objetivo foi identificar se essa via também é importante para o controle de *Legionella* flagelada in vivo. Os camundongos *Casp8*^{-/-} são embrionariamente letais, o que nos impede de gerar camundongos *Casp1/11/8*^{-/-} (Kaiser *et al.*, 2011). De acordo com os nossos dados ASC é essencial para a formação do inflamassoma de NLRC4/ASC/Caspase-8, a partir disso nós utilizamos como estratégia os animais *Asc/Casp1/11*^{-/-} para verificar a importância dessa via no controle da replicação de *Legionella* spp. in vivo. Com esse objetivo camundongos C57BL/6, *Nlrc4*^{-/-}, *Casp1/11*^{-/-} e *Asc/Casp1/11*^{-/-} foram infectados com 10⁵ bactérias/camundongo de *L. pneumophila flit*, *L. gratiana* e *L. micdadei*. Após 4, 48 ou 72 horas os pulmões dos animais foram retirados, homogeneizados e plaqueados em meio CYE para a determinação das UFCs/pulmão. Nós observamos que tanto na infecção com *flit* quanto com *L. gratiana* e *L. micdadei* os animais *Asc/Casp1/11*^{-/-} foram tão susceptíveis quando os *Nlrc4*^{-/-} (Fig. 12A-C). Como demonstrado nos macrófagos, os camundongos *Casp1/11*^{-/-} foram parcialmente restritivos à infecção por essas bactérias, entretanto os camundongos C57BL/6 foram altamente restritivos. Confirmando nossos dados, camundongos *Asc/Casp1/11*^{-/-} foram mais susceptíveis que os *Casp1/11*^{-/-} apenas quando infectados com *flit*, mas não com a *flaA*⁻ que não é reconhecida por nenhuma das vias de NLRC4 (Fig. 12D). Em conjunto esses dados indicam que o inflamassoma de NLRC4/ASC/Caspase-8 é formado em resposta à flagelina de *Legionella* spp. e opera no controle da sua replicação in vitro e in vivo em uma via independente de caspase-1/11.

Figura 12: ASC é essencial para o controle da replicação de *Legionella* flagelada mediado por NLRC4 e independente de caspase-1/11 in vivo. Camundongos C57BL/6 (círculo aberto), *Nlrc4*^{-/-} (quadrado fechado), *Casp1/11*^{-/-} (triângulo fechado) e *Asc/Casp1/11*^{-/-} (triângulo aberto) foram infectados por via intranasal com 10⁵ bactérias/camundongo de *fliI* (A e D), *L. gratiana* (B), *L. micdadei* (C) e *flaA* (D). Os animais foram sacrificados após 4, 48 ou 72 horas de infecção. Diluições dos pulmões homogeneizados foram plaqueados em meio CYE para a determinação das unidades formadoras de colônias (UFCs). Cada ponto representa um animal, e as linhas horizontais representam as médias dos valores. *, *P*<0.05. #, indica *P*<0.05 comparado com C57BL/6, *Mann Whitney test*. Os dados apresentam um experimento representativo de cinco (A), um (B-C) ou dois (D) experimentos com resultados similares. De: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

4.5 – AIM2 não participa no inflamassoma de NLRC4/ASC/Caspase-8 em resposta à infecção por *L. pneumophila*

Trabalhos tem demonstrado que AIM2, um sensor de dupla fita de DNA, induz a ativação de um inflamassoma pelo recrutamento de ASC, levando à formação de punctas, ativação de caspase-1, piroptose e secreção de citocinas pró-inflamatórias (Muruve *et al.*, 2008; Burckstummer *et al.*, 2009; Fernandes-Alnemri *et al.*, 2009; Hornung *et al.*, 2009; Roberts *et al.*, 2009). Em paralelo tem sido observado a participação de AIM2 em vias independentes de caspase-1 e dependentes de caspase-8 (Pierini *et al.*, 2012; Pierini *et al.*, 2013; Sagulenko *et al.*, 2013). Desta maneira, nós investigamos se AIM2 faz parte do inflamassoma de NLRC4/ASC/Caspase-8 em resposta à *L. pneumophila*. Para isso BMDMs de camundongos *Casp1/11*^{-/-} foram transduzidos com NLRC4-GFP e infectados com *flit* (MOI 10). Após 8 horas de infecção as células foram fixadas e marcadas com anti-AIM2, para análise da colocalização entre AIM2 e os punctas de NLRC4-GFP. Não foi observada uma colocalização significativa entre os punctas de NLRC4-GFP e AIM2, o que indica que AIM2 não está presente no inflamassoma de NLRC4/ASC/Caspase-8 (Fig. 13A). É importante destacar, que foram detectados punctas de AIM2 em resposta à *L. pneumophila*, como demonstrado na Fig. 13A, entretanto a maioria desses punctas não colocalizavam com os de NLRC4-GFP. Para confirmar esses dados, BMDMs de camundongos *Casp1/11*^{-/-} e *Aim2/Casp1/11*^{-/-} transduzidos com NLRC4-GFP foram infectados com WT Lp, *flit* ou *flaA*⁻ por 8 horas para a quantificação dos punctas de NLRC4-GFP. Nós não detectamos nenhuma diferença na formação de punctas de NLRC4-GFP entre os macrófagos de camundongos *Casp1/11*^{-/-} e *Aim2/Casp1/11*^{-/-} infectados com WT Lp ou *flit* (Fig. 13B). O mesmo foi observado quando comparamos a ativação de caspase-8 entre as células *Casp1/11*^{-/-} e *Aim2/Casp1/11*^{-/-}, indicando que AIM2 não é importante para a formação e ativação do inflamassoma de NLRC4/ASC/Caspase-8 (Fig. 13C).

Para corroborar os dados de que AIM2 não participa do inflamassoma de NLRC4/ASC/Caspase-8, nós investigamos o papel desse receptor no controle da replicação de *L. pneumophila* in vitro e in vivo na ausência de caspase-1/11. Para isso BMDMs de camundongos C57BL/6, *Nlrc4*^{-/-}, *Casp1/11*^{-/-} e *Aim2/Casp1/11*^{-/-} foram infectados com *flit* e lisados após 0, 24, 48 ou 72 horas para a determinação das UFCs. Não houve nenhuma diferença no crescimento bacteriano entre as células

Casp1/11^{-/-} e as *Aim2/Casp1/11^{-/-}*, indicando que AIM2 não é importante para o controle da replicação de *L. pneumophila* mediado por NLRC4 na ausência de caspase-1/11 (Fig. 14A). Para validar esses dados in vivo, camundongos C57BL/6, *Nlrc4^{-/-}*, *Casp1/11^{-/-}*, *Aim2/Casp1/11^{-/-}* e *Asc/Casp1/11^{-/-}* foram infectados com *fliH* (10^5 bactérias/camundongo) e após 4, 48 ou 72 horas os pulmões dos animais foram retirados, homogeneizados e plaqueados em meio CYE para a determinação das UFCs/pulmão. Como observado nos macrófagos, também in vivo não houve diferença no crescimento de bactérias no pulmão dos camundongos *Casp1/11^{-/-}* e *Aim2/Casp1/11^{-/-}* tanto no tempo de 48 quanto de 72 horas (Fig.14B-C). Entretanto, os camundongos *Nlrc4^{-/-}* e *Asc/Casp1/11^{-/-}* apresentaram uma alta susceptibilidade à infecção por *fliH*, como demonstrado anteriormente na figura 12A. Dessa maneira nós concluímos que AIM2 não faz parte do inflamassoma de NLRC4/ASC/Caspase-8.

Figura 13: AIM2 não participa da formação dos pontos de NLRC4-GFP e da ativação de caspase-8 em resposta à infecção por *L. pneumophila* com flagelina. (A-B) Macrófagos derivados de medula óssea (BMDMs) de *Casp1/11^{-/-}* (A-B) e *Aim2/Casp1/11^{-/-}* (B) foram transduzidos com retrovírus expressando NLRC4-GFP e infectados com *L. pneumophila* WT Lp (B) e *fliI⁻* (A-B) (MOI 10) por 8 horas. (A) As células foram fixadas e marcadas com anti-AIM2 (vermelho), o núcleo celular com DAPI (ciano) e os pontos de NLRC4-GFP estão representados em verde. A porcentagem de colocalização entre os pontos de NLRC4-GFP e os de AIM2 está representada no gráfico e foi quantificada com auxílio de microscópio de epifluorescência. As imagens foram adquiridas por microscopia de multifóton utilizando objetiva de óleo de imersão de aumento de 63X. As imagens foram analisadas utilizando o software *ImageJ*. Barra de escala, 10 μ m. (B) A quantificação das células transduzidas contendo pontos de NLRC4-GFP após 8 horas de infecção com WT Lp e *fliI⁻* (MOI 10), foi realizada com auxílio de microscópio de epifluorescência. (C) BMDMs de camundongos *Casp1/11^{-/-}* e *Aim2/Casp1/11^{-/-}* foram infectados com *fliI⁻* e *flaA⁻* (MOI 10). Após 8 horas de infecção a ativação de caspase-8 foi avaliada utilizando um substrato luminescente de caspase-8 (*Caspase-8 Glo Assay*). Dados demonstram a média $\pm$ o desvio padrão das triplicatas. *, $P < 0.05$, teste *t* não paramétrico. NI, não infectado. URL, unidades relativas de luminescência. NS, não significativo. Os dados apresentam um experimento representativo de dois experimentos com resultados similares. Adaptado de: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

Figura 14: AIM2 não é importante para o controle da replicação mediado por NLRC4 e independente de caspase-1/11 em macrófagos e in vivo. (A) Macrófagos derivados de medula óssea (BMDMs) de camundongos C57BL/6 (círculo aberto), *Nlrc4*^{-/-} (quadrado fechado), *Casp1/11*^{-/-} (triângulo fechado) e *Aim2/Casp1/11*^{-/-} (losango aberto) foram infectados com *flit* (MOI 10). As células foram lisadas após 0, 24, 48 ou 72 horas para a determinação das unidades formadoras de colônias (UFCs). (B-C) Camundongos C57BL/6 (círculo aberto), *Nlrc4*^{-/-} (quadrado fechado), *Casp1/11*^{-/-} (triângulo fechado), *Aim2/Casp1/11*^{-/-} (losango aberto) e *Asc/Casp1/11*^{-/-} (triângulo aberto) foram infectados por via intranasal com 10⁵ bactérias/camundongo de *flit*. Os animais foram sacrificados após 4, 48 ou 72 horas de infecção. Diluições dos pulmões homogeneizados foram plaqueados em meio CYE para a determinação das UFCs. Cada ponto representa um animal, e as linhas horizontais representam as médias dos valores. *, *P*<0.05. #, indica *P*<0.05 comparado com C57BL/6, ANOVA (A) Mann Whitney (B-C). Os dados apresentam um experimento representativo de três (A) e dois (B-C) experimentos com resultados similares. Adaptado de: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

4.6 – Naip5 é essencial para a ativação do inflamassoma de NLRC4/ASC/Caspase-8 em resposta à *L. pneumophila* flagelada na ausência de caspase-1/11

Nossos dados anteriores demonstraram que na ausência de caspase-1/11 o inflamassoma de NLRC4/ASC/Caspase-8 é ativado levando ao controle da replicação de *L. pneumophila*. Sabe-se que na via de NLRC4 dependente de caspase-1, o reconhecimento de flagelina ocorre pelo sensor Naip5. Esse receptor do tipo NLR reconhece o domínio C-terminal da flagelina, levando ao recrutamento de NLRC4 e ativação de caspase-1 (Amer *et al.*, 2006; Molofsky *et al.*, 2006; Ren *et al.*, 2006; Zamboni *et al.*, 2006; Lightfield *et al.*, 2008; Kofoed e Vance, 2011; Zhao *et al.*, 2011). Para avaliar se na via de NLRC4/ASC/Caspase-8, Naip5 também é o receptor de flagelina, foi utilizado um sistema retroviral expressando uma sequência de shRNA para o silenciamento de Naip5 em BMDMs *Casp1/11^{-/-}* e *Asc/Casp1/11^{-/-}*. Como controle foram adicionados aos experimentos retrovírus expressando uma sequência não relacionada (NT), utilizada anteriormente nos experimentos de silenciamento com caspase-8 (Fig. 10 e 11E-I). Por meio de análises de RT-qPCR, nós confirmamos o silenciamento de Naip5 pela diminuição da sua expressão nas células transduzidas com os retrovírus expressando a sequência de shRNA para Naip5 em comparação com a sequência NT (Fig. 15A). Após a confirmação do silenciamento esses macrófagos foram infectados com *L. pneumophila* WT Lp, *fliI⁻* e *flaA⁻* por 8 horas e foi avaliada a ativação de caspase-8 nessas células. Foi detectada uma alta ativação de caspase-8 nos macrófagos *Casp1/11^{-/-}* infectados com WT Lp e *fliI⁻* em comparação com os infectados com *flaA⁻*, como demonstrado anteriormente nas figuras 9A e 13C (Fig.15B). Entretanto, nas células silenciadas para Naip5 essa ativação foi reduzida, indicando que Naip5 é importante para a ativação de caspase-8 de maneira dependente de flagelina. Como demonstrado anteriormente nos macrófagos *Asc/Casp1/11^{-/-}*, a ativação de caspase-8 não foi significativa.

Para avaliar o papel de Naip5 no controle da replicação de *L. pneumophila* na via independente de caspase-1/11 os BMDMs *Casp1/11^{-/-}* e *Asc/Casp1/11^{-/-}* silenciados ou não para Naip5 foram infectados com *fliI⁻* e *flaA⁻*, após 0, 24, 48 ou 72 horas as células foram lisadas para a determinação das UFCs. Nós detectamos um aumento do número de bactérias nas células *Casp1/11^{-/-}* silenciadas infectadas com *fliI⁻* quando comparadas com as células não silenciadas, indicando que Naip5 é

importante para o controle na replicação mesmo na ausência de caspase-1/11 (Fig. 15C). Entretanto, o silenciamento de Naip5 não alterou o crescimento bacteriano nas células infectadas com *flaA*⁻ ou nas células *Asc/Casp1/11*^{-/-} infectadas tanto com *fliH* quanto com *flaA*⁻ (Fig. 15D-F). Esses dados indicam que Naip5 participa do inflamassoma de NLRC4/ASC levando à ativação de caspase-8 em resposta à flagelina de *L. pneumophila* em uma via independente de caspase-1/11.

Figura 15: A ativação do inflamassoma de NLRC4/ASC/Caspase-8 em resposta à infecção por *L. pneumophila* flagelada é dependente de Naip5. Macrófagos derivados de medula óssea (BMDMs) de *Casp1/11*^{-/-} e *Asc/Casp1/11*^{-/-} foram transduzidos com retrovírus expressando uma sequência de shRNA para o silenciamento de Naip5 e uma sequência não relacionada (NT- *non-target*). (A) O silenciamento foi confirmado por análises de qPCR em Tempo Real. Quantificação da expressão de Naip5 por RT-qPCR em macrófagos transduzidos com retrovírus contendo uma sequência de shRNA para o silenciamento de Naip5 e uma sequência não relacionada. O gene *Gapdh* foi utilizado como controle para normalização dos níveis de expressão. Os números acima das barras indicam a porcentagem de silenciamento comparado com a sequência NT pela equação: $\{1 - (2^{-\Delta\Delta Ct}) \times 100\}$. (B) BMDMs de *Casp1/11*^{-/-} e *Asc/Casp1/11*^{-/-} transduzidos foram infectados com *L. pneumophila flii*⁻ e *flaA*⁻ (MOI 10). Após 8 horas de infecção a ativação de caspase-8 foi avaliada pela adição um substrato luminescente de caspase-8 (*Caspase-8 Glo Assay*). (C-F) As células transduzidas com a sequência NT (triângulo fechado) ou shRNA Naip5 (quadrado aberto) foram infectadas com *flii*⁻ e *flaA*⁻ (MOI 10) e

lisadas após 0, 24, 48 ou 72 horas para a determinação das unidades formadoras de colônias (UFCs). Dados demonstram a média $\pm$ o desvio padrão das triplicatas. *, $P < 0.05$ comparado com as células transduzidas com a sequência NT ou como indicado, teste t não paramétrico (B) e ANOVA (C-F). NI, não infectado. NT, não relacionado (*non-target*). URL, unidades relativas de luminescência. Os dados apresentam um experimento representativo de um (B) ou três (C-F) experimentos com resultados similares. Adaptado de: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

4.7 – A ativação do inflamassoma de Naip5/NLRC4/ASC/Caspase-8 induz a formação de poro, mas não a secreção de IL-1 β

Foi relacionado a formação de poro e indução de piroptose como um mecanismos que desencadeiam o controle da replicação de bactérias intracelulares como *Salmonella* Typhimurium, *Burkholderia thailandensis* e *L. pneumophila* (Miao *et al.*, 2010; Jorgensen, Lopez, *et al.*, 2016; Jorgensen, Zhang, *et al.*, 2016). Nosso grupo demonstrou que *L. pneumophila* leva à formação de poro pela via de caspase-1 e caspase-11, pelo reconhecimento de flagelina e LPS respectivamente (Silveira e Zamboni, 2010; Case *et al.*, 2013; Cerqueira *et al.*, 2015). Mediante esses dados, nosso próximo objetivo foi avaliar se o inflamassoma de NLRC4/ASC/Caspase-8 induz a formação de poro em resposta a infecção por *L. pneumophila*. Para isso foi utilizado um ensaio de formação de poro em tempo real onde a fluorescência liberada pela incorporação de Iodeto de Propídio (PI) foi detectada por um fluorímetro de placa. O PI é um corante fluorescente não permeável, ou seja, só é incorporado em células com a membrana danificada, sendo assim uma bom indicador da formação de poros (Case *et al.*, 2013; Cerqueira *et al.*, 2015; Cunha *et al.*, 2015; Cunha *et al.*, 2017). Para esses experimentos foram utilizamos BMDMs C57BL/6, *Casp1/11*^{-/-} e *Asc/Casp1/11*^{-/-} infectados com bactérias *fliH*⁻ e *flaA*⁻. Essas células foram monitoradas por 8 horas de infecção em fluorímetro de placa para a detecção da incorporação de PI a cada 5 minutos. Nós não detectamos nenhuma formação de poros nas células não infectadas (Fig. 16A). Entretanto, observamos uma alta taxa de formação de poro nos macrófagos C57BL/6 infectados com *fliH*⁻, em decorrência da ativação do inflamassoma de Naip5/NLRC4/Caspase-1 (Fig. 16B). Essas mesmas células infectadas com *flaA*⁻ apresentaram um poro mais reduzido, resultado da ativação do inflamassoma de caspase-11 (Fig. 16C) (Case *et al.*, 2013). Notavelmente, nós detectamos uma significativa a formação de poro nos BMDMs *Casp1/11*^{-/-} infectados com *fliH*⁻, entretanto esse poro começou mais tardiamente quando comparado com o poro das células C57BL/6, indicando que existe uma via de morte celular independente de caspase-1/11 (Fig. 16B). Não foi detectada uma significativa formação de poros nas células *Casp1/11*^{-/-} infectadas com *flaA*⁻, ou nas células *Asc/Casp1/11*^{-/-} infectadas tanto com *fliH*⁻ quanto com *flaA*⁻. Esses dados indicam que

que a via NLRC4/ASC/Caspase-8 induz a formação de poros de maneira dependente de flagelina e ASC e independente de caspase-1/11.

Foi demonstrado que a formação de poros na membrana leva a um desbalanço iônico, o que gera entrada de água na célula e assim a sua lise e liberação da enzima lactato desidrogenase (LDH) no sobrenadante (Fink e Cookson, 2006). Para corroborar nossos dados, foi dosado a liberação de LDH no sobrenadante desses macrófagos após 8 horas de infecção. Tanto os BMDMs de camundongos C57BL/6 quanto *Casp1/11*^{-/-} infectados com *flii*⁻ apresentaram uma alta taxa de liberação de LDH, confirmando que existe uma via de morte celular independente de caspase-1/11 (Fig. 16D). Essas mesmas células infectadas com *flaA*⁻, apresentaram uma liberação de LDH reduzida, em resposta à ativação do inflamassoma de caspase-11 e de AIM2, recentemente demonstrado pelo nosso grupo (Case *et al.*, 2013; Cunha *et al.*, 2017). Não houve diferença na liberação de LDH nas células *Asc/Casp1/11*^{-/-} infectadas tanto com *flii*⁻ quanto com *flaA*⁻, já que a detecção de LDH foi baixa com ambas as infecções. Desta maneira nossos dados indicam que o inflamassoma de NLRC4/ASC/Caspase-8 induz a formação de poros e morte celular de maneira independente de caspase-1/11 e dependente do reconhecimento de flagelina.

Tem sido amplamente reportado a participação de caspase-8 e ASC em vias independentes de caspase-1 na secreção de citocinas pró-inflamatórias como IL-18 e IL-1 β (Man *et al.*, 2013; Man *et al.*, 2014; Antonopoulos *et al.*, 2015; Rauch *et al.*, 2017; Schneider *et al.*, 2017). Para avaliar se o inflamassoma de NLRC4/ASC/Caspase-8 também induz a secreção dessas citocinas em resposta à *L. pneumophila*, BMDMs C57BL/6, *Casp1/11*^{-/-} e *Asc/Casp1/11*^{-/-} foram infectados com WT Lp, *flii*⁻ e *flaA*⁻ por 24 horas quando então seus sobrenadantes foram coletados para detecção de IL-1 β e IL-12. Nós encontramos uma alta secreção de IL-1 β nos macrófagos C57BL/6 infectados tanto com WT Lp quanto com *flii*⁻. Entretanto, a secreção desta citocina nos BMDMs *Casp1/11*^{-/-} foi muito baixa e nos *Asc/Casp1/11*^{-/-} praticamente não foi detectada (Fig. 16E). A produção de IL-12 foi igual em todas as células infectadas, confirmando que todas estavam primadas e prontas para a responder à infecção (Fig. 16F). Desta maneira, nossos dados indicam que a via majoritária de secreção de IL-1 β em resposta à flagelina é a de Naip5/NLRC4/Caspase-1. Já a via

NLRC4/ASC/Caspase-8 não parece ser importante para a secreção desta citocina em resposta à infecção por *L. pneumophila*.

Nós demonstramos anteriormente que a flagelina de *L. pneumophila* induz formação de poros e morte celular, mas não a secreção de IL-1 β de maneira independente de caspase-1/11 (Figura 16). Para avaliar a participação de caspase-8 nessa formação de poro independente de caspase-1/11, nós utilizamos BMDMs *Casp1/11*^{-/-} e *Asc/Casp1/11*^{-/-} silenciados para caspase-8 e infectados com *flii*⁻ e *flaA*⁻ e analisamos a incorporação de PI por 6 horas. Os macrófagos não infectados não apresentaram formação de poro, confirmando que a transdução não leva à morte celular (Fig. 17A e D). A formação de poros foi evidente nas células *Casp1/11*^{-/-} infectadas com *flii*⁻ em comparação com as *Asc/Casp1/11*^{-/-} (Fig. 17B e E). Em paralelo, nós observamos uma diminuição na formação de poro nas células *Casp1/11*^{-/-} silenciadas para caspase-8 em relação às não silenciadas (Fig. 17B). Confirmando o papel de flagelina na ativação dessa via, apenas células *Casp1/11*^{-/-} infectadas com *flii*⁻, mas não com *flaA*⁻, induziram a formação de poros na membrana (Fig. 17B e C). Corroborando esses dados BMDMs *Casp1/11*^{-/-} tratados com Z-IETD, um inibidor de caspase-8, e infectados com *flii*⁻ apresentaram uma diminuição na formação de poros em relação às células tratadas com DMSO (Fig. 17H). Entretanto não houve diferença na formação de poros nas células tratadas infectadas com *flaA*⁻ ou não infectadas (Fig. 17G e I). Esses dados indicam que caspase-8 é importante para a formação de poros pela via independente de caspase-1/11 em resposta à infecção por *L. pneumophila*.

Para avaliar a participação de Naip5 na indução de morte celular induzida pela via NLRC4/ASC/Caspase-8 foram utilizados BMDMs *Casp1/11*^{-/-} e *Asc/Casp1/11*^{-/-} transduzidos com shRNA para o silenciamento de Naip5 e a sequência NT, e infectados com WT Lp, *flii*⁻ e *flaA*⁻. Nós detectamos uma diminuição na formação de poros nas células *Casp1/11*^{-/-} silenciadas para Naip5 e infectadas com WT Lp e *flii*⁻ em relação às não silenciadas (Fig. 18B e C). Entretanto, as células infectadas com *flaA*⁻, bem como os macrófagos *Asc/Casp1/11*^{-/-} infectados com WT Lp, *flii*⁻ ou *flaA*⁻ apresentaram uma formação de poros baixa, tanto nas células silenciadas quanto nas não silenciadas para Naip5 (Fig. 18D-H). Esses achados indicam que a flagelina de *L. pneumophila* desencadeia uma formação de poro e morte celular independente de caspase-1/11 pela via dependente de Naip5/NLRC4/ASC/Caspase-8.

Figura 16: A via de NLRC4/ASC/Caspase-8 é importante para a formação de poro, mas não secreção de IL-1β em resposta à *L. pneumophila* flagelada na ausência de caspase-1/11. (A-C) Macrófagos derivados de medula óssea (BMDMs) de camundongos C57BL/6 (círculo aberto), *Casp1/11^{-/-}* (triângulo fechado) e *Asc/Casp1/11^{-/-}* (triângulo aberto) foram infectados com *L. pneumophila flii⁻* ou *flaA⁻* (MOI 10). A formação de poro foi avaliada pela incorporação de iodeto de propídio (PI) em tempo real com o auxílio de um fluorímetro de placa (a cada 5 minutos). As porcentagens de URF foram estimadas em relação às células lisadas com Triton X-100. (D-F) BMDMs de camundongos C57BL/6, *Casp1/11^{-/-}* e *Asc/Casp1/11^{-/-}* foram infectados com WT Lp (E-F), *flii⁻* (D-F)

ou *flaA*⁻ (D-F) (MOI 10). Após 8 (D) ou 24 (E-F) horas o sobrenadante dessas células foi coletado para a detecção da liberação de lactato desidrogenase (LDH) (D) e citocinas (IL-1 β e IL-12 p40) (E-F), respectivamente. (D) Foi dosada a liberação de LDH no sobrenadante das células utilizando o *kit CytoTox96 LDH-release*. As porcentagens de liberação de LDH foram estimadas em relação as células lisadas com Triton X-100. (E-F) A produção de IL-1 β (E) e IL-12 p40 (F) na cultura as células foi estimada pelo método de ELISA. Dados demonstram a média $\pm$ o desvio padrão das triplicatas. *, $P < 0.05$, teste *t* não paramétrico. NI, não infectado. URF, unidades relativas de fluorescência. ND, não detectado. NS, não significativo. Os dados apresentam um experimento representativo de cinco (A-C), três (D) e dois (E-F) experimentos com resultados similares. Adaptado de: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

Figura 17: Poro induzido por ASC na ausência de caspase-1/11 é dependente de caspase-8. (A-F) Macrófagos derivados de medula óssea (BMDMs) de *Casp1/11*^{-/-} (A-C) e *Asc/Casp1/11*^{-/-} (D-F) foram transduzidos com retrovírus expressando duas sequências independentes de shRNA para o silenciamento de caspase-8 (Seq1 e Seq2) e uma sequência não relacionada (NT- *non-target*). Os macrófagos transduzidos foram infectados com *L. pneumophila flii*⁻ ou *flaA*⁻ (MOI 10). (G-I) BMDMs de camundongos *Casp1/11*^{-/-} foram tratados com Z-IETD, um inibidor de caspase-8 ou DMSO e então infectados com *flii*⁻ ou *flaA*⁻ (MOI 10). (A-I) A formação de poro foi avaliada pela incorporação de iodeto de propídio (PI) em tempo real com o auxílio de um fluorímetro de placa (a cada 5 minutos). As porcentagens de URF foram estimadas em relação às células lisadas com Triton X-100. Dados demonstram a média ± o desvio padrão das triplicatas. NI, não infectado. URF, unidades relativas de fluorescência. Os dados apresentam um experimento representativo de três (A-F) e dois (G-I) experimentos com resultados similares. Adaptado de: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

Figura 18: Naip5 é importante para a formação de poro pelo inflamassoma de NLRC4/ASC/Caspase-8 em resposta à *L. pneumophila* flagelada. Macrófagos derivados de medula óssea (BMDMs) de *Casp1/11^{-/-}* (A-D) e *Asc/Casp1/11^{-/-}* (E-H) foram transduzidos com retrovírus expressando uma sequência de shRNA para o silenciamento de Naip5 e uma sequência não relacionada (NT- *non-target*). Os macrófagos transduzidos foram infectados com *L. pneumophila* WT Lp, *flii⁻* ou *flaA⁻* (MOI 10). A formação de poro foi avaliada pela incorporação de iodeto de propídio (PI) em tempo real com o auxílio de um fluorímetro de placa (a cada 5 minutos). As porcentagens de URF foram estimadas em relação às células lisadas com Triton X-100. Dados demonstram a média $\pm$ o desvio padrão das triplicatas. NI, não infectado. URF, unidades relativas de fluorescência. Os dados apresentam um experimento representativo de dois experimentos com resultados similares. Adaptado de: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

4.8- Caspase-8 é recrutada para o inflamassoma de NLRC4/ASC/Caspase-1, mas só é ativada na ausência de caspase-1 ou gasdermina-D.

Anteriormente nós utilizamos como estratégia camundongos deficientes para *Casp1/11* para avaliar os mecanismos da via independente dessas proteínas. Entretanto, em condições normais as proteínas caspase-1 e a caspase-11 estão presentes nas células e são ativadas em resposta à *L. pneumophila*. Desta maneira nós investigamos como caspase-8 participa do inflamassoma de Naip5/NLRC4/ASC na presença de caspase-1/11 em resposta à infecção por *L. pneumophila*. Com esse objetivo, BMDMs de camundongos C57BL/6 foram infectados com *L. pneumophila flit* por 8 horas e então incubados com anti-ASC, anti-caspase-8 ou anti-caspase-1 para avaliar o recrutamento dessas proteínas endógenas para o inflamassoma. Nos macrófagos não infectados, a formação de punctas não foi significativa (Fig. 19A). Entretanto, nós detectamos a formação de punctas de ASC e o recrutamento de caspase-8 para esses punctas mesmo nas células C57BL/6 (Fig. 19B). Notavelmente, foi encontrada uma alta taxa de colocalização entre os punctas de caspase-1 e caspase-8 (mais de 60%), indicando que mesmo na presença de caspase-1, a caspase-8 também é recrutada para o inflamassoma de Naip5/NLRC4/ASC (Fig. 19C).

A partir desses dados, nosso próximo passo foi avaliar a ativação de caspase-8 na presença de caspase-1/11. Com essa finalidade, BMDMs de camundongos C57BL/6, *Casp1/11^{-/-}* e *Asc/Casp1/11^{-/-}* foram infectados com *flit* e *flaA⁻* por 8 horas para a determinação da ativação de caspase-8 nessas células. Como demonstrado nos dados anteriores, existe uma alta ativação de caspase-8 nos macrófagos *Casp1/11^{-/-}* infectados com *flit*, mas não nos infectados com *flaA⁻* ou nos *Asc/Casp1/11^{-/-}* (Fig. 19D). Entretanto, não foi possível identificar ativação de caspase-8 nos macrófagos C57BL/6 infectados tanto com *flit* quanto com *flaA⁻*, indicando que a caspase-8 não é ativada na presença de caspase-1/11. Para verificar se essa diferença na ativação de caspase-8 não é decorrente de um fundo genético ou microbiota diferentes, camundongos C57BL/6 foram cruzados com *Casp1/11^{-/-}* gerando uma prole F1 que foi então cruzada gerando camundongos F2 (*Casp1/11^{+/+}*, *Casp1/11^{+/-}* e *Casp1/11^{-/-}*). BMDMs dos camundongos F2 (*littermate control*) foram infectados com WT Lp, *flit* e *flaA⁻* para avaliação da ativação de caspase-8 após 8 horas de infecção. Nós detectamos ativação de caspase-8 somente nas células

Casp1/11^{-/-} infectadas com WT Lp e *flii*⁻, mas não nas *Casp1/11^{+/+}* e *Casp1/11^{+/-}*, indicando que a presença de caspase-1 ou caspase-11 impede a ativação de caspase-8 (Fig. 19E). Para verificar qual das caspases inflamatórias, caspase-1 ou caspase-11, estaria impedindo a ativação de caspase-8, nós utilizamos BMDMs de camundongos deficientes apenas para caspase-1 (*Casp1^{-/-}/Casp11^{tg}*) ou deficientes apenas para caspase-11 (*Casp11^{-/-}*) (Kayagaki *et al.*, 2011). A ativação de caspase-8 foi detectada apenas nas células deficientes para caspase-1 expressando caspase-11 como um transgene (*Casp1^{-/-}/Casp11^{tg}*) infectadas com WT Lp e *flii*⁻ (Fig. 19F). Entretanto a deficiência apenas na caspase-11 não foi o bastante para possibilitar a ativação de caspase-8 em resposta à infecção por *L. pneumophila*. Esses dados indicam que a caspase-8 é recrutada para o inflamassoma de NLRC4/ASC/Caspase-1, entretanto só é ativada na ausência de caspase-1.

Recentemente foi demonstrado que a ativação das caspases inflamatórias como caspase-1 e caspase-11 levam à clivagem de gasdermina-D (GSDMD) (He *et al.*, 2015; Kayagaki *et al.*, 2015; Shi *et al.*, 2015). Essa proteína quando clivada libera o seu domínio N-terminal que é o responsável por formar a estrutura do poro na membrana e posteriormente a morte celular por piroptose (Ding *et al.*, 2016; Liu *et al.*, 2016). Desta maneira nós avaliamos se a inibição de GSDMD em BMDMs contendo caspase-1 possibilitaria a ativação de caspase-8. Com esse objetivo, BMDMs C57BL/6 e *Casp1/11^{-/-}* foram transduzidos com retrovírus expressando duas sequências independentes de shRNA para o silenciamento de GSDMD e a sequência NT (não relacionada). Por meio de análises de qPCR em Tempo Real, nós confirmamos o silenciamento de GSDMD pela diminuição da sua expressão nas células transduzidas com os retrovírus expressando as sequências de shRNA para GSDMD em comparação com a sequência NT (Fig. 20A). Após a confirmação do silenciamento por essas sequências de shRNA, os macrófagos transduzidos foram infectados com WT Lp, *flii*⁻ e *flaA*⁻ por 8 horas para a detecção da ativação de caspase-8. Nós observamos que o silenciamento de GSDMD com as duas sequências de shRNA nas células C57BL/6, possibilitou a ativação de caspase-8 em resposta à *L. pneumophila* contendo flagelina, indicando que caspase-8 só é ativada na ausência de caspase-1 ou GSDMD (Fig. 21B). Não houve diferença na ativação de caspase-8 entre as células *Casp1/11^{-/-}* silenciadas ou não para GSDMD. Para corroborar esses dados nós avaliamos a ativação de caspase-8 em BMDMs de camundongos

deficientes para *Gsdmd*. Confirmando nossos dados anteriores nós observamos a ativação de caspase-8 somente em macrófagos *Casp1/11^{-/-}* e não em C57BL/6, *Casp11^{-/-}* e *Asc/Casp1/11^{-/-}* infectados com WT Lp e *flii^{-/-}* (Fig. 20C). Corroborando a hipótese de que a ausência de GSDMD possibilita a ativação de caspase-8, BMDMs de camundongos *Gsdmd^{-/-}* apresentaram uma ativação de caspase-8 similar aos *Casp1/11^{-/-}* infectados com WT Lp e *flii^{-/-}*, confirmando assim que caspase-8 só é ativada na ausência de caspase-1 ou GSDMD.

Por fim, nós avaliamos o papel de GSDMD no poro independente de caspase-1/11, induzido pela via NLRC4/ASC/Caspase-8 em resposta à infecção por *L. pneumophila*. Utilizando o sistema de shRNA, nenhuma formação de poro foi detectada nas células não infectadas silenciadas para GSDMD, confirmando que o silenciamento não leva à uma morte celular inespecífica (Fig. 21A e E). Os macrófagos C57BL/6 silenciados para GSDMD e infectados para WT Lp e *flii^{-/-}* apresentaram uma diminuição na formação de poros, confirmando que GSDMD é importante para a formação do poro pela via Naip5/NLRC4/Caspase-1 (Fig. 21B e C). Entretanto não houve diferença na formação de poros entre as células *Casp1/11^{-/-}* silenciadas ou não para GSDMD, indicando que a formação de poro pela via de NLRC4/ASC/Caspase-8 ocorre por uma via independente de GSDMD (Fig. 21F-H). Em conjunto esses dados demonstram que em resposta à flagelina de *Legionella* spp. caspase-8 é recrutada para o inflamassoma de NLRC4/ASC/Caspase-1, entretanto só é ativada na ausência de caspase-1 ou GSDMD. O inflamassoma de NLRC4/ASC/Caspase-8 induz a formação de poros independente de GSDMD, possivelmente pela clivagem de outra gasdermina.

Figura 19: Caspase-8 colocaliza com inflamassoma de NLRC4/ASC/Caspase-1, mas só é ativada na ausência de caspase-1. (A-C) Macrófagos derivados de medula óssea (BMDMs) de C57BL/6 não infectados (NI) ou infectados com *L. pneumophila flii*⁻ (MOI 10) (B-C) por 8 horas foram fixados e marcados com anti-caspase-1 (verde), anti-caspase-8 (vermelho), anti-ASC (verde) e o núcleo celular com DAPI (ciano). A porcentagem de colocalização entre os pontos de caspase-1 e caspase-8 está representada no gráfico e foi quantificada com auxílio de microscópio de epifluorescência. As imagens foram adquiridas por microscopia de multifóton utilizando objetiva de óleo de imersão de aumento de 63X. As imagens foram analisadas utilizando o software *ImageJ*. Barra de escala, 10 µm. (D) BMDMs de camundongos C57BL/6, *Casp1/11*^{-/-} e *Asc/Casp1/11*^{-/-} foram infectados com *flii*⁻ e *flaA*⁻ com MOI 10. (E) BMDMs gerados a partir de camundongos *Casp1/11*^{+/+}, *Casp1/11*^{+/-} e *Casp1/11*^{-/-} (Littermate Control) foram infectados com WT Lp, *flii*⁻ e *flaA*⁻ com MOI 10. (F) BMDMs de camundongos C57BL/6, *Casp1/Casp11*^{tg} e *Casp11*^{-/-} foram infectados com *flii*⁻ e *flaA*⁻ com MOI 10 (D-F) Após 8 horas de infecção a ativação de caspase-8 foi avaliada utilizando um substrato luminescente de caspase-8 (Caspase-8 Glo Assay). Dados demonstram a média ± o desvio padrão das triplicatas. *, *P* < 0.05, teste *t* não paramétrico. NI, não infectado. URL, unidades relativas de luminescência. Os dados apresentam um experimento representativo de dois (A-C e E) e cinco (D) experimentos com resultados similares. Adaptado de: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

Figura 20: Inibição ou deleção de gasdermina-D permite a ativação de caspase-8 em células contendo caspase-1. Macrófagos derivados de medula óssea (BMDMs) de C57BL/6 e *Casp1/11^{-/-}* foram transduzidos com retrovírus expressando duas sequências independentes de shRNA para o silenciamento de GSDMD (Seq1 e Seq2) e uma sequência não relacionada (NT- *non-target*). (A) O silenciamento foi confirmado por análises de qPCR em Tempo Real. Quantificação da expressão de GSDMD por RT-qPCR em macrófagos silenciados para duas sequências de shRNA para GSDMD e sequência não relacionada. O gene *Gapdh* foi utilizado como controle para normalização dos níveis de expressão. Os números acima das barras indicam a porcentagem de silenciamento comparado com a sequência NT pela equação: $\{1 - (2^{-\Delta\Delta Ct}) \times 100\}$. (B) BMDMs C57BL/6 e *Casp1/11^{-/-}* transduzidos foram infectados com *L. pneumophila* WT Lp, *fliI*⁻ e *flaA*⁻ com MOI 10. Após 8 horas de infecção a ativação de caspase-8 foi avaliada pela adição um substrato luminescente de caspase-8 (*Caspase-8 Glo Assay*) (C) BMDMs de camundongos C57BL/6, *Casp1/11^{-/-}*, *Casp11^{-/-}*, *Gsdmd^{-/-}* e *Asc/Casp1/11^{-/-}* foram infectados com WT Lp, *fliI*⁻ e *flaA*⁻ com MOI 10. Após 8 horas de infecção a ativação de caspase-8 foi avaliada pela adição um substrato luminescente de caspase-8 (*Caspase-8 Glo Assay*). Dados demonstram a média $\pm$ o desvio padrão das triplicatas. *, $P < 0.05$ comparado com as células infectadas com *flaA*⁻, teste *t* não paramétrico. NI, não infectado. NT, não relacionado (*non-target*). URL, unidades relativas de luminescência. Os dados apresentam um experimento representativo de dois experimentos com resultados similares. Adaptado de: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017. Figura C ainda não publicada.

