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*J Immunol* published online 10 February 2012
http://www.jimmunol.org/content/early/2012/02/10/jimmunol.1103051

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Supplementary Material
http://www.jimmunol.org/content/suppl/2012/02/13/jimmunol.1103051.S1.DC1

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Impaired NLRP3 Inflammasome Function in Elderly Mice during Influenza Infection Is Rescued by Treatment with Nigericin

Heather W. Stout-Delgado,* Sarah E. Vaughan,* Anushree C. Shirali,† Richard J. Jaramillo,* and Kevin S. Harrod*

The NLRP3 inflammasome is activated in the lung during influenza viral infection; however, the impact of aging on inflammasome function during influenza infection has not been examined. In this study, we show that elderly mice infected with a mouse-adapted strain of influenza produced lower levels of IL-1β during in vitro and in vivo infection. Dendritic cells from elderly mice exhibited decreased expression of ASC, NLRP3, and capase-1 but increased expression of pro–IL-1β, pro–IL-18, and pro–IL-33 compared with dendritic cells from young infected mice. Treatment with nigericin during influenza infection augmented IL-1β production, increased caspase-1 activity, and decreased morbidity and mortality in elderly mice. Our study demonstrates for the first time, to our knowledge, that during influenza viral infection, elderly mice have impaired NLRP3 inflammasome activity and that treatment with nigericin rescues NLRP3 activation in elderly hosts.

Influenza viral infections are responsible for significant morbidity and mortality involving approximately 5 million people worldwide, with the highest infections rates found in the elderly (>65 y) population (1). It has been well established that innate and adaptive immune responses to influenza are impaired with aging; however, the mechanisms that underlie this impairment and thereby contribute to higher levels of mortality and morbidity in elderly hosts have not been well elucidated (2–8). The innate immune system expresses several germline-encoded pattern recognition receptors, known as pattern recognition receptors, that recognize general motifs that are essential to the function of microbes (9). TLRs, NLRs, and RLRs are several classes of pattern recognition receptors that have been shown to be involved in the recognition of influenza virus (10–13). Previous work has highlighted age-associated decreased TLR function in dendritic cells, dysregulated signaling cascades, and decreased cytokine production, which contribute to impaired innate immune responses (14–17). Similarly, age-associated defects in RIG-I signaling, specifically impaired IFN signaling after RIG-I stimulation with West Nile virus, have been observed (18). Recent work has illustrated a critical role for NLRs in immunity against influenza; however, age-associated changes in this pathway have not been reported (11).

Influenza virus activates inflammasome-dependent innate and adaptive immune responses (11). Influenza infection stimulates dendritic cells and macrophages via TLR7, leading to the synthesis of pro–IL-1β, pro–IL-18, and pro–IL-33. Recent work has shown that the proton-specific ion channel, M2, which plays a key role both in fusion and during viral entry and synthesis of new virions, can provide a secondary signal that is sufficient to trigger inflammasome assembly and activation (19). Activation of the NLRP3 inflammasome can be attributed to changes in potassium efflux and is reactive oxygen species (ROS) dependent (20, 21). Stimulation of the P2X7 receptor by ATP or nigericin treatment can stimulate NLRP3 inflammasome function in LPS-primed dendritic cells (11, 21, 22). Once activated, NLRP3-dependent production of IL-1β by alveolar macrophages and dendritic cells leads to the production of chemokines and subsequent recruitment of inflammatory cells to the site of infection (11). Activation of the NLRP3 inflammasome during influenza viral infection leads to the migration of dendritic cells from the lung to the draining lymph node where subsequent T cell priming contributes to adaptive immune responses, specifically the establishment of Th1, CTL, and IgA responses to influenza viral infection (11, 23). Deficiency of NLRP3 inflammasome function results in decreased production of IL-1β and increased mortality upon challenge with a sublethal dose of influenza virus (11, 23, 24). Thus, NLRP3 inflammasome function is required for innate and adaptive immune responses that limit disease during influenza infection.

In the current study, we investigated the effect of aging on NLRP3 inflammasome activation in a murine model of influenza. Our results demonstrate that elderly hosts have impaired inflammasome activation, decreased gene expression of several key components of the NLRP3 inflammasome signaling pathway, decreased caspase-1 activity, and decreased production of IL-1β and IL-18 in response to in vitro and in vivo infection with HKx31, a mouse-adapted strain of influenza. Adoptive transfer of young dendritic cells into elderly influenza-infected hosts decreased morbidity, increased leukocyte infiltration, and increased IL-1β secretion compared with those in elderly influenza-infected mice that did not receive an adoptive transfer or were adoptively transferred with elderly dendritic cells. Notably, while pro–IL-1β,

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Received for publication October 26, 2011. Accepted for publication January 11, 2012.

This work was supported by the National Institutes of Health (K01AG034999-01A1 to H.W.S.-D.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: BAL: bronchial alveolar lavage; BMDC, bone marrow-derived dendritic cell; MOI, multiplicity of infection; PAMP, pathogen-associated molecular pattern; ROS, reactive oxygen species.

