Cytokine 120 (2019) 115–124

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Hyperactivation of the NLRP3 inflammasome protects mice against influenza A virus infection via IL-1β mediated neutrophil recruitment

Junling Niu[a,b,c,1], Shuxian Wu[b,c,1], Mingkuan Chen[b,c], Ke Xu[b,c], Qiuhong Guo[b,c], Ailing Lu[b,c], Liping Zhao[a], Bing Sun[b,c], Guangxun Meng[b,c,*]

[a] State Key Laboratory of Microbial Metabolism, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China
[b] CAS Key Laboratory of Molecular Virology & Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai 200031, China
[c] University of Chinese Academy of Sciences, Beijing 100039, China

ARTICLE INFO

Keywords:
NLRP3 inflammasome
Influenza
Neutrophil

ABSTRACT

Host innate immune system is critical for combating invading microbes including Influenza A virus (IAV). As an important arm of the innate immunity, the NLRP3 inflammasome has been found essential for protecting host against IAV challenge, while the mechanism remained elusive. Here we found that mice carrying a gain-of-function mutation in the *Nlrp3* gene (*Nlrp3*^{R258W}) are strongly resistant to IAV infection. Upon H1N1 IAV infection, the *Nlrp3*^{R258W} mice exhibited decreased weight loss, increased survival rate and attenuated lung damage compared with WT littermate controls. Mechanistically, the resistance of *Nlrp3*^{R258W} mice to IAV infection was dependent on IL-1β-mediated neutrophil recruitment. Upon IAV infection, mice carrying the *Nlrp3*^{R258W} mutation produced more IL-1β than WT mice in the lung, which enhanced neutrophil recruitment locally. The recruited neutrophils facilitated IAV clearance, so that the viral load in *Nlrp3*^{R258W} mice was lower than that in control mice. Conversely, neutrophil depletion in *Nlrp3*^{R258W} mice compromised IAV clearance. Taken together, our results demonstrate a previously undescribed mechanism by which hyperactivation of the NLRP3 Inflammasome protects mice from IAV infection through IL-1β mediated neutrophil recruitment, thus suggest that positively fine tuning the physiological function of NLRP3 inflammasome can be beneficial for a mammalian host against IAV challenge.

1. Introduction

A vertebrate host usually initiates immune responses through recognition of microbes via pattern recognition receptors (PRRs) expressed on innate immune cells [1]. These PRRs recognize microbial associated molecular patterns (MAMPs) from invading or resident microorganisms, as well as danger associated molecular patterns (DAMPs) from damaged host cells [1,2]. Two categories of PRRs have been identified, e.g. the membrane bound ones including TLRs (Toll-like receptors) and CLRs (C-type lectin like receptors), and the intracellular PRRs such as RLRs (RIG-I like receptors), NLRs (Nod-like receptors) and ALRs (AIM2 like receptors) [1,3]. A subgroup of NLRs and ALRs can assemble with adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1 to form a multiprotein complex named inflammasome, which leads to caspase-1 activation and subsequent cleavage of pro-IL-1β and/or pro-IL-18 for their maturation and secretion [3–5]. Each identified inflammasome usually recognizes certain ligands. For instance, the NLRP1 inflammasome recognizes anthrax lethal toxin, the Naip-NLRC4 inflammasome recognizes bacterial flagellin/needle proteins, and the AIM2 inflammasome recognizes DNA [6]. In contrast, the NLRP3 inflammasome can be activated by a broad spectrum of stimuli including but not limited to ATP, MSU, bacterial toxin and certain viral proteins [7,8]. Although the exact mechanism for the activation of NLRP3 inflammasome remains elusive, NLRP3 has been associated with many diseases and thus heavily investigated [9]. In addition, other diseases such as type II diabetes, Parkinson's disease, Alzheimer disease, inflammatory bowel disease, asthma, cancer, as well as bacterial, viral, parasitical and fungal infections all involve the activation of NLRP3 inflammasome [9,10]. Of note, gain-of-function mutations in the coding region of human *NLRP3* gene result in a group of diseases named cryopyrin-associated periodic syndromes (CAPS) characterized with auto-inflammation in skin, joints and eyes [11–14]. Notably, genetically modified mice carrying the *Nlrp3*^{R258W} mutation homologous to the human *NLRP3*^{R260W} site also

* Corresponding author at: Institut Pasteur of Shanghai, Chinese Academy of Sciences, 320 Yueyang Road, Life Science Research Building, Shanghai 200031, China.
E-mail address: gxmeng@ips.ac.cn (G. Meng).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.cyto.2019.04.019
Received 29 January 2019; Received in revised form 18 March 2019; Accepted 27 April 2019
Available online 02 May 2019

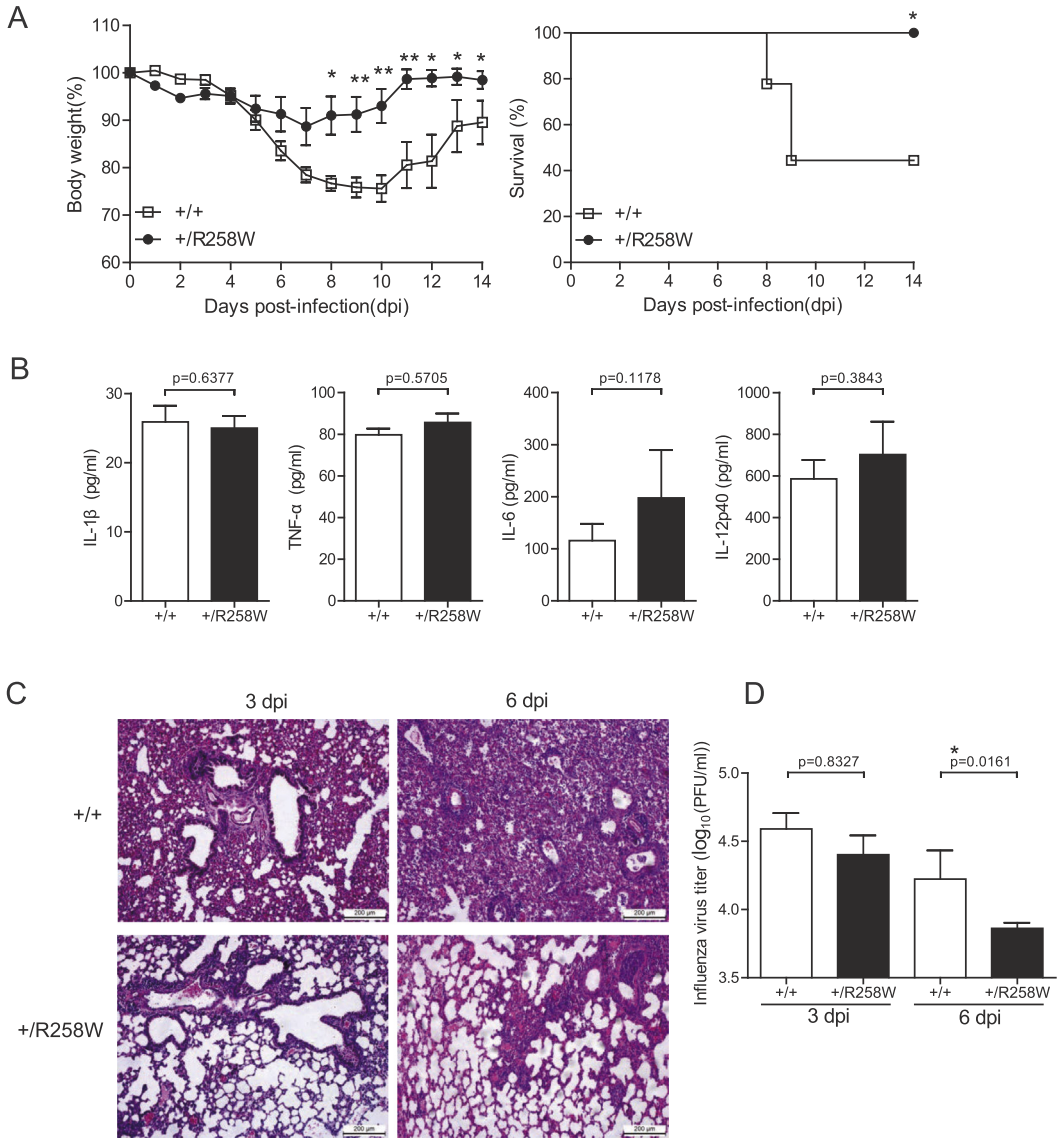

Fig. 1. *Nlrp3*^{R258W} mutation protects mice against influenza A virus infection. (A), WT (n = 9) and *Nlrp3*^{R258W} (n = 9) mice were intranasally infected with 50 PFU of IAV PR8, weight loss and survival were monitored daily for 14 days. (B), Cytokines in sera from infected WT (n = 4) and *Nlrp3*^{R258W} (n = 4) mice were analyzed via ELISA on day 6 post-infection. (C), The lungs from infected WT (n = 5 for 3 dpi, n = 5 for 6 dpi) and *Nlrp3*^{R258W} (n = 5 for 3 dpi, n = 5 for 6 dpi) mice were harvested on day 3 or 6 post-infection, sectioned, and then stained with hematoxylin and eosin. Representative images from each group are shown, scale bar = 200 μm. (D), The PR8 viral load in the lung homogenates from infected WT (n = 3 for 3 dpi, n = 4 for 6 dpi) and *Nlrp3*^{R258W} (n = 4 for 3 dpi, n = 5 for 6 dpi) was determined with the standard plaque assay on day 3 and 6 post-infection. Data shown are representative from three independent infection experiments. Values in (A) (left), (B) and (D) are the means ± standard error of the mean (SEM), two-tailed Student's *t* test. Survival rate shown in (A) (right) is analyzed with Log-rank (Mantel-Cox) test. Significant values are defined by $^{*}P < 0.05$ and $^{**}P < 0.01$.

