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Ion efflux and influenza infection trigger NLRP3 inflammasome signaling in human dendritic cells

Melissa Victoria Fernandez, Elizabeth Miller,† Florian Krammer,‡ Ramya Gopal,§ Benjamin D. Greenbaum,§ and Nina Bhardwaj§,1*

*Department of Pathology, New York University School of Medicine, New York, New York, USA; †Division of Infectious Diseases, Department of Medicine, ‡Department of Microbiology, and §Division of Hematology and Oncology, Hess Center for Science and Medicine, Icahn School of Medicine at Mount Sinai, New York, New York, USA

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ABSTRACT

The nucleotide-binding oligomerization domain-like receptor protein 3 inflammasome, a multiprotein complex, is an essential intracellular mediator of antiviral immunity. In murine dendritic cells, this complex responds to a wide array of signals, including ion efflux and influenza A virus infection, to activate caspase-1-mediated proteolysis of IL-1β and IL-18 into biologically active cytokines. However, the presence and function of the nucleotide-binding oligomerization domain-like receptor protein 3 inflammasome in human dendritic cells, in response to various triggers, including viral infection, has not been defined clearly. Here, we delineate the contribution of the nucleotide-binding oligomerization domain-like receptor protein 3 inflammasome to the secretion of IL-1β, IL-18, and IL-1α by human dendritic cells (monocyte-derived and primary conventional dendritic cells). Activation of the nucleotide-binding oligomerization domain-like receptor protein 3 inflammasome in human dendritic cells by various synthetic activators resulted in the secretion of bioactive IL-1β, IL-18, and IL-1α and induction of pyroptotic cell death. Cellular IL-1β release depended on potassium efflux and the activity of proteins nucleotide-binding oligomerization domain-like receptor protein 3 and caspase-1. Likewise, influenza A virus infection of dendritic cells resulted in priming and activation of the nucleotide-binding oligomerization domain-like receptor protein 3 inflammasome and secretion of IL-1β and IL-18 in an M2- and nucleotide-binding oligomerization domain-like receptor protein 3-dependent manner. The magnitude of priming by influenza A virus varied among different strains and inversely corresponded to type I IFN production. To our knowledge, this is the first report describing the existence and function of the nucleotide-binding oligomerization domain-like receptor protein 3 inflammasome in human dendritic cells and the ability of influenza A virus to prime and activate this pathway in human dendritic cells, with important implications for antiviral immunity and pathogenesis. *J. Leukoc. Biol.* 99: 723–734; 2016.

Introduction

Activation of innate immunity is required to stimulate and modulate adaptive immune responses to pathogens and vaccines. As the most potent APCs, DCs are central to the orchestration of adaptive responses serving to uptake antigens, migrate to lymphoid tissues, and activate naïve T cells. To enable rapid pathogen detection, the innate immune system uses numerous germline-encoded PRRs that recognize conserved pathogen-derived motifs or host-derived markers of cell stress or damage. Upon pathogen detection, these PRRs act as signaling receptors to induce the production of innate effector molecules. Among these signaling receptors are the cytosolic NLRs, which respond to a diverse range of pathogen-associated molecular patterns and danger-associated molecular patterns. After encountering pathogen-derived or "danger"-associated factors, DCs undergo a process of maturation that results in the up-regulation of surface costimulatory molecules and the production of proinflammatory cytokines, including members of the IL-1 family, which are dependent on NLR signaling for their production. DC maturation greatly enhances their efficiency in promoting T cell and NK cell activation through cell-to-cell contact and cytokine signaling, including IL-1β [1], thus greatly enhancing the inflammatory response at the site of infection.

Cytokines of the IL-1 family (e.g., IL-1β, IL-18, and IL-1α) are critical components of host defense against infection. Upon recognition of a microorganism, proinflammatory cytokines

Abbreviations: $\Delta\Delta C_T$ = difference in threshold cycle, 7AAD = 7-amino-actinomycin D, A.F. = allantoic fluid, ASC = apoptosis-associated speck-like protein containing a caspase activation and recruitment domain and pyrin domain, ATP_o = oxidized ATP, BMDM = bone marrow-derived macrophage, CARD = caspase activation and recruitment domain, CBA = cytokine bead array, cDC = conventional (myeloid) dendritic cell, DC = dendritic cell,

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The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. Correspondence: Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Pl., Box 1079, New York, NY 10029, USA. E-mail: nina.bhardwaj@mssm.edu

IL-1β and IL-18 are secreted and act as chemoattractants and activators of innate and adaptive immune cells. In vivo IL-1β is largely responsible for the acute-phase response, including fever and inflammatory cytokine synthesis [2], and is one of the most potent, endogenous fever-inducing molecules known to date. IL-1R signaling downstream of viral recognition plays a key role in induction of proinflammatory signals, as well as recruitment and activation of immune cells [3]. Meanwhile, IL-18 is essential for the induction of IFN-γ by Th1 and NK cells [4] and as a chemoattractant for certain immune cells. Production of IL-1β requires the inflammasome complex, which is necessary for activating the inflammatory caspase known as IL-1 converting enzyme (procaspase-1), allowing it to convert cytoplasmic pro-IL-1β and pro-IL-18 to bioactive cytokines [5]. The most extensively studied inflammasome complex is mediated by NLRP3 (or NALP3), a member of the NLR family. NLRP3 has the ability to initiate the formation of a multimolecular protein complex composed of 3 core proteins: homo-oligomerized NLRP3; the scaffold protein ASC (also known as PYRIN-PAAD-DAPIN domain and a CARD), which binds to NLRP3 N-terminal domains; and procaspase-1, which is recruited via CARDs on ASC [6, 7].

Successful secretion of IL-1β family cytokines requires sensing of 2 different and independent danger signals. Initially, cell priming via TLR ligation or cytokine receptor signaling (TNF-α or IL-1β) is required for production of intracellular pro-IL-1β in human MDDCs [8]. It is important to note that whereas constitutive IL-18 gene expression is present in human PBMCs [9], expression is up-regulated further upon TLR ligand sensing [10]. A second signal is then necessary for inflammasome complex formation upstream of procaspase-1 maturation. This leads to caspase-1 activation and cleavage of pro-IL-1β and pro-IL-18 to their bioactive forms. Previously described NLRP3 inflammasome-stimulating signals in murine cells include agents that promote K^+ efflux, such as: extracellular ATP, bacterial membrane pore-forming toxins (e.g., Nigericin), and lysosomal-disrupting factors (e.g., calcium phosphate, alum, cholesterol, and MSU) [11–15]. The precise mechanism leading to the activation of the NLRP3 inflammasome is still unclear, but a unifying hypothesis proposes that common intracellular activities, including induction of hypokalemia, reactive oxygen species, or calcium-dependent phospholipase-2, are indirectly activating the inflammasome [16].

DCs are present in the epithelial lining of conductive airways and are also rapidly mobilized from circulation to bronchial tissue following inhalation of microbial agents [17–20]. Therefore, DCs play a critical role in the afferent phase of the immune response to IAV infection [21]. Genomic IAV RNA is initially recognized during infection by the innate immune system through TLR7/8, leading to the synthesis of inflammatory cytokines TNF-α, IL-6, pro-IL-1β, IL-12p40, and IFN-α [22–24]. Upon replication of the virus, de novo M2 interacts with the *trans*-Golgi network to upset intracellular ion concentrations, an effect similar to the cellular K^+ efflux induced by the bacterial ionophore Nigericin. This ion perturbation activates the formation of the NLRP3 inflammasome and leads to the maturation of IL-1β, which has been shown to mediate acute pulmonary inflammatory pathology [25], whereas caspase-1 activation in the hematopoietic compartment is required to induce protective antiviral immunity during IAV infection [26, 27]. Therefore, whereas inflammasome activity may play a key role in antiviral immunity, it may simultaneously contribute to pathogenesis when activation is sustained or overwhelming. Indeed, there is a strong correlation between IAV-associated immunopathology and increased cytokine production [28]. Enhanced knowledge of the inflammatory responses generated by innate immune cells, specifically DCs, during IAV infection may reveal methods to prevent or treat infection-related complications once they occur.

Here, we identified the primary inflammasome pathway underlying IL-1β and IL-18 secretion from DCs, first by use of a model for viral infection that combines TLR7/8 ligation followed by triggering the NLRP3 inflammasome with agents known to induce cellular K^+ efflux. Our results demonstrate that TLR7/8 ligation potently stimulates the production of procytokines, including pro-IL-1β and pro-IL-18. Additionally, K^+ efflux induced by Nigericin and ATP results in the following: 1) NLRP3 inflammasome activity, 2) activation of caspase-1, 3) secretion of IL-1β and IL-18, and 4) induction of pyroptosis. Infection of human DCs with IAV both primed and activated the NLRP3 inflammasome, although the degree of priming varied significantly between different strains and inversely correlated with type I IFN production. These data collectively describe the presence of the NLRP3 inflammasome in human DCs and provide evidence of its role in the innate immune response to IAV infection in humans.

MATERIALS AND METHODS

Generation of MDDCs and primary DC isolation

Human MDDCs were differentiated from monocyte fractions of human PBMCs obtained from healthy volunteer blood donors, as described previously [8, 29, 30]. Human buffy coats served as the source of monocytes and were obtained from the New York Blood Center (New York, NY, USA). Human monocytes ($CD14^+CD11c^+$) were differentiated from human MDDC ($CD14^-CD11c^+$) by characterizing the cells with CD14 and CD11c antibodies.

Human cDCs were isolated using the CD1c $(BDCA\text{-}1)^+$ Dendritic Cell Isolation Kit (Miltenyi Biotec, San Diego, CA, USA) or the EasySep Human Myeloid DC Enrichment Kit ($CD11c^+$; Stemcell Technologies, Vancouver, BC, Canada) per the manufacturers' protocol. Primary human DCs were purified further by cell sorting for $Lineage^-HLA\text{-}DR^+CD14^-CD11c^+$ cDCs before performing analysis.

Cytokine quantification

IL-1β, IL-6, IL-10, TNF-α, and IL-12p70 cytokine secretion was analyzed using the Human Cytometric Bead Array (BD Biosciences, San Diego, CA, USA).

