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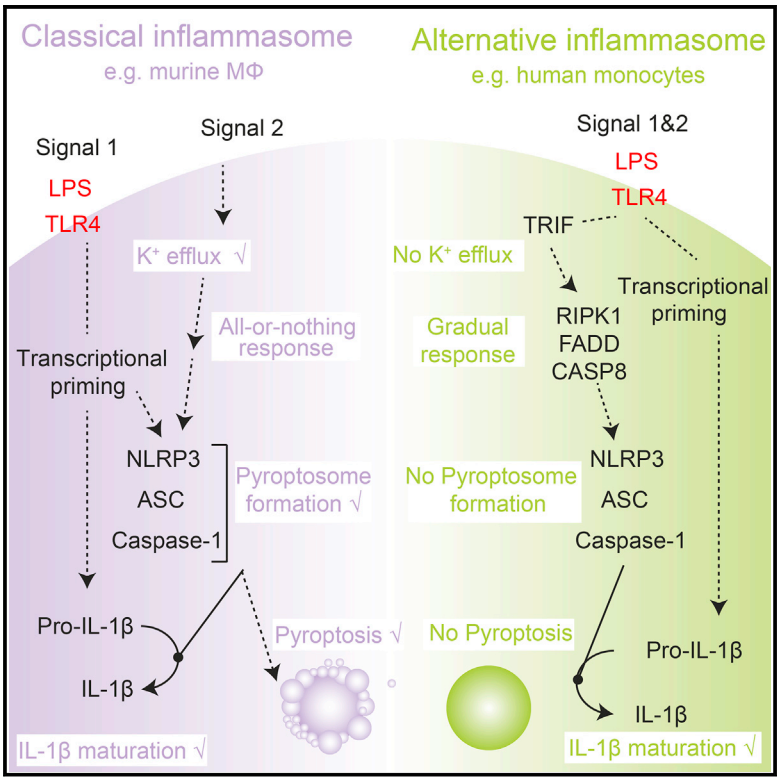
Immunity

Human Monocytes Engage an Alternative Inflammasome Pathway

Graphical Abstract



Highlights

- LPS by itself triggers IL-1β secretion in human, but not in murine monocytes
- Human monocytes activate an alternative inflammasome in response to LPS
- This proceeds independently of K^+ efflux, pyroptosome formation, and pyroptosis
- Alternative but not classical inflammasome signals via TLR4-TRIF-RIPK1-FADD-CASP8

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In Brief

How human monocytes secrete processed IL-1β upon LPS challenge is unknown. Hornung and colleagues report that LPS triggers an alternative NLRP3 inflammasome pathway in human monocytes. Unlike classical NLRP3 inflammasome signaling, alternative inflammasome activation proceeds independently of potassium efflux, pyroptosome formation, and pyroptosis, while it engages TLR4-TRIF-RIPK1-FADD-CASP8 upstream of NLRP3.

Gaidt et al., 2016, Immunity *44*, 833–846
April 19, 2016
http://dx.doi.org/10.1016/j.immuni.2016.01.012

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Human Monocytes Engage an Alternative Inflammasome Pathway

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http://dx.doi.org/10.1016/j.immuni.2016.01.012

SUMMARY

Interleukin-1β (IL-1β) is a cytokine whose bioactivity is controlled by activation of the inflammasome. However, in response to lipopolysaccharide, human monocytes secrete IL-1β independently of classical inflammasome stimuli. Here, we report that this constituted a species-specific response that is not observed in the murine system. Indeed, in human monocytes, lipopolysaccharide triggered an "alternative inflammasome" that relied on NLRP3-ASC-caspase-1 signaling, yet was devoid of any classical inflammasome characteristics including pyroptosome formation, pyroptosis induction, and K^+ efflux dependency. Genetic dissection of the underlying signaling pathway in a monocyte transdifferentiation system revealed that alternative inflammasome activation was propagated by TLR4-TRIF-RIPK1-FADD-CASP8 signaling upstream of NLRP3. Importantly, involvement of this signaling cascade was limited to alternative inflammasome activation and did not extend to classical NLRP3 activation. Because alternative inflammasome activation embraces both sensitivity and promiscuity of TLR4, we propose a pivotal role for this signaling cascade in TLR4-driven, IL-1β-mediated immune responses and immunopathology in humans.

INTRODUCTION

The cytokine interleukin-1β (IL-1β) plays a pivotal and pleiotropic role in orchestrating innate and adaptive immune responses. Besides its critical involvement in pathogen clearance and immune homeostasis, aberrant IL-1β production is associated with monogenetic auto-inflammatory diseases (Masters et al., 2009), but also with ligand-dependent, multifactorial, sterile inflammatory conditions, such as atherosclerosis, type 2 diabetes, Alzheimer's disease, or gout (Dinarello, 2009, 2011; Haneklaus and O'Neill, 2015). The bioactivity of IL-1β is controlled at the post-translational level by proteolytic maturation via the cysteine protease caspase-1, the effector molecule of a large cytosolic protein complex known as the inflammasome. Among others, NLRP3 represents the most-studied inflammasome sensor, due to its pivotal role in anti-microbial immunity and sterile inflammation. Activated inflammasome sensor proteins have been shown to seed the prion-like assembly of the inflammasome adaptor ASC into filamentous, high-molecular-weight complexes (Cai et al., 2014; Lu et al., 2014), for which the term pyroptosome has originally been coined (Fernandes-Alnemri et al., 2007). Pyroptosome complexes function as scaffolds to recruit pro-caspase-1, resulting in its auto-proteolytic activation and subsequent enzymatic activity. Next to the proteolytic maturation of cytokines of the IL-1 family, caspase-1 activation is furthermore associated with a special type of cell death known as pyroptosis (Latz et al., 2013). Single cell analysis in murine macrophages reveals that inflammasome-mediated caspase-1 activation is an all-or-nothing response, in which all cells with activated caspase-1 commit to pyroptosis and IL-1β release (Liu et al., 2014).

It is generally accepted that inflammasome activation leading to IL-1β maturation employs a two-step activation mechanism that is spatially and temporally separated. To this end, recognition of a first signal (signal 1) provides transcriptional upregulation of pro-IL-1β, e.g., via Toll-like receptor (TLR) signaling, whereas a second signal (signal 2) is recognized in order to activate the inflammasome (Guo et al., 2015; Lamkanfi and Dixit, 2014; Latz et al., 2013; Wen et al., 2013). The exact mechanism of NLRP3 inflammasome activation remains elusive, nevertheless, a common denominator of its activation seems to be the cytosolic efflux of K^+, which appears to be necessary and sufficient to trigger NLRP3 inflammasome activation (Muñoz-Planillo et al., 2013; Pétrilli et al., 2007). More recently, it has become evident that lipopolysaccharide (LPS) from gram-negative bacteria is directly sensed by caspase-11 or its human orthologs caspase-4 and caspase-5 in the cytosol to trigger cell death and activate the NLRP3 inflammasome (Kayagaki et al., 2011; Shi et al., 2014). To distinguish this unique pathway from conventional or canonical NLRP3 activation, the term non-canonical inflammasome has been coined (Lamkanfi and Dixit, 2014; Storek and Monack, 2015).

Before the concept of inflammasome activation became a particular focus of innate immunity research, IL-1β maturation and secretion have been extensively studied in human monocytes using LPS as a stimulus (Danis et al., 1990; Hazuda et al., 1988; Lonnemann et al., 1989; Schumann et al., 1998). In