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0022-1767/12/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1103051
pro–IL-18, and pro–IL-33 mRNA expression was preserved in elderly dendritic cells, expression of NLRP3, ASC, and caspase-1 mRNA was decreased during HKx31 infection. Stimulation with ATP or nigericin, which have been previously shown to promote K+ efflux, augmented IL-1β and IL-18 release by elderly dendritic cells and increased expression of NLRP3 inflammasome components and augmented caspase-1 activity. Treatment of HKx31-infected elderly mice with nigericin resulted in increased IL-1β secretion and improved morbidity and mortality during the course of infection. Taken together, our data provide the first evidence, to our knowledge, for an age-associated decline in NLRP3 inflammasome function, which confers increased susceptibility of elderly hosts to influenza viral infection, and an early intervention, such as treatment with nigericin, can rescue function and improve clinical outcomes.

Materials and Methods

Mice

Young (2–4 mo) and middle-aged (8–9 mo) male and female BALB/c mice were either purchased from Taconic (Hudson, NY) or the National Institute of Aging rodent facility (Charles River Laboratories, New York, NY). Elderly (15–16 mo) male and female BALB/c mice were purchased from the National Institute of Aging rodent facility (Charles River Laboratories). Upon receipt, mice were handled under identical husbandry conditions and fed certified commercial feed. Examination of NLRP3 inflammasome activation in young and middle-aged mice purchased from either Taconic or the National Institute of Aging rodent facility showed that activation of the NLRP3 inflammasome and production of IL-1β was consistent despite the source of the animals. Body weights were measured daily, and mice were humanely euthanized if they lost more than 20% of their starting body weight. The Institutional Animal Care and Use Committee of Lovelace Respiratory Research Institute approved the use of animals in this study. No animals were used in the study if they had evidence of skin lesions, weight loss, or lymphadenopathy.

Viral propagation

HKx31 virus was generously provided by Dr. Ralph Tripp (Department of Infectious Diseases, University of Georgia) and grown in MDCK cells (American Type Culture Collection, Manassas, VA) as previously described (25).

Primary bone marrow cell isolation and culture

Bone marrow cells were prepared from the femurs and tibiae of mice as previously described (26). Bone marrow cells were cultured in complete RPMI (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen) and 1× penicillin/streptomycin (Invitrogen) and 25 ng/ml GM-CSF (Cell Signal Technology, Danvers, MA) for 5 d at 37°C with 5% CO2. For in vitro stimulation experiments, the following treatments were given in complete RPMI for specified times: HKx31 (multiplicity of infection [MOI] = 10; 1, 2, or 24 h), ATP (5 mM; 0.5–1 h) (Invivogen, San Diego, CA), nigericin (1 μM; 0.5–1 h) (Invivogen), and/or LPS (100–500 ng/ml; 3 h) (Invivogen).

Treatment with rotenone. Cells were subsequently treated with rotenone (5 μM; Enzo Life Sciences, Plymouth Meeting, PA) for 1 h prior to ATP (5 mM; Invivogen) stimulation (30 min).

Treatment with mitoTEMPO. Cells were treated with mitoTEMPO (100 μM; Enzo Life Sciences) for 1 h prior to culture with LPS (100 ng/ml) or HKx31 (MOI = 10) for 4 h. Cells were then stimulated with ATP (5 mM; Invivogen) for 30 min.

Alum stimulation. Cells were stimulated with LPS (100 ng/ml) for 4 h prior to alum stimulation (250 μg/ml; 24 h; Invivogen).

Pulmonary dendritic cell isolation

Pulmonary lymphocytes were isolated as previously described (27). Briefly, lung samples were digested at 37°C for 1 h in freshly prepared collagenase digestion solution [300 U/ml collagenase type II (Roche), 10 ml PBS (Invitrogen), and 150 μl DNase I of a 10 mg/ml stock solution (Roche)] prior to passage through a 40-μm sieve. Cells were negatively selected using the EasySep dendritic cell selection kit (StemCell Technologies, Vancouver, Canada). Cell purity was assessed by flow cytometry (>95% purity).

RNA purification and real-time PCR

RNA samples were extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) and quantified by A260/A280 absorbance readings. Superscript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) was used according to the manufacturer’s instructions to assess primer-specific gene expression. QuantiTect Primer Assays were purchased from Qiagen and used at a 1× concentration per reaction. PCR was conducted in 96-well plates using the Applied Biosystems 7300 detection system (Applied Biosystems, Carlsbad, CA). All reactions were performed in triplicate. Relative levels of mRNA were calculated by the comparative cycle threshold method (User Bulletin No. 2; Applied Biosystems), and 18sRNA mRNA levels were used as the invariant control for each sample.

ELISA

Culture supernatants or serum were analyzed for IL-1β or IL-18 production using ELISA kits purchased from eBioscience (San Diego, CA) according to the manufacturer’s instructions.