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F/T = freeze thaw, FAM-FLICA = fluorescent-labeled inhibitor of caspase, H3N2 = A/Victoria/361/2011(H3N2), HAU/ml = hemagglutination units/ml, IAV = influenza A virus, IP-10 = IFNγ-inducible protein 10 (also known as CXCL10), K^+ = potassium ion, LDH = lactate dehydrogenase, MDDC = monocyte-derived dendritic cell, MOI = multiplicity of infection, MSU = monosodium urate crystal, NC = A/New Caledonia/20/99(H1N1), NLRP3 = nucleotide-binding oligomerization domain-like receptor protein 3 (also known as NALP3), ODN = oligodeoxyribonucleotide, P_2Rx7 = purinergic receptor P2X, ligand-gated ion channel, 7, PR/8 = A/Puerto Rico/8/34(H1N1), PRR = pattern recognition receptor, qPCR = quantitative real-time PCR, rhIFN = recombinant human IFN, RIG-I = retinoic acid-inducible gene 1, WSN = A/WSN/1/33(H1N1), Z-VAD-FMK = benzyloxycarbonyl-Val-Ala-Asp (O-methyl)-fluoromethylketone, Z-Wehd-FMK = benzyloxycarbonyl-Trp-Glu(OMe)-His-Asp(OMe)-fluoromethylketone

IL-1α (Abcam, Cambridge, MA, USA) and IL-18 (R&D Systems, Minneapolis, MN, USA, and Biosensis, Thebarton, South Australia) were analyzed by standard ELISA. Multisubtype IFN-α was detected by ELISA (PBL Assay Science, Piscataway, NJ, USA).

Gene expression analysis

Cytokine- and inflammasome-related gene expression was analyzed by qPCR (primers from Integrated DNA Technologies, Coralville, IA, USA). Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA, USA). First-strand cDNA synthesis (Applied Biosystems, Foster City, CA, USA) was performed before RT-PCR (Invitrogen, Carlsbad, CA, USA). A no-template control and minus reverse transcription control were included for quality control. Gene expression was normalized to unstimulated control and GAPDH using the $\Delta\Delta C_T$ method.

Protein detection

The following antibodies were used for Western blotting: α-IL-18, α-IL-1β, and α-IL-1β-FITC (R&D Systems); ASC, β-actin, and β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and caspase-1 (Cell Signaling Technology, Danvers, MA, USA, and Imgenex, Novus Biologicals, Littleton, CO, USA). IRDye secondary antibodies (LI-COR, Lincoln, NE, USA) were used for Western blot signal detection. IL-1β, IL-18 (R&D Systems), and caspase-1 p10 subunit (Imgenex, Novus Biologicals) were analyzed by Western blotting, transferred onto Immobulin-FL PVDF membrane (EMD Millipore, Billerica, MA, USA), and probed in 5% BSA or Odyssey blocking buffer (LI-COR). Blots were imaged using the LI-COR Odyssey scanner or chemiluminescence detection. Multiple protein probes on the same gel were detected using the same method within each experiment. Detection of intracellular proteins was performed by loading 2×10^5 cells/well. For the detection of caspase-1 p10 and IL-1β from supernatants, 1×10^6 cells were stimulated per condition in serum-free media. Protein concentration of extracellular proteins was performed with chloroform and methanol. The entire volume was loaded on a 15% SDS-PAGE gel. For detection of NLRP3 (bs-10021R; Bioss, Woburn, MA, USA), 50 ng total cellular protein derived from untreated cells was loaded on an 8% SDS-PAGE gel and detected by chemiluminescence. GAPDH (2118S; Cell Signaling Technology) was used as a loading control.

Cell death determination

MDDCs were treated as described, followed by staining with AnnexinV-FITC (BD PharMingen, San Diego, CA, USA) and 7AAD (Abcam) after 3 h of inflammasome stimulation for 10 min, and then washed before analysis by flow cytometry. Pyroptotic cells were distinguished from apoptotic and necrotic cells as those that stained double positive. Additionally, after MDDCs were treated to activate the inflammasome, LDH secretion was measured with a colorimetric LDH Assay (Abcam), according to the manufacturer's instructions. LDH secretion was represented as the percent of control cells that were 3 times F/T with dry ice.

Viral infection

Pyrogen-free influenza PR/8 and A.F. negative control was produced by Charles River Laboratories (Germantown, MD, USA) and used at 1000 HAU/ml [21, 31], unless otherwise indicated. The H1N1 strains WSN, NC, PR/8 (labeled Mount Sinai PR/8), and H3N2 were a gift from Dr. Peter Palese (Mount Sinai Hospital, New York, NY, USA) and used at an MOI of 1 unless otherwise indicated. An equivalent volume of A.F. was added to control conditions. R848 (5 μM) was used as a TLR7/8 ligand for 18 h, unless otherwise indicated. Cells were stimulated with live or inactivated virus continuously in complete media.

Inhibitor treatment

The following inhibitors of the inflammasome were added to cellular media after 18 h of TLR stimulation and before inflammasome stimulation or before IAV infection: caspase-1 (50 μM; Z-Wehd-FMK), pan-caspase (100 μM; Z-VAD-FMK), Glyburide (200 μM; ENZO Life Sciences, Farmingdale, NY, USA), K^+ (140 mM; Sigma, St. Louis, MO, USA), or ATP_o (5 mM; Sigma). Unbound Z-Wehd-FMK, Z-VAD-FMK, Glyburide, and ATP_o were washed out after a 30 min incubation with the cells. Amantadine and Rimantadine (200 μM) were added to conditions, 2 h after infection with PR/8, which was heat inactivated for 1 h in a water bath at 65°C to inhibit binding of the virus to the cell membrane and 56°C to prevent viral replication. Exposure to 1 J/cm^2 UV light, 4 times with a Stratalinker UV cross-linker (Agilent, Santa Clara, CA, USA), was performed to UV irradiate PR/8 before infection to block viral replication [32]. The phosphorothioate TLR inhibitory ODNs IRS661 and IR957 were synthesized, as described elsewhere [33, 34], and purified using Na^+ ion exchange and HPLC (Integrated DNA Technologies). They were added to cells at a final concentration of 1 μM before addition of R848.

Caspase activity detection

Caspase-1 activity assay (Abcam) was performed with 5×10^6 cells/condition and read on a UV spectrometer, according to the product manual. Caspase-1 FAM-FLICA (ImmunoChemistry Technologies, Bloomington, MN, USA) was added 30 min before activation of the inflammasome with Nigericin for indicated times. Data were acquired immediately by flow cytometry. Comparison of para-nitroaniline absorbance by spectrophotometer between Nigericin-treated samples and an untreated control allowed for determination of the fold increase in caspase-1 activity.

IFN neutralization and supplementation

MDDCs were incubated with rhIFN-β (1000 IU/ml), 6 h after priming with R848 for 12 h. Cells were then treated with Nigericin, and supernatants were collected 6 h later. Isotype control had no affect and is not shown.

Statistics

Statistical analyses were performed using unpaired Student's *t* tests, two tailed (GraphPad Prism version 5.0). Significance levels are indicated in figure legends.

Online Supplemental material

Supplemental Fig. 1 shows primary human DCs isolated from blood release IL-1 family cytokines in response to R848 and Nigericin treatment and that the IL-1β released is a mature cytokine. Supplemental Fig. 2 shows that both human MDDCs and human cDCs express intracellular NLRP3, ASC, and caspase-1. Supplemental Fig. 3 shows that caspase-1 activity is increased following activation with Nigericin, and this activity is increased further if the cells were primed with R848 (TLR ligand). Supplemental Fig. 4 shows (using markers for cell death) that ATP, Nigericin, MSU, and IAV all induce a pyroptotic phenotype in MDDCs. This phenotype is specific to the triggers used and dependent on NLRP3 activation.

RESULTS

Human DCs up-regulate IL-1 family cytokines upon TLR sensing of R848

We first evaluated the expression of IL-1 family members in primary human DCs. Immature DCs express low levels of surface costimulatory makers CD80, CD86, CD83, and CD40. Exposure to a TLR ligand, such as TLR7/8 agonist R848 (a synthetic purine also known as Resiquimod), results in maturation of DCs, a state characterized by the up-regulation of surface costimulatory molecules under NF-κB transcriptional control [35, 36]. TLR signaling also results in priming of DCs via inflammatory cytokine transcription and expression. To assess IL-1β and IL-18 expression, DCs were stimulated over the course of 4 h with R848, and RNA was extracted for qPCR analysis. Human MDDCs (**Fig. 1A**) and primary CD11c$^+$ cDCs (Fig. 1B) from healthy volunteer donors displayed TLR-induced transcriptional

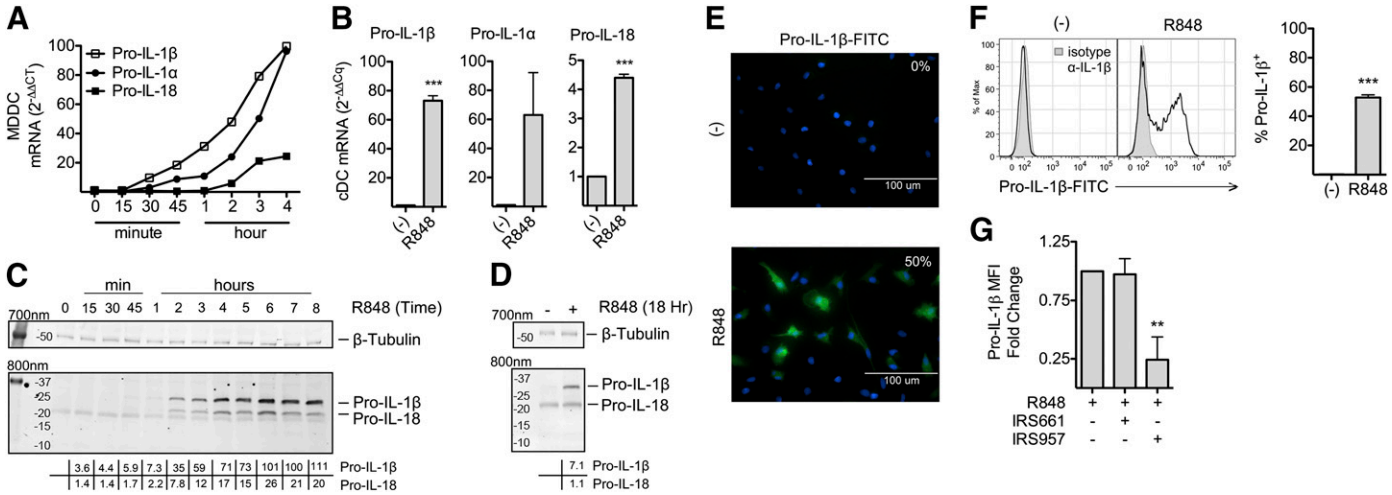

Figure 1. Human DCs up-regulate intracellular pro-IL-1 cytokines in response to R848 sensing by TLR. (A) MDDCs and (B) cDCs were primed with R848, and qPCR analysis of IL-1 cytokines was performed. Expression was compared with GAPDH. (C and D) MDDCs and cDCs were primed with R848, and Western blot, using LI-COR Odyssey Infrared Imaging System, was done for pro-IL-1β and pro-IL-18. Relative intracellular cytokine protein was normalized to β-tubulin and indicated below the corresponding band. (C) MDDCs were primed with R848 over the course of 8 h. (D) cDCs were primed for 18 h. (E and F) MDDCs were primed with R848 for 18 h and stained for pro-IL-1β-FITC and DAPI. Cells were imaged by epifluorescence microscopy. Isotype staining (not shown) revealed no background. (F) Representative histogram of flow cytometric analysis showing up-regulation of intracellular IL-1β. Percent of pro-IL-1β$^+$ cells was quantified for multiple donors. (G) MDDCs were primed with 1 μM IRS661 or IRS957 for 1 h before overnight stimulation with 10 μM R848. Data are representative of (A and C) 4, (B and D) 3, and 6 (E–G) independent donors. MFI, Mean fluorescence intensity. Error bars represent SD. $^{**}P < 0.01$; $^{***}P < 0.001$.

