Dynamin-related Irgm proteins modulate LPS-induced caspase-11 activation and septic shock

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Abstract

Inflammation associated with gram-negative bacterial infections is often instigated by the bacterial cell wall component lipopolysaccharide (LPS). LPS-induced inflammation and resulting life-threatening sepsis are mediated by the two distinct LPS receptors TLR4 and caspase-11 (caspase-4/-5 in humans). Whereas the regulation of TLR4 activation by extracellular and phago-endosomal LPS has been studied in great detail, auxiliary host factors that specifically modulate recognition of cytosolic LPS by caspase-11 are largely unknown. This study identifies autophagy-related and dynamin-related membrane remodeling proteins belonging to the family of Immunity-related GTPases M clade (IRGM) as negative regulators of caspase-11 activation in macrophages. Phagocytes lacking expression of mouse isoform *Irgm2* aberrantly activate caspase-11-dependent inflammatory responses when exposed to extracellular LPS, bacterial outer membrane vesicles, or gram-negative bacteria. Consequently, *Irgm2*-deficient mice display increased susceptibility to caspase-11-mediated septic shock *in vivo*. This *Irgm2* phenotype is partly reversed by the simultaneous genetic deletion of the two additional *Irgm* paralogs *Irgm1* and *Irgm3*, indicating that dysregulated *Irgm* isoform expression disrupts intracellular LPS processing pathways that limit LPS availability for caspase-11 activation.

Keywords autophagy; caspase-4; IRGM; lipopolysaccharide; noncanonical inflammasome

Subject Categories Autophagy & Cell Death; Immunology; Microbiology, Virology & Host Pathogen Interaction

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Introduction

Severe sepsis is an overwhelming microbe-induced inflammatory response resulting in organ dysfunction and constitutes a major public health concern due to high mortality rates and lack of treatment options (Polat *et al*, 2017). A chief sepsis-inducing agent is lipopolysaccharide (LPS, or endotoxin), an abundant building block of the cell wall of all gram-negative bacteria (Munford, 2008; Simpson & Trent, 2019). Extracellular and endocytosed LPS is sensed by the transmembrane receptor toll-like receptor 4 (TLR4), which signals to induce the expression of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF α) and pro-interleukin-1 beta (pro-IL-1 β) (Rosadini & Kagan, 2017). Cytosolic LPS on the other hand is sensed by the cysteine protease caspase-11 (caspase-4 and caspase-5 in humans), which upon direct binding to LPS oligomerizes and proteolytically activates the cell death-inducing protein gasdermin D (GSDMD) (Hagar *et al*, 2013; Kayagaki *et al*, 2013; Shi *et al*, 2014, 2015; Liu *et al*, 2016). GSDMD forms pores in the plasma membrane, causing pyroptosis directly as well as indirectly through potassium efflux-mediated activation of the canonical NLRP3 inflammasome (Ruhl & Broz, 2015; Shi *et al*, 2015; Liu *et al*, 2016). Activated caspase-1, the defining component of canonical inflammasomes, cleaves numerous substrates including pro-IL-1 β to produce mature IL-1 β for secretion (Broz & Dixit, 2016; Brewer *et al*, 2019).

Whereas regulation of TLR4 signaling has been extensively studied, mechanisms that prevent excessive caspase-11-induced inflammation are just beginning to be understood (Rathinam *et al*, 2019). The host-derived oxidized phospholipid oxPAPC as well as stearyl lysophosphatidylcholine competitively block binding of LPS to caspase-11 (Chu *et al*, 2018; Li *et al*, 2018). However, oxPAPC was also shown to function as a caspase-11 agonist in dendritic cells and skews LPS-primed macrophages toward pro-inflammatory

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metabolism (Zanoni *et al*, 2016, 2017; Di Gioia *et al*, 2020). Additional known checkpoints on caspase-11-triggered inflammation operate downstream from cytosolic LPS sensing and involve inhibition of caspase oligomerization, reduction in membrane lipid peroxidation and membrane repair by the ESCRT III pathway (Kang *et al*, 2018; Ruhl *et al*, 2018; Choi *et al*, 2019).

Similar to the limited number of studies exploring regulatory mechanisms that operate downstream from cytosolic LPS sensing, investigators only recently began to characterize the upstream pathways that control LPS accessibility to the cytosolic compartment (Rathinam *et al*, 2019). Phagocytosis of bacterial LPS-replete outer membrane vesicles (OMVs) triggers potent activation of caspase-11 (Vanaja *et al*, 2016). The mechanism by which LPS derived from endocytosed OMVs escapes into the cytosol to be detected by caspase-11 is not understood but depends on the function of interferon (IFN)-inducible host guanylate binding proteins (Gbps) (Finethy *et al*, 2017). Gbps are additionally required for fast-kinetics caspase-11 activation following cell transfection with purified LPS (Pilla *et al*, 2014), implying a role for Gbps in caspase-11 activation downstream from LPS release into the host cell cytosol. In support of this model, we recently demonstrated that human GBP1 binds directly to LPS and aggregates LPS *in vitro* (Kutsch *et al*, 2020), indicating a direct role for Gbps in cytosolic LPS recognition.

A second family of dynamin-like proteins, the Immunity-related GTPases (IRGs), bind to various intracellular membrane compartments, where they ostensibly modulate membrane dynamics (Pilla-Moffett *et al*, 2016), including fusion of autophagosomes with lysosomes (Kumar *et al*, 2018). IRGs are produced in response to IFNs as well as other immune stimuli including LPS-TLR4 signaling and mediate several immune programs in response to those agents (Coers, 2013; Pilla-Moffett *et al*, 2016). Variants of the human IRG M clade encoding *IRGM* gene are associated with increased risk for Crohn's Disease, as first demonstrated by two large genome wide association studies (Parkes *et al*, 2007; Weersma *et al*, 2009). Subsequent candidate gene association studies expanded the association of human *IRGM* variants to increased susceptibility to mycobacterial infection (Intemann *et al*, 2009; King *et al*, 2011), and most pertinently, poor outcomes from sepsis (Kimura *et al*, 2014). While these studies linked the *IRGM* gene to inflammatory diseases, the cellular mechanism by which IRGM suppresses inflammation during infection and the causative genetic alteration in human *IRGM* disease-associated alleles remain poorly defined.

The human genome encodes a single *IRGM* gene, which expresses at least 5 distinct hIRGM splice isoforms. The mouse genome on the other hand encodes 3 paralogous *Irgm* genes, named *Irgm1*, *Irgm2*, and *Irgm3* (Bekpen *et al*, 2005). The exact functional relationship between hIRGM isoforms and their mouse *Irgm* orthologs is currently not well defined. However, several recent studies demonstrated that hIRGM and mouse *Irgm1* commonly regulate cellular processes that include mitochondrial dynamics (Singh *et al*, 2010; Liu *et al*, 2013; Henry *et al*, 2014; Schmidt *et al*, 2017), autophagic flux (Gutierrez *et al*, 2004; Singh *et al*, 2006; Traver *et al*, 2011) and NLRP3 inflammasome activation (Mehto *et al*, 2019). The parallels between human and mouse IRGMs extend beyond these cell culture phenotypes: similar to hIRGM, *Irgm1* attenuates intestinal inflammation (Liu *et al*, 2013; Rogala *et al*, 2018) and promotes host survival during LPS-induced sepsis (Bafica *et al*, 2007). Increased mortality among endotoxemic *Irgm1*^{-/-} mice is

largely due to hyperresponsive TLR4 signaling in these animals, which leads to augmented TNF α production and inflammation (Bafica *et al*, 2007; Schmidt *et al*, 2017). Whereas the anti-inflammatory function of *Irgm1* is now firmly established, possible immune-modulatory functions of the two other *Irgm* paralogs have remained unexplored.

In this study, we systematically assessed the role of individual *Irgm* proteins in shaping endotoxemic inflammation and discovered a novel function for *Irgm2* as an inhibitor of caspase-11 activation following LPS ingestion by macrophages. Accordingly, *Irgm2*-deficient mice display caspase-11-dependent elevated IL-1 β serum levels and more readily succumb to LPS-induced sepsis. Unexpectedly, the simultaneous deletion of all *Irgm* paralogs partially restores immune homeostasis, thus revealing inter-regulatory relationships among *Irgm* proteins important for balancing inflammation during gram-negative infections.