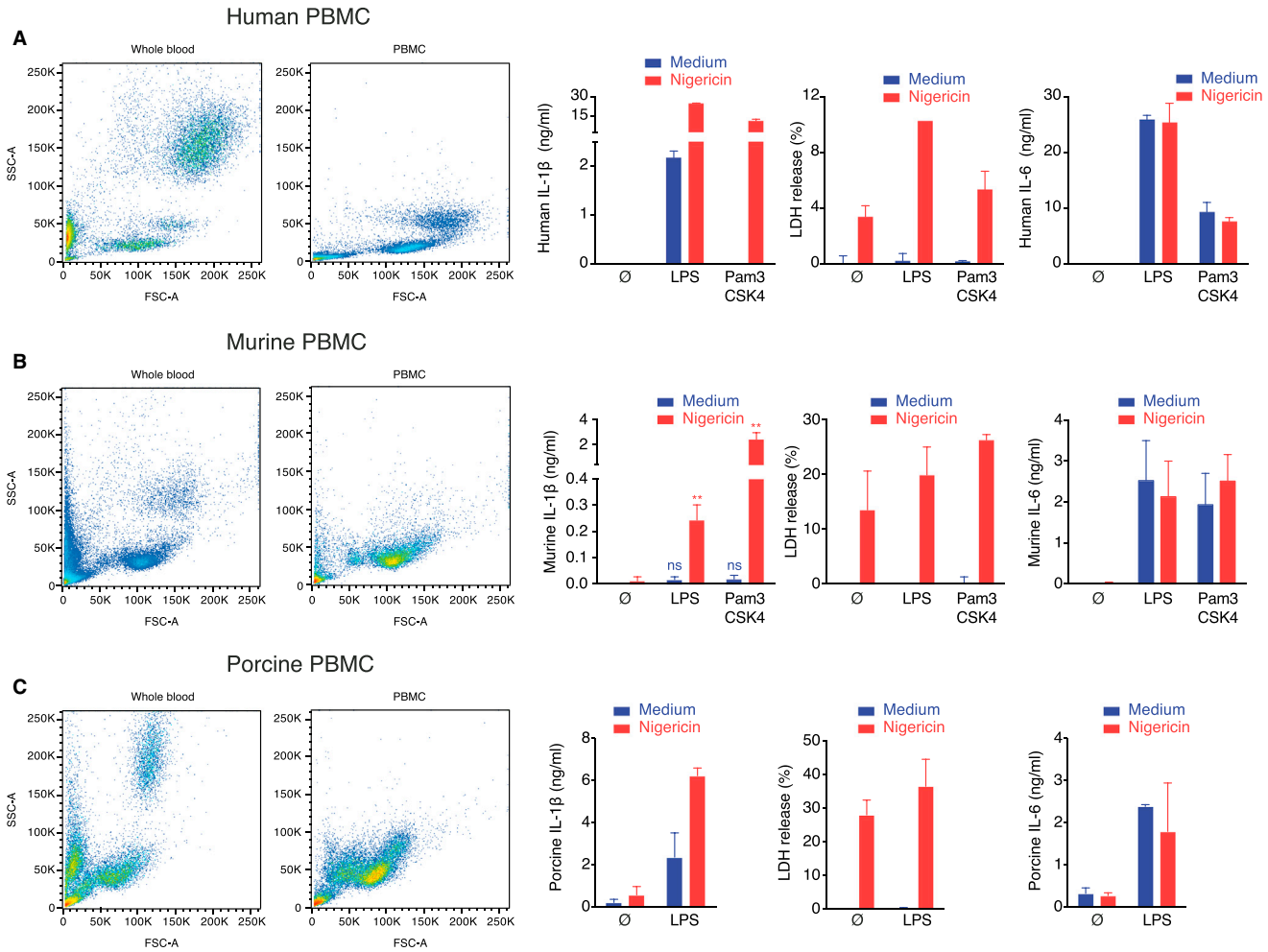


Figure 1. LPS Induces IL-1β in a Species-Specific Manner without Inducing Pyroptosis

Granulocyte-depleted PBMCs of human, murine or porcine origin were stimulated for 14 hr with 2 μg/ml LPS or Pam3CSK4 and subsequently stimulated for 2 hr with Nigericin (red) or left untreated (blue). LDH release and cytokine secretion is depicted as mean + SD of duplicate measurements from one representative donor of three (A) or as mean + SEM of five (B) or three (C) independent experiments. **p < 0.01, ns = not significant.

Please see also Figure S1.

this setting, LPS per se is sufficient to trigger caspase-1-dependent IL-1β maturation and secretion, thereby contradicting the above-described two-step activation dogma that has mainly been established in the murine system (Hogquist et al., 1991). However, owing to the limited availability of decisive loss-of-function tools in the human monocyte system, clear insight into this phenomenon remains limited and focus has been put on the murine system, in which genetic studies can be performed to study inflammasome biology. Nevertheless, in light of the fact that several studies have already indicated considerable differences between human and murine inflammasome activation (Khare et al., 2012; Sha et al., 2014; Wang et al., 2013) it remains unclear to what extent central conclusions drawn from murine inflammasome activation can be extrapolated to the human system. Here, we report a species-specific NLRP3 inflammasome pathway that existed in human and porcine peripheral blood mononuclear cells (PBMCs) but was absent from murine PBMCs. Because we have found both phenotypic and genetic distinctions from signal 2 mediated "classical" NLRP3 activation, we propose to call this new pathway "alternative inflammasome."

RESULTS

A Monocyte Transdifferentiation System Models Human Inflammasome Activation In Vitro

In order to recapitulate the unique LPS-mediated IL-1β secretion observed in human monocytes (Danis et al., 1990; Hazuda et al., 1988; Lonnemann et al., 1989; Schumann et al., 1998), PBMCs of human, murine, or porcine origin were isolated from peripheral blood to be devoid of contaminating granulocytes (Figure 1 left panels). As previously reported, LPS by itself induced considerable IL-1β secretion in human PBMCs in absence of a classical signal 2 stimulus for the NLRP3 inflammasome (Nigericin). Notably, TLR2 activation by Pam3CSK4 was not sufficient to induce IL-1β secretion, despite its capacity to prime cells for

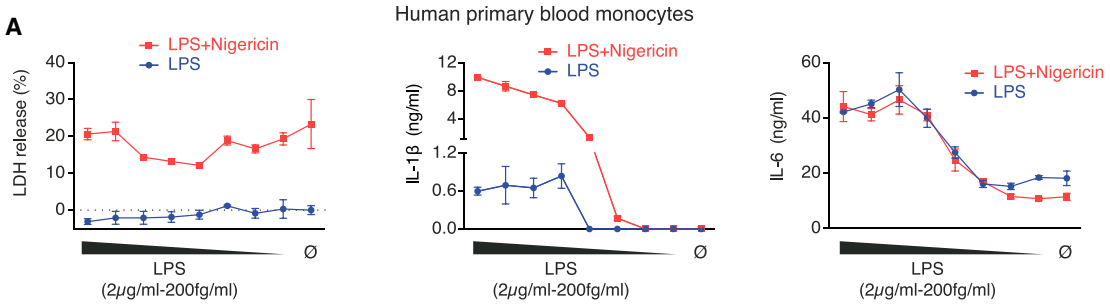
Nigericin-mediated IL-1β secretion. Additional Nigericin treatment also enhanced LPS-mediated IL-1β secretion by approximately 7-fold and induced concomitant pyroptotic cell death, as observed by lactate dehydrogenase (LDH) release (Figure 1A). Of note, cell viability was not compromised upon LPS-induced IL-1β secretion (Figure 1A). This unique, LPS-dependent inflammasome signaling cascade, which functions in the absence of a signal 2 stimulus, was not observed in the murine system. Instead, murine PBMCs required presence of a signal 2 stimulus to mount a considerable and significant IL-1β secretion (Figure 1B). Additionally, murine bone-marrow-derived macrophages (Figure S1A) and immortalized murine macrophages (data not shown) failed to secrete IL-1β in response to LPS treatment in the absence of additional Nigericin stimulation. Conversely, porcine PBMCs responded in a similar fashion as human PBMCs, readily secreting IL-1β in response to LPS only (Thorgersen et al., 2009), again in the absence of pyroptosis (Figure 1C).

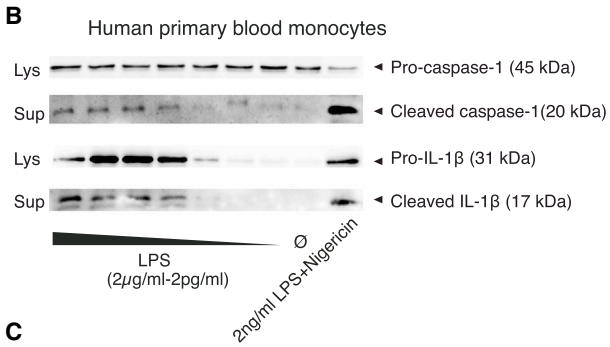
This LPS-dependent inflammasome response was also observed in isolated human monocytes, which constitute the main source for IL-1β within PBMCs (Netea et al., 2009). Solely upon LPS treatment, monocytes readily secreted IL-1β in the absence of pyroptosis (Figure 2A). Again, additional Nigericin treatment enhanced the LPS response by approximately 10-fold and led to concomitant pyroptotic cell death. Opposing to that, neither TLR2 nor TLR3 activation was sufficient to activate the inflammasome in primary human monocytes (Figure S1B). Studying IL-1β and caspase-1 by immunoblot in lysates and supernatants revealed that LPS stimulation by itself resulted in the release of mature, cleaved IL-1β, which was accompanied by the processing of caspase-1 (Figure 2B). Because established human monocyte-like cells lines, such as THP1 cells, did not show this unique IL-1β response (Figure S1C), we explored the possibility to employ a recently described transdifferentiation system, in which immortalized human B cells can be transdifferentiated into post-mitotic monocytes. This system employs the heterologous expression of the myeloid-specific transcription factor C/EBPα from a β-estradiol-translocatable construct (BLaER1 cells, Figure 2C) (Rapino et al., 2013). Upon transdifferentiation, BLaER1 cells stop proliferating, become adherent, acquire a monocyte or macrophage-like morphology, develop a myeloid gene-expression profile, and become highly phagocytic (Rapino et al., 2013) (Figures 2C and 2D). Comparing the cytokine response of LPS-stimulated BLaER1 cells before and after transdifferentiation to primary B cells and monocytes, we observed that BLaER1 transdifferentiation resulted in a cytokine response pattern closely resembling primary human monocytes (Figure S2). Most importantly, characterizing the inflammasome response using LPS alone or in conjunction with Nigericin in BLaER1 monocytes, we detected IL-1β secretion upon LPS treatment only, which was comparable in sensitivity and magnitude to primary human monocytes (Figure 2E). Again, this response was accompanied by caspase-1 activation and IL-1β maturation (Figure 2F). Analogous to primary monocytes, LPS-dependent inflammasome activation did not result in LDH release, whereas Nigericin mediated stimulation readily induced pyroptotic cell death. Mirroring the response in primary monocytes (Figure 2A), NLRP3 activation did not require priming, as both pyroptosis as well as caspase-1 cleavage could be induced by Nigericin alone (Figures 2E and 2F). Nevertheless, this response was strongly enhanced by adding LPS (Figure 2E). Altogether these results indicated that BLaER1 monocytes faithfully recapitulated this unique, species-specific signal 1 only inflammasome response that does not involve pyroptosis.