Caspase-1 assay

Cultured cells were lysed, and caspase-1 activity was assessed using the Caspase-1 Colorimetric Assay Kit from Abcam (Cambridge, MA) according to the manufacturer’s instructions. Fold increase in caspase-1 activity was determined by comparing the results of treated samples with the level of the untreated control.

Western blotting

Cells were washed and lysed in buffer containing 50 mM Tris, pH 7.5, 1% (v/v) Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, and a protease inhibitor mixture. Protein concentrations were assayed using the Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA) and using BSA as a standard. Equal amounts of protein (15 μg/lane) were separated on 4–15% bis-acrylamide gels (Invitrogen) by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane (Bio-Rad). Immunodetection was performed using primary rabbit anti-ASC (Genway Biotech, San Diego, CA), rabbit anti-NLRP3 (Abcam), rabbit anti-caspase-1 (Abcam), and secondary HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technologies, Cambridge, MA) and the ECL Western Blotting Analysis System (Santa Cruz Biologicals, Santa Cruz, CA). Images were acquired using Multi-Gauge software (Fujifilm, Greenwood, SC).

In vivo procedures

Influenza viral infection. All mice were anesthetized with isoflurane (5% for induction and 2% for maintenance) prior to intranasal instillation with 1 × 106 PFU HKx31 (50–100 μl volume in PBS). All mice were observed twice daily for 16 d after HKx31 administration. As previously described, the following clinical scores were assigned: 0 = normal, 1 = slightly ruffled, 2 = ruffled fur, 3 = ruffled fur and inactive, 4 = hunched/moribund, and 5 = dead (28).

Adoptive transfer. Mice were given 1 × 107 young or elderly bone marrow–derived dendritic cells (200–μl volume in PBS) iv. i.v. injection of the tail vein 1 d prior to influenza instillation.

Nigericin administration. Mice received a 250-μl volume of 0.005 mg/g body weight dose of nigericin (Invivogen) (1× PBS as vehicle) i.p. at 24 h after influenza viral infection. On completion of each of the above procedures, mice were monitored until normal breathing and postural reflexes returned.

Statistical analysis

Survival analysis between groups was calculated using the log rank method and the Mantel–Cox test. Comparison of means was performed using a two-tailed t test, one-way or two-way ANOVA, when appropriate, and repeated measures using ANOVA. All data were analyzed using GraphPad prism software (San Diego, CA). Statistical significance was considered by a p value <0.05.

Results

Increased morbidity in elderly mice during influenza viral infection with HKx31

It is well established that the elderly have increased morbidity and mortality to influenza infections, and it is believed that decreased immune function is the reason. Previous work has illustrated age-
associated impairments in immune responses during influenza viral infection with A/Puerto Rico/8/1934 (H1N1) (2, 29, 30). To examine this phenotype further, we infected young (2–4 mo of age), middle-aged (8–9 mo of age), and elderly (15 mo of age) BALB/c mice with HKx31 (H3N2), a mouse-adapted strain of influenza (29). During influenza viral infection, elderly mice also had increased viral load in the lung (two-way ANOVA, by plaque assay. As illustrated in Fig. 1D, elderly mice also had compared with those of young mice. Further, comparison between young mice, elderly mice had decreased cellular infiltration of leukocytes to the site of infection (Fig. 1C; t test, p < 0.01). At specific time points during influenza viral infection, lung tissue was harvested from young and elderly mice, and lung viral titers were assessed by plaque assay. As illustrated in Fig. 1D, elderly mice also had increased viral load in the lung (two-way ANOVA, p < 0.0001) compared with that in young virally infected mice. Taken together, these results illustrate that compared with young mice, elderly mice infected with HKx31 display increased morbidity with decreased leukocyte infiltration and increased viral load in the lung.

Decreased activation of IL-1β in elderly dendritic cells during influenza viral infection

The NLRP3 inflammasome activates caspase-1 in response to influenza viral infection resulting in the processing and secretion of IL-1β and IL-18 (11, 31–33). As production of IL-1β and IL-18 is an important component of the acute-phase responses to influenza, we examined if aging alters NLRP3 inflammasome-mediated activation of IL-1β and IL-18. Young and elderly bone marrow-derived dendritic cells (BMDCs) were infected with active or heat-inactivated HKx31 (MOI = 10) for 24 h, and the production of IL-1β and IL-18 was assessed by ELISA. As shown in Fig. 2, elderly BMDCs had significantly decreased secretion of IL-1β (Fig. 2A; two-way ANOVA, p < 0.0001) and IL-18 (Fig. 2B; two-way ANOVA, p = 0.009) in response to HKx31 compared with that of young BMDCs. Production of mature IL-1β and mature IL-18 by young and elderly BMDCs in response to HKx31 was caspase-1 dependent, as treatment with caspase-1 inhibitor (V-ZAD-FMK) decreased IL-1β and IL-18 secretion in both young and elderly BMDCs (data not shown). We next investigated IL-1β and IL-18 production in elderly mice during HKx31 viral infection. Young, middle-aged, and elderly BALB/c mice were infected with HKx31, and lung, serum, and bronchial alveolar lavage (BAL) were collected at specific time points during viral infection. Compared with young and middle-aged HKx31-infected groups, IL-1β and IL-18 production was significantly decreased in lung homogenates (Fig. 2C; two-way ANOVA, p = 0.0001; Fig. 2D; two-way ANOVA, p = 0.0003), serum (Fig. 2E; two-way ANOVA, p < 0.0001), and BAL (Fig. 2F; t test, p < 0.0001, Figure 2G; t test, p = 0.0041) isolated from elderly mice. Taken together, these data indicate that aging decreases both in vitro and in vivo production of IL-1β and IL-18 in response to influenza viral challenge.