Results

Irgm1* and *Irgm2* but not *Irgm3* limit the production of inflammatory cytokines in response to LPS *in vivo

Mice deficient for *Irgm1* and *Irgm3* were previously reported (Taylor *et al*, 2000; Collazo *et al*, 2001). To systematically dissect the physiological function of all three mouse *Irgm* isoforms, we generated an *Irgm2*-deficient mouse strain. Lack of *Irgm2* protein expression was confirmed by Western blotting and immunofluorescence (Appendix Fig S1A and B). To profile the *in vivo* function of individual *Irgm* isoforms during endotoxemia, we injected wild-type C57BL/6J (WT), *Irgm1*^{-/-}, *Irgm2*^{-/-}, and *Irgm3*^{-/-} mice with LPS at a concentration of 8 mg/kg bodyweight, collected serum at 4 h post-injection (hpi), and performed multiplex quantitative serum cytokine measurements. Whereas the LPS-induced cytokine profile of *Irgm3*^{-/-} mice was comparable to WT mice, serum concentrations of several cytokines were elevated in *Irgm1*^{-/-} and *Irgm2*^{-/-} relative to WT (Figs 1A and EV1). Consistent with the previously reported role for *Irgm1* as an attenuator of TLR4 signaling (Bafica *et al*, 2007), we detected significantly elevated TNF α serum concentrations in LPS-treated *Irgm1*^{-/-} mice. Notably, *Irgm2*^{-/-} mice exhibited WT-like TNF α serum levels but increased serum levels for the inflammasome-dependent cytokines IL-1 α and IL-1 β (Figs 1A and EV1). To validate our multiplex data and to further assess a potential role for *Irgm2* in regulating inflammasome-dependent cytokine production *in vivo*, we injected WT, *Irgm1*^{-/-}, *Irgm2*^{-/-}, and *Casp1*^{-/-}*Casp11*^{-/-} mice with LPS and assayed serum IL-18, an additional inflammasome-processed cytokine, as well as IL-1 β and TNF α concentrations via ELISA. We detected elevated concentrations of IL-1 β and IL-18 but not of TNF α in the serum of LPS-injected *Irgm2*^{-/-} mice by ELISA (Fig 1B), confirming our initial observations and thus implicating *Irgm2* in the suppression of LPS-induced inflammasome activation *in vivo*.

***Irgm2* suppresses inflammasome activation in LPS-treated macrophages**

To define the mechanism by which *Irgm2* regulates cytokine production, we employed bone marrow-derived macrophages

Figure 1. Irgm1 and Irgm2 but not Irgm3 limit the production of inflammatory cytokines in response to LPS *in vivo*.

A WT, *Irgm1*^{-/-}, *Irgm2*^{-/-}, and *Irgm3*^{-/-} mice ($n = 4$ mice/genotype) were injected *i.p.* with LPS (8 mg/kg). Serum was collected 4 h post-injection (hpi) and concentration of various cytokines determined via a preconfigured Luminex multiplex panel. Relative concentration (fold change relative mean of WT) is shown for the indicated cytokines (absolute cytokine concentrations of same experiment are shown in Fig EV1).

B WT ($n = 9$), *Irgm1*^{-/-} ($n = 7$), *Irgm2*^{-/-} ($n = 9$), and *Casp1*^{-/-}*Casp11*^{-/-} ($n = 7$) mice were injected *i.p.* with LPS (8 mg/kg). Serum was collected 4 hpi and concentration of IL-1 β , IL-18, and TNF α was measured via ELISA.

Data information: Data shown are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for indicated comparisons or comparison between WT and indicated genotype by one-way ANOVA with Dunnett's multiple comparison test. N.S., non significant.

(BMMs) as an established tissue culture model to study LPS-dependent IL-1 β secretion. Inflammasome activation in BMMs and consequential secretion of IL-1 β is dependent on two signals: the first signal is provided by extracellular damage- or pathogen-associated molecular patterns (DAMPs or PAMPs), such as LPS, that induce the expression of inflammasome components and cytokine proforms; cytoplasmic PAMPs or DAMPs act as a second signal to trigger inflammasome assembly (Broz & Dixit, 2016). To provide the first signal, we primed WT, *Irgm2*^{-/-}, and *Casp1*^{-/-} *Casp11*^{-/-} BMMs with LPS. In all genotypes, LPS priming induced WT levels of pro-IL-1 β and TNF α mRNA and also induced WT-like TNF α secretion, indicating that TLR4 signaling is unchanged in *Irgm2*^{-/-} BMMs (Fig 2A and B). Unexpectedly, 24 h priming with extracellular LPS at 1 μ g/ml was sufficient to induce robust secretion of IL-1 β and IL-18 in *Irgm2*^{-/-} but not WT BMMs (Fig 2B). We considered the hypothesis that *Irgm2*^{-/-} BMMs no longer required a second signal and would therefore secrete IL-1 β in response to a broad range of first signals. However, we found that among several tested TLR agonists only LPS was sufficient to trigger IL-1 β and IL-18 secretion in *Irgm2*^{-/-} BMMs (Fig 2C), suggesting that extracellular LPS, in the absence of Irgm2 expression, serves as both the first and second signal to activate inflammasomes.

To further test the hypothesis that Irgm2 blocks inflammasome-dependent cellular responses, we monitored pyroptotic cell death in LPS-primed cells. In congruence with our cytokine data, LPS priming resulted in significantly higher rates of cell death in *Irgm2*^{-/-} compared with WT BMMs (Fig 2D). Ectopic expression of Irgm2 in

Irgm2^{-/-} immortalized BMMs (iBMMs) restored cell viability to WT iBMM levels (Appendix Fig S2A and B), ruling out the possibility that the death phenotype is due to bystander mutations associated with the *Irgm2* loss-of-function allele. Deletion of the inflammatory caspases, caspase-1 and caspase-11, restored cell viability and abolished IL-1 β /IL-18 secretion in LPS-primed *Irgm2*^{-/-} BMMs (Fig 2D), indicating that Irgm2 expression suppresses canonical inflammasome activation by extracellularly supplied LPS. To directly visualize canonical inflammasome formation in BMMs, we stained cells for the canonical inflammasome adapter protein Apoptosis-associated Speck-like protein containing a CARD (ASC). During canonical inflammasome assembly, ASC polymerizes into a large bioactive protein complex that is visible as a single large speck per individual cell (Broz & Dixit, 2016). Consistent with our predictions, loss of Irgm2 enhanced LPS-induced ASC speck formation (Fig 2E) and increased the amount of autoproteolytically processed caspase-1 p20 (Fig 2F). Collectively, these observations reveal a novel role for Irgm2 in mitigating inflammasome responses.

Irgm2 suppresses caspase-11 activation in response to LPS and bacterial infections

Because LPS alone is sufficient to trigger inflammasome-mediated cellular responses in *Irgm2*^{-/-} BMMs, we hypothesized that the IFN-inducible cytosolic LPS sensor caspase-11 was activated by extracellularly supplied LPS in cells deficient for Irgm2. In support of this hypothesis, we noticed that IFN γ priming accelerated the

Figure 2.

Figure 2. Irgm2 suppresses inflammasome activation in LPS-treated macrophages.

- A qPCR measurement of IL-1 β and TNF- α mRNA levels in WT and *Irgm2*^{-/-} BMMs following 8-h stimulation with LPS (1 μ g/ml).
- B WT, *Irgm2*^{-/-}, and *Casp1*^{-/-}*Casp11*^{-/-} BMMs were treated for 24 h with LPS at indicated doses and supernatant TNF α , IL-1 β , and IL-18 was measured by ELISA.
- C IFN γ -primed WT and *Irgm2*^{-/-} BMMs were treated with LPS, Pam3CSK4, poly(I:C), or a combination of Pam3CSK4 and poly(I:C) (1 μ g/ml for all treatments) for 24 h and cell supernatant IL-1 β and IL-18 concentrations were assessed by ELISA.
- D WT, *Irgm2*^{-/-}, *Casp1*^{-/-}*Casp11*^{-/-}, and *Irgm2*^{-/-}*Casp1*^{-/-}*Casp11*^{-/-} BMMs were treated with LPS (1 μ g/ml) and IL-1 β , IL-18, and LDH release were assessed at 24 h post-treatment (hpt).
- E IFN γ -primed WT, *Irgm2*^{-/-}, *Casp1*^{-/-}*Casp11*^{-/-} and *Irgm2*^{-/-}*Casp1*^{-/-}*Casp11*^{-/-} BMMs were treated with LPS (5 μ g/ml) for 4 h and subsequently stained with anti-ASC antibody and Hoechst stain (DNA/nuclei). Representative images are shown with white arrows pointing at ASC specks. Number of ASC specks per nuclei was quantified. Scale bars: 20 μ m.
- F IFN γ -primed WT, *Irgm2*^{-/-}, *Casp1*^{-/-}*Casp11*^{-/-} and *Irgm2*^{-/-}*Casp1*^{-/-}*Casp11*^{-/-} BMMs were treated with LPS (1 μ g/ml) for 24 h and cell lysates and supernatants collected. Protein levels in cell lysates (caspase-1, and actin) and supernatants (caspase-1 p20) were visualized via immunoblotting.