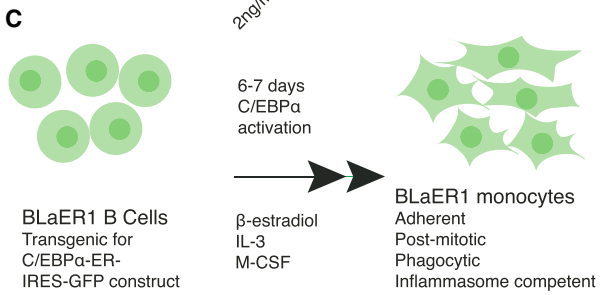
Alternative Inflammasome Activation Is Phenotypically Distinct from Classical Inflammasome Activation

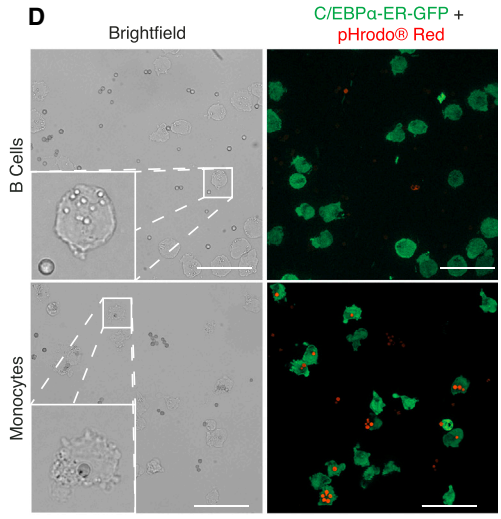
To dissect the underlying pathway of inflammasome activation in absence of a signal 2 stimulus, we generated gene deficiencies using CRISPR/Cas9 technology in proliferating B cell BLaER1 cells (Schmid-Burgk et al., 2014), which were identified by deep-sequencing and validated by immunoblot (Figure S3). Analyzing inflammasome activation upon transdifferentiation, we observed that non-canonical inflammasome activation was dispensable for IL-1β secretion in response to extracellular LPS, whereas TLR4 turned out to be a critical signaling component (Figures S4A and S4B). Nevertheless, to exclude potential cross talk with cytosolic LPS recognition (non-canonical inflammasome activation), which was mediated by caspase-4 in BLaER1 monocytes (Figure S4A), we conducted all of the following experiments in *CASP4*-deficient cells (from now on referred to as "Ctrl"). Subsequent experiments showed that classical inflammasome activation by Nigercin and LPS-mediated inflammasome activation shared the common downstream signaling components NLRP3, ASC, and CASP1, whereas only LPS-mediated IL-1β secretion required the TLR-adaptor TRIF (Figure 3A, Figure S4C). However, both LPS- and Nigericin-mediated IL-1β secretion was completely blunted in *MyD88*-deficient cells as they displayed a severe NF-κB priming defect (Figure S4D). To exclude an impact of TLR4 mediated inflammasome activation on Nigericin stimulation, we from now on used TLR2 priming by Pam3CSK4, whenever necessary. Purinergic receptor P2X, ligand gated ion channel, 7 (P2RX7) was not involved in LPS-mediated inflammasome activation in BLaER1 monocytes (Figure S4E) and pharmacological inhibition of its signaling did not block LPS-mediated inflammasome activation, while readily inhibiting ATP-induced IL-1β secretion in primary human monocytes (Figure S4F). Thus, a role for paracrine or autocrine ATP signaling in LPS-triggered inflammasome activation (Netea et al., 2009; Piccini et al., 2008) can be excluded.

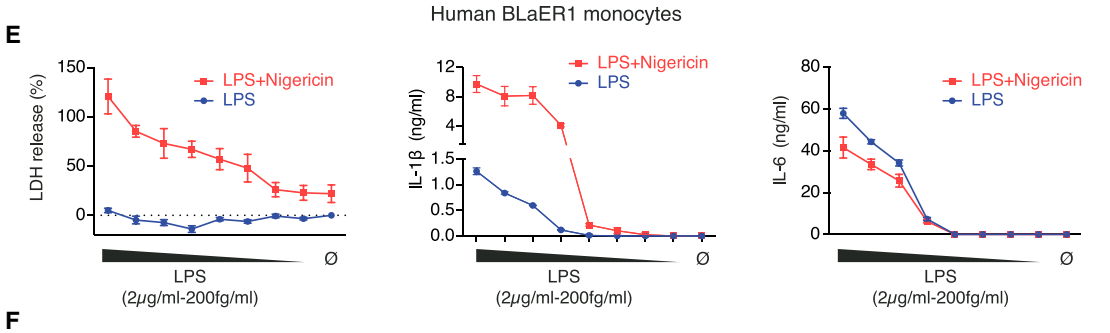
The fact that LPS-activated monocytes did not undergo pyroptosis yet displayed caspase-1 maturation, prompted us to further characterize differences in NLRP3 activation by LPS or by Nigericin. All known NLRP3 activators, including caspase-4 mediated non-canonical activation, can be blocked by culturing cells in high K^+ medium, which inhibits K^+ efflux (Baker et al., 2015; Rühl and Broz, 2015; Schmid-Burgk et al., 2015). Opposing to that, LPS-mediated inflammasome activation proceeded in the presence of elevated extracellular potassium concentrations that readily blocked Nigericin-mediated inflammasome activation (Figure 3B). Another hallmark of inflammasome signaling is the oligomerization of the adaptor-protein ASC into large cytosolic complexes called pyroptosomes (Fernandes-Alnemri et al., 2007). To monitor pyroptosome formation in BLaER1 monocytes, we generated an ASC-mRFP reporter cell line by introducing monomeric RFP C-terminal to the endogenous ASC locus. Following Nigericin treatment, BLaER1 monocytes either showed re-localization of ASC-RFP into one or

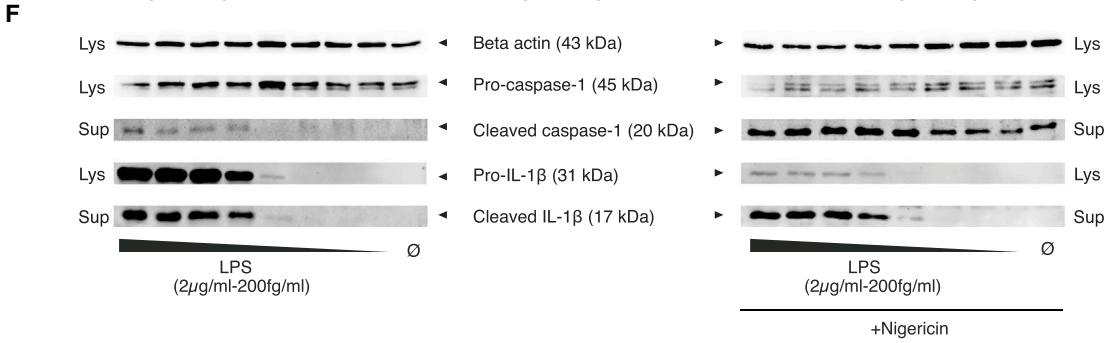












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multiple pyroptosomes per cell (6%) or they lost ASC-mRFP protein expression (94%), most likely attributable to membrane disintegration during pyroptosis. Preventing cell death using the caspase-1 inhibitor Z-YVAD-FMK increased the proportion of cells displaying ASC pyroptosomes upon Nigericin stimulation to 50%. Opposing to that, we could not detect pyroptosome formation during LPS-mediated inflammasome activation, neither in absence or presence of Z-YVAD-FMK (Figures 3C and 3D). These results were recapitulated in un-tagged BLaER1 monocytes using the chemical cross linker DSS, which only crosslinks ASC molecules that have been assembled into a pyroptosome due to spatial proximity (Fernandes-Alnemri et al., 2007). Confirming the absence of pyroptosomes in LPS-mediated inflammasome activation, we only detected cross-linked ASC-dimers upon Nigericin treatment, a phenomenon that was fully NLRP3 dependent (Figure 3E). To distinguish this signaling cascade from classical inflammasome activation (canonical or non-canonical), we refer to this pathway as "alternative inflammasome" activation in the following (Figure S4G).

RIPK1-FADD-CASP8 Signaling Genetically Dissects Alternative from Classical Inflammasome Signaling

With the proximal signaling events during alternative inflammasome activation remaining elusive, we genetically deleted a panel of known signaling hubs downstream of TRIF. Doing so, we identified a crucial role for RIPK1-FADD-CASP8 signaling upstream of alternative inflammasome activation. Of note, this axis could only be studied in necroptosis-deficient cells, as perturbation of *RIPK1*, *FADD*, or *CASP8* resulted in necroptosis-dependent IL-1β secretion that was accompanied by massive cell death and impaired IL-6 production (Figure 4A, left panel and see below). However, if necroptosis was prevented by additional *RIPK3* or *MLKL* deletion, the crucial involvement of the RIPK1-FADD-CASP8 axis for alternative inflammasome signaling became apparent: While *RIPK3* or *MLKL*-deficient cells displayed no defect in alternative inflammasome signaling, additional deletion of *RIPK1*, *FADD*, or *CASP8* completely blunted alternative inflammasome activation in necroptosis-deficient cells (Figure 4A, middle and right panel). Importantly, only alternative but not classical inflammasome activation engaged RIPK1-FADD-CASP8 signaling. To this end, deficiency in any of these components had no impact on Nigericin mediated inflammasome activation (Figure 4A, red bars; immunoblot in Figure S5A). Thus, under steady-state conditions, this signaling node served two functions: On the one hand it was required to trigger the alternative inflammasome, and on the other hand it was necessary to prevent necroptosis (see below). Notably, RIPK1-FADD-CASP8 alternative inflammasome signaling is reminiscent of tumor necrosis factor receptor (TNFR) signaling, leading to complex IIb (ripoptosome) formation (Vanden Berghe et al., 2014). However, we could not detect an involvement of autocrine TNF signaling in the course of alternative inflammasome activation (Figure S5B) and TNF by itself was not sufficient to induce the alternative inflammasome (data not shown). Additionally, caspase-8-mediated NLRP3 activation did not display an apoptotic feedback loop because it proceeded unperturbed in BLaER1 monocytes devoid of the executioner caspases *CASP3*, *CASP6*, and *CASP7* (Figure 4B). Because these cells displayed a severe NF-κB signaling defect upon LPS stimulation we normalized the IL-1β secretion in relation to the IL-6 response to take the diminished pro-IL-1β amount in these cells into account.