up-regulation of pro-IL-1β, pro-IL-1α, and pro-IL-18. All donors displayed a poststimulation, time-dependent up-regulation of cytokine RNA, but the degree of expression varied among them. Transcriptional up-regulation of these cytokines was detected 30 min after exposure to R848, indicating their involvement in the acute-phase response of DCs to TLR ligation (Fig. 1A). These results were consistent for all donors of MDDC and cDC (Fig. 1B).

Western blot was performed to determine whether translational expression of these cytokines occurred at the protein level (Fig. 1C and D). Intracellular protein expression of pro-IL-1β (33 kDa) and pro-IL-18 (24 kDa) in MDDCs mirrored the change in expression of RNA over time (Fig. 1C). Whereas there was baseline expression of all cytokine RNA in MDDC, both pro-IL-18 and pro-IL-1β were minimally detectable by Western blot until 2 h after addition of R848 to appropriate conditions. Notably, mature forms of cytokines were not elicited by R848 alone. In contrast, cDCs expressed baseline pro-IL-18, but not pro-IL-1β, in freshly isolated cells, and these proteins were increased further upon R848 priming overnight (Fig. 1D), consistent with the literature [9, 10].

MDDCs were stained for intracellular IL-1β protein expression after overnight R848 priming to determine if pro-IL-1β was uniformly expressed throughout the MDDC ($CD11c^+CD14^-$) population. Staining for IL-1β was evident in ~50% of R848-primed MDDCs (Fig. 1E and F). Primary cDCs had similar levels (~50%) of intracellular IL-1β expression (data not shown).

To verify the specificity of TLR signaling upstream of cytokine expression in response to R848, MDDCs were treated with TLR-blocking agents IRS661 (TLR7 inhibitor ODN) or IRS957 (TLR8 inhibitor ODN; Fig. 1G). MDDCs express TLR8 but not TLR7 [37, 38]; therefore, expression of pro-IL-1β should be prevented when pretreating the cells with IRS957. As expected, up-regulation of intracellular pro-IL-1β in the presence of these endosomal TLR inhibitors was prevented with IRS957 pre-treatment but not IRS661, in agreement with the concept that specific TLR triggering is required to initiate inflammasome priming.

In summary, immature DCs require a priming signal delivered through TLR ligation, such as R848 on TLR7 (expressed by cDCs) or TLR8 (expressed by MDDCs) to up-regulate expression of costimulatory markers, as well as transcription and cytosolic expression of pro-IL-1β, pro-IL-18, and pro-IL-1α. Expression of these cytokines following treatment with R848 occurs rapidly, within 2 h, in approximately half of the MDDC and cDC population. These data indicate that both human-derived DCs and primary cDCs are similar in their ability to express pro-IL-1β and pro-IL-18 in response to TLR7/8 sensing of R848.

Inflammasome activation results in IL-1 family cytokine secretion

To assess IL-1 production by inflammasome stimulation, MDDCs were primed overnight with 5 or 10 μM R848, and inflammasome activation was induced with Nigericin, MSU, or ATP. Upon activation of the inflammasome, MDDCs secreted IL-1β, IL-18, and IL-1α (**Fig. 2A–C**). Secretion of IL-1 cytokines occurred in a priming- and activation-dependent manner. The inflammasome independent cytokine, TNF-α, was also measured (Fig. 2D). Secretion of TNF-α was dependent on R848 but not on inflammasome activation. Primary blood $CD11c^+$ DCs secreted IL-1β, IL-18, and IL-1α with R848 priming alone, and this secretion was similarly potentiated by Nigericin activation

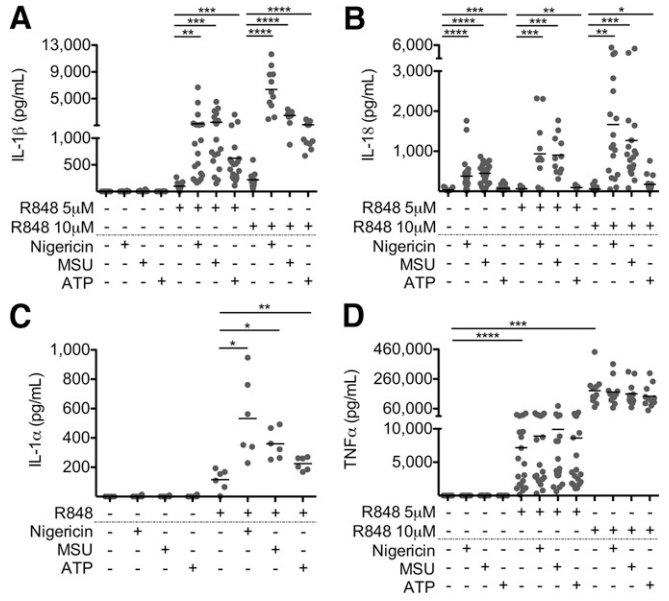

Figure 2. Human DCs secrete IL-1 cytokines in response to NLRP3 inflammasome activators. MDDCs were primed with R848 overnight, followed by activation with Nigericin, MSU, or ATP. (A–D) Secreted IL-1β, IL-18, and TNF-α was measured by CBA and IL-1α by ELISA. Data are representative of at least (A, B, and D) 11 and (C) 3 independent donors. All experiments were performed in triplicate. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.001$.

(Supplemental Fig. 1A). The inflammasome-independent cytokine, TNF-α, was also dependent on priming but not inflammasome activity in $CD11c^+$ cells.

Reduction of intracellular pro-IL-1β and increase in the secretion of mature IL-1β product are characteristics of inflammasome activation. Western blot of total $CD1c^+$ cDC and MDDC lysates displayed an increase in intracellular pro-IL-1β with priming and a decrease upon Nigericin activation (Supplemental Fig. 1B). In mirroring the changes in intracellular IL-1β, Western blotting of secreted IL-1β in the supernatant verified the presence of the mature form of IL-1β only, following R848 priming and subsequent Nigericin activation.

In R848-stimulated human DCs, expression of NLRP3 and associated inflammasome proteins, ASC and procaspase-1, was investigated. These proteins were all expressed at baseline in unstimulated DCs, and modest up-regulation of NLRP3 RNA was observed following R848 stimulation in cDCs, whereas MDDCs experienced ~2-fold up-regulation in ASC RNA (Supplemental Fig. 2A). Despite these changes observed at the transcriptional level in NLRP3 and ASC, Western blot analysis revealed that cDC and MDDC expressed NLRP3, ASC, and procaspase-1 proteins in their basal state, which was largely unchanged following overnight incubation with R848 (Supplemental Fig. 2B and C). These data indicate that DCs constitutively express NLRP3, ASC, and procaspase-1, possibly to assemble rapidly the inflammasome complex following activation.

The blocking of the inflammasome can be accomplished with irreversible chemical inhibitors specific for polycaspases (Z-VAD-FMK), caspase-1 (Z-Wehd-FMK), or NLRP3 inflammasome formation (Glyburide; **Fig. 3A**). We next confirmed the capacity of these inhibitors to block IL-1β release from DCs.

K^+ efflux upstream of NLRP3 inflammasome formation is required during Nigericin- and ATP-induced inflammasome formation through different mechanisms [39]. ATP_o has been shown to prevent inflammasome activation by irreversibly blocking the ATP-binding sites on P_2Rx7 and other purinergic membrane-bound receptors, thus reducing K^+ efflux in the presence of 5 mM ATP [40–42]. Previous studies have shown the expression of surface membrane P_2Rx7 on human DCs [43, 44]. To investigate the role of P_2Rx7, MDDCs were primed with R848 overnight, treated with ATP_o for 1 h, and activated with ATP (Fig. 3B, left). As expected, inhibition of purinergic receptor signaling with ATP_o prevented secretion of IL-1β.

As a result of its nonspecific activity, activation of the inflammasome with Nigericin results in purinergic receptor-independent K^+ efflux. To determine the necessity of K^+ efflux during Nigericin activation of the inflammasome, MDDCs were primed with R848 overnight and supplemented with extracellular KCl before treatment with Nigericin. Extracellular K^+ prevented the secretion of IL-1β during Nigericin and ATP activation (Fig. 3B, middle).

