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Materials and Methods

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Mice and *in vivo* challenges

Wild-type C57BL/6 (Jackson Laboratory), *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ (*23*, *24*), *Casp1*$^{-/-}$ *Casp11*$^{129mt/129mt}$ referred to as *Casp1*$^{-/-}$*Casp11*$^{-/-}$ (*25*), *Casp11*$^{-/-}$ (*3*), *Tlr4*$^{lps\text{-}del/lps\text{-}del}$ referred to as *Tlr4*$^{-/-}$ (Jackson # 007227) mice were used in this study. Mice were housed in a specific pathogen–free facility. All protocols were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill and met guidelines of the US National Institutes of Health for the humane care of animals.

For study of lethal endotoxia, mice were challenged via intraperitoneal (i.p.) injection with the indicated quantities of LPS in OptiMEM. To prepare LPS challenge doses, serial dilutions of 1mg/mL LPS were carried out, where each dilution was allowed to equilibrate at room temperature for at least 5 minutes before further dilution. In some experiments, mice were primed with LPS (400μg/kg) or poly(I:C) (10μg/kg) for 6-8 hours. In COX-1 inhibition experiments, mice were given SC-560 (5 mg/kg, Calbiochem) 1 hour prior to LPS challenge. In some experiments, rectal temperatures were recorded at the indicated time points using a MicroTherma 2T thermometer (Braintree Scientific) with a lubricated RET-3 probe. The presence of blood in the intestinal lumen was confirmed using the Sure-Vue Fecal Occult Blood Test (Guaic test). Mice were considered moribund when unable to right themselves.

Bacterial growth conditions

All bacterial strains were grown in Luria-Bertani medium (LB) overnight at 37°C, except for *L. monocytogenes* and *F. novicida*, which were grown in BHI at 30°C or BHI + 0.1% cystine at 37°C, respectively.

Bacterial lysates and lipid A preparation

For generation of bacterial lysates, 1mL of overnight culture normalized to an OD_{600} of 1.0 was pelleted, resuspended in 50μL PBS, and boiled 10 minutes. Insoluble lysate components were pelleted at 20,000 x *g* for 10 minutes, and the supernatant was collected. In some experiments, supernatants were treated with DNase (10μg/mL), RNase (10μg/mL)**,** Proteinase K (100μg/mL), and/or lysozyme (100μg/mL) for 3 hours, or adjusted to contain 30% ammonium hydroxide, incubated overnight at room temperature, air-dried, and resuspended in PBS.

LPS was extracted essentially as previously described (Westphal O, Journal of medicinal and pharmaceutical chemistry 1961). Briefly, 500ml overnight cultures were pelleted at 5000 x*g* for 10 minutes. Bacterial pellets were then resuspended in 40mL of sterile water and 90% aqueous phenol at a 1:1 ratio. Mixtures were incubated in a rotating oven at 65°C for 1 hour. Samples were then cooled on ice for 5 minutes and centrifuged at 3000 x*g* for 30 minutes. The

aqueous layer was transferred to a new tube. 20mL of sterile water was then added to the phenol layer, mixed, and the aqueous layer extracted a second time. The aqueous fractions were combined and then dialyzed for 24 hours against water in 1000 MWC tubing. After dialysis, insoluble material was removed by centrifugation at 17,000 x*g* for 20 minutes. The LPS sample was then frozen and lyophilized overnight. The dried sample was resuspended in 10mL of 10mM Tris, pH 8.0 containing 25μg/mL RNaseI and 100μg/mL DNaseI and incubated for 2 hours at 37°C. To digest protein, Proteinase K was added at 100μg/mL and the incubation was continued at 37°C for 2 additional hours. 5mL of water-saturated phenol was then added and the mixture was centrifuged at 3000 x*g* for 30 minutes. The aqueous layer was removed and dialyzed for 24 hours against water in 1000 MWC tubing. The final LPS sample was then frozen and lyophilized overnight.

