IL-36 receptor deletion attenuates lung injury and decreases mortality in murine influenza pneumonia

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Influenza virus causes a respiratory disease in humans that can progress to lung injury with fatal outcome. The interleukin (IL)-36 cytokines are newly described IL-1 family cytokines that promote inflammatory responses via binding to the IL-36 receptor (IL-36R). The mechanism of expression and the role of IL-36 cytokines are poorly understood. Here, we investigated the role of IL-36 cytokines in modulating the innate inflammatory response during influenza virus-induced pneumonia in mice. The intranasal administration of influenza virus upregulated IL-36α mRNA and protein production in the lungs. *In vitro*, influenza virus-mediated IL-36α but not IL-36γ is induced and secreted from alveolar epithelial cells (AECs) through both a caspase-1 and caspase-3/7 dependent pathway. IL-36α was detected in microparticles shed from AECs and promoted the production of pro-inflammatory cytokines and chemokines in respiratory cells. IL-36R-deficient mice were protected from influenza virus-induced lung injury and mortality. Decreased mortality was associated with significantly reduced early accumulation of neutrophils and monocytes/macrophages, activation of lymphocytes, production of pro-inflammatory cytokines and chemokines, and permeability of the alveolar–epithelial barrier in despite impaired viral clearance. Taken together, these data indicate that IL-36 ligands exacerbate lung injury during influenza virus infection.

INTRODUCTION

Influenza virus-induced pneumonia and resultant acute respiratory distress syndrome are associated with high mortality in critical care patients.[1] High viral load, excessive pro-inflammatory cytokines and chemokine expression and robust inflammatory cells influx into the lungs are associated with disease severity in influenza virus infection.[2–4] Imbalance between viral pathogenicity and host immune response is believed to contribute to influenza virus-induced respiratory failure.

Interleukin (IL)-36 cytokines, including three agonist proteins IL-36α, IL-36β, and IL-36γ, along with the antagonist protein IL-36Ra, are novel members of IL-1 family of cytokines. IL-36 agonists bind the same receptor complex, consisting of the IL-36 receptor (IL-36R) and IL-1receptor accessory protein (IL-1RAcP), a protein that is shared with the IL-1 receptor and the IL-33 receptor. Binding to this complex stimulates inflammatory signals through nuclear factor-κB and mitogen-activated protein kinase.[5] The IL-36 agonists similarly induce pro-inflammatory cytokines and chemokines and contribute to neutrophils accumulation, dendritic cell activation, and polarization of T helper 1 and IL-17-producing T cells.[5–7] IL-36 cytokines are expressed by keratinocytes and other epithelial cells, as well as macrophages and monocytes. Conversely, IL-36R is widely expressed throughout the body.[5] It was well characterized that IL-36 cytokines are involved in the pathogenesis of human psoriasis[8,9] and animal models of skin inflammation.[10,11] However, little is known about the role of IL-36 cytokines in the lung. Transcript products of IL-36γ have been shown to be upregulated in bronchial epithelial cells by stimulation of pro-inflammatory cytokines, bacteria, and viral-associated molecular patterns.[12,13] Intratracheal administration of IL-36α or IL-36γ in mice induce a rapid influx of neutrophils into the lungs and the expression of pro-inflammatory cytokines and chemokines.[14,15] These previous findings suggest that IL-36 cytokines may play an important

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role in host defense against respiratory pathogens and contribute to leukocyte recruitment during infection. To date, no studies have investigated IL-36 cytokines in the host defense against influenza virus or as mediators of virus-induced lung injury.

Secretion mechanism of IL-36 cytokines from stimulated cells is not well understood. IL-36 cytokines, like other IL-1 family cytokines, lack a signal peptide and do not utilize a classical endoplasmic reticulum-to-Golgi secretion pathway, requiring alternative mechanisms for their release. Extracellular adenosine triphosphate (ATP) has been shown to be necessary for the secretion of intracellularly produced IL-36 cytokines.[8,16,17] Stimulation of P2X7 receptor by extracellular ATP induced NOD-like receptor family, pyrin containing 3 (NLRP3) inflammasome activation, and caspase-1-dependent processing and release of IL-1β and IL-18.[18] Moreover, P2X7 signaling induces the release of extracellular microvesicles or microparticles (MPs), and secreted IL-1β and IL-18 are packaged within MPs.[19,20] MPs are membrane-bound vesicles ranging in size from 0.1 to 1 μm released by a variety of cells. MPs contain proteins, bioactive lipids, nucleic acid, and organelles, and are recognized to be a biomarker and effector of cell-to-cell signaling in inflammatory diseases.[21] We have recently shown that IL-36γ protein shed from macrophages stimulated with bacteria and ATP was detected within MPs.[22] However, the role and secretion mechanism of IL-36 cytokines in the pathogenesis of lung inflammation is unknown.

In the current study, we hypothesized that IL-36 cytokines and their receptor, IL-36R, can contribute to the pathogenesis of influenza virus-induced lung injury. Influenza virus infection in mice resulted in the selective upregulation of IL-36α, but not IL-36β and γ, in the lungs. IL-36α was secreted by alveolar epithelial cells (AECs) in MPs, exosomes (EXs), and directly into the extracellular milieu in response to influenza virus, and AEC-derived MPs directly stimulate cytokine and chemokine production in pulmonary macrophages (PMs) *in vitro* in an IL-36R-dependent fashion. As compared with wild-type (WT) controls, IL-36R$^{-/-}$ mice display decreased early accumulation and activation of inflammatory cells, production of pro-inflammatory cytokines and chemokines, and reduced alveolar–epithelial barrier leak, which is associated with reduced mortality in response to influenza virus challenge. Taken together, these data demonstrate for the first time an important role of IL-36R ligands in the pathogenesis of influenza virus-induced lung injury.

RESULTS

IL-36α is selectively induced in the lungs during influenza virus infection

We first examined the transcript products of IL-36 cytokines in the lungs of WT C57B/6 mice infected with influenza virus (Influenza A/Puerto Rico/8/34 virus). Both IL-36α and IL-36γ mRNA levels were significantly elevated in lung at day 2 after influenza administration, and this increase in IL-36α mRNA, but not IL-36γ mRNA, persisted through day 6 (**Figure 1a**). Conversely, IL-36β mRNA was not detected in influenza virus-

infected lungs (data not shown). The protein levels of IL-36α in bronchoalveolar lavage (BAL) fluid, but not IL-36γ, were significantly higher at day 2 compared with untreated mice (**Figure 1b**).

We have recently shown that IL-36γ protein released from primary PMs stimulated with *Streptococcus pneumoniae* was primarily packaged within MPs and EXs. We therefore examined whether IL-36α in BAL fluid post influenza virus challenge was present in MPs and EXs. To assess this, BAL was performed at day 3 after flu administration, then MPs and/or EXs in BAL fluid were isolated by three-step centrifugation.[22] IL-36α was detected by enzyme-linked immunosorbent assay within the MPs, EXs, and post-spin fluid fraction, and was significantly higher in the MP and post-spin fractions in influenza virus-infected mice as compared with untreated mice (**Figure 1c**).