Data information: Graphs in (A–E) show means $\pm$ SEM from $n = 3$ independent experiments. *** $P < 0.001$ for indicated comparisons or comparison between WT and indicated genotype by two-way ANOVA with Sidak's (A, C) or Tukey's (B, D, E) multiple comparisons test. Images in panel E are representative of one of three independent experiments. Panel F is representative of $n = 2$ independent experiments. N.S., non significant.

Source data are available online for this figure.

kinetics of pyroptotic cell death in *Irgm2*^{-/-} BMMs (Appendix Fig S3A and B). However, to test our hypothesis directly, we monitored inflammasome-dependent responses in BMMs obtained from cross-bred *Irgm2*^{-/-}*Casp11*^{-/-} mice. Deletion of caspase-11 in *Irgm2*^{-/-} BMMs repressed ASC foci formation, restored cell viability, and abrogated LPS-elicited IL-1 β /IL-18 secretion (Fig 3A and B). Together, these data show that extracellularly supplied LPS is able to robustly activate the cytosolic LPS sensor caspase-11 when cells lack Irgm2 expression.

To determine whether Irgm2-mediated regulation of caspase-11 was unique to purified LPS or extended to other caspase-11-activating stimuli, we tested whether Irgm2 could regulate the inflammatory response to bacterial infections. We confirmed our previous observations (Finethy et al, 2017) that nonvirulent *Escherichia coli* K12 induces caspase-11 activation in BMMs in a dose-dependent manner, and found that K12-elicited responses were significantly exacerbated in *Irgm2*^{-/-} BMMs (Fig 3C and D). Similarly, we found that Irgm2 lessened caspase-11 activation in BMMs infected with virulent *E. coli* species, including adherent invasive *E. coli* (AIEC) and uropathogenic *E. coli* (UPEC) (Fig EV2), demonstrating that Irgm2 attenuates caspase-11 activation in response to both virulent and avirulent *E. coli*.

Irgm proteins regulate the subcellular localization and function of members of a second family of IFN-inducible dynamin-like GTPase, the Gbps (Traver et al, 2011; Halder et al, 2013). Gbps colocalize with phagocytosed bacteria expressing virulence-inducing protein secretion systems (Coers, 2017; Feeley et al, 2017), promote the bacteriolytic destruction of these targeted virulent bacteria, and thereby release PAMPs from lysed bacteria resulting in the activation of cytosolic pattern recognition receptors (Man et al, 2015; Meunier et al, 2015; Liu et al, 2018; Fisch et al, 2019). Although Gbps fail to colocalize with avirulent *E. coli*, Gbps still promote caspase-11 activation in BMMs exposed to non-pathogenic bacteria through a process that may be facilitated by the Gbp-mediated recognition and processing of bacterially secreted OMVs (Vanaja et al, 2016; Finethy et al, 2017). We therefore asked whether Irgm2 could regulate the inflammatory response to OMVs. As with our infection experiments, we observed that loss of Irgm2 expression resulted in higher rates of cell death across a range of OMV concentrations (Fig 3E) and similarly amplified OMV-stimulated IL-1 β and IL-18 secretion (Fig 3F). These data thus define Irgm2 as an inhibitor of LPS-, OMV-, and infection-induced caspase-11 activation.

Irgm2 deficiency elevates caspase-11 activity in the absence of chromosome-3 encoded GBPs

In light of the functional interactions that exist between Gbp and Irgm proteins (Coers et al, 2018), we considered the hypothesis that Irgm2 could attenuate inflammasome activation by acting as a Gbp antagonist. We first monitored expression of Gbp2, a protein required for activation of both the noncanonical caspase-11 and the canonical absent in melanoma 2 (AIM2) inflammasomes (Man et al, 2017; Gomes et al, 2019). Gbp2 and caspase-11 were expressed normally in *Irgm2*^{-/-} BMMs (Fig 4A). To functionally test whether Irgm2 modulates Gbp2-dependent activation of AIM2, we infected WT, *Aim2*^{-/-}, *Gbp*^{chr3-/-} (lacking expression of *Gbp1*, *Gbp2*, *Gbp3*, *Gbp5*, and *Gbp7* but still expressing Gbps encoded on chromosome 5), and *Irgm2*^{-/-} BMMs with the cytosol-invading gram-negative bacterium *Francisella novicida*. We observed that *F. novicida* induced AIM2-mediated cell death in a Gbp-dependent manner (Fig 4B), thus supporting previous findings that Gbp-induced bacteriolysis liberates bacterial DNA for recognition by AIM2 (Man et al, 2015; Meunier et al, 2015). Whereas *F. novicida*-induced pyroptosis was reduced in *Gbp*^{chr3-/-} BMMs, cell death remained unchanged in *Irgm2*^{-/-} BMMs (Fig 4B), demonstrating that the absence of Irgm2 leaves Gbp-dependent AIM2 activation undisturbed.

We next considered the hypothesis that Irgm2 more narrowly regulates Gbp functions specifically tailored toward the activation of caspase-11. To address this hypothesis, we monitored caspase-11-dependent activation in BMMs taken from cross-bred *Irgm2*^{-/-}*Gbp*^{chr3-/-} mice. We found that loss of Irgm2 on a *Gbp*^{chr3-/-} background led to an increase in cell death, enhanced caspase-1 processing and elevated IL-1 β /IL-18 secretion (Fig 4C and D), albeit not to the same level as observed in *Irgm2*^{-/-} BMMs. While these data do not necessarily allow us to rule out a potential role for Irgm2 in modulating the GBP^{chr3}-dependent caspase-11 pathway, we can conclude that Irgm2 suppresses an additional GBP^{chr3}-independent caspase-11 activation pathway (Fig 4E).

Irgm2 interferes with caspase-11-dependent but not caspase-11-independent NLRP3 activation

Based on a previous report describing Gbp5 as a potential regulator of the NLRP3 inflammasome (Shenoy et al, 2012), we asked whether lack of Irgm2 expression directly impacted NLRP3

Figure 3. Irgm2 suppresses caspase-11 activation in response to LPS and bacterial infections.

A IFN γ primed WT, *Irgm2*^{-/-}, *Casp11*^{-/-} and *Irgm2*^{-/-}*Casp11*^{-/-} BMMs were treated with LPS (5 μ g/ml) for 4 h. Following treatment, cells were stained with anti-ASC antibody and Hoechst (DNA/nuclei). Representative images of ASC specks (white arrows point at specks) are shown and number of ASC specks per nuclei quantified. Scale bars: 20 μ m.

B WT, *Irgm2*^{-/-}, *Casp11*^{-/-} and *Irgm2*^{-/-}*Casp11*^{-/-} BMMs were treated with LPS (1 μ g/ml). IL-1 β , IL-18, and LDH release were assessed at 24 h post-treatment (hpt).

C WT, *Irgm2*^{-/-}, *Casp11*^{-/-} and *Irgm2*^{-/-}*Casp11*^{-/-} BMMs were infected with *E. coli* K-12 at indicated MOIs and cell viability assessed via CellTiter-Glo. Cell death was calculated as a function of relative viability to uninfected cells.

D WT, *Irgm2*^{-/-}, *Casp11*^{-/-} and *Irgm2*^{-/-}*Casp11*^{-/-} BMMs were infected with *E. coli* K-12 (MOI 25) and 24 hpi supernatant IL-1 β and IL-18 levels were measured by ELISA.

E WT, *Irgm2*^{-/-}, *Casp11*^{-/-} and *Irgm2*^{-/-}*Casp11*^{-/-} BMMs were treated with OMVs at indicated concentrations for 24 h. Cell viability was assessed via CellTiter-Glo, and cell death was calculated as a function of relative viability to untreated cells.

F WT, *Irgm2*^{-/-}, *Casp11*^{-/-} and *Irgm2*^{-/-}*Casp11*^{-/-} BMMs were treated with OMVs (1 μ g/ml) and 24 hpt cell supernatant IL-1 β and IL-18 levels were measured via ELISA.