Figura 21: Gasdermina-D é importante para a formação de poro em BMDMs C57BL/6, mas não em *Casp1/11*^{-/-}. Macrófagos derivados de medula óssea (BMDMs) de C57BL/6 e *Casp1/11*^{-/-} foram transduzidos com retrovírus expressando duas sequências independentes de shRNA para o silenciamento de GSDMD (Seq1 e Seq2) e uma sequência não relacionada (NT- *non-target*). Os macrófagos transduzidos foram infectados com *L. pneumophila* WT Lp, *flii*⁻ ou *flaA*⁻ (MOI 10). A formação de poro foi avaliada pela incorporação de iodeto de propídio (PI) em tempo real com o auxílio de um fluorímetro de placa (a cada 5 minutos). As porcentagens de URF foram estimadas em relação às células lisadas com Triton X-100. Dados demonstram a média $\pm$ o desvio padrão das triplicatas. NI, não infectado. URF, unidades relativas de fluorescência. Os dados apresentam um experimento representativo de dois experimentos com resultados similares. De: MASCARENHAS, Danielle PA et al. *PLoS pathogens*, 2017.

Discussão

5 – DISCUSSÃO

L. pneumophila é uma bactéria intracelular Gram-negativa flagelada, causadora da doença dos legionários e febre de Pontiac (Mcdade *et al.*, 1977). Durante sua história evolutiva, esta bactéria seguiu parasitando protozoários intracelulares como amebas de vida livre, entretanto os humanos são considerados seus hospedeiros acidentais (Rowbotham, 1980; Fliermans *et al.*, 1981). Consequentemente, quando é fagocitada por macrófagos alveolares, *L. pneumophila* é reconhecida por uma série de PRRs, como os TLRs e os NLRs, sendo um excelente modelo de estudo para a ativação das vias do sistema imune inato (Massis e Zamboni, 2011; Mascarenhas e Zamboni, 2017). Diversos TLRs e NLRs são ativados em resposta à *L. pneumophila* (Massis e Zamboni, 2011). A ativação de certos NLRs leva à formação de um complexo proteico citoplasmático denominado de inflamassoma (Ting *et al.*, 2008; Guo *et al.*, 2015). A formação desse complexo, com a proteína adaptadora ASC, leva à ativação e clivagem de caspase-1 com consequente morte celular por piroptose e secreção de citocinas pró-inflamatórias como IL-18 e IL-1 β (Martinon *et al.*, 2002).

O inflamassoma mais importante para o controle da replicação de *L. pneumophila* em células de camundongos é o de Naip5/NLRC4/Caspase-1 (Yamamoto *et al.*, 1988; Zamboni *et al.*, 2006). Nesse inflamassoma o receptor Naip5 reconhece a porção C-terminal da flagelina pelo seu domínio NBD (Zamboni *et al.*, 2006; Lightfield *et al.*, 2008; Tenthorey *et al.*, 2014). Após o reconhecimento de flagelina, Naip5 se oligomeriza e recruta NLRC4, levando à ativação de caspase-1 (Lightfield *et al.*, 2008; Kofoed e Vance, 2011; Zhao *et al.*, 2011). A ativação dessa protease leva à morte celular por piroptose e controle da replicação da bactéria (Silveira e Zamboni, 2010; Pereira, Marques, *et al.*, 2011).

Trabalhos reportaram respostas induzidas por NLRC4 de maneira independente de caspase-1, como controle da replicação e indução de morte celular (Gao e Abu Kwaik, 1999; Coers *et al.*, 2007; Akhter *et al.*, 2009; Fortier *et al.*, 2009; Abdelaziz, Gavrilin, Akhter, Caution, Kotrange, Khweek, Abdulrahman, Grandhi, *et al.*, 2011; Abdelaziz, Gavrilin, Akhter, Caution, Kotrange, Khweek, Abdulrahman, Hassan, *et al.*, 2011; Pereira, Morgantetti, *et al.*, 2011; Zhu *et al.*, 2013). Pereira, Morgantetti, *et al.* (2011) demonstraram que além do inflamassoma de Naip5/NLRC4/Caspase-1, existe uma outra via independente de caspase-1/11 e dependente de NLRC4 em

resposta à flagelina de *Legionella* spp. Neste trabalho foi verificado que camundongos deficientes para *Nlrc4* são mais susceptíveis do que camundongos deficientes para *Casp1/11* (Pereira, Morgantetti, *et al.*, 2011). Esta foi uma observação muito importante, já que por muito tempo foi considerado que NLRC4 e caspase-1 faziam parte de uma via única de controle da replicação de bactérias flageladas.

Foi demonstrada a participação de caspases apoptóticas, como caspase-3, caspase-7 e caspase-8, nas funções induzidas pelos inflamassomas, induzindo morte celular e secreção de citocinas pró-inflamatórias (Masumoto *et al.*, 2003; Hasegawa *et al.*, 2005; Akhter *et al.*, 2009; Kumar *et al.*, 2010; Abdelaziz, Gavrilin, Akhter, Caution, Kotrange, Khweek, Abdulrahman, Hassan, *et al.*, 2011; Motani *et al.*, 2011; Pierini *et al.*, 2012; Man *et al.*, 2013; Sagulenko *et al.*, 2013; Man *et al.*, 2014; Vajjhala *et al.*, 2015; Kawamoto *et al.*, 2017; Rauch *et al.*, 2017). A partir da hipótese de que alguma dessas caspases poderia fazer parte do inflamassoma independente de caspase-1/11, nós realizamos ensaios de colocalização entre NLRC4-GFP e essas caspases. A partir desses experimentos nós determinamos que esse novo inflamassoma é composto por NLRC4/ASC/Caspase-8 em resposta ao reconhecimento de flagelina de *L. pneumophila* (Fig. 7 e 8). Nós especulamos que essa via também é ativada em resposta à outras bactérias flageladas, já que foi reportado a interação de NLRC4 e caspase-8 em outras espécies como *S. Typhimurium* e *Listeria monocytogenes* (Man *et al.*, 2013; Sagulenko *et al.*, 2013; Uchiyama *et al.*, 2013; Rauch *et al.*, 2017).

Além do recrutamento para o puncta de NLRC4 e ASC em resposta à *L. pneumophila* flagelada, demonstramos que caspase-8 é ativada de maneira independente de caspase-1/11 e dependente de ASC (Fig. 9). Nós observamos a ativação de caspase-8 pela detecção de um substrato luminescente desta protease e também pela sua clivagem em p18 por Western Blot. A utilização destas duas técnicas é importante uma vez que foi demonstrado que, assim como a caspase-1, a caspase-8 também apresenta uma forma ativa e não clivada importante nas respostas contra patógenos (Philip *et al.*, 2016).

Além de caspase-1, trabalhos demonstraram a participação de caspase-3 e caspase-7 em respostas induzidas por *L. pneumophila* (Gao e Abu Kwaik, 1999; Akhter *et al.*, 2009; Abdelaziz, Gavrilin, Akhter, Caution, Kotrange, Khweek, Abdulrahman, Hassan, *et al.*, 2011; Zhu *et al.*, 2013). Entretanto, nos nossos

experimentos não observamos nenhuma colocalização entre os punctas de NLRC4-GFP, caspase-3 e caspase-7, sugerindo que essas caspases não interagem diretamente com o inflamassoma de NLRC4/ASC. De maneira muito interessante dados preliminares realizados após este trabalho, sugerem que caspase-7 também faz parte deste complexo. Nós especulamos que, como ocorre nos mecanismos de apoptose, na indução de morte celular pelo inflamassoma de NLRC4/ASC/Caspase-8, a caspase-8 também atua como uma caspase iniciadora levando à ativação de caspase-7, uma caspase efetora (Shi, 2002; Li e Yuan, 2008; Creagh, 2014). Esses dados não contradizem o que demonstramos nos experimentos de colocalização, já que utilizamos Z-VAD para a inibição da ativação das caspases e melhor visualização dos punctas.

Por meio da utilização de shRNA para o silenciamento de caspase-8 e o camundongo *Asc/Casp1/11^{-/-}*, nós demonstramos que o inflamassoma NLRC4/ASC/Caspase-8 é essencial para o controle da replicação de *Legionella spp.* flagelada in vitro e in vivo (Fig. 10-12). Esses dados são muito relevantes, uma vez que nunca foi demonstrada uma resposta relacionada à caspase-8 e essa bactéria. É importante dar destaque para a estratégia que utilizamos de shRNA para o silenciamento de caspase-8, visto que camundongos deficientes para caspase-8 não sobrevivem pela indução da via de necroptose, sendo necessária a deleção também de Ripk3 nesses animais (Varfolomeev *et al.*, 1998; Kaiser *et al.*, 2011). Além disso, caspase-8 está envolvida em diversas vias e processos celulares, dentre eles a indução das vias de apoptose, o *priming* pelos TLRs, e a produção de citocinas como TNF e IL-12 (Li e Yuan, 2008; Parrish *et al.*, 2013; Creagh, 2014; Mace *et al.*, 2014; Antonopoulos *et al.*, 2015; Kang *et al.*, 2015; Philip *et al.*, 2016). Desta maneira, a utilização dos camundongos *Asc/Casp1/11^{-/-}* é mais adequada para a avaliação do controle da replicação neste inflamassoma dependente de Naip5/NLRC4.

Trabalhos primordiais no estudo de *L. pneumophila* identificaram que Naip5 é essencial para controlar a replicação desta bactéria (Yamamoto *et al.*, 1988; Diez *et al.*, 2000; Diez *et al.*, 2003; Wright *et al.*, 2003). Além da via de reconhecimento de flagelina com Naip5 e Naip6, NLRC4 também interage com Naip1 e Naip2 quando ocorre o reconhecimento de proteínas *needle* e *rod* (respectivamente) dos sistemas de secreção do Tipo III de bactérias (Kofoed e Vance, 2011; Rayamajhi *et al.*, 2013; Rauch *et al.*, 2016). Entretanto, no controle da replicação de *L. pneumophila*, o

receptor Naip5 parece ser o único importante dentre os receptores do tipo Naip (Rauch *et al.*, 2016; Zhao *et al.*, 2016). Por meio do silenciamento de Naip5 por shRNA nós observamos que Naip5 é essencial no controle da replicação de *L. pneumophila* também na via independente de caspase-1/11. Nós detectamos uma diminuição na ativação de caspase-8 e formação de poros em macrófagos silenciados para Naip5, confirmando que o reconhecimento de flagelina por esse receptor é essencial para a ativação do inflamassoma de NLRC4/ASC/Caspase-8 na ausência de caspase-1/11.

Diversos trabalhos sugerem que a morte celular por piroptose pode resultar no controle bacteriano (Miao *et al.*, 2010; Larock e Cookson, 2013). Foi proposto recentemente que a formação de PITs (*Pore Intracellular Traps*) é importante para o controle da infecção de bactérias intracelulares (Jorgensen, Zhang, *et al.*, 2016). Os PITs são formados de restos de células que passaram por piroptose, onde as bactérias ficam presas, impedindo que infectem novas células. Foi demonstrado que as bactérias que passaram por essas células em piroptose, apresentam uma menor capacidade de infectar novas células, são mais susceptíveis à ROS e antibióticos. Nossos dados sugerem que a flagelina de *Legionella* spp. induz morte celular tanto pela via dependente quanto independente de caspase-1, pela ativação de caspase-8. Tanto as vias dependentes de caspase-1 ou caspase-8 levam ao controle da replicação de *L. pneumophila*. Desta maneira nós especulamos que a formação dos PITs é responsável pelo controle da replicação de *Legionella* spp. flagelada de maneira dependente de caspase-1 e caspase-8.

Foi demonstrado que a piroptose leva à um extravasamento do conteúdo citoplasmático, com liberação de DAMPs, citocinas pró-inflamatórias e mediadores lipídicos recrutando novas células para o sítio de infecção (Jorgensen, Lopez, *et al.*, 2016). Um trabalho publicado pelo nosso grupo em 2014, demonstra que existe uma fase de replicação inicial que pode ser controlada pelo inflamassoma de NLRC4 e uma fase da infecção mais tardia que é controlada pelo recrutamento de células, levando ao “*clearance*” das bactérias (Mascarenhas *et al.*, 2015). Desta maneira, nós sugerimos que a ativação dos inflamassomas, como o de NLRC4/ASC/Caspase-8, induz piroptose com formação dos PITs, esse mecanismo controla a replicação inicial das bactérias. O recrutamento de novas células para o sítio de infecção,

principalmente neutrófilos leva a um controle da infecção dessas bactérias ou *clearance*.

Um trabalho utilizando mutantes de NLRC4 para a região LRR, identificou uma morte celular induzida por NLRC4 independente de caspase-1 e dependente de caspase-8 (Kumar *et al.*, 2010). Neste trabalho os autores identificaram que a proteína Sug1, componente do proteassoma 26S, se liga à NLRC4 levando a sua ubiquitinação e com isso ao recrutamento e ativação de caspase-8. Desta maneira, é muito possível que Sug1 também faça parte do inflamassoma de NLRC4/ASC/Caspase-8 colaborando na indução de morte celular (Raghawan *et al.*, 2017).

Man *et al.*, 2013 observaram a colocalização de ASC e caspase-8 em resposta à infecção por *S. Typhimurium* (Man *et al.*, 2013). Contudo, diferente dos nossos achados, a ativação de caspase-8 induz somente a secreção de IL-1 β e não a indução de morte celular em resposta à infecção por *S. Typhimurium*. Essa diferença de fenótipos pode ser explicada, já que os autores do trabalho utilizaram apenas células de camundongos C57BL/6. Provavelmente a diminuição que observaram nas células *Casp8Ripk3*^{-/-} na indução de IL-1 β é referente à ativação do inflamassoma de NLRP3, já que é bem descrito que caspase-8 é essencial para o priming e ativação deste inflamassoma (Gurung *et al.*, 2014; Antonopoulos *et al.*, 2015; Kang *et al.*, 2015). No presente trabalho utilizamos como estratégia macrófagos de camundongos *Casp1/11*^{-/-} para o estudo desta via independente de caspase-1/11. Esses macrófagos já secretam uma baixa quantidade de IL-1 β em resposta à infecção por *L. pneumophila*, indicando que a via de NLRC4/ASC/Caspase-8 é ineficiente para a secreção desta citocina (Zamboni *et al.*, 2006; Case *et al.*, 2009; Case e Roy, 2011; Kayagaki *et al.*, 2011; Casson *et al.*, 2013; Cerqueira *et al.*, 2015).

AIM2 é caracterizado como um receptor que leva à ativação de caspase-8 em um processo dependente de ASC. Entretanto, nossos dados com AIM2 sugerem que AIM2 não faz parte do inflamassoma composto por NLRC4/ASC/Caspase-8. Observamos que AIM2 não colocaliza com os punctas de NLRC4-GFP e não é importante para a formação dessas estruturas (Fig. 13). Também foi determinado que AIM2 não é importante para o controle da replicação em macrófagos e in vivo em resposta à infecção por *L. pneumophila* flagelada (Fig. 14). É interessante notar, que foram encontrados punctas de AIM2, já que tem sido reportada a ativação do

inflamassoma de AIM2 em resposta à *L. pneumophila* (Ge *et al.*, 2012; Cunha *et al.*, 2017). Entretanto esses punctas não colocalizavam com os de NLRC4-GFP. Este é um dado interessante, pois demonstra que o puncta se forma de maneira específica e não são todas as moléculas ativadas dos inflamassomas em um mesmo puncta. Uma vez que foi reportado que em resposta à infecção por *S. Typhimurium* os inflamassomas de NLRP3 e NLRC4 são recrutados para o mesmo complexo (Man *et al.*, 2014). Em dados preliminares, nós observamos que os punctas de NLRP3 também não colocalizam com os de NLRC4/ASC/Caspase-8.

Além de verificarmos que o inflamassoma independente de caspase-1/11 é composto por NLRC4/ASC/Caspase-8, também determinamos que neste inflamassoma a formação de punctas e ativação de caspase-8 é dependente da proteína adaptadora ASC (Fig. 6B). Dado que vai de acordo com o demonstrado em outros trabalhos de que ASC é essencial para a nucleação dos inflamassomas (Case e Roy, 2011). Um trabalho publicado em 2015 demonstrou que o domínio PYD de ASC interage com o domínio DED da caspase-8 gerando uma estrutura filamentosa muito parecida com o que demonstramos na figura 8C (Vajjhala *et al.*, 2015). A interação entre esses dois domínios é essencial para a nucleação e autoprocessamento da caspase-8. Já a ligação entre ASC e NLRC4 ocorre pelos domínios CARD das duas proteínas (Schroder e Tschopp, 2010). A determinação das funções de ASC no inflamassoma de NLRC4/ASC/Caspase-8 é muito importante para esclarecer a controvérsia por trás do papel de ASC nas funções induzidas por NLRC4.

Trabalhos demonstram que ASC não é importante para o controle da infecção de *L. pneumophila* em células de camundongos (Zamboni *et al.*, 2006; Case *et al.*, 2009). Entretanto, outros trabalhos reportaram que células *Asc*^{-/-} são mais susceptíveis à infecção por essa bactéria (Abdelaziz, Gavrilin, Akhter, Caution, Kotrange, Khweek, Abdulrahman, Grandhi, *et al.*, 2011; Abdelaziz, Gavrilin, Akhter, Caution, Kotrange, Khweek, Abdulrahman, Hassan, *et al.*, 2011). Nós iniciamos o trabalho testando os BMDMs *Asc*^{-/-} e não detectamos nenhuma diferença em relação ao crescimento bacteriano quando comparados aos C57BL/6, confirmando que ASC não é importante para o controle da replicação de *L. pneumophila* em condições normais (Fig. 5). Entretanto, na ausência de caspase-1/11 essa proteína adaptadora se mostrou essencial para o controle da replicação desta bactéria, tanto em

macrófagos quanto in vivo, já que os camundongos *Asc/Casp1/11^{-/-}* foram mais susceptíveis que os *Casp1/11^{-/-}*. Em 2010, um trabalho utilizou formas mutantes de caspase-1 para avaliar as funções de ASC (Broz *et al.*, 2010). Foi observado que existem dois tipos de complexos formados na ativação dos inflamassomas. Um primeiro complexo sem a presença de ASC, que leva apenas à ativação de caspase-1 e não sua clivagem. A caspase-1 ativa e não clivada é capaz de induzir a formação de poros, morte celular, e consequentemente controle da replicação. É importante destacar que esse primeiro complexo não leva à uma eficiente clivagem de caspase-1 e secreção de citocinas. O recrutamento de ASC para o complexo leva à formação dos punctas, e clivagem das caspases por proximidade, gerando assim a secreção de citocinas pró-inflamatórias como IL-1 β . De maneira muito interessante, após a morte celular por piroptose, são liberados DAMPs para meio extracelular, dentre eles os punctas formados, que parecem ativar novas células, causando uma propagação da inflamação e assim formando novos punctas (Franklin *et al.*, 2014; Franklin *et al.*, 2018). Desta maneira, diferente do que ocorre no inflamassoma dependente de caspase-1 onde ASC só é importante para a formação dos punctas e clivagem de citocinas inflamatórias, na via dependente de caspase-8 a proteína adaptadora ASC também é essencial para a formação de poro e controle da replicação de *Legionella* spp.

Nossos dados sugerem que em condições normais, na presença de caspase-1/11, a caspase-8 é recrutada para este puncta contendo NLRC4/ASC/Caspase-1, inflamassoma que é altamente eficiente na secreção de IL-1 β . Entretanto a caspase-8 só é ativada na ausência e/ou inibição de caspase-1 ou GSDMD (Fig. 19 e 20). Um trabalho publicado alguns meses após a publicação do nosso estudo, encontrou dados muito semelhantes (Schneider *et al.*, 2017). Neste trabalho os autores observaram que em resposta à diversos estímulos ocorre a ativação dos inflamassomas de NLRP3, AIM2 e NLRC4, com recrutamento de caspase-1 e caspase-8 para os punctas de ASC. Através da utilização de um mutante enzimaticamente inativo de caspase-1, foi observado que a ativação de caspase-8 só ocorre na ausência de caspase-1 ativa ou de GSDMD. Tudo indica que essa inibição da ativação de caspase-8 é tempo dependente. Isso porque a indução de piroptose pela via de NLRC4/Caspase-1 é muito rápida, começando com menos de uma hora de infecção (Fig. 16B). De acordo com alguns dados preliminares nossos, com 2

horas de infecção a taxa de ativação de caspase-8 ainda é muito baixa em resposta à *L. pneumophila*. Além disso, a adição de glicina antes da infecção por essa bactéria, que inibe a lise da célula por piroptose, parece aumentar a ativação de caspase-8 nas células C57BL/6. Entretanto, os mecanismos pelos quais ocorre essa inibição da ativação de caspase-8 na presença de caspase-1 ou GSDMD ainda precisam ser melhor estudados. Nossos dados indicam que essa via de NLRC4/ASC/Caspase-8 é uma via alternativa de controle da replicação de patógenos quando a via dependente de caspase-1 não pode ser ativada. Essa via alternativa de sinalização abaixo de NLRC4 pode ser muito importante no caso de infecções com vírus ou outros organismos que possam inibir ativação da caspase-1 ou de GSDMD. Inibição que já foi reportada por alguns trabalhos (Callus e Vaux, 2007; Jamieson *et al.*, 2013; Lei *et al.*, 2017).

Além da formação de poro dependente de caspase-1 e caspase-11, nós demonstramos que existe uma formação de poros e morte celular independente de caspase-1/11 com características muito similares as de piroptose. Em contrapartida, diferente do que ocorre nos macrófagos de camundongos C57BL/6, nas células deficientes para *Casp1/11* essa morte celular não é dependente de GSDMD, o que também foi observado em outros estudos (Rauch *et al.*, 2017; Schneider *et al.*, 2017). É possível que essa morte celular induzida por caspase-8 é dependente de outra gasdermina, ou até mesmo outra proteína formadora de poro. São conhecidas até hoje dez gasderminas em camundongos: GSDMA (GSDMA1, GSDMA2, GSDMA3), GSDMC (GSDMC1, GSDMC2, GSDMC3 e GSDMC4), GSDMD, GSDME (também conhecida como dfna5) e GSDMF (também conhecida como dfnb59) (Kovacs e Miao, 2017). A maioria dessas gasderminas já teve seu papel descrito ligado à indução de poro e morte celular (Shi *et al.*, 2015). Inclusive já foi demonstrado que GSDME é clivada por caspase-3, levando a indução de piroptose em resposta à drogas quimioterápicas (Rogers *et al.*, 2017; Wang *et al.*, 2017; Wang *et al.*, 2018). Entretanto, ainda não há relatos acerca da resposta à patógenos e a indução de piroptose por outras gasderminas. Neste contexto, estudos futuros acerca do papel de outras gasderminas na morte celular induzida pelo inflamassoma de NLRC4/ASC/Caspase-8 são de extrema importância.

De forma geral os dados deste trabalho em conjunto com o que foi demonstrado por outros estudos, sugere que em resposta à flagelina de *Legionella*

spp. é formado um primeiro complexo com Naip5/NLRC4 e sem ASC onde ocorre a ativação de caspase-1 (Fig. 22) (Broz *et al.*, 2010). Essa ativação leva à clivagem de GSDMD, com consequente formação de poro e morte celular por piroptose (He *et al.*, 2015; Kayagaki *et al.*, 2015; Shi *et al.*, 2015; Aglietti *et al.*, 2016; Liu *et al.*, 2016; Sborgi *et al.*, 2016; Kovacs e Miao, 2017). A indução de piroptose tem sido ligada ao controle da replicação dessas bactérias, principalmente pela formação de PITs (Jorgensen, Zhang, *et al.*, 2016). Após o recrutamento de ASC, ocorre a formação dos punctas e o autoprocessamento de caspase-1 levando à secreção de citocinas pró-inflamatórias como IL-1 β e IL-18 (Broz *et al.*, 2010). Nossos dados sugerem que em resposta à infecção por espécies de *Legionella* flageladas caspase-8 também é recrutada para o puncta contendo NLRC4/ASC/Caspase-1 (Fig. 22). Em caso de inibição de caspase-1 ou degradação de GSDMD por vírus ou outros microrganismos, a caspase-8 é ativada levando à clivagem de uma outra proteína indutora de poro, possivelmente uma gasdermina que não GSDMD (Callus e Vaux, 2007; Jamieson *et al.*, 2013; Lei *et al.*, 2017). Nós sugerimos que essa indução de morte celular pela via NLRC4/ASC/Caspase-8 induz o controle da replicação de *L. pneumophila* tanto em macrófagos quanto in vivo. Além da ativação do inflamassoma por flagelina também ocorre a ativação do inflamassoma de AIM2 por dupla fita de DNA e de caspase-11 por LPS de *L. pneumophila*. Em ambos os inflamassomas a clivagem de caspase-1, assim como de caspase-11 induz piroptose dependente de GSDMD e com isso um efluxo de potássio que levará à ativação do inflamassoma de NLRP3 (Cunha *et al.*, 2017). É possível especular que assim como ocorre com os inflamassomas de AIM2, caspase-11 e NLRC4/Caspase-1, a morte celular induzida por NLRC4/ASC/Caspase-8 promove o efluxo de potássio que é necessário para a ativação do inflamassoma de NLRP3. Dessa forma, estudos futuros serão necessários para determinar se caspase-8 contribui para ativação de uma via não canônica de ativação de NLRP3.

Figura 22: Modelo proposto. O reconhecimento da porção C-terminal da flagelina por Naip5 induz a sua oligomerização e recrutamento de NLRC4 e caspase-1, formando um primeiro complexo. Caspase-1 é ativada, levando à clivagem de GSDMD e consequente formação de poro, piroptose e controle da replicação de *L. pneumophila* flagelada. O recrutamento de ASC induz a formação dos punctas e clivagem de caspase-1 por autoprocessamento. A clivagem de caspase-1 gera a secreção de citocinas pró-inflamatórias como IL-1β e IL-18. Nosso trabalho demonstrou que caspase-8 é recrutada para esse complexo (circulado em linhas tracejadas) NLRC4/ASC/Caspase-1. Caspase-8 será ativada caso ocorra a inibição da ativação de caspase-1 ou degradação de GSDMD. Nossos dados indicam que a ativação de caspase-8 induz a formação de poro e morte celular de maneira independente de GSDMD, com a possível participação de outra proteína formadora de poro. A ativação do inflamassoma de NLRC4/ASC/Caspase-8 é essencial para o controle da replicação de *L. pneumophila* independente de caspase-1/11. Além dos inflamassomas ativados por flagelina, o inflamassoma de AIM2 é ativado por fita dupla de DNA e o de caspase-11 por LPS de *L. pneumophila* e outras bactérias Gram-negativas. A clivagem de caspase-1 e caspase-11 por esses inflamassomas, assim como o de NLRC4/Caspase-1 leva à piroptose, efluxo de potássio com consequente ativação do inflamassoma de NLRP3.

Sumário

6 – SUMÁRIO

- Um puncta de NLRC4, dependente de ASC e independente de caspase-1/11 é formado em resposta à *L. pneumophila* que expressa flagelina.
- O inflamassoma independente de caspase-1/11 formado em resposta à infecção por *L. pneumophila* contendo flagelina é composto por NLRC4/ASC/Caspase-8.
- ASC é essencial para a ativação de caspase-8 em resposta à infecção por *L. pneumophila*.
- Caspase-8 é importante para o controle da replicação de *Legionella* flagelada em macrófagos por uma via independente de caspase-1/11.
- ASC é importante para o controle da replicação mediado por NLRC4 de *Legionella* flagelada in vivo.
- AIM2 não participa do inflamassoma de NLRC4/ASC/Caspase-8 em resposta à *L. pneumophila*.
- Reconhecimento de flagelina por Naip5 é essencial para os mecanismos efetores do inflamassoma de NLRC4/ASC/Caspase-8.
- *L. pneumophila* expressando flagelina desencadeia a formação de poro e morte celular independente de caspase-1/11 e dependente da via de Naip5/NLRC4/ASC/Caspase-8.
- A via de NLRC4/ASC/Caspase-8 não contribui de forma significativa para a secreção de IL-1 β .
- Em resposta à flagelina caspase-8 é recrutada para o inflamassoma de NLRC4/ASC/Caspase-1.
- Caspase-8 está presente no inflamassoma composto por Naip5/NLRC4, mas só é ativada na ausência de caspase-1 ou GSDMD.
- A morte celular induzida pelo inflamassoma composto por NLRC4/ASC/Caspase-8 ocorre de maneira independente de GSDMD.

Conclusão

7 – CONCLUSÃO

A bactéria intracelular flagelada *L. pneumophila* desencadeia a ativação de um inflamassoma contendo Naip5, NLRC4, ASC, caspase-1 e caspase-8. Entretanto, a ativação de caspase-8 ocorre somente na ausência de caspase-1 ou GSDMD, funcionando como uma via alternativa no controle da replicação de *L. pneumophila* em macrófagos e in vivo.

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Anexos

9 – ANEXO I: Trabalhos de primeira autoria*

*Trabalhos de primeira autoria em ordem de importância para o entendimento deste trabalho.

9.1 – Anexo 1 - Artigo publicado (MASCARENHAS, Danielle PA et al. *Plos Pathogens*, 2017)

“Inhibition of caspase-1 or gasdermin-D enable caspase-8 activation in the Naip5/NLRC4/ASC inflammasome”

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RESEARCH ARTICLE

Inhibition of caspase-1 or gasdermin-D enable caspase-8 activation in the Naip5/NLRC4/ASC inflammasome

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Abstract

Legionella pneumophila is a Gram-negative, flagellated bacterium that survives in phagocytes and causes Legionnaires' disease. Upon infection of mammalian macrophages, cytosolic flagellin triggers the activation of Naip/NLRC4 inflammasome, which culminates in pyroptosis and restriction of bacterial replication. Although NLRC4 and caspase-1 participate in the same inflammasome, *Nlrc4*^{-/-} mice and their macrophages are more permissive to *L. pneumophila* replication compared with *Casp1/11*^{-/-}. This feature supports the existence of a pathway that is NLRC4-dependent and caspase-1/11-independent. Here, we demonstrate that caspase-8 is recruited to the Naip5/NLRC4/ASC inflammasome in response to flagellin-positive bacteria. Accordingly, caspase-8 is activated in *Casp1/11*^{-/-} macrophages in a process dependent on flagellin, Naip5, NLRC4 and ASC. Silencing caspase-8 in *Casp1/11*^{-/-} cells culminated in macrophages that were as susceptible as *Nlrc4*^{-/-} for the restriction of *L. pneumophila* replication. Accordingly, macrophages and mice deficient in *Asc/Casp1/11*^{-/-} were more susceptible than *Casp1/11*^{-/-} and as susceptible as *Nlrc4*^{-/-} for the restriction of infection. Mechanistically, we found that caspase-8 activation triggers gasdermin-D-independent pore formation and cell death. Interestingly, caspase-8 is recruited to the Naip5/NLRC4/ASC inflammasome in wild-type macrophages, but it is only activated when caspase-1 or gasdermin-D is inhibited. Our data suggest that caspase-8 activation in the Naip5/NLRC4/ASC inflammasome enable induction of cell death when caspase-1 or gasdermin-D is suppressed.

Author summary

Legionella pneumophila is the causative agent of Legionnaires' disease, an atypical pneumophila that affects people worldwide. Besides the clinical importance, *L. pneumophila* is a very useful model of pathogenic bacteria for investigation of the interactions of innate immune cells with bacterial pathogens. Studies using *L. pneumophila* demonstrated that Naip5 and NLRC4 activate caspase-1 and this inflammasome is activated by bacterial

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flagellin. However, macrophages and mice deficient in NLRC4 are more susceptible for *L. pneumophila* replication than those deficient in caspase-1, indicating that the flagellin/Naip5/NLRC4 inflammasome triggers responses that are independent on caspase-1. Here, we used *L. pneumophila* to investigate this novel pathway and found that caspase-8 interacts with NLRC4 in a process that is dependent on ASC and independent of caspase-1 and caspase-11. Although caspase-8 is recruited to the Naip5/NLRC4/ASC inflammasome, it is only activated when caspase-1 or gasdermin-D is inhibited. Our data suggest that caspase-8 activation in the Naip5/NLRC4/ASC inflammasome may favor host responses during infections against pathogens that inhibit components of the pyroptotic cell death including caspase-1 and gasdermin-D.

Introduction

Legionella pneumophila is the causative agent of Legionnaires' disease. It was identified for the first time in 1976, after an atypical pneumonia affected the participants of the American Legion Convention in Philadelphia, United States [1]. After isolation, *L. pneumophila* were characterized as Gram-negative, flagellated, intracellular facultative bacteria [2,3]. The species of *Legionella* were found mainly in freshwater and soil environments, including lakes and irrigation systems [4]. Infection of humans occurs upon inhalation of water droplets derived from these environments containing *Legionella* [5]. After inhalation, *L. pneumophila* can subvert the normal vesicle traffic within alveolar macrophages and form LCV (*Legionella*-containing vacuoles), a process that is mediated by the injection of hundreds of bacterial effectors through a type IV secretion system called Dot/Icm [6–9]. During its evolution, *L. pneumophila* were selected based on their replication in protozoa but not in humans, which are accidental hosts [10]. Consequently, *L. pneumophila* can be recognized by many innate immune receptors in mammalian cells, including proteins from the family of the nucleotide-binding domain and leucine-rich repeat-containing proteins (NLRs). These characteristics make *L. pneumophila* an excellent model for the study of innate immunity, including intracellular signaling pathways and inflammasomes.

The major inflammasome that leads to the restriction of *Legionella* replication in macrophages is Naip5/NLRC4. This pathway was discovered in mouse cells upon observations that macrophages from the A/J mouse strain, but not cells from other mice strains, are susceptible to *L. pneumophila* replication [11]. The resistance was mapped to the *Lgn1* locus, which encodes several copies of *Naip* genes, including *Naip5* (*Birc1e*), which is the gene responsible for resistance [12–16]. Successful lines of investigation culminated in the demonstration that Naip5 recognizes bacterial flagellin and interacts with NLRC4 for caspase-1 activation and the restriction of bacterial replication [17–20]. This platform was named the Naip5/NLRC4 inflammasome and triggers pore formation and pyroptosis, which has been considered one of the most important mechanisms for the restriction of intracellular pathogen replication via inflammasomes [21–24]. Host cell death via pyroptosis eliminates intracellular parasite replication and traps intracellular microbes in pyroptotic cells, facilitating microbial destruction by additional phagocytes [23,25–29]. Pyroptosis occurs concomitantly with the secretion of inflammatory cytokines such as IL-1 β and IL-18, a process that requires the adaptor molecule ASC and the formation of NLRC4/ASC puncta [20,30]. ASC also functions as an adaptor protein for other inflammasomes, including AIM2 and NLRP3, which triggers the processing of caspase-1 and caspase-8 [21,31–34]. Of note, Naip5/NLRC4 appears to be the only inflammasome required for the restriction of *L. pneumophila* replication. Macrophages that are deficient

in NLRP3 or AIM2 can efficiently restrict *L. pneumophila* replication [20,21,23,35]. However, the participation of ASC in the resistance of *L. pneumophila* infection is controversial. In murine macrophages, ASC is dispensable for the induction of pyroptosis and the restriction of bacterial replication [20,21]. By contrast, experiments performed with human monocytes indicate that ASC silencing leads to an increase in bacterial replication [36,37]. Thus, the role of ASC in the restriction of *L. pneumophila* replication is still unclear.

We have previously demonstrated the existence of a pathway that is dependent on flagellin and NLRC4 but independent of caspase-1 [38]. Here, we used macrophages and *Casp1/11*^{-/-} mice to systematically assess this pathway. By searching for additional components that operate in the NLRC4 inflammasome independently of caspase-1/11, we found that caspase-8 interacts with NLRC4 in a process that is dependent on ASC. This pathway effectively accounts for resistance to infection in macrophages and in vivo when caspase-1 is absent. In wild-type cells, caspase-8 is recruited to the Naip5/NLRC4/ASC/caspase-1 inflammasome, but is not activated. Caspase-8 activation in this platform only occurs when caspase-1 or gasdermin-D is inhibited, suggesting that this pathway may be important when pyroptosis is inhibited.

Results

Restriction of *L. pneumophila* replication in BMDMs is flagellin/NLRC4-dependent, ASC-independent and partially caspase-1/11-dependent

We have previously demonstrated that activation of the flagellin/NLRC4 inflammasome triggers caspase-1-dependent and independent responses to restrict *Legionella* replication in macrophages and in mouse lungs [38]. However, the caspase-1-independent mechanisms underlying this pathway are unknown. To further characterize this pathway, we performed growth curves using high and very low multiplicity of infections (MOIs) in bone marrow-derived macrophages (BMDMs). Macrophages were infected with wild-type *L. pneumophila* in the JR32 background (WT Lp) and the isogenic mutants *flaA*⁻ and *fliI*⁻. *FliI* is an ATPase that is required for the secretion of flagellin through the flagellar apparatus [39]. Consequently, *fliI*⁻ mutants express flagellin but are non-motile and non-flagellated, making them an appropriate control for *flaA*⁻ mutants for investigations related to the role of flagellin. We found that BMDMs from C57BL/6 and *Asc*^{-/-} mice fully restrict the replication of WT Lp and *fliI*⁻ bacteria at low and high MOIs. In contrast, *Nlrc4*^{-/-} cells are permissive and *Casp1/11*^{-/-} cells are partially restrictive (S1 Fig). Bacterial mutants for flagellin bypass NLRC4-mediated growth restriction and replicate in all macrophages as previously described [17–19,40]. These data support previous reports showing that ASC is not required for the restriction of *L. pneumophila* replication in the presence of caspase-1/11 [20,21]. In addition, these data further support our previous assertion that flagellin triggers an uncharacterized pathway that is dependent on NLRC4 and independent of caspase-1 and caspase-11 [38]. We decided to use BMDMs from *Casp1/11*^{-/-} mice to further investigate this NLRC4-dependent and caspase-1/11-independent pathway.