IL-36α is released from influenza virus stimulated AECs through caspase-1- and caspase-3/7-dependent pathways

Respiratory epithelial cells and macrophages are important innate immune cells during influenza virus infection. To determine the primary cellular source of IL-36α during influenza, primary AECs and PMs isolated from WT mice were incubated with various doses of influenza virus. Lipopolysaccharide (LPS) stimulation was used as a control immune activator. Treatment with LPS induced IL-36α mRNA in both AECs and PMs. Though the expression level of IL-36α mRNA was significantly elevated in influenza virus-treated AECs in a dose dependent manner, IL-36α mRNA was not induced in PMs by influenza virus (**Figure 2a**). Heat killed (fusion inhibiting) influenza virus failed to induce IL-36α mRNA in AECs (data not shown). Incubation with ultraviolet-inactivated influenza virus (replication inhibiting)-induced IL-36α mRNA in AECs; its level was significantly lower as compared with live virus (see **Supplementary Figure S1A** online).

Two distinct toll-like receptors (TLRs)-3 and TLR-7 recognize the double stranded and single stranded viral RNA of influenza virus, respectively, within endosomes and play key roles in the innate immune responses against virus.[23] To examine whether these pathogen recognition receptors contribute to induction and secretion of IL-36α, we first compared mRNA expression of IL-36α in AECs treated with the TLR-3 agonist poly (I:C); the TLR-7 agonist R843; the combination of poly (I:C) plus R843; influenza virus; or LPS. Unlike a previous study,[17] treatment with poly (I:C) alone did not induce IL-36α mRNA. Conversely, treatment with R843 alone enhanced expression of IL-36α mRNA, and its level tended to be enhanced by co-stimulation with poly (I:C) treatment (**Figure 2b**). To evaluate secretion of IL-36α protein, IL-36α was measured in culture medium (CM) by enzyme-linked immunosorbent assay. The production of IL-36α was significantly elevated in AECs treated with live influenza virus or with the combination of poly (I:C) plus R843, but not poly (I:C) or R843 alone (**Figure 2c**). These data suggest that both TLR-3 and TLR-7 signaling were required for secretion of
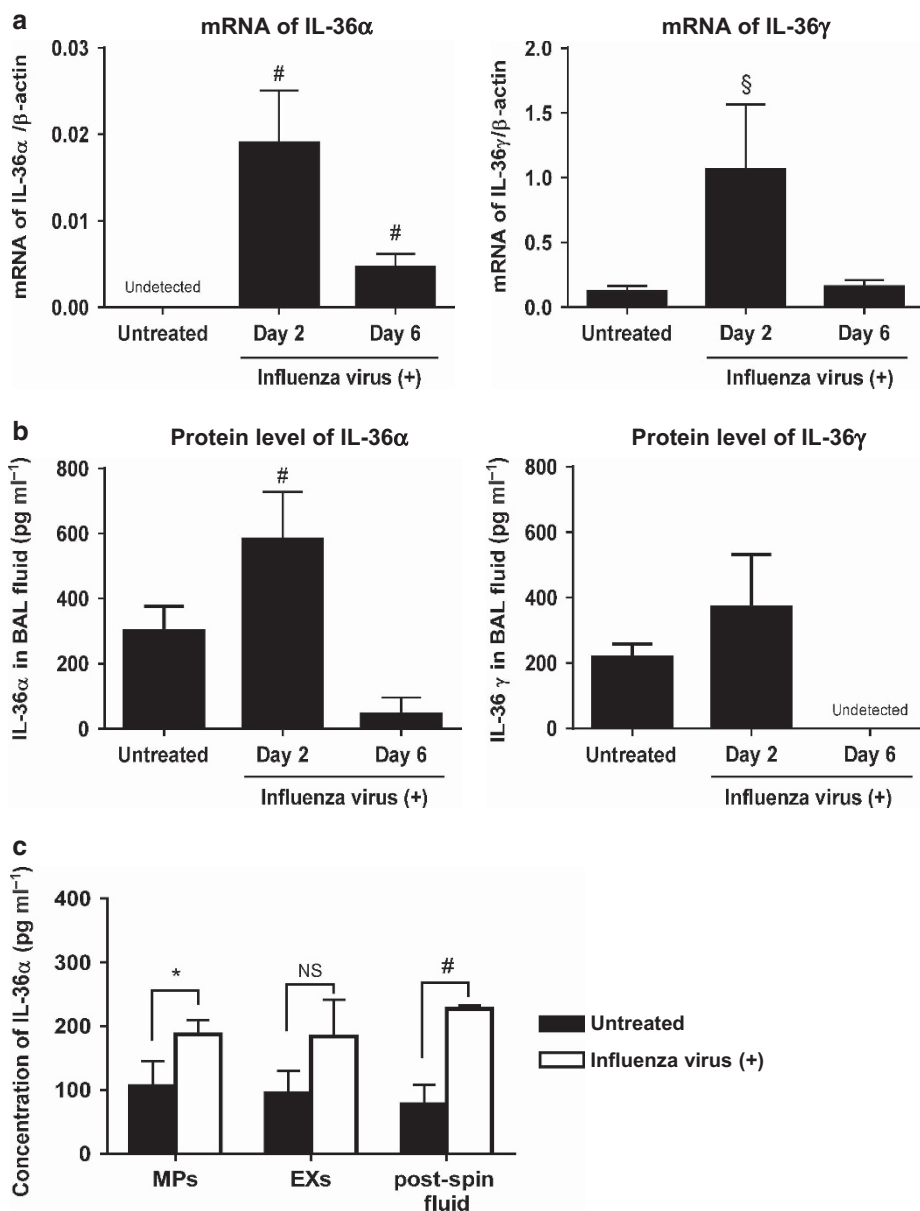
Figure 1 Expression of IL-36 cytokines in lung of WT mice infected with influenza virus. (**a**) Transcript products of IL-36α (left panel) and IL-36γ (right panel) in the lungs of untreated, day 2, and day 6 after influenza virus infection. mRNA was analyzed by real-time PCR. (**b**) Protein expression of IL-36α (left panel) and IL-36γ (right panel) in BAL was quantified by ELISA. (**c**) MPs and EXs in BAL fluid were isolated by three-step centrifugation. Production of IL-36α in MPs, EXs and post-spin fluid in which MPs and EXs fractions were removed were measured by ELISA. Data are shown as means ± s.d. of five mice per group. $*P<0.05$, $^{\#}P<0.01$, $^{§}P<0.001$, significant compared with untreated mice or as indicated. BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assay; IL-36, interleukin-36; WT, wild type.

IL-36α by AECs. Moreover, we did not observe secretion of IL-36α by AECs incubated with ultraviolet -inactivated influenza virus (see **Supplementary Figure S1B**). These data indicate that viral replication is required for maximal induction and secretion of IL-36α from virus-infected AECs.

We next investigated the influence of influenza-induced caspase-1 and caspase-3/7 activation on induction and secretion of IL-36α. Influenza virus has previously been shown to induce the expression of IL-1 like family members such as IL-1β and IL-18 in respiratory cells by a caspase-1-dependent manner.[18] Moreover, the secretion of IL-36 cytokines from