Previous work has illustrated that activation of the NLRP3 inflammasome and subsequent processing of IL-1β by caspase-1 requires two distinct signals. The first signal involves TLR activation, which induces the synthesis of pro–IL-1β, pro–IL-18, and pro–IL-33 (11). A second signal leads to the activation of the NLRP3 inflammasome and subsequent processing of IL-1β, IL-

**FIGURE 1.** Increased morbidity and mortality in elderly mice during viral infection with HKx31. Young (2–4 mo), middle-aged (8–9 mo), and elderly (15 mo) BALB/c mice were infected with 1 × 10^6 PFU HKx31 via intranasal instillation. Throughout the duration of HKx31 infection, weights and clinical scores were recorded. (A) Clinical scores were recorded for young and elderly BALB/c mice after infection with HKx31 (one-way ANOVA, p = 0.0108). Young and elderly PBS controls did not exhibit a significant change in clinical scores. (B) Survival of young and elderly BALB/c mice during HKx31 infection (Mantel–Cox test, p = 0.0015). No lethality occurred in young and elderly PBS-treated mice. (C) Lungs were harvested from HKx31-infected young and elderly BALB/c on day 5 of infection (t test, **p < 0.01). Lung tissue was digested with collagenase A, and the number of leukocytes is shown. (D) Viral titer was evaluated in lung homogenates from young and elderly mice by plaque assay (two-way ANOVA, p < 0.0001). Similar results were obtained from at least three independent experiments with greater than n = 5 per group, and results are shown as the mean ± SEM.
To examine if decreased IL-1β and IL-18 production by elderly dendritic cells was due to impaired TLR activation, we examined the expression of pro–IL-1β and pro–IL-18 during HKx31 infection. As shown in Fig. 3A, synthesis of pro–IL-1β mRNA in elderly BMDCs was significantly higher during HKx31 infection compared with that in young BMDCs (t test, \( p = 0.0053 \)). Synthesis of pro–IL-18 and pro–IL-33 mRNA during HKx31 infection was similar between young and elderly BMDCs (Fig. 3B, 3C, respectively). Nuclear translocation of the p65 subunit of NF-κB (data not shown) and the synthesis of IL-6 and TNF-α mRNA in response to HKx31 infection was similar between young and elderly BMDCs (data not shown). Next, we examined the expression of pro–IL-1β and pro–IL-18 in pulmonary dendritic cells isolated from young, middle-aged, and elderly control and HKx31-infected mice. As shown in Fig. 3D, pro–IL-1β mRNA expression levels in pulmonary dendritic cells isolated from HKx31-infected mice were similar between all age groups. Compared with young, expression of pro–IL-18 and pro–IL-33 in pulmonary dendritic cells during HKx31 infection was increased in middle-aged and elderly mice (Fig. 3E, 3F, respectively). Similarly, elderly pulmonary dendritic cells isolated from HKx31-infected mice had higher expression of IL-6 and TNF-α mRNA compared with that of young pulmonary dendritic cells (data not shown). Our results indicate that despite increased mRNA transcript levels, IL-1β and IL-18 production by elderly dendritic cells is decreased during influenza viral infection.

Next, we examined if activation of the NLRP3 inflammasome and subsequent activation of caspase-1 was altered in elderly dendritic cells during influenza infection. To this extent, young and elderly BMDCs were infected with HKx31, and gene expression of ASC, NLRP3, and caspase-1 was examined. As shown in Fig. 4A, compared with young BMDCs, elderly BMDCs have decreased synthesis of ASC (t test, \( p = 0.0025 \)), NLRP3 (t test, \( p = 0.0085 \)), and caspase-1 (t test, \( p = 0.0258 \)) mRNA during HKx31 infection. We next investigated if caspase-1 activity in response to HKx31 infection was altered in elderly dendritic cells. As shown in Fig. 4D, caspase-1 activity in elderly BMDCs during HKx31 infection was decreased compared with that in young HKx31-infected BMDCs (t test, \( p = 0.0015 \)). To expand upon these results, we examined ASC, NLRP3, and caspase-1 mRNA synthesis in young, middle-aged, and elderly pulmonary dendritic cells during HKx31 infection. Dendritic cells isolated from lungs of elderly HKx31-infected mice had decreased expression of ASC, NLRP3, and caspase-1 compared with that of dendritic cells isolated from lungs of young HKx31-infected mice (data not shown).
Next, we examined if adoptive transfer of young dendritic cells to elderly hosts could rescue impaired NLRP3 function in elderly mice during influenza infection. To test this, 1 d prior to influenza viral infection, young and elderly BALB/c mice were adoptively transferred with dendritic cells isolated from either young or elderly BALB/c mice. As shown in Fig. 5A, adoptive transfer of young dendritic cells to elderly mice attenuated the decreased weight loss compared with that of HKx31-infected elderly controls.