Distinct processing requirements for different functions of caspase-8 have been described. Whereas extrinsic apoptosis downstream of TNFR or FasR requires both catalytic caspase activity and auto-proteolysis, caspase-8-mediated inhibition of TNF-mediated necroptosis was shown to require catalytic caspase activity only, with a heterodimer of caspase-8:cFLIP being operational in this setting (Oberst and Green, 2011; Oberst et al., 2011). To study the processing requirements of caspase-8 in the context of alternative inflammasome activation, we complemented *CASP8*$^{-/-}$ × *RIPK3*$^{-/-}$ cells with a wild-type, a catalytically dead or an auto-processing-deficient caspase-8 construct (Figure 4C, Figures S5C and S5D). These studies revealed that both catalytic activity and auto-proteolysis of caspase-8 were required for alternative inflammasome signaling to occur (Figure 4C). Studying the caspase-8 constructs in necroptosis-competent cells (*CASP8*$^{-/-}$) revealed that caspase-8-mediated inhibition of necroptotic cell death only required the catalytic activity of caspase-8, but not its auto-processing activity. To this end, both wild-type and non-cleavable caspase-8 inhibited LDH release in response to TNF and LPS stimulation, whereas this was not observed for the catalytically dead mutant (Figure S5E). Of note, in the context of heterologous caspase-8 expression, we observed a slight LDH release and increased IL-6 production following LPS stimulation (Figure 4C), which was absent in cells with endogenously expressed caspase-8 (Figure 4A). These responses required both the catalytic and auto-processing activity of caspase-8 (Figure 4C) and are likely attributable to an artificial apoptosis response caused by overexpression of caspase-8. Importantly, immunoblotting of active endogenous caspase-8 was detectable after LPS stimulation in BLaER1 monocytes (Figure 4D), which confirmed proteolytic activation of caspase-8 in the course of alternative inflammasome activation. Whereas pharmacological inhibition of NLRP3 by a small-molecule inhibitor (MCC950) (Coll et al., 2015) readily

Figure 2. LPS Activates the Inflammasome in Primary and BLaER1 Human Monocytes

(A and B) Human monocytes from peripheral blood were treated for 14 hr with ascending amounts of LPS or left untreated and stimulated for 2 hr with Nigericin (red) or left untreated (blue). LDH release and cytokine secretion are depicted as mean + SD from one representative donor out of three. Immunoblotting from one representative donor of two is depicted.

(C) BLaER1 transdifferentiation system.

(D) Fluorescence microscopy of BLaER1 cells before (upper panel) and after (lower panel) transdifferentiation that were incubated with pHrodo® beads. Scale bars denote 50 μm.

(E and F) BLaER1 monocytes were stimulated as indicated. LDH release and cytokine secretion are depicted as mean + SEM of three independent experiments. Immunoblotting from one representative experiment of three is shown.

Please see also Figures S1 and S2.

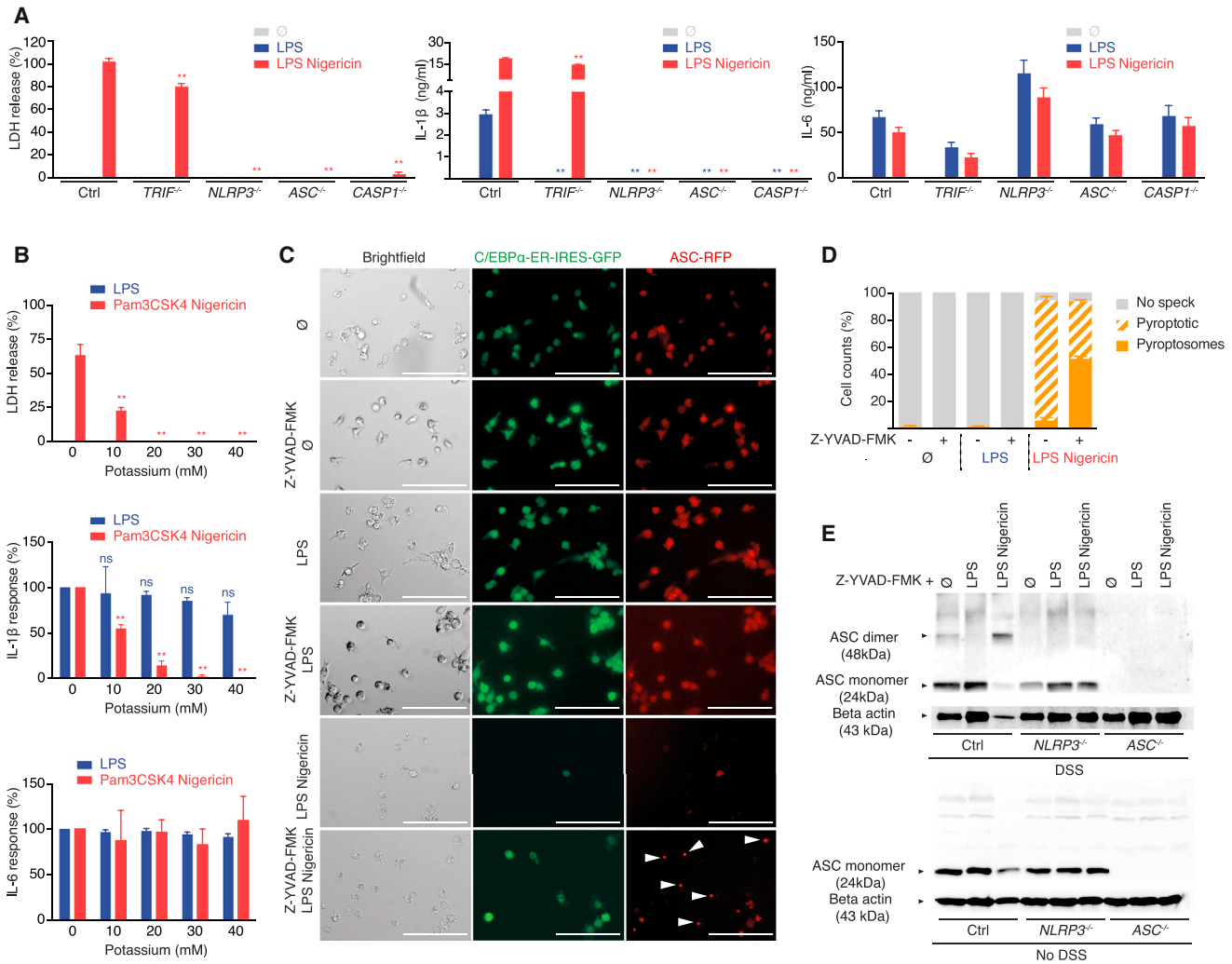


Figure 3. LPS Activates an Alternative NLRP3 Inflammasome in BLaER1 Monocytes

(A) BLaER1 monocytes of the indicated genotype were stimulated for 14 hr with LPS and for 6 hr with Nigericin or left untreated. LDH release and cytokine secretion from one representative clone of two are depicted as mean + SEM of three independent experiments. *p < 0.05, **p < 0.01, ns = not significant (tested against control).

(B) BLaER1 monocytes were stimulated as indicated in presence of increasing concentrations of K^+ with LPS for 14 hr or with Pam3CSK4 for 14 hr and with Nigericin for 6 hr. LDH release and cytokine secretion are depicted as mean + SEM of three independent experiments. *p < 0.05, **p < 0.01, ns = not significant (against the 0 mM K^+ condition).

(C and D) BLaER1 ASC-RFP monocytes were stimulated as indicated and analyzed by light microscopy. Micrographs of one representative of three experiments are depicted. Pyroptosome formation is depicted as mean + SEM of three independent experiments. Scale bars denote 100 μm.

(E) BLaER1 monocytes of indicated genotype were stimulated in presence of Z-YVAD-FMK for 14 hr with LPS and for 6 hr with Nigericin or left untreated. Immunoblot analysis against ASC of cell lysates with or without DSS crosslinking is depicted from one representative experiment of two.

Please see also Figures S3 and S4.

blocked IL-1β secretion, it did not prevent caspase-8 maturation (Figure 4D). This firmly establishes the epistatic role of caspase-8 upstream of alternative NLRP3 activation (Figure S5F). Although the RIPK1-FADD-CASP8 signaling axis had a minor impact on LPS-mediated NF-kB activation (decreased IL-6 secretion in Figure 4A), a critical involvement of this signaling axis on transcriptional priming of NLRP3 (signal 1) can be excluded, since respective gene targeted BLaER1 clones remained fully responsive to classical inflammasome activation (Figure 4A) and did not show impaired NLRP3 or pro-IL-1β expression (Figure 4E).