stimulated cells has been shown to require extracellular ATP treatment (which activates caspase-1)[8,16,17] or caspase-3/7 activity.[17] We confirmed that live influenza virus activated caspase-1 and caspase-3/7 in AECs (see **Supplementary Figure 2C, D**). While treatment with caspase-3/7 inhibitor did not alter IL-36α mRNA levels (**Figure 2d**), the secretion of IL-36α in CM was significantly reduced by caspase-3/7 inhibitor (**Figure 2e**). Alternatively, caspase-1 inhibition suppressed both mRNA and extracellular protein expression of IL-36α (**Figures 2d** and **e**). These data indicated that live influenza virus-induced caspase-1 activation contributed to
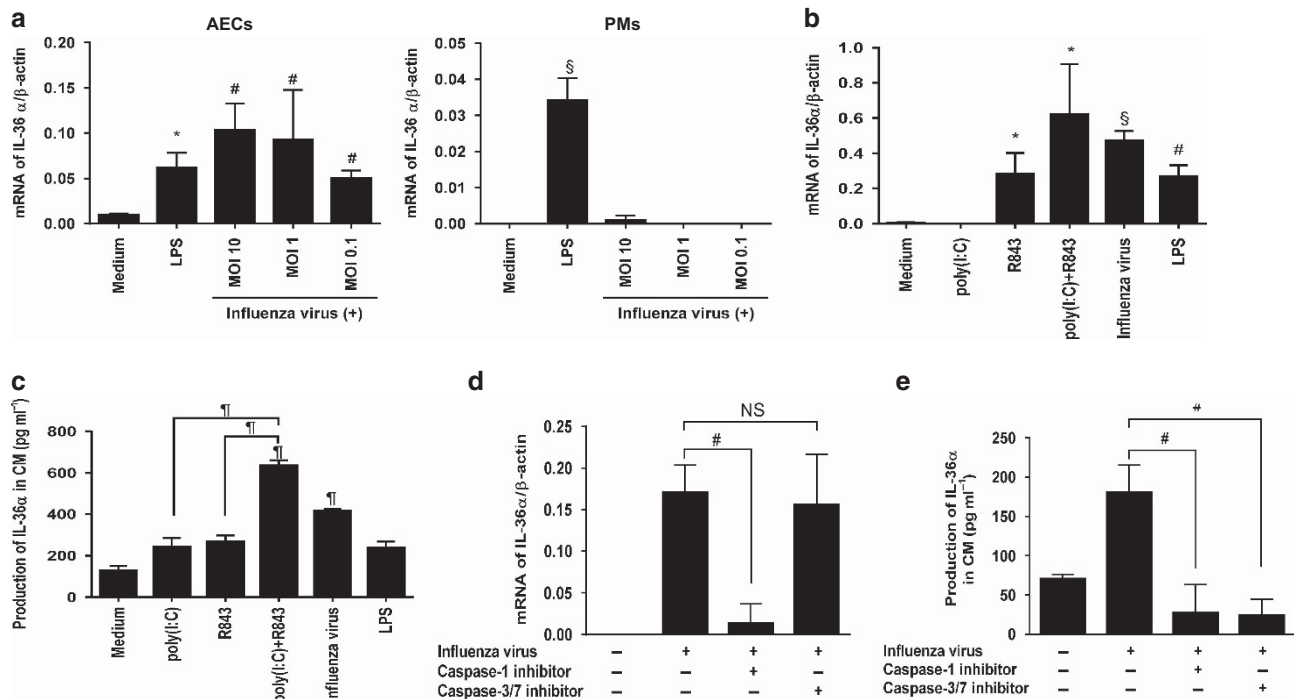
Figure 2 Mechanisms of IL-36α protein synthesis and secretion from AEC in response to influenza virus, TLR-3, and TLR-7 agonists. (a) Primary AECs (right panel) and PMs (left panel) isolated from WT mice were treated with LPS (1 mg ml^{-1}) or different doses of influenza virus (MOI 10–0.1). After 24 h incubation, expression of IL-36α mRNA was analyzed by real-time PCR. (b,c) AECs were treated with poly (I:C) (25 μg ml^{-1}), R843 (25 μg ml^{-1}), both poly (I:C) (25 μg ml^{-1}) and R843 (25 μg ml^{-1}), influenza virus (MOI 10), or LPS (1 μg ml^{-1}). (b) After 24 h incubation, expression of IL-36α mRNA was analyzed by real-time PCR. (c) The protein levels of IL-36α in CM were measured by ELISA. (d,e) AECs were pre-treated with Caspase-1 (20 μM) or Caspase-3/7 (100 μM) inhibitor 1 h before influenza virus stimulation. After 24 h incubation, IL-36α mRNA (d) and protein production in CM (e) were analyzed. Data (means ± s.d.) are representative of two independent experiments.*$P < 0.05$, #$P < 0.01$, §$P < 0.001$, ¶$P < 0.0001$, NS, not significant, compared with medium only or as indicated. AEC, alveolar epithelial cell; CM, culture medium; ELISA, enzyme-linked immunosorbent assay; IL-36, interleukin-36; LPS, lipopolysaccharide; MOI, multiplicity of infection; PM, pulmonary macrophage; TLR, toll-like receptor; WT, wild type.

induction of IL-36α, whereas caspase-3/7 was required for the secretion of IL-36α from AECs. Interestingly, we did not observe apoptosis of AECs at the 24 h post infection (as determined by Annexin V + staining), a time point in which IL-36α was detected in conditioned media (data not shown), suggesting that caspase-1 and caspase-3/7 activity, but not cell death, is required for the induction and secretion of IL-36α in influenza virus-infected AECs.

Influenza virus induced IL-36α from AECs is contained within MPs

As shown previously, a percentage of IL-36α in BAL was secreted within MPs during influenza virus infection (Figure 1c), and the secretion of IL-36α from AECs was dependent upon influenza virus-induced activation of caspase-3/7 (Figure 2e). We next examined whether IL-36α secreted from influenza virus-treated AECs was packaged within extracellular vesicles. AECs were treated with or without influenza virus for 48 h, then MPs and EXs isolated from CM by three-step centrifugation. Although there was no difference of number of MPs released by influenza virus-treated AECs as compared with vehicle-treated cells, the size of MPs shed from influenza virus-treated AECs were larger than that from untreated AECs (Figure 3a). On the other hand, no difference was observed in the number and size of EXs between the two

groups. As shown **Figure 3b**, the quantity of IL-36α protein contained within influenza virus-induced MPs was > 2-fold higher than that of MPs recovered from uninfected cells, whereas no difference in IL-36α levels was observed in EXs and post spin fluid (**Figure 3c**). To address the size difference in MPs shed from influenza virus-infected cells as compared with untreated control, we examined whether or not influenza virus was contained within MPs. MPs were isolated from CM with or without influenza virus-treated AECs by ultracentrifugation and then washed twice to remove virus binding to the surface of MPs. Immunoblotting showed the presence of both IL-36α and influenza virus M2 protein within MPs from influenza-infected AECs (**Figure 3d**), and virus contained with these MPs could be grown in MDCK cells (**Figure 3e**).

IL-36R$^{-/-}$ mice were protected from influenza virus-induced lung injury and mortality