develop auto-inflammation in skin [15]. NLRP3 can interact with the ubiquitin ligase–associated protein SGT1 and heat-shock protein 90 (HSP90) before it is activated to form NLRP3 inflammasome [16]. *Nlrp3*^{R258W} mutation may change the configuration or conformation of the protein. As a result, the interaction of NLRP3^{R258W} with SGT1 and HSP90 becomes weak or disappeared, which further leads to easier activation of the inflammasome when encountering activation signals.

Epidemics and pandemics caused by influenza virus infection are major global health challenges, and approximately 300,000 deaths and more than 4 million hospitalizations are directly related to influenza virus infection worldwide every year [17]. Both innate and adaptive immune responses are necessary for humans to combat influenza virus. Upon influenza viral infection, multiple PRRs are engaged to initiate innate immune responses for protection [18]. The endosomal TLR3 or TLR7 that senses double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA) respectively, the cytosolic RIG-I that senses 5′-triphosphate RNA, as well as the cytosolic NLRP3 that senses multiple stimuli, are all involved in this process [18–21].

Thomas PG et al. showed that NLRP3 protects against influenza A virus-induced lung damage through regulating neutrophil and monocyte recruitment [22]. Allen IC et al. indicated that NLRP3 recognizes influenza A viral RNA and mediates *in vivo* immune protection [23]. In addition, Stout-Delgado HW et al. found that the function of NLRP3 inflammasome is compromised in aged mice, and nigericin, a NLRP3 agonist, rescues the capability of aged mice against influenza challenge [24]. The results shown in these works indicated that the activation of NLRP3 inflammasome is essential for the host to defeat IAV infection. However, the mechanism remained largely elusive.

In the current work, we employed the *Nlrp3*^{R258W} mice, in which the NLRP3 inflammasome is hyperactive, to investigate the mechanism by which the inflammasome protects against influenza A virus infection. We found that the NLRP3 inflammasome-IL-1β signaling cascade

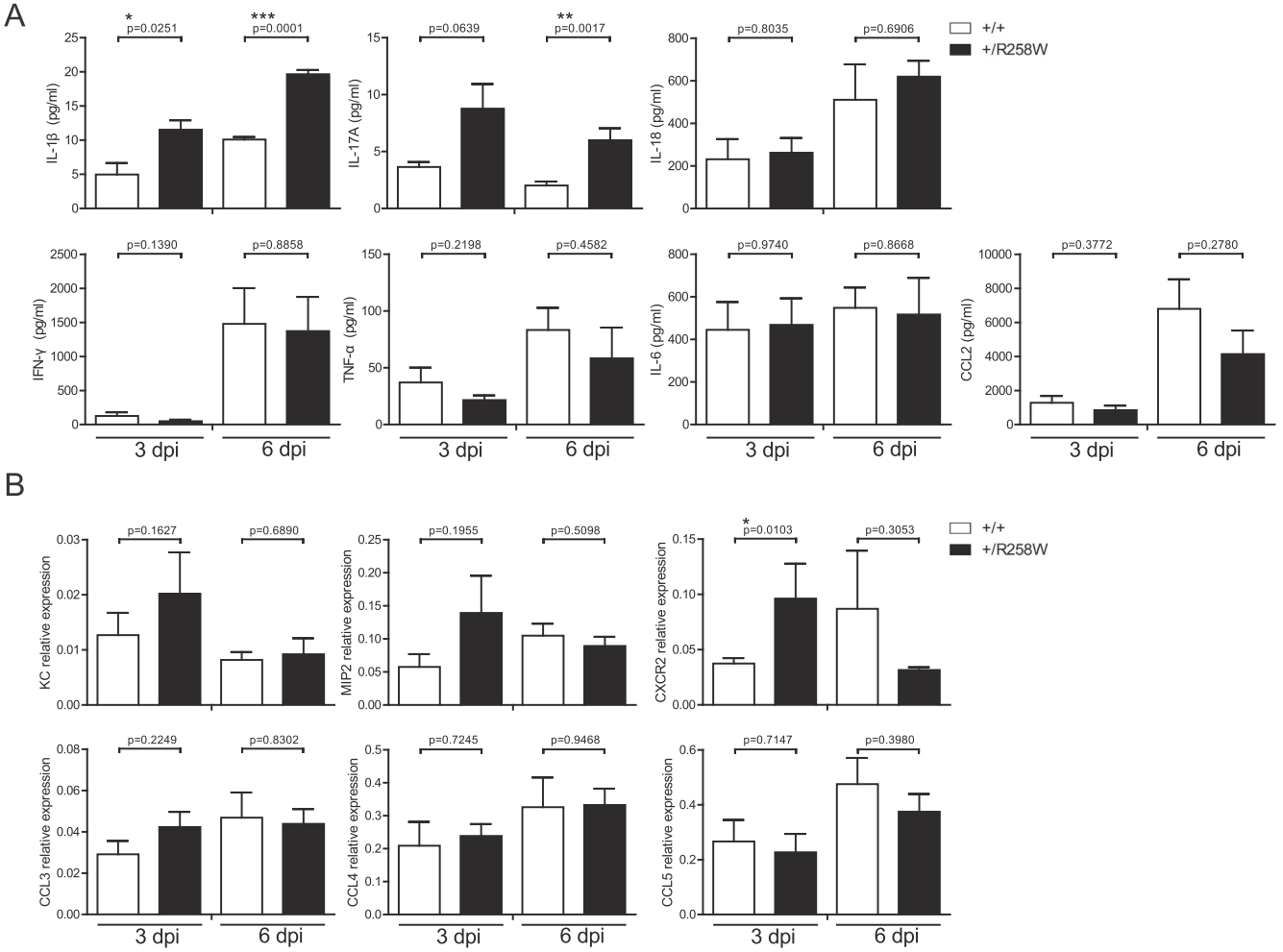

Fig. 2. *Nlrp3*^{R258W} mice exhibit elevated pro-inflammatory responses upon influenza virus infection. (A), WT (n = 5 for 3 dpi, n = 6 for 6 dpi) and *Nlrp3*^{R258W} (n = 6 for 3 dpi, n = 7 for 6 dpi) mice were intranasally infected with 50 PFU of IAV PR8, and pro-inflammatory cytokines and chemokines in BALF from infected mice were analyzed by ELISA on day 3 and 6 post-infection. (B), The expression of chemokines and chemokine receptors in the lung homogenates from PR8 infected WT (n = 5 for 3 dpi, n = 6 for 6 dpi) and *Nlrp3*^{R258W} (n = 5 for 3 dpi, n = 6 for 6 dpi) mice on day 3 and 6 post-infection. Data shown are representative from three independent infection experiments. Values in (A) and (B) are the means ± SEM. Significant values are defined by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (two-tailed Student's *t* test).

mediated neutrophil recruitment is vital for virus clearance and host survival following influenza A virus infection.

2. Materials and methods

2.1. Virus

A/Puerto Rico/8/34(H1N1) virus was cultured and titrated in Madin-Darby Canine Kidney (MDCK) cells as described before [25]. All viral infection experiments were performed in the biosafety level 2 laboratory at Institut Pasteur of Shanghai following the standard operating protocols approved by the institutional biosafety committee.

2.2. Mice

Nlrp3^{R258W} mice have been described before [15]. *Nlrp3*^{R258W} mice were maintained as heterozygotes by crossing with WT C57BL/6J mice from the Jackson Laboratory (Bar Harbor, ME, USA). *Nlrp3*^{R258W} mice (+/R258W) and WT (+/+) mice in this study are littermate controls. *Il1r1*$^{-/-}$ mice were from the Jackson Laboratory. All animals were maintained in the specific pathogen-free (SPF) facility at Institut Pasteur of Shanghai. Animal care, use and experimental procedures complied with national guidelines and were approved by the Animal Care and Use Committee of Institut Pasteur of Shanghai, Chinese Academy of Sciences.