Flagellated *L. pneumophila* triggers NLRC4 puncta that associate with caspase-8 in a process that is ASC-dependent

The transduction of BMDMs with a retrovirus encoding NLRC4 fused to GFP allows the visualization of NLRC4 puncta in the cytoplasm of macrophages infected with flagellated bacteria [30]. Here, we used this retroviral system to investigate the formation of the NLRC4 inflammasome in the absence of caspase-1/11. BMDMs from *Casp1/11*^{-/-} mice were transduced with

NLRC4-GFP and infected with WT Lp, *fliI*⁻ and *flaA*⁻ at different MOIs and time points. We found that WT Lp and *fliI* triggered the formation of NLRC4 puncta in the absence of caspase-1/11 (Fig 1A). The formation of NLRC4 puncta was influenced by the MOI and significantly diminished in response to *flaA*⁻ bacteria (Fig 1A). Next, we evaluated the requirement of ASC for the formation of NLRC4 puncta in the absence of caspase-1/11. We constructed a mouse that was deficient in ASC and caspase-1/11 and found that whereas the formation of

Fig 1. *Legionella* triggers the formation of NLRC4/caspase-8 puncta in a process that is dependent on ASC and flagellin and independent of caspase-1/11. Bone marrow-derived macrophages (BMDMs) obtained from *Casp1/11*^{-/-} and *Asc/Casp1/11*^{-/-} mice were transduced with retrovirus encoding NLRC4-GFP. Cells were infected with wild-type *L. pneumophila* (WT Lp), motility-deficient mutants expressing flagellin (*fliI*) or with flagellin-deficient mutants (*flaA*⁻) at a MOI of 1, 3 or 10. (A) After 2, 4 and 8 hours of infection, the cells were fixed, and the percentage of transduced cells containing NLRC4-GFP puncta were determined using an epifluorescence microscope. *, *P* < 0.05 compared with BMDMs infected with *flaA*⁻, Student's *t* test. (B-D) Transduced cells were infected with *fliI* (MOI 10) and fixed after 8 hours of infection. (B) The percentages of transduced *Casp1/11*^{-/-} and *Asc/Casp1/11*^{-/-} cells containing NLRC4-GFP puncta were determined. (C) The percentages of NLRC4-GFP puncta that colocalized with ASC and caspase-8 were determined. (D) Representative images of a transduced BMDM infected with *fliI* at a MOI of 10. The cultures were stained with anti-caspase-8 (red) and anti-ASC (purple). The cell nuclei were stained with DAPI (cyan); NLRC4-GFP is shown in green. The images show the colocalization of NLRC4-GFP, ASC and caspase-8 in *Casp1/11*^{-/-} BMDMs. The images were acquired by multiphoton microscopy using a 63x oil immersion objective and analyzed using ImageJ Software. Scale bar, 10μm. Data show the average ± SD of triplicate wells. NI, uninfected. Data are presented for one representative experiment of five (A) and two (B-D) experiments with similar results.

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NLRC4 puncta occurred in the absence of caspase-1/11, ASC was essential for formation of the NLRC4 puncta (Fig 1B). These data are in agreement with previous findings indicating that ASC is critical for the nucleation of several inflammasomes, including AIM2, NLRP3 and NLRC4 [30,32,33,41–46]. Our results confirm that ASC is essential to NLRC4 puncta formation formed in the absence of caspase-1/11. Next, we used this NLRC4-GFP system to identify additional components of the NLRC4 inflammasome that operates in the absence of caspase-1/11. Non-inflammatory caspases have been previously shown to participate in the assembly of inflammasomes and to interact with ASC, including caspase-3, caspase-7 and caspase-8 [32–34,37,44,46–51]. Thus, we transduced *Casp1/11*^{−/−} macrophages with a retrovirus encoding NLRC4-GFP and evaluated the colocalization of NLRC4 with these caspases. In this experiment, we used the pan-caspase inhibitor Z-VAD to block caspase activation and to visualize puncta formation. We did not detect significant numbers of NLRC4 or ASC puncta containing caspase-3 and caspase-7 (S2 Fig). In contrast, caspase-8 and ASC was present in more than 90% of the NLRC4 puncta (Fig 1C and S2 Fig). These data are in agreement with our findings indicating that ASC is required for NLRC4 puncta formation, accordingly, endogenous ASC colocalizes with NLRC4 and caspase-8 in the same puncta (Fig 1D). To evaluate the participation of caspase-8 in the NLRC4 inflammasome, we transduced BMDMs from *Casp1/11*^{−/−} mice with a retrovirus encoding ASC fused to GFP (ASC-GFP) and analyzed ASC puncta colocalization with caspase-8. We found that ASC puncta formed readily after the infection and that this process occurred in response to WT Lp and *fliI* but not *flaA*[−] (Fig 2A). After 8 hours of infection, the formation of ASC puncta was partially dependent on flagellin (Fig 2A). We stained caspase-8 in macrophages transduced with retrovirus encoding ASC-GFP and found that caspase-8 colocalized with ASC puncta in response to infection with flagellated bacteria (Fig 2B). Collectively, these results indicate that flagellin triggers the assembly of an inflammasome composed of NLRC4 and ASC, which colocalizes with caspase-8.

The double-stranded DNA sensor AIM2 is known to recruit ASC to trigger puncta formation in response to infection, leading to caspase-1 activation and IL-1 β and IL-18 release [31,52–55]. The role of AIM2 inflammasome in the recognition of *L. pneumophila* has been demonstrated using *sdhA*[−] deficient bacteria. In the absence of SdhA, bacteria do not maintain vacuole integrity and localize in the macrophage cytoplasm, triggering activation of the AIM2 inflammasome [56,57]. In addition, the AIM2 inflammasome has been shown to trigger caspase-8 activation independently of caspase-1 [33,34,58]. Thus, we investigated whether AIM2 is present in the NLRC4/ASC/caspase-8 inflammasome that is formed in response to flagellin-positive *L. pneumophila*. We stained AIM2 in macrophages transduced with retrovirus encoding NLRC4-GFP and found no AIM2 in the NLRC4 puncta (S3A Fig). Moreover, we generated *Aim2/Casp1/11*^{−/−} mice and found that AIM2 was dispensable for the formation of NLRC4 puncta in response to flagellin-positive bacteria (S3B Fig).

The NLRC4/ASC/caspase-8 inflammasome accounts for flagellin-dependent restriction of *Legionella* replication in macrophages in the absence of caspase-1/11

Our data are consistent with the hypothesis that caspase-8 is a part of the inflammasome composed of NLRC4 and ASC. Thus, we investigated whether caspase-8 is activated during infection. We found that caspase-8 was strongly activated in *Casp1/11*^{−/−} BMDMs in response to *fliI* but not *flaA*[−] bacteria (Fig 3A). In agreement with the requirement of ASC for the assembly of the NLRC4/ASC/caspase-8 inflammasome, we found that caspase-8 activation was abolished in *Asc/Casp1/11*^{−/−} cells (Fig 3A). Caspase-8 activation occurred normally in *Aim2/Casp1/11*^{−/−} cells, indicating that AIM2 was not involved in the activation of caspase-8 through the

flagellin/NLRC4/ASC inflammasome (S4 Fig). We also evaluated caspase-8 activation by western blot analysis by measuring the cleavage of p55 and the production of p18 isoforms. We found that flagellated bacteria triggered caspase-8 activation in *Casp1/11*^{-/-} but not in *Asc/Casp1/11*^{-/-} cells. This phenomenon was evident by the reduction in p55 and increased production of p18 in *Casp1/11*^{-/-} BMDMs infected with *fliI*⁻ but not *flaA*⁻ bacteria (Fig 3B).

Next, we evaluated the participation of caspase-8 in caspase-1/11-independent restriction of *L. pneumophila* replication in macrophages, a process that was dependent on flagellin and NLRC4. Endogenous caspase-8 was silenced in *Casp1/11*^{-/-} BMDMs using two independent retrovirus encoding shRNA to target caspase-8. A non-target sequence was used as a control (NT). By western blotting, we detected reduced caspase-8 expression in *Casp1/11*^{-/-} transduced cells. The shRNA Casp8 Seq1 was more efficient than Seq2 for silencing caspase-8 as determined by western blot (Fig 4A and S5 Fig) and RT-PCR (Fig 4B). Importantly, complete silencing of caspase-8 cannot be achieved because caspase-8 expression is required for macrophage survival [59,60]. Nonetheless, using the described silencing conditions, we did not detect signs of cell death or LDH in the supernatant of the transduced macrophages. To evaluate the efficiency of caspase-8 silencing, we quantified caspase-8-containing puncta formation and caspase-8 activation in macrophages infected with flagellated *L. pneumophila*. We found that the frequency of puncta containing caspase-8 and caspase-8 activation was reduced in caspase-

Fig 2. ASC puncta are induced in response to flagellated bacteria and colocalize with caspase-8. Bone marrow-derived macrophages (BMDMs) from *Casp1/11*^{-/-} mice were transduced with retrovirus encoding ASC-GFP. (A) Cells were infected with wild-type *L. pneumophila* (WT Lp), motility-deficient mutants expressing flagellin (*fliI*⁻) or with flagellin-deficient mutants (*flaA*⁻) at a MOI of 10. After 1 or 8 hours of infection, the cells were fixed, and the number of transduced cells containing ASC-GFP puncta was determined using an epifluorescence microscope. (B) Representative images of a transduced BMDM infected with *fliI*⁻ at a MOI of 10. The cultures were stained with anti-caspase-8 (red), cell nuclei were stained with DAPI (cyan) and ASC-GFP is shown in green. The images show the colocalization between ASC-GFP and caspase-8 in *Casp1/11*^{-/-} BMDMs infected for 8 hours. The images were acquired by multiphoton microscopy with a 63x oil immersion objective and analyzed using ImageJ Software. Scale bar, 10μm. Data show the average ± SD of triplicate wells. *, *P*<0.05, Student's *t* test. NI, uninfected. Data are presented for one representative experiment of three (A) and two (B) experiments with similar results.

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8-silenced cells (Fig 4C and 4D). Next, we evaluated the effect of caspase-8 for the restriction of *L. pneumophila* replication in *Casp1/11*^{-/-} BMDMs. We found that silencing caspase-8 culminated in increased replication of *fliI*⁺ but not *flaA*⁻ bacteria (Fig 4E and 4F). These data indicated that caspase-8 contributed to the restriction of bacterial replication in a process that was dependent on flagellin, supporting the hypothesis that caspase-8 functionally participates in responses that are NLRC4/ASC-dependent and caspase-1/11-independent.

ASC is required for NLRC4/caspase-8-mediated restriction of *Legionella* replication in the absence of caspase-1/11

Our data indicate that caspase-8 is part of the NLRC4/ASC inflammasome and that ASC is essential for the assembly of this inflammasome. Thus, we reasoned that in the absence of

Fig 3. Caspase-8 is activated in response to flagellated bacteria in a process that is ASC-dependent and caspase-1/11-independent. Bone marrow-derived macrophages (BMDMs) from *Casp1/11*^{-/-} and *Asc/Casp1/11*^{-/-} mice were infected with motility-deficient *L. pneumophila* mutants expressing flagellin (*fliI*⁺) or with flagellin-deficient bacteria (*flaA*⁻) at a MOI of 10 for 8 hours. The activity of caspase-8 was measured using the Caspase-8 Glo Assay (A) or by western blot analysis (B). The pro-caspase-8 p55 and the processed form of caspase-8 p18 are indicated. Data show the average ± SD of triplicate wells. *, *P* < 0.05, Student's *t* test. RLU, relative luminescence units; NI, uninfected. Data are presented for one representative experiment of three (A) and two (B) experiments with similar results.

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Fig 4. Caspase-8 is important for NLRC4-mediated restriction of *L. pneumophila* replication independently of caspase-1/11. Bone marrow-derived macrophages (BMDMs) generated from *Casp1/11*^{-/-} mice were transduced with a retrovirus encoding shRNA sequences to target caspase-8 (Seq1, Seq2) and a non-target shRNA sequence (NT). (A) The silencing was confirmed by western blot analysis and Real Time qPCR. (A) Cell lysates were separated by SDS-PAGE, blotted and probed with anti-caspase-8 (pro-caspase-8 p55) and anti-α-actin. (B) Quantification of the *casp8* gene expression by Real Time qPCR. *Actin beta* gene was used as a control for normalization of expression levels. The number above the bars indicates the percentage of silencing compared to the NT sequence. (C-D) The transduced *Casp1/11*^{-/-} BMDMs were infected with motility-deficient *L. pneumophila* mutants expressing flagellin (*flil*) at a MOI of 10. After 8 hours the cells were fixed, the number of caspase-8 puncta was quantified using an epifluorescence microscope (C) and the activity of caspase-8 was measured using the Caspase-8 Glo Assay (D). (E-F) The transduced *Casp1/11*^{-/-} BMDMs were infected with *flil*⁻ (E) and *flaA*⁻ (F) at a MOI of 10 to evaluate bacterial replication. The cells were incubated for 24, 48 and 72 hours for CFU determination. Data show the average ± SD of triplicate wells. * *P* < 0.05, compared with NT. (C-D) Student's *t* test, (E-F) ANOVA. RLU, relative luminescence units; NI, uninfected. Data are presented for one representative experiment of three experiments with similar results.

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ASC, the NLRC4/ASC/caspase-8 inflammasome would not be functional. If this hypothesis is correct, *Asc/Casp1/11*^{-/-} macrophages should be more permissive than *Casp1/11*^{-/-} and as permissive as *Nlrc4*^{-/-}. We infected C57BL/6, *Casp1/11*^{-/-}, *Asc/Casp1/11*^{-/-} and *Nlrc4*^{-/-} BMDMs

with *fliI*⁻ and *flaA*⁻, and evaluated bacterial replication after 24, 48 and 72 hours. Using flagellin-positive bacteria, we confirmed that C57BL/6 BMDMs were restrictive to bacterial growth, *Nlrc4*^{-/-} were permissive and *Casp1/11*^{-/-} were partially restrictive (Fig 5A). Importantly, *Asc*/

Fig 5. ASC is important for NLRC4/caspase-8-mediated restriction of *L. pneumophila* replication independently of caspase-1/11. (A-D) Bone marrow-derived macrophages (BMDMs) from C57BL/6 (open circles), *Nlrc4*^{-/-} (closed squares), *Casp1/11*^{-/-} (closed triangles) and *Asc/Casp1/11*^{-/-} (open triangles) mice were infected with motility-deficient mutants expressing flagellin (*fliI*⁻, A), with flagellin-deficient mutants (*flaA*⁻, B), *L. gratiana* (C) or with *L. micdadei* (D) at a MOI of 10. The cells were incubated for 24, 48 and 72 hours for CFU determination. Data show the average $\pm$ SD of triplicate wells. *, $P < 0.05$ compared with *Casp1/11*^{-/-} BMDMs. #, $P < 0.05$ compared with C57BL/6 BMDMs, ANOVA. (E-I) BMDMs from *Casp1/11*^{-/-} and *Asc/Casp1/11*^{-/-} mice were transduced with a retrovirus encoding shRNA sequences to target caspase-8 (Seq1, Seq2) and a non-target shRNA sequence (NT). (E) Caspase-8 silencing was confirmed by western blot analysis. Cell lysates were separated by SDS-PAGE, blotted and probed with anti-caspase-8 (pro-caspase-8 p55) and anti- α -actin. (F-I) Transduced *Casp1/11*^{-/-} (F, H) and *Asc/Casp1/11*^{-/-} (G, I) BMDMs were infected with *fliI*⁻ (F, G) or *flaA*⁻ (H, I) at a MOI of 10 and incubated for 24, 48 and 72 hours for CFU determination. Data show the average $\pm$ SD of triplicate wells. *, $P < 0.05$ compared with NT shRNA, ANOVA. NT, non-target shRNA. Data are presented for one representative experiment of four (A), two (B-D) and one (F-I) experiments performed with similar results.

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Casp1/11^{-/-} cells were highly permissive and phenocopied the *Nlrc4*^{-/-} cells (Fig 5A). As predicted, flagellin mutants bypassed the NLRC4 and replicated in all macrophages evaluated (Fig 5B). These data further confirmed that *Casp1/11*^{-/-} cells were more restrictive than *Nlrc4*^{-/-} macrophages, possibly due to the presence of the NLRC4/ASC/caspase-8 inflammasome. We also used non-pneumophila species to compare bacterial replication in *Nlrc4*^{-/-} and *Asc/Casp1/11*^{-/-} macrophages. Infections performed with *L. gratiana* and *L. micdadei* indicated that *Asc/Casp1/11*^{-/-} macrophages were as susceptible as *Nlrc4*^{-/-}, whereas *Casp1/11*^{-/-} cells were partially restrictive (Fig 5C and 5D). Similar experiments performed with *Aim2/Casp1/11*^{-/-} macrophages did not support the role of AIM2 in the NLRC4/ASC-dependent growth restriction that occurred in the absence of caspase-1/11 (S6 Fig). These data indicate that flagellated species of *Legionellae* trigger NLRC4 responses that are independent of caspase-1/11 but dependent on ASC. To further confirm the participation of caspase-8 in this NLRC4/ASC inflammasome, we silenced caspase-8 in *Casp1/11*^{-/-} and *Asc/Casp1/11*^{-/-} macrophages. We confirmed the silencing by western blot analysis (Fig 5E and S7 Fig) and found that the reduction in caspase-8 expression impaired the restriction of bacterial replication in *Casp1/11*^{-/-} infected with *fliI* (Fig 5F). Inhibition of caspase-8 expression affected neither the replication of *fliI* in *Asc/Casp1/11*^{-/-} cells (Fig 5G) nor the replication of *flaA* in *Casp1/11*^{-/-} and in *Asc/Casp1/11*^{-/-} cells (Fig 5H and 5I). Collectively, these data are consistent with the hypothesis that flagellin activates a response that is dependent on NLRC4, ASC and caspase-8 and occurs in the absence of caspase-1/11.

Casp8^{-/-} mice are embryonic lethal [59], and we were not able to generate *Casp8/1/11*^{-/-} mice. Since the deletion of ASC impairs the assembly of the NLRC4/ASC/caspase-8 inflammasome and caspase-8 activation, we used *Asc/Casp1/11*^{-/-} mice to assess the role of the NLRC4/ASC/caspase-8 inflammasome in the restriction of *Legionella* replication in vivo. Using flagellin-positive bacteria such as *L. pneumophila*, *L. gratiana* and *L. micdadei*, we demonstrated that *Asc/Casp1/11*^{-/-} mice were highly permissive to bacterial replication and phenocopied infection of *Nlrc4*^{-/-} mice (Fig 6A–6C). *Casp1/11*^{-/-} mice were more permissive than C57BL/6, but they were less permissive than *Nlrc4*^{-/-} mice (Fig 6A–6C). Experiments performed with *fliI* and *flaA* indicated that *Asc/Casp1/11*^{-/-} were more permissive than *Casp1/11*^{-/-} when infected with *fliI* but not *flaA* (Fig 6D). To determine whether AIM2 accounted for the restriction of bacterial replication in the absence of caspase-1/11, we compared infection of *Aim2/Casp1/11*^{-/-} with *Casp1/11*^{-/-}. We found that *Aim2/Casp1/11*^{-/-} and *Casp1/11*^{-/-} supported similar replication levels of *fliI* *L. pneumophila* in the lungs. In contrast, *Asc/Casp1/11*^{-/-} and *Nlrc4*^{-/-} mice were significantly more permissive to bacterial replication (S8 Fig). Collectively, these data indicate that AIM2 is dispensable for the functions of the NLRC4/ASC/caspase-8 inflammasome. This molecular platform is assembled in response to flagellin-positive bacteria and operates to restrict bacterial replication in vitro and in vivo in a process that is independent of both caspase-1 and caspase-11.

Activation of the NLRC4/ASC/caspase-8 inflammasome triggers pore formation and cell death

Activation of caspase-1 inflammasomes induces pyroptosis and contributes to the restriction of infection by flagellated bacteria such as *L. pneumophila*, *Salmonella typhimurium* and *Burkholderia thailandensis* [23]. Accordingly, we have previously demonstrated that *L. pneumophila* trigger pyroptosis in a process mediated by caspase-1 and caspase-11, which are activated in response to flagellin and LPS, respectively [20,24,35,61]. Thus, we investigated whether activation of the NLRC4/ASC/caspase-8 inflammasome could trigger cell death in *Casp1/11*^{-/-} macrophages. We assessed membrane permeabilization fluorometrically in real time via the

Fig 6. ASC is essential for NLRC4-mediated restriction of *L. pneumophila* replication independently of caspase-1/11 in vivo. C57BL/6 (open circles), *Nlrc4*^{-/-} (closed squares), *Casp1/11*^{-/-} (closed triangles) and *Asc/Casp1/11*^{-/-} (open triangles) mice were infected with motility-deficient *L. pneumophila* mutants expressing flagellin (*fliI*, A, D), with flagellin-deficient *L. pneumophila* (*flaA*, D), with *L. gratiana* (B) or with *L. micdadei* (C) at a dose of 10⁵ bacteria/mice. The mice were euthanized at 4, 48 or 72 hours after infection. Dilutions of the lung homogenates were added to charcoal-yeast extract agar plates for colony-forming unit determination. Each dot represents a single animal, and the horizontal lines represent the averages. *, *P*<0.05. #, indicates *P*<0.05 compared with C57BL/6, Mann Whitney test. Data are presented for one representative experiment of five (A), one (B-C) and two (D) experiments performed with similar results.

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uptake of propidium iodide. Macrophages were infected with *fliI* or *flaA*, and pore formation was monitored in real time for 6 hours. C57BL/6 macrophages triggered robust pore formation in response to infection with *fliI* and reduced pore formation in response to *flaA* (Fig 7A–7C). The pore formation observed in C57BL/6 macrophages infected with *flaA* mutants was dependent on caspase-11 [61] and will not be addressed herein. Importantly, despite the absence of both caspase-1 and caspase-11, we detected significant pore formation in *Casp1/11*^{-/-} cells infected with *fliI* (Fig 7B). This response was not detected in *Casp1/11*^{-/-} cells infected with *flaA* or in *Asc/Casp1/11*^{-/-} macrophages infected with *fliI* or *flaA* (Fig 7B and 7C). These data support the hypothesis that NLRC4/ASC/caspase-8 induces pore formation. Experiments

Fig 7. The NLRC4/ASC/caspase-8 inflammasome is important for pore formation and cell death independently of caspase-1/11. (A-D; K-M) Bone marrow-derived macrophages (BMDMs) were generated from C57BL/6, *Casp1/11*^{-/-}, *Aim2/Casp1/11*^{-/-} and *Asc/Casp1/11*^{-/-} mice and infected with motility-deficient *L. pneumophila* mutants expressing flagellin (*flit*) or with flagellin-deficient bacteria (*flaA*) at a MOI of 5. (E-J) BMDMs were transduced with retrovirus encoding shRNA sequences to target caspase-8 (Seq1, Seq2) and a non-target shRNA sequence (NT) and infected with *flit* or *flaA* at a MOI of 5. (A-C; E-J) Pore formation was assessed fluorometrically in real time by the uptake of propidium iodide. RFU (%) represents the percentage of RFU estimated with cells lysed with Triton X-100. (D) LDH release was measured using the CytoTox 96 LDH-release kit. The LDH release (%) represents the percentage of LDH release estimated with cells lysed with Triton X-100. (K-M) BMDMs were treated with 50 μ M of Z-IETD or DMSO for 1 hour and infected with (L) *flit* or (M) *flaA* at a MOI of 5. Pore formation was assessed fluorometrically in real time by the uptake of propidium iodide. Data show the average $\pm$ SD of triplicate wells. *, $P < 0.05$, Student's *t* test. NS, not significant; RFUs, relative fluorescence units; NI, uninfected. Data are presented for one representative experiment of five (A-C), three (E-J) and two (D and K-M) experiments with similar results.

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performed using *Aim2/Casp1/11*^{-/-} macrophages corroborate our previous findings, indicating that AIM2 is not required for the activities of the NLRC4/ASC/caspase-8 inflammasome (Fig 7B and 7C). Pore formation induced in response to caspase-1 and caspase-11 activation

culminates with the induction of macrophage lysis, a process that can be assessed by the presence of LDH in tissue culture supernatants [24,61,62]. Thus, we measured LDH release in the supernatants of macrophages infected with *fliI*⁺ and *flaA*⁺ for 8 hours. By comparing *Casp1/11*^{-/-} and *Asc/Casp1/11*^{-/-} cells, we found that macrophage lysis occurred despite the absence of caspase-1/11 (Fig 7D). Cell death was flagellin-dependent because infections with *fliI*⁺ but not *flaA*⁺ induced LDH release (Fig 7D). The participation of caspase-8 in pore formation and cell death induced in caspase-1/11-deficient macrophages was evident using both MOI 5 (Fig 7) and MOI 10 (S9 Fig). Importantly, cell death was not observed in *Asc/Casp1/11*^{-/-}, a feature that corroborates the pore formation studies and indicates that the NLRC4/ASC/caspase-8 inflammasome triggers pore formation and lysis of infected cells. We also assessed whether the NLRC4/ASC/caspase-8 inflammasome was important for the activation of inflammatory cytokines. We found that whereas C57BL/6 macrophages readily triggered the production of IL-1 β after 24 hours of infection with flagellated bacteria, the *Casp1/11*^{-/-} or *Asc/Casp1/11*^{-/-} deficient cells do not trigger a IL-1 β production (S10A Fig). The production of IL-12p40 by these cells confirmed that all macrophages were primed and could respond to *L. pneumophila* infection (S10B Fig). To evaluate the participation of caspase-8 in cell death induced by the NLRC4/ASC/caspase-8 inflammasome, we silenced endogenous caspase-8 using shRNA. Macrophages that were transduced with retrovirus encoding shRNA did not exhibit pore formation before infection, indicating that transduction itself did not trigger cell death (Fig 7E and 7H). In contrast, pore formation was evident in *Casp1/11*^{-/-} but not in *Asc/Casp1/11*^{-/-} macrophages in response to *fliI*⁺ infection (Fig 7F and 7I). Pore formation induced in *Casp1/11*^{-/-} was diminished in caspase-8-silenced cells (Fig 7F). In support of the role of flagellin for triggering these responses, we did not detect pore formation in cells infected with *flaA*⁺ (Fig 7G and 7J). To further evaluate the participation of caspase-8 in pore formation induced by flagellin, we performed pore formation assays using Z-IETD, a cell permeable peptide that binds irreversibly to the catalytic site of caspase-8 [63–65]. We found that treatment of *Casp1/11*^{-/-} macrophages with DMSO or Z-IETD did not cause pore formation in uninfected cells (Fig 7K). However, Z-IETD treatment reduced the pore formation induced by *fliI*⁺ (Fig 7L) but not by *flaA*⁺ (Fig 7M). Collectively, these data indicate that flagellin-positive bacteria trigger pore formation and cell death-independent of caspase-1/11 via a process that requires ASC and caspase-8.

Naip5 is required for NLRC4/ASC/caspase-8 inflammasome activation in response to flagellated *Legionella*

Our data reveal that the NLRC4/ASC/caspase-8 inflammasome is activated in *Casp1/11*^{-/-} macrophages in response to infection with flagellated *Legionella*. To evaluate if Naip5 is required for activation of this inflammasome, we used shRNA to silence endogenous Naip5. In Naip5 silenced *Casp1/11*^{-/-} macrophages, we detected a reduced activation of caspase-8 in response to WT Lp and *fliI*⁺ bacteria (Fig 8A). Naip5 silencing was confirmed by RT-PCR (Fig 8B). We also tested if Naip5 is important for pore formation induced via caspase-8 in *Casp1/11*^{-/-} macrophages. By evaluating pore formation, we found that Naip5 is important for efficient pore formation in response to WT Lp and *fliI*⁺ bacteria (Fig 8D–8E). As previously reported, no pore formation was detected in response to *flaA*⁺ bacteria (Fig 8F) or in *Asc/Casp1/11*^{-/-} macrophages (Fig 8G–8J). Finally, we tested if Naip5 is important for restriction of *L. pneumophila* replication in *Casp1/11*^{-/-} macrophages. We found that Naip5 is important for restriction of flagellin-positive *L. pneumophila* replication in *Casp1/11*^{-/-} macrophages (Fig 8K). Naip5 silencing did not affect the replication of *flaA*⁺ bacteria in *Casp1/11*^{-/-} macrophages (Fig 8L). As predicted, *Asc/Casp1/11*^{-/-} macrophages were permissive to replication of both *flaA*⁺ and *fliI*⁺ bacteria and Naip5 did not influenced this process (Fig 8M and 8N). Taken together, these

Fig 8. Naip5 is required for the functions of the NLRC4/ASC/Caspase-8 inflammasome independently of caspase-1/11. Bone marrow-derived macrophages (BMDMs) generated from *Casp1/11*^{-/-} and *Asc/Casp1/11*^{-/-} mice were transduced with a retrovirus encoding shRNA sequence to target Naip5 and a non-target shRNA sequence (NT). Transduced BMDMs were infected with wild-type *L. pneumophila* (WT Lp), motility-deficient mutants expressing flagellin (*fliI*⁻) or with flagellin-deficient mutants (*flaA*⁻) at a MOI of 10. (A) 8 hours after infection the activity of caspase-8 was measured using the Caspase-8 Glo Assay. (B) Quantification of the *Naip5* gene expression by Real Time qPCR. *Gapdh* gene was used as a control for normalization of expression levels. The number above the bars indicates the percentage of silencing compared to the NT shRNA (open bars). (C–J) Pore formation was assessed fluorometrically in real time by the uptake of propidium iodide. The RFU (%) represents the percentage of RFU estimated with cells lysed with Triton X-100. (K–N) The cells were infected with *fliI*⁻ or *flaA*⁻ for 24, 48 and 72 hours for CFU determination. Data show the average ± SD of triplicate wells. *, *P* < 0.05, Student's *t* test (A) and ANOVA (K–N). NS, not significant; RFU, relative fluorescence units; NI, uninfected. Data are presented for one representative experiment of two (A–J) and three (K–N) experiments with similar results.

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data indicates that Naip5 participate of the NLRC4/ASC inflammasome that trigger caspase-8 activation in the absence of caspase-1/11.

Caspase-8 is recruited to the NLRC4/ASC/caspase-1 inflammasome but it is only activated when caspase-1 or gasdermin-D is suppressed

In the experiments shown thus far, we used *Casp1/11*^{-/-} macrophage as a tool to assess the caspase-8 effects without the interference of caspase-1. However, because caspase-1 is present in natural conditions, we tested if caspase-8 participates in the NLRC4/ASC inflammasome in

the presence of caspase-1. First, we infected C57BL/6 macrophages with flagellin-positive *L. pneumophila* to assess if endogenous caspase-8 colocalizes with the Naip5/NLRC4/ASC inflammasome. In uninfected conditions, we detected no significant puncta formation (Fig 9A). However, in response to *fliI* bacteria, caspase-8 colocalizes with ASC (Fig 9B) and caspase-1 (Fig 9C). We determined that caspase-8 is present in more than 60% puncta containing caspase-1 (Fig 9C). These data indicates that regardless to the presence of caspase-1, the caspase-8 is recruited to the inflammasome during activation.

Next, we evaluated caspase-8 activation in wild-type macrophages. We found that caspase-8 activation does not occur in C57BL/6 macrophages infected with *L. pneumophila*. As expected caspase-8 is readily activated in *Casp1/11^{-/-}*, but not in *Asc/Casp1/11^{-/-}* macrophages infected with *fliI* bacteria (Fig 9D). To ensure similar genetic background, we intercrossed a F1 progeny of *Casp1/11^{-/-}* x C57BL/6 to obtain F2 littermate controls. Infections in macrophages from littermate control mice indicated that caspase-8 activation occur in *Casp1/11^{-/-}*, but not in *Casp1/11^{+/-}* and *Casp1/11^{+/+}* macrophages (Fig 9E). To further test whether the deficiency in caspase-1 or caspase-11 enable caspase-8 activation, we performed experiments using mice single deficient in caspase-1 or caspase-11 as previously described [35,66]. We found that caspase-11 deficiency alone is not sufficient to enable caspase-8 activation in response to *L. pneumophila* infection (Fig 9F). In contrast, caspase-1-deficient cells expressing caspase-11 as a transgene (*Casp1^{-/-}Casp11^{tg}*) effectively trigger caspase-8 activation in response to *fliI* *L. pneumophila* (Fig 9F). These data indicate that caspase-1 but not caspase-11 is required to prevent caspase-8 activation.

Caspase-1 activation via the NLRC4 inflammasome is known to trigger activation of gasdermin-D (GSDMD) to induce cell death [67,68]. Thus, we tested if inhibition of GSDMD is sufficient to enable caspase-8 activation in the presence of caspase-1. To achieve this, we inhibited endogenous GSDMD using shRNA and found that despite the presence of caspase-1, caspase-8 is robustly activated when GSDMD is inhibited (Fig 9G). RT-PCR was used to confirm the silencing of the two different sequences of shRNA used (Fig 9H). To further confirm the GSDMD silencing, we performed pore formation assay in C57BL/6 macrophages infected with *L. pneumophila*. We found that GSDMD silencing inhibited the caspase-1-mediated pore formation induced by WT Lp and *fliI* *L. pneumophila* (S11A–S11D Fig). However, GSDMD did not participate of pore formation induced via caspase-8 that occurs in *Casp1/11^{-/-}* macrophages (S11E–S11H Fig). Collectively, these data indicates that despite the presence of caspase-1, caspase-8 activation occur in the Naip5/NLRC4/ASC inflammasome when GSDMD is inhibited.

Discussion

The recognition of *Legionella* flagellin by the Naip5/NLRC4 inflammasome in macrophages is a major mechanism for the restriction of bacterial replication in mouse cells [17–20,22,69,70]. It is well accepted in the field that not all NLRC4 functions require caspase-1 [29,38,71,72]. This conclusion is supported by the observation that *Nlrc4^{-/-}* mice (and their macrophages) are significantly more permissive to *L. pneumophila* replication than *Casp1/11^{-/-}* mice [29,38]. Here, we unraveled this caspase-1/11-independent pathway and characterized an inflammasome composed of Naip5, NLRC4, ASC and caspase-8, which operates in the absence of caspase-1 and caspase-11. This inflammasome effectively participates in the mechanisms involved in the restriction of bacterial replication in macrophages and in vivo. Previous biochemistry studies using yeast two-hybrid screening showed that NLRC4 is ubiquitinated by Sug1, a process that facilitates the activation of caspase-8 [51]. Thus, it is possible that Sug1 is also a component of this NLRC4 inflammasome. In addition, previous studies using the *Salmonella*

Fig 9. Caspase-8 colocalizes with the NLRC4/ASC/Caspase-1 inflammasome, but it is only activated if caspase-1 or gasdermin-D is inhibited. (A-C) Bone marrow-derived macrophages (BMDMs) generated from C57BL/6 mice were infected with motility-deficient *L. pneumophila* mutants expressing flagellin (*flii*) at a MOI of 10 for 8 h. The cultures were stained with anti-caspase-1 (green) (A, C) or anti-ASC (green) (B), anti-caspase-8 (red). Cell nuclei were stained with DAPI (cyan). Images were acquired by multiphoton microscopy with a 63x oil immersion objective and analyzed using ImageJ software. The images are the maximal projection of a z project. Scale bars, 10μm. (C) The percentage of the caspase-1 puncta containing caspase-8 was determined using an epifluorescence microscope. (D) BMDMs were generated from C57BL/6, *Casp1*^{11-/-} and *Asc/Casp1*^{11-/-} mice and infected with *flii*⁻ or *flaA*⁻. (E) BMDMs were generated from *Casp1*^{11+/+}, *Casp1*^{11-/-} and *Casp1*^{11-/-} littermate control mice and infected with WT, *flii*⁻ and *flaA*⁻. (F) BMDMs were generated from C57BL/6, *Casp1*^{11-/-} and *Casp11*^{tg} mice and infected with WT, *flii*⁻ and *flaA*⁻. (G-H) BMDMs generated from C57BL/6 and *Casp1*^{11-/-} mice were transduced with a retrovirus encoding shRNA sequences to target Gasdermin D (GSDMD) (Seq1, Seq2) and a non-target shRNA sequence (NT). (G) Transduced cells were infected with WT Lp, *flii*⁻ and *flaA*⁻. (D-G) Cells were infected with an MOI of 10 and after 8 hours the activity of caspase-8 was measured using the Caspase-8 Glo Assay. (H) GSDMD silencing was confirmed by Real Time qPCR. *Gapdh* gene was used as a control for normalization of expression levels. The numbers above the bars indicate the percentage of silencing compared to the NT sequence. Data show the average ± SD of triplicate wells. *, *P* < 0.05, Student's *t* test in relation to *flaA*⁻. RLU, relative luminescence units; NI, non-infected. Data are presented for one representative experiment of two (A-C and E-H) and five (D) experiments with similar results.

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enterica serovar Typhimurium have indicated that both caspase-8 and caspase-1 are recruited to the ASC puncta in response to infection. However, caspase-8 is involved in the synthesis of pro-IL-1 β and is dispensable for *Salmonella*-induced cell death [32]. These data contrast with published data using *L. pneumophila*, which indicate that in the absence of caspase-1/11, no inflammatory cytokines are produced [20,21,25,30,35,66]. Accordingly, our data indicates that this Naip5/NLRC4/ASC/Caspase-8 inflammasome is very inefficient to trigger IL-1 β maturation when caspase-1 is not present.

Importantly, AIM2 is not part of this NLRC4/ASC/caspase-8 inflammasome. AIM2 is well-known to trigger caspase-8 activation via ASC [33,34,58]. Our data unequivocally demonstrate that AIM2 is neither a component of this inflammasome, nor is it required for inflammasome functions. AIM2 did not colocalize with the NLRC4/ASC/caspase-8 puncta, it was dispensable for the activation of caspase-8 in response to flagellated *L. pneumophila* and for the induction of cell death and restriction of *L. pneumophila* replication. The pyrin domain of ASC can bind to the death domain of caspase-8 [46]. Thus, it is possible that the interactions of the ASC pyrin domain with the caspase-8 dead domain are critical for the recruitment of caspase-8 to the complex. Consistent with this hypothesis, our studies unequivocally show that ASC is required for the assembly and function of this NLRC4 inflammasome. Importantly, the characterization of this NLRC4/ASC/caspase-8 inflammasome accounted to clarify a controversy in the field concerning the participation of ASC in the NLRC4 inflammasome. Studies utilizing biochemistry and cells from gene-deficient mice have demonstrated that ASC is dispensable for NLRC4 functions, including pyroptosis and the restriction of *L. pneumophila* replication [20,21,29]. However, ASC is essential for caspase-1 cleavage and the processing of inflammatory cytokines in response to flagellated *L. pneumophila*, a process that is NLRC4-dependent and NLRP3-independent [20,21,25,30]. In addition, ASC participates in the restriction of intracellular replication of *L. pneumophila* under certain circumstances [36,37]. Our studies provide data that help to consolidate these data in a cohesive model. NLRC4 can operate to form an inflammasome in absence of ASC that triggers pore formation and the restriction of bacterial replication [20,21,25,30]. This platform does not form puncta and is ineffective for triggering caspase-1 cleavage and processing inflammatory cytokines. When ASC is present, NLRC4 inflammasome associates with ASC and recruit caspase-1 and caspase-8 to the puncta. This inflammasome is very efficient to cleave caspase-1 and inflammatory cytokines such as IL-1 β . Interestingly, our data indicate that caspase-8 is not activated when caspase-1 is present. However, when caspase-1 is missing or when Gasdermin-D is inhibited, we detected a robust caspase-8 activation. These data suggest that activation of caspase-8 in the Naip5/NLRC4/ASC inflammasome functions as a backup strategy to guarantee cell death when key components of the pyroptotic cell death are inhibited. Interestingly, our data and previously published data indicate that gasdermin-D is dispensable for caspase-8-induced cell death [73]. Therefore, when gasdermin-D is inhibited, caspase-8 engages gasdermin-D-independent cell death. It is possible that caspase-8 induces caspase-3 and caspase-7 to targeted gasdermin-E (also known as DFNA5) to induce pore formation and cell death independent of gasdermin-D [74,75]. This may guarantee appropriate responses to pathogens that inhibit canonical components of pyroptotic cell death such as caspase-1 or gasdermin-D.