To liberate lipid A from the core and O-antigen of LPS, 500μL of 10mM sodium acetate + 1% SDS was added to the dried LPS sample. This mixture was incubated at 100°C for 1 hour. This sample was then frozen and lyophilized overnight. The dried sample was resuspended in 100 μL of water along and then added to 1mL of acidified ethanol (100 μL 4N HCl in 20 mL of 95% ethanol). This sample was then centrifuged at 5000 x*g* for 5 minutes and the supernatant discarded. The sample was then washed with 1mL of 95% ethanol 3 times (centrifugation at 5000 x *g* for 5 minutes between washes, supernatant discarded) to remove residual SDS. Following the last centrifugation step, the remaining pellet was resuspended in 500μL of water and lyophilized overnight.

Contaminating lipids were removed following the protocol of Folch *et al*. (Folch J, JBC, 1957) Briefly, lipid A sample was resuspended 1-2% in chloroform/methanol (2:1). Samples were vortexed for 1 minute and then centrifuged at 10,000 x*g* for 10 minutes at 4°C. Supernatants were removed and then this extraction step was repeated. The remaining pellet was suspended in 500μL of water and lyophilized overnight.

Contaminating proteins were removed following the protocol of Hirschfeld *et al.* (Hirschfeld, JI, 2000). Briefly, 5mg of lipid A was resuspended in 1mL of water containing 0.2% triethylamine (TEA). Deoxycholate (DOC) was added to a final concentration of 0.5% and followed addition of 500μL of water-saturated phenol. Samples were vortexed for 5 minutes and then the phases were allowed to separate at room temperature for 5 minutes. Samples were cooled on ice and then centrifuged at 10,000 x*g* for 2 minutes. The aqueous layer was removed to a new tube and the phenol layer was reextracted with 500μL of water containing 0.2%TEA and 0.5% DOC. The aqueous layers were pooled and adjusted to contain a final concentration of 75% ethanol and 30mM sodium acetate. The sample was then allowed to precipitate at -20°C for 1 hour. Precipitates were centrifuged at 10,000 x*g* for 10 minutes at 4°C, washed in 100% ethanol and then air-dried. The precipitates were then resuspended in 500μL of water containing 0.2% TEA and lyophilized overnight.

Liposome preparation and transfections

Bacterial lysate transfection complexes were generated as follows: For transfection of $5x10^4$ BMMs, 0.25μL lysate suspended in 25μL OptiMEM and 0.25μL Lipofectamine 2000 suspended in 25μL were allowed to equilibrate at room temperature for 5 minutes. Suspensions were mixed and incubated at room temperature for 20 minutes. For LPS and lipid A transfection complexes, 75ng of LPS or lipid A suspended in 2μL OptiMEM and 375ng DOTAP suspended in 2μL OptiMEM were allowed to equilibrate at room temperature for 5 minutes. Suspensions

were the mixed and incubated for 30 minutes at room temperature. Reaction volumes were then brought up to 50μL with OptiMEM.

Macrophage culture, treatment, and analysis of inflammasome activation

BMMs were prepared as described (*26*). For experiments, macrophages were seeded into 96-well tissue culture treated plates at a density of $5x10^4$ cells/well. When indicated, macrophages were primed with lipopolysaccharide (50 ng/ml), poly(I:C) (1μg/mL) or IFN-γ (8 ng/ml) overnight. For infections, culture media was replaced with OptiMEM and then bacteria were added to BMMs at MOI 200 (*F. novicida*) or MOI 5 (*L. monocytogenes*), centrifuged for 5 min at 200 x *g*, and incubated at 37°C. After 1 hour, extracellular bacterial growth was stopped by addition of 20 μg/ml gentamicin. In some experiments, 1μg/mL LPS or lipid A was included during the infection. For transfections, culture media was replaced with OptiMEM and then LPS and lysate transfection complexes were added to BMMs and centrifuged for 5 min at 200 x *g*. For CTB experiments, macrophages were primed for 5h with poly(I:C) and Pam_3CSK_4 (100μg/mL). Media was replaced with OptiMEM containing poly(I:C), Pam_3CSK_4, and combinations of CTB, LPS, and PrgJ as indicated in the figure legends. Supernatant samples were collected at the indicated time points. Cytotoxicity was defined as the percentage of total lactate dehydrogenase released into the supernatant and was determined as described (*27*). IL-1β secretion was determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems). PrgJ was purified as described (*11*). *S. minnesota* LPS was obtained from List Biologicals. *E. coli* O111:B4 LPS was from Sigma.