2.3. Viral infection of mice and sampling

Mice were anesthetized with Avertin (2,2,2-tribromoethanol, Sigma-Aldrich) and infected intranasally with 50pfu of PR8 virus in 25ul of PBS (1LD50 for WT mice). Mice were either weighed and monitored daily and euthanized on day 14, or sacrificed at various intervals for sampling. The infected mice of more than 25% weight loss were sacrificed and treated as death. Weight loss and survival data were analyzed using the GraphPad-Prism 5.0 software unless stated otherwise.

2.4. BALF related experiments

The bonchoalveolar lavage fluid (BALF) was harvested in 3 × 1 ml washes on either day 3 or day 6 post infection and centrifuged at 400g for 5 min at 4 °C. The supernatants were collected and frozen at −80 °C for enzyme-linked immunosorbent assay (ELISA). The red blood cells were lysed, and remaining cells were counted with a hemocytometer and stained with fluorochrome-conjugated antibodies. Data were acquired using a BD Fortessa flow cytometer and then analyzed with

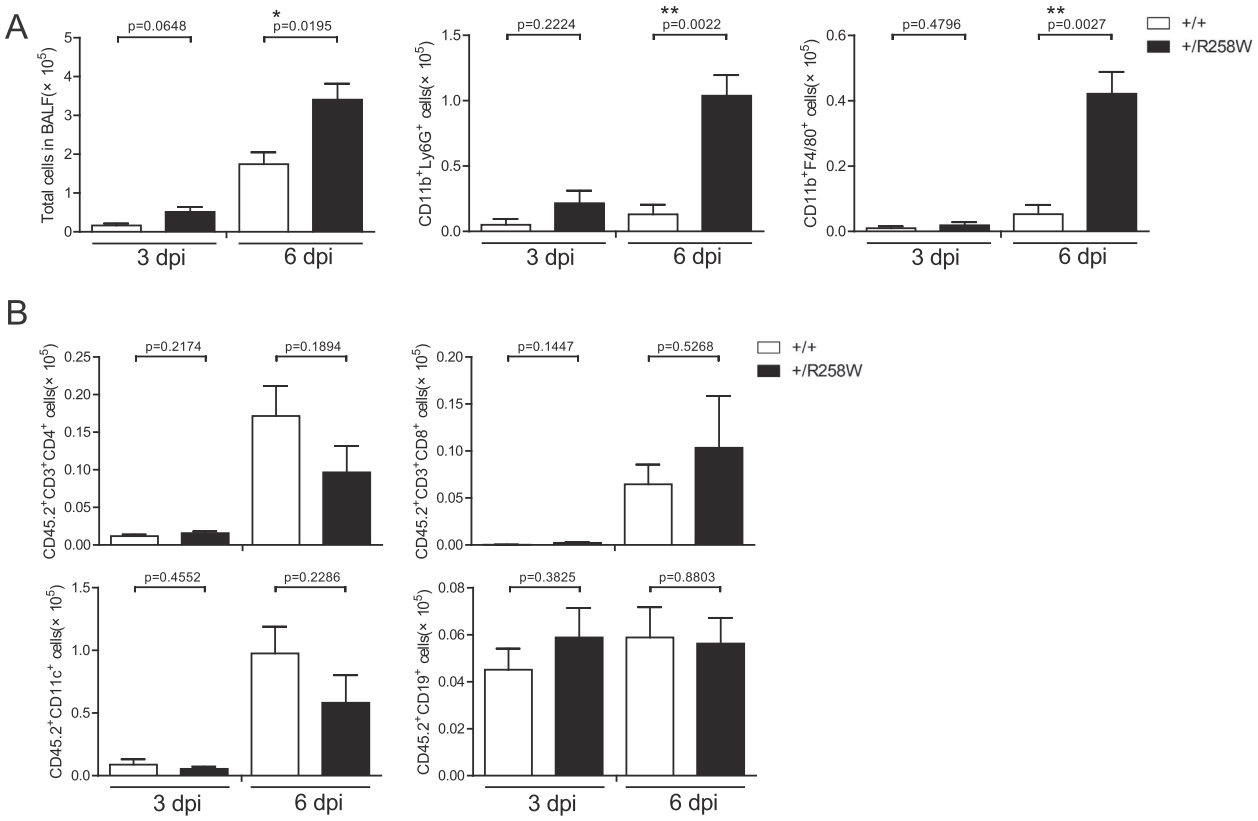

Fig. 3. Analysis for the immune cell infiltration to the lungs of *Nlrp3*^{R258W} and WT mice upon IAV infection. (A and B), The total cells, CD11b$^+$Ly6G$^+$ neutrophils, CD11b$^+$F4/80$^+$ macrophages, CD4$^+$ T cells, CD8$^+$ T cells, CD11c$^+$ DCs and CD19$^+$ B cells in BALF from PR8 infected WT (n = 4 for 3 dpi, n = 5 for 6 dpi) and *Nlrp3*^{R258W} (n = 5 for 3 dpi, n = 6 for 6 dpi) mice were analyzed via FACS on day 3 and 6 post-infection. Data shown are representative from three independent infection experiments. Values in (A) and (B) are the means ± SEM. Significant values are defined by $^*P < 0.05$ and $^{**}P < 0.01$ (two-tailed Student's *t* test).

FlowJo_V10 software.

2.5. Antibodies for flow cytometric analysis

Fluorochrome-conjugated anti-mouse antibodies against F4/80 (PE), Ly6G (APC), and CD11c (PE) were purchased form eBiosciences, San Diego, CA, USA. Fluorochrome-conjugated anti-mouse antibodies against CD11b (FITC), CD45.2 (BV605), CD3e (BV421), CD4 (V500), and CD8a (APC) were obtained from BD Biosciences, San Jose, CA, USA, and CD19 (PerCP Cy5.5) was purchased from Biolegend, San Diego, CA.

2.6. Quantitative real-time PCR

Total RNA was extracted from homogenized lung with TRIzol reagent (Sigam-Aldrich) according to the manufacturer's instructions. Synthesis of cDNA was performed with a GoSript™ Reverse Transcription kit (Promega). Real time quantitative PCR was performed with the SYBR Green qPCR Master Mix (TOYOBO) on an ABI 7900 HT Fast Real-Time cycler (Applied Biosystems). The expression of target genes was normalized to expression of housekeeping gene *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase). The primers used in this study were: MIP2, 5′- CCAACCACCAGGCTACAGG-(forward) 3′, 5′-GCGTCACACTCAAGCTCTG-(reverse) 3′; KC, 5′-CTGGGATTCACCTCAAGAACATC-(forward) 3′, 5′-CAGGGTCAAGGCAAGCCTC-(reverse) 3′;Cxcr2, 5′- ATGCCCTCTATTCTGCCAGAT-(forward) 3′, 5′- GTGCTCCGGTTGTATAAGATGAC-(reverse) 3′; Ccl2, 5′- TTAAAAACCTGGATCGGAACCAA-(forward) 3′, 5′- GCATTAGCTTCAGATTTACGGGT-(reverse) 3′; Ccl3, 5′- TGTACCATGACACTCTGCAAC-(forward) 3′, 5′- CAACGATGAATTGGCGTGGAA-(reverse) 3′; Ccl4, 5′- TTCCTGCTGTTTCTCTTACACCT-(forward) 3′, 5′- CTGTCTGCCTCTTTTGGTCAG-(reverse) 3′; Ccl5, 5′- GCTGCTTTGCCTACCTCTCC-(forward) 3′, 5′- TCGAGTGACAAACACGACTGC-(reverse) 3′; Ccl20, 5′- ACTGTTGCCTCTCGTACATACA-(forward) 3′, 5′- GAGGAGGTTCACAGCCCTTTT-(reverse) 3′; Ccl28, 5′-AGAGTGAGTTCATGCAGCATCC -(forward) 3′, 5′- CTGCTTCAAAGTACGATTGTGCG-(reverse) 3′; Sele, 5′- CCAATCTGAAACATTCACCGAGT -(forward) 3′, 5′- GAGTCTTTGGTTCGTTGGATGTA-(reverse) 3′; Selp, 5′- CATCTGGTTCAGTGCTTTGATCT -(forward) 3′, 5′- ACCCGTGAGTTATTCCATGAGT-(reverse) 3′; Icam1, 5′- TGCCTCTGAAGCTCGGATATAC -(forward) 3′, 5′- TCTGTCGAACTCCTCAGTCAC-(reverse) 3′; Gapdh, 5′-AGGTCGGTGTGAACGGATTTG-(forward) 3′, 5′-TGTAGACCATGTAGTTGAGGTCA-(reverse) 3′.