Materials and methods

Bacterial culture

The *L. pneumophila* bacteria used were JR32 and isogenic clean deletion mutants for motility (*fliT*) and flagellin (*flaA*) [19,38]. *L. micdadei* (ATCC 33218) and *L. gratiana* (ATCC 49413) were used to generate streptomycin-resistant strains. *RpsL* mutants of *L. micdadei* and *L.*

gratiana were isolated by plating these strains on CYE agar containing 100 µg/ml of streptomycin. All bacteria were grown on buffered charcoal-yeast extract (CYE) agar plates [1% yeast extract, 1% MOPS, 3.3 mM L-cysteine, 0.33 mM Fe(NO₃)₃, 1.5% Bacto agar and 0.2% activated charcoal, pH 6.9] [76].

Macrophages

Bone Marrow derived macrophages (BMDMs) were generated from mice as previously described [77]. Briefly, bone marrow cells were harvested from femurs and differentiated with RPMI 1640 (Gibco) containing 20% fetal bovine serum (FBS—Invitrogen) and 30% L-929 cell-conditioned medium (LCCM), 2 mM L-glutamine (Sigma-Aldrich), 15 mM Hepes (Gibco) and 100 U/ml penicillin-streptomycin (Sigma-Aldrich) at 37°C with 5% CO₂ [77]. BMDMs were seeded at 2 X 10⁵ cells/well in 24-well plates and cultivated in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 5% LCCM, 2 mM L-glutamine and 15 mM Hepes.

In vitro infections and CFU determination

For the in vitro infections, the cultures were infected at a multiplicity of infection of 0.015, 5 or 10 followed by centrifugation for 5 minutes at 300 X g at room temperature and incubation at 37°C in a 5% CO₂ atmosphere. In the colony-forming units (CFU) experiments, cultures infected at a MOI of 10 were washed two times with PBS, and 1 ml of medium was added to each well. For CFU determination, the cultures were lysed in sterile water, and the cell lysates were combined with the cell culture supernatant from the respective wells. Lysates plus supernatants from each well were diluted in water, plated on CYE agar plates, and incubated for 4 days at 37°C for CFU determination as described previously [28,38].

Retroviral transduction and quantification of NLRC4-GFP and ASC-GFP puncta

Murine *Nlrc4* or *Asc* were cloned into the pEGFP (N2) vector (Clontech) using XhoI and BamHI restriction sites as previously described [30]. NLRC4-GFP or ASC-GFP and GFP were cloned into the pMSCV2.2 murine-specific retroviral vector (Clontech). The pCL vector system 51 was used to package the retroviruses in transfected monolayers of Hek Peak cells (ATCC CRL-2828), which were maintained in RPMI with 10% FBS. The supernatant from the Hek Peak cells containing retrovirus was collected three days after transfection, filtered using a 0.45-µm filter and used for BMDM transduction. BMDMs were obtained from *Casp1/11*^{-/-}, *Asc/Casp1/11*^{-/-} and *Aim2/Casp1/11*^{-/-} mice and seeded in differentiation medium. On day 3 of differentiation, the supernatants containing retroviral were added to BMDMs in 20% FBS and 25% LCCM. After differentiation, the BMDMs were seeded at 2 X 10⁵ cells/well in 24-well plates containing 12-mm glass cover slides and cultivated in RPMI 1640 medium supplemented with 10% FBS and 5% LCCM. For the caspase colocalization experiments, the cultures were treated with 20 µM of Z-VAD for 1 hour and infected at a MOI of 1, 3 or 10. After infection, the plates were centrifuged for 5 minutes at 300 X g at room temperature and incubated at 37°C in a 5% CO₂ atmosphere. At 1, 2, 4 and 8 hours after infection, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.05% saponin, stained with DAPI and mounted on glass slides using Prolong Gold Antifade Reagent (Invitrogen). For the colocalization assay, the cells were stained with rat anti-caspase-8 (Enzo—1G12; 1:50), rabbit anti-cleaved caspase-8 (Cell signaling- 8592; 1:800), rabbit anti-cleaved caspase-3 (Cell signaling- 9664; 1:400); rabbit anti-cleaved caspase-7 (Cell signaling-8438; 1:400), anti-ASC (Adipogen- AL177; 1:250), goat anti-ASC (Santa Cruz—sc33958; 1:50), rabbit anti-caspase-1 (Santa Cruz- sc514;

1:500) or anti-AIM2 (Cell signaling- 13095; 1:400), followed by Alexa 594-conjugated goat anti-rabbit secondary Ab (Invitrogen; 1:3000), Alexa 594-conjugated goat anti-rat secondary Ab (Invitrogen; 1:3000) or Alexa 647-conjugated chicken anti-rabbit secondary Ab (Invitrogen; 1:2000) and DAPI and mounted on glass slides using Prolong Gold Antifade Reagent (Invitrogen). The images were processed using LAS AF software (Leica Microsystems) and analyzed under fluorescence using a Leica DMI 4000B inverted microscope with a 100X oil objective. The number of NLRC4-GFP or ASC-GFP puncta in the transduced cells and the colocalization were quantified. Bacteria were not stained. Therefore, the whole cell population was scored. Multiphoton microscopy images were acquired using an LSM 780 Zeiss AxioObserver microscope equipped with a 63X oil immersion objective and analyzed using ImageJ software.

Retroviral silencing of caspase-8, Naip5 and GSDMD

For retroviral silencing of caspase-8, Naip5 and GSDMD (Gasdermin D), Hek Peak cells were transfected with lentiviral vectors encoding a small hairpin RNA (shRNA) targeting caspase-8 [Sigma- Seq1: TRCN0000231279 (Sequence- CCGGTCATCTCACAAGAACTATATTCTCG AGAATATAGTTCTTGTGAGATGATTTTGG); Seq2: TRCN0000231281 (Sequence-CCGG TCCTGACTGGCGTGAAGTATGCTCGAGCATAGTTCACGCCAGTCAGGATTTTGG)], Naip5 [Sigma- TRCN0000114742 (Sequence—CCGGCGCTTGATTATCTTCTGGAAACT CGAGTTTCCAGAAGATAATCAAGCGTTTGG)], GSDMD [Sigma- TRCN0000219619 (Sequence—CCGGGATTGATGAGGAGGAATTAATCTCGAGATTAATTCCTCCTCATC AATCTTTTGG); TRCN0000219620 (Sequence—CCGGCCTAAGGCTGCAGGTAGAATC CTCGAGGATTCTACCTGCAGCCTTAGGTTTTGG)] and a negative control vector that included a non-target shRNA sequence (NT). The plates were treated with polyethylenimine (Sigma-Aldrich) (Corning). Transduced cells were maintained in RPMI 1640 medium supplemented with 10% FBS at 37°C and 5% CO₂. Lentiviruses expressing shRNAs were collected, filtered using a 0.45-μm filter and added to the BMDMs. After selection with puromycin, the resistant cells were seeded at 2 X 10⁵ cells/well in 24-well plates and infected with *fliI* or *flaA* for CFU determination. The caspase-8 silencing efficiency was measured by immunoblotting: 1 X 10⁶ cells were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, and 0.1% SDS) in the presence of a protease inhibitor cocktail (Roche). The lysates were suspended in 4X Laemmli buffer, boiled for 5 minutes, resolved by 15% SDS PAGE and transferred (Semidry Transfer Cell, Bio-Rad) to 0.22-μm nitrocellulose membranes (GE Healthcare). The membranes were blocked in Tris-buffered saline (TBS) with 0.01% Tween-20 and 5% non-fat dry milk for 1 hour. Rat anti-caspase-8 p55 (Enzo- 1G12; 1:500) and anti-rat peroxidase-conjugated antibody (KPL, 1:3000) were diluted in blocking buffer for the incubations (overnight for anti-caspase-8 and 1 hour for the secondary antibody). The ECL luminol reagent (GE Healthcare) was used for antibody detection. For evaluation of caspase-8 silencing by immunoblots, Image J software were used to estimate the ratio of caspase-8 p55 to α-actin.

Caspase-8 activation

To assess caspase-8 activation, BMDMs were infected with *fliI* or *flaA* for 8 hours, and the activity of caspase-8 was measured using the Caspase-8 Glo Assay (Promega) according to manufacturer's recommendations. To evaluate caspase-8 activation by western blot analysis, 1 X 10⁷ cells were infected at a MOI of 10 and lysed 8 hours after infection in RIPA buffer with protease inhibitor, as previously described. The lysates were suspended in 4X Laemmli buffer, boiled for 5 minutes, resolved by 15% SDS PAGE and transferred to 0.22-μm nitrocellulose

membranes. The membranes were blocked in Tris-buffered saline (TBS) with 0.01% Tween-20 and 5% non-fat dry milk or for 1 hour. The mouse anti-caspase-8 (Enzo- 1G12) and anti-rabbit peroxidase-conjugated antibody (KPL; 1:3000) were diluted in blocking buffer for the incubations (overnight for anti-cleaved caspase-8 and 1 hour for the secondary antibody). ECL luminol reagent (GE Healthcare) was used for antibody detection.

Real Time q-PCR

Total RNA was extracted from 2×10^6 macrophages using total RNA isolation kit (illustra RNAspin, GE Healthcare, UK), according to manufacturer's instructions. After extraction, an aliquot of 2 μ l was used to determine the RNA concentration in NanoDrop (Thermo Fisher Scientific) and 1 μ g of the extracted RNA was used for the cDNA conversion using the iScriptTM cDNA Synthesis kit (BIO-RAD) in a thermal cycler. The cDNA (10 ng) was used for the quantification of the Caspase 8 gene expression (TaqMan Assay: Casp 8—Mm01255716_m1) by real-time PCR using TaqMan Fast Advanced Master Mix, according to the manufacturer's instruction (Applied Biosystems). Actin beta (Actb) gene (TaqMan Assay: Actb-Mm00607939_s1) was used as a control for normalization of expression levels. The quantification of GSDMD and Naip5 were performed using 25 ng of cDNA and 10 μ M of each primer, 1X SYBR Green (Applied Biosystems), and was normalized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh). The specificity of the PCR products was assessed by melting curve analysis for all samples. The following primers were used: Gapdh, FWD- AGGTCGGTG TGAACGGATTG, REV-TGTAGACCATGTAGTTGAGGTCA, GSDMD, FWD-TCATGT GTCAACCTGTCAATCAAGGACAT, REV- CATCGACGACATCAGAGACTTTGAAGGA, NAIP5, FWD- GTTGAGATTGGAGAAGACCTCG, REV-CACACGTGAAAGCAACCATGG. The real-time quantitative reaction was performed in the Viia 7 Real-Time PCR System (Applied Biosystems). The results were analyzed using the $2^{-\Delta\Delta CT}$ method and are expressed in relation to the reference group. The percentage of silencing knockdown was estimated using the $[1-(2^{-\Delta\Delta CT}) \times 100]$ equation [78].

Mice and in vivo infections

Mice used in this study were breed and maintained in institutional animal facilities. Mice used were C57BL/6 (Jax 000664), *Nlrp4*^{-/-} [79], *Casp1*¹¹^{-/-} [80], *Asc*^{-/-} [81], *Casp1*¹¹^{-/-} and *Casp1*¹¹^{tg} [66] were. Double-deficient mice were generated by intercrossing a F1 progeny of the parental strains. All mice were matched by sex and age (all were at least 8 weeks old at the time of infection) and were in a C57BL/6 mouse genetic background. For the in vivo experiments, approximately 5–7 mice per group were used, as indicated in the figures. For in vivo infections, the mice were anesthetized with ketamine and xylazine (50 mg/kg and 10 mg/kg, respectively) by intraperitoneal injection followed by intranasal inoculation with 40 μ l of phosphate-buffered saline (PBS) containing 1×10^5 bacteria per mouse. For CFU determination, the lungs were harvested and homogenized in 5 ml of sterile water for 30 seconds using a tissue homogenizer (Power Gen 125; Thermo Scientific). Lung homogenates were diluted in sterile water and plated on CYE agar plates for CFU determination as previously described [28,38].

Pore formation assay

Pore formation in BMDMs was quantified based on the permeability to propidium iodide (PI) in damaged cells as previously described [61]. BMDMs were seeded in a black, clear-bottom 96-well plate (1×10^5 cells/well). Before infection, the medium was replaced with 10% RPMI without phenol red, 0.038 g/ml NaHCO₃, 6 μ l/ml PI and anti-*L. pneumophila* (1:1000). Infected BMDMs were maintained at 37 °C, and PI was excited at 538 nm. The fluorescence

emission was read at 617 nm every 5 minutes using a plate fluorometer (SpectraMax i3x, Molecular Devices). Total pore formation was determined by lysing cells with Triton X-100.

Lactate dehydrogenase release assay and ELISA

BMDMs were seeded in 24-well plates (5×10^5 cells/well). Infections were performed in RPMI1640 medium without phenol red, 15 mM HEPES and 2 g/l NaHCO_3 supplemented with 10% FBS. After 8 hours of infection, the supernatants were collected for analysis of lactate dehydrogenase (LDH) release. Total LDH was determined by lysing the cultures with Triton X-100. LDH was quantified using the CytoTox 96 LDH-release kit (Promega). For cytokine determination, enzyme-linked immunosorbent assay (ELISA) were used. BMDMs were seeded into 24-well plates (5×10^5 cells/well) and infected with WT Lp, *fliI*⁻ and *flaA*⁻ (MOI 10) for 24 hours. BMDMs supernatant was assessed using ELISA kits according to manufacturer's recommendations (BD Biosciences).

Ethics statement

The care of the mice was in compliance with the institutional guidelines on ethics in animal experiments; approved by CETEA (Comissão de Ética em Experimentação Animal da Faculdade de Medicina de Ribeirão Preto, approved protocol number 218/2014). CETEA follow the Brazilian national guidelines recommended by CONCEA (Conselho Nacional de Controle em Experimentação o Animal). For euthanasia, the mice were treated with ketamine and xylazine (50 mg/kg and 10 mg/kg, respectively) by intravenous injection.

Statistical analysis

The data were plotted and analyzed using GraphPad Prism 5.0 software. The statistical significance was calculated using the Student's t-test or analysis of variance (ANOVA). Nonparametric test Mann-Whitney U test were used for analysis of in vivo experiments. Differences were considered statistically significant when *P* was <0.05, as indicated by an asterisk in the figures.

Supporting information

S1 Fig. Restriction of *L. pneumophila* replication is fully dependent on NLRC4 and flagellin, and partially dependent on caspase-1/11. Bone marrow-derived macrophages (BMDMs) from C57BL/6 (open circles), *Nlrc4*^{-/-} (closed squares), *Casp1/11*^{-/-} (closed triangles) and *Asc*^{-/-} (open inverted triangles) mice were infected with *L. pneumophila* for CFU determination. (A, B) Cells were infected with wild-type *L. pneumophila* (WT Lp). (C, D) Cells were infected with motility-deficient mutants expressing flagellin (*fliI*⁻). (E, F) Cells were infected with flagellin-deficient mutants (*flaA*⁻). BMDMs were infected with 3×10^3 (MOI 0.015) or 2×10^5 (MOI 10) bacteria per well and incubated for 24, 48, 72 and 96 hours for CFU determination. Data show the average $\pm$ SD of triplicate wells. *, *P*<0.05 compared with *Casp1/11*^{-/-} BMDMs, ANOVA. Data are presented for one representative experiment of two experiments with similar results. (TIF)

S2 Fig. Caspase-8 but not caspase-3 and caspase-7 colocalize with NLRC4-GFP and ASC-GFP puncta. Bone marrow-derived macrophages (BMDMs) generated from *Casp1/11*^{-/-} mice were transduced with retrovirus encoding NLRC4-GFP (A) or ASC-GFP (B) and infected with motility-deficient *L. pneumophila* mutants expressing flagellin (*fliI*⁻) at a MOI of 10 for 8 h. (A-B) The percentage of colocalization of caspase-3, caspase-7 and caspase-8 with NLRC4-GFP and ASC-GFP was determined using an epifluorescence microscope. (C-E) BMDMs generated from *Casp1/11*^{-/-} mice were infected with *fliI*⁻ at a MOI of 10 for 8 h. The

cultures were fixed and stained with anti-ASC (green), anti-caspase-3 (red) (C), anti-caspase-7 (red) (D), anti-caspase-8 (red) (E). Cell nuclei were stained with DAPI (cyan). Images were acquired by multiphoton microscopy with a 63x oil immersion objective and analyzed using ImageJ software. The images are the maximal projection of a z project. Scale bar, 10µm. Data show the average ± SD of triplicate wells. *, $P < 0.05$, Student's t test. Data are presented for one representative experiment of two experiments with similar results.

(TIF)

S3 Fig. AIM2 does not colocalize and is not required for NLRC4-GFP puncta formation.

Bone marrow-derived macrophages (BMDMs) generated from *Casp1/11^{-/-}* and *Aim2/Casp1/11^{-/-}* mice were transduced with retrovirus encoding NLRC4-GFP and infected with wild-type *L. pneumophila* (WT) or with motility-deficient *L. pneumophila* mutants expressing flagellin (*fliI*) at a MOI of 10 for 8 h. (A) The cultures were stained with anti-AIM2 (red), the cell nuclei were stained with DAPI (cyan) and the NLRC4-GFP puncta is shown in green. The percentage of colocalization of AIM2 with NLRC4-GFP is shown. Images were acquired by multiphoton microscopy with a 63x oil immersion objective and analyzed using ImageJ software. Scale bar, 10µm. (B) Quantification of the number of transduced cells containing NLRC4-GFP in response to WT or *fliI* infection was estimated in *Casp1/11^{-/-}* and *Aim2/Casp1/11^{-/-}* BMDMs. Data show the average ± SD of triplicate wells. NS, not significant, Student's t test. NI, uninfected. Data are presented for one representative experiment of two experiments with similar results.

(TIF)

S4 Fig. AIM2 is not required for caspase-8 activation in response to flagellated *L. pneumophila*.

Bone marrow-derived macrophages (BMDMs) generated from *Casp1/11^{-/-}* and *Aim2/Casp1/11^{-/-}* mice were infected with motility-deficient *L. pneumophila* mutants expressing flagellin (*fliI*) or with flagellin-deficient bacteria (*flaA*) at a MOI of 10 for 8 hours. The activity of caspase-8 was measured using the Caspase-8 Glo Assay. Data show the average ± SD of triplicate wells. *, $P < 0.05$, Student's t test. NI, uninfected. Data are presented for one representative experiment of two experiments with similar results.

(TIF)

S5 Fig. Caspase-8 quantification in the western blot shown in Fig 4A.

Bone marrow-derived macrophages (BMDMs) generated from *Casp1/11^{-/-}* mice were transduced with a retrovirus encoding shRNA sequences to target caspase-8 (Seq1, Seq2) and a non-target shRNA sequence (NT). The silencing was confirmed by western blot analysis (Fig 4A). Cell lysates were separated by SDS-PAGE, blotted and probed with anti-caspase-8 (pro-caspase-8 p55) and anti-α-actin. Immunoblots were analyzed in Image J software and the caspase-8 p55 to α-actin ratio is shown.

(TIF)

S6 Fig. AIM2 is not required for NLRC4-mediated restriction of *L. pneumophila* replication in macrophages.

Bone marrow-derived macrophages (BMDMs) from C57BL/6, *Nlrc4^{-/-}*, *Casp1/11^{-/-}* and *Aim2/Casp1/11^{-/-}* mice were infected with motility-deficient *L. pneumophila* mutants expressing flagellin (*fliI*) at a MOI of 0.015. The cultures were incubated for 24, 48, 72 and 96 hours after infection for CFU determination. Data show the averages ± SD of triplicate wells. *, $P < 0.05$, compared with *Casp1/11^{-/-}* cells. Student's t test. Data are presented for one representative experiment of three experiments with similar results.

(TIF)

S7 Fig. Caspase-8 quantification in the western blot shown in Fig 5E.

Bone marrow-derived macrophages (BMDMs) generated from *Casp1/11^{-/-}* and *Asc/Casp1/11^{-/-}* mice were transduced with a retrovirus encoding shRNA sequences to target caspase-8 (Seq1, Seq2) and a non-target

shRNA sequence (NT). The silencing was confirmed by western blot analysis (Fig 5E). Cell lysates were separated by SDS-PAGE, blotted and probed with anti-caspase-8 (pro-caspase-8 p55) and anti- α -actin. Immunoblots were analyzed in Image J software and the caspase-8 p55 to α -actin ratio is shown.

(TIF)

S8 Fig. AIM2 is not required for NLRC4-mediated restriction of *L. pneumophila* infection in vivo. C57BL/6 (open circles), *Nlr4*^{-/-} (closed squares), *Casp1/11*^{-/-} (closed triangles), *Aim2/Casp1/11*^{-/-} (open diamond) and *Asc/Casp1/11*^{-/-} (closed triangles) mice were infected intranasally with 1x10⁵ motility-deficient *L. pneumophila* mutants expressing flagellin (*fliT*). The mice were euthanatized at 4 and 48 hours after infection. Dilutions of the lung homogenates were added to charcoal-yeast extract agar plates for colony-forming unit determination. Each dot represents a single animal, and the horizontal lines represent averages. *, *P*<0.05, Student's *t* test. NS, not significant.

(TIF)

S9 Fig. The NLRC4/ASC/caspase-8 inflammasome is important for pore formation and cell death independently of caspase-1/11. Bone marrow-derived macrophages (BMDMs) were generated from C57BL/6, *Casp1/11*^{-/-} and *Asc/Casp1/11*^{-/-} mice and infected with motility-deficient *L. pneumophila* mutants expressing flagellin (*fliT*) or with flagellin-deficient bacteria (*flaA*) at a MOI of 10. (A-C) Pore formation was assessed fluorometrically in real time by the uptake of propidium iodide. RFU (%) represents the percentage of RFU estimated with cells lysed with Triton X-100. (D) After 8 hours the LDH release was measured using the CytoTox 96 LDH-release kit. The LDH release (%) represents the percentage of LDH released compared with cells lysed with Triton X-100. Data show the average $\pm$ SD of triplicate wells. *, *P*<0.05, Student's *t* test. NS, not significant; RFU, relative fluorescence units; NI, uninfected. Data are presented for one representative experiment of five (A-C) and two (D) experiments with similar results.

(TIF)

S10 Fig. IL-1 β is not efficiently produced by *Casp1/11*^{-/-} and *Asc/Casp1/11*^{-/-} macrophages. Bone marrow-derived macrophages (BMDMs) were generated from C57BL/6, *Casp1/11*^{-/-} and *Asc/Casp1/11*^{-/-} mice and infected with wild-type *L. pneumophila* (WT Lp), motility-deficient mutants expressing flagellin (*fliT*) or with flagellin-deficient mutants (*flaA*) at a MOI of 10. The production of IL-1 β (A) and IL-12p40 (B) in the tissue culture supernatants was estimated by ELISA at 24 hours after infection. Data show the average $\pm$ SD of triplicate wells. *, *P*<0.05, Student's *t* test. nd, not detected; RFU, relative fluorescence units; NI, uninfected. Data are presented for one representative experiment of two experiments with similar results.

(TIF)

S11 Fig. Gasdermin-D is important for pore formation in C57BL/6 but not in *Casp1/11*^{-/-} macrophages. BMDMs generated from C57BL/6 (A-D) and *Casp1/11*^{-/-} (E-H) mice were transduced with a retrovirus encoding shRNA sequence to target Gasdermin D (GSDMD) (Seq1) and a non-target shRNA sequence (NT). Transduced cells were infected with wild-type *L. pneumophila* (WT Lp) (B and F), motility-deficient mutants expressing flagellin (*fliT*) (C and G) or with flagellin-deficient mutants (*flaA*) (D and H) at a MOI of 10. Pore formation was assessed fluorometrically in real time by the uptake of propidium iodide. The RFU (%) represents the percentage of RFU compared with cells lysed with Triton X-100. Data show the average $\pm$ SD of triplicate wells. RFU, relative fluorescence units; NI, uninfected. Data are presented for one representative experiment of two experiments with similar results.

(TIF)

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Supplementary Fig.1 Mascarenhas et al.

Supplementary Fig.2. Mascarenhas et al.

Supplementary Fig.3. Mascarenhas et al.

Supplementary Fig.4. Mascarenhas et al.

Supplementary Fig.5. Mascarenhas et al.

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Supplementary Fig. 8. Mascarenhas et al.

Supplementary Fig.9 Mascarenhas et al.

Supplementary Fig.10 Mascarenhas et al.

Supplementary Fig.11 Mascarenhas et al.

9.2 – Anexo 2 - Artigo publicado (MASCARENHAS, Danielle PA; ZAMBONI, Dario S. *Journal of Leukocyte Biology*, 2017)

“Inflammasome biology taught by *Legionella pneumophila*”

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Inflammasome biology taught by *Legionella pneumophila*

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ABSTRACT

Inflammasomes are multimeric protein complexes that assemble in the cytosol of many types of cells, including innate immune cells. The inflammasomes can be activated in response to infection or in response to stress signals that induce damage in the host cell membranes. These platforms trigger inflammatory processes, cell death, and the control of microbial replication. Many inflammasomes have been described so far, including NLRP3, NAIP/NLRC4, caspase-11, and AIM2. The ligand for NLRP3 is still unidentified, but the efflux of K⁺ is essential for NLRP3 activation. By contrast, inflammasomes, such as those composed of NAIP/NLRC4, caspase-11, and AIM2, can be activated by bacterial flagellin, LPS, and dsDNA. The knowledge of inflammasome biology has advanced tremendously in the last decade, fostered by the use of model organisms, such as *Legionella pneumophila*. This bacterium evolved, infecting unicellular protozoa in freshwater environments, and the human infection is accidental. Thus, *L. pneumophila* did not evolve sophisticated mechanisms to inhibit mammalian innate immunity. For this reason, it has emerged as a very appropriate model of a pathogenic microbe for the investigation of inflammasome biology. In this review, we highlight the current information regarding the biology of inflammasomes and emphasize the advances achieved using *L. pneumophila*. We also describe the inflammasomes activated in response to *L. pneumophila* infection and discuss the effector mechanisms that operate to clear the infection. *J. Leukoc. Biol.* 101: 000–000; 2017.

INTRODUCTION TO LEGIONELLA

L. pneumophila is an intracellular bacteria that was first identified in 1976 after an outbreak of pneumonia occurred in Philadelphia, PA, USA, among the participants of an American Legion convention [1]. The development of an axenic media favored

the isolation of many species of legionellae [2, 3]. The subsequent investigation characterized *L. pneumophila* as a Gram-negative, flagellated organism that is the causative agent of 2 forms of disease in humans, Legionnaire's disease, and Pontiac fever [4–6]. Legionnaire's disease is a severe form of pneumonia. This condition has an incubation period of 2–10 d, followed by lung damage, and affects mainly immunocompromised individuals [6, 7]. Pontiac fever is the most common clinical manifestation and causes typical symptoms with characteristics of influenza infection, including fever, chills, and headache following a short incubation period [5, 8].

This bacterium is found in freshwater and soil environments, where it infects various species of unicellular protozoa, including several species of free-living amoebae [9]. Currently, >60 species of the genus *Legionella* have been isolated from environmental samples, and approximately half of these have been isolated from human samples at least once [4, 10]. Human infection occurs upon the inhalation of contaminated water droplets or soil aerosols. After inhalation, *Legionella* can infect and multiply in alveolar macrophages. The bacteria inhabit a vacuole, called the LCV, and replicate intracellularly. *Legionella* can replicate by subverting the intracellular traffic of vesicles to impair the fusion of the LCV with endocytic vesicles, including lysosomes (reviewed in ref. [11]). This process is strictly dependent on the T4BSS, called Dot/Icm, which translocates hundreds of effector proteins directly into the cytoplasm of the host cell. These effector proteins are responsible for the development of an intracellular compartment that supports the *Legionella* replication in mammalian macrophages and in amoebae [11–13].

L. pneumophila evolved by replicating within unicellular protozoa in freshwater environments. Thus, in the absence of interaction with mammalian hosts during the evolutionary process, *L. pneumophila* did not encounter selective pressure for the development of mechanisms to inhibit or bypass the PRRs in mammalian cells. For this reason, *L. pneumophila* serves as an excellent model of infection for the assessment of the biology of such mammalian receptors. Indeed, much information

Abbreviations: ^{-/-} = deficient, AIM2 = absent in melanoma 2, ASC = apoptosis-associated speck-like protein containing a caspase activation and recruitment domain, BIR = baculovirus inhibitor of apoptosis repeat, CARD = caspase activation and recruitment domain, *Casp1/7/11* = caspase-1/7/11, DAMP = damage-associated molecular pattern, Dot/Icm = defect in organelle trafficking/intracellular multiplication, GSDMD = gasdermin-D,

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regarding the biology and functions of the intracellular receptors has been revealed in studies using *L. pneumophila*.

INTRODUCTION TO INTRACELLULAR PRRs

PRRs are central molecules of innate immunity that facilitate the recognition of conserved molecules of microbes (called PAMPs). PRRs also recognize stress- and damage-induced molecules (called DAMPs) [14, 15]. A family of intracellular molecules that contains a NBD and LRR has a major role in the recognition of intracellular pathogens. This family of sensors exhibits 3 main characteristic domains: 1) LRR in the C-terminus that regulates the receptor activation; 2) a centrally located NBD that causes oligomerization in response to ATP; and 3) a variable N-terminal domain that is important for signaling and/or the recruitment of additional molecules. Among the N-terminal domains of the NLRs are the CARD, PYD, and BIRs [16–19].

The stimulation of certain NLRs by PAMPs and DAMPs leads to the activation and oligomerization of the sensor protein, which in turn, induces the formation of a cytosolic platform of proteins, called the inflammasome [20, 21]. This complex can facilitate the activation and cleavage of caspase-1 and in some cases, caspase-8. Many inflammasomes have been described (reviewed in ref. [22]), most of which require the presence of an adaptor protein, known as ASC, which contains 2 death domains that are important for the homologous protein–protein interactions: the PYD of ASC binds to the PYD of the NLRPs, whereas the CARD of ASC binds to the CARD of caspases [23]. Therefore, ASC facilitates the nucleation of a multimeric complex, the inflammasome, which contains inflammatory caspases. These complexes can be visualized by light microscopy and are called “puncta” or “specks” [24]. The formation of the inflammasome leads to the autoproteolysis of caspase-1, a process that leads to the cleavage and activation of IL-1 β and IL-18 and to a specific inflammatory form of cell death, called “pyroptosis” [21, 24–28]. Inflammasome activation has been demonstrated to play a critical role in the control of the replication of many pathogens, including the Gram-negative bacteria *L. pneumophila*. As previously mentioned, *L. pneumophila* has become a very useful model of infection for assessment of the biology of such mammalian PRRs. Much information regarding the biology and functions of the intracellular receptors and inflammasomes has been revealed by studies using *L. pneumophila*. Herein, we review the current information related to the biology of inflammasomes that has been revealed using *L. pneumophila*. In addition, we highlight the inflammasomes that are activated in response to

L. pneumophila and discuss the effector mechanisms that operate to control and resolve the infection.

NAIP5/NLRC4 INFLAMMASOME TAUGHT BY LEGIONELLA

Early studies performed in the 1980s using macrophages and *L. pneumophila* launched a successful series of investigations that culminated in the revelation of the biology and functions of the NAIP5/NLRC4 inflammasome (Fig. 1). In 1988, it was reported that macrophages from most strains of mice, with the exception of A/J mice, are highly restrictive to *L. pneumophila* replication [29]. Through classic genetics, the resistance was mapped to an autosomal locus, named *Lgn1* on chromosome 13 [30, 31]. In 2000, it was reported that the genes contained in *Lgn1* are expressed in macrophages. These genes included *Naip1*, *Naip3*, *Naip6*, *Naip5*, and *Naip2*, but only *Naip1*, *Naip2*, *Naip5*, and *Naip6* are functional in C57BL/6 mice [32]. In 2003, 2 important articles defined *Naip5* as the gene within *Lgn1* that is responsible for the macrophage resistance to *L. pneumophila* [33, 34]. The function of *Naip5* was finally demonstrated in 2006 [35]. *Naip5* was reported to sense *L. pneumophila* in a Dot/Icm-dependent manner and trigger caspase-1 activation (Fig. 2). *Naip5* was also shown to interact with NLRC4. Accordingly, macrophages and mice that expressed the defective *Naip5* allele from the A/J strain were permissive for *L. pneumophila* replication. In contrast, the expression of the restrictive C57BL/6 *Naip5* impaired the bacterial replication in macrophages and in mouse lungs [35]. Interestingly, mice in the C57BL/6 genetic background that were genetically deficient in NLRC4 or caspase-1 were susceptible to *L. pneumophila* infection [35]. The development of the *Naip5*^{−/−} mice unequivocally confirmed the important role for *Naip5* in the activation of caspase-1 in response to *L. pneumophila* [36]. After the demonstration that *Naip5* activates caspase-1, 3 independent groups used *L. pneumophila* to demonstrate that flagellin is required for the activation of *Naip5*, NLRC4, and caspase-1 [37–39]. In 2008, it was demonstrated that the same C-terminal region of flagellin is required for the activation of *Naip5* and NLRC4 signaling [36], which supported the hypothesis that 1 of these proteins was the receptor and that the other was required for signaling. The final demonstration that *Naip5* is the flagellin sensor was published in 2011, when 2 independent groups demonstrated that *Naip5* is the flagellin sensor and requires NLRC4 to activate caspase-1 [40, 41]. These articles also reported that although *Naip5* is a sensor of flagellin, *Naip2* senses the rod (PrgJ) proteins from the bacterial T3SS [40, 41]. Furthermore, *Naip1* was reported to be a cytoplasmic receptor of the T3SS needle protein (PrgI) and also to use NLRC4 as an adaptor to activate caspase-1 [42, 43]. Thus, *Naip1*, *Naip2*, and *Naip5* are receptors that require NLRC4 to trigger caspase-1 activation. *L. pneumophila* does not encode a T3SS; therefore, only *Naip5* operates to activate NLRC4 in response to this bacterium (Fig. 3). Nonetheless, *Naip6* has also been shown to function as a flagellin receptor, and thus, both *Naip5* and *Naip6* sense *L. pneumophila* flagellin, with *Naip5* being more effective [40, 41]. The recent development of a mouse deficient in several *Naips* further confirmed that *Naip6* (although it is significantly less efficient than *Naip5*) also accounts for flagellin

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HIN = hematopoietic IFN-inducible nuclear domain, hNAIP = human nucleotide-binding domain leucine-rich repeat-containing protein family of apoptosis inhibitory protein, LCV = *Legionella*-containing vacuole, LRR = leucine-rich repeat, NAIP = nucleotide-binding domain leucine-rich repeat-containing protein family of apoptosis inhibitory protein, NBD = nucleotide-binding and oligomerization domain, NLR = nucleotide-binding domain leucine-rich repeat-containing protein, NLRC4 = nucleotide-binding domain leucine-rich repeat-containing protein family caspase activation and recruitment domain-containing protein 4, NLRP = nucleotide-binding domain leucine-rich repeat-containing protein family pyrin domain-containing protein, PAAMP = pathogen-associated molecular pattern, PRR = pattern recognition receptor, PYD = pyrin domain, T3/4SS = type 3/4 secretion system

Figure 1. Timeline for the *L. pneumophila* investigations that culminated with the discovery of the Naip5/NLRC4 inflammasome. The indicated references are as follows: Yamamoto et al., 1988 [29]; Dietrich et al., 1995 [31]; Beckers et al., 1995 [30]; Diez et al., 2000 [32]; Wright et al., 2003 [34]; Diez et al., 2003 [33]; Zamboni et al., 2006 [35]; Ren et al., 2006 [38]; Molofsky et al., 2006 [39]; Amer et al., 2006 [37]; Lightfield et al., 2008 [36]; Pereira et al., 2011 [53]; Kofoed and Vance, 2011 [40]; Zhao et al., 2011 [41]; Kortmann et al., 2015 [78]; Rauch et al., 2016 [44].

recognition [44, 45]. It is worth mentioning that chimeric Naip2 and Naip5 constructs were produced, and it was determined that the ligand specificity was not mediated by the LRR domain but by a region present in the internal NBD [46]. In addition, it was demonstrated that the NBD is important for the Naip oligomerization and its interactions with the adaptor protein NLRC4 during inflammasome activation [46].

The recognition of intracellular flagellin during the responses to intracellular pathogens has been studied extensively since the initial description of the Naip5/NLRC4 inflammasome. As previously mentioned, the original demonstration that flagellin is recognized by the Naip5/NLRC4 inflammasome emerged in 2006 from the work published by several groups using *L. pneumophila* [37–39]. Many studies using various additional bacteria, such as *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Shigella flexneri*, have confirmed the important roles for Naip5 and NLRC4 in the recognition of bacterial flagellin [39, 47–51]. In addition, the flagellin from nonpneumophila *Legionella* species, including *L. bonzemani*, *L. gratiana*, *L. micdadei*, *L. jordanis*, and *L. rubrilucens*, is also able to trigger this pathway [52–54].

NAIP5/NLRC4 INFLAMMASOME AND THE EFFECTOR MECHANISMS FOR THE CONTROL OF *LEGIONELLA* REPLICATION

The activation of the Naip5/NLRC4 inflammasome triggers a robust, autonomous cellular process that culminates with the suppression of *L. pneumophila* replication in macrophages [29, 35]. The effector mechanisms underlying this Naip5/NLRC4-mediated control of the *L. pneumophila* replication in macrophages are not completely understood. It has been demonstrated that this inflammasome plays an important role in the regulation of the LCV

maturation [37, 55]. On the other hand, work published in 2004 proposed that the mechanisms for resistance are multifactorial and involve the regulation of phagosome maturation, lysis, and cell death in response to infection [56]. Autophagy has also been shown to be important for the control of *L. pneumophila* replication by Naip5/NLRC4 [57, 58]. In this context, it is interesting that some strains of *L. pneumophila* inhibit autophagy through specific Dot/Icm effector proteins [59, 60]. Another hypothesis to explain the mechanism underlying the Naip5/NLRC4-mediated suppression of the growth emerged from the observation that *L. pneumophila* leads to an inflammatory cell death. Pyroptosis is defined as an inflammatory form of cell death that is characterized by the formation of pores in the membrane, swelling, and osmotic lysis that results in the release of inflammatory cytokines, such as IL-1 β and IL-18 [25, 26, 61]. The mechanism underlying the inflammasome-mediated control of infection by pyroptosis involves the death of the host cells, followed by phagocytosis by neutrophils, in a process that involves reactive oxygen species [62]. Very likely, the effector mechanisms that are involved in the control of infection are dependent on the cytokine production induced by PRRs and the recruitment of neutrophils to the infection sites [63–66].