To examine whether IL-36R-mediated signaling is involved in the host response against influenza infection, WT IL-36R$^{-/-}$ were inoculated intranasally with 2LD$_{50}$ dose of influenza virus, survival and body weight change assessed out to 14 days. Because IL-36 cytokine have previously been shown to enhance the expression of other IL-36 family members,[11] and we observed upregulation of IL-36γ mRNA in lungs of influenza virus infected mice at day 2, we included IL-36γ$^{-/-}$ as an
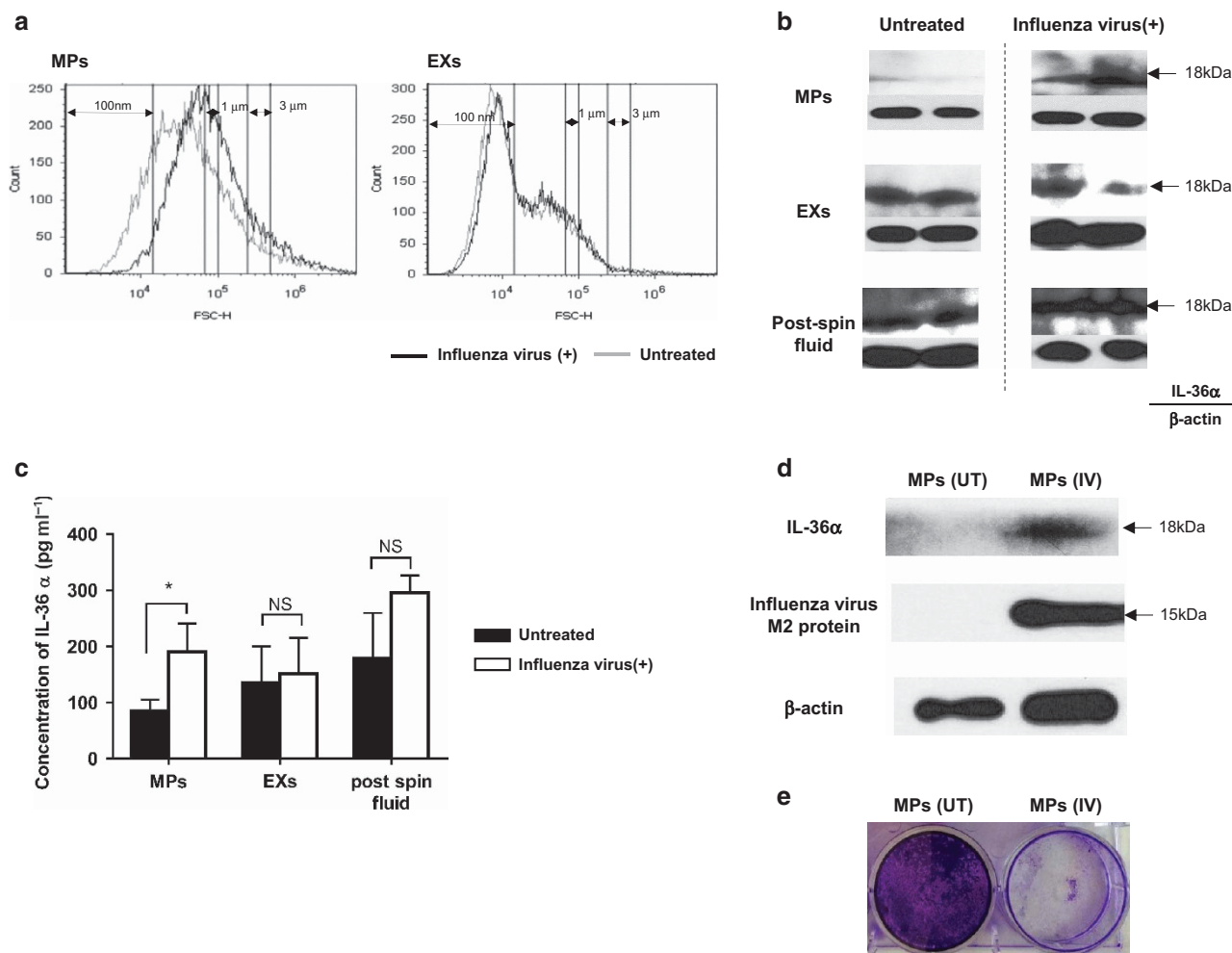
Figure 3 IL-36α released from influenza virus stimulated AECs contained within MPs. AECs were treated with or without influenza virus for 48 h incubation, and then MPs and EXs were isolated from CM by three-step differential centrifugation. (**a**) MPs and EXs were identified as Annexin V-positive cells. The size of MPs (left panel) and EXs (right panel) was assessed by flow cytometry for size relative to submicron calibration beads. (**b**) The protein expression of IL-36α in MPs, EXs, and post-spin fluid was detected by western blotting. Arrows showed the expression of IL-36α at 18 kDa (upper panel). Lower panel showed β-actin at 42 kDa. (**c**) The production of IL-36α in MPs, EXs, and post-spin fluid were measured by ELISA. Data are shown as means ± s.d.. *$P < 0.05$, NS, not significant as indicated. (**d**, **e**) MPs isolated from CM were washed twice to remove surface-bound virus. The protein expression of IL-36α and influenza virus M2 antigen in MPs were detected by western blotting. Arrow shows the expression of IL-36α at 18 kDa (upper panel), influenza virus M2 antigen at 15 kDa (middle panel), and β-actin at 42 kDa (lower panel) (**d**). MPs were cultured with MDCK cells (**e**). AEC, alveolar epithelial cell; CM, culture medium; ELISA, enzyme-linked immunosorbent assay; EX, exosomes; IL-36α, interleukin-36α; MP, microparticle; MPs (UT), MPs from untreated AECs; MP (IV), MPs from influenza virus-treated AECs.

additional control group. Body weight was not significantly different among the three groups (**Figure 4a**). However, all of WT and IL-36γ$^{-/-}$ mice were dead within 11 days post infection, whereas 50% of IL-36R$^{-/-}$ mice survived beyond day 14 post infection (**Figure 4b**). We next examined viral clearance after influenza virus challenge by quantitating lung viral titers in WT and IL-36R$^{-/-}$ mice on days 2 and 6 after infectious challenge. There was no difference in viral titers observed between WT and IL-36R$^{-/-}$ mice at day 2 (**Figure 4c**, left panel). However, by day 6 there was a 10-fold higher viral burden in IL-36R$^{-/-}$ mice as compared with infected WT mice (**Figure 4c**, right panel).