2.7. ELISA

Sera and BALF supernatants were measured with ELISA kits (eBioscience) according to the manufacturer's instructions.

2.8. Pulmonary histopathology

For histopathologic examination, lungs were collected from mice at the indicated days, fixed through immersion in 10% buffered formalin for at least one week at 4 °C, processed, embedded in paraffin, sectioned, and then stained with hematoxylin and eosin(H&E).

2.9. Plaque assay

Lungs were homogenized in 1 ml of PBS and the homogenates were centrifuged at 400*g* for 5 min at 4 °C. The supernatants containing influenza virus were diluted serially with 0.2% BSA (bovine serum albumin) in PBS. MDCK cells were grown in 48-well cell culture plates to produce a confluent monolayer, and then washed with PBS, infected

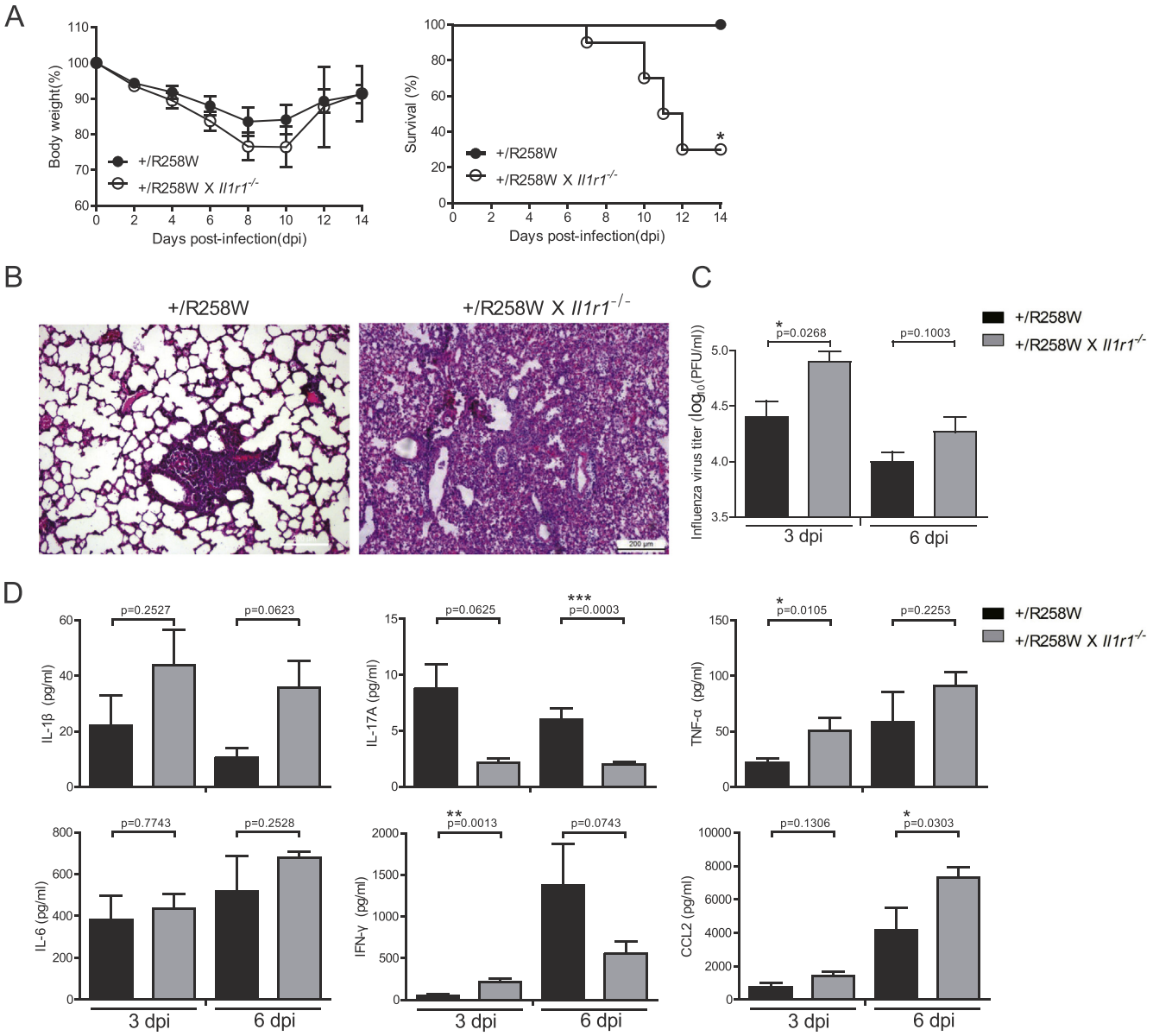

Fig. 4. IL-1β signaling is required for the *Nlrp3*^{R258W} mice against IAV infection. (A), *Nlrp3*^{R258W} (n = 11) and *Nlrp3*^{R258W} X *Il1r1*$^{-/-}$ (n = 10) mice were intranasally infected with 50 PFU of IAV PR8, weight loss and survival were monitored for 14 days. (B), The lungs harvested from *Nlrp3*^{R258W} (n = 5) and *Nlrp3*^{R258W} X *Il1r1*$^{-/-}$ (n = 5) mice on day 6 post-infection were sectioned, and then stained with hematoxylin and eosin, scale bar = 200um. (C), The PR8 viral load in the lung homogenates from infected *Nlrp3*^{R258W} (n = 4 for 3 dpi, n = 5 for 6 dpi) and *Nlrp3*^{R258W} X *Il1r1*$^{-/-}$ (n = 4 for 3 dpi, n = 5 for 6 dpi) was determined with the standard plaque assay on day 3 and 6 post-infection. (D), The cytokines and chemokines in BALF from infected *Nlrp3*^{R258W} (n = 6 for 3 dpi, n = 8 for 6 dpi) and *Nlrp3*^{R258W} X *Il1r1*$^{-/-}$ (n = 5 for 3 dpi, n = 9 for 6 dpi) mice were analyzed through ELISA on day 3 and 6 post-infection. Data shown are representative from three independent infection experiments. Values in (A) (left), (C) and (D) are the means ± SEM, two-tailed Student's *t* test. Survival rate is shown in (A) (right), Log-rank (Mantel-Cox) test. Significant values are defined by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

with serially diluted supernatants, and incubated at 37 °C with 5% CO_2 for 1 h for viral absorption. Unabsorbed virus particles were washed away with PBS, and then 0.5 ml of 50% 2 × DMEM media, supplemented with 0.4% BSA, 2% Penicillin & Streptomycin, 50% Avecil (2.4%) and 2μg/ml TPCK-treated trypsin, was added to each well. After incubation for 72 h at 37 °C in 5% CO_2 incubator, the MDCK cells were fixed with 4% paraformaldehyde, and then incubated with rabbit anti-NP (H1N1) antibody followed by goat-anti-rabbit HRP. Color development was performed with True Blue™ Peroxidase Substrate (KPL), plaques were counted, and the virus titers were determined.

2.10. Depletion of neutrophils in vivo

Mice were intravenously injected with isotype control rat IgG_{2a} (BioXcell) or rat anti-mouse Ly-6G monoclonal antibody (clone 1A8, BioXcell) one day prior to infection, and on day 1 and 3 post infection (2 mg/kg).

2.11. Statistical analysis

GraphPad Prism 5.0 software (La Jolla, CA, USA) was used for data analysis. Statistically significant difference was determined by two-tailed Student's *t* test for two groups or one-way ANOVA for three or more groups. Survival curves were compared with the Log-rank (Mantel-Cox) test. $P < 0.05$ was considered a statistically significant.