Although murine PBMC are not capable of alternative inflammasome activation (Figure 1B), murine bone-marrow-derived dendritic cells (BMDC) have been described to secrete IL-1β in response to LPS alone (He et al., 2013; Moriwaki et al., 2015). Confirming these results, we characterized this response to be fully dependent on Ripk3 and only minimally dependent on Nlrp3 (Figure S6A). Importantly, LPS treatment induced cell death in murine BMDC (Figure S6A), whereas the Nlrp3 response resulted in pyroptosome formation in these cells (Figure S6B). Altogether, these results clearly discerned the LPS response

pathway in murine BMDCs from alternative inflammasome activation.

Necroptosis Triggers Classical Inflammasome Signaling

As noted above, alternative inflammasome insufficient cells (*RIPK1*$^{-/-}$, *FADD*$^{-/-}$, or *CASP8*$^{-/-}$ cells) succumbed to necroptosis in response to LPS (Figures 4A and 5A; Movie S1; Figure S7A), which was accompanied by robust IL-1β secretion. This is consistent with the previously proposed role of necroptosis in inflammasome activation (Kang et al., 2013; Vince et al., 2012). Of note, necroptosis also required the presence of the TLR adaptor TRIF (Figure S7B) and the NLRP3 inflammasome to secrete IL-1β (Figure 5B; Figure S7C). Indeed, genetic ablation of *NLRP3* completely prevented caspase-1 and IL-1β maturation in the context of necroptosis, with substantial amounts of pro-IL-1β being secreted from inflammasome-deficient necroptotic cells (Figure S7C). Of note, the impaired IL-6 secretion in necroptotic cells was partially restored upon NLRP3 inflammasome deficiency (Figure 5B), indicating that NLRP3 inflammasome signaling impeded pro-inflammatory gene expression under these conditions. In contrast to previous reports, dynamin 1-like (DNM1L) (Wang et al., 2014) and PGAM family member 5 (PGAM5) (Kang et al., 2013) were dispensable downstream of RIPK3 for necroptosis-mediated NLRP3 activation (Figure S7D). Instead, necroptosis-mediated inflammasome signaling was preceded by K^+ efflux (Figure S7E) and blocking K^+ efflux decreased caspase-1 and IL-1β maturation in these cells (Figure 5C). In line with these data, LPS triggered pyroptosome formation in caspase-8 deficient BLaER1 monocytes (Figure 5D). Thus, in contrast to ''alternative inflammasome'' activation, classical NLRP3 inflammasome activation is at play if induced in the course of necroptosis (Figure S7F).

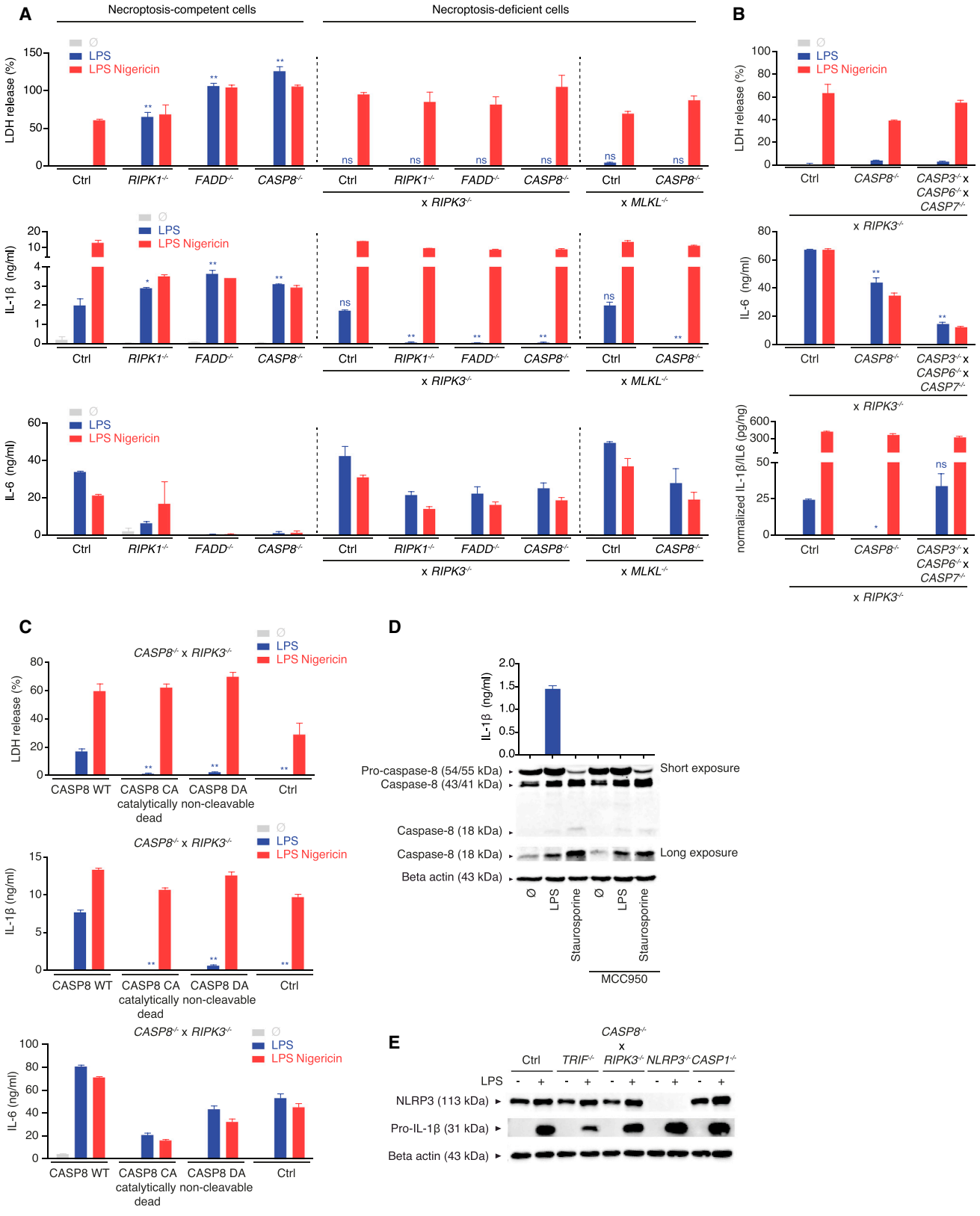
LPS Activates the Alternative Inflammasome Pathway in Primary Monocytes

Next, we wanted to address the existence of the here-established ''alternative inflammasome'' pathway in primary human monocytes. To this end, we made use of small-molecule inhibitors to target NLRP3 (MCC950) (Coll et al., 2015), caspase-1 (Z-YVAD-FMK), caspase-8 (Z-IETD-FMK), pan-caspases (Z-VAD-FMK), and RIPK3 (GSK872) (Figure S7G). Inhibition of NLRP3 (MCC950) completely abrogated IL-1β secretion in response to LPS, as well as Nigericin-mediated IL-1β secretion and LDH release. Caspase-1 inhibition (Z-YVAD-FMK) had comparable, albeit weaker effects (Figure 6A). These results confirmed that alternative and classical inflammasome activation were fully dependent on the NLRP3 inflammasome in primary monocytes. To evaluate the dual function in alternative inflammasome signaling and necroptosis inhibition of caspase-8, we employed its inhibitor Z-IETD-FMK at different concentrations. At low concentrations, it inhibited LPS induced IL-1β secretion, without inducing cell death (Figure 6B; +). If, however, high doses of the caspase-8 inhibitor Z-IETD-FMK or the pan-caspase inhibitor Z-VAD-FMK were applied, human monocytes responded with a massive cell death and IL-1β secretion in response to LPS stimulation (Figure 6B; ++). In line with the BLaER1 monocyte model, this could be blocked with the RIPK3 inhibitor GSK872, indicating that LPS-stimulated monocytes responded with necroptosis-driven inflammasome activation upon caspase-8 perturbation. The fact that only Z-VAD-FMK but not Z-IETD-FMK inhibited IL-1β secretion after Nigericin stimulation in presence of GSK872, confirmed specificity of caspase-8 inhibition in this setting. Of note, high doses of Z-IETD-FMK and Z-VAD-FMK also increased cell death following Pam3CSK4 priming and Nigericin stimulation. As this could be blocked with the RIPK3 inhibitor, we attribute this to necroptosis induction via an autocrine TNF loop caused by TLR2 stimulation. Another trait of alternative inflammasome activation in BLaER1 monocytes was the requirement of caspase-8 cleavage. Analyzing this activation step in primary monocytes by immunoblotting, we observed increased abundance of activated caspase-8 p18 after LPS stimulation (Figure 6C), confirming a pivotal role of caspase-8 in LPS-treated primary monocytes.

In line with the phenotypic traits of alternative inflammasome activation, we observed that LPS-triggered IL-1β secretion proceeded independently of K^+ efflux in primary monocytes, whereas classical NLRP3 activation could be easily inhibited using increasing amounts of extracellular K^+ (Figure 7A). Moreover, by staining pyroptosomes in human monocytes following activation, we only detected an increase in pyroptosome-positive cells after classical but not after alternative inflammasome (Figure 7B). At the same time, pyroptosome formation could be readily monitored in classically activated cells by DSS crosslinking, yet it was absent in LPS-stimulated primary monocytes (Figure 7C). Of note, we could exclude that the validity of our pyroptosome assays was compromised by the lack of sensitivity to detect the inherently lower response of alternative inflammasome activation. In fact, IL-1β ELISPOT analysis showed that a similar number of cells were activated to secrete IL-1β during classical and alternative inflammasome activation (Figure 7D). As pyroptosome formation is an all or nothing response at the single cell level (Liu et al., 2014), this supported the validity of our assay. Moreover, this also indicated that the inflammasome response was gradually lower at the single cell level, but not at the proportion of cells being activated. We conclude, that apart from classical, signal 2 dependent NLRP3 activation, alternative inflammasome activation depicts a distinct, species-specific signaling identity, which we could discriminate both phenotypically and genetically from each other (Table S1). Because alternative inflammasome activation embraces both sensitivity and promiscuity of TLR4, we propose a pivotal role for this signaling cascade in TLR4-driven, IL-1β-mediated immune responses and immunopathology in humans.