Data information: Shown are means $\pm$ SEM from $n = 4$ (A) or $n = 3$ (B-F) independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for indicated comparisons by two-way ANOVA with Tukey's multiple comparisons test. Images in panel A are representative of one of four independent experiments. N.S., non significant.

Figure 4. *Irgm2* deficiency restores caspase-11 activity in the absence of chromosome-3 encoded GBPs.

A WT and *Irgm2*^{-/-} BMMs were stimulated overnight with IFN γ or left untreated and cell lysates were collected. Lysates were assessed for Casp11, Gbp2, and actin protein levels via immunoblotting.
B IFN γ -primed and unprimed WT, *Irgm2*^{-/-}, *Gbp*^{chr3-/-} and *Aim2*^{-/-} BMMs were infected with *Francisella novicida* (MOI 10) and LDH release measured at 4 hpi.
C WT, *Irgm2*^{-/-}, *Gbp*^{chr3-/-} and *Irgm2*^{-/-} *Gbp*^{chr3-/-} BMMs were treated with LPS (1 μ g/ml). IL-1 β , IL-18, and LDH release were assessed at 24 hpt.
D IFN γ -primed WT, *Irgm2*^{-/-}, *Gbp*^{chr3-/-} and *Irgm2*^{-/-} *Gbp*^{chr3-/-} BMMs were treated with LPS (1 μ g/ml) for 24 h and cell lysates and supernatants (Caspase-1 p20) were visualized via immunoblotting.
E Model depicting regulation of caspase-11 activation by *Irgm2* and GBPs.

Data information: Graphs show means $\pm$ SEM from $n = 3$ (B, C) independent experiments. ** $P < 0.01$, *** $P < 0.001$ for indicated comparisons by two-way ANOVA with Tukey's multiple comparisons test. (A) and (D) represent one of two independent experiments. N.S., non significant.

Source data are available online for this figure.

activation. We found that treatment of cells with the NLRP3 activator nigericin induced the secretion of equivalent amounts of IL-1 β and comparable levels of cell death in WT and *Irgm2*^{-/-} BMMs (Fig 5A). As expected, treatment of cells with the NLRP3 inhibitor MCC950 effectively blocked nigericin-induced IL-1 β secretion and cell death (Fig 5B). MCC950 treatment of LPS-activated *Irgm2*^{-/-} BMMs on the other hand only blocked IL-1 β secretion but failed to suppress pyroptosis (Fig 5C). Further arguing against a regulatory role for *Irgm2* in canonical NLRP3 activation, we found levels of mitochondrial radical oxygen species, known activators of the NLRP3 inflammasome, to be comparable between *Irgm2*^{-/-} and WT

BMMs under different experimental conditions (Fig EV3A). Overall, our data thus demonstrate that *Irgm2* specifically interferes with caspase-11 and associated NLRP3 activation triggered by LPS treatment.

Direct cytosolic delivery of LPS overcomes *Irgm2*-mediated suppression of caspase-11 activation

We next asked whether *Irgm2* could exert its anti-inflammatory effect simply through the inhibition of LPS ingestion. We therefore monitored the uptake of fluorescently labeled LPS in both unprimed

Figure 5. Irgm2 interferes with caspase-11-dependent but not caspase-11-independent NLRP3 activation upstream of cytosolic LPS accessibility.

A WT, *Irgm2*^{-/-}, and *Nlrp3*^{-/-} BMMs were treated with LPS (0.1 μg/ml) for 3 h followed by nigericin for 1 h and IL-1β/LDH release was measured.
 B WT, *Irgm2*^{-/-}, *Casp11*^{-/-} and *Irgm2*^{-/-}*Casp11*^{-/-} BMMs were treated with LPS (0.1 μg/ml) for 3 h followed by nigericin and/or MCC950 for 1 h and IL-1β/LDH release was measured.
 C WT, *Irgm2*^{-/-}, *Casp11*^{-/-} and *Irgm2*^{-/-}*Casp11*^{-/-} BMMs were treated with LPS (1 μg/ml) and/or MCC950 for 24 h and IL-1β/LDH release was measured.
 D IFNγ-primed WT, *Irgm2*^{-/-}, *Casp11*^{-/-} and *Irgm2*^{-/-}*Casp11*^{-/-} BMMs were transfected with LPS using lipofectamine LTX and LDH release was measured at 2 hpt.
 E IFNγ-primed WT, *Irgm2*^{-/-}, *Casp11*^{-/-} and *Irgm2*^{-/-}*Casp11*^{-/-} BMMs were co-treated with LPS (indicated doses) and *Listeria monocytogenes* (MOI 5) and LDH release was measured at 4 hpt.

Data information: Shown are means ± SEM from *n* = 5 (A) or *n* = 3 (B, C, D, E) independent experiments. ***P* < 0.01, ****P* < 0.001 for indicated comparisons by two-way ANOVA with Tukey's multiple comparisons test. N.S., non-significant.

and IFNγ-primed *Casp11*^{-/-} and *Irgm2*^{-/-}*Casp11*^{-/-} BMMs, choosing caspase-11-deficient cells to avoid compounding effects resulting from pyroptotic cell death. We found that lack of *Irgm2* expression

failed to increase LPS ingestion (Fig EV3B and C). However, deletion of an important mediator of LPS uptake, the glycosylphosphatidylinositol (GPI)-anchored LPS-binding protein CD14, did not

reduce cell death or IL-18 secretion in LPS-treated *Irgm2*^{-/-} BMMs (Fig EV3D and E). Because LPS bound to hepatocyte-secreted high mobility group box 1 (HMGB1) protein is endocytosed by macrophages via the receptor for advanced glycation end products (RAGE) and subsequently released into the host cell cytosol through destabilization of LPS-containing endosomes (Deng *et al*, 2018), we also tested whether HMGB1/RAGE expression as well as signaling were altered in *Irgm2*^{-/-} BMMs. However, we found protein expression of HMGB1 and RAGE as well as RAGE agonist-induced TNF α secretion to be comparable between WT and *Irgm2*^{-/-} BMMs (Appendix Fig S4A and B). Furthermore, the presence of *Irgm2*-deficient BMMs did not increase the rate of LPS-induced cell death in WT cells during co-culture (Appendix Fig S4C), ruling out increased secretion of HMGB1 as the mechanisms underlying the enhanced LPS responsiveness of *Irgm2*^{-/-} BMMs. Together, these data suggest that a CD14- and HMGB1-independent pathway delivers LPS for caspase-11 sensing in cultured *Irgm2*^{-/-} BMMs.

Having established that *Irgm2*^{-/-} BMMs ingest LPS at levels comparable to WT BMMs, we next investigated whether *Irgm2* was directly interfering with the recognition of cytosolic LPS by caspase-11. To do so, we transfected WT, *Irgm2*^{-/-}, *Casp11*^{-/-}, and *Irgm2*^{-/-}*Casp11*^{-/-} BMMs with LPS and measured pyroptotic death. As expected, caspase-11-deficient BMMs were resistant to LPS-mediated cell death, whereas WT BMMs succumbed to cytosolically delivered LPS (Fig 5D). Remarkably, we found that cell death was equivalent between LPS-transfected WT and *Irgm2*^{-/-} BMMs (Fig 5D). To further substantiate these findings, we deposited LPS into the host cell cytosol via co-delivery with the vacuole-disrupting gram-positive bacterium *Listeria monocytogenes*. Using this alternative approach, we again found that WT and *Irgm2*^{-/-} BMMs were equally susceptible to LPS-triggered pyroptotic cell death across a 1-log range of LPS concentrations (Fig 5E). These data showed that cytosolic delivery of LPS circumnavigates the inhibitory function *Irgm2* exerts on caspase-11.

Atg-deficient macrophages undergo NLRP3-independent pyroptosis in response to extracellular LPS

IRGM proteins control membrane fusion events through direct interactions with members of the autophagy-related protein 8 (Atg8) family (Kumar *et al*, 2018) and Atg-deficient macrophages were previously shown to secrete elevated levels of IL-1 β in response to LPS treatment (Saitoh *et al*, 2008; Harris *et al*, 2011). In agreement with these studies, we found that LPS-treated autophagy-deficient *Atg5*^{-/-} and *Atg14*^{-/-} BMMs secreted elevated amounts of IL-1 β and IL-18, formed ASC specks more frequently and underwent cell death more readily when compared to WT BMMs, thus resembling the response we observed in *Irgm2*^{-/-} BMMs (Fig EV4A and B). Treatment of *Atg5*^{-/-} BMMs with the NLRP3 inhibitor MCC950 blocked LPS-induced IL-1 β secretion but not pyroptotic cell death (Fig EV4C), thus paralleling our observations made in *Irgm2*^{-/-} BMMs. To address whether enhanced LPS accessibility was also primarily responsible for the LPS hypersusceptibility phenotype of Atg-deficient BMMs, we transfected these cells with LPS and monitored cell death. We found that LPS transfection induced comparable levels of cell death in *Atg5*^{-/-}, *Atg14*^{-/-}, *Irgm2*^{-/-}, and WT BMMs (Fig EV4D), suggesting that Atg5, Atg14, and *Irgm2* are likewise involved in blocking LPS accessibility to the cytosolic sensor

caspase-11 rather than the direct regulation of caspase-11 function itself.