Complementation and knockdown of *Casp1* and *Casp11*

Bone marrow derived macrophages were immortalized (iBMM) as described (*28*). For complementation of *Casp1* and *Casp11* in $Casp1^{-/-}Casp11^{-/-}$ iBMMs, macrophages were transduced with pMXsIP (*29*) derived retrovirus carrying *Casp1* or *Casp11*.

Western blots

Total protein from lysates and supernatants of 5×10^4 cells was analyzed by Western blot. Caspase-11 content and processing was analyzed using anti-caspase-11 antibody (17D9, Novus) diluted 1:500. Caspase-1 processing was determined using an anti-caspase-1 antibody (clone 4B4, Genentech). Blots were stripped and equivalent loading of protein was ensured by Western blot using anti-β-actin HRP antibody (Cat. # 20272, AbCam) diluted 1:20,000.

Supplementary Figure Legend

Fig. S1. Schematic of canonical and non-canonical inflammasome pathways. The canonical inflammasomes (shown here: NLRP3, AIM2, and NLRC4) activate caspase-1, whereas a hypothetical non-canonical inflammasome activates caspase-11. Both caspase-1 and caspase-11 initiate pyroptosis. Via its CARD domain, NLRC4 can directly bind caspase-1 to mediate its activation. In contrast, Pyrin domain containing inflammasomes, such as NLRP3 and AIM2,

cannot directly bind caspase-1; rather, they interact indirectly via the adaptor protein ASC, which polymerizes into a structure called the ASC focus. Caspase-1 processes IL-1β and IL-18 to their mature secreted forms in the ASC focus. In contrast, caspase-11 does not process IL-1β alone, rather it activates the NLRP3/ASC/caspase-1 pathway (arching arrow). In the absence of *Nlrc4* and *Asc*, macrophages are unable to signal via any known canonical inflammasome pathways; however, they retain the ability to activate caspase-11, which functions independently of the canonical inflammasomes. *Casp1*$^{-/-}$*Casp11*$^{-/-}$ macrophages, on the other hand, are deficient in all inflammasome pathways.

Fig. S2. Upregulation of caspase-11 potentiates pyroptosis but not proteolytic processing. (**A**, left) Overexposure of caspase-11 western blot from Fig. 1F reveals minor processing of caspase-11 in LPS primed C57BL/6 BMMs 2 hours after transfection with LPS. (**A**, right) *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ BMMs (2 x 10^5 cells in 5μL endotoxin free water + protease inhibitor) were subjected to three freeze thaw cycles. Insoluble components were pelleted and supernatants were then incubated for 5 minutes at 37°C. SDS-sample buffer was added to the reaction and then processed for western blotting. Significant spontaneous processing was observed in the lysates. Interpretation of (**A**, left): Although the minor processed band observed after LPS transfection could represent caspase-11 processing in response to cytoplasmic LPS, it could also be due to spontaneous processing that occurs in the media after pyroptotic cell lysis. (**B**) Upregulation of full-length caspase-11 in unprimed BMMs, or BMMs primed overnight with LPS (50ng/mL), poly(I:C) (1000ng/mL), or IFN-γ (8ng/mL), as revealed by western blot.

Supplementary Table:

Name of Strain	Designation	Notes	Reference
S. typhimurium	ATCC 14028s	wild type	www.atcc.org
B. thailandensis	E264	wild type	www.atcc.org
P. aeruginosa	PAO1	wild type	Joseph Mougous
UPEC	ATCC 19110	wild type	www.atcc.org
C. rodentium	ATCC 51459	wild type	www.atcc.org
S. flexneri	M90T	wild type	Alan Aderem
L. monocytogenes	10403S		Dan Portnoy
L. monocytogenes Δ*hly*			Dan Portnoy
L. innocua			www.atcc.org
B. subtilis			Tony Richardson
S. aureus	ATCC25923	wild type	www.atcc.org
S. pyogenes	MGAS-5005	wild type	Tony Richardson
E. faecalis	V583	wild type	Tony Richardson
F. novicida	U112	wild type	www.nwrce.org
F. novicida lpxF	TBE264	*lpxF* mutant, hexa-acyl lipid A	(*14*)
F. novicida flmK	TBE229	*flmK* mutant, tetracyl lipid A	(*16*)
Y. pestis	KIM6	WT	Joe Hinnebusch

Table S1. Strain list.