DISCUSSION

The current understanding of IL-1β secretion involves a two-step model, in which the first stimulus is integrated to induce transcription and translation of pro-IL-1β, and a subsequent second stimulus is required for proteolytic maturation of pro-IL-1β, most commonly by inflammasome-activated caspase-1. Here, we report an alternative pathway for NLRP3 inflammasome activation, which is induced by TLR4 signaling only, without the necessity for a second stimulus. Although alternative inflammasome activation required ASC and yielded mature and secreted caspase-1 and IL-1β, it proceeded independently of pyroptosome formation, pyroptosis, and signaled independently of K^+ efflux,



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a common denominator of classical NLRP3 stimuli. While these features clearly discriminate alternative from classical inflammasome signaling, we could also genetically dissect alternative from classical inflammasome activation. To this end, we identified a non-redundant role for TRIF-RIPK1-FADD-CASP8 signaling resulting in caspase-8 catalytic activity upstream of NLRP3 in this unique pathway. Although this is, to our knowledge, the best described molecular activation mechanism upstream of NLRP3, the connection between caspase-8 and NLRP3 remains elusive. Because the NLRP3 protein did not undergo direct cleavage by caspase-8 (data not shown), we speculate that caspase-8-mediated cleavage of an unknown, intermediate protein is required for alternative inflammasome activation.

The addition of this alternative mode of activation to inflammasome biology adds versatility to its role in host defense and challenges the conventional view of it as a non-reversible all-or-nothing response. Indeed, this pathway allows the cell to respond with a gradual release of IL-1β without committing to non-reversible cell death. Thus, alternative inflammasome activation and associated IL-1β secretion could be pivotal in a setting where cell activation and effector function are temporally separated. In line with this, the uncoupling of inflammasome activation and pyroptosis, as seen in alternative inflammasome activation, might enable pro-inflammatory IL-1β signaling while avoiding pyroptosis-associated effector functions (Miao et al., 2010; von Moltke et al., 2012). Of note, uncoupling of pyroptosis from inflammasome activation has already been described in murine neutrophils, yet here it appears that these cells harbor a general resistance to inflammasome-induced pyroptosis per se (Chen et al., 2014). However, in monocytes, it is the specific signaling via the alternative inflammasome that omits cell death in otherwise pyroptosis-competent cells. Apart from that pyroptosis-independent release of IL-1β indicates that passive release of mature IL-1β in the course of a lytic cell death is not the only form of IL-1β secretion. Indeed, other unconventional secretory pathways have to be employed to release IL-1β from living cells. This might involve threading of IL-1β into early stage phagophores (Zhang et al., 2015) and the autophagy machinery (Dupont et al., 2011).

Of note, while a role for caspase-8 in IL-1β maturation (Maelfait et al., 2008; Moriwaki et al., 2015; Vince et al., 2012; Yabal et al., 2014) and NLRP3 priming (signal 1) (Gurung et al., 2014) has been described before, a unique role for caspase-8 upstream of NLRP3 at the posttranslational level, as observed for alternative inflammasome signaling, has not been described yet. In murine cells, caspase-8 has been shown to directly mature IL-1β (Maelfait et al., 2008) and also TLR signaling is able to activate caspase-8 via Trif-Ripk3 in murine cells to drive IL-1β maturation directly (Moriwaki et al., 2015; Vince et al., 2012; Yabal et al., 2014). In the course of alternative inflammasome activation, we could not find any indication of caspase-8 directly cleaving IL-1β, because monocytes cells failed to secret IL-1β in response to LPS, when NLRP3, ASC, or caspase-1 were absent. Moreover, the NLRP3 specific inhibitor MCC950 completely blocked alternative inflammasome activation in primary monocytes. Of note, because BLaER1 monocytes are in principle able to cleave IL-1β by caspase-8 in the course of cIAP depletion-mediated necroptosis (data not shown), one can conclude that this pathway is specifically omitted during alternative inflammasome activation. On the other hand, caspase-8 signaling has been suggested to act upstream of Nlrp3 in both canonical and non-canonical activation in murine macrophages (Gurung et al., 2014). However, in this system the proposed upstream activation of Nlrp3 by caspase-8 in the context of classical inflammasome activation can be attributed to a priming defect (signal 1) in the murine macrophage system under study (Gurung et al., 2014). In BLaER1 monocytes, caspase-8 deficiency had no impact on NLRP3 priming and respective gene-deficient cells had no defect in classical inflammasome activation, clearly ruling out an involvement RIPK1-FADD-CASP8 signaling in the course of classical inflammasome activation. These results are in stark contrast to the alternative inflammasome cascade, in which this signaling hub is critically required upstream of NLRP3. Experiments in primary monocytes corroborated these findings, showing that the caspase-8 inhibitor Z-IETD-FMK blocked alternative, but not classical NLRP3 activation. Altogether these data indicate that caspase-8 is specifically involved upstream of alternative, but not classical inflammasome activation.

In summary, the data presented here reveal that TLR4 plays a critical role in NLRP3 inflammasome activation in the human system. While TLR4-signaling has already been implicated in the priming of NLRP3 responses, these data now position TLR4 mechanistically upstream of NLRP3 inflammasome activation in human monocytes. Given the high promiscuity and sensitivity of the TLR4 signaling cascade, especially in the human system, these data have important implications for our understanding of IL-1-driven immune responses. For example, it is well known that humans are far more prone to disease pathology in the course of endotoxemia than mice (Munford, 2010) and we speculate that the functionality of an alternative inflammasome pathway in humans might in part be responsible for this phenomenon. On the other hand, the role of TLR4 extends

Figure 4. Alternative Inflammasome Activation Is Propagated via RIPK1-FADD-CASP8 and Requires Caspase-8 activity and Auto-Cleavage

(A and B) BLaER1 monocytes of indicated genotype were stimulated with LPS (14 hr) and with Nigericin (6 hr) or left untreated. LDH release and cytokine secretion are depicted as mean + SEM of two (A) or three (B) independent experiments from one representative clone of two. *p < 0.05, **p < 0.01, ns = not significant (against ctrl).

(C) *CASP8*$^{-/-}$ × *RIPK3*$^{-/-}$ BLaER1 monocytes were reconstituted with caspase-8 coding variants and stimulated with LPS (14h) and Nigericin (6 hr) or left untreated. LDH release and IL-1β production of polyclonal cells are depicted as mean + SEM of three independent experiments. *p < 0.05, **p < 0.01, ns = not significant (against reconstitution with wild-type caspase-8).

(D) *TNFRSF1A*$^{-/-}$ BLaER1 monocytes were stimulated in presence or absence of the NLRP3 inhibitor MCC950 for 10 hr. Immunoblot and ELISA data (mean + SEM) show one representative experiment of two.

(E) Immunoblot analysis of BLaER1 monocytes of indicated genotype that were stimulated with LPS (14 hr). One representative experiment of two is depicted. Please see also Figures S5 and S6.