Because *Atg5*^{-/-}, *Atg14*^{-/-}, and *Irgm2*^{-/-} BMMs display comparable phenotypes, we asked whether the observed LPS sensitization of *Irgm2*^{-/-} BMMs could be the result of a defect in canonical autophagy. To test this hypothesis, we monitored LC3 lipidation and autophagic flux-dependent degradation of the ubiquitin-binding adapter protein p62 in WT, *Irgm2*^{-/-}, *Atg5*^{-/-}, and *Atg14*-deficient BMMs. LC3 lipidation and p62 degradation were comparable in WT and *Irgm2*^{-/-} BMMs, essentially excluding a prominent role for *Irgm2* in modulating canonical autophagy (Fig EV4E), and instead suggesting a possible role for *Irgm2* in a noncanonical autophagy-related pathway. This model is further supported by findings reported in the accompanying manuscript by Meunier and colleagues (Eren *et al*, 2020), which shows that *Irgm2* interacts with the Atg8 protein Gabarapl2 to attenuate caspase-11 activation.

Irgm2 protects against caspase-11-driven septic shock during endotoxemia

Because cytosolic sensing of LPS by caspase-11 promotes septic shock in a mouse model (Hagar *et al*, 2013; Kayagaki *et al*, 2013), we hypothesized that loss of *Irgm2* would increase susceptibility to an LPS challenge *in vivo*. To test our hypothesis, WT, *Irgm2*^{-/-}, *Casp11*^{-/-}, and *Irgm2*^{-/-}*Casp11*^{-/-} mice were intraperitoneally injected with 8 mg/kg bodyweight LPS and monitored for 48 h. As predicted, *Irgm2*^{-/-} mice displayed decreased survival rates relative to WT mice and loss of caspase-11 restored survival in *Irgm2*-deficient and WT animals alike (Fig 6A). Similarly, deletion of caspase-11 dramatically reduced IL-1 β and IL-18 serum levels in LPS-challenged *Irgm2*^{-/-} mice (Fig 6B). These findings demonstrate that *Irgm2* blocks caspase-11-dependent inflammatory responses initiated by circulating serum LPS.

Immune homeostasis is partially restored in mice deficient for all *Irgm* paralogs

We previously provided evidence in favor of a model in which distinct *Irgm* isoforms have antagonistic relationships with each other. For example, we showed that T cell homeostasis disrupted in *Irgm1*^{-/-} animals was restored through the simultaneous deletion of *Irgm3*, a second *Irgm* family member (Feng *et al*, 2008; Henry *et al*, 2009; Coers *et al*, 2011). To determine whether the disturbance of these inter-regulatory relationships among *Irgm* proteins could contribute to the elevated inflammatory state of LPS-treated *Irgm2*^{-/-} cells and mice, we generated a novel mouse strain deficient for the expression of all 3 *Irgm* isoforms, henceforth referred to as the pan*Irgm*^{-/-} strain (Fig EV5A). We found that lack of *Irgm1* and *Irgm3* expression in pan*Irgm*^{-/-} BMMs substantially moderated the inflammatory response of *Irgm2*-deficient macrophages exposed to extracellular LPS or *E. coli*: specifically, we observed that pan*Irgm*^{-/-} BMMs secreted significantly less IL-1 β and were more resistant to pyroptotic cell death than *Irgm2*^{-/-} BMMs (Fig 7A and B). Nonetheless, pan*Irgm*^{-/-} BMMs remained more responsive to LPS and *E. coli* than WT BMMs under IFN γ -primed conditions (Fig 7A and B). Restoring *Irgm1* expression in BMMs obtained from a pan*Irgm*^{-/-}-derived *Irgm2*^{-/-}*Irgm3*^{-/-} recombinant backcross (Fig EV5A) again reverted the

Figure 6. Irgm2 protects against caspase-11-driven septic shock during endotoxemia.

A WT ($n = 9$), $Irgm2^{-/-}$ ($n = 10$), $Casp11^{-/-}$ ($n = 7$), and $Irgm2^{-/-}Casp11^{-/-}$ ($n = 8$) mice were injected *i.p.* with LPS (8 mg/kg). Morbidity and mortality were observed for 48 h at 3-h intervals.

B WT ($n = 9$), $Irgm2^{-/-}$ ($n = 8$), $Casp11^{-/-}$ ($n = 7$), and $Irgm2^{-/-}Casp11^{-/-}$ ($n = 10$) mice were injected *i.p.* with LPS (8 mg/kg). Serum was collected 4 hpi and concentration of IL-1 β , and IL-18 was measured by ELISA. Data shown are from 2 pooled experiments.

Data information: Graphs (B) show means $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$ for indicated comparisons by log-rank Mantel-Cox test (A) or one-way ANOVA with Tukey's multiple comparisons test (B). N.S., non significant.

$panIrgm^{-/-}$ phenotype and restored elevated LPS-induced cell death and IL-1 β secretion, as seen in $Irgm2^{-/-}$ BMMs (Fig 7A and B). Cell death and IL-1 β secretion in $Irgm3^{-/-}$ BMMs was comparable to WT (Fig 7A and B). Together, these observations indicate that the expression of endogenous Irgm1 is required for the hyper-inflammatory state of Irgm2-deficient macrophages.

Lastly and in agreement with our cell culture observations, we found that deletion of Irgm1 and Irgm3 partially reversed the caspase-11-driven hyperinflammatory response of Irgm2-deficient animals, as evidenced by the intermediate IL-1 β serum levels of endotoxemic $panIrgm^{-/-}$ relative to WT and $Irgm2^{-/-}$ mice (Fig 7C). TNF α serum levels were comparable across the different genotypes tested (Fig 7C), indicating that deletion of all Irgm isoforms not only partially normalized the sensitized caspase-11 pathway of $Irgm2^{-/-}$ mice but also tempered the excessive TLR4 signaling pathway of $Irgm1^{-/-}$ mice (Bafica *et al*, 2007; Schmidt *et al*, 2017). Lastly, we found that the deletion of all Irgm genes increased survival of endotoxemic mice to WT or near WT levels (Figs 7D and EV5B), further supporting a model, according to which dysregulated Irgm isoform expression rather than merely Irgm2 loss-of-function drives the pathological inflammation in endotoxemic $Irgm2^{-/-}$ mice.

Discussion

Infections elicit robust inflammatory responses, which benefit the host by boosting protective antimicrobial immunity. However,

dysregulated or overly robust inflammation can cause substantial host morbidity and mortality. Host organisms including humans thus evolved complex regulatory mechanisms to calibrate inflammation so that infections can be contained while collateral damage to infected tissue is minimized (Casadevall & Pirofski, 1999). Here, we define a novel pathway responsible for regulating and minimizing inflammation elicited by the cytosolic LPS sensor caspase-11. Our studies characterize mouse Irgm proteins as novel rheostats calibrating the sensitivity of cells and whole animals to LPS. We demonstrate that deletion of Irgm2 leads to excessive activation of the noncanonical caspase-11 inflammasome pathway. We further show that simultaneous deletion of all Irgm paralogs largely restores immune homeostasis in Irgm2-deficient animals, thus indicating that the disturbance of the Irgm expression network is largely responsible for LPS hyperresponsiveness and inflammation in these animals.

While caspase-11 is known to sense LPS in the cytosol, how LPS becomes accessible to its cytosolic sensor is not well understood. The majority of "free" LPS is internalized via receptor-mediated endocytosis or macropinocytosis and then trafficked to the lysosomal compartment for detoxification (Kitchens *et al*, 1998; Poussin *et al*, 1998; Deng *et al*, 2018). How LPS exits membrane-bound compartments has remained mostly unstudied owing in part to the observation that when WT macrophages are exposed to extracellular LPS in cell culture, only minimal caspase-11 activation is detectable (Hagar *et al*, 2013; Kayagaki *et al*, 2013). Here, we demonstrate that extracellular LPS can potentially activate the inflammasome in murine macrophages deficient for Irgm2.