The mechanism by which MSU activates the inflammasome is less clear. The current accepted model describes phagocytic uptake of various particulate matter along with a concurrent drop in intracellular K^+ as a requirement, preceding the release of IL-1β [39, 45]. In contrast to Nigericin and ATP, MSU

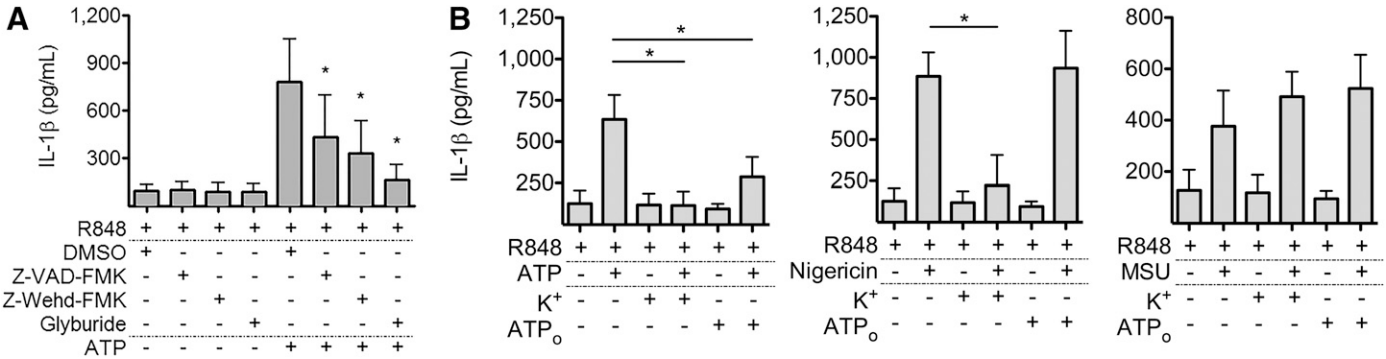

Figure 3. IL-1β secretion is attenuated when blocking NLRP3 activation. (A) MDDCs were treated with inhibitors before activation with ATP. Secreted IL-1β was measured by CBA. (B) MDDCs were treated with K^+ or ATP_o before activation. CBA was performed to detect secreted IL-1β. Data are representative of 3 independent donors. Each donor was tested in triplicate. Error bars represent SD. $*P < 0.05$.

activation during treatment with ATP_o or K^+ did not inhibit IL-1β secretion from MDDCs (Fig. 3B, right). In support of this conflicting finding, a recent report from a separate group uncovered a role for MSU-triggered ATP release, which potentiates the secretion of IL-1β from murine macrophages [46]. Additionally, another study found that K^+ efflux was not required for IL-1β maturation [47], and suggests that cellular volume increase causes concurrent dilution of intracellular K^+. Therefore, ATP release downstream of MSU phagocytosis or dilution of intracellular K^+ may provide compensatory mechanisms for IL-1β maturation during blockage of K^+ cellular efflux.

NLRP3 inflammasome activation results in caspase-1 activity

The hallmark of inflammasome activity is to recruit and induce procaspase-1 autoproteolysis. Active caspase-1 formation can be measured via irreversible binding of a probe composed of an inhibitor peptide sequence containing a fluorescent tag, referred to as FAM-FLICA. Therefore, increased staining in the FITC channel indicates the generation of active caspase-1.

First, a time course was performed over 2 h, wherein cells were primed with R848 overnight, followed by incubation with FAM-FLICA before activation with Nigericin (Supplemental Fig. 3A). Unactivated cells, both immature and primed, had low background staining for FAM-FLICA (Supplemental Fig. 3A, top). At 15 min post-Nigericin activation, almost 100% of DCs stained for active caspase-1, labeled FAM-FLICA$^+$. Interestingly, at 60 min, a new, more intense FAM-FLICA population appeared, labeled FAM-FLICA^{++} (Supplemental Fig. 3A, compare middle with bottom), presumably representing further activation of caspase-1 within these cells. Both immature and R848-primed MDDCs were capable of activating caspase-1 in response to Nigericin-induced inflammasome activation, but R848-primed cells induced double the population of FAM-FLICA^{++} compared with unprimed DCs (Supplemental Fig. 3A, bottom). Although TLR priming with R848 produced no obvious change in overall procaspase-1 expression when measured by Western blot, p10 (a cleavage product of inflammasome activity) could be detected in the supernatants of primed MDDCs (Supplemental Fig. 3B). These findings indicate that MDDCs primed with R848 followed by activation with Nigericin resulted in more expression of p10 compared with cells stimulated with Nigericin alone.

Finally, caspase-1 enzymatic activity was also measured with a specific enzymatic assay (Supplemental Fig. 3C). Caspase-1 activity increased over the course of 2 h for both immature and primed DCs. R848 alone was able to induce caspase-1 activity that was increased further with Nigericin treatment, in agreement with the FAM-FLICA experiments. Altogether, our results indicate that the proteins required for inflammasome activation are functionally active in MDDCs following a suitable trigger and are enhanced by pretreatment with R848.

Human MDDCs undergo pyroptosis following NLRP3 activation

Necrosis, apoptosis, and pyroptosis are morphologically distinct forms of cell death [48]. The morphology of pyroptosis is still incompletely defined; however, differentiating markers include AnnexinV$^+$7AAD$^+$ staining, LDH release [49], secretion of IL-1β, activation of caspase-1, and morphologic changes, such as cellular swelling [50, 51].

Inflammasome activation results in the induction of a specialized programmed inflammatory cell death, termed pyroptosis. Human DCs were assessed for expression of pyroptotic morphologic hallmarks after induction of cellular death with Nigericin. MDDCs were first primed with R848 overnight and activated with Nigericin for 3 h (Supplemental Fig. 4A). Nigericin exposure resulted in increased secretion of LDH from cells compared with unactivated cells in immature and R848-primed MDDCs.

During pyroptosis, the cell membrane forms pores to permit AnnexinV to stain the inner leaflet of the membrane, allowing membrane impermeant fluorescent dyes, such as 7AAD, to stain DNA. Pyroptotic cells appeared only upon activation with Nigericin or ATP (Supplemental Fig. 4B). In keeping with the pattern of increased caspase-1 activity following priming with R848, the percentage of pyroptotic cells (labeled AnnexinV$^+$7AAD$^+$) was increased when DCs were first primed with R848.

Expansion of cell volume is induced upstream of NLRP3 inflammasome formation, and therefore, pyroptosis is characterized morphologically by cellular swelling [52], which is accurately detected using light-scatter analysis [53]. Glyburide, a well-established inhibitor of K^+ efflux, protects cells from ATP-induced membrane damage and cellular swelling, while preventing inflammasome formation [54]. Cells were primed with R848 overnight and incubated with Glyburide for 1 h before activating with Nigericin for 3 h (Supplemental Fig. 4C). Inhibition of the NLRP3 inflammasome formation with Glyburide prevented expansion in cellular size induced by R848 priming and Nigericin within the time frame measured.

To determine whether Nigericin specifically induced an inflammatory cell death, characterized by IL-1β release, MDDCs were primed with R848 overnight, followed by induction of cell death with Nigericin, UV exposure, or multiple F/T cycles for 6 h to initiate pyroptotic, apoptotic, and necrotic cell death, respectively (Supplemental Fig. 4D). Treatment with Nigericin following R848 priming resulted in potent IL-1β release. Exposure to UV radiation also resulted in secretion of IL-1β, as described previously [55] but to a much lesser extent [56]. As expected, multiple F/T cycles induced no secretion of IL-1β above the R848-only control.

Our studies show that MDDCs undergo inflammatory cellular death characterized by LDH secretion, AnnexinV and 7AAD costaining, IL-1β release, caspase-1 activation, and cellular swelling when activated with Nigericin.

IAV primes the NLRP3 inflammasome in human DCs

Upon IAV infection, monocytes and DCs express viral proteins, up-regulate costimulatory molecules [57] and proinflammatory cytokines [58], and undergo eventual cellular death [31, 58, 59], including pyroptosis (Supplemental Fig. 4A and B). Therefore, we evaluated MDDCs for their capacity to respond to live IAV infection. To determine whether MDDCs were capable of maturing in response to IAV infection, cells were infected with PR/8 for 18 h and stained for the costimulatory molecule CD86 (**Fig. 4A**). Influenza infection resulted in the increase of these

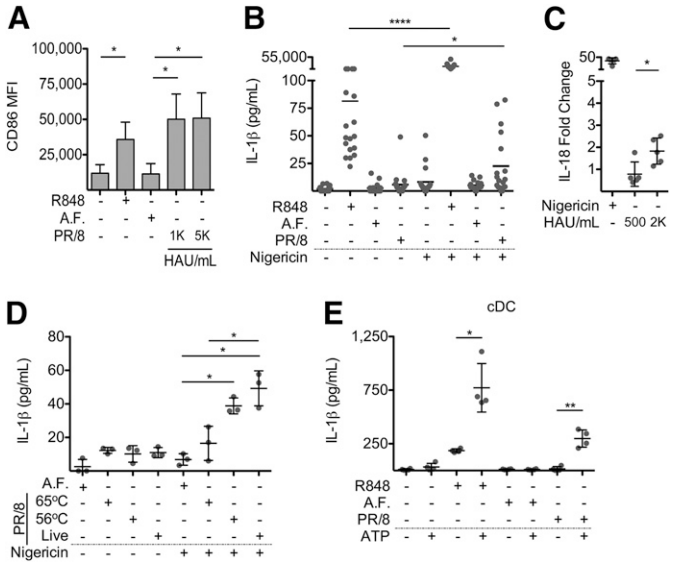

Figure 4. IAV primes the expression of IL-1β and IL-18 in human DCs. (A) MDDCs were infected for 16 h with various doses of PR/8 generated by Charles River Laboratories. Cells were then fixed and stained for surface expression of CD86 before flow cytometry analysis. (B) MDDCs were infected overnight and activated with Nigericin for 6 h. CBA was performed to detect secretion of IL-1β. (C) MDDCs were infected with live virus alone for 24 h. Secreted IL-18 was measured by ELISA. Data were normalized to A.F. control. (D) MDDCs were infected with heat-inactivated IAV followed by Nigericin for 6 h. Secretion of IL-1β was detected by CBA. (E) cDCs were primed with IAV overnight and activated with Nigericin 6 h. Data are representative of (A) 18, (B) 5, (C) 3, and (D) 4 independent donors. C was performed in triplicate. Error bars represent SD. $*P < 0.05$; $**P < 0.01$; $****P < 0.001$.

markers, but CD86 expression was not dose dependent in the range tested.

IAV has been shown to prime murine BMDMs via TLR7 stimulation for the production of IL-1β [22–24]. PR/8 was evaluated as a priming stimulus in MDDCs before activation with Nigericin to promote secretion of IL-1β (Fig. 4B). Virus alone resulted in very weak to no induction of IL-1β release in vitro, whereas the addition of Nigericin allowed for its significant increase. Additionally, PR/8 infection alone weakly induced IL-18 secretion (Fig. 4C).