ADDITIONAL FUNCTIONS OF NAIP5/NLRC4 IN RESPONSE TO *L. PNEUMOPHILA*

Although caspase-1 activation is a major consequence of Naip5/NLRC4 activation, caspase-1-independent responses have also been described [67–71]. Pereira et al. [53] demonstrated that activation of the Naip5/NLRC4 inflammasome triggers responses that are independent of caspase-1/11. This explains why *Nlrp4*^{-/-} mice are more susceptible than *Casp1*/11^{-/-} mice to

Figure 2. Inflammasomes activated in response to *L. pneumophila*. Various inflammasomes are activated in response to *L. pneumophila*. (Upper left) NAIP/NLRC4 is activated by cytosolic flagellin. Murine Naip5 and Naip6 or hNAIP sense flagellin and recruit NLRC4 to activate caspase-1. This process leads to pyroptosis, the cleavage of caspase-1, the production of IL-1 β , and the restriction of *L. pneumophila* in macrophages. (Upper right) *L. pneumophila* LPS binds and activates caspase-11. The active caspase-11 triggers pore formation and partially accounts for the IL-1 α secretion. The pore formation induced by caspase-11 facilitates the efflux of K⁺ and the noncanonical activation of NLRP3. (Lower left) The AIM2 inflammasome is activated in response to dsDNA from *L. pneumophila*. AIM2 binds ASC to activate caspase-1. This process leads to pyroptosis and IL-1 β secretion. (Lower right) The canonical NLRP3 inflammasome requires a pore-forming agent or a molecule that damages the host cell membranes (such as a crystal) to trigger K⁺ efflux. The reduced concentration of cytosolic K⁺ activates NLRP3, which recruits ASC to activate caspase-1. This process leads to pyroptosis and the induction of inflammatory cytokines, such as IL-1 β and IL-18. During an *L. pneumophila* infection, the NLRP3 inflammasome is activated only via the noncanonical pathway, which requires caspase-11 activation by LPS. This process leads to pore formation and the activation of the NLRP3 inflammasome. The indicated domains are as follows: HIN, PYD, NBD, LRR, BIR, CARD, and Caspase.

the replication of *L. pneumophila* in macrophages and in vivo [53]. The mechanisms that underlie the caspase-1/11-independent suppression of *L. pneumophila* replication via the flagellin/Naip5/Nlrc4 inflammasome are still unknown. In addition, one study reported an important role for caspase-7 in the activation of caspase-1 via the Naip5/NLRC4 inflammasome [67]. Accordingly, the *Casp7*^{-/-} macrophages had a higher bacterial load, which suggested that caspase-7 is involved in suppression of *L. pneumophila* replication [67].

Another question that is still unanswered involves the participation of ASC in the Naip5/NLRC4 inflammasome. Studies of *L. pneumophila* replication in human cells have demonstrated that ASC is important for the control of the bacterial replication [72]. However, this contrasts with studies that used mouse macrophages, which indicated that ASC is not required for some functions of the NLRC4 inflammasome, including induction of pore formation, pyroptosis, and the suppression of *L. pneumophila* replication [35, 52, 53, 73] (Fig. 3). Furthermore, it was demonstrated that ASC is essential for certain NLRC4 functions, including puncta formation, as well as the cleavage and release of caspase-1, IL-1 β , and IL-18 [73, 74]. An elegant study demonstrated that the formation of ASC puncta is critical for caspase-1 autoproteolysis. Moreover, cleaved caspase-1 is responsible for the release of IL-1 β and IL-18. On the other hand, an active but uncleaved form of caspase-1 can induce pore formation and pyroptosis, a process that does not require ASC [75]. Therefore, ASC is important for the recruitment of the additional molecules to the inflammasome in the puncta-like structure, and this process leads to the cleavage and secretion of caspase-1 and inflammatory cytokines, such as IL-1 β and IL-18 [73–75]. In conclusion, the published data are consistent with the hypothesis that ASC can be part of the Naip5/NLRC4 inflammasome that induces caspase-1 cleavage and cytokine

Figure 3. Functional consequences of the inflammasome activation induced by *L. pneumophila*. The recognition of flagellin by the Naip5/NLRC4 inflammasome is a Dot/Icm-dependent process and induces the formation of 2 platforms. One is ASC independent and leads to pore formation and the suppression of bacterial replication. The other is ASC dependent and induces caspase-1 cleavage and the production of inflammatory cytokines. dsDNA from *L. pneumophila* is recognized by AIM2 and recruits ASC to trigger the activation of the AIM2 inflammasome. The *L. pneumophila* LPS is recognized by caspase-11 in a process that induces pore formation and K⁺ efflux and leads to the noncanonical activation of the NLRP3/ASC. Caspase-11 cleaves GSDMD in response to LPS. The N-terminal domain of GSDMD binds to lipids in the inner part of the plasma membrane to promote the formation of pores that are essential for K⁺ efflux and pyroptosis induction.

production (Fig. 3). Nonetheless, the function of Naip5/NLRC4 is independent of ASC with respect to the activation of caspase-1 (without cleavage) to induce pyroptosis and the suppression of *L. pneumophila* replication. These data are very clear in that *Asc*^{-/-} macrophages are fully sufficient to induce pyroptosis and suppression of *L. pneumophila* replication via the flagellin/Naip5/NLRC4 inflammasome [35, 52, 74].

THE hNAIP

Human macrophages, in contrast to the mouse cells, are highly permissive for *L. pneumophila* replication and only express 1 NAIP gene (hNAIP) [33]. Murine Naip5 is the gene responsible for flagellin recognition and the suppression of *L. pneumophila* replication in mouse cells; therefore, it was believed that the homologous hNAIP does not recognize flagellin. This hypothesis was supported by the demonstration that the hNAIP recognizes PrgI, a needle protein from the bacterial T3SS [41–43]. In addition, immortalized monocytic-derived cell lines, such as THP-1 and U937, do not respond to flagellin, which argues that hNAIP is not involved in flagellin recognition. However, previous reports indicated that hNAIP and NLRC4 are involved in the restriction of *L. pneumophila* replication in human cells [76]. Consistent with this view, certain human populations express several copies of hNAIP, and the level of expression inversely correlates with the resistance to *L. pneumophila* [77]. A recently published article clarified this issue and supported the important role of hNAIP in flagellin sensing [78]. This study demonstrated that flagellin detection requires a full-length isoform of hNAIP, which is highly expressed in primary human macrophages but not in immortalized human cell lines, such as THP-1 and U937. Thus, these cell lines are insensitive to flagellin, whereas hNAIP is able to sense flagellin in primary human macrophages [78]. Notably, hNAIP has several annotated transcript variants that encode several NAIP isoforms, and 1 specific transcript encodes an isoform with a high level of identity with murine Naip5 [78]. Thus, although humans encode only a single Naip gene, different isoforms may guarantee the ability to recognize both flagellin and bacterial T3SS effectors.

RESPONSES OF THE NLRP3 INFLAMMASOME TO *LEGIONELLA*

NLRP3 (previously known as *Nalp3/cryopyrin/cold-induced auto-inflammatory syndrome 1*) is a member of the NLRP class of intracellular sensors and is characterized by the PYD in its N-terminal domain. This domain is important for its interaction with ASC, which leads to activation of caspase-1 [79]. The first study that addressed the role of the NLRP3 inflammasome in the recognition of and responses to *L. pneumophila* was published in 2006. Macrophages deficient in ASC were defective in the induction of inflammatory cytokines, such as IL-1 β , but were fully competent for the induction of pyroptosis and the suppression of the *L. pneumophila* replication [35]. In the same year, several groups demonstrated that the NLRP3 inflammasome is activated in response to various compounds, including monosodium urate crystals, bacterial RNA, ATP, toxins that lead to pore formation

(nigericin and maitotoxin), and calcium pyrophosphate dehydrate [80–84]. Surprisingly, although ASC is essential for the inflammasome-mediated induction of inflammatory cytokines [35], NLRP3^{-/-} macrophages produced IL-1 β normally in response to *L. pneumophila* [84]. Further investigation indicated that NLRP3 is indeed unnecessary for caspase-1 activation and the suppression of *L. pneumophila* replication in macrophages and in mouse lungs [73]. However, the NLRP3-independent caspase-1 cleavage in response to *L. pneumophila* occurred in response to only flagellin-positive *L. pneumophila*. Studies comparing wild-type and flagellin-deficient *L. pneumophila* strains demonstrated that NLRP3 is essential for caspase-1 cleavage in the absence of the NLRC4 inflammasome [85]. Activation of the NLRC4 inflammasome by flagellin leads to caspase-1 cleavage and IL-1 β production in a process that is ASC dependent and NLRP3 independent [85]. In contrast, in the absence of flagellin, caspase-1 cleavage and IL-1 β production are dependent on caspase-11 and NLRP3 [64, 85–87]. This process is the so-called noncanonical pathway for the activation of the NLRP3 inflammasome.

CASPASE-11 AND NONCANONICAL ACTIVATION OF THE NLRP3 INFLAMMASOME

In 2011, an elegant study performed by Dixit and colleagues [88] demonstrated that caspase-11 is required for caspase-1 activation in certain conditions. It was demonstrated that caspase-11^{-/-} mice fail to activate caspase-1 and to secrete IL-1 β in response to gram-negative bacteria, but the response to canonical NLRP3 activators, such as ATP and nigericin, is normal. In addition, caspase-11 was demonstrated to trigger responses that are independent of NLRP3, including pore formation, cell death, and the release of IL-1 α [85, 88]. Investigations using *L. pneumophila* demonstrated that caspase-11 is required for the activation of NLRP3 in response to this organism [64, 85]. Caspase-11 activation in response to *L. pneumophila* is flagellin independent; accordingly, the caspase-11 pathway cooperates with the flagellin/NLRC4 pathway to induce caspase-1 cleavage and IL-1 β production [64, 85]. Caspase-11 studies performed using *L. pneumophila* indicated that caspase-11 promoted the fusion of the *L. pneumophila* vacuole with lysosomes by modulating actin polymerization through cofilin [89, 90]. However, the role of caspase-11 in the suppression of *L. pneumophila* replication is still controversial. Experiments performed with caspase-11^{-/-} mice in various mouse genetic backgrounds indicated that caspase-11 is not required for the suppression of *L. pneumophila* replication in macrophages and in vivo [91].

The mechanism by which *L. pneumophila* LPS reaches the host cytoplasm to trigger caspase-11 activation is also unclear. This process is certainly dependent on the bacterial T4BSS, Dot/Icm [64, 85]. One hypothesis is that LPS leaks via the Dot/Icm. In addition, it was reported that guanylate-binding proteins promote vacuolar lysis, which releases the LPS into the macrophage cytoplasm [92, 93]. It is also possible that bacterial outer-membrane vesicles fuse with the host cells and deliver LPS into the cytosol to induce caspase-11 activation [94]. Regardless of

how LPS reaches the cytoplasm, it is clear that murine caspase-11 (and human caspase-4) is activated in response to *L. pneumophila* LPS and facilitates pore formation. This process is important in triggering K⁺ efflux, a feature that is critical to enable NLRP3 activation [64, 85, 93, 95]. Thus, the “noncanonical pathway for the activation of the NLRP3 inflammasome” occurs when activated caspase-11 induces pore formation, enabling K⁺ efflux and NLRP3 activation [96–98].

Until 2015, the mechanism by which caspase-11 triggers pore formation and cell death was unclear, but independent studies using genetic screening identified GSDMD as a mediator for pyroptosis and caspase-1 activation via the noncanonical activation of the NLRP3 inflammasome. These studies demonstrated that caspase-11 leads to GSDMD cleavage, which results in an N-terminal fragment that is responsible for the downstream signaling [99, 100]. Recently, new studies demonstrated that the cleaved N-terminal fragment (p30) of GSDMD is localized in the lipid bilayer, whereas the C-terminal fragment remains intracellular. This N-terminal fragment forms a ring-like structure where Ca²⁺ secretion occurs, suggesting that p30 forms pores in the membranes [101–104]. Whether GSDMD is activated in response to *L. pneumophila* and facilitates pore formation and pyroptosis in response to infection is a matter for future investigation.

THE AIM2 INFLAMMASOME IN RESPONSE TO LEGIONELLA

Four independent groups reported in 2009 that a cytosolic protein containing an HIN/helicase domain oligomerizes upon the interaction with dsDNA in the cytosol of innate immune cells [20, 105–107]. This HIN-containing protein is AIM2, which contains a PYD and HIN. The latter binds dsDNA in a length-dependent manner and exposes the PYD, which binds to the PYD of ASC through a homotypic domain interaction. ASC clustering allows procaspase-1 recruitment via a CARD–CARD interaction. This complex of proteins is the AIM2 inflammasome, which is activated in response to dsDNA (Fig. 2).

A few years before the AIM2 inflammasome was identified, a study performed with *L. pneumophila* reported that a Dot/Icm effector protein, called SdhA, is important in the prevention of macrophage cell death and thus, facilitates bacterial replication [108]. In 2012, SdhA was shown to regulate the vacuole integrity on the basis that infections performed with *sdhA*^{−/−} bacteria lead to vacuole destabilization and disruption with the consequent induction of host cell death [109]. This process was shown to be independent of flagellin and bacterial degradation. Moreover, it was demonstrated that the secreted phospholipase, PlaA, another Dot/Icm effector, regulates vacuole rupture and cell death in the absence of SdhA [109]. Notably, a strain of *L. pneumophila* with a mutation in the *sdhA* gene localizes in the cytosol and triggers multiple responses, including the induction of type I IFN and the activation of caspase-1 [109, 110]. Additional evidence that the effector protein SdhA is critical to the maintenance of vacuolar integrity, bacterial replication, and prevention of the host cell death was provided by subsequent studies that reported that the expression of SdhA inhibits the activation of the AIM2 inflammasome. Ge and colleagues [111] demonstrated that

L. pneumophila DNA is released into the host cytoplasm, and this was increased in the infection with *sdhA*^{−/−} bacteria. Subsequently, the bacterial DNA is recognized by the AIM2 inflammasome, a process that leads to caspase-1 activation, IL-1β release, and the pyroptosis of mouse and human cells [111]. The activation of caspase-1 via the AIM2 inflammasome is independent of type I IFN and TNF-α, in contrast to the activation of AIM2 in response to *Francisella tularensis* [111–113].

Notably, SdhA regulates the vacuolar integrity in various hosts [114], regardless of the presence or absence of the AIM2 inflammasome. This is consistent with the fact that *L. pneumophila* evolved in freshwater environments in which it infected protozoa, and human infection is incidental. We speculate that the evolutionary pressure to maintain SdhA expression is likely related to the essential requirement to regulate vacuolar integrity in the unicellular protozoa, rather than a mechanism that evolved to evade innate immune activation and inflammasomes in higher eukaryotes.

CONCLUDING REMARKS

Over the last decade, *L. pneumophila* has been used very successfully to reveal specific aspects of PRR signaling and inflammasome activation in response to infection. These studies using *L. pneumophila* revealed relevant information regarding the mechanisms of the function of the inflammasomes and also generated important information that contributed to our understanding of the mechanisms used by innate immune cells to recognize and fight infectious diseases, including those caused by *Legionella* species. We envisage that additional future studies will provide exciting information regarding the function of uncharacterized or poorly characterized inflammasomes and their intracellular sensors. In addition, future investigations involving *L. pneumophila* may help to answer open questions regarding the biology of well-studied inflammasomes, including NLRP3, NLRC4, and AIM2.

AUTHORSHIP

D.P.A.M. and D.S.Z. wrote the manuscript and prepared the figures.

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DISCLOSURES

The authors declare no conflicts of interest related to this work.

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KEY WORDS:

caspase-1 · caspase-11 · NLRC4 · NLRP3 · ASC · AIM2

9.3 – Anexo 3 – Manuscrito enviado por convite da revista (MASCARENHAS, Danielle PA; ZAMBONI, Dario S. *Methods in Molecular Biology*/2018)

“Inflammasome activation in *Legionella*-infected macrophages”

1 **Title: Inflammasome Activation in *Legionella*-infected Macrophages**

2

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17 **Keywords: Macrophages, Inflammasome, Caspase-1, Caspase-11, *Legionella***

18 **Word count: 5227**

19 **Figures: 1**

20

21 **Abstract:**

22 *Legionella pneumophila* is a gram-negative bacterium that infects many species of
23 unicellular protozoa in freshwater environments. The human infection is accidental and the
24 bacteria may not evolved strategies to bypass innate immune signaling in mammalian
25 macrophages. Thus, *L. pneumophila* triggers many innate immune pathways including
26 inflammasome activation. The inflammasomes are multimolecular platform assembled in
27 the host cell cytoplasm that leads to activation of inflammatory caspases. Inflammasome
28 activation leads to secretion of inflammatory cytokines, such as IL-1 β and IL-18, and an
29 inflammatory form of cell death called pyroptosis, which initiates with the induction of a
30 pore in the macrophage membranes. In this chapter we provide detailed protocols to
31 evaluate *Legionella*-induced inflammasome activation in macrophages, including real-time
32 pore formation assay, western blotting to detect activation of inflammatory caspases
33 (cleavage and pull-down) and the measurement of inflammatory cytokines.

34

35

36 **1- Introduction**

37 *Legionella pneumophila* has been widely used as a model of intracellular pathogen to
38 assess inflammasome biology (reviewed in [17]). Legionellae evolved infecting various
39 species of unicellular protozoa in freshwater environments and the infection in mammals
40 is accidental [21, 26]. Therefore, the bacteria did not evolved mechanisms to bypass or
41 inhibit activation of mammalian innate immune signaling, a feature that is consistent with
42 the fact that *Legionella* triggers many innate immune pathways (reviewed in [19]). For this
43 reason, *L. pneumophila* emerged as an excellent model for investigating the innate immune
44 pathways in mammalian cells including inflammasome activation.

45

46 Inflammasomes are multimeric protein complexes formed in the host cell cytoplasm in
47 response to specific stimuli. It culminates with activation and cleavage of inflammatory
48 caspases and the release of inflammatory cytokines [16]. In the case of *L. pneumophila*
49 infection, upon the inhalation, the bacteria are phagocytosed by alveolar macrophages and
50 can subvert the normal vesicular trafficking by injecting hundreds of effectors proteins via
51 the Dot/Icm type IV secretion system [2, 3, 13, 24]. Although the Dot/Icm is critical for
52 survival of the bacteria in macrophages, it allows the activation of multiple immune
53 pathways including the inflammasome activation [28].

54

55 The most important inflammasome for restriction of *L. pneumophila* replication is the
56 NAIP5/NLRC4 inflammasome. The NAIP5 receptor can recognize *Legionella* flagellin and

57 induces activation and oligomerization of NLRC4, which leads to caspase-1 activation [1,
58 14, 20, 23, 27, 28]. The activation and cleavage of caspase-1 leads to pore formation and a
59 specific form of cell death called pyroptosis, resulting in the restriction of *Legionella*
60 replication in macrophages [20, 23, 25, 28]. The real time analysis of pore formation by the
61 incorporation of iodide propidium, a non-permeable fluorescent dye, and the western blot
62 of cleaved caspase-1 are easy and fast methods to measure the activation of this
63 inflammasome [4, 6, 18]. Beyond pore formation, the activation of caspase-1 also leads the
64 cleavage and release of IL-1 β and IL-18, which provides additional readouts to assess
65 inflammasome activation in macrophages.

66

67 In addition to NAIP5/NLRC4 inflammasome, others inflammasome pathways are activated
68 in response to *L. pneumophila* and others species of *Legionella*. Caspase-11 is also highly
69 activated by the recognition of *L. pneumophila* LPS (Lipopolysaccharide), a process that
70 leads to pore formation, efflux of K⁺ and the non-canonical pathway for activation of the
71 NLRP3 inflammasome [4, 5, 6]. Furthermore, activation of the AIM2 inflammasome in
72 response to *L. pneumophila* has also been shown [8, 11]. Both the NAIP5/NLRC4, AIM2
73 and caspase-11 activation can be measured by pore formation assay and analysis of caspase
74 cleavage. Using this strategy, it was recently demonstrated that pore formation and
75 pyroptosis are also induced by caspase-8 in response to *L. pneumophila* infection, a process
76 that is flagellin and NAIP5/NLRC4-dependent and caspase-1/11-independent ([18, 22] and
77 Figure 1). Here, we provide detailed protocols for measurements of inflammasome
78 activation in infected macrophages, including real-time pore formation assay, western blot

79 of cleaved caspases, western blot for pull-down of active caspase-1/11 and detection of
80 inflammatory cytokine release.

81

82 **2- Materials**

83 **2.1- Bone marrow-derived macrophages (BMDMs)**

84 1. Sterile endo-free phosphate-buffered saline (PBS) for cell culture.

85 2. RPMI endo-free for cell culture.

86 3. RPMI 20/30:

87 ✓ RPMI endo-free for cell culture;

88 ✓ 15 mM HEPES;

89 ✓ 2 mM L-glutamine;

90 ✓ 100 U/ml penicillin-streptomycin;

91 ✓ 20% of fetal bovine serum (FBS);

92 ✓ 30% of L929 cell-conditioned medium (see Note 2).

93 4. RPMI 10%:

94 ✓ RPMI endo-free for cell culture;

95 ✓ 15 mM HEPES;

96 ✓ 2mM L-glutamine;

97 ✓ 10% FBS.

- 98 5. Mice (see Note 3).
- 99 6. Sterile scissors and tweezers.
- 100 7. Ethanol 70%.
- 101 8. Pieces of gauze.
- 102 9. 2 ml sterile endo-free microcentrifuge tube.
- 103 10. Ice.
- 104 11. Laminar flow cabinet.
- 105 12. 15 and 50 ml sterile endo-free conical centrifuge tubes.
- 106 13. Sterile syringe and needles (we recommended the needle of 0.45 x 13 mm for tibia and
- 107 0.70 mm x 25 mm for femur).
- 108 14. Centrifuge for conical tubes.
- 109 15. Untreated endo-free cell culture plates 100 mm x 20 mm (see Note 4).
- 110 16. Sterile 5 ml, 10 ml and 25 ml serological pipette.
- 111 17. Micropipettes (p20: 2–20 µl, p200: 20–200 µl and p1000: 100–1000 µl).
- 112 18. Sterile tips for p20, p200, and p1000 micropipettes.
- 113 19. Cell incubator at 36°C and 5% CO₂.
- 114 20. Vacuum pump.

115

116 **2.2- Preparation of *L. pneumophila* inoculum**

- 117 1. *L. pneumophila*.
- 118 2. CYE (Charcoal-yeast extract) agar plate [10]:
- 119 ✓ 1% MOPS or ACES;
- 120 ✓ 1% yeast extract;
- 121 ✓ pH 6.9
- 122 ✓ 3.3 mM L-cysteine;
- 123 ✓ 0.33 mM Fe(NO₃)₃;
- 124 ✓ 1.5% bacto agar;
- 125 ✓ 0.2% activated charcoal.
- 126 3. 37°C bacterial incubator.
- 127 4. Spectrometer
- 128 5. Sterile distilled water.
- 129 6. 1.5 ml sterile endo-free microcentrifuge tube.
- 130 7. Micropipettes (p20: 2–20 µl, p200: 20–200 µl and p1000: 100–1000 µl).
- 131 8. Sterile tips for p20, p200, and p1000 micropipettes.
- 132 9. Vortex.
- 133

134 **2.3- Pore formation assay**

- 135 1. 96 well cell culture plate (Treated, Black with clear-bottom).
- 136 2. RPMI 10%:
 - 137 ✓ RPMI endo-free for cell culture;
 - 138 ✓ 15 mM Hepes;
 - 139 ✓ 2 mM L-glutamine;
 - 140 ✓ 10% fetal bovine serum (FBS).
- 141 3. Pore medium (see Note 5):
 - 142 ✓ RPMI endo-free without phenol red containing just 0.038 g/ml NaHCO₃;
 - 143 ✓ 15 mM Hepes;
 - 144 ✓ 2 mM L-glutamine;
 - 145 ✓ 2% of FBS.
- 146 4. Lipopolysaccharide (LPS).
- 147 5. *L. pneumophila* inoculum.
- 148 6. Laminar flow cabinet.
- 149 7. Centrifuge for plates.
- 150 8. 2 ml sterile endo-free microcentrifuge tube.
- 151 9. 15 and 50 ml sterile endo-free conical centrifuge tubes.
- 152 10. Sterile 5 ml, 10 ml and 25 ml serological pipette.

- 153 11. Micropipettes (p20: 2–20 µl, p200: 20–200 µl and p1000: 100–1000 µl).
- 154 12. Sterile tips for p20, p200, and p1000 micropipettes.
- 155 13. Cell incubator at 36°C and 5% CO₂.
- 156 14. Vacuum pump.
- 157 15. Iodide propidium 1mg/ml (PI).
- 158 16. Antibody anti-*L. pneumophila*.
- 159 17. Triton X-100 9%.
- 160 18. Fluorometer.
- 161

162 **2.4- Active caspase-1 and caspase-11 immunoblot assay**

- 163 1. 48 well cell culture plate (tissue culture-treated).
- 164 2. RPMI 10%:
- 165 ✓ RPMI endo-free for cell culture;
- 166 ✓ 15 mM Hepes;
- 167 ✓ 2 mM L-glutamine;
- 168 ✓ 10% fetal bovine serum (FBS).
- 169 3. Lipopolysaccharide (LPS).
- 170 4. *L. pneumophila* inoculum.

- 171 5. Laminar flow cabinet.
- 172 6. Centrifuge for plates.
- 173 7. 2 ml sterile endo-free microcentrifuge tube.
- 174 8. 15 and 50 ml sterile endo-free conical centrifuge tubes.
- 175 9. Sterile 5 ml, 10 ml and 2 ml serological pipette.
- 176 10. Micropipettes (p20: 2–20 μ l, p200: 20–200 μ l and p1000: 100–1000 μ l).
- 177 11. Sterile tips for p20, p200, and p1000 micropipettes.
- 178 12. Cell incubator at 36°C and 5% CO₂.
- 179 13. Vacuum pump.
- 180 14. Ultra-low temperature freezer (-70 to -80).
- 181 15. RIPA buffer:
- 182 ✓ 150 mM NaCl;
- 183 ✓ 0.5% DOC (Deoxycholic acid - deoxycholate);
- 184 ✓ 1% NP-40;
- 185 ✓ 0.1% SDS;
- 186 ✓ 50 mM Tris pH 8.0.
- 187 16. Protease inhibitor (stock 25X)
- 188 17. Laemmli 4X buffer:

- 189 ✓ 2.8 ml distilled H₂O ;
- 190 ✓ 2,4 ml 1M Tris HCl pH 6.8;
- 191 ✓ 0.8 g SDS (Sodium Dodecyl Sulfate);
- 192 ✓ 4 ml glycerol;
- 193 ✓ 1 ml β-mercaptoethanol;
- 194 ✓ 200 μl blue bromophenol 2%.

195

196 **2.4.1- Western Blot**

- 197 1. Electrophoresis vertical system.
- 198 2. Gel system with combs, short plate and spacer plate.
- 199 3. 50 ml conical centrifuge tubes.
- 200 4. 5 ml, 10 ml and 25 ml serological pipette.
- 201 5. Micropipettes (p20: 2–20 μl, p200: 20–200 μl and p1000: 100–1000 μl).
- 202 6. Tips for p20, p200, and p1000 micropipettes.
- 203 7. Distilled H₂O;
- 204 8. Polyacrylamide mix:
- 205 ✓ 29 g Acrylamide;
- 206 ✓ 1 g Bis-acrylamide;

- 207 ✓ 100 ml distilled H₂O.
- 208 9. 1.5 M Tris HCl pH 8.8.
- 209 10. 1 M Tris HCl pH 6.8.
- 210 11. SDS 10%.
- 211 12. Ammonium persulfate 10%.
- 212 13. TEMED.
- 213 14. Ethanol 100%.
- 214 15. Protein ladder.
- 215 16. Running Buffer 10X:
- 216 ✓ 1L distilled H₂O;
- 217 ✓ 30.2 g Tris-base;
- 218 ✓ 188 g glycine;
- 219 ✓ 10 g SDS.
- 220 17. Western Blot transfer system.
- 221 18. Transfer Buffer (specific for the transfer system).
- 222 19. Power Supply.
- 223 20. Nitrocellulose membrane 0.22μm.
- 224 21. Tris-buffered saline 10X (TBS 10X):

- 225 ✓ 1L distilled H₂O;
- 226 ✓ 87.6 g NaCl;
- 227 ✓ 60.5 g Tris-base;
- 228 ✓ Adjust the pH to 8.0 with HCl.
- 229 22. Tween 20.
- 230 23. Non-fat dry milk or bovine serum albumin (BSA) (according with the primary
- 231 antibody).
- 232 24. Primary antibody for cleaved caspase-1 or caspase-11.
- 233 25. Secondary antibody conjugated with peroxidase (HRP).
- 234 26. Chemiluminescent Luminol Reagent.
- 235 27. Gel imaging system.
- 236
- 237 **2.5- Active caspase-1/11 pull-down assay**
- 238 1. 6 wells cell culture plate (tissue culture-treated).
- 239 2. RPMI 10%:
- 240 ✓ RPMI endo-free for cell culture;
- 241 ✓ 15 mM HEPES;
- 242 ✓ 2 mM L-glutamine;
- 243 ✓ 10% fetal bovine serum (FBS).

- 244 3. Pam3Cys.
- 245 4. Biotin-VAD-FMK or biotin-YVAD-FMK (specific for caspase-1).
- 246 5. *L. pneumophila* inoculum.
- 247 6. Laminar flow cabinet.
- 248 7. Centrifuge for plates.
- 249 8. 2 ml sterile endo-free microcentrifuge tube.
- 250 9. 15 and 50 ml sterile endo-free conical centrifuge tubes.
- 251 10. Sterile 5 ml, 10 ml and 2 ml serological pipette.
- 252 11. Micropipettes (p20: 2–20 μ l, p200: 20–200 μ l and p1000: 100–1000 μ l).
- 253 12. Sterile tips for p20, p200, and p1000 micropipettes.
- 254 13. Cell incubator at 36°C and 5% CO₂.
- 255 14. Vacuum pump.
- 256 15. RIPA buffer:
- 257 ✓ 150 mM NaCl;
- 258 ✓ 0.5% DOC (Deoxycholic acid - deoxycholate);
- 259 ✓ 1% NP-40;
- 260 ✓ 0.1% SDS;
- 261 ✓ 50 mM Tris pH 8.0.

- 262 16. Protease inhibitor (stock 25X).
- 263 17. Protein quantification method.
- 264 18. Streptavidin-agarose beads.
- 265 19. Laemmli 4X buffer:
- 266 ✓ 2.8 ml distilled H₂O;
- 267 ✓ 2,4 ml 1M Tris HCl pH 6.8;
- 268 ✓ 0.8 g SDS (Sodium Dodecyl Sulfate);
- 269 ✓ 4 ml glycerol;
- 270 ✓ 1 ml β -mercaptoethanol;
- 271 ✓ 200 μ l blue bromophenol 2%.
- 272 20. Materials from step 2.4.1.

273

274 **2.6- Release of IL-1 β or IL-18**

- 275 1. 24 well cell culture plate (tissue culture-treated).
- 276 2. RPMI 10%:
- 277 ✓ RPMI endo-free for cell culture;
- 278 ✓ 15 mM HEPES;
- 279 ✓ 2 mM L-glutamine;

- 280 ✓ 10% of fetal bovine serum (FBS).
- 281 3. Lipopolysaccharide (LPS).
- 282 4. *L. pneumophila* inoculum.
- 283 5. Laminar flow cabinet
- 284 6. Centrifuge for plates.
- 285 7. 2 ml sterile endo-free microcentrifuge tube.
- 286 8. 15 and 50 ml sterile endo-free conical centrifuge tubes.
- 287 9. Sterile 5 ml, 10 ml and 25 ml serological pipette.
- 288 10. Micropipettes (p20: 2–20 µl, p200: 20–200 µl and p1000: 100–1000 µl).
- 289 11. Sterile tips for p20, p200, and p1000 micropipettes.
- 290 12. Cell incubator at 36°C and 5% CO₂.
- 291 13. Vacuum pump.
- 292 14. Ultra-low temperature freezer (-70 to -80).
- 293 15. ELISA kits for IL-1β and/or IL-18.

294

295 **3- Methods**

296 **3.1- Bone marrow-derived macrophages (BMDMs)**

- 297 1. Remove femur and tibias of the mouse and store in 2 ml microcentrifuge tube with
298 1.5ml of PBS 1X on ice. You can use a piece of gauze with ethanol 70% to help clean
299 the bones.
- 300 2. In laminar flow cabinet, prepare the materials to remove the bone marrow:
- 301 • 15 ml tube with 4 ml of ethanol 70%;
- 302 • 15 ml tube with 4ml of RPMI or PBS;
- 303 • 50 ml tube with ethanol 70% with a sterile scissor and tweezer;
- 304 • Syringe with RPMI (usually 3-5 ml/bone) and needle.
- 305 • 15 ml conical centrifuge tube to collect the bone marrow (one for each group).
- 306 3. Quickly wash the bones in ethanol 70% and transfer it to the tube with RPMI or PBS.
- 307 4. Open the syringe and the collect tube for the removal of the bone marrow. Next, cut the
308 both ends of the bone, insert the syringe needle into one end and remove the bone
309 marrow by flushing RPMI through the bone. Store in ice or refrigerator at 4°C.
- 310 5. Centrifuge at 200 x g for 5 minutes.
- 311 6. Discard the supernatant and suspend bone marrow in RPMI 20/30.
- 312 7. Distribute the supernatant with the cells into non-tissue culture-treated plates with a
313 final volume of 10 ml (1 femur renders approximately 2-3 plates and 1 tibia renders
314 approximately 1-2 plates with at least 1×10^7 cells).
- 315 8. Incubate at 36°C and 5% CO₂.
- 316 9. After 4 days in culture add 10 ml of RPMI 20/30 (the RPMI must be at 36-37°C).

- 317 10. On the seventh day of culture, the cells are ready to be released from the plate for use in
318 the experiments. For this, aspirate the plate supernatant with a sterile tip and vacuum
319 pump or with a pipette.
- 320 11. Wash the plate with 10 ml of sterile endo-free PBS 1X (avoid throwing the PBS
321 directly into the cells, just in the wall of the plate). Discard the PBS.
- 322 12. Add 10 ml of sterile endo-free PBS and place the plate on ice or refrigerator 4°C for 10
323 minutes.
- 324 13. Store the plate in room temperature for 5 minutes.
- 325 14. Remove the cell pipetting PBS in the cells and collect in 15 ml or 50 ml conical tube.
- 326 15. Centrifuge at 200 x g for 5 minutes.
- 327 16. Discard the supernatant and suspend cells in RPMI 10% FBS.
- 328 17. Determine the number of the cells/ml and dilute the cell suspension in RPMI 10% FBS
329 to reach the desired cell concentration/ml.
- 330 18. Distribute cell suspension in the appropriate plates for the experiments and incubate at
331 36°C in a 95% air, 5% CO₂ incubator for at least 4 hours before the experiment starts.

332

333 **3.2- Preparation of *L. pneumophila* inoculum**

- 334 1. Defrost the *L. pneumophila* 2 days after starting macrophage differentiation. For this,
335 scrape with a sterile tip or inoculation loop the frozen *Legionella* stock and streak in

336 CYE agar plate to isolate single colonies. Incubate the plate at 37°C for 4 days to isolate
337 single colonies [10, 12].

338 2. After 4 days pick up a single colony and inoculate in CYE agar plate doing a "heavy
339 patch" of about 1 x 1cm and incubate at 37°C for 2 days.

340 3. On the day of the infection, scrape a little of the "2 days heavy patch" with a sterile tip
341 and add in a 1.5 ml microcentrifuge tube with 1 ml of sterile distilled water.

342 Homogenize the bacterial suspension using a vortex mix.

343 4. Measure the optical density (O.D.) at 600nm using a spectrometer. Note that each
344 spectrometer has an optimum O.D. range, therefore, if the bacterial concentration is
345 high it will be necessary to dilute the bacteria in water for appropriate measurements.

346 5. Estimate the final bacteria concentration (an O.D. of 1.0 corresponds to 10^9 bacteria).

347 6. Calculate how much of each bacteria should be added to each well of the experiment:

348

349 **3.3- Pore formation assay**

350 1. Plate 1×10^5 cells/well in a black, clear-bottom 96 well treated plate with 200 μ l/well of
351 RPMI 10% FBS (see Note 6).

352 2. Incubate at 36°C and 5% CO₂ for 1 day.

353 3. This assay can be performed with or without priming with LPS or another TLR agonist
354 (see Note 7). In the case of LPS, sonicate the LPS for 10 minutes and then add in the
355 plate 1 μ g/ml for 3-4 hours (see Note 8).

- 356 4. In the moment of the infection, replace the medium for the “pore medium” without
357 phenol red containing propidium iodide (PI) and anti-*L. pneumophila* (200 µl/well) (see
358 Note 5).
- 359 • Make a mix of pore medium with PI (6µl/ml) and anti-*L. pneumophila* for all the wells.
- 360 • Make the *L. pneumophila* inoculum as described in the step 3.2.
- 361 5. Aspirate the well medium with a sterile tip (it is important not to leave any RPMI with
362 phenol red in the well).
- 363 6. Add 200µl of the medium of the step 4 and the medium containing the bacteria in the
364 specific wells.
- 365 7. Centrifuge at 200 x g for 5 minutes at room temperature.
- 366 8. Add 34µl of Triton X-100 9% in the specific wells .
- 367 9. Place the plate in a fluorometer and setup with:
- 368 • Temperature: 36°C.
- 369 • Excitation reading: 535nm.
- 370 • Emission reading: 617nm.
- 371 • Reading from the bottom.
- 372 • Time interval of your choice.

373

374 **3.4- Active caspase-1 and caspase-11 immunoblot assay**

- 375 1. Plate 1×10^6 cells/well in a 48 well treated plate with 500 μ l of RPMI 10% FBS.
- 376 2. Incubate at 36°C and 5% CO₂ for 1 day.
- 377 3. This assay can be performed with or without priming with LPS or another TLR agonist
378 (see Note 7). In the case of LPS, sonicate the LPS for 10 minutes and then add in the
379 plate 1 μ g/ml (with 100 μ l/well of RPMI 10% FBS) for 3-4 hours (see Note 8).
- 380 4. Replace the medium for RPMI 10% FBS (100 μ l/well).
- 381 5. Make the *L. pneumophila* inoculum as shown in step 3.2 and add the bacteria into each
382 specific well.
- 383 6. Centrifuge at 200 x g for 5 minutes at room temperature.
- 384 7. Incubate at 36°C and 5% CO₂ for 2 hours for samples with priming or 9 hours for
385 samples without priming with LPS.
- 386 8. Collect the supernatant in microcentrifuge tube or well plates and store at -70°C (if you
387 do not have a -70°C available go to step 9).
- 388 9. Add Laemmli 4X buffer (25 μ l) in the supernatant of the step 8, boil for 5 minutes and
389 store in -20°C.
- 390 10. For the cell lysate samples, put the plate on the ice and add 50 μ l of RIPA buffer with
391 protease inhibitor 25X in each well (See Note 9).
- 392 11. With a sterile tip scrape the well for the cells release and collect in a microcentrifuge
393 tube.
- 394 12. Centrifuge at 20.500 x g 4°C for 15 minutes and collect the supernatant.

395 13. Add Laemmli 4X buffer, boil the sample for 5 minutes and store in -20°C.

396

397 **3.4.1- Western Blot**

398 1. Mount the gel system with the short plate and spacer plate.