**FIGURE 3.** Expression of pro–IL-1β, pro–IL-18, and pro–IL-33 is preserved in elderly dendritic cells during HKx31 infection. Young and elderly bone marrow cells were cultured with GM-CSF (25 ng/ml) for 5 d in 37°C, 5% CO2. On day 5, cells were cultured in media alone or media containing HKx31 (MOI = 10) for 24 h. RNA was isolated, and (A) pro–IL-1β (t test, *p = 0.0053), (B) pro–IL-18, and (C) pro–IL-33 mRNA expression was assessed by real-time PCR. Pulmonary dendritic cells were isolated from lung tissue collected from control and HKx31-infected young and elderly BALB/c mice on day 5 of infection. RNA was isolated, and (D) pro–IL-1β, (E) pro–IL-18, and (F) pro–IL-33 (t test, *p = 0.0306) mRNA expression was assessed by real-time PCR. Similar results were obtained from three or more independent experiments with an n = 3 or greater per experiment and are expressed as the mean ± SEM.

**FIGURE 4.** NLRP3 inflammasome-mediated activation of IL-1β in elderly dendritic cells during influenza viral infection is decreased. Young and elderly bone marrow cells were cultured with GM-CSF (25 ng/ml) for 5 d in 37°C, 5% CO2. On day 5, cells were cultured in media alone or media containing HKx31 (MOI = 10) for 24 h. RNA was isolated, and (A) ASC (t test, **p = 0.0025), (B) NLRP3 (t test, **p = 0.0085), and (C) caspase-1 (t test, *p = 0.0258) mRNA expression was assessed by real-time PCR. (D) Caspase-1 activity was measured in young and elderly BMDCs culture with HKx31 for 4 h (37°C, 5% CO2) (t test, **p = 0.0015). Similar results were obtained from three or more independent experiments with an n = 3 or greater per experiment and are expressed as the mean ± SEM.
trols (two-way ANOVA, p < 0.0001). Further, elderly mice receiving an adoptive transfer of young dendritic cells prior to influenza viral infection had increased cellular infiltration, as illustrated by increased lung weight (Fig. 5B; two-way ANOVA, p < 0.0001) and total leukocyte numbers (Fig. 5C; two-way ANOVA, p = 0.0002), compared with that of elderly HKx31-infected controls. We next examined if adoptive transfer of young dendritic cells increased production of IL-1β in elderly hosts during HKx31 infection. As shown in Fig. 5D and 5E, elderly mice receiving an adoptive transfer of young dendritic cells had increased IL-1β release in serum (Fig. 5D; two-way ANOVA, p < 0.0001) and lung homogenates (Fig. 5E; two-way ANOVA, p < 0.0001) during influenza infection compared with that of elderly HKx31-infected controls.

Decreased NLRP3 inflammasome-mediated production of IL-1β by elderly dendritic cells is not unique to influenza viral infection

Previous work has illustrated that in the presence of pathogen-associated molecular patterns (PAMPs), alum crystals can activate the NLRP3 inflammasome (11, 33–37). Activation of the NLRP3 inflammasome by alum requires phagocytosis, which causes lysosomal swelling and damage and involves cathepsin B, a lysosomal cysteine protease (35–37). To this extent, we examined production of IL-1β by response to alum in LPS-stimulated young and elderly BMDCs. As shown in Supplemental Fig. 1A, despite similar lysosomal trafficking and cathepsin B mRNA expression (data not shown), treatment of LPS-stimulated elderly BMDCs with alum resulted in decreased production of IL-1β compared with that of similarly stimulated young BMDCs (t test, p = 0.0015). Further, compared with young BMDCs, NLRP3 mRNA expression (Supplemental Fig. 1B; t test, p = 0.0066) and caspase-1 activity (Supplemental Fig. 1C; t test, p = 0.0328) were also decreased in elderly BMDCs after alum stimulation.