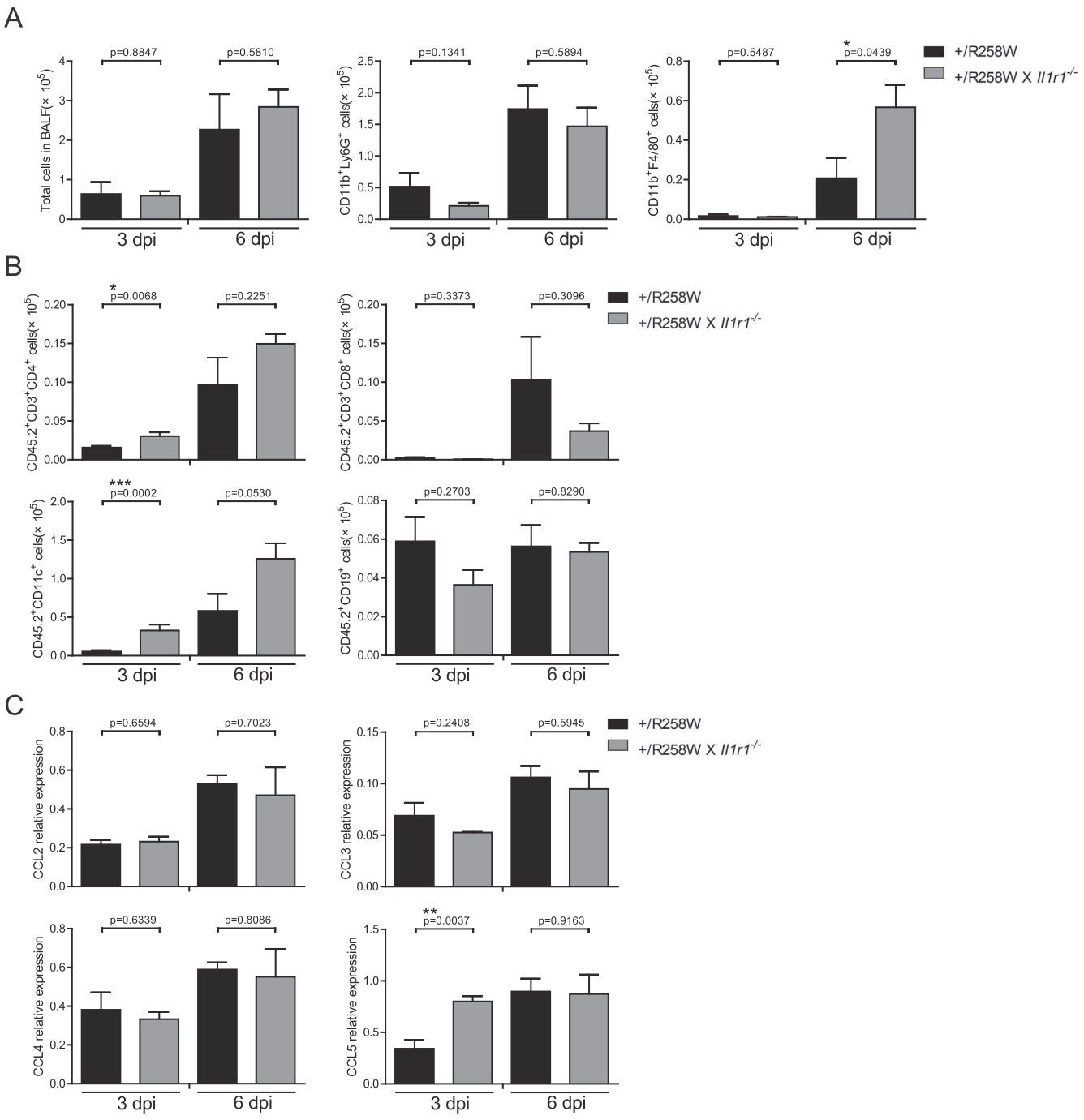

Fig. 5. The augmented neutrophil recruitment in the lung of *Nlrp3*^{R258W} mice requires IL-1β signaling. (A and B), The total cells, CD11b$^+$Ly6G$^+$ neutrophils, CD11b$^+$F4/80$^+$ macrophages, CD4$^+$ T cells, CD8$^+$ T cells, CD11c$^+$ DCs and CD19$^+$ B cells in BALF from PR8 infected *Nlrp3*^{R258W} (n = 5 for 3 dpi, n = 5 for 6 dpi) and *Nlrp3*^{R258W} X *Il1r1*$^{-/-}$ (n = 5 for 3 dpi, n = 5 for 6 dpi) mice were analyzed via FACS on day 3 and 6 post-infection. (C), Real time quantitative PCR analysis for the expression of pro-inflammatory chemokines in the lung homogenates from PR8 infected *Nlrp3*^{R258W} (n = 4 for 3 dpi, n = 5 for 6 dpi) and *Nlrp3*^{R258W} X *Il1r1*$^{-/-}$ (n = 4 for 3 dpi, n = 5 for 6 dpi) mice on day 3 and 6 post-infection. Data are representative from three independent infection experiments. Values in (A), (B) and (C) are the means ± SEM. Significant values are defined by $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (two-tailed Student's *t* test).

3. Results

3.1. *Nlrp3*^{R258W} protects mice against IAV-induced morbidity and mortality

An upregulated inflammatory response can be either detrimental or beneficial to the mammalian host during IAV infection [21,26]. To determine whether the enhanced inflammasome activity in *Nlrp3*^{R258W} mice has any physiological function during influenza A viral challenge, we infected WT and *Nlrp3*^{R258W} mice with the H1N1 IAV strain PR8. Of note, *Nlrp3*^{R258W} animals showed significantly decreased body weight loss and better survival compared with WT mice (Fig. 1A). Of note, the level of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and IL-12p40 on day 6 post infection (6 dpi) from the sera of *Nlrp3*^{R258W} mice did not show a clear difference compared with that from WT control animals (Fig. 1B), indicating that the effect of R258W mutation on NLRP3 was likely exerting the anti-IAV function in the lung locally instead of systemically.