399 2. Prepare the lower gel, in a 50 ml tube add:

400 • Lower gel (15% polyacrylamide-SDS gel):

401 ✓ 2.3 ml distilled H₂O;

402 ✓ 5.0 ml Polyacrylamide mix;

403 ✓ 2.5 ml 1.5 M Tris HCl pH 8.8;

404 ✓ 100 µl SDS 10%;

405 ✓ 100 µl Ammonium persulfate 10% (add last)

406 ✓ 4 µl TEMED (add last).

407 3. After vortex, put the mix in the gel space and complete with ethanol 100% or distilled
408 water.

409 4. Wait 20 minutes for the gel solidification.

410 5. Discard the ethanol 100% and wash 3 times the free space with distilled H₂O.

411 6. Prepare the upper gel, in a 50 ml tube:

412 • Upper part:

413 ✓ 2.7 ml distilled H₂O;

- 414 ✓ 670 µl Polyacrylamide mix;
- 415 ✓ 500 µl 1.0 M Tris HCl pH 6.8;
- 416 ✓ 40 µl SDS 10%;
- 417 ✓ 40 µl Ammonium persulfate 10% (add last);
- 418 ✓ 4 µl TEMED (add last);
- 419 7. After vortex, put the mix in the free gel space and add the comb.
- 420 8. After 20 minutes, mount the electrophoresis system with the running buffer 1X.
- 421 9. Apply the samples and the protein ladder.
- 422 10. Run the gel for around 80V until the samples reach the upper gel, and then it is possible
- 423 to run by 100-150V.
- 424 11. Run the gel until the 10kDa band reach in the edge (be careful not run out the sample of
- 425 the gel, the band of active caspase-1 and caspase-11 that has around 20kDa).
- 426 12. Transfer the gel for a 0.22 µm nitrocellulose membrane with a transfer system of your
- 427 choice.
- 428 13. Block the membrane for 1-2 hours with a block buffer (TBS 1X with 0.01% Tween and
- 429 5% non-fat dry milk or BSA, according with the primary antibody).
- 430 14. Discard the block buffer in the membrane.
- 431 15. Add the primary antibody for caspase-1 or caspase-11 diluted with a block buffer and
- 432 incubated overnight at 4°C or the best time for your primary antibody according with
- 433 the datasheet.

- 434 16. Wash 3 times the membrane with TBS 0.01% Tween for 5 minutes.
- 435 17. Add the secondary antibody diluted in the block buffer and incubate at room
- 436 temperature for 40-60 minutes or the best time for your secondary antibody according
- 437 with the datasheet.
- 438 18. Wash 3 times the membrane with TBS 0.01% Tween for 5 minutes.
- 439 19. Add chemiluminescent luminol reagent.
- 440 20. Revel in a gel imaging system of your choice.

441

442 **3.5- Active caspase-1/11 pull-down assay**

- 443 1. Plate 5×10^6 cells/well in a 6 well treated plate with 2 ml of RPMI 10% FBS (3 wells for
- 444 each condition with a total of 1.5×10^7 cells/condition).
- 445 2. Incubate at 36°C and 5% CO₂ for 1 day.
- 446 3. This assay can be performed with or without priming with TNF- α or a TLR agonist (see
- 447 Note 7). In the case of Pam3Cys, use 500 ng/ml (with 2 ml/well of RPMI 10% FBS) for
- 448 3-4 hours (see Note 10).
- 449 4. Replace the medium for RPMI 10% FBS (500 μ l/well) containing 20 μ M biotin-VAD-
- 450 FMK or biotin-YVAD-FMK (specific for caspase-1). Incubate at 36°C and 5% CO₂ for
- 451 15 minutes.
- 452 5. Make the *L. pneumophila* inoculum as shown in step 3.2 and add the bacteria in each
- 453 specific well.

- 454 6. Centrifuge at 200 x g for 5 minutes at room temperature.
- 455 7. Incubate at 36°C and 5% CO₂ for 2 hours for samples with priming or 6-9 hours for
456 samples without priming with Pam3Cys (for MOI 10).
- 457 8. Discard the supernatant.
- 458 9. Put the plate on the ice and lyse the cells with 500 µl/condition (3 wells together) of
459 RIPA buffer with protease inhibitor.
- 460 10. Centrifuge at 20.500 x g 4°C for 15 minutes and collect the supernatant.
- 461 11. Measure the protein number by the method of your choice.
- 462 12. Save 30 µl of the sample for the Western Blot control. This will be the “input”.
- 463 13. Wash 100 µl of streptavidin-agarose beads for each sample, 3 times with cold RIPA
464 buffer (centrifuge at 11.000 x g for 2 minutes and 4°C). In all the washings, carefully
465 remove the supernatant.
- 466 14. Incubate the sample with the beads for 3-4 hours at 4°C and low rotation.
- 467 15. Wash sample with beads 3 times with cold RIPA buffer (centrifuge at 11.000 x g for 2
468 minutes and 4°C).
- 469 16. After the last wash, suspend the supernatant in Laemmli 4X buffer (30 µl), boil the
470 sample for 5 minutes.
- 471 17. Centrifuge at 11.000 x g for 2 minutes and collect the supernatant.
- 472 18. Apply in the Western Blot assay as shown in step 3.4.1.
- 473

474

475 **3.6- Release of IL-1 β or IL-18**

476 1. Plate 5x10⁵ cells/well in a 24 well cell treated plate with a total of 1ml of RPMI 10%
477 FBS.

478 2. Incubate at 36°C and 5% CO₂ for 1 day.

479 3. This assay can be performed with or without priming with LPS or another TLR agonist
480 (see Note 7). In the case of LPS, sonicate the LPS for 10 minutes and then add in the
481 plate 1 μ g/ml (500 μ l/well of RPMI 10% FBS) for 3-4 hours (see Note 8).

482 4. Replace the medium for RPMI 10% FBS (500 μ l/well).

483 5. Make the *L. pneumophila* inoculum as shown step 3.2 and add the bacteria in each
484 specific well.

485 6. Centrifuge at 200 x g for 5 minutes at room temperature.

486 7. Incubate at 37°C and 5% CO₂ for 2 hours for samples with priming or 9 hours for
487 samples without priming (MOI 10).

488 8. Centrifuge at 200 x g for 5 minutes at room temperature.

489 9. Collect the supernatant in microcentrifuge tube or well plates and store at -70°C.

490 10. Use the supernatant for the measurement of IL-1 β and IL-18 using ELISA kits
491 according to manufacturer's recommendations.

492

493 **4- Notes**

- 494 1. It is important for BMDMs differentiation to work with endo-free reagents. Any
495 endotoxin contamination, such as with LPS, can alter the experimental results.
- 496 2. The supernatant of L929 cells is rich in MCSF (mouse colony-stimulating factor) which
497 leads to the development of precursor cells in BMDMs [9].
- 498 3. The protocols must be in compliance and approved by the local institutional guidelines
499 on ethics for animal experiments.
- 500 4. BMDMs are adherent cells, therefore it is recommended for its differentiation the use of
501 non-tissue culture-treated plates (not treated) as described [15].
- 502 5. It is very important replace the medium for the “Pore medium” without phenol red and
503 with a low ratio of FBS, in pore formation assay. This is important because phenol red
504 and FBS cause a background of fluorescence and can interfere in the result of the
505 experiment.
- 506 6. In the pore formation assay, it is recommended to add at least 3 wells for each group of
507 BMDM or bacteria, including the non-infected wells and wells for Triton X-100
508 samples. The Triton X-100 can be used for normalize the experiment for the total of cell
509 death (the wells with Triton X-100 should not be next to the other samples, because
510 they spread to adjacent wells) [18].
- 511 7. Some inflammasome components like caspase-1, caspase-11, pro-IL-1 β and pro-IL-18
512 have a low expression in BMDMs not stimulated or not primed. As such, macrophage
513 treatment with Tumor necrosis factor (TNF) or TLRs agonists provides the “first
514 signal” to induce the expression of these molecules through the activation of NF- κ B

515 pathway. In the case of Gram-negative bacteria, such as *L. pneumophila*, their own LPS
516 lead to “priming” and activation of NF- κ B pathway [4].

517 8. For ultrapure LPS add 500 ng/ml.

518 9. Cleaved caspase-1 and caspase-11 will be found in cells supernatant. Meanwhile, the
519 cell lysate can be collected for detection of pro-caspase-1 and pro-caspase-11 [6, 7].

520 10. In the active caspase-11 pull-down assay, we not recommended the priming with LPS,
521 because it leads to the detection of a non-specific activation.

522

523

524

525 **Figure Captions**

526 **Fig.1. Pore formation in macrophages infected with *Legionella pneumophila*.** Bone
527 marrow-derived macrophages (BMDMs) generated from C57BL/6 (blue squares) and
528 *Casp1/11*^{-/-} (red circles) mice were infected with *L. pneumophila* at an MOI of 10 bacteria
529 per cell (filled symbols). Pore formation was assessed fluorometrically in real time by the
530 uptake of propidium iodide. Data show the average $\pm$ SD of triplicate wells. RFUs, relative
531 fluorescence units; NI, uninfected (open symbols).

532

533

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540

541

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9.4 – Anexo 4 – Artigo publicado (MASCARENHAS, Danielle PA et al. *Journal of Infectious Diseases*, 2014)

“Interleukin 1 Receptor-Driven Neutrophil Recruitment Accounts to MyD88-dependent Pulmonary Clearance of *Legionella pneumophila* Infection In Vivo”

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Interleukin 1 Receptor–Driven Neutrophil Recruitment Accounts to MyD88-Dependent Pulmonary Clearance of *Legionella pneumophila* Infection In Vivo

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Legionella pneumophila, the etiological agent of Legionnaires' disease, triggers activation of multiple innate immune pathways that lead to the restriction of bacterial replication in vivo. Despite the critical role for MyD88 in infection clearance, the receptors and mechanisms responsible for MyD88-mediated pulmonary bacterial clearance are still unclear. Here, we used flagellin mutants of *L. pneumophila*, which bypass the NAIP5/NLRC4-mediated restriction of bacterial replication, to assess the receptors involved in MyD88-mediated pulmonary bacterial clearance. By systematically comparing pulmonary clearance of *L. pneumophila* in C57BL/6 MyD88^{-/-}, TLR2^{-/-}, TLR3^{-/-}, TLR4^{-/-}, TLR9^{-/-}, IL-1R^{-/-}, and IL-18^{-/-} mice, we found that, while the knockout of a single Toll-like receptor or interleukin 18 resulted only in minor impairment of bacterial clearance, deficiency in the interleukin 1 (IL-1) receptor led to a significant impairment. IL-1/MyD88-mediated pulmonary bacterial clearance occurs via processes involving the recruitment of neutrophils. Collectively, our data contribute to the understanding of the effector mechanisms involved in MyD88-mediated pulmonary bacterial clearance.

Keywords. *Legionella pneumophila*; pulmonary bacterial clearance; neutrophils; MyD88; IL-1 receptor.

Legionella pneumophila is a gram-negative, facultative, intracellular bacterium that is the main cause of a severe form of pneumonia called Legionnaires' disease [1, 2]. This bacterium survives in freshwater environments and multiplies intracellularly in different species of unicellular protozoa. When droplets of water contaminated by this bacterium are inhaled by humans, *L. pneumophila* is able to infect and replicate within alveolar macrophages, a process that may culminate in the development of Legionnaires' disease if an innate immune response is not readily triggered [1, 3–5]. The inter-

action of *L. pneumophila* with mammalian alveolar macrophages readily triggers the activation of multiple innate immune signaling pathways that are important for the early restriction of bacterial replication and for the recruitment of additional phagocytes to the infection sites [6–12]. Among the recruited phagocytes are the neutrophils, which are critical for infection control. Recruitment of neutrophils to pulmonary sites has been extensively reported during human infection and in animal models of Legionnaires' disease [6–10, 13–15]. The importance of neutrophils for human resistance to this disease is also corroborated by data indicating that neutropenia is a risk factor for the development of Legionnaires' disease [16–18]. Neutrophil recruitment has been reported to be triggered when microbial molecules trigger innate immune receptors, such as Nod-like receptors (NLRs) and Toll-like receptors (TLRs), and also via novel and unidentified pathways [6, 7, 9, 13, 15, 19, 20].

Unlike human macrophages, mouse macrophages are highly restrictive for the replication of *L. pneumophila*.

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In mice, restriction of bacterial replication is triggered when bacterial flagellin gains access to the host macrophage cytoplasm, possibly via the Dot/Icm type IV secretion system. This leads to the activation of a molecular platform composed of the NLRs NAIP5 and NLRC4, which triggers caspase-1 activation [21–27]. We and others have reported that the use of flagellin mutants of *L. pneumophila* in mice leads to an efficient pulmonary infection, in which *Legionella* is able to freely replicate in the lungs during the first 2 days of infection but is restricted and cleared from the lungs 2–5 days after infection by mechanisms that may operate similarly in nonimmunocompromised infected humans [6, 11, 21, 28, 29].

Infections using WT *L. pneumophila* have been previously performed in mice deficient in single or multiple TLRs. In vitro and ex vivo studies have demonstrated that TLR2, TLR5, and TLR9 effectively participate in bacterial recognition [10, 28, 30–37]. TLR5 has been proposed to play an important role in human infection, although TLR5^{−/−} mice show no defects in cytokine production and restriction of pulmonary bacterial infection [8, 10, 28, 34]. Similar to that reported with TLR5, the deletion of mouse TLR4, which senses bacterial lipopolysaccharide, does not interfere with cytokine production or with pulmonary bacterial replication [30, 31]. Infections performed in mice deficient in TLR2 or TLR9 or in mice double-deficient in TLR2 and TLR9 indicate that these molecules are partially required for cytokine production [28, 31, 33, 35, 36]. However, the absence of these molecules results in only a modest increase in bacterial replication in mouse lungs, compared with that in WT animals [28, 31, 32, 35]. In contrast, several independent groups have shown that loss of the TLR-adaptor protein MyD88 in mice results in impaired cytokine production, failure in triggering pulmonary neutrophil recruitment, and impaired ability to restrict bacterial replication [14, 28, 31, 35]. Notably, the striking susceptibility of MyD88-deficient mice to infection was observed neither in mice deficient in individual TLRs nor in those deficient in multiple TLRs. These observations raise the possibility that additional receptors besides TLRs account for MyD88-mediated resistance to *L. pneumophila* infection.

Here, we used a virulent strain of *L. pneumophila* deficient in flagellin to dissect MyD88-mediated clearance of *L. pneumophila* in mouse models of infection. We found that while the deficiency in TLR2 and TLR9 results in only a minor impairment in bacterial clearance, the deficiency in the interleukin 1 receptor (IL-1R) signaling led to a significant impairment in bacterial clearance in the later stages of infection. IL-1R-mediated bacterial clearance is dependent on recruitment of neutrophils to mouse lungs. Collectively, our data provide a basis for understanding MyD88-mediated processes that lead to the restriction of bacterial replication during the acute replication phase and the effector mechanisms involved in pulmonary bacterial clearance.

MATERIALS AND METHODS

Bacterial Cultures

The *L. pneumophila* bacteria used were JR32 (WT) and isogenic, clean deletion mutants for the flagellin (*flaA*[−]) gene [25]. All bacteria were grown on buffered charcoal-yeast extract (CYE) agar as described [38].

Mice and in vivo Infections

Mice were matched by sex and age (all were at least 8 weeks old at the time of infection) and had a C57BL/6 mouse genetic background. C57BL/6 and IL-1R^{−/−} mice were from Jackson (stock numbers 000664 and 003245, respectively). MyD88^{−/−} [39], TLR2^{−/−} [40], TLR3^{−/−} [41], TLR4^{−/−} [42], TLR9^{−/−} [43], and IL-18^{−/−} [44] mice were previously described. For in vivo experiments, approximately 6–8 mice per group were used, as indicated in the figure legends. The care of mice was in compliance with the institutional guidelines on ethics in animal experiments approved by CETEA (Comissão de Ética em Experimentação Animal da Faculdade de Medicina de Ribeirão Preto; approved protocol number 084/2013). For in vivo infections, the mice were anesthetized with ketamine and xylazine (90 mg/kg and 5 mg/kg, respectively) by intraperitoneal injection, followed by intranasal inoculation with 40 μ L of phosphate-buffered saline (PBS) containing 1×10^5 bacteria per mouse. For determination of the number of colony-forming units (CFU), the lungs were harvested and homogenized in 5 mL of sterile water for 30 seconds, using a tissue homogenizer (Power Gen 125; Thermo Scientific, Waltham, MA). Lung homogenates were diluted in sterile water and plated on CYE agar plates for CFU determination.

Macrophages and Infections

C57BL/6, MyD88^{−/−}, IL-1R^{−/−}, and TLR2^{−/−} mice were used to generate bone-marrow-derived macrophages (BMDMs) in vitro, using L929 cell-conditioned medium as previously described [45]. BMDMs were seeded at 2×10^5 cells/well in 24-well plates and cultivated in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum and 5% L929 cell-conditioned medium. Cultures were infected at a multiplicity of infection of 0.015, centrifuged for 5 minutes at 200 $\times$ g at room temperature, and incubated at 37°C in an atmosphere of 5% CO₂. For CFU determinations, cultures were exposed to sterile water for cell lysis, and cell lysates were combined with cell culture supernatant from the respective well. Lysates plus supernatants from each well were diluted in water, plated on CYE agar plates, and incubated for 4 days at 37°C for CFU determination.

Determination of Leukocyte Recruitment and Myeloperoxidase (MPO) Activity and Depletion of Neutrophils

One milliliter of PBS containing 5 mM ethylenediaminetetraacetic acid was injected in the trachea of each mouse, and the

Figure 1. The use of a flagellin-mutant strain of *Legionella pneumophila* supports the assessment of the critical role for MyD88 in bacterial clearance in the latter stages of infection. C57BL/6 mice were infected intranasally with 1×10^5 wild-type (WT; closed circles) or *flaA*⁻ *L. pneumophila* (open circles). MyD88^{-/-} mice were infected intranasally with 1×10^5 *flaA*⁻ *L. pneumophila* (closed squares). Mice were euthanized 4, 48, or 96 hours after infection, and dilutions of the lung homogenates were added to charcoal-yeast extract agar plates for colony-forming units determination. Uniquely for this figure, the data represent a pool of 2 independent experiments, which include 7 mice per group. For appropriate comparisons, data are plotted as fold-change. Abbreviation: Lp, *Legionella pneumophila*.

bronchoalveolar lavage (BAL) fluid was used for determination of the total number of cells, using a Cell Counter (Mindray Coulter). The percentages of monocytes and neutrophils were estimated by microscopic enumeration of cells in the BAL fluid that were stained by hematoxylin and eosin. Neutrophils (NE) accumulation in the lung tissue was evaluated by assaying MPO activity as previously described [9]. Neutrophil depletions were preformed with 100 μ g anti-Gr1 antibody (clone RB6-8C5) in a volume of 200 μ L injected intraperitoneally. The first injection was 24 hours before infection and was performed on alternate days during the course of infection.

Statistical Analysis

Statistical significance was calculated using the unpaired Student *t* test or analysis of variance. Differences were considered statistically significant when $P < .05$.

RESULTS

Use of *flaA*⁻ *L. pneumophila* Reveals a Role for MyD88 in Pulmonary Bacterial Clearance in Mice

By using WT and *flaA*⁻ *L. pneumophila* for in vivo infections, we found that use of *flaA*⁻ but not WT *L. pneumophila* results in at least 2 logs of bacterial replication during the first 48 hours of infection (indicated as acute replication phase). The acute replication phase is followed by the elimination of bacteria from the infected lungs (indicated as clearance phase). In contrast, infection of MyD88^{-/-} mice with *flaA*⁻ *L. pneumophila* results in a much more robust bacterial replication during the

replication phase that cannot be eliminated during the clearance phase (Figure 1). The use of *flaA*⁻ *L. pneumophila* for infections in C57BL/6 mice supports previous findings demonstrating an essential role of MyD88 in pulmonary clearance of *L. pneumophila* and provides us with an appropriate model to assess host components involved in MyD88-mediated clearance of *L. pneumophila* infection in vivo.

Deficiency in Individual TLRs in Mice Does Not Affect Efficiency of Pulmonary Bacterial Clearance

We used mice with a C57BL/6 genetic background that were deficient in individual TLRs to evaluate the participation of individual TLRs in flagellin/NLRC4-independent pulmonary bacterial clearance. To this end, we used *flaA*⁻ *L. pneumophila* to systematically infect mice strains deficient in TLRs. Age- and sex-matched WT (C57BL/6) and MyD88^{-/-} mice were used in all experiments for appropriate comparisons of MyD88-mediated bacterial clearance. We assessed bacterial replication and clearance by evaluating the bacterial loads in mouse lungs 4, 48, and 96 hours after infection. By assessing the CFUs at 4 and 48 hours, we found that *flaA*⁻ *L. pneumophila* replicated effectively and similarly during the replication phase in the lungs of C57BL/6 mice and in the lungs of mice deficient in MyD88, TLR2, TLR3, TLR4, or TLR9. Notably, we found that TLR2^{-/-}, TLR3^{-/-}, TLR4^{-/-}, or TLR9^{-/-} mice did not show major defects to clear pulmonary bacteria, as assessed by enumeration of CFUs at 48 and 96 hours of infection (Figure 2). Mice deficient in TLR2 and TLR9 were partially able to clear pulmonary bacteria; statistically significant differences from C57BL/6 mice were found at 96

Figure 2. Mice deficient in Toll-like receptor 2 (TLR2), TLR3, TLR4, and TLR9 but not in MyD88 are effective in triggering pulmonary bacterial clearance. C57BL/6, MyD88^{-/-}, and TLR2^{-/-} mice (A), TLR3^{-/-} mice (B), TLR4^{-/-} mice (C), and TLR9^{-/-} mice (D) were infected intranasally with 1×10^5 *flaA*⁻ *Legionella pneumophila*. Mice were euthanized 4 hours (closed squares), 48 hours (closed circles), or 96 hours (open circles) after infection. Dilutions of the lung homogenates were added to charcoal-yeast extract agar plates for colony-forming units determination. Each dot represents a single animal, and the horizontal lines represent the averages. Data are representative of those found in 1–3 independent experiments for each of the TLR-deficient mice strain. * $P < .05$, by the Student *t* test. Abbreviation: NS, not significant.

hours of infection (Figure 2A and 2D). The minor deficiencies of TLR2^{-/-} or TLR9^{-/-} mice in restricting pulmonary replication of *L. pneumophila* are consistent with data previously published using infections with flagellated bacteria [28, 30, 31, 35].

Mice Deficient in IL-1R but Not in Interleukin 18 (IL-18) Fail to Trigger Pulmonary Bacterial Clearance

We investigated the involvement of additional receptors that are not TLR members but signal via MyD88. Initially, we evaluated the clearance of *flaA*⁻ *L. pneumophila* in mice deficient in the gene for IL-18, which signals via MyD88 and was previously reported to be important for interferon γ production by natural killer cells in response to infection with WT *L. pneumophila* [28, 39]. By infecting IL-18^{-/-} mice, we found that, despite the absence of IL-18, the mice were fully able to trigger bacterial clearance by 96 hours of infection (Figure 3A). Similar to IL-18 signaling, IL-1R signaling is also dependent on MyD88 [46]. Thus, we investigated the effect of IL-1R signaling on the flagellin-independent clearance of *L. pneumophila* infection. By infecting IL-1R^{-/-} mice with *flaA*⁻ *L. pneumophila*, we found that, in the absence of IL-1R signaling, the mice showed severely impaired pulmonary bacterial clearance, as the bacterial loads found in the lungs of IL-1R^{-/-} mice were similar to those

found in MyD88^{-/-} mice (Figure 3B), a feature not previously detected in TLR-deficient mice. These results support the hypothesis that IL-1R signaling is critical for the clearance of *L. pneumophila* and may provide a mechanistic explanation for the impaired MyD88-mediated pulmonary bacterial clearance.

IL-1R Signaling Is Not Required for Macrophage Restriction of Bacterial Replication but Favors Recruitment of Neutrophils to Mouse Lungs

Next, we investigated whether the impaired bacterial clearance in the lungs of mice deficient in MyD88 and IL-1R was caused by an intrinsic macrophage defect in the restriction of bacterial replication. To test this hypothesis, we generated BMDMs from C57BL/6, MyD88^{-/-}, and IL-1R^{-/-} mice and infected them with a flagellin-deficient strain of *L. pneumophila*. Macrophages deficient in TLR2 were also used because TLR2-deficient mice were partially able to clear pulmonary bacteria, compared with C57BL/6 mice (Figure 2A). We found that *flaA*⁻ *L. pneumophila* effectively replicate in the BMDMs regardless of the presence of TLRs, MyD88, or IL-1R signaling (Figure 4A). Accordingly, BMDMs obtained from C57BL/6, TLR2^{-/-}, MyD88^{-/-}, and IL-1R^{-/-} mice effectively restricted the replication of flagellated *L. pneumophila* (Figure 4B). These data support the finding that

Figure 3. Mice deficient in interleukin 1 receptor (IL-1R), but not those deficient in interleukin 18 (IL-18), fail to trigger pulmonary bacterial clearance. C57BL/6, MyD88^{-/-}, and IL-18^{-/-} mice (A) or IL-1R^{-/-} mice (B) were infected intranasally with 1×10^5 *flaA*⁻ *Legionella pneumophila*. Mice were euthanized 4 hours (closed squares), 48 hours (closed circles), or 96 hours (open circles) after infection, and dilutions of the lung homogenates were added to charcoal-yeast extract agar plates for colony-forming units determination. Each dot represents a single animal, and the horizontal lines represent the averages. Data are representative of those found in 2 (A) and 5 (B) independent experiments. **P* < .05, by the Student *t* test. Abbreviation: NS, not significant.

MyD88-dependent signaling pathways are not required for NAIP5/NLRC4-mediated restriction of bacterial replication in macrophages.

Since MyD88-dependent restriction of bacterial replication was not associated with an intrinsic macrophage activity, we investigated the effect of MyD88 signaling on the total cell population obtained from the BAL fluid of mice infected for 24 and 48 hours with *flaA*⁻ *L. pneumophila*. We found that mice deficient in MyD88 and IL-1R but not those deficient in TLR2 contain a reduced amount of cells in the pulmonary cavity at 48 hours of infection, compared with WT mice (Figure 5A). Microscopy of these cells indicated that, while the macrophage population is similar in C57BL/6, TLR2^{-/-}, MyD88^{-/-}, and IL-1R^{-/-} mice, the neutrophil population is highly reduced in MyD88^{-/-} and IL-1R^{-/-} mice (Figure 5B and 5C). By measuring the neutrophil MPO activity in the lung tissue, we found an accumulation of neutrophils in the pulmonary tissue 48 hours

Figure 4. Interleukin 1 receptor (IL-1R)/MyD88-mediated restriction of *Legionella pneumophila* replication is not detectable in isolated macrophages. Bone-marrow-derived macrophages were generated from C57BL/6 mice (closed circles), MyD88^{-/-} mice (closed squares), TLR2^{-/-} mice (open squares), and IL-1R^{-/-} mice (open circles). Cells were plated in 24-well plates and infected with *flaA*⁻ mutants (A) or wild-type *L. pneumophila* (B). Each well was infected with 3000 bacteria and further incubated at 37°C for 24, 48, 72, and 96 hours for colony-forming units (CFU) determination. The data show the averages ± standard deviation of triplicate wells. Data are representative of those found in 3 independent experiments. **P* < .05, by analysis of variance.

after infection and a decrease 96 hours after infection, which is consistent with the increase in neutrophils in the BAL fluid at 48 hours (Figure 5C and 5D). Accordingly, mice deficient in MyD88 and IL-1R but not those deficient in TLR2 had reduced neutrophil counts in the lung tissue 48 hours after infection (Figure 5C). Collectively, these data indicate that the IL-1/MyD88-signaling pathway contributes to neutrophil recruitment to the mouse lungs in response to infection with *flaA*⁻ mutants of *L. pneumophila*.

IL-1/MyD88-Mediated Neutrophil Recruitment Accounts for Pulmonary Bacterial Clearance

To evaluate the effect of neutrophil recruitment in the IL-1/MyD88-dependent bacterial clearance, we used anti-granulocyte receptor-1 (α -GR1) neutralizing antibodies for neutrophil depletion in C57BL/6, IL-1R^{-/-}, and MyD88^{-/-} mice. As expected, α -GR1 treatment resulted in a decrease in the numbers of neutrophils in the lungs of both C57BL/6 and IL-1R^{-/-} mice, whereas it did not influence the macrophage population in pulmonary cavities (Figure 6A and 6B). By measuring bacterial replication in neutrophil-depleted mice, we found that neutrophils play a minor role during the acute replication phase, as the CFU loads were similar 48 hours infection (Figure 6C). In

Figure 5. Interleukin 1 receptor signaling is required for MyD88-mediated neutrophil recruitment to the lungs of *Legionella pneumophila*-infected mice. C57BL/6, MyD88^{-/-}, TLR2^{-/-}, and IL-1R^{-/-} mice were infected intranasally with 1×10^5 *flaA*⁻ *L. pneumophila*. Mice were euthanized, and the lungs and bronchoalveolar fluid lavage (BAL) fluid were collected 24 hours (closed circles) and 48 hours (open circles) after infection for evaluation of cell recruitment to the lungs. Total cells (A), monocytes (B), and neutrophils (C) were microscopically scored from BAL fluid. The activity of neutrophil myeloperoxidase enzyme (MPO) was measured in lung tissue homogenates (D). Each dot represents a single animal, and the horizontal lines represent the averages. Data are representative of those found in 3 independent experiments. * $P < .05$, by the Student *t* test. Abbreviation: NI, not infected.

contrast, at 96 hours of infection, the CFU loads were significantly higher in neutrophil-depleted mice, thus suggesting that neutrophils may play an important role during the clearance phase (Figure 6C). Notably, the reduced bacterial clearance observed in neutrophil-depleted mice was less pronounced in IL-1R^{-/-} mice, compared with C57BL/6 mice, a result that is consistent with the requirement for IL-1R signaling in neutrophil recruitment in response to pulmonary *L. pneumophila* infection (Figure 6C). Taken together, these data indicate that IL-1R signaling effectively contributes to MyD88-mediated bacterial clearance by mechanisms involving the recruitment of neutrophils to the infection sites.

Figure 6. Neutrophil recruitment is required for interleukin 1 receptor-mediated pulmonary bacterial clearance. C57BL/6, IL-1R^{-/-}, and MyD88^{-/-} mice were infected intranasally with 1×10^5 *flaA*⁻ *Legionella pneumophila*. When indicated, mice were treated with 100 μ g of α -GR1 on alternate days during the course of infection. A and B, Mice were euthanized, and bronchoalveolar lavage (BAL) fluid was collected 48 hours after infection for evaluation of the recruitment of neutrophils and monocytes to the lungs of infected mice. Neutrophils (A) and monocytes (B) were microscopically scored from BAL fluid. C, Mice were euthanized 4, 48, or 96 hours after infection, and dilutions of the lung homogenates were added to charcoal-yeast extract agar plates for colony-forming units determination. Each dot represents a single animal, and the horizontal lines represent the averages. Data are representative of those found in 2 independent experiments. * $P < .05$, by the Student *t* test. Abbreviation: NS, not significant.

Although experiments comparing the bacterial clearance in IL-1R^{-/-} and MyD88^{-/-} mice often reveal similar bacterial CFU counts during the clearance phase (assessed here at 96 hours), we investigated whether, at later time points, IL-1R^{-/-} and MyD88^{-/-} mice were similar in their bacterial clearance capability. Thus, we infected a large cohort of C57BL/6, IL-1R^{-/-}, and MyD88^{-/-} mice with *flaA*⁻ *L. pneumophila* and systematically measured the CFU counts at 24, 48, 72, 96, 120, and 144 hours. We found that although IL-1R is clearly required for efficient bacterial clearance, IL-1R signaling does not solely explain MyD88-mediated restriction of pulmonary bacterial clearance (Figure 7). Taken together, our data indicate that, while IL-1R signaling effectively contributes to MyD88-

Figure 7. The interleukin 1 receptor (IL-1R) signaling effectively accounts for pulmonary bacterial clearance, but IL-1R-independent mechanisms also operate in MyD88-mediated resistance to *Legionella pneumophila* infection. C57BL/6 mice (closed squares), IL-1R^{-/-} mice (open circles), and MyD88^{-/-} mice (closed circles) were infected intranasally with 1×10^5 *flaA*⁻ *L. pneumophila*. Mice were euthanized 4, 24, 48, 72, 96, 120, or 144 hours after infection, and dilutions of the lung homogenates were added to charcoal-yeast extract agar plates for colony-forming units (CFU) determination. The averages $\pm$ standard deviation of CFU/lung of approximately 5 mice per group are shown. * $P < .05$, by analysis of variance.

mediated bacterial clearance by mechanisms dependent on neutrophil recruitment, additional receptors, possibly consisting of a combination of multiple TLRs, also account for MyD88-mediated clearance of pulmonary bacterial replication.

DISCUSSION

Infections in mice with WT strains of *L. pneumophila* culminate with flagellin-mediated activation of the NAIP5/NLRC4 inflammasome. This process readily triggers the restriction of bacterial replication during the acute phase of infection in mice. Although identification of this flagellin-sensing platform effectively contributed to our understanding of the mechanistic functions of Naip genes, the flagellin/NAIP5/NLRC4-mediated growth restriction does not appear to operate in humans, a feature that explains the reports that mice are not an appropriate animal model for Legionnaires' disease. In this study, we used flagellin mutants of *L. pneumophila* for infections in mice from the C57BL/6 background. By systematically infecting several knockout mice from the C57BL/6 background with *flaA* mutants of *L. pneumophila*, we studied the previously reported MyD88-mediated resistance to infection [14, 28, 31, 35]. We detected a key role for IL-1R signaling in MyD88-mediated pulmonary bacterial clearance in the later stages of infection. The mechanism underlying IL-1R-mediated resistance involves neutrophil recruitment to the lungs of the infected mice. Our data are consistent with recent reports showing that IL-1 α is important for neutrophil recruitment and mouse resistance in response to WT *L. pneumophila* infection [19, 47]. Thus, our data showing that IL-1R^{-/-} mice fail to recruit neutrophils support the hypothesis that MyD88-dependent neutrophil recruitment

is mediated by IL-1R signaling, which is known to occur via MyD88 [46, 48]. In addition, by counting the pulmonary CFUs at later time points after infection in neutrophil-depleted mice, we provided data in support of the role of neutrophils in the IL-1R-mediated resistance to infection. These data provide a mechanistic explanation for the well-described MyD88-mediated pulmonary bacterial clearance.

The comparisons of mice deficient in MyD88 to those deficient in IL-1R for up to 6 days after infection indicated that MyD88-mediated host resistance is only partially dependent on IL-1R (Figure 7). This is in agreement with previous results showing that MyD88-dependent responses involving TLRs are effectively triggered in mice infected with *L. pneumophila* [10, 28, 30–33, 35–37]. Although it is known that *L. pneumophila* triggers activation of TLR2, TLR4, TLR5, and TLR9, deletion of these genes has little influence on resistance of the mice to infection in vivo. Among the TLR-deficient mice that are significantly more permissive to *L. pneumophila* infection are TLR2^{-/-} and TLR9^{-/-} mice [28, 31, 35]. However, although these TLR-deficient mice are slightly more permissive than the WT mice, their reduced restriction of bacterial replication are considerably different from those observed in MyD88^{-/-} mice [28, 31]. It is possible that the redundancy among TLRs would compensate for the deficiency in a single TLR. In a massive effort to overcome this redundancy, mice double deficient in TLR5 and TLR2 and mice deficient in TLR2 along with either TLR5 or TLR9 were generated and infected with *L. pneumophila*. It was concluded that even in the absence of functional TLR1, TLR2, TLR5, TLR6, and TLR9 signaling, the mice were overall competent in controlling bacterial replication and had no apparent defects in cytokine production when compared with control mice [28]. It is possible that IL-1R signaling,

which requires MyD88, will compensate for the absence of individual TLRs during the infections. According to this hypothesis, our data indicate that IL1R signaling does not fully account for MyD88-mediated resistance (Figure 7). It is possible that signaling via multiple TLRs together with signaling via IL-1R and IL-18 will account for the total MyD88-mediated resistance. In this context, CRISPR-mediated multiple gene deletion may provide us with appropriate tools to further identify the individual molecules that contribute to MyD88-mediated host resistance. Regardless of the complex cooperation between these genes, the deletion of a single gene (*il1r*) results in mice that are significantly more susceptible to *L. pneumophila* infection than any single-TLR- or multiple-TLR-deficient mice generated so far.

Regardless of the receptors accounting for MyD88-mediated host resistance, our data effectively contribute to the elucidation of the host cell processes that lead to neutrophil-mediated resistance in Legionnaires' disease. IL-1R is unique in that it is activated both by IL-1 β and IL-1 α [49]; neither IL-1 β nor IL-1 α functions as a chemokine for direct neutrophil recruitment. It was recently shown that hematopoietic cells are required for IL-1 α production in response to *L. pneumophila* infection in vivo [19]. It is possible that these cytokines are secreted when the infected alveolar macrophages are targeted by virulent *L. pneumophila* [19, 47, 50]. In this scenario, the macrophage-induced IL-1 α may target specific host cells for the production of specific chemokines, which may ultimately facilitate neutrophil recruitment. Although the mechanisms involved in IL-1 α secretion by infected macrophages and the processes underlying IL-1 α -mediated neutrophil recruitment remain obscure, our data provide a mechanistic explanation for the 2 process that have long been known to be important for host resistance in Legionnaires' disease: MyD88-mediated host resistance to infection and recruitment of neutrophils to the lungs of infected individuals. Further investigation into these processes will be important for a comprehensive understanding of the mechanisms governing health and disease during Legionnaires' disease.

Notes

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10 – ANEXO II: Trabalhos de colaboração

10.1 – Anexo 5 - Artigo publicado (CUNHA, Larissa D; SILVA, Alexandre LN; RIBEIRO, Juliana M; MASCARENHAS, Danielle PA; QUIRINO, Gustavo FS; SANTOS, Leonardo L; FLAVELL, Richard A; ZAMBONI, Dario S. *Cell Reports*, 2017)

“AIM2 Engages Active but Unprocessed Caspase-1 to Induce Noncanonical Activation of the NLRP3 Inflammasome”

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AIM2 Engages Active but Unprocessed Caspase-1 to Induce Noncanonical Activation of the NLRP3 Inflammasome

Graphical Abstract

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In Brief

Cunha et al. find that the AIM2 inflammasome is activated in response to *Legionella* and cooperates with caspase-11 to trigger host resistance. Mechanistically, AIM2 engages active but unprocessed caspase-1 to trigger pore formation and K⁺ efflux, which also converges into NLRP3 activation. Thus, the AIM2 inflammasome cooperates with caspase-11 to amplify the signals of infection and triggers noncanonical activation of the NLRP3 inflammasome.