An alteration in mitochondrial ROS does not enhance the production of IL-1β by elderly dendritic cells

Mitochondrial ROS are required for the formation of the NLRP3 inflammasome (20, 21). As aging is associated with increased levels of ROS, we next examined the impact of aging on mitochondrial integrity and production of ROS on NLRP3 activation (38). Young and elderly BMDCs cultured with LPS or HKx31 were treated with rotenone, a mitochondrial complex I inhibitor, followed by stimulation with ATP. As shown in Supplemental Fig.
2A, both young and elderly LPS-primed BMDCs that received rotenone treatment had increased release of IL-1β after ATP stimulation. Despite an overall increase in IL-1β production, compared with young LPS-primed BMDCs, elderly LPS-primed BMDCs had significantly decreased levels of IL-1β regardless of treatment with rotenone (Supplemental Fig. 2A; \( t \) test, \( p = 0.0030 \)), stimulation with ATP (Supplemental Fig. 2A; \( t \) test, \( p = 0.0095 \)), or treatment with both rotenone and ATP (Supplemental Fig. 2A; \( t \) test, \( p = 0.0058 \)). Whereas rotenone treatment during stimulation with LPS and ATP increased NLRP3 mRNA expression in both young and elderly BMDCs, NLRP3 expression in elderly BMDCs was still significantly decreased compared with that of young BMDCs after rotenone treatment (Supplemental Fig. 2B; two-way ANOVA, \( p = 0.0011 \)). To investigate whether alterations in mitochondrial integrity could enhance NLRP3 inflammasome activity in elderly BMDCs during influenza viral infection, we next examined IL-1β production after treatment with rotenone. As illustrated in Supplemental Fig. 2C, treatment with rotenone stimulated IL-1β production by elderly BMDCs. In the presence of ATP, IL-1β production by both young and elderly BMDCs during HKx31 infection was increased, and levels were similar between the groups (Supplemental Fig. 2C). Rotenone treatment during HKx31 infection increased NLRP3 mRNA expression similarly in both young and elderly BMDCs (Supplemental Fig. 2D). Notably, ATP stimulation of HKx31-infected elderly BMDCs resulted in a significant upregulation of NLRP3 mRNA compared with that of ATP-stimulated young HKx31-infected BMDCs (Supplemental Fig. 2D; \( t \) test, \( p = 0.0381 \)). Given these findings, we next examined NLRP3-mediated production of IL-1β in the absence of mitochondrial ROS. Thus, prior to LPS stimulation or HKx31 infection, we treated both young and elderly BMDCs with mitoTEMPO, a mitochondrial-targeted antioxidant. As shown in Supplemental Fig. 2E, treatment with mitoTEMPO decreased IL-1β production in both young and elderly BMDCs during both LPS stimulation (Supplemental Fig. 2E; \( t \) test, \( p = 0.0250 \)) and HKx31 infection (Supplemental Fig. 2F). Taken together, these results illustrate that IL-1β production by young and elderly dendritic cells is ROS dependent, and decreased mitochondrial-specific generation of ROS equally impaired IL-1β release by young and elderly BMDCs.

**Caspase-1 activity in elderly dendritic cells during influenza viral infection is recovered by depletion of intracellular potassium (\( K^+ \))**

Previous work has illustrated that stimulation of the P2X7 receptor can provide a signal that leads to the maturation and release of active IL-1β (21, 22, 39, 40). Upon activation, the P2X7 receptor induces a rapid potassium efflux from the cytosol (39, 40). Although potassium efflux alone is not sufficient for activation of the NLRP3 inflammasome, association with pannexin-1 and subsequent opening of a larger pore that mediates the delivery of PAMPs to the cytosol can lead to the activation of caspase-1 and release of IL-1β (40, 41). As stimulation of the P2X7 receptor can serve as a strong second signal for NLRP3 inflammasome activation, we examined if stimulation of the P2X7 receptor with ATP could augment caspase-1 activation and release of IL-1β by elderly dendritic cells during influenza infection. Young and elderly BMDCs were infected with HKx31 followed by stimulation with ATP, and NLRP3 mRNA expression was evaluated. Compared with young HKx31-infected BMDCs, ATP stimulation significantly increased NLRP3 mRNA expression in elderly HKx31-infected BMDCs (Fig. 6A; \( t \) test, \( *p = 0.0175 \) and \( **p = 0.0381 \)). We next examined if ATP stimulation could increase caspase-1 activity in elderly HKx31-infected BMDCs. As shown in Fig. 6, compared with HKx31 infection alone, stimulation of elderly HKx31-infected BMDCs with ATP significantly increased caspase-1 activity (Fig. 6B; \( t \) test, \( p = 0.0015 \)), IL-1β release (Fig. 6C; \( t \) test, \( p = 0.0278 \)), and IL-18 production (Fig. 6D, two-way ANOVA, \( p < 0.0001 \)). Notably, despite increased P2X7 receptor expression in elderly BMDCs (data not shown), compared with young HKx31-infected BMDCs, ATP stimulation of elderly HKx31-infected BMDCs resulted in similar caspase-1 activity, IL-1β release, and IL-18 production (Fig. 6B–D, respectively).

We next investigated if nigericin, a pore-forming toxin that has been shown to promote P2X7 receptor-independent potassium efflux, could increase NLRP3 activation in elderly BMDCs during influenza viral infection (21). Young and elderly HKx31-infected BMDCs were stimulated with nigericin, and caspase-1 activity was assessed. Treatment with nigericin resulted in a significant increase in caspase-1 activity in elderly HKx31-infected BMDCs (Fig. 6E; \( t \) test, \( p = 0.0117 \)) compared with that of untreated, elderly HKx31-infected BMDCs. Compared with young HKx31-infected BMDCs, nigericin stimulation of elderly HKx31-infected BMDCs resulted in significantly increased IL-1β release (Fig. 6F; two-way ANOVA, \( p < 0.0001 \)) and IL-18 production (Fig. 6G; two-way ANOVA, \( p < 0.0001 \)).