Figure 7. Restoring expression of endogenous Irgm1 is sufficient to hypersensitize *panIrgm*^{-/-} macrophages for caspase-11 activation.

A WT, *Irgm2*^{-/-}, *Irgm3*^{-/-}, *Irgm2*^{-/-}*Irgm3*^{-/-}, *panIrgm*^{-/-}, and *Casp11*^{-/-} BMMs were treated with LPS (1 μ g/ml) or infected with *E. coli* K-12 (MOI 25) and IL-1 β , and LDH release were assessed at 24 hpt ($n = 3$ independent experiments).

B IFN γ -primed WT, *Irgm2*^{-/-}, *Irgm3*^{-/-}, *Irgm2*^{-/-}*Irgm3*^{-/-}, *panIrgm*^{-/-}, and *Casp11*^{-/-} BMMs were treated with LPS (1 μ g/ml) or infected with *E. coli* K-12 (MOI 25) and IL-1 β , and LDH release were assessed at 8 hpt/hpi ($n = 3$ independent experiments).

C WT ($n = 15$), *Irgm2*^{-/-} ($n = 12$), and *panIrgm*^{-/-} ($n = 13$) mice were injected i.p. with LPS (8 mg/kg). Serum was collected 4 hpi and concentration of IL-1 β and TNF α was measured via ELISA. Data shown are from 3 pooled experiments.

D WT, *Irgm2*^{-/-}, *panIrgm*^{-/-}, and *Casp11*^{-/-} mice ($n = 8$ mice/genotype) were injected i.p. with LPS (8 mg/kg body weight). Morbidity and mortality were observed for 48 h at 3-h intervals.

Data information: Graphs show means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for indicated comparisons by two-way ANOVA (A, B) or one-way ANOVA (C) with Tukey's multiple comparison test, or log-rank Mantel-Cox test (D). N.S., non significant.

There are a variety of nodes in the LPS uptake and processing pathway, which could potentially be modulated by Irgm2 including (i) lysosomal LPS detoxification, (ii) LPS internalization, and (iii) LPS accessibility. The lysosomal enzyme acyloxyacyl hydrolase (AOAH) deacylates LPS and thereby inactivates LPS as a caspase-11 and TLR4 agonist. Consequently, deletion of AOAH results in prolonged reactions to LPS *in vivo* (Lu *et al*, 2008, 2013). However, AOAH operates with slow kinetics and

inactivates only approximately 25% of LPS over a 12-h period (Ojogun *et al*, 2009). We therefore predict that even a complete loss of AOAH could only have a minor effect on caspase-11 activation in the shorter time courses used in this study. A more likely model would predict a role for Irgm2 in LPS internalization. However, arguing against this model, we found that bulk LPS uptake by macrophages was not noticeably changed in *Irgm2*-deficient macrophages. As an alternative model, we propose that

Irgm2 controls cytosolic accessibility of LPS. As far as we know, no mammalian LPS transmembrane transporter exists. However, a recent report demonstrated that hepatocyte-secreted HMGB1 binds to circulating serum LPS, promotes LPS uptake into lysosomes and facilitates LPS delivery from lysosomes into the host cell cytosol (Deng *et al*, 2018). Based in part on the observation that amphipathic HMGB1 can destabilize liposomes at an acidic pH *in vitro*, it was proposed that HMGB1 disrupts lysosomal membranes and thereby promotes cytosolic leakage of LPS (Deng *et al*, 2018). In addition to HMGB1 it is also conceivable that amphipathic LPS itself or other host-derived molecules such as Irgm1 could induce moderate vacuolar destabilization of LPS-containing compartments. We predict that Irgm2 minimizes membrane damage of LPS-containing vacuoles and thereby blocks LPS accessibility for caspase-11. The expression of Irgm2 could promote vacuolar stability through a number of potential mechanisms such as repairing damaged endosomes or sorting LPS-containing endosomes away from rupture-prone compartments. Additional studies are necessary to define if and how Irgm2 regulates each of these processes.

While our results reveal a novel function for mouse Irgm2 in controlling caspase-11 activation, it is unclear whether Irgm2 shares this function with human IRGM. Prior studies of mouse Irgm proteins almost exclusively focused on Irgm1-deficient mice and revealed shared functions of mouse Irgm1 and human IRGM, especially regarding the regulation of mitochondrial dynamics (Coers *et al*, 2018). Mitochondrial dysfunction and the associated release of oxidized mitochondrial DNA provide compelling molecular models to account for the increased inflammation observed in Irgm1-deficient cells and animals, and may also underlie some of the autoinflammation associated with human IRGM disease alleles (Coers *et al*, 2018; Holley & Schroder, 2020). In addition to controlling mitochondrial remodeling and autophagy, Golgi-resident human IRGM also regulates Golgi fragmentation in response to viral infections (Hansen *et al*, 2017). Whether mouse Irgm proteins execute similar functions has not been explored, but the localization of both Irgm1 and Irgm2 to the Golgi Apparatus ((Henry *et al*, 2014; Martens & Howard, 2006) and Appendix Fig S1) indicates that murine IRGM homologs may play similar roles in shaping Golgi membrane remodeling events. An accompanying study (Eren *et al*, 2020) shows that the Irgm2-interaction partner Gabarapl2, another Golgi-resident protein (Sagiv *et al*, 2000), cooperates with Irgm2 in mouse macrophages and blocks caspase-11 activation in both mouse and human macrophages. Thus, the same anti-inflammatory pathway disrupted in *Irgm2*^{-/-} BMMs appears to also operate in human cells. Future studies will be aimed at defining this pathway in detail through the systematic discovery and characterization of the molecular players and their activities, many of which are likely shared between mouse and human macrophages.

Too much or too little inflammation is host detrimental. The mammalian host thus evolved complex regulatory networks, which fine-tune the inflammatory response during an infection. The identity of many of these regulatory circuits and how they function remain to be determined. Here, we report the discovery of a novel regulatory pathway, which attenuates cytosolic LPS sensing and moderates LPS-induced inflammation. Future studies will expand our mechanistic understanding of this

regulatory pathway and may open up avenues for therapeutic interventions to alleviate disease during gram-negative bacterial infections.

Materials and Methods

Production of the *Irgm2*^{-/-} mouse line

To generate *Irgm2*^{-/-} mice, we inserted a *loxP* site together with a *neo* marker (*floxneo*) flanked by two flippase recognition target sites (*frt*) before the ATG of *Irgm2* and a *loxP* site after the stop codon of *Irgm2* using backbone vector pEZ-Frt-lox-DT (Addgene plasmid #11736). We placed this cassette between 5' and 3' *Irgm2* homology arms. The *Irgm2* targeting construct was transfected into C57BL/6-derived PRX-B6N ES cells (Primogenix). Properly targeted ES cell clones were identified by PCR screening, confirmed by Southern blotting and used for blastocyst injection. The resulting chimeras were crossed with C57BL/6J mice and germline transmission of targeted ES clones was obtained. We performed Southern blot analysis to confirm the proper targeting in F2 mice. These mice were crossed with a germline flippase-expressing strain B6(C3)-Tg(Pgk1-FLPo)10Sykr/J (Wu *et al*, 2009) to remove the neomycin cassette. Offspring from this cross harbored a floxed *Irgm2* allele (*Irgm2*^{flox}). *Irgm2*^{flox} mice were crossed with the germline-expressing Cre strain B6.FVB-Tg(EIIa-cre)C5379Lmgd/J (Lakso *et al*, 1996) to generate *Irgm2*^{-/-} mice. Mice were genotyped by PCR using primers Irgm2_543 5'- GATCTGAAAGCCTGGACAA-3' and Irgm2_542 5'- GGGTACACATGGGGTACAGG-3' which yield a 1.4 kb (WT) and 0.2 kb (*Irgm2*^{-/-}) DNA product.