Highlights

- AIM2 triggers caspase-1 activation, but not cleavage, in response to *Legionella* infection
- AIM2 engages active but unprocessed caspase-1 to induce pore formation and K⁺ efflux
- AIM2 amplifies infection signals to trigger activation of the NLRP3 inflammasome
- AIM2 and caspase-11 cooperate to induce host resistance to *Legionella* infection

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AIM2 Engages Active but Unprocessed Caspase-1 to Induce Noncanonical Activation of the NLRP3 Inflammasome

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SUMMARY

Inflammasomes are multimeric protein complexes that initiate inflammatory cascades. Their activation is a hallmark of many infectious or inflammatory diseases. Their composition and activity are specified by proinflammatory stimuli. For example, the NLRP3 inflammasome is activated in response to cell damage and K⁺ efflux, whereas the AIM2 inflammasome is activated in response to cytosolic DNA. We used *Legionella pneumophila*, an intracellular bacterial pathogen that activates multiple inflammasomes, to elucidate the molecular mechanisms regulating inflammasome activation during infection. Upon infection, the AIM2 inflammasome engaged caspase-1 to induce pore formation in the cell membrane, which then caused K⁺-efflux-mediated activation of NLRP3. Thus, the AIM2 inflammasome amplifies signals of infection, triggering noncanonical activation of NLRP3. During infection, AIM2 and caspase-11 induced membrane damage, which was sufficient and essential for activating the NLRP3 inflammasome. Our data reveal that different inflammasomes regulate one another's activity to ensure an effective immune response to infection.

INTRODUCTION

Inflammation is a protective response triggered by the innate immune system in response to pathogens or damage caused by potentially harmful stimuli. A key contributor to the inflammatory response is the intracellular activation of inflammasomes, which are large multimeric protein complexes that serve primarily as an activating scaffold for caspase-1. Upon sensing harmful stimuli such as pathogens or irritants, pattern-recognition receptors assemble distinct inflammasome structures that recruit and activate caspase-1. Activated caspase-1 then initiates a large proinflammatory cascade by cleaving various

molecules, including inflammatory cytokines, into their bioactive forms. Inflammasome function and activity is dictated by the particular pattern-recognition receptor sensing the stimulus. Although the mechanisms leading to activation of inflammasomes are well characterized, the mechanisms by which distinct inflammasome types are able to compartmentalize their activity are not.

Inflammasomes are often studied in genetically engineered mice lacking a single gene. High concentrations of synthetic or purified molecules that activate distinct inflammasome types are also used. This approach provides valuable information about the specific functions of different inflammasome types; however, it does not provide a comprehensive understanding of the cooperation between inflammasomes during infection or the effect of one inflammasome type on the regulation of another. In this context, the use of relevant pathogens that target inflammasomes to model the physiologic response to infection is essential to assess the role of inflammasomes in infection and immunity. This is particularly important for activation of the pattern-recognition receptor absent in melanoma 2 (AIM2), which recruits the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) to promote caspase-1 activation in response to cytosolic DNA (Bürckstümmer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009). The AIM2 inflammasome is broadly involved in pathogen recognition, but the physiologic functions of this inflammasome type remain obscure.

Legionella pneumophila is a gram-negative bacterium that causes a severe form of pneumonia called Legionnaires disease. *L. pneumophila* evolved in freshwater environments, where it replicates in several protozoa species. Mammalian infection is inadvertent and considered a “dead end” for the bacteria. Therefore, *L. pneumophila* has not adapted a mechanism to bypass/inhibit innate immune signaling pathways that are not present in amoeba. Because *L. pneumophila* initiates the activation of multiple inflammasome types, it is an appropriate model of pathogenic microbial infection to investigate functions of the innate immune system (Cunha and Zamboni, 2014; Fontana and Vance, 2011; Massis and Zamboni, 2011).

L. pneumophila expresses Dot/Icm, a type IV secretion system that delivers bacterial proteins into the cytoplasm of host cells. Dot/Icm is essential for bacterial replication in macrophages, which also enables the innate immune system to identify pathogens and activate multiple innate immune pathways (Shin, 2012; Vance, 2010). Dot/Icm facilitates the delivery of bacterial flagellin into the cytoplasm, which is an agonist that stimulates intracellular Nod-like receptors (NLRs) such as NAIP5 that initiate the assembly an inflammasome composed of NLRC4 and caspase-1 (Amer et al., 2006; Molofsky et al., 2006; Ren et al., 2006). *L. pneumophila* flagellin mutants (*flaA*[−]) bypass NAIP5/NLRC4 inflammasome-mediated growth restriction and freely multiply in restrictive macrophages. Therefore, NAIP5 and NLRC4 inflammasomes are required to restrict bacterial replication in macrophages (Amer et al., 2006; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006).

The AIM2 inflammasome is also activated in response to *L. pneumophila* infection, which also depends on Dot/Icm. SdhA, an effector protein secreted by Dot/Icm, stabilizes *L. pneumophila*-containing vacuoles and consequently reduces AIM2 activation (Creasey and Isberg, 2012; Ge et al., 2012). Deletion of the *sdhA* gene strongly activates AIM2-mediated cell death. Nevertheless, AIM2 is activated in wild-type (WT) *L. pneumophila* containing SdhA (Ge et al., 2012).

Caspase-11 is activated in response to *L. pneumophila* infection (Aachoui et al., 2013; Akhter et al., 2012; Case et al., 2013; Casson et al., 2013; Pilla et al., 2014) through a mechanism independent of bacterial flagellin, NLRC4, or caspase-1. However, this occurs after NLRC4 inflammasome activation (Case et al., 2013) most likely because caspase-11 activation requires macrophage priming to trigger its transcriptional upregulation (Kayagaki et al., 2011). Once activated, caspase-11 triggers macrophage pyroptosis (i.e., inflammation-associated programmed cell death) and noncanonical activation of the NACHT-, LRR-, and PYD-domain-containing protein 3 (NLRP3) inflammasome. Caspase-11-induced NLRP3 inflammasome formation requires ASC and culminates in caspase-1 activation and inflammatory cytokine secretion (Case et al., 2013; Casson et al., 2013; Kayagaki et al., 2011).

Lipopolysaccharide (LPS), flagellin, and bacterial DNA are agonists for caspase-11, NAIP5/NLRC4/caspase-1, and AIM2/ASC/caspase-1 inflammasomes, respectively. However, the specific agonist and mechanisms underlying the activation of NLRP3/ASC/caspase-1 remain obscure, which is surprising given that NLRP3 is the most thoroughly studied inflammasome type. NLRP3 is also involved in the pathogenesis of several inflammatory and infectious diseases (reviewed in Wen et al., 2012). Although several molecules activate NLRP3 (e.g., reactive oxygen species, lysosomal cathepsins, bacterial RNA, and mitochondrial DNA), K⁺ efflux appears to be essential for NLRP3 activation (Baker et al., 2015; Muñoz-Planillo et al., 2013; Perregaux and Gabel, 1994; Pétrilli et al., 2007; Rühl and Broz, 2015; Schmid-Burgk et al., 2015).

We used the bacterial pathogen *L. pneumophila* as a relevant model of infection to assess the coordination of inflammasome functions during infection. We hypothesize that the AIM2 inflammasome does not directly cooperate with NLRP3 to trigger caspase-1 activation. Instead, AIM2 indirectly activates the

NLRP3 inflammasome during the physiologic response to infection by inducing membrane damage and K⁺ efflux, thereby amplifying the signals of infection. Thus, AIM2 induces noncanonical activation of the NLRP3 inflammasome, a process that ensures robust activation of inflammasomes and an effective innate immune response to infection.

RESULTS

Pore Formation Can Occur Independently of Flagellin/NLRC4 and LPS/Caspase-11 Inflammasomes in *L. pneumophila*-Infected Macrophages

Infection of mouse macrophages with WT *L. pneumophila* activates the NAIP5/NLRC4 inflammasome, which induces pore formation via mechanisms dependent on caspase-1 and flagellin (Case et al., 2009; Silveira and Zamboni, 2010; Zamboni et al., 2006). In addition, caspase-11 mediates pore formation and pyroptosis independently of flagellin/NLRC4 and caspase-1 but requires upregulation of *Casp11* through macrophage priming (Case et al., 2013). To assess the role of caspase-1 and caspase-11 in pyroptosis, we examined pore formation by evaluating the loss of membrane integrity in *Legionella*-infected bone-marrow-derived macrophages (BMDMs) using a real-time fluorimetric assay of propidium iodide (PI) uptake. We primed the cells with LPS and observed that pore formation induced by WT *L. pneumophila* (JR32 WT) partially depended on caspase-1 and was abolished in BMDMs derived from *Casp11/11*-deficient mice (Figures 1A and 1C). As previously reported (Case et al., 2013), pore formation was reduced overall and depended on caspase-11, but not caspase-1, in response to *flaA*[−] *L. pneumophila* (Figure 1B). Experiments performed using *L. pneumophila dotA*[−] mutants, which have a defective Dot/Icm system, indicated that the bacterial type IV secretion system is essential for pore formation in macrophages (Figure 1D). We obtained similar results for BMDMs primed with tumor necrosis factor alpha (TNF-α), indicating that regardless of the molecule used for priming, caspase-1 activation requires flagellin and caspase-11 does not (Figures 1E–1G). Furthermore, *flaA*[−] *L. pneumophila* triggered pore formation in *Casp11*^{−/−} BMDMs, suggesting that a pathway independent of flagellin/NLRC4 and caspase-11 can mediate pore formation (Figures 1B and 1F). This unidentified pore-formation pathway presumably depends on caspase-1, because it was abolished in *Casp11/11*-deficient BMDMs and reduced in *Casp1*^{−/−} BMDMs compared to WT BMDMs (C57BL/6) (Figures 1B and 1F).

These data support the idea that *Legionella*-induced pore formation and pyroptosis involve multiple pathways. One pathway depends on flagellin/NLRC4 and caspase-1, another depends on caspase-11, but not flagellin, and a third is independent of flagellin and caspase-11.

The AIM2 Inflammasome Promotes Pore Formation, Pyroptosis, and Caspase-1 Activation in Response to *L. pneumophila*

We determined the molecules comprising the pathway that functions independently of flagellin/NLRC4 and LPS/caspase-11 with *flaA*[−] *L. pneumophila*. The AIM2 inflammasome is activated

Figure 1. AIM2 Induces NLRC4 and Caspase-11-Independent Pore Formation and Pyroptosis in Response to *L. pneumophila* Infection
(A–K) Fluorometric quantification of PI uptake over time, expressed in relative fluorescence units (RFUs). BMDMs BL/6, *Casp11*^{-/-}, *Casp1*^{-/-}, and *Casp1/11*^{-/-} pretreated with LPS (500 ng/mL) for 3 hr were either not infected (NI) (A) or infected at MOI20 with *flaA*⁻ mutant strain of *L. pneumophila* (JR32 *flaA*) (B), WT *L. pneumophila* (JR32 WT) (C), or *dotA*⁻ mutant strain (JR32 *dotA*) (D). BMDMs BL/6, *Casp11*^{-/-}, *Casp1*^{-/-}, and *Casp1/11*^{-/-} pretreated with TNF-α (10 ng/mL) for 3 hr were either NI (E), infected at MOI 20 with JR32 *flaA* (F), or JR32 WT (G). BMDMs BL/6, *Casp11*^{-/-}, *Aim2*^{-/-}, and *Casp1/11*^{-/-} pretreated with LPS (500 ng/mL) for 3 hr were NI (H) or infected at MOI 20 with JR32 *flaA* (I). BMDMs BL/6, *Casp11*^{-/-}, *Aim2*^{-/-}/*Casp11*^{-/-} and *Casp1/11*^{-/-} pretreated with LPS (500 ng/mL) for 3 hr were NI (J) or infected at MOI 20 with JR32 *flaA* (K). Data are representative of four independent experiments and are expressed as the average of triplicate wells.
(L and M) Cytotoxicity was measured in an LDH-release assay. BMDMs pretreated with LPS (500 ng/mL) for 3 hr were either NI (L) or infected with JR32 *flaA* at MOI 20 (M). Data are representative of three independent experiments and are expressed as the mean of triplicate wells. Error bars depict SEM. *p < 0.05 versus *Casp1/11*^{-/-} BMDMs.
See also Figure S1.

Figure 2. AIM2 and Caspase-11 Cooperate to Trigger Caspase-1 Cleavage in Response to *L. pneumophila* Infection

(A and B) Immunoblots of caspase-1, IL-1β, and caspase-11 in the supernatant (SN) and cell extract (CE) of BMDMs. BMDMs pretreated for 3 hr with LPS (500 ng/mL) (A) or TNF-α (10 ng/mL) (B) were either not infected (NI) or infected for 2 hr with JR32 *flaA* (MOI 20). Data are representative of three independent experiments.

(C and D) IL-1β released in BMDM SN, as measured by ELISA. BMDMs pretreated for 3 hr with LPS (500 ng/mL) (C) or TNF-α (10 ng/mL) (D) were infected for 4 hr with JR32 *flaA* (MOI 20). Data are representative of three independent experiments and are expressed as the mean of triplicate wells. Error bars depict ± SEM. *p < 0.05 versus NI. See also Figure S2.

in response to *L. pneumophila* via a process that requires Dot/Icm and activates caspase-1 (Ge et al., 2012). Therefore, we tested whether AIM2 was required for *L. pneumophila*-induced pore formation. We found that the absence of AIM2 in BMDMs from *Aim2*^{-/-} mice generated in a C57BL/6 background did not affect caspase-11-dependent pore formation in response to *L. pneumophila* (Figures 1H and 1I). The caspase-1-dependent, LPS/caspase-11-independent, and flagellin/NLRC4-independent pathway was evident, as *flaA*⁻ *L. pneumophila* triggered pore formation in *Casp11*^{-/-} BMDMs, but not in *Casp11*^{-/-} BMDMs (Figures 1H and 1I). To further test whether AIM2 operates in this pathway, we used mice double deficient for *Aim2* and *Casp11*. Caspase-11-independent pore formation was abolished in these macrophages (Figures 1J and 1K). Similar results were obtained by infection with Lp01, a widely used laboratory strain of *L. pneumophila* that was isolated from a historic outbreak of Legionnaires disease in Philadelphia in 1976 (Figures S1A and S1B).

L. pneumophila-induced pore formation culminates in macrophage pyroptosis. Therefore, we measured lactate dehydroge-

nase (LDH) release to assess macrophage pyroptosis in response to *flaA*⁻ *L. pneumophila*. In agreement with our pore-formation findings, AIM2 and caspase-11 induced pyroptosis in response to *L. pneumophila* (Figures 1L and 1M). In immortalized C57BL/6 macrophages that express an AIM2-citrine fusion construct, *flaA*⁻, but not *dotA*⁻, *L. pneumophila* induced AIM2 oligomerization (Figures S1C and S1D). This corroborates previous reports of AIM2 activation in response to *L. pneumophila* infections (Ge et al., 2012).

Caspase-11 activates the noncanonical pathway of NLRP3 inflammasome activation (Kayagaki et al., 2011; Rathinam et al., 2012). Therefore, we tested whether AIM2 cooperates with caspase-11 to trigger the noncanonical pathway, which induces caspase-1 proteolysis and proteolytic maturation and secretion of interleukin-1 beta (IL-1β). Compared to that in WT C57BL/6 BMDMs, *Casp11*^{-/-} BMDMs exhibited reduced caspase-1 cleavage in response to infection with *flaA*⁻ *L. pneumophila* in cells primed with LPS or TNF-α (Figures 2A and 2B). Deficiency of *Aim2* alone had little effect on caspase-1 processing; however, caspase-1 proteolysis was strongly reduced in BMDMs that were double deficient in *Aim2* and *Casp11* (Figures 2A and 2B). Similar results were observed for IL-1β processing and secretion by BMDMs, both of which depend on caspase-1 activation and proteolysis (Figures 2C and 2D). To ensure that these results were not caused by differences in microbiota or mouse genetic backgrounds, we performed experiments with littermate controls. BMDMs from *Aim2*^{-/-} *Casp11*^{+/-} mice displayed no defects in caspase-1 processing or IL-1β secretion, but those derived from *Aim2*^{-/-} *Casp11*^{-/-} mice were partially defective, and BMDMs derived from *Aim2*^{-/-} *Casp11*^{-/-} mice displayed severe impairment in both processes (Figure S2).

Figure 3. AIM2 and Caspase-11 Cooperate to Induce K $^{+}$ Efflux that Enables NLRP3-Induced Cleavage of Caspase-1 in Response to Infection

(A) Immunoblot of caspase-1 in the supernatant (SN) and cell extract (CE) of BMDMs. BMDMs pretreated for 3 hr with LPS (500 ng/mL) were either not infected (NI) or infected for 2 hr with JR32 *flaA* (MOI 20). Data are representative of three independent experiments.

(B) IL-1 β released in BMDM SN, as measured by ELISA. BMDMs pretreated for 3 hr with LPS (500 ng/mL) were infected for 4 hr with JR32 *flaA* (MOI 20). Data shown are representative of three independent experiments and are expressed as the mean of triplicate wells. Error bars depict $\pm$ SEM. * p < 0.05 versus NI.

NLRP3 Activation and K $^{+}$ Efflux Are Required for Caspase-1 Proteolysis, but Not Pore Formation, in Response to *L. pneumophila*

Caspase-11 activation facilitates the maturation of caspase-1 and the secretion of IL-1 β in response to *flaA* $^{-}$ *L. pneumophila* (Case et al., 2013). This suggests that caspase-11 plays a role in mediating the noncanonical activation of NLRP3, a process that culminates with caspase-1 cleavage and IL-1 β secretion in response to gram-negative bacteria (Kayagaki et al., 2011; Rathinam et al., 2012). Our findings indicated that AIM2 or caspase-11 is required for caspase-1 proteolysis. Therefore, we tested whether AIM2 participates in the noncanonical pathway to activate NLRP3. We initially used *flaA* $^{-}$ *L. pneumophila* to assess whether NLRP3 inflammasome activation was required for caspase-1 proteolysis in response to infection in the presence of AIM2 and caspase-11. NLRP3 activation was essential for caspase-1 proteolysis and IL-1 β secretion, regardless of the presence of caspase-11 and AIM2 (Figures 3A and 3B). In the absence of both, caspase-1 proteolysis and IL-1 β secretion were abolished, regardless of the presence of NLRP3. These results are consistent with a model in which caspase-11 or AIM2 is required to activate NLRP3. To determine whether AIM2 and NLRP3 function in two independent inflammasome types or in the same type, we infected BMDMs with *flaA* $^{-}$ *L. pneumophila* and performed immunostaining for endogenous NLRP3 and AIM2. Both AIM2 and NLRP3 foci formed in response to infection, but they did not colocalize (Figure S3). Therefore, AIM2 and NLRP3 function in discrete molecular complexes. These results support the hypothesis that AIM2 mediates a process resulting in noncanonical activation of NLRP3 via a mechanism similar to yet distinct from that of caspase-11.

To investigate whether the AIM2 inflammasome is sufficient to trigger caspase-1 proteolysis in the absence of NLRP3, we evaluated caspase-1 proteolysis in high extracellular K $^{+}$ conditions, which inhibits the activation of NLRP3, but not that of AIM2. Caspase-1 proteolysis and IL-1 β maturation and secretion were abrogated in the presence of KCl, but not NaCl, in all BMDMs tested (Figures 3C–3F). These data support a model in which NLRP3 is a key factor in caspase-1 proteolysis and IL-1 β secretion. According to this model, caspase-11 or AIM2 is required to trigger NLRP3-mediated caspase-1 cleavage and IL-1 β

(C and D) Immunoblot of caspase-1 and IL-1 β in the SN and CE of BMDMs. BMDMs pretreated for 3 hr with LPS (500 ng/mL) were NI or infected for 2 hr with JR32 *flaA* (MOI 20), in the presence of NaCl (C) or KCl (D). Data shown are representative of three independent experiments.

(E and F) IL-1 β released in BMDM SN, as measured by ELISA. BMDMs pretreated for 3 hr with LPS (500 ng/mL) were infected for 4 hr with JR32 *flaA* (MOI 20), in the presence of NaCl (E) or KCl (F). Data shown are representative of three independent experiments and are expressed as the mean of triplicate wells. Error bars depict $\pm$ SEM. * p < 0.05 versus NI.

(G) Assessment of intracellular K $^{+}$ levels, as measured by the fluorescent K $^{+}$ probe APG-2. BMDMs were NI or were infected for 2 hr with JR32 *flaA* (MOI 5). Each dot represents the percentage of APG-2 fluorescence intensity in relation to the average fluorescence of control cells, and the bars represent the mean of all analyzed cells. Error bars depict $\pm$ SEM. * p < 0.05 versus NI. Data shown are representative of three independent experiments. See also Figures S3 and S4.

Figure 4. AIM2 Triggers Caspase-1 Cleavage Independent of NLRP3 in Response to Cytosolic DNA, but NLRP3 Is Essential for AIM2-Induced Caspase-1 Cleavage in Response to *L. pneumophila* Infection

(A–D) Fluorometric quantification of PI uptake over time, expressed in RFUs. BMDMs pretreated with LPS (500 ng/mL) for 3 hr were either not infected (NI) (A), infected with JR32 *flaA* (MOI 20) (B), stimulated with 20 μM nigericin (C), or stimulated with 500 ng/mL poly(dA:dT) (D). Data are representative of three independent experiments and are expressed as the average of triplicate wells. (E and F) Immunoblots of caspase-1 in the supernatant (SN) of BMDMs. BMDMs pretreated for 3 hr with LPS (500 ng/mL) were NI or infected at the different time points with JR32 *flaA* (MOI 20) (E). As a control, BMDMs were infected with JR32 WT (MOI 20), treated with 20 μM nigericin, or stimulated with 500 ng/mL poly(dA:dT) (F). Data shown are representative of three independent experiments.

(Figures S4A and S4B). Conversely, attenuating K⁺ efflux did not affect pore formation in response to infection in either WT or *Casp11*^{-/-} BMDMs (Figures S4C–S4F), which confirmed that NLRP3 is not required for this process. In agreement with these findings, pyroptosis was not affected by the absence of NLRP3, as LDH levels and K⁺ efflux were unaffected in *Nlrp3*^{-/-} BMDMs (Figures S4G–S4I). These findings also support the hypothesis that caspase-11- or AIM2-mediated pore formation does not require NLRP3. Finally, reducing K⁺ efflux did not affect the induction of the AIM2 inflammasome, as indicated by AIM2-citrine oligomerization in response to infection with *flaA*⁻ *L. pneumophila* or transfection with double-stranded DNA (Figures S4J and S4K). Collectively, our data support a

secretion. To further test this hypothesis, we determined whether AIM2 and caspase-11 are required for K⁺ efflux in macrophages infected with *flaA*⁻ *L. pneumophila*. The intracellular K⁺ concentration decreased in response to infection, and this was independent of ASC and NLRP3 and partially dependent on AIM2 and caspase-11 (Figure 3G). We detected no K⁺ efflux in *Aim2*^{-/-}/*Casp11*^{-/-} or *Casp11*^{-/-} BMDMs (Figure 3G). These data are consistent with the hypothesis that AIM2 or caspase-11 is required for pore formation, leading to the noncanonical activation of NLRP3 via K⁺ efflux.

Our data demonstrate that NLRP3 is required to induce caspase-1 proteolysis via either AIM2 or caspase-11. Therefore, we investigated whether NLRP3 is likewise required for the functions of caspase-11 and AIM2 inflammasomes (i.e., pore formation, pyroptosis, and inflammasome assembly) in infected BMDMs. NLRP3 was not required for pore formation

model in which AIM2 or caspase-11 is required to induce pore formation and activate NLRP3. Conversely, NLRP3 is dispensable for the functions of AIM2 and caspase-11.

Activation of AIM2 Inflammasome Preferentially Induces Pore Formation, and NLRP3 Is Essential for Caspase-1 Cleavage

To evaluate the requirement of NLRP3 for caspase-1 cleavage and pore formation, we established mouse strains that are double deficient for *Nlrp3* and *Casp11* or *Nlrp3* and *Aim2*. Measuring pore formation in response to *flaA*⁻ *L. pneumophila*, we found that *Nlrp3*^{-/-}/*Casp11*^{-/-} BMDMs phenocopied *Casp11*^{-/-} cells, confirming that *Nlrp3* does not affect pore formation in response to infection (Figures 4A and 4B). We treated BMDMs with nigericin or transfected them with poly(dA:dT) to confirm that pore formation is induced by canonical activation

of NLRP3 (Figure 4C) or AIM2 (Figure 4D), as previously established (Kayagaki et al., 2011). We also confirmed that caspase-1 cleavage in response to infection depends on NLRP3. *Nlrp3*^{-/-}/*Casp1*¹¹^{-/-} and *Aim2*^{-/-}/*Nlrp3*^{-/-} BMDMs did not process caspase-1 (Figure 4E), whereas NLRC4 activation triggered caspase-1 cleavage in WT BMDMs (Figure 4F). Furthermore, caspase-1 cleavage induced by nigericin depended only on NLRP3, whereas cleavage induced by poly(dA:dT) depended only on AIM2 (Figure 4F). These data support the hypothesis that AIM2 inflammasome promotes the activation and cleavage of caspase-1 in the presence of high amounts of cytosolic DNA. However, during the physiologic response to infection, AIM2 preferentially triggers pore formation to induce caspase-1 cleavage by NLRP3.

AIM2-Induced Pore Formation Requires Caspase-1 Activity, but Not Proteolysis

NLRP3 is essential for caspase-1 proteolysis in the physiologic response to *L. pneumophila* infection. AIM2-mediated pore formation requires caspase-1 but occurs in the absence of NLRP3. Therefore, we hypothesized that AIM2 requires caspase-1 but triggers pyroptosis independently of caspase-1 proteolysis. An uncleavable yet catalytically active variant of caspase-1 (*Casp1* 6DN) is sufficient to induce pyroptosis (Broz et al., 2010). To test the hypothesis that AIM2 employs an active but uncleaved form of caspase-1 to trigger pore formation independently of caspase-11, we transduced *Casp1*¹¹^{-/-} BMDMs with a lentivirus encoding WT caspase-1 (*Casp1* WT), *Casp1* 6DN, or empty virus (vector) and then assayed pore formation. Western blot analysis confirmed that *Casp1* WT, but not *Casp1* 6DN or *Casp1* C285A, underwent autoproteolysis in response to infection with *flaA*⁻ *L. pneumophila* (Figure 5A). We then verified the caspase-1 requirements for pore formation in immortalized *Casp1*¹¹^{-/-} BMDMs that stably overexpress *Casp1* WT or *Casp1* 6DN. Overexpression of either variant failed to induce spontaneous pore formation in uninfected cells. Likewise, infection with *dotA*⁻ *L. pneumophila* did not trigger pore formation (Figures 5B–5D). In contrast, infection with WT or *flaA*⁻ *L. pneumophila* readily induced pore formation in BMDMs expressing *Casp1* WT or *Casp1* 6DN, but not empty virus (Figures 5B–5D). Similar results were obtained when we used a lentivirus to express *Casp1* WT and *Casp1* 6DN in primary macrophages from *Casp1*¹¹^{-/-} mice (Figures S5A–S5D). Overexpression of *Casp1* WT or *Casp1* 6DN did not restore pore formation in *Asc/Casp1/Casp1*¹¹^{-/-} triple-knockout BMDMs in response to infection, suggesting that the activation of uncleavable caspase-1 requires ASC (Figures S5E–S5G).

To determine whether AIM2 engages unprocessed caspase-1 to trigger pore formation in the absence of caspase-11, we transduced BMDMs from *Casp1*¹¹^{-/-} or *Aim2*^{-/-}/*Casp1*¹¹^{-/-} mice with a lentivirus encoding *Casp1* WT, *Casp1* 6DN, or vector and then assayed the cells for pore formation. We also included a lentivirus encoding the catalytically inactive form of caspase-1 (*Casp1* C285A) (Figure 5A). Overexpression of *Casp1* WT and *Casp1* 6DN restored pore formation in *Casp1*¹¹^{-/-} BMDMs infected with *flaA*⁻ *L. pneumophila* (Figures 5E–5G). In contrast, *Casp1*¹¹^{-/-} BMDMs expressing *Casp1* C285A did not trigger pore formation (Figure 5F). Therefore, cleavage is dispensable,

but the C285 active site is essential for caspase-1-induced pore formation.

When we used *Aim2*^{-/-}/*Casp1*¹¹^{-/-} BMDMs, no pores formed in response to *flaA*⁻ *L. pneumophila* (Figures 5H and 5I). In this experiment, we included infections with WT *L. pneumophila* as a positive control, because the NLRC4 inflammasome triggers caspase-1-dependent pore formation. WT *L. pneumophila* induced pore formation in *Casp1/Casp1*¹¹^{-/-} and *Aim2*^{-/-}/*Casp1/Casp1*¹¹^{-/-} BMDMs when the cells expressed *Casp1* WT or *Casp1* 6DN, but not *Casp1* C285A (Figure 5J). These results indicate that AIM2 is dispensable for NLRC4-induced pore formation, and caspase-1 activation without proteolysis is sufficient to induce pore formation via AIM2 or NLRC4 inflammasomes. These results imply that these inflammasomes engage an active, uncleaved form of caspase-1 to trigger pore formation. This process is also necessary and sufficient to induce the noncanonical activation of the NLRP3 inflammasome.

AIM2- and Caspase-11-Induced Pore Formation Contributes to the Restriction of Pathogen Replication In Vivo

We next evaluated the consequences of activating inflammasomes on the infection ability of *L. pneumophila* in macrophages and in vivo. In Legionnaires disease, NAIP5/NLRC4 (but not NLRP3) inflammasome mediates resistance to pulmonary infection (Amer et al., 2006; Case et al., 2009; Molofsky et al., 2006; Pereira et al., 2011; Zamboni et al., 2006). Therefore, we used *flaA*⁻ *L. pneumophila* to evaluate whether mice deficient in the signaling pathways that mediate pore formation are more susceptible to infection. WT and *Aim2*^{-/-}/*Casp1*¹¹^{-/-} mice were infected with *flaA*⁻ *L. pneumophila* at a very low MOI (10³ per mouse). Bacterial replication was higher in *Aim2*^{-/-}/*Casp1*¹¹^{-/-} mice than in WT mice (Figure 6A). Although loss of AIM2 or caspase-11 individually in littermate control mice did not influence the restriction of *L. pneumophila* replication, *Aim2*^{-/-}/*Casp1*¹¹^{-/-} mice were more susceptible than WT mice (Figure 6B). Because the innate immune pathways that restrict *L. pneumophila* infections in vivo are extensively redundant, these data indicate that pore formation induced by caspase-11 and AIM2 contributes to the restriction of pulmonary bacterial replication in a murine model of Legionnaires disease.

DISCUSSION

L. pneumophila is a genetically tractable bacterial pathogen that targets multiple inflammasome types. We determined that the activation of NLRP3 by *L. pneumophila* is regulated by other inflammasome types. Cell damage induced by at least one inflammasome type (NLRC4, caspase-11, or AIM2) is required and sufficient to engage NLRP3 and to independently mediate resistance to *L. pneumophila* infection.

Membrane-disrupting pathogens (e.g., *Listeria*, *Shigella*, *Francisella*, and *Rickettsia*) and many toxin-producing bacteria damage host cell membranes. Likewise, certain fungi and the protozoan parasite *Trypanosoma cruzi* cause cell damage (Silva et al., 2013; Wellington et al., 2014; Zamboni and Lima-Junior, 2015). Therefore, these pathogens may activate NLRP3 via a

Figure 5. Caspase-1 Activity, but Not Proteolysis, Is Necessary and Sufficient to Trigger AIM2-Dependent Pore Formation in Response to *L. pneumophila* Infection

(A) Immunoblot of caspase-1 in the precipitated supernatant (SN) and cell extract (CE) of BMDMs. The BMDMs from *Casp1/11*^{-/-} and *Aim2*^{-/-}/*Casp1/11*^{-/-} mice were transduced with viruses encoding empty vector (Vector), WT caspase-1 (*Casp1* WT), 6DN mutant (*Casp1* 6DN), or catalytically inactive C285A mutant (*Casp1* C285A). Cells pretreated for 3 hr with LPS (500 ng/mL) were either not infected (NI) or infected for 2 hr with JR32 *flaA* (MOI 20).

(legend continued on next page)

Figure 6. Caspase-11 and AIM2 Inflammasomes Contribute to Restriction of *L. pneumophila* Infection in Mouse Lungs

(A) WT C57BL/6 and *Aim2*^{-/-}/*Casp11*^{-/-} mice were intranasally infected with 1×10^3 *flaA*. Mice were euthanized at the indicated time points postinfection, and dilutions of lung homogenates were added to charcoal yeast extract agar plates for CFU determination. Data shown are representative of two independent experiments.

(B) *Aim2*^{+/-}/*Casp11*^{+/-}, *Aim2*^{-/-}/*Casp11*^{+/-}, *Aim2*^{+/-}/*Casp11*^{-/-} and *Aim2*^{-/-}/*Casp11*^{-/-} mice were infected as in (A). Each symbol represents a single animal, and the horizontal lines represent mean of all animals. Error bars depict $\pm$ SEM. * $p < 0.05$. Data shown are representative of two independent experiments.

process that does not first require inflammasomes to damage host cells. This hypothesis is consistent with the fact that crystals and pore-forming molecules are bona fide activators of NLRP3. Of note, the crystal- and toxin-mediated canonical activation of the NLRP3 inflammasome requires host cell priming to regulate the transcription of *Nlrp3* and the pro-forms of inflammatory cytokines (e.g., *Il1b* and *Il18*), in addition to the posttranscriptional mechanisms of NLRP3 degradation (Bauernfeind et al., 2009; Ghonime et al., 2014; Juliana et al., 2012; Mariathasan et al., 2004; Perregaux et al., 2000). Therefore, host cell priming in combination with a membrane-disrupting agent may mimic host cell infection by virulent pathogens by damaging membranes. In contrast, bona fide intracellular pathogens are usually nonlytic to host cells and may even protect cells from damage (Ashida et al., 2011; Cunha and Zamboni, 2013; Galluzzi et al., 2008; Rodrigues et al., 2012). In this case, innate immune cells may employ additional cellular components to induce cell

damage and K⁺ efflux, which is essential to activate NLRP3. As shown in this study, additional inflammasome types are activated by intracellular pathogen-associated molecular patterns and can trigger pore formation, thereby facilitating the engagement of the NLRP3 inflammasome. We have shown previously and here that flagellin/NLRC4, LPS/caspase-11, and DNA/AIM2 can induce cell damage, which enables K⁺ efflux and activates NLRP3 and caspase-1 proteolysis (Baker et al., 2015; Rühl and Broz, 2015; Schmid-Burgk et al., 2015). We found that caspase-11, NLRC4, AIM2, or a combination thereof is necessary and sufficient to trigger NLRP3 inflammasome in response to *L. pneumophila* infection. Therefore, several inflammasome types provide innate immune cells with multiple platforms from which to detect distinct cytosolic pathogens and trigger the canonical activation of NLRP3, a process that culminates in caspase-1 cleavage and inflammatory cytokine induction.

Cell membrane permeabilization and consequent pyroptosis is required for inflammasome-mediated restriction of infection by intracellular pathogens (Miao et al., 2010). Although recognition of flagellin by NAIP/NLRC4 is the main pathway triggering pyroptosis and control of *L. pneumophila* replication, NLRP3 is dispensable for restricting bacterial replication in vivo (Case et al., 2009). Our data reveal a role for AIM2 and caspase-11 in control of infection by *L. pneumophila* in vivo, suggesting that the induction of pyroptosis by these inflammasomes ensures bacterial restriction independently of their role in the downstream activation of NLRP3 (Figure 7).

Our results suggest that AIM2 operates via a different multi-protein platform than does NLRP3. We did not detect colocalization of NLRP3 and AIM2 in infected cells, indicating that two distinct inflammasome types are assembled in response to infection. These data are consistent with the fact that NLRP3 is dispensable for either the assembly of AIM2 inflammasome or AIM2-induced pore formation. Recent reports have shown the colocalization of proteins from different inflammasome types (Karki et al., 2015; Man et al., 2014). Therefore, the lytic activities of pathogens may simultaneously activate the two types of inflammasomes, leading to the colocalization of AIM2 and NLRP3. In contrast, *L. pneumophila* did not activate NLRP3 in the absence of caspase-11, AIM2, or NLRC4, which suggests that at least one of these inflammasome types is required to activate NLRP3. This temporal delay in inflammasome activation may not favor the recruitment of AIM2 and NLRP3 in the same protein complex. Therefore, our data support a model in which AIM2 and NLRP3 are assembled in two distinct inflammasome types.

An important aspect of caspase-1 activation that we have demonstrated here and others have shown is that caspase-1 can function in an active but uncleaved form (Broz et al., 2010; Guey et al., 2014; Van Opdenbosch et al., 2014). Active,

(B–J) Fluorometric quantification of PI uptake over time, expressed in RFUs. *Casp1/11*^{-/-} immortalized bone-marrow-derived macrophages (iBMDMs) transduced with vector (B), *Casp1* WT (C), or *Casp1* 6DN (D) and pretreated with LPS (500 ng/mL) for 3 hr were not infected (NI) or were infected with JR32 WT, JR32 *flaA*, or JR32 *dotA* (MOI 20). *Casp1/11*^{-/-} BMDMs transduced with the different caspase-1 constructs were pretreated with LPS (500 ng/mL) and either NI (E) or were infected at MOI 20 with JR32 *flaA* (F), or JR32 WT (G). *Aim2*^{-/-}/*Casp1/11*^{-/-} BMDMs transduced with the different caspase-1 constructs were pretreated with LPS (500 ng/mL) and either NI (H) or were infected at MOI 20 with JR32 *flaA* (I) or JR32 WT (J). Data shown are representative of three independent experiments.

See also Figure S5.

Figure 7. AIM2 Engages Active but Uncleaved Caspase-1 to Trigger the Noncanonical Activation of NLRP3 Inflammasome

Schematic of noncanonical activation of the NLRP3 inflammasome, which occurs via membrane damage caused by upstream inflammasomes, including the AIM2 and caspase-11. Activation of AIM2 inflammasome during infection leads to pore formation via a process that requires the activation, but not cleavage of caspase-1. Pore formation and pyroptosis lead to infection control. The membrane damage that is induced by the AIM2 inflammasome further triggers K⁺-efflux-mediated activation of NLRP3 inflammasome, a process that culminates in caspase-1 proteolysis and the secretion of the inflammatory cytokines IL-1 β and IL-18.

uncleaved caspase-1 is recruited by NLRC4 and AIM2 to trigger pore formation and pyroptosis. These inflammasomes function in cells that express a catalytically null form of caspase-1. In contrast, once recruited by the NLRP3 inflammasome, caspase-1 undergoes proteolytic cleavage and may become less available to AIM2. In addition to producing inflammatory cytokines, NLRP3 may negatively regulate the activation of AIM2, which is important for the induction of pyroptosis. Therefore, NLRP3 may represent a key regulator of the balance between the induction of inflammatory cell death and that of inflammatory cytokines.

EXPERIMENTAL PROCEDURES

Further details and an outline of resources used in this work can be found in [Supplemental Experimental Procedures](#).

Experimental Model Details

Institutional Permission

All mice were bred and maintained under specific-pathogen-free conditions in the Animal Facilities of the Medical School Ribeirão Preto (FMRP-USP). The care of the mice was in compliance with institutional guidelines on ethics in animal experiments approved by CETEA (Comissão de Ética em Experimentação Animal da Faculdade de Medicina de Ribeirão Preto; protocol number 130/2014).