Based on these findings, we next investigated if treatment of elderly BMDCs with ATP or nigericin could rescue NLRP3, ASC, or caspase-1 protein expression during influenza infection. To this extent, young and elderly BMDCs were infected with HKx31 followed by stimulation with either ATP or nigericin, and protein expression of ASC, NLRP3, and caspase-1 was examined by Western blot. As shown in Fig. 6H, protein expression of NLRP3, ASC, and caspase-1 was increased in young BMDCs during HKx31 infection and in response to subsequent ATP or nigericin stimulation. In contrast, NLRP3, ASC, and caspase-1 levels are only detectable in elderly HKx31-infected BMDCs after ATP or nigericin stimulation (Fig. 6H).

As nigericin treatment can increase caspase-1 activity and IL-1β release during in vitro influenza viral infection, we examined if administration of nigericin during influenza viral infection would stimulate NLRP3 inflammasome-mediated production of IL-1β in elderly hosts. At 24 h after influenza administration, elderly mice were treated with PBS or nigericin (0.005 mg/kg). As shown in Fig. 7, elderly mice that received nigericin had decreased morbidity and mortality during influenza infection, as illustrated by decreased weight loss (data not shown), decreased clinical scores (Fig. 7A, two-way ANOVA, \( p < 0.0001 \)), and decreased lethality (Fig. 7B; Mantel–Cox test, \( p = 0.0004 \)) compared with that of untreated, HKx31-infected elderly controls. Further, compared with PBS-treated controls, elderly mice that received nigericin treatment had increased infiltration of leukocytes (Fig. 7C; \( t \) test, \( p = 0.009 \)) and increased IL-1β release (Fig. 7D; \( t \) test, \( p = 0.007 \)). Taken together, these results illustrate that nigericin treatment during early influenza infection augments IL-1β production and improves clinical outcomes in elderly mice.

**Discussion**

Although the increased burden of influenza infections in the elderly (>65 y) population is well known, the molecular mechanisms that influence this increased susceptibility have not been well elucidated. In the current study, we examined the impact of aging on inflammasome activation during influenza viral infection. We found that NLRP3 inflammasome-mediated activation of IL-1β by elderly dendritic cells is decreased during influenza infection (Fig. 2). This was not due to impaired NF-κB-mediated gene transcription, as expression of the proforms of IL-1β, IL-18, and IL-33 and of IL-6 and TNF-α mRNAs was elevated in both young and
During influenza infection, treatment with ATP or nigericin can enhance caspase-1 activity and expression of mature IL-1β and IL-18 (Fig. 6). After ligation, the channels formed by the P2X7 receptor rapidly transform to pores that allow passage of PAMPs and damage-associated molecular patterns into the cytosol. Therefore, treatment of elderly dendritic cells with ATP may also further amplify TLR stimulation and increase inflammasome activity. Of note, mRNA and protein expression of NLRP3, ASC, and caspase-1 in elderly dendritic cells was enhanced with ATP or nigericin stimulation, indicating that transcription and translation of these inflammasome components is not impaired in aging (Fig. 6). Further, activation of caspase-1 and expression of IL-1β and IL-18 was also enhanced by ATP or nigericin stimulation, illustrating that expression and activity of these components is also conserved in elderly dendritic cells (Fig. 6).

The NLRP3 inflammasome signaling pathway is a multifaceted signaling pathway that requires two signals to induce inflammasome complex formation and secretion of IL-1β and IL-18. Based on our current results, LPS priming and secondary stimulation with ATP does not fully rescue impaired inflammasome activation in elderly dendritic cells, and this may be due to a residual age-induced impairment in NLRP3 activation. ATP stimulation leads to changes in ionic concentrations within the cytoplasm, and
NLRP3 senses these disturbances, thus leading to inflammasome complex formation. We have examined NLRP3 mRNA expression in response to LPS priming and subsequent ATP stimulation and have found that NLRP3 expression in elderly dendritic cells does increase (~15-fold), but these levels are lower than those detected in similarly treated, young dendritic cells (Supplemental Fig. 2). Previous work has shown that inflammasome activation during influenza viral infection is mediated by the M2 ion channel, which via pH neutralization of the trans-Golgi network results in potassium efflux and increased ROS production (27). Hence, influenza can stimulate the NLRP3 inflammasome via TLR7 stimulation and M2 ion channel-mediated activation of NLRP3. Treatment of young and elderly dendritic cells with heat-inactivated HK×31 resulted in a significant decrease in IL-1β and IL-18 expression (Fig. 2) indicating that actively replicating HK×31 was necessary for IL-1β expression during influenza infection (Supplemental Fig. 2). Notably, enhancement of ROS by treatment of young and elderly dendritic cells with rotenone resulted in similar levels of IL-1β expression during influenza infection (Supplemental Fig. 2). Treatment with mitoTEMPO equally decreased IL-1β expression by young and elderly dendritic cells during influenza infection (Supplemental Fig. 2). Taken together, these results illustrate that although ROS synthesis is necessary for inflammasome formation and function, age-induced impairments in caspase-1 activity and IL-1β production during influenza infection were not solely due to an increased accumulation of ROS within elderly dendritic cells.