Production of the *panIrgm*^{-/-} and *Irgm2*^{-/-}*Irgm3*^{-/-} mouse lines

To generate *panIrgm*^{-/-} mice, CRISPR sgRNA-Cas9 protein ribonucleoprotein complexes were injected into pronuclear-stage mouse embryos derived from previously reported double-knockout (*Irgm1*^{-/-}*Irgm3*^{-/-}) mice on a C57BL/6J genetic background (Henry *et al*, 2009; Coers *et al*, 2011). The following pair of sgRNA were used for the injection: sgRNApan1 5'-GACTGCCGTGAAA-GAAGGGG-3' and sgRNApan4 5'-GGTGGCTCTATAGGTTTG-3'. The genomic *Irgm2* locus of *panIrgm*^{-/-} founder mice was sequenced and a founder line with 925 bp deletion and nonsense mutation was backcrossed to C57BL/6J and then intercrossed to obtained animals homozygous for the deletion alleles of all 3 *Irgm* paralogs, as confirmed by PCR genotyping and Western blotting. *PanIrgm*^{-/-} mice were backcrossed to C57BL/6J mice and screened for recombinants by PCR. A *Irgm2*^{-/-}*Irgm3*^{-/-} recombinant was intercrossed to obtain homozygous mice.

Additional mouse strains used

WT (C57BL/6J) mice were originally purchased from Jackson Laboratories. *Irgm1*^{-/-}, *Irgm3*^{-/-}, *Atg5*^{fl/t}, *LysMCre-Atg5*^{fl/t}, *Atg14*^{fl/t}, *LysMCre-Atg14*^{fl/t}, *Casp1*^{-/-}*Casp11*^{-/-}, *Casp11*^{-/-}, *CD14*^{-/-}, *Aim2*^{-/-}, *Nlrp3*^{-/-}, and *Gbp*^{chr3-/-} mice were previously described (Kuida *et al*, 1995; Moore *et al*, 2000; Taylor *et al*, 2000; Collazo *et al*, 2001; Zhao *et al*, 2008; Rathinam *et al*, 2010; Kayagaki *et al*, 2011; Inoue *et al*, 2012; Yamamoto *et al*, 2012; Choi *et al*, 2014). The

mouse strains *Irgm2*^{-/-}*Casp1*^{-/-}*Casp11*^{-/-}, *Irgm2*^{-/-}*Casp11*^{-/-}, *Irgm2*^{-/-}*GBP^{chr3}*^{-/-}, and *Irgm2*^{-/-}*CD14*^{-/-} mice were generated through intercrossing of aforementioned mouse lines.

Mouse husbandry and *in vivo* experiments

All mouse lines were maintained at animal facilities at Duke University Medical Center. Animal protocols were approved by the Institutional Animal Care and Use Committees at Duke University. Mice were between 7 and 14 weeks of age with the exception of the Luminex experiment (age range was 7–20 weeks). Approximately equal numbers of male ($n = 122$, weight (mean $\pm$ SD) = 24.18 ± 2.45 g) and female ($n = 105$ mice, weight (mean $\pm$ SD) = 19.18 ± 1.91 g) mice were used with both sexes utilized in each set of experiments. Mice were injected intraperitoneally with 2 or 8 mg *E. coli* LPS (O111:B4 LPS; L3024; Sigma)/kg bodyweight or with PBS control, as indicated. For cytokine monitoring, serum was collected 4 h after LPS injection. Cytokine concentrations were measured by ELISA or Luminex Multiplex Assay (Invitrogen Mouse 20-plex). To determine host susceptibility to endotoxemia, mice were monitored every 3 h for up to 48 h following LPS injection. Mice were considered moribund and euthanized when their body weight dropped below 80% of starting weight, or the mice exhibited convulsions, or uncontrollable shivering, or severe ataxia as indicated by lack of righting response. We used approximately equal numbers of male and female mice for experiments.

Production and culturing of bone marrow-derived macrophages (BMMs)

Bone marrow-derived macrophages (BMMs) were generated essentially as previously described (Pilla et al, 2014; Finethy et al, 2017). Briefly, bone marrow was flushed out from mouse femurs and tibia using a 23-gauge needle and then cultured in RPMI 1640, 20% FBS, 2-mercaptoethanol, and 14% conditioned media containing macrophage colony-stimulating factor at 37°C in 5% (vol/vol) CO₂ in nontissue culture-treated dishes for 5–7 days. Differentiated BMMs were harvested, frozen, and then thawed again for use in experiments. Immortalized BMMs (iBMMs) were generated through infection with J2 virus as previously described (Pilla et al, 2014).

Ectopic expression of *Irgm2*

Irgm2 was inserted into the tetracycline-inducible vector pInducer20 via Gateway recombination (Meerbrey et al, 2011). To this end, the *Irgm2* cDNA was amplified from RNA isolated from IFN γ -stimulated WT BMMs using the primer pair attB1-IRGM2-Forward/attB2-IRGM2-Reverse, which add both flanking attB-recombination sites and a 5'-Kozak consensus sequence. The resulting PCR product was inserted into pDONR221 via Gateway BP recombination, followed by Gateway LR recombination into pInducer20. Viral particles were prepared in HEK-293T cells and transduced into iBMMs as previously described (Feeley et al, 2017). Expression of *Irgm2* was induced by overnight incubation with cell culture medium containing anhydrotetracycline (aTc, 2 μ g/ml). Primers: attB1-*Irgm2*-Forward, 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCACCATGGAAGAGGCAGTTGAGTCACC-3'; attB2-*Irgm2*-Reverse, 5'-GGGGA

CCACTTTGTACAAGAAAGCTGGGTCCTAAGGATGAGGAATGGAGAGTCTCAGG-3'

Bacterial culture and OMV production

E. coli K-12 BW5113 (K12), uropathogenic *E. coli* CFT073 (UPEC), and *E. coli* O18:K1:H7 strain C5 (AIEC) were cultured overnight in Luria-Bertani broth (LB) at 37°C with agitation. *Francisella novicida* strain U112 (ATTC catalog No. NR-13) was grown overnight in Brain Heart Infusion (BHI) broth shaking at 37°C. For LPS delivery experiments, *Listeria monocytogenes* strain DH-L1039 was grown overnight in BHI at 30°C, as previously reported (Pilla et al, 2014). OMVs were isolated from culture supernatants via ultracentrifugation and ultrafiltration as previously described (Finethy et al, 2017).

Tissue culture infection and treatment protocols

For all infections, bacteria cultures were diluted in cell culture medium to achieve the desired multiplicity of infection (MOI) and then added directly to cells. Cells were then centrifuged at 700 g for 10 min. Following 1-h incubation at 37°C, gentamicin was added to wells at a final concentration of 100 μ g/ml and cells were assayed at indicated time points. Where indicated, cells were primed with 100 U/ml of mouse IFN γ (EMD Millipore) overnight. Ultrapure *S. minnesota* R595 LPS (List Biologicals) at a concentration of 1 μ g/ml was used for all cell culture LPS treatments unless otherwise noted. Where described, cells were treated with 5 μ M MCC950 (Invivogen), 1 μ g/ml poly(I:C) (Sigma-Aldrich), 1 μ g/ml Pam3CSK4 (Invivogen) alone or in combination with other reagents. Where noted, cells were treated with indicated concentrations of advanced glycation end product-bovine serum albumin (AGE-BSA, Sigma-Aldrich) for 3 h. Unless otherwise mentioned, agonists were diluted in complete cell culture medium and added directly to cells for designated times. For qPCR experiments, cells were treated with LPS or left untreated for 8 h prior to harvesting of RNA. For immunoblot experiments, cells were washed 3 times with PBS before addition of LPS diluted in Opti-MEM (Gibco). Cell lysates and supernatants were collected 24 h post-treatment (hpt). To visualize ASC specks, LPS was diluted to a concentration of 5 μ g/ml in Opti-MEM and cells were fixed 4 hpt. To activate the NLRP3 inflammasome, BMMs were first primed with LPS in cell culture medium or left unstimulated for 3 h before cells were treated with 5 μ M nigericin (Sigma-Aldrich) for 1 h. At that time, cell supernatants were collected for analysis by ELISA or LDH release assays. For monitoring autophagy, IFN γ primed and unprimed BMMs were washed with PBS and then incubated for 2 h in cell culture media, media containing Bafilomycin-A1 (100 nM, Sigma-Aldrich), or Hank's balanced salt solution (HBSS, Thermo Fisher) prior to harvesting of cell lysates.