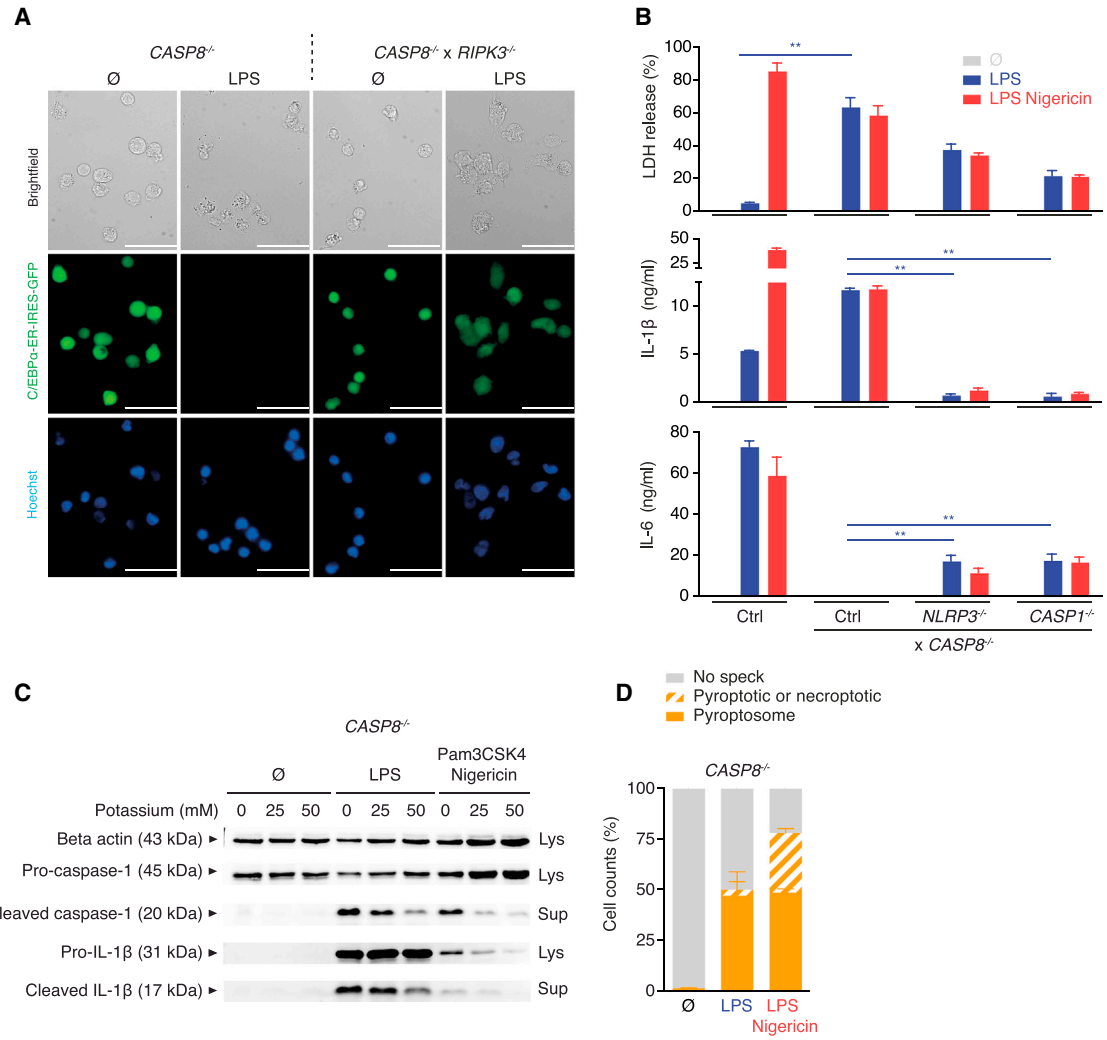

Figure 5. LPS-Mediated Necroptosis Activates the Classical NLRP3 Inflammasome via K^+ Efflux upon Caspase-8 Deficiency

(A) Fluorescence micrographs of BLaER1 monocytes of indicated genotype stimulated with LPS (4 hr). GFP expression and Hoechst DNA staining is depicted from one representative of two experiments. Scale bars denote 50 μm.

(B) BLaER1 monocytes of indicated genotype were stimulated with LPS (14 hr) and with Nigericin (6 hr) or left untreated. LDH release and cytokine secretion are depicted as mean + SEM of three independent experiments from one representative clone of two. *$p < 0.05$, **$p < 0.01$, ns = not significant.

(C) *CASP8*$^{-/-}$ BLaER1 monocytes were stimulated in presence of increasing concentrations of K^+ with LPS (14 hr) or with Pam3CSK4 (14 hr) and with Nigericin (6 hr). Immunoblot analysis of cell lysates and supernatants of one representative experiment of two is depicted.

(D) *CASP8*$^{-/-}$ BLaER1 ASC-RFP monocytes were stimulated as indicated. Pyroptosome formation is depicted as mean + SEM of three independent experiments from one representative clone of two.

Please see also Figure S7 and Movie S1.

far beyond the recognition of LPS. To this end it has been shown that TLR4 can be activated by a plethora of endogenous DAMPs (Chen and Nuñez, 2010) and that this recognition plays a decisive role in many sterile (auto-)inflammatory conditions, in which IL-1 signaling is at play (Dinarello, 2011; McGettrick and O'Neill, 2013). Our data suggest that low-grade, alternative inflammasome rather than classical inflammasome activation might be involved. To this end, we would consider it important to re-evaluate the role of classical inflammasome activation by signal 2 in these disease entities. Indeed, since the species-specific requirements of alternative inflammasome activation are apparently not met in mice, other model organisms, like the pig, should be considered to address the role of the inflammasome pathways in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture

BLaER1 and THP1 cells were cultivated in RPMI Medium 1640 supplemented with L-glutamine, sodium pyruvate, 10% (v/v) FCS (all Life Technologies), and 10 μg/ml ciprofloxacin (Fresenius Kabi). BLaER1 cells were transdifferentiated into monocytes for 6–7 days in medium containing 10 ng/ml of hrIL-3, 10 ng/ml hr-CSF-1 (M-CSF) (both PeproTech), and 100 nM β-Estradiol (Sigma-Aldrich) at 7×10^4 cells per 96 well. Cells were stimulated in fresh medium as indicated. THP1 cells were differentiated overnight with 100 ng/ml

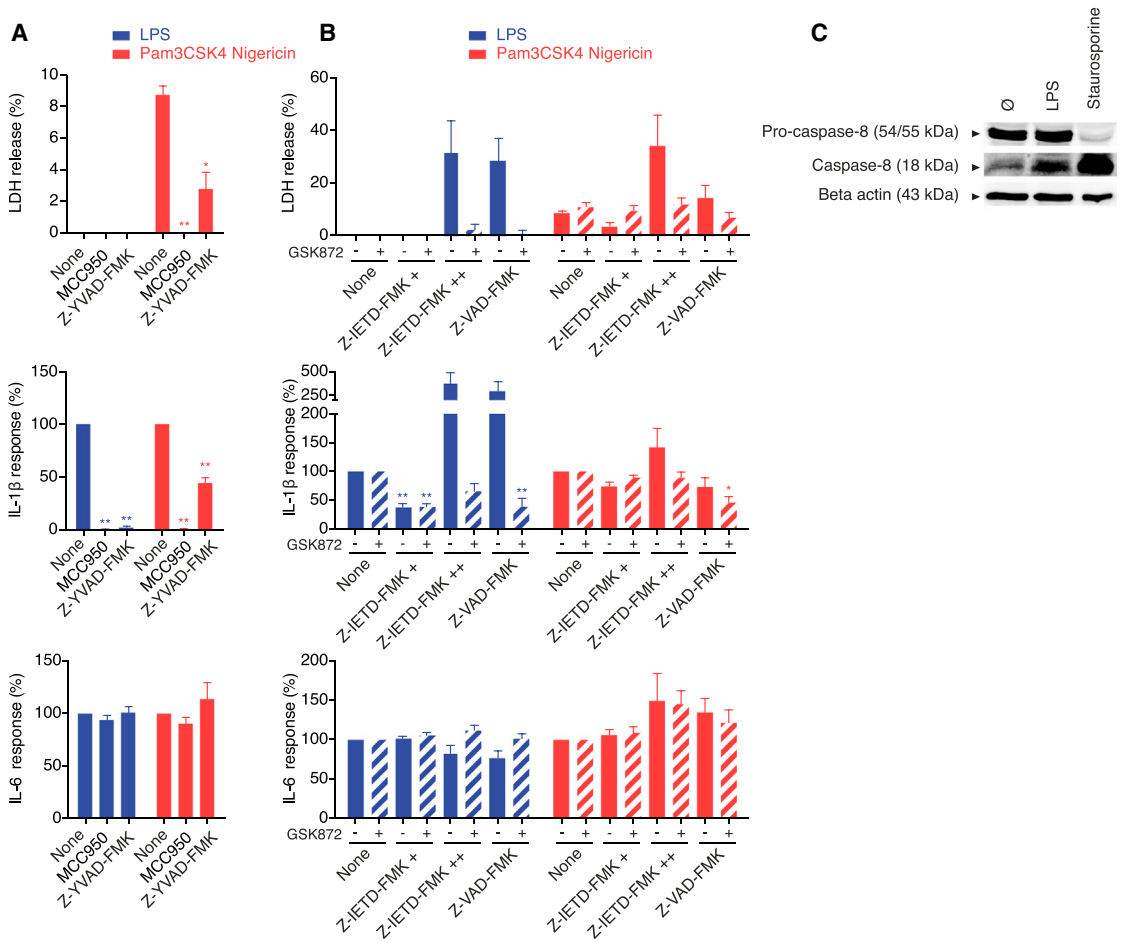

Figure 6. Dual Function for Caspase-8 in Inflammasome Activation and Necroptosis Inhibition in Primary Monocytes

(A and B) Human monocytes from peripheral blood were pretreated with indicated inhibitors and stimulated with Pam3CSK4 or LPS (14 hr) and Nigericin (2 hr) or left untreated. LDH release and cytokine secretion are depicted as mean + SEM of five donors. *$p < 0.05$; **$p < 0.01$; ns = not significant.

(C) Primary human monocytes were stimulated with LPS or with Staurosporine (10 hr). Caspase-8 activation was analyzed by immunoblotting from cell lysates. One representative donor out of five is depicted.

Please see also Figure S7.

PMA (Sigma-Aldrich), washed three times with ice-cold PBS and re-plated at 7×10^4 cells per 96 well. Murine BMM and BMDC were cultured in DMEM containing the same additives, whereas primary human monocytes and B cells were cultivated in RPMI Medium 1640 supplemented with L-glutamine, sodium pyruvate, 10% (v/v) FCS (all Life Technologies), and Pen-Strep (GIBCO). For background sensitive assays in primary monocytes (caspase-8 immunoblot, ELISPOT), medium was additionally supplemented with another 10% (v/v) FCS, MEM Non-Essential Amino Acids (Life Technologies), 500 U/ml GM-CSF (ImmunoTools), and 5 μg/ml Infliximab (MSD). All cell lines and respective gene targeted clones were routinely tested to be free of mycoplasma contaminations.