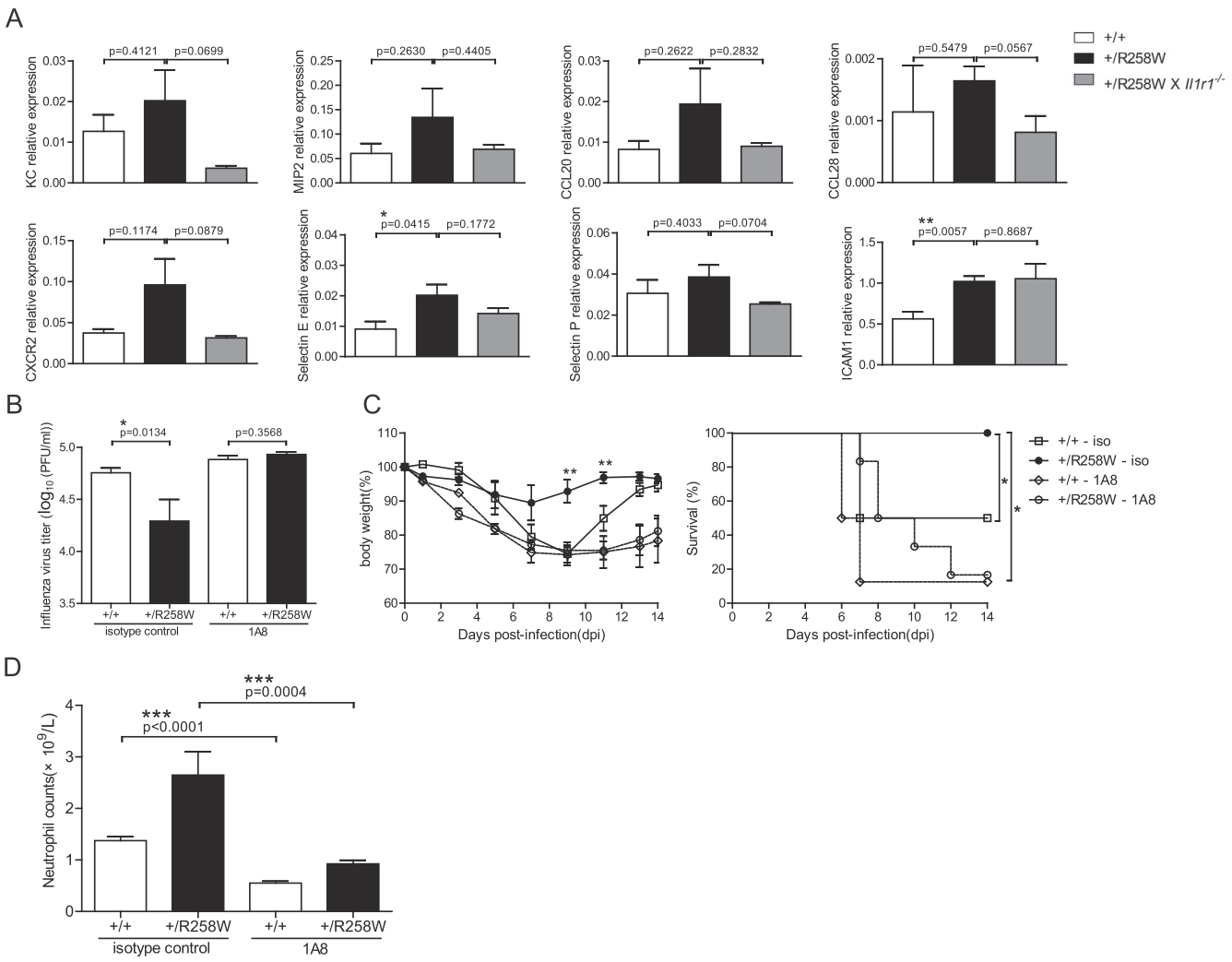

Fig. 6. IL-1β signaling-dependent neutrophil recruitment is critical for *Nlrp3*^{R258W} mice against influenza virus challenge. (A), The expression of chemokines, chemokines receptors, selectins and integrin ligand ICAM-1 in lung homogenates from PR8 infected WT (n = 4), *Nlrp3*^{R258W} (n = 4) and *Nlrp3*^{R258W} X *Il1r1*$^{-/-}$ (n = 4) mice on day 3 post-infection. (B), The viral titers in lung homogenates from infected WT (n = 7 for isotype control, n = 8 for 1A8) and *Nlrp3*^{R258W} (n = 4 for isotype control, n = 7 for 1A8) mice on day 5 post-infection, which had been treated with isotype control IgG_{2a} or neutrophil specific anti-Ly6G antibody (clone 1A8) prior to and during the infection. (C), The weight loss and survival curves of infected WT (n = 4 for isotype control, n = 8 for 1A8) and *Nlrp3*^{R258W} (n = 4 for isotype control, n = 6 for 1A8) mice injected with indicated antibodies prior to and during the infection. (D), Neutrophil depletion was confirmed by counting neutrophils in blood from WT (n = 7 for isotype control, n = 8 for 1A8) and *Nlrp3*^{R258W} (n = 4 for isotype control, n = 7 for 1A8) mice injected with indicated antibodies the same as in (B). Data are representative from two independent infection experiments. Values in (A), (B), (C) (left) and (D) are the means ± SEM. Significant values are defined by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (one-way ANOVA for (A), two-tailed Student's *t* test for (B), (C) (left) and (D)). Survival rate shown in (C) (right) is analyzed with Log-rank (Mantel-Cox) test.

3.2. Nlrp3^{R258W} attenuates lung damage and enhances viral clearance during IAV infection

When assessed for IAV-induced lung damage, WT mice exhibited obviously thickened and ruptured alveolar septa on 3 and 6 dpi, and necrotic cells and inflammatory exudate in brochioli on 6 dpi, while *Nlrp3*^{R258W} mice attenuated the aforementioned tissue damage through a distinct cell infiltration into bronchiole on 6 dpi (Fig. 1C). A previous report indicated that the NLRP3 inflammasome is involved in IAV clearance [23]. To determine whether the protection of *Nlrp3*^{R258W} against IAV was due to enhanced viral clearance, virus replication was monitored in our experiments. Notably, it was found that the viral load in the lung of *Nlrp3*^{R258W} mice was clearly decreased compared with that from WT controls, especially on 6 dpi (Fig. 1D).

3.3. Nlrp3^{R258W} mice exhibit elevated pro-inflammatory responses in the lung upon IAV infection

When infected with IAV, the host recruits inflammatory cells to the lung for safeguarding, which is largely mediated by inflammatory chemokines and cytokines [27]. We determined the cytokine levels in the bronchoalveolar lavage fluid (BALF) of our experimental animals, and found that the *Nlrp3*^{R258W} mice produced significantly higher amounts of IL-1β and IL-17A than WT mice on 3 and 6 dpi (Fig. 2A). However, the production of IL-18, a cytokine also dependent on inflammasome for maturation in certain cells, was comparable between WT and *Nlrp3*^{R258W} mice (Fig. 2A). Other inflammasome-independent cytokines such as IFN-γ, TNF-α and IL-6 showed similar secretion between WT and *Nlrp3*^{R258W} mice on 3 and 6 dpi (Fig. 2A). Similarly, the production of the chemokine CCL2 did not show any difference between WT and *Nlrp3*^{R258W} mice on 3 or 6 dpi (Fig. 2A). In addition, the neutrophil-recruiting chemokines KC [28], MIP2, and chemokine receptor CXCR2 [29] in the *Nlrp3*^{R258W} mice were moderately higher than

those in WT mice on 3 dpi (Fig. 2B), while other chemokines such as CCL3, CCL4 and CCL5 were all comparable between the two lines of experimental animals (Fig. 2B).

IL-1β is a pro-inflammatory cytokine that can function as a pyrogen to induce the expression of adhesion molecules on endothelial cells to promote the infiltration of leukocytes [30,31]. Additionally, IL-17A can also recruit neutrophils to the sites of infection [27]. The increased production of IL-1β and IL-17A in *Nlrp3*^{R258W} mice might have led to augmented leukocyte infiltration into the airways during influenza virus infection. Indeed, the numbers of total cells, CD11b$^+$Ly6G$^+$ neutrophils and CD11b$^+$F4/80$^+$ macrophages were all significantly higher in the BALF of *Nlrp3*^{R258W} on 6 dpi, although they were comparable on 3 dpi (Fig. 3A). The numbers of other cell populations including CD45.2$^+$CD3$^+$CD4$^+$ T helper cells, CD45.2$^+$CD3$^+$CD8$^+$ cytotoxic T cells, CD45.2$^+$CD11c$^+$ dendritic cells and CD45.2$^+$CD19$^+$ B cells were all comparable on 3 dpi between *Nlrp3*^{R258W} and WT mice, and the numbers of CD4$^+$ T helper cells and CD11c$^+$ dendritic cells were even a bit lower in *Nlrp3*^{R258W} mice on 6 dpi (Fig. 3B). Taken together, these results demonstrated that the *Nlrp3*^{R258W} mice produced more IL-1β and IL-17A, and recruited adequate neutrophils, which might have mediated virus clearance and thus attenuated IAV- induced lung damage.

3.4. IL-1β signaling contributes to the protection of *Nlrp3*^{R258W} mutation against IAV infection

Both IL-1β and IL-18 are the downstream effector cytokines whose maturation depends on inflammasome activation [5]. The *Nlrp3*^{R258W} mice secreted more IL-1β during IAV infection, while the production of IL-18 was comparable between WT and *Nlrp3*^{R258W} mice (Fig. 2A). This indicated that instead of IL-18, IL-1β was likely responsible for *Nlrp3*^{R258W} mice against influenza virus infection. Indeed, when the *Nlrp3*^{R258W} mice were crossed with the *Il1r1*$^{-/-}$ mice, the protective role of *Nlrp3*^{R258W} mutation was largely lost: *Nlrp3*^{R258W} × *Il1r1*$^{-/-}$ mice, characterized with more severe lung damage (Fig. 4B) and compromised virus clearance (Fig. 4C), developed more weight loss and mortality (Fig. 4A) than *Nlrp3*^{R258W} mice. We also monitored the levels of pro-inflammatory mediators in the BALF. As expected, the deficiency of IL-1R1 didn't affect IL-1β secretion, but significantly decreased the production of IL-17A in the *Nlrp3*^{R258W} × *Il1r1*$^{-/-}$ mice (Fig. 4D). Other cytokines such as TNF-α and IFN-γ in the *Nlrp3*^{R258W} × *Il1r1*$^{-/-}$ mice were upregulated on 3 dpi compared with those in *Nlrp3*^{R258W} mice (Fig. 4D), but the level of IL-6 was comparable between these two groups of mice (Fig. 4D). Of note, the chemokine CCL2 kept at a higher level in the *Nlrp3*^{R258W} × *Il1r1*$^{-/-}$ mice (Fig. 4D).

Additionally, although the deficiency of IL-1R1 didn't directly affect the number of the total recruited cells to bronchioalveoli, and even increased the number of CD11b$^+$F4/80$^+$ macrophages on 6 dpi (Fig. 5A), it obviously decreased CD11b$^+$Ly6G$^+$ neutrophil infiltration to bronchioalveoli in the *Nlrp3*^{R258W}x *Il1r1*$^{-/-}$ mice on both 3 dpi (Fig. 5A). The numbers of CD8$^+$ cytotoxic T cells and CD19$^+$ B cells didn't show a clear difference between these two groups of mice, while the numbers of CD4$^+$ T helper cells and dendritic cells were even augmented in the *Nlrp3*^{R258W} × *Il1r1*$^{-/-}$ mice (Fig. 5B). What's more, *Nlrp3*^{R258W} × *Il1r1*$^{-/-}$ mice showed comparable chemokine expression with *Nlrp3*^{R258W} mice, except for the CCL5 expression that was increased in *Nlrp3*^{R258W} × *Il1r1*$^{-/-}$ mice 3 dpi (Fig. 5C). CCL5 can be produced by dendritic cells and promotes T cell chemotaxis and activation [32], higher level of dendritic cells in *Nlrp3*^{R258W} × *Il1r1*$^{-/-}$ mice may account for the enhanced CCL5 expression. Taken together, these results suggest that the downstream IL-1β signaling of NLRP3 inflammasome protects against IAV infection in the *Nlrp3*^{R258W} mice.