Heat-inactivated IAV at temperature of 65 and 56°C selectively denatures viral hemagglutinin and abrogates replication capacity, respectively [26, 32, 60, 61]. The incubation of IAV at 65°C reduced its capacity to prime MDDCs before Nigericin activation compared with live virus (Fig. 4D). Heat inactivation at 56°C did not significantly reduce the capacity of IAV to prime MDDCs, as the virus retains the capacity to be endocytosed into TLR7-containing endosomes and to access the cellular cytoplasm through fusion. Similar to MDDCs, cDCs did not secrete IL-1β in response to infection with IAV alone and required a second signal in vitro, such as ATP, to induce secretion of IL-1β (Fig. 4E).

To determine how other H1N1 strains compared with PR/8 in their capacity to prime the inflammasome, MDDCs were infected with WSN and NC at an MOI of 1, and PR/8 (Charles River Laboratories) at 1000 HAU/ml. The NC strain resulted in more IL-1β but less TNF-α secreted from MDDCs when compared with PR/8 (**Fig. 5A** and **B**). The WSN strain, at equal MOI, induced the opposite pattern of cytokine expression—low IL-1β and high TNF-α.

To resolve if there was a correspondence between IL-1β secretion and the production of type 1 IFN, as has been described previously [62], we measured IFN-α and IFN-β production in MDDCs in response to IAV (Fig. 5C). IFN-β was not detected following infection of MDDC with IAV strains, but significant amounts of IFN-α were secreted. Notably, high IFN-α secretion by PR/8 and WSN corresponded to lower IL-1β secretion.

Murine bone marrow-derived DCs and BMDMs treated with rhIFN-β during activation with Alum blocked the secretion of IL-1β, IL-18, and IL-1α by suppressing the activation of caspase-1 [62], possibly through the induction of IL-10 as an intermediate step to decrease the intracellular pro-IL-1β cytokine pool. Additionally, primary monocytes from healthy donors exposed to rhIFN-β displayed diminished IL-1β secretion. To investigate whether type I IFN exerts the same anti-inflammatory effects on human MDDCs, cells were primed with R848 for 6 h, cultured overnight with rhIFN-β, followed by activation with Nigericin. Consistent with this previous report, incubation with rhIFN-β effectively diminished Nigericin-induced IL-1β secretion (**Fig. 6A**). Attempts to neutralize IFN were unsuccessful as a result of high levels of endotoxin in commercial-blocking antibodies. Additionally, in agreement with Guarda et al. [62], release of IL-10 increased in the presence of rhIFN-β (Fig. 6B), concurrent with a decrease in TNF-α and increase in IP-10 release (Fig. 6C). Our results indicate that IAV strains can vary in their ability to activate inflammasomes and that this may be partially dependent on their intrinsic ability to induce type I IFNs.

IAV activates the NLRP3 inflammasome in human DCs

In a recent study, commensal bacteria were found to regulate immunity by priming DCs to up-regulate cytoplasmic pro-IL-1β

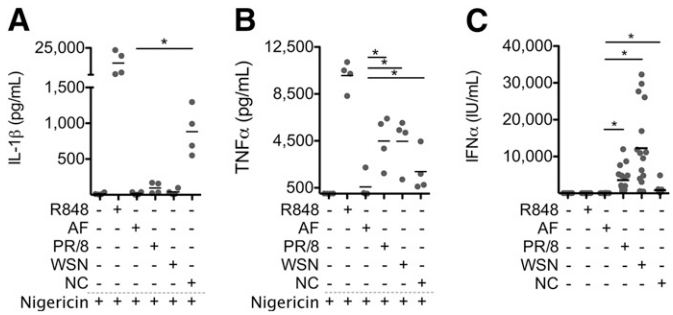

Figure 5. Expression of inflammatory cytokines depends on the IAV strain. MDDCs were primed with R848 overnight or activated with IAV for 18 h followed by Nigericin for 6 h. IAV strains were used at an MOI of 1, and the PR/8 used was produced by Charles River Laboratories at a concentration of 1000 HAU/ml. CBA was performed to detect secretion of (A) IL-1β and (B) TNF-α. (C) Multisubtype IFN-α was measured by ELISA. Data are representative of (A and B) 4 and (C) 16 independent donors. $*P < 0.05$.

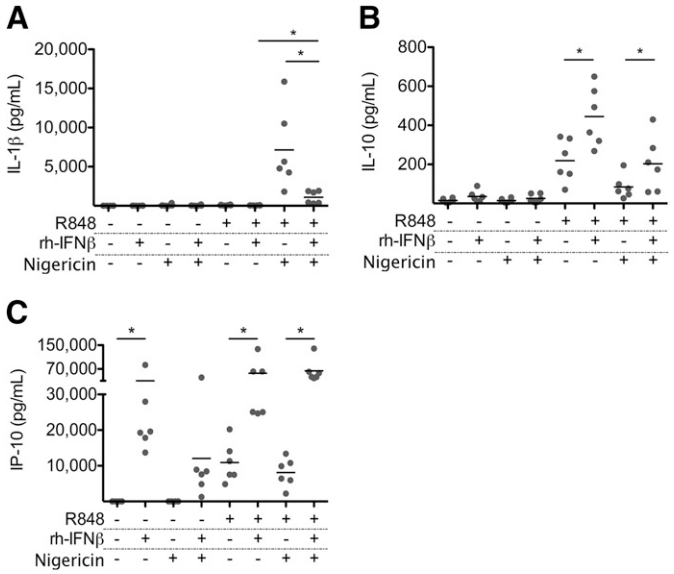

Figure 6. Expression of IL-1β is attenuated by IFN signaling. Cells were primed with R848, supplemented with rhIFN-β for 6 h, and activated with Nigericin. Secreted IL-1β (A), IL-10 (B), and IP-10 (C) were measured by CBA. Data are representative of 6 independent donors. $*P < 0.05$.

expression [63]. Following treatment with antibiotics, mice were infected with a sublethal dose of PR/8, which resulted in IL-1β cytokine secretion, suggesting that IAV can also function as an intrinsic activator of the inflammasome. To test this, MDDCs were primed with R848 overnight before infection with IAV. We found that secretion of IL-1β was dose dependent (**Fig. 7A**), confirming that IAV can serve as an effective secondary trigger for IL-1β release from DCs.

To determine if there were differences in the capacity of IAV strains to activate the inflammasome, infection with PR/8 was compared with WSN, NC, and a recent IAV H3N2 isolate, Victoria. Infection with all 3 strains resulted in different levels of IL-1β secretion (Fig. 7B). The NC strain caused the most cytokine release, similar to its function as a priming agent, whereas the H3N2 strain resulted in lower IL-1β secretion compared with the A.F. control. Additionally, the PR/8 strain from Charles River Laboratories was compared with the Mount Sinai strain. Surprisingly, the Charles River Laboratories strain caused a significant increase in secreted IL-1β, whereas the Mount Sinai strain did not induce IL-1β release compared with control, including at higher doses.

IAV-induced NLRP3 inflammasome activation reportedly requires the production of de novo viral M2 production [23]. Among its many functions, M2 is thought to interact with the *trans*-Golgi network, where it forms intracellular proton channels. The activity of these channels disturbs intracellular ion concentrations, which promotes the formation of the NLRP3 inflammasome and subsequent IL-1β release. To investigate further the role of M2 in activation of the inflammasome, MDDCs were primed with R848 to up-regulate intracellular pro-IL-1β, followed by infection with PR/8 in the presence of M2 inhibitors, the adamantane-based drugs Amantadine or Rimantadine. Both inhibitors resulted in attenuation of IL-1β secretion compared with the IAV, infected only control when added 2 h after initial infection (**Fig. 8A**). The release of another TLR-induced proinflammatory cytokine, TNF-α, was not inhibited significantly by Amantadine treatment during IAV infection, consistent with the ability of IAV to retain DC-activating function. Additionally, replication-incompetent, UV-treated PR/8 resulted in significantly less IL-1β secretion from MDDCs compared with control, likely as a result of its inability to replicate efficiently and produce de novo M2 protein [26].

Finally, to confirm the role of NLRP3 in IL-1β secretion elicited by IAV, MDDCs were primed with R848 and infected with virus in the presence of Glyburide (Fig. 8B). Secretion of IL-1β was attenuated when cells were infected in its presence. Altogether, these data show that DCs infected with IAV require viral entry, endosomal fusion, and replication activity to prime and activate optimally the NLRP3 inflammasome.

DISCUSSION

IL-1 family cytokines have long been associated with acute and chronic inflammation, playing an essential role in the proinflammatory innate response to bacterial and viral infection. Among their biologic functions, these cytokines increase acute-phase signaling, leukocyte trafficking, epithelial cell activation, and secondary cytokine production. Of the 11 cytokines that compose this family, IL-1 is the key proinflammatory mediator during infection and primarily a product of monocytes, macrophages, and DCs. Recent studies observed rapid activation of NLRP3 and IL-1 production, resulting in the mass infiltration of leukocytes, particularly neutrophils, to the inflamed lung during early IAV infection [25, 27, 64, 65]. Together with lung epithelial cells, along with resident and recruited immune cells, secretion of proinflammatory cytokines is continued. This potent inflammatory response leads to a cytokine storm, mediated, in part, by IL-1β. These findings highlight how IAV infection results

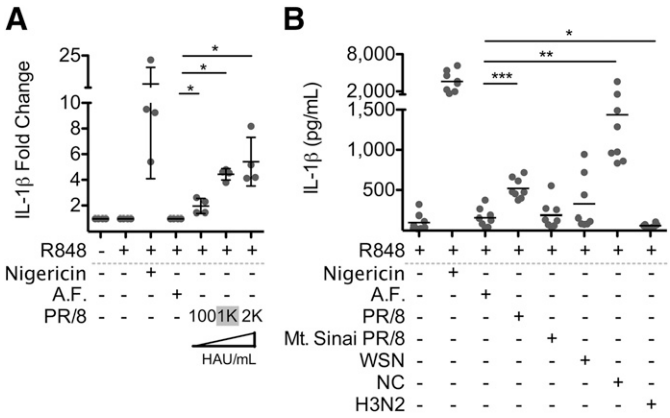

Figure 7. IAV activates the secretion of IL-1β in a strain-dependent manner. (A) MDDCs were primed with R848 overnight and activated with Charles River Laboratories PR/8 at various doses for 18 h. CBA was performed to detect secretion of IL-1β. Data were represented as fold change to A.F. control to normalize the data. (B) Cells were primed with R848 overnight before activation with various strains of live IAV for 18 h. Data are representative of (A) 4 and (B) 8 independent donors. Error bars represent SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

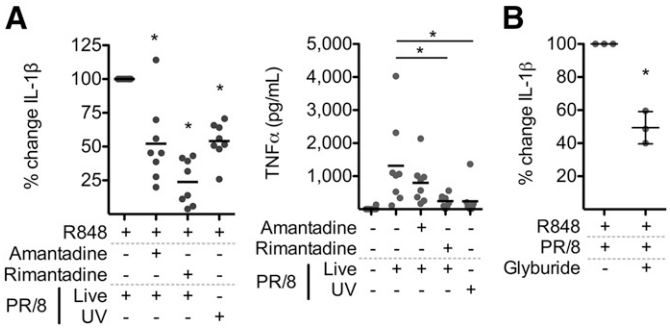

Figure 8. Amantadine and Rimantadine attenuate IL-1β production. (A) Cells were treated 2 h after infection with live or UV-irradiated PR/8 produced by Charles River Laboratories with Amantadine and Rimantadine. (B) MDDCs were primed with R848 and infected with live IAV for 6 h before inhibiting the inflammasome with Glyburide. Data are representative of (A) 8 and (B) 3 independent donors. Error bars represent SD. $*P < 0.05$.

in activation of the inflammasome and subsequent release of IL-1β that lends to severe, potentially deleterious inflammatory lung pathology.