Pneumonia has become an increasingly significant cause of morbidity and mortality in the aging population (44–46). Although primary influenza infection alone can lead to adverse outcomes, secondary bacterial infections during and shortly after recovery from influenza infections are more common reasons for influenza-associated illness (47). Previous work has illustrated multiple bacterial pathogens can activate the NLRP3 inflammasome. Secretion of pneumolysin by Streptococcus pneumoniae can induce NLRP3 inflammasome activation through potassium efflux and lysosomal leakage induction, and absence of NLRP3 expression stimulation, although it does increase NLRP3 activation and IL-1β production, does not provide a robust enough signal that fully restores this aged-induced impairment.

Aging is associated with an accumulation of ROS within cells, which augments oxidative stress and subsequently impairs cellular functions (43). Similar to previously published studies, our data demonstrate that mitochondrial generation of ROS is important for NLRP3 inflammasome-mediated activation of IL-1β (20). Notably, enhancement of ROS by treatment of young and elderly dendritic cells with rotenone resulted in similar levels of IL-1β expression during influenza infection (Supplemental Fig. 2). Treatment with mitoTEMPO equally decreased IL-1β expression by young and elderly dendritic cells during influenza infection (Supplemental Fig. 2). Taken together, these results illustrate that although ROS synthesis is necessary for inflammasome formation and function, age-induced impairments in caspase-1 activity and IL-1β production during influenza infection were not solely due to an increased accumulation of ROS within elderly dendritic cells.

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resulted in impaired bacterial clearance (48). As NLRP3 inflammasome activity during influenza infection is impaired, it will be important to examine whether elderly hosts display altered responses to secondary bacterial infections after primary influenza infection.

In summary, our findings show that NLRP3 inflammasome activation during influenza viral infection is impaired in elderly dendritic cells. An increase in potassium efflux by ATP or nigericin stimulation rescued this defect. The data presented in this study provide new evidence as to why older persons are more susceptible to influenza viral infection and provide a possible mechanism to enhance these responses, thereby decreasing morbidity and mortality in this population.

Acknowledgments
We thank Ralph Tripp for the HKx31 virus, Jennifer Tipper for propagation of the HKx31 virus, Steven Belinsky, Yohannes Tesfaegi, Julie Wilder, and Mohan Sopori for valuable discussions, and Zennmie Pollock, Dana Mittel, and Jessie vanWestrienen for assistance with experimental techniques.

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Disclosures
The authors have no financial conflicts of interest.
Supplemental Figure 1: *Decreased NLRP3 inflammasome mediated production of IL-1 by elderly dendritic cells is not unique to influenza viral infection.*

Young and elderly BMDCs were stimulated with LPS (100ng/mL) for 4 hours prior to treatment with alum (250μg/ml for 24 hours). (A) Cell culture supernatants were collected and the production of IL-1β was examined by ELISA (t-test, p=0.0015). (B) RNA was isolated from cultured cells and NLRP3 mRNA expression was assessed by real time PCR (t-test, p=0.0066). (C) Cells were collected and caspase-1 activity was assessed (t-test, p=0.0328). Similar results were obtained from two or more independent experiments with an N=3 or greater and are expressed as the mean ± SEM.

Supplemental Figure 2: *Alterations in mitochondrial integrity do not enhance IL-1β expression by elderly dendritic cells during HKx31 infection.*

Young and elderly bone marrow cells were cultured with GM-CSF (25ng/mL) for 5 days in 37°C, 5% CO2. (A-C) On day 5, cells were treated with LPS (100ng/mL) (A) or HKx31 (MOI=10) (B-C) for 4 hours. Cells were subsequently treated with rotenone (5μM) for 1 hour prior to ATP (5mM) stimulation (30 minutes). Cell culture supernatants were collected and IL-1β expression was assessed by ELISA. (C) Cultured cells were collected and NLRP3 mRNA expression was assessed by real time PCR. (D-E) On day 5, cells were treated with mitoTEMPO (100μM) for 1 hour prior to culture with LPS (100ng/mL) (D) or HKx31 (MOI=10) (E) for 4 hours. Cells were then stimulated with ATP (5mM) for 30 minutes. Cell culture supernatants were collected and IL-1β expression was assessed by
ELISA. Similar results were obtained from two or more independent experiments with an N=3 or greater and are expressed as the mean ± SEM.
Decreased NLRP3 inflammasome mediated production of IL-1β by elderly dendritic cells is not unique to influenza viral infection.
An alteration in mitochondrial ROS does not enhance the production of IL-1β by elderly dendritic cells

Supplemental Figure 2