Isolation of PBMCs, Primary Human Monocytes, and B Cells

PBMCs were isolated from heparinized peripheral blood from informed, consenting, and healthy volunteers according to the principles of the Declaration of Helsinki and were approved by the responsible ethics committee (Ethics committee of the Medical Faculty, University of Bonn). Human primary monocytes were isolated from human PBMCs using CD14 MACS microbeads or Monocyte Isolation Kit II (Miltenyi), in case primary B cells were isolated by CD19 microbeads beforehand, according to the provider's protocol. For ELISA and LDH experiments, cells were plated at 3×10^5 cells per well. Murine or porcine PBMCs were obtained from heparinized blood from C57BL/6 mice or from pigs (German Landrace) by density gradient centrifugation using Biocoll separating solution (Biochrom) and subjected to short erythrocyte lysis (BD Pharm Lyse). Porcine PBMCs were enriched for adherent cells for 4 hr in a plastic dish. Experiments were conducted in accordance with the Principles of Laboratory Animal Care guidelines and were approved by the Local Animal Care Commission of North Rhine-Westphalia. Animal experiments and handling were also supervised by Institutional Animal Care and Use Committee (IACUC) of the medical faculty Bonn (HET, House of Experimental Therapy, University Hospital, University of Bonn).

Cell Stimulation

If not otherwise indicated, cells were stimulated with 200 ng/ml ultrapure LPS from *E. coli* (Invivogen) for 14 hr. Pam3CSK4 (Invivogen) was added at 2 μg/ml into monocyte cultures and at 20 μg/ml in BLaER1 monocyte cultures for 14 hr. After priming with the respective stimulus, Nigericin (Sigma-Aldrich) was added to a final concentration of 6.5 μM for 2 hr or in case of BLaER1 cells to a final concentration of 2.167 μM for 6 hr or ATP (Sigma Aldrich) was added to a final concentration of 5 mM for 2 hr. For LPS lipofection 2 μg of ultrapure LPS per 96 well were transfected using LF2000 (Life Technologies) according to the manufacturer's instructions. Supernatant were analyzed after 20 hr of stimulation. 100 ng/ml hTNF (PeproTech), 1 μM Birinapant (BioCat), and 20 μM Z-VAD-FMK (Peptide Institute, Inc) or 0.5 μM Staurosporine (Santa

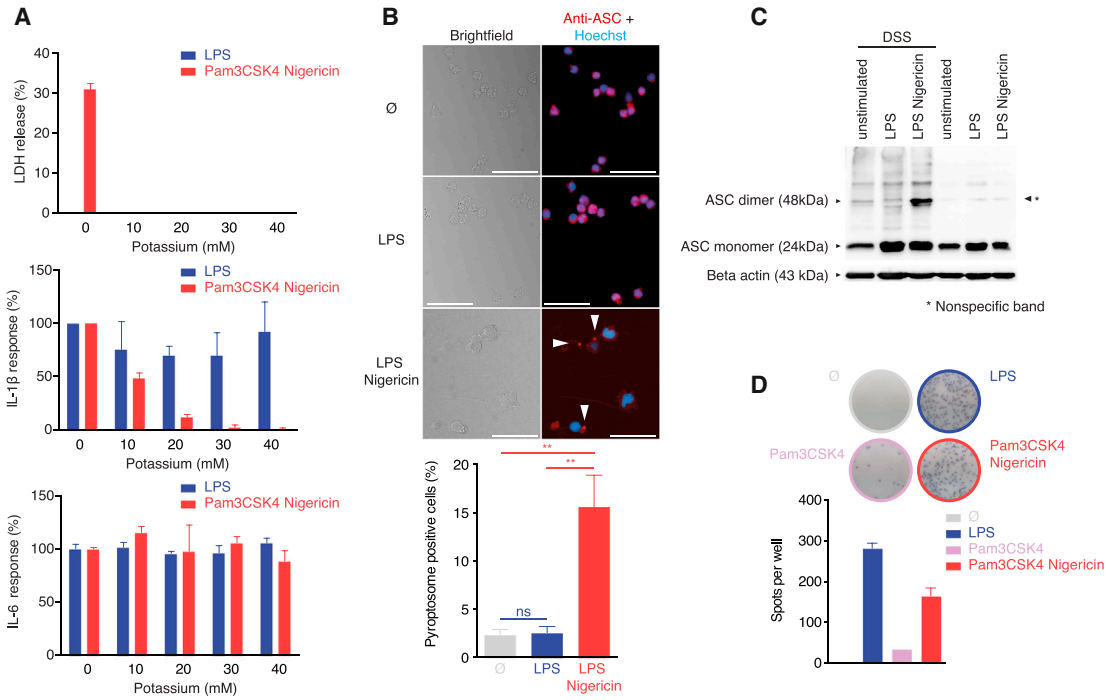

Figure 7. Alternative Inflammasome Activation in Primary Monocytes

(A) Primary human monocytes were stimulated in increasing concentrations of extracellular K^+ with LPS or Pam3CSK4 (14 hr) and with Nigericin (2 hr). LDH release and cytokine secretion are depicted as mean + SD of duplicates from one representative donor out of three.

(B) Fluorescence micrographs from primary human monocytes stimulated with LPS (14 hr) and then left untreated or stimulated with Nigericin (2 hr). Pyroptosomes were detected by immunostaining for ASC. Representative images from one representative donor out of eight are depicted. Quantification is depicted as mean + SEM of eight independent donors. **$p < 0.01$; ns = not significant. Scale bars denote 50 μm.

(C) Immunoblot analysis of ASC in cell lysates with or without DSS crosslinking is depicted from one representative donor out of three.

(D) IL-1β ELISPOT assay from primary monocytes stimulated with LPS or Pam3CSK4 (6 hr) and left untreated or stimulated with Nigericin (2 hr). Data is depicted as mean + SD of duplicates from one representative donor of two.

Please see also Table S1.

Cruz) were added for 14 hr to cells. For TLR3 activation poly(I:C) RNA (Invivogen) was added for 16 hr at indicated concentrations. For inhibition of potassium efflux, cells were re-suspended in medium, which was diluted with 150 mM potassium chloride (Roth) to contain indicated potassium concentrations. For small molecule inhibition, compounds were added 1 hr before stimulation of cells at following concentrations: high 20 μM/low 5 μM Z-IETD-FMK (BD Biosciences), 20 μM Z-VAD-FMK (Peptide Institute, Inc), 20 μM Z-YVAD-FMK (R&D Systems), 3 μM GSK872 (Aobious), 5 μM MCC950, 300 μM oxATP (Sigma-Aldrich), 10 μM KN-62 (Abcam), and 10 μM A438079 (Abcam).

CRISPR/Cas9 Mediated Gene Targeting

Gene-deficient BLaER1 cells were generated using a previously described protocol (Schmid-Burgk et al., 2014). Briefly, sgRNAs (20 or 18-mer), specific for the indicated genes, were designed to target an early coding exon of the respective gene. BLaER1 cells were electroporated with U6-sgRNA and CMV-mCherry-T2A-Cas9 expression plasmids using a Biorad GenePulser device. Flow cytometry sorted mCherry-positive cells were plated under limiting dilution conditions. Sub-cloned monoclonal cell lines were identified, rearranged, and duplicated. One half was used for further cell culture and the other half was genotyped via deep sequencing. Gene targeted clones contained all-allelic frameshift mutations without any wild-type reads. Two independent single-cell clones, in which both alleles were successfully disrupted, were analyzed per genotype.

Statistical Tests

If indicated, results were tested for statistical significance using one-way ANOVA and Bonferroni's test to correct for multiple comparisons if multiple genotypes, stimuli, or conditions were to be compared. If two genotypes, stimuli, or conditions were to be compared, two-tailed Student's t tests were used. Statistical analyses of normalized data were performed using a one-sample Student's t test. All data calculations were performed using GraphPad Prism. If not otherwise indicated all conditions were tested against the corresponding wild-type clone. * $p < 0.05$, ** $p < 0.01$, ns = not significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, Supplemental Experimental Procedures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.01.012.

AUTHOR CONTRIBUTIONS

M.M.G. and V.H. conceived this study and designed the experiments. M.M.G. and T.S.E. performed most of the experiments, and D.C. performed the experiments involving murine BMMs and BMDCs. T.S. and J.L.S.-B. established the gene targeting and generated the CRISPR constructs. A.A.B.R. and M.A.C. provided MCC950 and F.R. and T.G. provided BLaER1 cells. M.M.G. and V.H. wrote the manuscript with the input from all authors. V.H. supervised the study.

ACKNOWLEDGMENTS

We kindly thank Karin Pelka for providing pHrodo® beads, Gabor Horvath for imaging support, Bernardo Franklin for the ASC-staining protocol (all Institute

of Innate Immunity, University of Bonn), Michael Zwick for porcine blood (Haus für Experimentelle Therapie, University of Bonn), and Andreas Jaensch and Harald Färber for support with K^+ measurements (Institute for Hygiene and Public Health, University of Bonn). We kindly thank Elmar Endl and his team for FACSorting, Sarah Zuber for great technical support, Grethe Brügmann for obtaining blood samples, and Christopher Jakobs and Björn Hiller for help with murine PBMC isolation (all Institute of Molecular Medicine, University of Bonn). We furthermore kindly thank Vishva Dixit (Genentech) and Egil Lien (UMass Medical School) for providing Nlrp3 or Ripk3-deficient animals. This work was supported by grants from the German Research Foundation (SFB704 and SFB670) to V.H. V.H. is a member of the Excellence cluster ImmunoSensation.

Received: September 15, 2015
Revised: December 4, 2015
Accepted: January 20, 2016
Published: March 29, 2016

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