To investigate the ability of human DCs to produce inflammasome-dependent cytokines, the TLR7/8 ligand R848 (a viral mimic) was used to prime DCs, thus allowing for the measure of key inflammasome substrates, primarily pro-IL-1β. Both human MDDCs and primary cDCs rapidly produced pro-IL-1β, pro-IL-18, and pro-IL-1α in response to R848 priming in a time-, dose-, and TLR-dependent fashion. Similar to IL-1β, IL-18 is synthesized as an inactive precursor, but unlike IL-1β, we found that the IL-18 precursor is constitutively present in primary cDCs.

Caspase-1 is responsible for the conversion of pro-IL-1β to bioactive IL-1β, which is then secreted via the nonclassic secretory pathway [66]. Subsequent to TLR priming, DCs require an additional trigger to process the zymogen form of caspase-1 to the enzymatically active form [40] by the NLRP3 inflammasome complex. Caspase-1 activity was measured in unstimulated and R848-primed cells. Primed DCs resulted in greater caspase-1 activity and pyroptosis soon after inflammasome activation with inducers of cellular K^+ efflux, such as Nigericin and ATP. This phenotype may be a result of the regulation of an important cofactor that modulates inflammasome kinetics, such as thioredoxin-interacting protein [67]. Secretion of IL-1α and bioactive IL-1β and IL-18 occurred in a NLRP3- and caspase-1-dependent manner, as shown by inhibition with caspase inhibitors and Glyburide. Release of IL-1β, in response to Nigericin and ATP, was also dependent on K^+ efflux, which presumably occurs upstream of caspase-1 formation. It has been shown by several studies conducted in THP-1 and murine macrophages that cellular K^+ efflux is a requirement for NLRP3 inflammasome activation triggered by all known activators, including MSU [15, 16]. In this study, MSU activation of the inflammasome did not depend on P_2Rx7 sensing of ATP nor K^+ efflux. The rigorous study by Muñoz-Planillo et al. [39] tested various particulate matter, with the exception of MSU, to define the role of K^+ efflux during uptake of particulate matter and found a consistent trend between tested NLRP3 agonists—a decrease in intracellular K^+ concentrations with concurrent release of mature IL-1β. More work is necessary to determine the role of K^+ efflux in MSU-dependent activation of the NLRP3 inflammasome from human DCs to define definitively the pathway of NLRP3 activation.

In addition to the conversion of pro-IL-1β and pro-IL-18 to their bioactive form, caspase-1 activity induces proinflammatory pyroptotic cell death. Activation of MDDCs with Nigericin and ATP induced a typical pyroptotic phenotype defined by LDH secretion and $AnnexinV^+7AAD^+$ costaining. Conversely, the majority (>70%) of MDDCs infected with PR/8 at 1000 HAU/ml, without a prepriming signal, expressed markers of apoptotic cell death within 18 h (Supplemental Fig. 4A and B). At later time points, IAV infection induced LDH release (WSN, NC, and PR/8 strains), as well as the production and release of several proinflammatory cytokines, including TNF-α, IL-1β, IFN-α, and IL-12 (data not shown). Multiple cell death programs are induced in a single dying cell during infection; thus, cell death is a continuum rather than a clearly defined pathway [48], whose kinetics may be influenced by the cellular environment. Consequently, it is possible for the predominant morphologic features of death during IAV infection to be apoptotic and simultaneously proinflammatory in stimulated populations. These data suggest that differences among PR/8, WSN, NC, and H3N2 are unlikely to be a result of large variations in the degree of infection and/or replication but rather, a function of cell viability.

Up-regulated IL-1β activity causes considerable damage in patients with hyperactive inflammasome activity [69] and viral infection [70, 71] and has been shown to mediate acute pulmonary inflammatory pathology, while also enhancing survival during IAV infection [25]. Genomic IAV RNA is sensed initially during infection by DCs through TLR7/8, leading to the synthesis of pro-IL-1β [22–24]. Likewise, HIV-1 has been shown to induce intracellular pro-IL-1β expression in human monocyte-derived macrophages [71] and healthy donor MDDCs [72], presumably through viral RNA detection activating the NF-κB signaling pathway. We not only defined the ability of human DCs to produce and secrete IL-1 cytokines but also determined that early events in IAV infection primed DCs to synthesize IL-1β. The more recent H1N1 isolate tested in our experiments, NC, secreted the highest levels of IL-1β when compared with the IAV isolates from the 1930s—PR/8 and WSN—despite all strains inducing similar expression of CD80, viral influenza nucleoprotein A synthesis. NC also induced the lowest levels of IFN-α (Fig. 5C), providing a possible explanation for this result. Viral NS1 encoded by the IAV has many functions, including inhibition of IFN-α production during infection [73]. Sequence analysis comparing the aligned NS1 reference protein sequences of these H1N1 strains tested here found a 10.82% aa difference between NC and PR/8, possibly explaining the differences in IFN-α secretion caused by these virus strains.

In keeping with the model of type I IFN attenuation of the inflammasome pathway [62], a separate study performed by Pontillo et al. [74] compared MDDCs derived using IFN-α in place of IL-4 from healthy donors. Their study found significant reduction in the induction of IL-1β from IFN-α-derived MDDCs infected with reverse transcription-incompetent aldrithiol-2 treated (AT-2) HIV-1 [74]. However, in this same study, MDDCs derived

from HIV$^+$ individuals were not able to induce significant IL-1β production compared with untreated cells. This response is possibly a result of the well-known, elevated and sustained type I IFN responses of HIV$^+$ patients that may impact inflammasome function [75]. Further studies of the relationship between type I IFN and IL-1β secretion will be useful to determine the differential secretion of these cytokines between seasonal and pandemic strains. Our results indicate intrinsic differences between strains in their ability to be sensed by the host cell, but the cause and full effects of these differences have yet to be determined.

The current model for IL-1β secretion during IAV infection requires that de novo-synthesized viral M2 interacts with the *trans*-Golgi network to alter intracellular proton concentrations, leading to NLRP3 inflammasome formation and secretion of mature IL-1β [23]. The blocking of M2 ion conductance is achieved with Rimantadine or Amantadine during infection with IAV. M2 inhibition resulted in attenuation of IL-1β release from DCs, supporting the model of M2 activity for the activation of the NLRP3 inflammasome. Given that M2 blockers were added 2 h following initial IAV infection, we cannot exclude the possibility that in addition to inhibiting neosynthesis of M2, there was blockade of viral replication.

To confirm further the role of active viral protein production in inflammasome activity, UV irradiation was used to prevent the replication of virus and subsequently, the production of M2. The exposure of IAV to UV radiation resulted in decreased IL-1β secretion from DCs, suggesting that new protein synthesis is necessary to activate optimally the inflammasome. To show the necessity of NLRP3 in IL-1β release during infection, NLRP3 inflammasome formation was blocked with Glyburide during infection, resulting in attenuated IL-1β secretion from MDDCs. RIG-I has been implicated recently in activating an inflammasome during IAV infection of primary lung epithelial cells [76]. Whereas our data support the model of de novo M2 ion channel activity upstream of NLRP3 inflammasome formation and subsequent secretion of mature IL-1β in human DCs, we cannot exclude the possible redundant role of another inflammasome in our system, such as the RIG-I inflammasome.

Resistance to adamantane-based drugs in H1N1 IAV strains is conferred through viral M2 mutations in the transmembrane helix responsible for viral ion channel activity [23, 77], specifically at amino acid residues Val27 and Ser31 within the central cavity, among others [78–80]. Therefore, it is possible that mutations in this region could affect activation of the inflammasome during IAV infection by altering ion conductance. A sequence analysis of M2 was performed in PR/8, WSN, and NC. Of note, the Mount Sinai PR/8 strain (Accession #AF389121.1) contained 2 mutations in the transmembrane region: Val27Ala and Ser31Asp. Functionally the Ser31Asp substitution is hypothesized to decrease proton current at low pH and thus, ion conductance by the M2 channel [81]. Additionally, Val27 is thought to function as a secondary gate of the channel controlling proton conductance [82]. Therefore, it is possible that the mutations found in the Mount Sinai strain M2 protein could account for its inability to activate the inflammasome in our system (Fig. 8B).

In summary, we have identified that the stimuli, components, and mechanics of NLRP3-dependent inflammasome activation, as well as consequent cytokine production in primary human cDCs, are modeled by human MDDCs. Moreover, we show how IAV can act as an activator of this pathway. Strikingly, our findings also suggest that differential expression of inflammatory cytokines and induction of cell death depend on the IAV strain. The optimal balance of IL-1β signaling appears to be critical for protection from viral infection; either too much or too little IL-1β promotes the development of disease. These findings may have therapeutic implications, suggesting that prophylactic drug therapy to limit the inflammatory response by suppressing the inflammasome may help to reduce the lung pathology associated with severe IAV infection.