Histological examination of lungs on day 2 post influenza virus administration revealed accumulation of neutrophils in a peribronchial distribution in WT mice, and no difference in inflammatory cells influx into alveolar space between WT and IL-36R$^{-/-}$ mice at that time point. At day 7 in WT mice, there was massive infiltration of neutrophils not only into the intra-alveolar septa but into the alveolus, as well as alveolar hemorrhage, alveolar septa edema, and proteinaceous debris in the alveolar space. These inflammatory changes were strikingly attenuated in IL-36R$^{-/-}$ mice. We quantified the histological changes of tissue injury using a standardized lung injury scoring system.[24] The lung injury score was significantly lower in IL-36R$^{-/-}$ mice as compared with infected WT mice (**Figure 4e**). We next assessed the permeability of the alveolar-capillary membrane by measurement of albumin concentration in BAL fluid. At
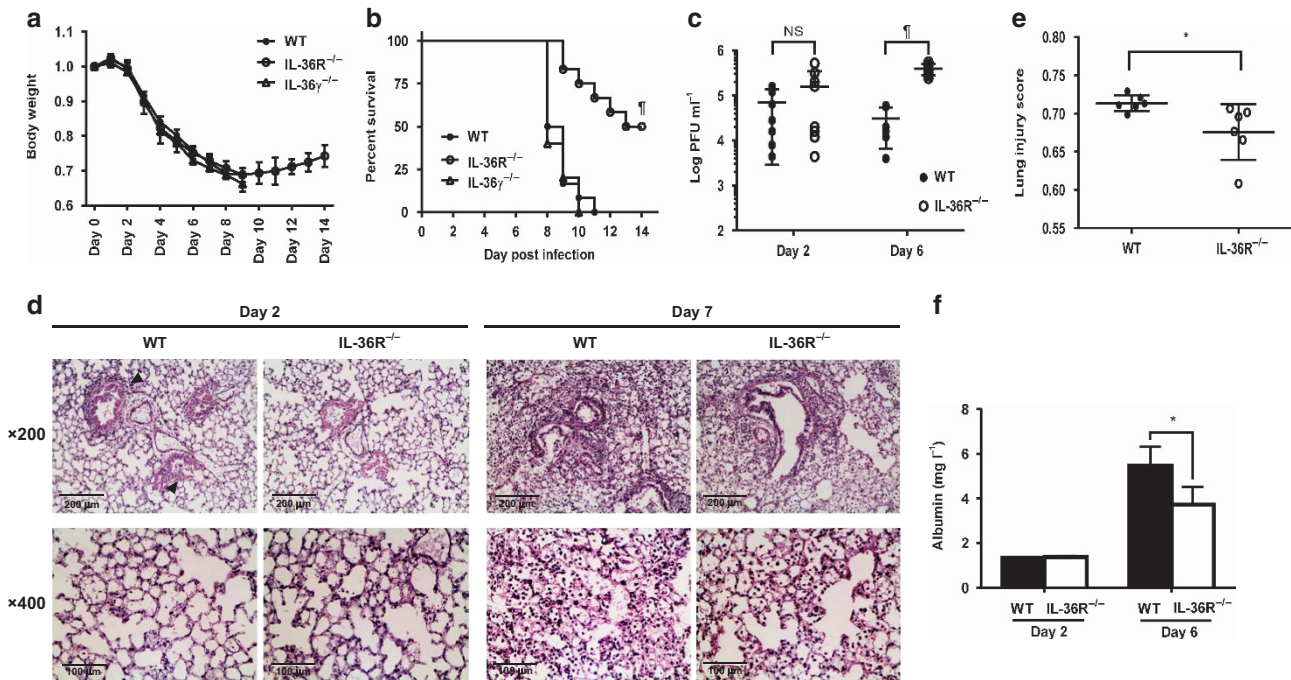
Figure 4 Effect of IL-36R gene deletion on mortality, viral clearance, and lung injury in influenza virus infection *in vivo*. Weight loss (**a**) and survival rate (**b**) were assessed daily following influenza virus infection. Each group consisted of 6–12 mice. (**c**) Virus titers was assessed on day 2 and day 6 after infection ($n = 8$ per each group). (**d**) Lung histopathological analysis was performed on day 2 and day 7 after infection. H&E-stained lung tissue at magnification of $\times 200$ (upper) and $\times 400$ (lower). Arrow showed neutrophils around bronchus. (**e**) The lung injury scores of lung sections from infected-WT and IL-36R$^{-/-}$ mice at day 7 were evaluated as previously described ($n = 6$ per each group). Lung injury scoring system parameters include the presence of neutrophils in the alveolar space (i), neutrophils in the interstitial space (ii), hyaline membranes (iii), proteinaceous debris filling the airspaces (iv), and alveolar septal thickening (v). At least 20 random regions were scored 0–2 independently and the final lung injury score was calculated as below; score $= ((20 \times i) + (14 \times ii) + (7 \times iii) + (7 \times iv) + (2 \times v))/(\text{number of fields} \times 100)$. (**f**) Lung permeability was quantified by albumin concentration in BAL fluid from 2 and 6 days after infection ($n = 4$ per each group). Data (means $\pm$ s.d.) are representative of two independent experiment. *$P < 0.05$, ¶$P < 0.0001$, NS, not significant, compared with infected in WT mice or as indicated. BAL, bronchoalveolar lavage; IL-36, interleukin-36; H&E, hematoxylin and eosin; WT, wild type.

day 6, albumin levels in BAL fluid were significantly reduced in IL-36R$^{-/-}$ mice compared with WT mice. Reduced alveolar permeability in influenza virus-infected IL-36R$^{-/-}$ mice was associated with a significant decrease in TUNEL positive apoptotic cells in IL-36R$^{-/-}$ mice compared with WT mice (see **Supplementary Figure S2A,B**). These data suggested that IL-36R ligands contributed to pathogenesis of lung injury and mortality induced by influenza virus despite a beneficial role in viral clearance.

Reduced lung leukocyte influx and activation in IL-36R$^{-/-}$ mice during influenza virus infection

We next quantified differences in lung inflammatory cell accumulation in WT and IL-36R$^{-/-}$ mice during influenza virus infection. No difference in number of total BAL leukocyte and proportion of monocytes/macrophages were observed in uninfected WT and IL-36R$^{-/-}$ mice at baseline. After inoculation of influenza virus, total number of lung leukocytes in BAL fluid increased in a time-dependent manner (**Figure 5a**). At day 2 and day 6, the number of total leukocyte, neutrophils, and monocytes/macrophages in BAL fluid was significantly reduced in IL-36R$^{-/-}$ mice as compared with their WT counterparts.

Lymphocytes, especially CD8 T cells, contribute to influenza virus clearance during infection. Murine lymphocytes including CD4 T cells, CD8 T cells, NK cells, and γδT cells express the IL-36 receptor, and IL-36 cytokines can directly influence activation and proliferation of selected T-cell populations.[25] We therefore examined the accumulation and activation of specific lymphocyte populations by flow cytometry. At day 2 of influenza virus infection, the numbers of CD4+ T cells, CD8+ T cells, NK cells, NKT cells, and γδ T cells were significantly reduced in infected IL-36R$^{-/-}$ mice than in WT animals. Moreover, decreased numbers of activated lymphocyte populations, as identified by co-expression of activation marker CD69, were observed in IL-36R deficient mice. In contrast, total numbers and numbers of activated CD4+ and CD8+ T cell were considerably greater in IL-36R$^{-/-}$ mice than in WT mice at day 6 (**Figure 5b**).

IL-36R$^{-/-}$ mice exhibit reduced early cytokine and chemokine production during influenza virus infection