Mice

We used male and female 8- to 12-week-old C57BL/6 mice, including WT (Jackson Laboratory, stock number 000664), *Casp11*^{-/-} (Kayagaki et al., 2011), *Casp11/11*^{-/-} (Kuida et al., 1995), *Casp11/11*^{-/-}/*Casp11*^{Tg} (Kayagaki et al., 2011) (herein called *Casp1*^{-/-}), *Aim2*^{-/-} (Jones et al., 2010), *Aim2*^{-/-}/*Casp11*^{-/-} (Rathinam et al., 2010), *Asc*^{-/-} (Sutterwala et al., 2006), *Nlrp3*^{-/-} (Lara-Tejero et al., 2006), and *Nlrp3*^{-/-} (Mariathasan et al., 2006) mice in these experiments. *Asc*^{-/-}/*Casp11/11*^{-/-}, *Aim2*^{-/-}/*Casp11/11*^{-/-}, *Nlrp3*^{-/-}/*Casp11*^{-/-}, and *Aim2*^{-/-}/*Nlrp3*^{-/-} mice were generated in-house by intercrosses. To ensure a similar genetic background between experimental mice, we crossed *Aim2*^{-/-}/*Casp11*^{-/-} mice with C57BL/6 mice and with *Aim2*^{-/-} and *Casp11*^{-/-} mice to obtain *Aim2*^{+/-}/*Casp11*^{+/-}, *Aim2*^{-/-}/*Casp11*^{+/-}, and *Aim2*^{+/-}/*Casp11*^{-/-} mice, respectively.

Bone-Marrow-Derived Macrophages

BMDMs were obtained as previously described (Marim et al., 2010). Briefly, mice were euthanized and bone marrow cells were harvested from the femurs and differentiated in RPMI 1640 (Sigma) containing 20% fetal bovine serum (FBS), 30% L-929 cell-conditioned media (LCCM), 1% L-glutamine (200 mM), and 1% penicillin-streptomycin (100 U/mL; Sigma). BMDMs were harvested and seeded on tissue culture plates 1 day before infection and maintained in RPMI 1640 media containing 10% FBS and 5% LCCM. All cells used in this study were cultivated at 37°C with 5% CO₂.

In Vivo Infections

For *L. pneumophila* infections, mice were anesthetized with ketamine and xylazine (50 mg/kg and 10 mg/kg, respectively) by intraperitoneal injection, followed by intranasal inoculation with 40 μ L PBS containing 10³ JR32 *flaA*⁻ per mouse. To determine the number of colony-forming units, mice were euthanized, the lungs harvested and homogenized in sterile water. Lung homogenates were then diluted in sterile water and plated on CYE agar plates for colony-forming unit (CFU) determination.

Quantification and Statistical Analysis

Data were plotted and analyzed with GraphPad Prism 7.0 software. Statistical significance was determined using a two-tailed, unpaired t test. An F test was performed to evaluate significant difference in variance between the compared groups. The number of replicate wells and the number of experiments performed are described in the figure legends. Differences were considered statistically significant when the p value was less than 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.06.086>.

AUTHOR CONTRIBUTIONS

L.D.C. and D.S.Z. designed the experiments. L.D.C., A.L.N.S., and J.M.R. performed the experiments and analyzed the data. D.P.A.M., G.F.S.Q., and L.L.S. performed and analyzed specific experiments. R.A.F. provided reagents and discussed hypotheses. L.D.C. and D.S.Z. wrote the manuscript, and all authors reviewed and approved the final text.

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“Peripheral NLRC4 inflammasome participates in the genesis of acute inflammatory pain”

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Peripheral NLR4 inflammasome participates in the genesis of acute inflammatory pain

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Abstract

Inflammatory hyperalgesia is a complex process that depends on the sensitization of primary nociceptive neurons triggered by proinflammatory mediators, such as interleukin 1 β (IL-1 β). Recently, the peripheral activation of caspase-1 (previously known as IL-1 β -converting enzyme) was implicated in the induction of acute inflammatory pain by promoting the processing of IL-1 β from its precursor form, pro-IL-1 β . Caspase-1 activation in several systems requires the assembly of an intracellular molecular platform called an inflammasome. Inflammasomes consist of 1 nucleotide-binding oligomerization domain-like receptor (NLR), the adapter molecule apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC), and caspase-1. NLRP3 and NLR4 inflammasomes are well described. However, the identity of the inflammasome that is involved in the peripheral activation of caspase-1 that accounts for acute inflammatory hyperalgesia has not been described. The present findings demonstrated that mice deficient in NLR4 or ASC, but not in NLRP3, present reduced mechanical and thermal acute inflammatory hyperalgesia induced by carrageenan. The reduced hyperalgesia was accompanied by significant impairments in the levels of mature forms of IL-1 β (p17) and caspase-1 (p20) compared to wild-type mice at the inflammatory site. Therefore, these results identified the inflammasome components NLR4 and ASC as the molecular platform involved in the peripheral activation of caspase-1 and IL-1 β maturation, which are responsible for the induction of acute inflammatory pain. In conclusion, our study provides new therapeutic targets for the control of acute inflammatory pain.

Keywords: Inflammatory pain, Inflammasome, IL-1 β , Caspase-1, NLR4, NLRP3

1. Introduction

Inflammatory pain is a common clinical symptom of inflammatory diseases characterized by hyperalgesia due to the sensitization of primary nociceptive neurons (PNN).⁴³ It is well established that mediators (eg, prostaglandins and sympathomimetic amines) released into the inflammatory site are responsible for the direct sensitization of PNN.^{18,32,43}

The production of prostaglandins and sympathomimetic amines is preceded by the release of several pronociceptive cytokines.^{10,53} Among the pronociceptive cytokines, tumor necrosis factor- α (TNF- α), keratinocyte-derived chemokine (KC/CXCL1)¹⁰ and interleukin 1 β (IL-1 β) seem to be the most relevant in the induction of prostaglandin production.¹⁰ Furthermore, chemokines are responsible for the stimulation of the sympathomimetic component

of inflammatory hyperalgesia.¹⁰ Notably, peripheral TNF- α and KC/CXCL1 mediate inflammatory hyperalgesia due to their ability to promote neutrophil recruitment to inflammatory sites whereas IL-1 β seems to act on neutrophils to induce the production of prostaglandins that ultimately sensitize PNN.^{11–13} Notwithstanding, these cytokines can act directly on receptors expressed by PNN causing their sensitization.^{3,27,56}

As mentioned above, IL-1 β is an important pronociceptive cytokine that is responsible for the induction of inflammatory hyperalgesia.¹⁹ Therefore, the control of IL-1 β production/release is a promising therapeutic target of great relevance.⁵³ IL-1 β is synthesized intracellularly as the inactive cytokine pro-IL-1 β (p35; 35 kDa). Pro-IL-1 β requires cleaving by cysteine protease to yield the biological active form, IL-1 β (p17; 17 kDa).⁷ Caspase-1 is the well-known cysteine protease involved in the processing of IL-1 β .^{7, 55}

Caspase-1 is synthesized as an inactive zymogen (pro-caspase-1 p45; 45 kDa), and it becomes proteolytically active when the controlled oligomerization of several different molecules occur.⁴² This assembly process generates an intracellular molecular platform called an inflammasome, which promotes the auto-cleavage of pro-caspase-1 to generate active caspase-1 (p20; 20 kDa).^{4,15} Inflammasome complexes are composed of intracellular nucleotide-binding oligomerization domain-like receptors (NLRs), the adapter molecule apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC), and caspase-1. NLRP3 and NLR4 inflammasomes are well described.^{39,41} NLRP3 seems to be activated primarily by microbes and cell stress products, and NLR4 has been described to operate when neuronal apoptosis inhibitory proteins (Naip) sense bacterial-derived products such as flagellin.^{41,39,38}

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Recently, we have shown that caspase-1 is involved in the peripheral processing of IL-1 β , and it accounts for the genesis of carrageenan-induced acute inflammatory hyperalgesia.¹⁴ However, the identity of the inflammasome that is involved in the peripheral activation of caspase-1 and IL-1 β maturation and accounts for the induction of acute inflammatory hyperalgesia induced by carrageenan has not been investigated.

The hypothesis that caspase-1 participates in the genesis of inflammatory hyperalgesia was based primarily on the use of mice deficient in caspase-1.¹⁴ Nevertheless, further studies described that these mice are also deficient in caspase-11.²⁹ Based on this evidence, this study investigated the participation of the NLRP3 and NLRC4 inflammasomes in the genesis of acute inflammatory hyperalgesia and focused on their role in the peripheral maturation of IL-1 β . The dependence of inflammatory pain on caspase-1 and/or caspase-11 was also investigated.

2. Materials and methods

2.1. Animals

The experiments were performed using male C57BL/6 mice (wild type, WT, 20–25 g) and mice deficient in the following proteins: double caspase-1 and 11 knockout ($\text{Casp1/11}^{-/-}$) mice ($\text{Casp1/11}^{-/-}$) (previously described as caspase-1 null mice),²⁹ $\text{Casp1/11}^{-/-}$ that had caspase-11 transgenically introduced to become caspase-1 single knockout mice ($\text{Casp1}^{-/-}/\text{Tg Casp11}$),²⁹ single Caspase-11 $\text{Casp11}^{-/-}$ mice ($\text{Casp11}^{-/-}$), interleukin 1 receptor type 1 (IL-1R1 $\text{IL-1R1}^{-/-}$), NLRP3 (NLRP3 $\text{NLRP3}^{-/-}$), NLRC4 (NLRC4 $\text{NLRC4}^{-/-}$), and ASC (ASC $\text{ASC}^{-/-}$). $\text{Casp1/11}^{-/-}$ ³¹ and NLRC4 $\text{NLRC4}^{-/-}$ ³³ mice were previously described and backcrossed to C57BL/6 mice for 8 generations. ASC $\text{ASC}^{-/-}$ mice were previously described and backcrossed to C57BL/6 mice for 9 generations.³³ These deficient mice were backcrossed until 10 generations to the C57BL/6 at the animal facilities at the Medical School of Ribeirão Preto. NLRP3 $\text{NLRP3}^{-/-}$ mice were constructed in the C57BL/6 background.⁴⁰ The $\text{Casp1}^{-/-}/\text{Tg Casp11}$ mice were created by the introduction of a Casp11 BAC transgene into $\text{Casp1/11}^{-/-}$ embryos.⁵⁴ The mice were housed in the animal care facility of the Ribeirão Preto Medical School and taken to the testing room at least 1 hour before experiments. Food and water were available *ad libitum*. All behavioral tests were performed between 9:00 AM and 5:00 PM. Animal care and handling procedures were in accordance with the guidelines of the International Association for the Study of Pain (IASP) on the use of animals in pain research. Approval for ethical animal care and use was obtained before this study commenced (Process No. 149/2011).

2.2. Mechanical nociceptive paw test

Mechanical hyperalgesia was tested using an electronic version of the von Frey test in mice as reported previously.⁹ Mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors in a quiet room 15 to 30 minutes before the start of testing. The test consisted of evoking a hind paw flexion reflex with a handheld force transducer (electronic esthesiometer; IITC Life Science, Woodland Hills, CA) adapted with a 0.5-mm² polypropylene tip. The investigator (blinded to group allocation) was trained to apply the tip perpendicularly to the central area of the plantar hind paw with a gradual increase in pressure. The gradual increase in pressure was manually performed in blinded experiments. The upper limit pressure was 15 g. The end point was characterized by the removal of the paw followed by clear flinching movements. The intensity of the pressure that elicited paw withdrawal was automatically recorded, and the final value for the

response was obtained by averaging 3 measurements. The animals were tested before and after treatments. The results are expressed as the nociceptive withdrawal threshold (in grams, mean of 3 measurements) before and at the indicated times after drug or solvent (control) injections. The nociceptive withdrawal threshold of naive mice was 8.5 ± 0.2 g (mean ± SEM; n = 30) before injection of the solvent or hypernociceptive agents.

2.3. Thermal nociceptive paw test (Hargreaves test)

The latency of paw withdrawal to a radiant heat stimulus was measured using a plantar test (model 400; IITC Life Science) as described previously.²⁵ Mice can move freely in this apparatus in which plastic boxes are placed on an elevated glass surface. Mice were acclimated for 1 hour before testing until they became calm and motionless. A calibrated infrared light source of high intensity was applied perpendicularly to the plantar surface of the hind paw of each mouse. The end point was characterized by the removal of the paw followed by clear flinching movements. Latency to paw withdrawal was automatically recorded. Each hind paw was tested alternately with an interval of 5 minutes for 4 trials.

2.4. Reagents

The following drugs were used in this study: prostaglandin E2 (PGE2) (P5640 from Sigma-Aldrich, St. Louis, MO), nigericin (N7143 from Sigma-Aldrich), IL-1 β (NIBSC; South Mimms, Hertfordshire, United Kingdom), and carrageenan (FMC, Philadelphia, PA). A stock solution of PGE2 (1 $\mu\text{g}/\mu\text{L}$) was prepared in 10% ethanol, and dilutions were made in 0.9% NaCl (saline); the final concentration of ethanol was 1%.

2.5. Cytokine measurements using enzyme-linked immunosorbent assays

Animals were terminally anesthetized 3 hours after an intraplantar (i.pl.) injection of carrageenan or saline, and the skin tissues of the plantar region were removed from the injected and control paws (saline). The samples were triturated and homogenized in 500 μL of the appropriate buffer (phosphate-buffered saline containing 0.05% Tween 20, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 kallikrein international units of aprotinin A) followed by a 10-minute centrifugation at 2000g. The supernatants were used to determine the levels of IL-1 β , TNF- α , and CXCL1/KC using ELISA Duo-Set R&D Systems following the manufacturer's instructions (Mouse IL-1 beta/IL-1F2 DY401; Mouse TNF- α DY210; Mouse CXCL1/KC DY453). The results are expressed as picograms (pg) of each cytokine per paw.

2.6. Determination of tissue myeloperoxidase activity

Neutrophil migration to the hind paw plantar tissues of mice was evaluated using a myeloperoxidase (MPO) kinetic colorimetric assay as previously described.^{13,52} Samples of subcutaneous plantar tissue were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide and kept at -80°C until use. Samples were homogenized using a Polytron (PT3100) and centrifuged at 16,100g for 4 minutes. The resulting supernatant was assayed for MPO activity spectrophotometrically at 450 nm (SpectraMax, Molecular Devices, San Francisco, CA), with 3 readings in 1 minute. The MPO activity of samples was compared with a standard curve of neutrophils. Briefly, 10 μL of the sample was mixed with 200 μL of 50 mM phosphate buffer, pH

6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The results are presented as MPO activity (number of neutrophils per milligram of tissue).

2.7. Western blot analysis

Mice were terminally anesthetized 5 hours after an i.pl. injection of carrageenan or saline, and skin tissues were removed from the plantar region of paws. The samples were homogenized, and the expression of the immature form of pro-IL-1 β (~35 kDa), the mature form of IL-1 β (~17 kDa), the immature form of pro-caspase-1 (~45 kDa), and the mature form of caspase-1 (~20 kDa) were evaluated using Western blot analyses. Briefly, samples were homogenized in a lysis buffer containing a mixture of proteinase inhibitors (Tris-HCl 50 mM, pH 7.4; NP-40 1%; Na-deoxycholate, 0.25%; NaCl, 150 mM; EDTA, 1 mM; PMSF, 1 mM; aprotinin, leupeptin, and pepstatin, 1 μ g/mL each). Proteins were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE-15%) and trans-blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). The membranes were blocked with 5% dry milk (overnight) and incubated overnight at 4°C with a rabbit polyclonal antibody against caspase-1 (1:300; Sigma-Aldrich, Saint Louis, MO), goat polyclonal antibody against IL-1 β (1:200, I3767; Sigma-Aldrich), and anti-ASC, pAb (AL177) (1:1000; Adipogen, San Diego, CA). The membranes were washed and incubated for 2 hours at room temperature with an HRP-conjugated secondary antibody (1:10,000; Jackson ImmunoResearch, Philadelphia, PA). Immunodetection was performed using an enhanced chemiluminescence light-detecting kit (Amersham Pharmacia Biotech) for 1 minute. A mouse monoclonal antibody against β -actin (1:10,000; Sigma-Aldrich) was used for loading controls. Western blot runs were performed with protein samples from saline-injected WT mouse paws and carrageenan-injected paws of WT, NLRC4 $^{-/-}$, ASC $^{-/-}$, NLRP3 $^{-/-}$, and Casp1/11 $^{-/-}$ mice. Images were used as representative blots. Densitometric data were measured after normalization to the control (house-keeping gene) using Scientific Imaging Systems (Image Lab 3.0 software; Biorad Laboratories, Hercules, CA).

2.8. Statistical analysis

Data are reported as the mean $\pm$ SEM and are representative of 2 separate experiments. Two-way analysis of variance followed by Bonferroni *t* test was used to compare groups when the

hyperalgesic responses were measured at different times after the stimulus injection. Alternatively, when the responses (thermal hyperalgesia, neutrophil migration and cytokine production, western blotting) were measured once after the stimulus injection, the differences were evaluated by 1-way analysis of variance followed by Bonferroni *t* test (for 3 or more groups) comparing all pairs of columns. The *P* values <0.05 were considered significant. Statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, CA).

3. Results

3.1. IL-1R1 and caspase-1 mediates mechanical inflammatory hyperalgesia

It was shown previously that the local or systemic treatment of experimental animals with an IL-1 receptor antagonist (IL-1ra) reduced acute inflammatory hyperalgesia.^{8,14} The current results confirmed the importance of IL-1 β in the genesis of acute inflammatory hyperalgesia because the mechanical hyperalgesia induced by carrageenan was reduced in IL-1R1 $^{-/-}$ mice compared with WT mice (Fig. 1A).

As mentioned before, our previous study that described the involvement of caspase-1 in the induction of acute inflammatory hyperalgesia used Casp1/11 $^{-/-}$ mice.¹⁴ Herein, inflammatory hyperalgesia induced by carrageenan was induced in Casp1 $^{-/-}$ /Tg Casp11 mice in an attempt to elucidate whether caspase-1 is indeed involved in the genesis of inflammatory hyperalgesia or whether this phenotype was because of the lack of caspase-11. Corroborating our previous study,¹⁴ carrageenan-induced mechanical inflammatory hyperalgesia was reduced in Casp1/11 $^{-/-}$ mice when compared with WT mice (Fig. 1B). Furthermore, carrageenan-induced mechanical inflammatory hyperalgesia was also reduced in Casp1 $^{-/-}$ /Tg Casp11 mice to the same level compared with Casp1/11 $^{-/-}$ mice (Fig. 1B). In agreement, it was observed that single Casp11 $^{-/-}$ mice developed similar mechanical inflammatory hyperalgesia compared with WT mice (Fig. 1C). Altogether, these results confirm the specific involvement of caspase-1 in the induction of acute inflammatory hyperalgesia induced by carrageenan.

3.2. Acute inflammatory hyperalgesia is not dependent on the NLRP3 inflammasome

Next, we investigated the identity of the inflammasomes that are involved in the peripheral activation of caspase-1, which

Figure 1. Acute inflammatory hyperalgesia depends on IL-1-R and caspase-1 but not caspase-11. (A) Mechanical nociceptive threshold was measured before (BL) and after (1, 3, and 5 hours) intraplantar injection of carrageenan (Cg: 100 μ g/paw) or vehicle (Sal-saline) in wild type (WT) or IL-1R $^{-/-}$ mice. (B) Mechanical nociceptive threshold was measured before (BL) and after (1, 3, and 5 hours) intraplantar injection of Cg (100 μ g/paw) or vehicle in WT, Casp1/11 $^{-/-}$ or Casp1 $^{-/-}$ /Tg Casp11 mice. (C) Mechanical nociceptive threshold was measured before (BL) and after (1, 3, and 5 hours) intraplantar injection of Cg (100 μ g/paw) or vehicle in WT or Casp11 $^{-/-}$ mice. Data are expressed as mean $\pm$ SEM, 5 mice per group. #Indicates statistical significance (*P* < 0.05) compared with the saline paw injected group. *Indicates statistical significance (*P* < 0.05) compared with the WT group injected with Cg. Two-way analysis of variance followed by the Bonferroni test. ns = no significance.

accounts for the genesis of acute inflammatory hyperalgesia. The NLRP3 inflammasome was tested first. The mechanical nociceptive threshold of NLRP3^{-/-} naive mice did not differ from the threshold of WT naive mice (Fig. 2A). NLRP3^{-/-} mice also presented mechanical hyperalgesia induced by carrageenan similarly to WT mice (Fig. 2A). The thermal inflammatory hyperalgesia induced by carrageenan was also similar in NLRP3^{-/-} mice compared with WT mice (Fig. 2B). Neutrophil migration triggered by carrageenan is not altered in NLRP3^{-/-} mice compared with WT mice (Fig. 2C). Furthermore, the expression of active caspase-1 (p20 kDa; Fig. 2D) and mature IL-1 β (p17 kDa; Fig. 2E) induced by i.pl. carrageenan was similar in NLRP3^{-/-} and WT mice. These results indicate that the NLRP3 inflammasome is not involved in the peripheral activation of caspase-1 and IL-1 β maturation, and consequently, it did not participate in the induction of carrageenan-induced inflammatory hyperalgesia.

3.3. Acute inflammatory hyperalgesia depends on the NLRC4/ASC inflammasome

The observation that the NLRP3 inflammasome is not involved in the induction of the acute inflammatory hyperalgesia prompted us to consider whether another important inflammasome, NLRC4 and its adapter molecule ASC, might play a role. The mechanical nociceptive threshold of NLRC4^{-/-} naive mice was similar to WT naive mice (Fig. 3A). However, mechanical inflammatory hyperalgesia induced by carrageenan was reduced in NLRC4^{-/-} mice compared with WT mice (Fig. 3A). The mechanical nociceptive threshold of ASC^{-/-} naive mice was similar to the WT mice, but carrageenan-induced acute mechanical inflammatory pain was reduced in ASC^{-/-} mice compared with WT mice (Fig. 3B). Importantly, thermal inflammatory hyperalgesia induced by carrageenan was also reduced in NLRC4^{-/-} and ASC^{-/-} mice compared with WT mice (Fig. 3C). Interestingly, paw injection of carrageenan upregulated ASC expression in a time-dependent manner (Fig. 3D). Paw tissue samples from ASC^{-/-} mice were used as a negative control.

Figure 2. The NLRP3 inflammasome is not involved in the induction of acute inflammatory hyperalgesia. (A) Mechanical nociceptive threshold was measured before (BL) and after (1, 3, and 5 hours) intraplantar injection of carrageenan (Cg; 100 μ g/paw) or vehicle (Sal-saline) in wild-type (WT) or NLRP3^{-/-} mice. (B) Thermal nociceptive threshold was measured before (BL) and after (3 hours) intraplantar injection of Cg (100 μ g/paw) or vehicle (Sal-saline) in WT or NLRP3^{-/-} mice. (C) MPO activity was determined in the WT or NLRP3^{-/-} mice plantar tissue 5 hours after Cg or saline injection. (D and E) WT and NLRP3^{-/-} mice received an intraplantar injection of carrageenan (100 μ g/paw) or saline. At 5 hours after Cg injection, plantar tissue samples were collected, and the level of mature (~20 kDa) and immature forms (~45 kDa) of caspase-1 and mature (~17 kDa) and immature forms (~35 kDa) of IL-1 β were determined using Western blot analysis. Representative blot and relative quantification. Beta actin levels were used as an internal control. Data are expressed as mean $\pm$ SEM, 5 mice per group. #Indicates statistical significance ($P < 0.05$) compared with the saline paw injected group. No statistical significance (ns; $P < 0.05$) was identified between WT and NLRP3^{-/-} mice groups that received Cg. Two-way or 1-way analysis of variance followed by Bonferroni test.

Figure 3. The NLRC4 inflammasome and its adapter molecule ASC participate in acute inflammatory hyperalgesia. (A) Mechanical nociceptive threshold was measured before (BL) and after (1, 3, and 5 hours) intraplantar injection of carrageenan (Cg; 100 μ g/paw) or vehicle (Sal-saline) in wild-type (WT) or NLRC4^{-/-} mice. (B) Mechanical nociceptive threshold was measured before (BL) and after (1, 3, and 5 hours) intraplantar injection of carrageenan (Cg; 100 μ g/paw) or vehicle (Sal-saline) in wild-type (WT) or ASC^{-/-} mice. (C) Thermal nociceptive threshold was measured before (BL) and after (3 hours) intraplantar injection of Cg (100 μ g/paw) or vehicle (Sal-saline) in WT, NLRC4^{-/-}, or ASC^{-/-} mice. (D) ASC (25 kDa) expression was determined in the paw samples from WT (1, 3, and 5 hours) and ASC^{-/-} (5 hour) mice after intraplantar injection of Cg (100 μ g/paw) or saline. Beta-actin levels were used as an internal control. Representative blot and relative quantification. Data are expressed as mean $\pm$ SEM of 5 animals per group. #Indicates statistical significance ($P < 0.05$) compared with the group injected with saline. *Indicates statistical significance ($P < 0.05$) compared with the WT group injected with Cg. Two-way or 1-way analysis of variance followed by Bonferroni test.

3.4. The NLRC4/ASC inflammasome is neither involved in the peripheral production of pronociceptive cytokines (TNF- α and CXCL1/KC) nor neutrophil migration during acute inflammation

We investigated the role of the NLRC4/ASC inflammasome in the production of pronociceptive cytokines, TNF- α , and KC/CXCL1 and in the recruitment of neutrophils to inflammatory sites to delineate the mechanisms by which the NLRC4/ASC inflammasome mediates carrageenan-induced acute inflammatory hyperalgesia. The intraplantar injection of carrageenan increased the levels of TNF- α , KC/CXCL1 and the infiltration of neutrophils into WT mice paws; these results were not different in NLRC4^{-/-} and ASC^{-/-} mice (Figs. 4A–C).

3.5. The NLRC4/ASC/caspase-1 inflammasome mediates the peripheral maturation of IL-1 β during acute inflammation

We tested the hypothesis that the NLRC4/ASC inflammasome mediates acute inflammatory hyperalgesia through its role in caspase-1 activation and IL-1 β processing. Western blotting analyses of paw samples showed that the expression of the active form of caspase-1 (caspase-1 p20) and mature IL-1 β (p17; ~17 kDa) increased in WT mice after carrageenan injection and

was reduced in NLRC4^{-/-} and ASC^{-/-} mice (Figs. 5A and B). The increase in the production of mature IL-1 β was also reduced in Casp1/11^{-/-} mice, which confirms our previous results.¹⁴ Notably, the increase in the production of pro-IL-1 β (p35; ~35 kDa) triggered by peripheral acute inflammation appeared similar in NLRC4^{-/-}, ASC^{-/-}, and Casp1/11^{-/-} mice compared with WT mice (Fig. 5A). Supernatants of LPS-primed macrophages activated with nigericin⁴⁴ were used as a positive control for the mature form of caspase-1 (Fig. 5B).

The intraplantar injection of IL-1 β and PGE2 produced mechanical hyperalgesia in ASC^{-/-} and NLRC4^{-/-} mice, and these mice exhibited similar intensities and durations as WT mice (Figs. 6A and B). These results support the hypothesis that the NLRC4/ASC inflammasome mediates carrageenan-induced acute inflammatory hyperalgesia through a mechanism that is dependent on peripheral caspase-1 activation and IL-1 β maturation.

4. Discussion

The production of proinflammatory cytokines at the inflammatory site mediates inflammatory hyperalgesia.⁵³ Among the peripheral pronociceptive cytokines, IL-1 β is one of the most important.^{10,53} This study established the molecular mechanisms involved in the

Figure 4. Carrageenan-induced pronociceptive cytokines production and neutrophil infiltration are not mediated by NLRC4/ASC inflammasome. Wild-type (WT), NLRC4^{-/-}, or ASC^{-/-} mice received an intraplantar injection of carrageenan (Cg; 100 µg/paw) or saline. At 3 hour after Cg injection, plantar tissue samples were collected, and the levels of (A) TNF-α and (B) KC/CXCL1 were determined using enzyme-linked immunospecific assay. (C) MPO activity was determined in mice plantar tissue 3 hours after Cg (100 µg/paw) injection in WT, NLRC4^{-/-}, or ASC^{-/-}. Data are expressed as mean ± SEM of 5 animals per group. #Indicates statistical significance ($P < 0.05$) compared with the group injected with saline. No statistical significance (ns; $P < 0.05$) was identified between WT, ASC^{-/-}, or NLRC4^{-/-} mice groups that received Cg. One-way analysis of variance followed by Bonferroni test.

production of the mature form of IL-1β that accounted for the genesis of acute inflammatory hyperalgesia. For example, the peripheral stimulation of the NLRC4/ASC inflammasome platform, but not the NLRP3 inflammasome, was involved in the cascade of events that finally promoted the production of the active form of IL-1β and acute inflammatory hyperalgesia. Additionally, the NLRC4/ASC inflammasome assembly triggered specific caspase-1-dependent inflammatory hyperalgesia, but the caspase-11-dependent noncanonical pathway was not involved.

IL-1β was the first cytokine implicated in the genesis of pain processes.¹⁹ The peripheral injections of small doses of IL-1β induce mechanical hyperalgesia in the rat paw through

a prostaglandin-dependent mechanism.¹⁹ In this context, IL-1β became an interesting target in the control of inflammatory diseases, including inflammatory hyperalgesia. In fact, the pharmacologic blockade of IL-1R1 with IL-1ra reduces inflammatory hyperalgesia.^{8,10} The results of this study confirmed previous studies because IL-1R1 null mice presented reduced inflammatory hyperalgesia compared with their littermates. Notably, IL-1β-targeting therapies are clinically effective in different inflammatory conditions associated with severe pain, such as rheumatoid arthritis, gout, and other autoimmune diseases.²¹

The discovery that the production of the biologically active form of IL-1β from pro-IL-1β cleavage is dependent on intracellular

Figure 5. The NLRC4/ASC inflammasome is involved in caspase-1 activation and peripheral maturation of IL-1β in carrageenan-induced paw inflammation. Wild-type (WT), ASC^{-/-}, or NLRC4^{-/-} mice received an intraplantar injection of carrageenan (Cg; 100 µg/paw) or saline. At 5 hours after Cg injection, plantar tissue samples were collected, and the levels of the (A) mature (~17 kDa) and immature forms (~35 kDa) of IL-1β and the (B) mature (~20 kDa) and immature forms (~45 kDa) of caspase-1 were determined using Western blot analyses. Representative blot and relative quantification. Beta-actin levels were used as an internal control. Samples from casp1/11^{-/-} mice were also used as a negative control. Supernatant samples from LPS plus nigericin-stimulated macrophages were used as a positive control of mature caspase-1 (C⁺). Data are expressed as mean ± SEM, 5 mice per group. #Indicates statistical significance ($P < 0.05$) compared with the group injected with saline. *Indicates statistical significance ($P < 0.05$) compared with the WT group injected with Cg. One-way analysis of variance followed by the Bonferroni test.

Figure 6. IL-1 β and PGE2 induced mechanical hyperalgesia in NLRC4 $^{-/-}$ and ASC $^{-/-}$ mice similar to wild-type (WT) mice. Mechanical nociceptive threshold was measured before (BL) and after (1, 1.5, and 3 hours) intraplantar injection of (A) IL-1 β (1 ng/paw), (B) PGE2 (100 ng/paw) or vehicle (Sal-saline) in WT, NLRC4 $^{-/-}$, or ASC $^{-/-}$ mice. Data are expressed as mean $\pm$ SEM, 5 mice per group. #Indicates statistical significance ($P < 0.05$) compared with the saline paw injected group. No statistical significance ($P < 0.05$) was identified between WT, ASC $^{-/-}$, and NLRC4 $^{-/-}$ mice groups that received Cg. Two-way analysis of variance followed by Bonferroni test.

cysteine protease, caspase-1, prompted the development of caspase-1-targeting therapies.^{16,24,35} We have shown that peripheral caspase-1 activation mediates acute inflammatory pain through the maturation of IL-1 β .¹⁴ This conclusion was based on data obtained with caspase-1 null mice. However, subsequent studies have shown that these mice also have the caspase-11 gene removed.^{29,54} Nevertheless, the present results confirmed that acute inflammatory hyperalgesia is controlled specifically by caspase-1 but not caspase-11 because Casp1/11 $^{-/-}$ and Casp1 $^{-/-}$ /Tg Casp11 mice exhibited similar reductions in carrageenan-induced acute inflammatory hyperalgesia. Moreover, single Casp11 $^{-/-}$ mice developed similar inflammatory hyperalgesia when compared with WT mice. Corroborating these findings is the fact that a selective inhibitor of caspase-1 also reduced acute inflammatory hyperalgesia in our previous study.¹⁴ These results strongly support the peripheral participation of caspase-1 in the mediation of carrageenan-induced acute inflammatory hyperalgesia.

Knowledge of the mechanisms related to caspase-1 activation and the maturation of IL-1 β has increased in the last years.⁴ Currently, it is described that caspase-1 activation and IL-1 β processing require the assembling of inflammasome platforms, which subsequently promotes caspase-1 activation.⁵¹ The NLRP3 inflammasome is the best characterized inflammasome that has been most implicated as a sensor of sterile injury.^{51,6} NLRP3 is proposed as the sensor of a wide range of structurally diverse DAMPs.⁴⁹ For example, both monosodium urate and calcium pyrophosphate crystals, which are inflammatory drivers of gout and pseudogout, activate NLRP3 inflammasome.⁴¹ These crystals are also suggested to be a general inflammatory signal that is released from dead and dying cells.¹⁷ Based on these data, NLRP3 was suggested to play an important role in carrageenan-induced acute inflammatory hyperalgesia. Surprisingly, NLRP3 $^{-/-}$ mice present similar acute inflammatory hyperalgesia as WT mice. The behavioral data were further supported by the similar expression of mature caspase-1 and IL-1 β in samples from NLRP3 $^{-/-}$ and WT mice.

In contrast, the peripheral NLRC4 inflammasome assembly was implicated in the genesis of carrageenan-induced acute mechanical and thermal inflammatory hyperalgesia. Peripheral NLRC4 inflammasomes seem to mediate this process through the activation of caspase-1 and the maturation of IL-1 β . In fact, the mature forms of caspase-1 and IL-1 β did not increase in the inflamed NLRC4 $^{-/-}$ and ASC $^{-/-}$ mice paws compared with WT mice, but the production of pro-IL-1 β was not changed. The production of TNF- α and CXCL1/KC chemokine and the recruitment of neutrophils were similar in NLRC4 $^{-/-}$ and ASC $^{-/-}$

mice during acute inflammation, which confirmed IL-1 β maturation as the mechanism by which the NLRC4 inflammasome participated in carrageenan-induced inflammatory pain. Remarkably, the release of TNF- α and CXCL1/KC is an upstream event related to IL-1 β production in the cascade of cytokines that mediate carrageenan-induced inflammatory pain in mice.¹⁰ Furthermore, TNF- α and CXCL1/KC chemokines mediate acute inflammatory pain because they are involved in the recruitment of neutrophils to the inflammatory site, but IL-1 β seems to act on recruited neutrophils to promote their activation and to stimulate the production of prostaglandins.^{10,13,14} These data are consistent with a recent finding showing that caspase-1 does not control the release of TNF- α and CXCL1/KC and neutrophil migration to the inflammatory site.¹⁴ Finally, NLRC4 $^{-/-}$ and ASC $^{-/-}$ mice developed similar intensities of hyperalgesia triggered by IL-1 β and PGE2 as WT mice, which indicated that the NLRC4 inflammasome is the upstream of these mediators and that NLRC4 $^{-/-}$ and ASC $^{-/-}$ mice did not have intrinsic problems with presenting inflammatory hyperalgesia. The importance of peripheral caspase-1 activation was also identified in a rat fracture model of complex regional pain syndrome.³⁶ However, caspase-1 action is triggered by NLRP1 in this model,³⁶ which suggests differences in the activated inflammasome depending on the stimuli that trigger pain.

Important questions emerging from our results are the mechanisms involved in the peripheral activation of the NLRC4 inflammasome and in which cell type this activation occurs. Classically, NLRC4 is an important cytosolic member of the NLR family, which is an indirect sensor of bacterial flagellin and bacterial type III secretion systems, and responds by assembling an inflammasome complex that promotes caspase-1 activation and interacts with ASC.^{39,57} The activation of NLRC4 by bacterial products seems to be dependent on apoptosis inhibitory proteins (NAIPs), which engage NLRC4 after interacting directly with flagellin or T3SS components, such as PrgJ.^{30,57} In certain cases, NLRC4 activation requires Naip5 because Naip5 $^{-/-}$ mice fail to activate NLRC4 or caspase-1 in response to infection with *Legionella pneumophila* or the C-terminus of flagellin.^{37,38} Nevertheless, acute inflammatory hyperalgesia developed similar to C57BL/6 mice in A/J mice, which have a defective Naip5 signaling (data not shown). Therefore, it is plausible that NLRC4 might be activated by endogenous stimuli because our experimental model did not include infectious stimuli.

One alternative explanation for NLRC4 activation in the present experimental model could be that extracellular stimuli might trigger intracellular signaling pathways that culminate with NLRC4 activation. Data in the literature showed that NLRC4 is

activated by phosphorylation at the Ser533 residue.⁴⁵ Furthermore, screening with kinase inhibitors identified PKC δ as a candidate for NLRC4 phosphorylation.⁴⁵ Therefore, it is plausible that extracellular signaling through PKC δ could be involved in the activation of NLRC4 that accounts for acute inflammatory pain.

Regarding the resident paw cells responsible for carrageenan-induced IL-1 β production and maturation, there is evidence showing that in mice paws, keratinocytes are the main source of caspase-1.³⁶ Thus, they could be involved in carrageenan-induced IL-1 β production and inflammatory hyperalgesia. Nevertheless, these processes could be mediated by more than 1 cell type. For instance, there are data showing that inflammasome complex can be secreted and process IL-1 β in the extracellular space.^{2,20} Thus, we could not discard the possibility that, in carrageenan-induced paw inflammation, inflammasome components can be produced by 1 cell type and pro-IL-1 β by another one.

Although the present results suggest that the peripheral NLRC4 assembly is important for the induction of carrageenan-induced inflammatory pain, we cannot exclude the possibility that central NLRC4 might play a role. Indeed, inflammatory pain is mediated by the production of IL-1 β at the level of the dorsal root ganglion and spinal cord.^{34,47,48} In addition, functional NLRC4 inflammasome has been shown in microglia of the central nervous system.²⁶ Therefore, further studies are necessary to elucidate whether central inflammasomes also play a role in the genesis of inflammatory pain.

Although the present findings might be specific for carrageenan-induced inflammatory hyperalgesia, they are important because IL-1 β processing could be mediated in an inflammasome-independent way including metalloproteases, elastases, and proteinases.^{50,21,22,28} Furthermore, there are data showing that mutations in inflammasomes components (including NLRC4) might trigger autoinflammatory diseases in which pain is an important symptom.^{5,46,1,23} In addition, carrageenan is one of the most well-described models of inflammatory pain and a model that is often used for the development of novel analgesic and anti-inflammatory drugs. Therefore, knowledge of the pathophysiologic mechanisms of this model potentially could be quite helpful in the development of novel analgesic drugs.

In summary, this study provides novel evidence that the peripheral assembly of the NLRC4/ASC inflammasome, but not NLRP3, participates in the genesis of carrageenan-induced acute inflammatory hyperalgesia. Furthermore, the NLRC4/ASC inflammasome mediates this process through the activation of caspase-1 and the maturation of IL-1 β , but it is not involved in the production of proinflammatory cytokines (TNF- α , CXCL1/KC) or in the recruitment of neutrophils. These results add new information about the physiopathologic mechanisms of inflammatory pain. Finally, from a prospective point of view, this study suggests that inhibition of NLRC4 assembly or caspase-1 might be effective in the control of acute inflammatory hyperalgesia without affecting other protective mechanisms of inflammation such as the production of proinflammatory cytokines and neutrophil migration.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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