AUTHORSHIP

M.V.F. was the lead investigator, responsible for all major areas of concept formation, data collection, and analysis, as well as the majority of manuscript composition. E.M. was involved in the later stages of concept formation and contributed significantly to manuscript edits. F.K. provided the influenza strains WSN, NC, PR/8, and H3N2 for experiments. R.G. performed Western blots on NLRP3, shown in the Supplemental figures. B.D.G. guided the amino acid sequence analysis on influenza proteins M2, NS1, and PB1-F2, while also contributing to the manuscript methodology and editing the discussion to include the authors' findings. N.B. was the supervisory author on this project and was involved throughout the project in concept formation and manuscript edits.

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DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

1. Bhardwaj, N., Lau, L. L., Friedman, S. M., Crow, M. K., Steinman, R. M. (1989) Interleukin 1 production during accessory cell-dependent mitogenesis of T lymphocytes. *J. Exp. Med.* **169,** 1121–1136.
2. Dinarello, C. A. (1996) Cytokines as mediators in the pathogenesis of septic shock. *Curr. Top. Microbiol. Immunol.* **216,** 133–165.
3. Piper, S. C., Ferguson, J., Kay, L., Parker, L. C., Sabroe, I., Sleeman, M. A., Briend, E., Finch, D. K. (2013) The role of interleukin-1 and interleukin-18 in pro-inflammatory and anti-viral responses to rhinovirus in primary bronchial epithelial cells. *PLoS One* **8,** e63365.
4. Dinarello, C. A. (1999) IL-18: a TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. *J. Allergy Clin. Immunol.* **103,** 11–24.
5. Gross, O., Thomas, C. J., Guarda, G., Tschopp, J. (2011) The inflammasome: an integrated view. *Immunol. Rev.* **243,** 136–151.
6. Martinon, F., Tschopp, J. (2004) Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* **117,** 561–574.
7. Martinon, F., Tschopp, J. (2005) NLRs join TLRs as innate sensors of pathogens. *Trends Immunol.* **26,** 447–454.

8. Fernandez, M. V., Miller, E. A., Bhardwaj, N. (2014) Activation and measurement of NLRP3 inflammasome activity using IL-1β in human monocyte-derived dendritic cells. *J. Vis. Exp.* **87,** e51284.
9. Puren, A. J., Fantuzzi, G., Dinarello, C. A. (1999) Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1beta are differentially regulated in human blood mononuclear cells and mouse spleen cells. *Proc. Natl. Acad. Sci. USA* **96,** 2256–2261.
10. Pirhonen, J., Sareneva, T., Kurimoto, M., Julkunen, I., Matikainen, S. (1999) Virus infection activates IL-1 beta and IL-18 production in human macrophages by a caspase-1-dependent pathway. *J. Immunol.* **162,** 7322–7329.
11. Martinon, F., Agostini, L., Meylan, E., Tschopp, J. (2004) Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. *Curr. Biol.* **14,** 1929–1934.
12. Kanneganti, T. D., Ozören, N., Body-Malapel, M., Amer, A., Park, J. H., Franchi, L., Whitfield, J., Barchet, W., Colonna, M., Vandenabeele, P., Bertin, J., Coyle, A., Grant, E. P., Akira, S., Núñez, G. (2006) Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* **440,** 233–236.
13. Mariathasan, S., Weiss, D. S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W. P., Weinrauch, Y., Monack, D. M., Dixit, V. M. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* **440,** 228–232.
14. Eisenbarth, S. C., Colegio, O. R., O'Connor, W., Sutterwala, F. S., Flavell, R. A. (2008) Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* **453,** 1122–1126.
15. Dostert, C., Pétrilli, V., Van Bruggen, R., Steele, C., Mossman, B. T., Tschopp, J. (2008) Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* **320,** 674–677.
16. Pétrilli, V., Papin, S., Dostert, C., Mayor, A., Martinon, F., Tschopp, J. (2007) Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ.* **14,** 1583–1589.
17. McWilliam, A. S., Nelson, D., Thomas, J. A., Holt, P. G. (1994) Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *J. Exp. Med.* **179,** 1331–1336.
18. Xia, W., Pinto, C. E., Kradin, R. L. (1995) The antigen-presenting activities of Ia+ dendritic cells shift dynamically from lung to lymph node after an airway challenge with soluble antigen. *J. Exp. Med.* **181,** 1275–1283.
19. Holt, P. G., Schon-Hegrad, M. A., Oliver, J., Holt, B. J., McMenamin, P. G. (1990) A contiguous network of dendritic antigen-presenting cells within the respiratory epithelium. *Int. Arch. Allergy Appl. Immunol.* **91,** 155–159.
20. Holt, P. G., Haining, S., Nelson, D. J., Sedgwick, J. D. (1994) Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. *J. Immunol.* **153,** 256–261.
21. Bender, A., Albert, M., Reddy, A., Feldman, M., Sauter, B., Kaplan, G., Hellman, W., Bhardwaj, N. (1998) The distinctive features of influenza virus infection of dendritic cells. *Immunobiology* **198,** 552–567.
22. Lund, J. M., Alexopoulou, L., Sato, A., Karow, M., Adams, N. C., Gale, N. W., Iwasaki, A., Flavell, R. A. (2004) Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA* **101,** 5598–5603.
23. Ichinohe, T., Pang, I. K., Iwasaki, A. (2010) Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nat. Immunol.* **11,** 404–410.
24. Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S., Reis e Sousa, C. (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303,** 1529–1531.
25. Schmitz, N., Kurrer, M., Bachmann, M. F., Kopf, M. (2005) Interleukin-1 is responsible for acute lung immunopathology but increases survival of respiratory influenza virus infection. *J. Virol.* **79,** 6441–6448.
26. Ichinohe, T., Lee, H. K., Ogura, Y., Flavell, R., Iwasaki, A. (2009) Inflammasome recognition of influenza virus is essential for adaptive immune responses. *J. Exp. Med.* **206,** 79–87.
27. Cillóniz, C., Shinya, K., Peng, X., Korth, M. J., Proll, S. C., Aicher, L. D., Carter, V. S., Chang, J. H., Kobasa, D., Feldmann, F., Strong, J. E., Feldmann, H., Kawaoka, Y., Katze, M. G. (2009) Lethal influenza virus infection in macaques is associated with early dysregulation of inflammatory related genes. *PLoS Pathog.* **5,** e1000604.
28. La Gruta, N. L., Kedzierska, K., Stambas, J., Doherty, P. C. (2007) A question of self-preservation: immunopathology in influenza virus infection. *Immunol. Cell Biol.* **85,** 85–92.
29. O'Neill, D. W., Bhardwaj, N. (2005) Differentiation of peripheral blood monocytes into dendritic cells. *Curr. Protoc. Immunol.* Chapter 22, Unit 22F 4.
30. Sabado, R. L., Miller, E., Spadaccia, M., Vengco, I., Hasan, F., Bhardwaj, N. (2013) Preparation of tumor antigen-loaded mature dendritic cells for immunotherapy. *J. Vis. Exp.* **78,** e50085.
31. Bhardwaj, N., Bender, A., Gonzalez, N., Bui, L. K., Garrett, M. C., Steinman, R. M. (1994) Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human CD8+ T cells. *J. Clin. Invest.* **94,** 797–807.
32. Bender, A., Bui, L. K., Feldman, M. A., Larsson, M., Bhardwaj, N. (1995) Inactivated influenza virus, when presented on dendritic cells, elicits human CD8+ cytolytic T cell responses. *J. Exp. Med.* **182,** 1663–1671.
33. Duramad, O., Fearon, K. L., Chan, J. H., Kanzler, H., Marshall, J. D., Coffman, R. L., Barrat, F. J. (2003) IL-10 regulates plasmacytoid dendritic cell response to CpG-containing immunostimulatory sequences. *Blood* **102,** 4487–4492.
34. Barrat, F. J., Meeker, T., Gregorio, J., Chan, J. H., Uematsu, S., Akira, S., Chang, B., Duramad, O., Coffman, R. L. (2005) Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J. Exp. Med.* **202,** 1131–1139.
35. Ade, N., Antonios, D., Kerdine-Romer, S., Boisleve, F., Rousset, F., Pallardy, M. (2007) NF-kappaB plays a major role in the maturation of human dendritic cells induced by NiSO(4) but not by DNCB. *Toxicol. Sci.* **99,** 488–501.
36. Ardeshna, K. M., Pizzey, A. R., Devereux, S., Khwaja, A. (2000) The PI3 kinase, p38 SAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. *Blood* **96,** 1039–1046.
37. Renn, C. N., Sanchez, D. J., Ochoa, M. T., Legaspi, A. J., Oh, C. K., Liu, P. T., Krutzik, S. R., Sieling, P. A., Cheng, G., Modlin, R. L. (2006) TLR activation of Langerhans cell-like dendritic cells triggers an antiviral immune response. *J. Immunol.* **177,** 298–305.
38. Gorden, K. B., Gorski, K. S., Gibson, S. J., Kedl, R. M., Kieper, W. C., Qiu, X., Tomai, M. A., Alkan, S. S., Vasilakos, J. P. (2005) Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. *J. Immunol.* **174,** 1259–1268.
39. Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendiran, T. M., Núñez, G. (2013) K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* **38,** 1142–1153.
40. Netea, M. G., Nold-Petry, C. A., Nold, M. F., Joosten, L. A., Opitz, B., van der Meer, J. H., van de Veerdonk, F. L., Ferwerda, G., Heinhuis, B., Devesa, I., Funk, C. J., Mason, R. J., Kullberg, B. J., Rubartelli, A., van der Meer, J. W., Dinarello, C. A. (2009) Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* **113,** 2324–2335.
41. Vergani, A., Fotino, C., D'Addio, F., Tezza, S., Podetta, M., Gatti, F., Chin, M., Bassi, R., Molano, R. D., Corradi, D., Gatti, R., Ferrero, M. E., Secchi, A., Grassi, F., Ricordi, C., Sayegh, M. H., Maffi, P., Pileggi, A., Fiorina, P. (2013) Effect of the purinergic inhibitor oxidized ATP in a model of islet allograft rejection. *Diabetes* **62,** 1665–1675.
42. Murgia, M., Hanau, S., Pizzo, P., Rippa, M., Di Virgilio, F. (1993) Oxidized ATP. An irreversible inhibitor of the macrophage purinergic P2Z receptor. *J. Biol. Chem.* **268,** 8199–8203.
43. Di Virgilio, F. (2005) Purinergic mechanism in the immune system: a signal of danger for dendritic cells. *Purinergic Signal.* **1,** 205–209.
44. Baroni, M., Pizzirani, C., Pinotti, M., Ferrari, D., Adinolfi, E., Calzavarini, S., Caruso, P., Bernardi, F., Di Virgilio, F. (2007) Stimulation of P2 (P2X7) receptors in human dendritic cells induces the release of tissue factor-bearing microparticles. *FASEB J.* **21,** 1926–1933.
45. Hari, A., Zhang, Y., Tu, Z., Detampel, P., Stenner, M., Ganguly, A., Shi, Y. (2014) Activation of NLRP3 inflammasome by crystalline structures via cell surface contact. *Sci. Rep.* **4,** 7281.
46. Riteau, N., Baron, L., Villeret, B., Guillou, N., Savigny, F., Ryffel, B., Rassendren, F., Le Bert, M., Gombault, A., Couillin, I. (2012) ATP release and purinergic signaling: a common pathway for particle-mediated inflammasome activation. *Cell Death Dis.* **3,** e403.
47. Schorn, C., Frey, B., Lauber, K., Janko, C., Strysio, M., Keppeler, H., Gaipl, U. S., Voll, R. E., Springer, E., Munoz, L. E., Schett, G., Herrmann, M. (2011) Sodium overload and water influx activate the NALP3 inflammasome. *J. Biol. Chem.* **286,** 35–41.
48. Fink, S. L., Cookson, B. T. (2005) Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect. Immun.* **73,** 1907–1916.
49. Rayamajhi, M., Zhang, Y., Miao, E. A. (2013) Detection of pyroptosis by measuring released lactate dehydrogenase activity. *Methods Mol. Biol.* **1040,** 85–90.
50. Lamkanfi, M. (2011) Emerging inflammasome effector mechanisms. *Nat. Rev. Immunol.* **11,** 213–220.
51. Labbé, K., Saleh, M. (2011) Pyroptosis: a caspase-1-dependent programmed cell death and a barrier to infection. In *The Inflammasomes* (I. Couillin, V. Pétrilli, F. Martinon, eds.), Springer Basel AG, Basel, Switzerland, 17–36.
52. Compan, V., Baroja-Mazo, A., López-Castejón, G., Gomez, A. I., Martínez, C. M., Angosto, D., Montero, M. T., Herranz, A. S., Bazán, E., Reimers, D., Mulero, V., Pelegrín, P. (2012) Cell volume regulation modulates NLRP3 inflammasome activation. *Immunity* **37,** 487–500.
53. Williams, O. (2004) Flow cytometry-based methods for apoptosis detection in lymphoid cells. *Methods Mol. Biol.* **282,** 31–42.
54. Lamkanfi, M., Mueller, J. L., Vitari, A. C., Misaghi, S., Fedorova, A., Deshayes, K., Lee, W. P., Hoffman, H. M., Dixit, V. M. (2009) Glyburide inhibits the cryopyrin/Nalp3 inflammasome. *J. Cell Biol.* **187,** 61–70.