Cytoplasmic delivery of LPS

Cells were primed overnight with 100 U/ml of IFN γ prior to LPS treatments. LPS was transfected into BMMs using Lipofectamine LTX with Plus Reagent (Thermo Fisher). BMMs were seeded in 96-well flat bottom plates at approximately 4×10^5 cells per well. For each well, 375 ng of Lipofectamine LTX and 75 ng of LPS alongside 75 ng of Plus Reagent were each diluted in 2 μ l of Opti-MEM. Following a 10-min incubation period at room temperature (RT), solutions were

mixed and incubated for an additional 30 min at RT before the total reaction volume was brought up to 50 μ l with media. Solutions were added to wells and plates centrifuged at 700 g for 10 min. Cells were then incubated at 37°C for indicated times and cytotoxicity assessed via LDH release assay. For co-delivery with *L. monocytogenes*, bacteria were grown overnight at 30°C and then diluted to give an MOI of 5 in Opti-MEM. LPS was added at indicated concentrations and mixtures spun onto cells at 700 g for 10 min. Cells were incubated at 37°C for 1 h, gentamicin was added to a final concentration of 15 μ g/ml and cells were returned to the incubator for an additional 3 h before LDH release was assessed.

Fluorescence-associated cell sorting

For LPS internalization studies, cells were treated with 2.5 μ g/ml Alexa Fluor 488-conjugated LPS (L23351, Thermo Fisher) or unlabeled control LPS for indicated times. Following treatment, cells were washed twice with PBS and collected in PBS containing 2% FBS. For measuring mitochondrial reactive oxygen species, IFN γ primed and unprimed cells were treated with LPS (1 μ g/ml) for 3 h, antimycin A (100 μ M) for 30 min, or were left untreated. Following treatments, cells were washed twice with PBS, incubated for 30 min with mitoSOX red (5 μ M, Invitrogen) in Opti-MEM, washed thrice with PBS and were collected in PBS containing 2% FBS. Fluorescence intensity data was collected a BD FACSCanto II system (BD Biosciences) and analysis was carried out using FlowJo Software.

Cytotoxicity, viability, and cytokine measurements

LDH release was measured using the CytoTox One homogenous membrane integrity assay (Promega). Cytotoxicity was calculated as a function of LDH release using the following formula: (sample – untreated control)/(100% lysis control – untreated control) $\times$ 100. In some instances, cell viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) and changes in viability relative untreated or uninfected cells was used to calculate cell death. Measurements of propidium iodide (PI) uptake were used to assess cell death over time. In these instances, treatments were performed in Opti-MEM containing 6 μ g/ml PI and fluorescence measured at indicated time points. Relative fluorescence was calculated by subtracting PI fluorescence at time of initial treatment. For all cytotoxicity and viability assays, measurements were performed via Enspire 2300 (PerkinElmer) Multilabel Reader.

Cytokine concentrations were determined via enzyme-linked immunosorbent assay (ELISA) or Luminex Multiplex Assay as indicated in figure legends. ELISAs were performed per manufacturer's instructions. The following ELISA kits were used: IL-1 β Mouse ELISA kit (Thermo Fisher), IL-18 Mouse ELISA kit (Thermo Fisher), and ELISA MAX Deluxe Set Mouse TNF α (Biolegend). Measurements were performed via Enspire 2300 (PerkinElmer) Multilabel Reader. Luminex Multiplex Assay (Mouse 20-plex; Invitrogen) was performed in the Immunology Unit of the Regional Biocontainment Laboratory at Duke using a BioPlex 200 instrument.

RNA isolation and quantitative PCR

RNA was isolated using a RNeasy Mini Kit (Qiagen) and reverse transcription accomplished using an iScript cDNA synthesis kit (Bio-

Rad). Quantitative PCR was performed with QuantaFast SYBR Green PCR mix (Qiagen) on a 7500 Fast Real-Time PCR system (Applied Biosystems). The following primers were used:

mGAPDH F, 5'-GGTCCTCAGTGTAGCCCAAG-3';
mGAPDH R, 5'-AATGTGTCCGTCGTGGATCT-3';
mIL-1 β F, 5'-GCAACTGTTCTGAACTCAACT-3';
mIL-1 β R, 5'-ATCTTTGGGGTCCGTCAACT-3';
mTNF F, 5'-CATCTTCTCAAAATTCGAGTGACAA-3';
mTNF R, 5'-TGGGAGTAGACAAGGTACAACCC-3'

Immunocytochemistry

For visualizing ASC specks, cells were fixed for 5 min with ice-cold methanol. Coverslips were then washed 3 times with PBS, and cells were blocked with PBS containing 5% Bovine Serum Albumin (BSA) for 30 min at RT. Cells were stained with rabbit anti-ASC antibody (AG-25B-0006; Adipogen; 1:1,000) diluted in blocking buffer overnight at 4°C. Coverslips were then washed three times with PBS containing 5% BSA and 0.1% saponin, and cells were next incubated with anti-rabbit Alexa Fluor 568-conjugated secondary antibody (Invitrogen; 1:1,000) and Hoechst for 1 h at RT followed by an additional three washes. For visualizing Gm130 and Irgm2, cells were fixed for 15 min at RT with 4% (wt/vol) paraformaldehyde. Coverslips were washed 3 times with PBS and then blocked with 5% BSA and 0.3 M glycine in PBS-saponin (0.1%) for 30 min at RT. Cells were stained with mouse anti-Gm130 (610823; BD Transduction Laboratories; 1:200) and rabbit anti-Irgm2 (1:500) diluted in blocking buffer for 1 h at RT. Coverslips were then washed 3 times with PBS-saponin and stained with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 568-conjugated secondary antibodies (Invitrogen), and Hoechst for 1 h at RT. Imaging was performed using a Zeiss Axioskop 2 upright epifluorescence microscope, a Zeiss 780 upright confocal microscope, or a Zeiss 880 Airyscan Fast Inverted Confocal. The custom-made polyclonal rabbit anti-Irgm2 antibody was raised against the N-terminal peptide KEAVESPEVKEFEYFS.

Immunoblotting

Whole-cell lysates were harvested in RIPA lysis buffer (Sigma-Aldrich) containing 1 $\times$ protease inhibitor cocktail (Sigma-Aldrich). For visualizing cleaved caspase-1, supernatants were collected and protein concentrated via trichloroacetic acid (TCA) precipitation. Samples were mixed with a 100% (wt/vol) TCA solution in water to a final TCA concentration of 10% and incubated on ice overnight. Samples were then centrifuged at 21,130 g for 10 min. Following centrifugation, supernatant was discarded, and pellets washed twice with ice-cold acetone. Pellets were air dried and resuspended in 8 M urea. Samples were loaded on 4–20% gradient SDS-PAGE gels and transferred to nitrocellulose or PVDF. Membranes were blocked for 1 h at RT in Tris-buffered saline-0.1% Tween 20 (TBST) containing 5% BSA and incubated overnight at 4°C with indicated primary antibodies. Membranes were washed thrice with TBST and then incubated for 1 h at RT with appropriate HRP-conjugated secondary antibodies. Membranes were washed and visualized on film. The following antibodies and dilutions were used: rabbit anti-Irm1 1B2 ((Butcher *et al.*, 2005); 1:500), rabbit anti-Irgm2 (raised against peptides KEAVESPEVKEFEYFS or KPESHDFPKLRETLQKDL; 1:500),

rabbit anti-Irgm3 927B0 ((Taylor *et al*, 1996); 1:500), rabbit anti-GBP2 ((Coers *et al*, 2011); 1:1,000), rabbit anti-caspase-1 (AG-20B-0042; Adipogen; 1:1,000), mouse anti- β -actin (A2228; Sigma-Aldrich; 1:1,000), rabbit anti-P62 (PM045; MBL; 1:1,000), rabbit anti-LC3 (2775; Cell Signaling Technology; 1:500), goat anti-CD14 (AF982; Novus Biologicals; 1:500), and rat anti-Casp11 (17D9; Novus Biologicals; 1:500), mouse anti-HMGB1 (GT348; Invitrogen, 1:1,000), and rat anti-RAGE (MAB11795; R&D Systems, 1:2,000).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.00 software. Significance was determined using one-way analysis of variance (ANOVA) or two-way ANOVA with multiple comparisons tests as indicated in figure legends. Log-rank Mantel-Cox test was used for determining significance in survival experiments. * $P < 0.5$, ** $P < 0.01$, *** $P < 0.001$. N.S, non-significant.

Data availability

This study includes no data deposited in external repositories.

Expanded View for this article is available online.

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Author contributions

RF and JC conceived the project with input from GAT. RF, JD, MK, GW, ASP, AKH, and SL conducted the experiments and acquired data. RF, JD, MK, GW, SL, and JC analyzed data. RF and JC wrote the manuscript. All authors contributed to manuscript reviewing and editing. SH, NO-R, JM, MJK, SW, and GAT provided reagents. JC and GAT supervised the project and acquired funding related to the project.

Conflict of interest

The authors declare that they have no conflict of interest.

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