3.5. IL-1β-dependent neutrophil recruitment is responsible for *Nlrp3*^{R258W} mice against influenza A virus infection

Being discovered as an endogenous pyrogen, IL-1β is a potent inflammatory cytokine which induces the expression of adhesion molecules on endothelial cells and promotes lymphocyte infiltration to the sites of infection or damage [31]. As described above, elevated neutrophil infiltration to bronchioalveoli in *Nlrp3*^{R258W} mice was dependent on IL-1β (Fig. 5A). In addition, the expressions of chemokines KC, MIP2, CCL20 and CCL28, the chemokine receptor CXCR2, Selectin E and Selectin P of endothelial cells, and integrin ligand ICAM1 in lung homogenates were all elevated in the *Nlrp3*^{R258W} mice in an IL-1β signaling-dependent manner on 3 dpi (Fig. 6A). Neutrophils can be recruited to the respiratory tract at the early stage of influenza virus infection, then bind to and take up influenza virus directly [28,33,34]. We thus hypothesized that neutrophils recruited to the lung via IL-1β signaling might contribute to the protection of *Nlrp3*^{R258W} mutation against influenza virus infection. To test this, we depleted neutrophils with anti-Ly6G antibody 1A8. The significant differences in viral load, weight loss and survival rate between *Nlrp3*^{R258W} and WT mice disappeared following 1A8 treatment (Fig. 6B and C). Neutrophil counting in total blood cells demonstrated that neutrophils had been successfully depleted (Fig. 6D). Taken together, our results demonstrate that the protection of *Nlrp3*^{R258W} mutation against influenza A virus is attributed to recruited neutrophils that confer virus clearance.

4. Discussion

Although previous work demonstrated that the NLRP3 inflammasome plays a critical role against influenza A virus infection [22,23], the detailed mechanism was not clear. Our current study uncovered the cellular mechanism for the protective effect of NLRP3 inflammasome against IAV challenge: influenza virus induces NLRP3 inflammasome activation in the lung resulting in IL-1β production, the latter induces the expression of chemokines and cell surface attachment/adhesion molecules, which leads to neutrophil recruitment to the infected sites for viral clearance.

Three research groups indicated that NLRP3 inflammasome played a critical role during influenza A virus infection. All these studies showed that the NLRP3 inflammasome component knock-out mice had decreased IL-1β and IL-18, less inflammatory cell infiltrations and more severe damage [22,23,35]. Notably, a recent study from Tate and colleagues demonstrated that MCC950, a specific inhibitor for the NLRP3 inflammasome [36], regulates the host immune responses to influenza viral infection in a temporal manner. Inhibition of NLRP3 inflammasome at the early stage of infection exaggerated the disease due to insufficient host immune responses, while the inhibition at the late stage protected mice from virus-induced disease [37]. Overall, all these studies emphasized the critical role of the NLRP3 inflammasome in combating influenza virus infection.

Both IL-1β and IL-18 are downstream cytokines of NLRP3 inflammasome activation [5]. The expression of IL-1β can be induced by NF-κB activation, while IL-18 is constitutively expressed in many types of cells [38]. Of note, although NLRP3 is expressed in various immune cells, more and more evidence showed that IL-18 is expressed in cells that do not contain NLRP3. For example, a recent study showed that human intestinal epithelial cells express high levels of IL-18, but not NLRP3 or IL-1β; while gut lamina propria cells express NLRP3 and IL-1β, but not IL-18 [39]. Our lab also found this phenomenon in mice, wherein intestinal lamina propria phagocytes express NLRP3 and IL-1β, but not IL-18; and gut epithelial cells express IL-18, but not NLRP3 or IL-1β [40]. This may well explain why the production of IL-1β was clearly different, but the secretion of IL-18 was similar between *Nlrp3*^{R258W} and WT mice (Fig. 2A). Since IL-1β but not IL-18 is expressed in the same type of cells as NLRP3, it is reasonable to see the elevation of IL-1β but not IL-18 when NLRP3 was mutated for hyper

inflammasome activation. Furthermore, it is apparent that IL-1β, instead of IL-18, is responsible for the protection of *Nlrp3*^{R258W} mutation against IAV challenge. This was proved to be the case, because the *Nlrp3*^{R258W} × *Il1r1*$^{-/-}$ mice diminished the protection against influenza virus, due to compromised neutrophil recruitment. Indeed, the viral loads in the *Nlrp3*^{R258W} × *Il1r1*$^{-/-}$ and neutrophil-depleted *Nlrp3*^{R258W} mice were both clearly higher than that in the original *Nlrp3*^{R258W} mice (Figs. 1D, 4C and 6B).

IL-1β induces the expression of adhesion molecules on the endothelial cells lining blood vessels, and thus promotes the infiltration of lymphocytes from blood to the sites of infection, which is a critical inflammatory process for virus control [31,41]. In response to the invading microbes, tissue resident macrophages or mast cells produce TNF-α, IL-1β and other cytokines which activate endothelial cells to capture neutrophils, mediating the neutrophil infiltration to the infected sites [42]. In addition, Fas ligand-induced massive neutrophil infiltration is also IL-1β dependent [43]. IL-1β mediated anti-tumor effect also depends on the recruited neutrophils [44]. Upon influenza virus challenge, mice deficient in IL-1β exhibited lower body temperature and higher mortality compared with WT controls [45]. The *Il1r1*$^{-/-}$ mice displayed significantly increased mortality and increased lung viral load, due to the impaired neutrophil recruitment, IgM antibody responses and CD4^{+} T cell infiltration to the infection sites [35]. Overall, IL-1β signaling is protective against influenza virus infection due to its positive regulation of innate and adaptive immune responses.

Neutrophils constitute 60% of all leukocytes in blood vessel and function as the primary effector cells fighting against invading microorganisms. These cells infiltrate into the inflammatory sites at the early stage of infection, especially for the acute inflammation [46]. Neutrophils combat invading microbes through phagocytosing bacteria, fungi and virions. Such cells can be recruited to the respiratory tract in the early phase of influenza virus infection, then bind and take up IAV directly [33,47]. In our study, the mutated mice showed amplified neutrophil infiltration, which promoted virus clearance more effectively.

Taken together, we identified a cellular mechanism for NLRP3 inflammasome fighting against influenza A virus. The *Nlrp3*^{R258W} mice resisted influenza A virus infection due to enhanced inflammasome activity, elevated IL-1β signal and augmented neutrophil mediated suppression of virus replication. These findings shed new lights on the development of therapeutic strategies to combat influenza A virus via manipulating host inflammatory responses.

Author contributions

G.M. conceived the project, J. N. and S.W. conducted the experiments, M.C., K.X., Q.G., A. L. and L.Z. helped with experiments, B.S. provided critical experimental materials, J. N., S.W. and G.M. analyzed the data and wrote the manuscript, G.M. supervised the study.

Competing interests

The authors have no financial conflicts of interest.

Acknowledgements

This work was supported by grants from National Key Basic Research Programs (2015CB554302, 2016YFE0133500, 2018YFA0507300), Natural Science Foundation of China (81830049, 81761128012, 31570895), Strategic Priority Research Program (XDB29030303) and International Partnership Program (153831KYSB20160009) of the Chinese Academy of Sciences, as well as the Shanghai Natural Science Foundation (16ZR1439900). We are grateful to Dr. Warren Strober for providing the *Nlrp3*^{R258W} mice.