Cytokines and chemokines contribute to control of viral replication via activation of both myeloid and parenchymal cells. However, these cytokines can also contribute to pathophysiology in patients with severe influenza virus infection.[26] Therefore, we examined whether IL-36R genetic
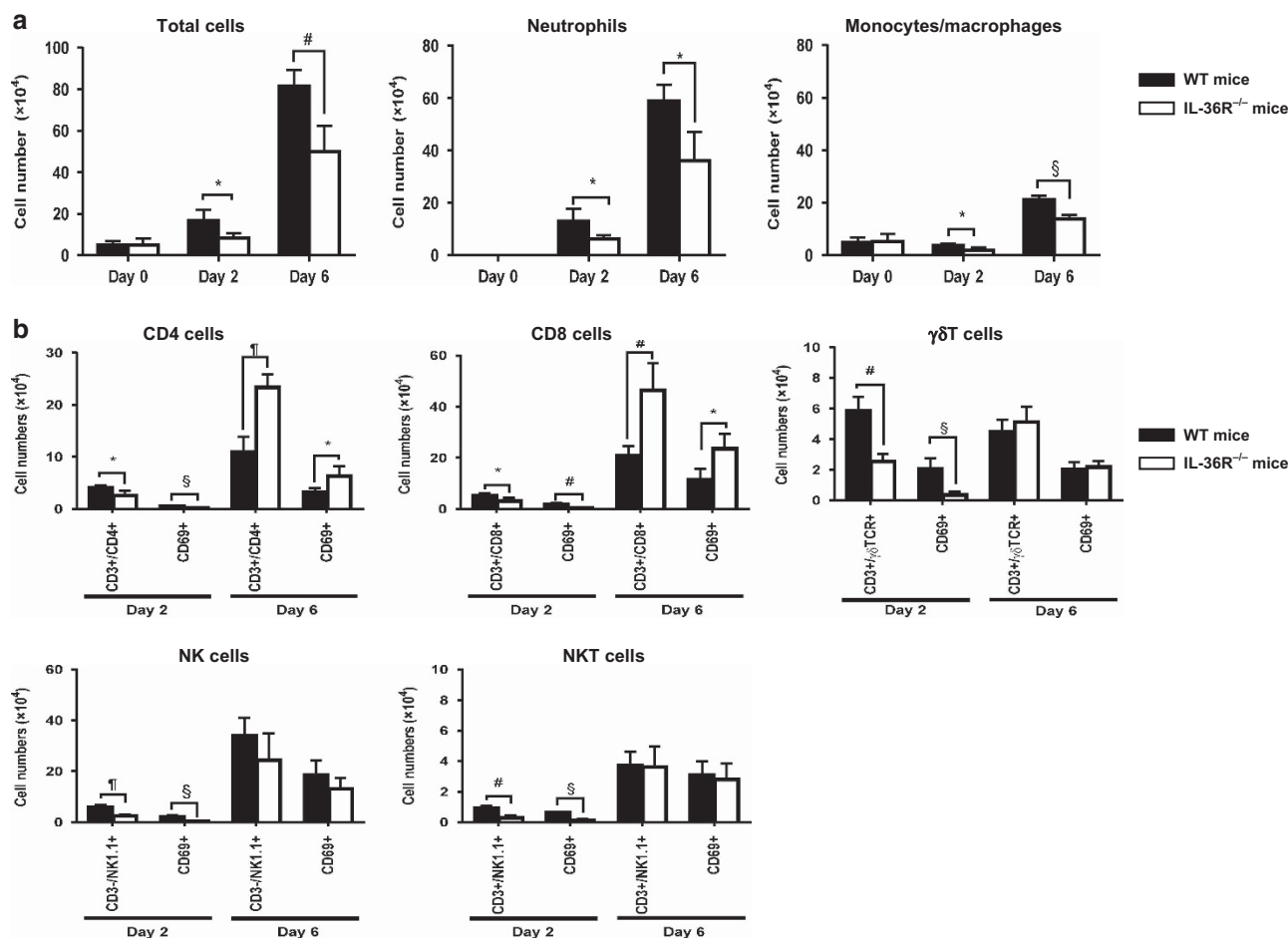
Figure 5 Effect of IL-36R gene deletion on lung leukocytes influx and lymphocyte activation during influenza virus infection. (**a**) BAL sample were collected at day 0, day 2, and day 6 after influenza virus infection and the number of total cell, neutrophils, and monocytes and macrophages were counted. (**b**) The number of CD4 + T cells, CD8 + T cells, NK cells, NKT cells, and $\gamma\delta$ T cells, and CD69-positive lymphocytes was determined by flow cytometry. Lung digests were collected at day 2 and day 6 post-infection. Each group consisted of four mice. Data (means ± s.d.) are representative of two independent experiments. *$P < 0.05$, #$P < 0.01$, §$P < 0.001$, ¶$P < 0.0001$ as indicated. BAL, bronchoalveolar lavage; IL-36, interleukin-36.

deficiency altered cytokines/chemokine production during influenza virus infection. BAL samples were collected from influenza virus-infected mice at day 2 and day 6. At day 2, BAL levels of IL-6, IL-17, KC/CXCL1, and IP-10/CXCL10 were significantly lower in IL-36R $^{-/-}$ mice compared with infected WT mice (**Figure 6**). By comparison, there was no difference in BAL fluid IFN-γ, TNF-α, and CCR2/MCP-1 levels (see **Supplementary Figure S2A**) between WT and IL-36R $^{-/-}$ mice during the course of infection. No difference in the levels of cytokines/chemokines was noted at day 6.

IL-36α enhanced cytokine/chemokine production from AECs and PMs

To examine whether IL-36α might promote the production of cytokines and chemokines by cells present within the alveolus in either an autocrine or paracrine fashion, primary AECs and PMs were isolated from WT and IL-36R $^{-/-}$ mice, then treated with mrIL-36α for 24 h, and the production of IL-6 and KC quantitated. Cells from IL-36R $^{-/-}$ mice were used as a control to exclude non-specific stimulation (e.g. LPS contamination).

Treatment with rIL-36α induced the production of IL-6 and CXCL1/KC from WT AECs and PMs. This effect was dependent upon IL-36R signaling, as no induction of IL-6 and KC was observed in AECs and PMs isolated from IL-36R $^{-/-}$ mice (**Figure 7a,b**).

Finally, using an *in vitro model* of cell-to-cell networking, we examined whether MPs released from influenza-infected AECs might stimulate the production of IL-6 and KC by PMs in an IL-36R-dependent fashion. AECs isolated from WT mice were treated with or without influenza virus for 48 h, then MPs isolated from CM by ultracentrifugation. We next incubated primary PMs isolated from WT and IL-36R $^{-/-}$ mice with AECs-derived MPs. Because influenza virus-mediated MPs contained live influenza virus (**Figure 3d,e**), we first examined the production of IL-6 and KC in response to influenza virus between PMs isolated from WT and IL-36R $^{-/-}$ mice, and no differences were observed (data not shown). The induction of IL-6 and KC from PMs was significantly greater when incubated with MPs recovered from influenza virus-treated AECs as compared with MPs isolated from uninfected AEC.
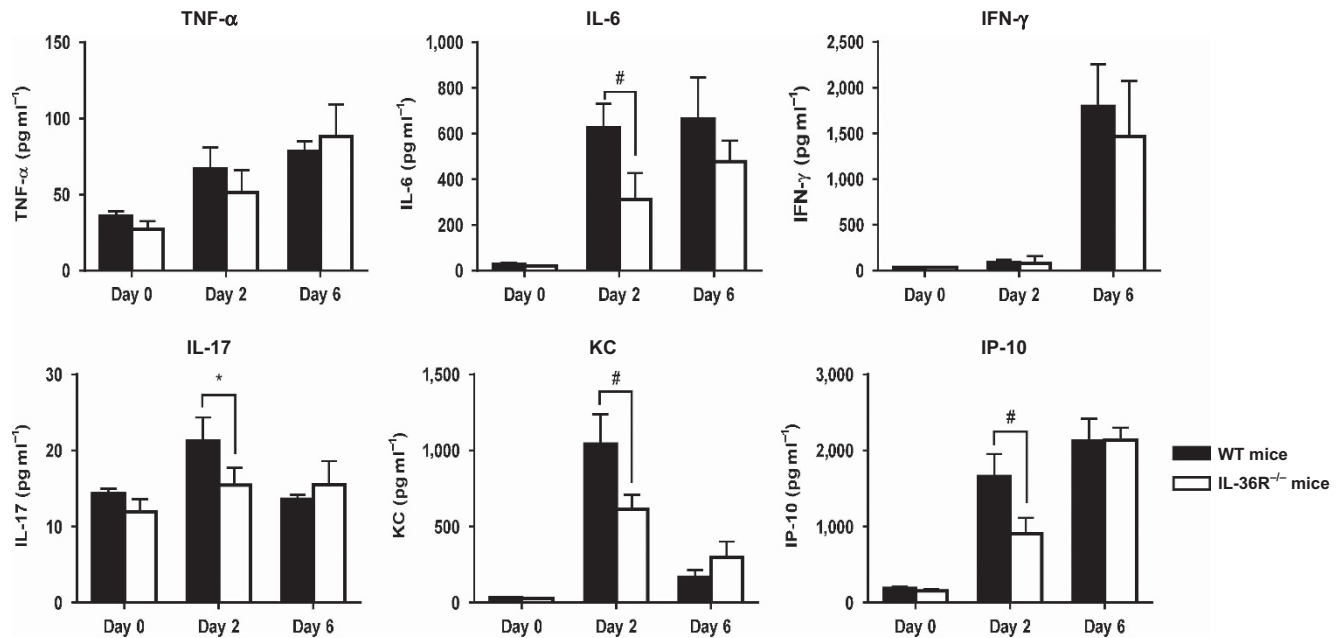
Figure 6 Effect of IL-36R gene deletion on the production of pro-inflammatory cytokines and chemokines during influenza virus infection. BAL sample were collected at day 0, day 2, and day 6 after influenza challenge. Levels of immunoreactive TNF-α, IL-6, IFN-γ, IL-17, KC/CXCL1, and IP-10/CXCL10 were measured by ELISA. Each group is consisted of four mice. Data (means ± s.d.) are representative of two independent experiments. *$P < 0.05$, #$P < 0.01$ as indicated. BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; TNF-α, tumor necrosis factor-α.