55. Feldmeyer, L., Keller, M., Niklaus, G., Hohl, D., Werner, S., Beer, H. D. (2007) The inflammasome mediates UVB-induced activation and secretion of interleukin-1beta by keratinocytes. *Curr. Biol.* **17,** 1140–1145.
56. Nasti, T. H., Timares, L. (2012) Inflammasome activation of IL-1 family mediators in response to cutaneous photodamage. *Photochem. Photobiol.* **88,** 1111–1125.
57. Fernandez-Sesma, A., Marukian, S., Ebersole, B. J., Kaminski, D., Park, M. S., Yuen, T., Sealfon, S. C., García-Sastre, A., Moran, T. M. (2006) Influenza virus evades innate and adaptive immunity via the NS1 protein. *J. Virol.* **80,** 6295–6304.
58. Hofmann, P., Sprenger, H., Kaufmann, A., Bender, A., Hasse, C., Nain, M., Gemsa, D. (1997) Susceptibility of mononuclear phagocytes to influenza A virus infection and possible role in the antiviral response. *J. Leukoc. Biol.* **61,** 408–414.
59. Fesq, H., Bacher, M., Nain, M., Gemsa, D. (1994) Programmed cell death (apoptosis) in human monocytes infected by influenza A virus. *Immunobiology* **190,** 175–182.
60. Epand, R. M., Epand, R. F. (2002) Thermal denaturation of influenza virus and its relationship to membrane fusion. *Biochem. J.* **365,** 841–848.
61. Ruigrok, R. W., Martin, S. R., Wharton, S. A., Skehel, J. J., Bayley, P. M., Wiley, D. C. (1986) Conformational changes in the hemagglutinin of influenza virus which accompany heat-induced fusion of virus with liposomes. *Virology* **155,** 484–497.
62. Guarda, G., Braun, M., Staehli, F., Tardivel, A., Mattmann, C., Förster, I., Farlik, M., Decker, T., Du Pasquier, R. A., Romero, P., Tschopp, J. (2011) Type I interferon inhibits interleukin-1 production and inflammasome activation. *Immunity* **34,** 213–223.
63. Ichinohe, T., Pang, I. K., Kumamoto, Y., Peaper, D. R., Ho, J. H., Murray, T. S., Iwasaki, A. (2011) Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc. Natl. Acad. Sci. USA* **108,** 5354–5359.
64. Allen, I. C., Scull, M. A., Moore, C. B., Holl, E. K., McElvania-TeKippe, E., Taxman, D. J., Guthrie, E. H., Pickles, R. J., Ting, J. P. (2009) The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity* **30,** 556–565.
65. Thomas, P. G., Dash, P., Aldridge, Jr., J. R., Ellebedy, A. H., Reynolds, C., Funk, A. J., Martin, W. J., Lamkanfi, M., Webby, R. J., Boyd, K. L., Doherty, P. C., Kanneganti, T. D. (2009) The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* **30,** 566–575.
66. Qu, Y., Franchi, L., Nunez, G., Dubyak, G. R. (2007) Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages. *J. Immunol.* **179,** 1913–1925.
67. Zhou, R., Tardivel, A., Thorens, B., Choi, I., Tschopp, J. (2010) Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat. Immunol.* **11,** 136–140.
68. McDermott, M. F., Tschopp, J. (2007) From inflammasomes to fevers, crystals and hypertension: how basic research explains inflammatory diseases. *Trends Mol. Med.* **13,** 381–388.
69. Morales-García, G., Falfán-Valencia, R., García-Ramírez, R. A., Camarena, Á., Ramirez-Venegas, A., Castillejos-López, M., Pérez-Rodríguez, M., González-Bonilla, C., Grajales-Muñíz, C., Borja-Aburto, V., Mejía-Aranguré, J. M. (2012) Pandemic influenza A/H1N1 virus infection and TNF, LTA, IL1B, IL6, IL8, and CCL polymorphisms in Mexican population: a case-control study. *BMC Infect. Dis.* **12,** 299.
70. Liu, Y., Li, S., Zhang, G., Nie, G., Meng, Z., Mao, D., Chen, C., Chen, X., Zhou, B., Zeng, G. (2013) Genetic variants in IL1A and IL1B contribute to the susceptibility to 2009 pandemic H1N1 influenza A virus. *BMC Immunol.* **14,** 37.
71. Hernandez, J. C., Latz, E., Urcuqui-Inchima, S. (2014) HIV-1 induces the first signal to activate the NLRP3 inflammasome in monocyte-derived macrophages. *Intervirology* **57,** 36–42.
72. Pontillo, A., Silva, L. T., Oshiro, T. M., Finazzo, C., Crovella, S., Duarte, A. J. (2012) HIV-1 induces NALP3-inflammasome expression and interleukin-1β secretion in dendritic cells from healthy individuals but not from HIV-positive patients. *AIDS* **26,** 11–18.
73. Hale, B. G., Randall, R. E., Ortín, J., Jackson, D. (2008) The multifunctional NS1 protein of influenza A viruses. *J. Gen. Virol.* **89,** 2359–2376.
74. Pontillo, A., Santillo, B. T., Duarte, A. J., Oshiro, T. M. (2013) Differential inflammasome expression and IL-1β secretion in monocyte-derived dendritic cells differentiated with IL-4 or IFN-α. *AIDS Res. Ther.* **10,** 35.
75. Sabado, R. L., O'Brien, M., Subedi, A., Qin, L., Hu, N., Taylor, E., Dibben, O., Stacey, A., Fellay, J., Shianna, K. V., Siegal, F., Shodell, M., Shah, K., Larsson, M., Lifson, J., Nadas, A., Marmor, M., Hutt, R., Margolis, D., Garmon, D., Markowitz, M., Valentine, F., Borrow, P., Bhardwaj, N. (2010) Evidence of dysregulation of dendritic cells in primary HIV infection. *Blood* **116,** 3839–3852.
76. Pothlichet, J., Meunier, I., Davis, B. K., Ting, J. P., Skamene, E., von Messling, V., Vidal, S. M. (2013) Type I IFN triggers RIG-I/TLR3/NLRP3-dependent inflammasome activation in influenza A virus infected cells. *PLoS Pathog.* **9,** e1003256.
77. Wang, C., Takeuchi, K., Pinto, L. H., Lamb, R. A. (1993) Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. *J. Virol.* **67,** 5585–5594.
78. Hu, F., Luo, W., Cady, S. D., Hong, M. (2011) Conformational plasticity of the influenza A M2 transmembrane helix in lipid bilayers under varying pH, drug binding, and membrane thickness. *Biochim. Biophys. Acta* **1808,** 415–423.
79. Suzuki, H., Saito, R., Masuda, H., Oshitani, H., Sato, M., Sato, I. (2003) Emergence of amantadine-resistant influenza A viruses: epidemiological study. *J. Infect. Chemother.* **9,** 195–200.
80. Shiraishi, K., Mitamura, K., Sakai-Tagawa, Y., Goto, H., Sugaya, N., Kawaoka, Y. (2003) High frequency of resistant viruses harboring different mutations in amantadine-treated children with influenza. *J. Infect. Dis.* **188,** 57–61.
81. Pinto, L. H., Dieckmann, G. R., Gandhi, C. S., Papworth, C. G., Braman, J., Shaughnessy, M. A., Lear, J. D., Lamb, R. A., DeGrado, W. F. (1997) A functionally defined model for the M2 proton channel of influenza A virus suggests a mechanism for its ion selectivity. *Proc. Natl. Acad. Sci. USA* **94,** 11301–11306.
82. Zhou, H. X. (2011) A theory for the proton transport of the influenza virus M2 protein: extensive test against conductance data. *Biophys. J.* **100,** 912–921.

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