References

[1] O. Takeuchi, S. Akira, Pattern recognition receptors and inflammation, Cell 140 (2010) 805–820.
[2] G. Eberl, Immunity by equilibrium, Nat. Rev. Immunol. 16 (2016) 524–532.
[3] D.E. Place, T.D. Kanneganti, Recent advances in inflammasome biology, Curr. Opin. Immunol. 50 (2017) 32–38.
[4] K. Schroder, J. Tschopp, The inflammasomes, Cell 140 (2010) 821–832.
[5] F. Bauernfeind, A. Ablasser, E. Bartok, S. Kim, J. Schmid-Burgk, T. Cavlar, et al., Inflammasomes: current understanding and open questions, Cell. Mol. Life Sci. 68 (2011) 765–783.
[6] M.R. de Zoete, N.W. Palm, S. Zhu, R.A. Flavell, Inflammasomes, Cold Spring Harbor Perspect. Biol. (2014).
[7] P. Broz, V.M. Dixit, Inflammasomes: mechanism of assembly, regulation and signalling, Nat. Rev. Immunol. 16 (2016) 407–420.
[8] D. Sharma, T.D. Kanneganti, The cell biology of inflammasomes: mechanisms of inflammasome activation and regulation, J. Cell Biol. 213 (2016) 617–629.
[9] H. Guo, J.B. Callaway, J.P. Ting, Inflammasomes: mechanism of action, role in disease, and therapeutics, Nat. Med. 21 (2015) 677–687.
[10] L. Franchi, T. Eigenbrod, R. Munoz-Planillo, G. Nunez, The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis, Nat Immunol. 10 (2009) 241–247.
[11] E. Aganna, F. Martinon, P.N. Hawkins, J.B. Ross, D.C. Swan, D.R. Booth, et al., Association of mutations in the NALP3/CIAS1/PYPAF1 gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness, and AA amyloidosis, Arthritis Rheum. 46 (2002) 2445–2452.
[12] C. Dodé, N. Le Dû, L. Cuisset, F. Letourneur, J.-M.M. Berthelot, G. Vaudour, et al., New mutations of CIAS1 that are responsible for Muckle-Wells syndrome and familial cold urticaria: a novel mutation underlies both syndromes, Am. J. Hum. Genet. 70 (2002) 1498–1506.
[13] H.M. Hoffman, J.L. Mueller, D.H. Broide, A.A. Wanderer, R.D. Kolodner, Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome, Nat. Genet. 29 (2001) 301–305.
[14] Z. Jacobs, C.E. Ciaccio, Periodic fever syndromes, Curr. Allergy Asthma Rep. 10 (2010) 398–404.
[15] G. Meng, F. Zhang, I. Fuss, A. Kitani, W. Strober, A mutation in the Nlrp3 gene causing inflammasome hyperactivation potentiates Th17 cell-dominant immune responses, Immunity 30 (2009) 860–874.
[16] A. Mayor, F. Martinon, T. De Smedt, V. Petrilli, J. Tschopp, A crucial function of SGT1 and HSP90 in inflammasome activity links mammalian and plant innate immune responses, Nat Immunol. 8 (2007) 497–503.
[17] WHO, Influenza (seasonal) fact sheet, 2014, Available from: < http://www.who.int/mediacentre/factsheets/fs211/en/ > .
[18] R.B. Seth, L. Sun, Z.J. Chen, Antiviral innate immunity pathways, Cell Res. 16 (2006) 141–147.
[19] S.S. Diebold, T. Kaisho, H. Hemmi, S. Akira, C. Reis e Sousa, Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA, Science 303 (2004) 1529–1531.
[20] C. Lupfer, T.D. Kanneganti, The expanding role of NLRs in antiviral immunity, Immunol. Rev. 255 (2013) 13–24.
[21] A. Iwasaki, P.S. Pillai, Innate immunity to influenza virus infection, Nat. Rev. Immunol. 14 (2014) 315–328.
[22] P.G. Thomas, P. Dash, J.R. Aldridge Jr., A.H. Ellebedy, C. Reynolds, A.J. Funk, et al., The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1, Immunity 30 (2009) 566–575.
[23] I.C. Allen, M.A. Scull, C.B. Moore, E.K. Holl, E. McElvania-TeKippe, D.J. Taxman, et al., The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA, Immunity 30 (2009) 556–565.
[24] H.W. Stout-Delgado, S.E. Vaughan, A.C. Shirali, R.J. Jaramillo, K.S. Harrod, Impaired NLRP3 inflammasome function in elderly mice during influenza infection is rescued by treatment with nigericin, J. Immunol. 188 (2012) 2815–2824.
[25] K. Xu, C. Klenk, B. Liu, B. Keiner, J. Cheng, B.J. Zheng, et al., Modification of nonstructural protein 1 of influenza A virus by SUMO1, J. Virol. 85 (2011) 1086–1098.
[26] J. Maelfait, K. Roose, P. Bogaert, M. Sze, X. Saelens, M. Pasparakis, et al., A20 (Tnfaip3) deficiency in myeloid cells protects against influenza A virus infection, PLoS Pathog. 8 (2012) e1002570.
[27] K. Van Reeth, Cytokines in the pathogenesis of influenza, Vet. Microbiol. 74 (2000) 109–116.
[28] B.F. Drescher, Bai, Neutrophil in viral infections, friend or foe? Virus Res. 171 (2013) 1–7.
[29] D.R. Nagarkar, Q. Wang, J. Shim, Y. Zhao, W.C. Tsai, N.W. Lukacs, et al., CXCR2 is required for neutrophilic airway inflammation and hyperresponsiveness in a mouse model of human rhinovirus infection, J. Immunol. 183 (2009) 6698–6707.
[30] C.A. Dinarello, Interleukin-1beta and the autoinflammatory diseases, N. Engl. J. Med. 360 (2009) 2467–2470.
[31] C.A. Dinarello, The IL-1 family and inflammatory diseases, Clin. Exp. Rheumatol. 20 (2002) S1–S13.
[32] T.T. Murooka, R. Rahbar, L.C. Platanias, E.N. Fish, CCL5-mediated T-cell chemotaxis involves the initiation of mRNA translation through mTOR/4E-BP1, Blood 111 (2008) 4892–4901.
[33] H. Fujisawa, S. Tsuru, M. Taniguchi, Y. Zinnaka, K. Nomoto, Protective mechanisms against pulmonary infection with influenza virus. I. Relative contribution of polymorphonuclear leukocytes and of alveolar macrophages to protection during the

early phase of intranasal infection, J. Gen. Virol. 68 (Pt 2) (1987) 425–432.
[34] T.M. Tumpey, A. Garcia-Sastre, J.K. Taubenberger, P. Palese, D.E. Swayne, M.J. Pantin-Jackwood, et al., Pathogenicity of influenza viruses with genes from the pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice, J. Virol. 79 (2005) (1918) 14933–14944.
[35] T. Ichinohe, H.K. Lee, Y. Ogura, R. FlavellA, Iwasaki, Inflammasome recognition of influenza virus is essential for adaptive immune responses, J. Exp. Med. 206 (2009) 79–87.
[36] R.C. Coll, A.A. Robertson, J.J. Chae, S.C. Higgins, R. Munoz-Planillo, M.C. Inserra, et al., A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases, Nat. Med. 21 (2015) 248–255.
[37] M.D. Tate, J.D. Ong, J.K. Dowling, J.L. McAuley, A.B. Robertson, E. Latz, et al., Reassessing the role of the NLRP3 inflammasome during pathogenic influenza A virus infection via temporal inhibition, Sci. Rep. 6 (2016) 27912.
[38] C.A. Dinarello, Interleukin 1 and interleukin 18 as mediators of inflammation and the aging process, Am. J. Clin. Nutr. 83 (2006) 447S–455S.
[39] S. Zhu, S. Ding, P. Wang, Z. Wei, W. Pan, N.W. Palm, et al., Nlrp9b inflammasome restricts rotavirus infection in intestinal epithelial cells, Nature 546 (2017) 667–670.
[40] X. Yao, C. Zhang, Y. Xing, G. Xue, Q. Zhang, F. Pan, et al., Remodelling of the gut microbiota by hyperactive NLRP3 induces regulatory T cells to maintain homeostasis, Nat. Commun. 8 (2017) 1896.
[41] M.D. Wewers, H.A. Dare, A.V. Winnard, J.M. Parker, D.K. Miller, IL-1 beta-converting enzyme (ICE) is present and functional in human alveolar macrophages: macrophage IL-1 beta release limitation is ICE independent, J. Immunol. 159 (1997) 5964–5972.
[42] E. Kolaczkowska, P. Kubes, Neutrophil recruitment and function in health and inflammation, Nat. Rev. Immunol. 13 (2013) 159–175.
[43] K. Miwa, M. Asano, R. Horai, Y. Iwakura, S. Nagata, T. Suda, Caspase 1-independent IL-1beta release and inflammation induced by the apoptosis inducer Fas ligand, Nat. Med. 4 (1998) 1287–1292.
[44] L.C. Chen, L.J. Wang, N.M. Tsang, D.M. Ojcius, C.C. Chen, C.N. Ouyang, et al., Tumour inflammasome-derived IL-1beta recruits neutrophils and improves local recurrence-free survival in EBV-induced nasopharyngeal carcinoma, EMBO Mol. Med. 4 (2012) 1276–1293.
[45] W. Kozak, H. Zheng, C.A. Conn, D. Soszynski, L.H. van der Ploeg, M.J. Kluger, Thermal and behavioral effects of lipopolysaccharide and influenza in interleukin-1 beta-deficient mice, Am. J. Physiol. 269 (1995) R969–R977.
[46] K. Beiter, F. Wartha, B. Albiger, S. Normark, A. Zychlinsky, B. Henriques-Normark, An endonuclease allows Streptococcus pneumoniae to escape from neutrophil extracellular traps, Curr. Biol. 16 (2006) 401–407.
[47] R. Gonzalez-Dosal, K.A. Horan, S.H. Rahbek, H. Ichijo, Z.J. Chen, J.J. Mieyal, et al., HSV infection induces production of ROS, which potentiate signaling from pattern recognition receptors: role for S-glutathionylation of TRAF3 and 6, PLoS Pathog. 7 (2011) e1002250.