Moreover, we found that the MP-stimulated production of IL-6 and KC from PMs was IL-36R dependent, as MPs failed to induce cytokine production in PMs isolated from IL-36R$^{-/-}$ mice (**Figure 7c**).

DISCUSSION

Influenza virus-induced lung injury is characterized by the disruption of the alveolar-capillary membranes and pulmonary edema.[26] Both bronchial and AECs play an important role in the immune response against influenza virus. If the host immune response and virus replication is not adequately controlled at infectious site, excessive inflammation can lead to epithelial cell death and tissue damage. In this study, we demonstrate that IL-36R ligands released from AECs and possibly other lung cells contribute to influenza virus-induced lung injury.

Influenza virus is single-stranded (ss) RNA virus, but produces double-stranded (ds) RNA intermediates during replication. TLR-3 and TLR-7 recognized ssRNA and dsRNA, respectively, and two distinct innate immune receptor-mediated signaling pathway, TLR-3-TRIF and TLR-7-MyD88, induced production of type-1 interferon or pro-inflammatory cytokines.[27] We have previously demonstrated that the induction of IL-36γ in PMs by LPS and whole bacteria was MyD88 dependent.[22] Our results indicate that TLR-4 and TLR-7, but not TLR-3, mediated the upregulation IL-36α mRNA in AECs (**Figure 2b**), and suggest that MyD88-, rather than TRIF signaling is required for induction of IL-36α message in AECs. It is known that secretion of IL-1β and IL-18 from influenza virus-infected cells depends on intracellular

caspase-1 activity via NLRP-3 inflammasome components.[27] We observed that treatment with a caspase-1 inhibitor suppressed IL-36α mRNA expression in response to influenza virus (**Figure 2d**), suggesting that induction of IL-36α mRNA in influenza virus-infected AECs is regulated by caspase-1 activity. Previous studies have demonstrated that caspase-3/7 activation induced by either ATP[8,16] or TLR-3[17] signal was required for secretion of IL-36 cytokines. Our results are consistent with previous studies demonstrating the requirement for viral replication, indicating a contribution of both TLR-7 and TLR-3 signal for maximal secretion of IL-36α.

MPs have been detected in patients with sepsis and acute respiratory distress syndrome. These structures are released from both apoptotic and activated cells in response to various stimuli such as LPS, cytokines and cellular stress. During sepsis, MPs have been associated with endothelial injury and vascular tone through pro-inflammatory pathways, pro-coagulation activity, and both oxidative and nitrogen stress.[21] However, little is known regarding the role of pathogen-mediated MPs in infectious disease. We found that influenza virus-induced IL-36α was contained within MPs shed from AECs (**Figure 3b,c**), as well as MPs in BAL fluid during influenza virus infection *in vivo* (**Figure 1c**). Furthermore, IL-36α packaged within MPs enhanced the production of IL-6 and KC by PMs raising the possibility of autocrine and paracrine networking between cells within the alveolar space (**Figure 7c**). In addition, we found that live influenza virus was packaged within virus-induced MPs (**Figure 3c, d**). Thus, MPs containing both IL-36α and influenza virus together have the potential of amplifying
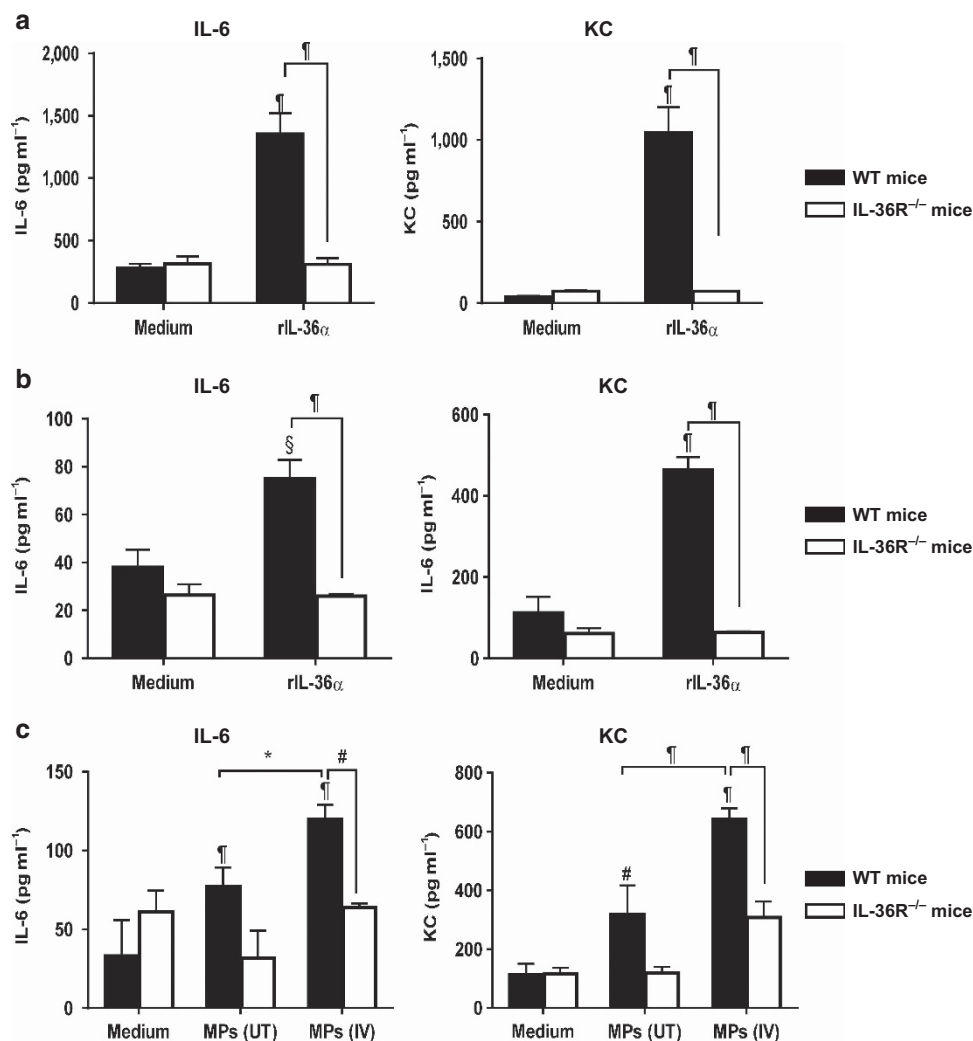
Figure 7 IL-36α induced production of cytokine and chemokines from primary AECs and PMs. Primary AECs and PMs isolated from WT and IL-36R$^{-/-}$ mice were treated with recombinant IL-36α (100 ng ml^{-1}) for 24 h (**a,b**). The production of IL-6 and KC by AECs (**a**) and PMs (**b**) were measured by ELISA. (**c**) AECs isolated from WT mice were treated with or without influenza virus. After 48 h incubation, MPs were isolated by ultracentrifugation method. Primary PMs isolated from WT and IL-36R$^{-/-}$ mice were treated with influenza virus-mediated and untreated MPs for 24 h. The production of IL-6 and KC in CM was measured by ELISA. rIL-36α, recombinant IL-36α; MPs (UT), MPs from untreated AECs; MP (IV), MPs from influenza virus-treated AECs. Data (means ± s.d.). *$P<0.05$, #$P<0.01$, §$P<0.001$, ¶$P<0.0001$ compared with medium or as indicated as indicated. AEC, alveolar epithelial cell; ELISA, enzyme-linked immunosorbent assay; IL-36α, interleukin36α; MP, microparticle; PM, PM, pulmonary macrophage: WT, wild type.

inflammatory responses in the lungs, which in the setting of influenza infection results in adverse consequences.

We found that lung injury was mitigated in IL-36R$^{-/-}$ mice, as indicated by less albumin concentration (**Figure 4f**), histological lung injury score (**Figure 4d, e**), and lung epithelial cell apoptosis (see **Supplementary Figure 2A,B**). Reduction in lung injury in IL-36R$^{-/-}$ mice was associated with significantly decreased early accumulation and activation of inflammatory cells, including monocytes, neutrophils, NK, and lymphocyte populations. Previous studies demonstrated that CCR2-positive exudative monocytes influx into alveolar space and contributed to lung epithelial cell damage through TRAIL-mediated apoptosis after influenza virus infection.[28,29] However, we found no differences in the CCR2-ligand MCP-1 production or in the expression level of TRAIL mRNA in the

lungs between influenza virus-infected lungs of WT and IL-36R$^{-/-}$ mice (see **Supplementary Figure 3A,B**). Macrophages and monocytes express IL-36 receptor, and changes in macrophage activational state may be IL-36-dependent. However, there was no difference in NO activity, which is an activate marker of macrophages/monocytes, between two groups (see **Supplementary data S3C**). These data suggest that the mechanism of reduced injury/apoptosis of lung epithelial cells in influenza virus-infected IL-36R$^{-/-}$ mice is not due to differences in TRAIL-mediated apoptosis.

A reduction in neutrophil influx was noted in the lungs of IL-36R deficient mice during influenza infection (**Figure 5a**). Exuberant recruitment and activation of neutrophils is considered to play a key role in progression of acute lung

injury/acute respiratory distress syndrome in several disease conditions.[30] In influenza infection, neutrophils have been shown to promote epithelial–endothelial barrier damage by the production of neutrophils extracellular traps,[31] reactive oxygen species,[32] and myeloperoxidase.[33] In flu-induced acute respiratory distress syndrome patients, IL-17, CXCL8/ IL-8, and CXCL10/IP-10 have been shown to be elevated in serum and BAL fluid and levels correlated with disease severity.[3,34] IL-17 and CXC chemokines are known to be key mediators of neutrophil migration. In addition, neutrophils express CXCR3, and IP-10/CXCL10 can stimulate the respiratory burst in neutrophils.[35] IL-17 is known to be responsible for upregulation of CXCR2 ligands such as KC[36] as well as the CXCR3 ligand IP-10.[37] In murine influenza infection, both IL-17 receptor and IL-17A-deficient mice showed increased survival rate and decreased lung injury, which occurred in association with reduced neutrophil accumulation.[38,39] In addition, mice treated with IP-10 antibody and IP-10 KO mice attenuated the mortality of influenza virus induced lung injury.[35,40] We found that the early production of IL-17, KC, and IP-10 at day 2 was significantly lower in IL-36R$^{-/-}$ mice as compared with influenza-infected WT mice. In influenza virus infection, the cellular source of IL-17 in the lungs is believed to be $\gamma\delta$T cells.[38,41] These cells also express the IL-36 receptor, and stimulation with IL-36 cytokine directly promotes IFN-γ production.[25] Importantly, we found that the absolute number of $\gamma\delta$ T cells and CD69 + $\gamma\delta$ T cells in the lungs are much lower in IL-36R$^{-/-}$ compared with WT at day 2 post infection, which may account for attenuated production of IL-17 in IL-36R$^{-/-}$ mice after influenza virus infection. We also observed that IL-36α directly induces KC production from AECs and PMs (**Figure 7a,b**). Since murine neutrophils do not express the IL-36 receptor,[42] IL-36R ligands are unlikely to directly mediate neutrophil accumulation and activation.

Of interest, we observed impaired viral clearance in IL-36R$^{-/-}$ mice. Our results are in line with the findings of others showing that influenza virus induced mortality does not necessarily correlate with lung viral clearance.[43–45] A fine balance exists between optimal immune clearance of virus and immune-mediated lung tissue damage. Neutrophils and exudate macrophages contribute to viral clearance, and reductions in these cell populations in IL-36R$^{-/-}$ mice may account for both attenuated lung injury responses and reduced viral clearance. A previous *in vitro* study demonstrated that IL-36γ directly promotes naïve CD8 + T cells proliferation and differentiation to effector CD8 + T cells,[25] thus IL-36 receptor ligands may be considered to contribute to virus clearance due to lymphocytes accumulation and antigen-specific activation at the infectious site. It is tempting to postulate that viral clearance was delayed in IL-36R$^{-/-}$ mice due to early reduction in the number of activated CD4 + and CD8 + T lymphocytes, cells known to contribute to viral clearance. On the other hand, at day 6 after infection, the number of total and CD69 + CD4 + and CD8 + T cells was much greater in IL-36R$^{-/-}$ mice. This paradoxical increase in CD4 and CD8 accumulation in IL-36-deficient mice at later time points remains unexplained. One possibility is that higher viral titers as a consequence of reduced early inflammatory cell accumulation (PMN, mononuclear cells) may drive lymphocyte accumulation/activation at later time points, which occurs in an IL-36-independent fashion. Finally, we observed reductions in number and activation of $\gamma\delta$ T cells and more modest reductions in NK and NKT cells at early time points in IL-36R-deficient mice, although the contribution of these cell populations to viral clearance and influenza-induced lung injury is less well defined. Regardless, our findings indicate that although IL-36 agonists promote antiviral clearance responses in the lung, they also promote deleterious lung injury culminating in death.

This study has some limitations. Most notably, we did not precisely identify the IL-36 receptor ligand responsible for the effects observed in our model. Because influenza infection did not stimulate IL-36γ secretion by virus-infected AECs and PMs, and mortality in IL-36γ KO mice did not differ from WT mice during influenza virus infection, we speculate that IL-36α rather than IL-36γ plays a central role in influenza virus-induced lung injury. Unfortunately, the polyclonal IL-36α Ab generated for antigen detection was non-neutralizing and no IL-36α KO mice is yet available. In addition, there is some uncertainty as to cellular sources of IL-36α other than AECs in our model. A recent study demonstrated that neutrophils expressed IL-36 cytokines in a murine autoimmune encephalitis model.[42]

In conclusion, our findings indicate that IL-36 agonists released from AECs and almost certainly other lung cells play a pivotal role in influenza virus-induced lung injury. Further studies will focus on identifying IL-36R ligands in humans with influenza virus-induced lung injury and correlation with relevant clinical outcomes.

METHODS

Mice. Specific pathogen-free age- and sex-matched C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-36R$^{-/-}$ mice on C57BL/6 background were provided by Jennifer Towne from Amgen (Thousand Oaks, CA).[10] A colony of IL-36$\gamma$$^{-/-}$ mice bred on a C57BL/6 background was established at the University of Michigan (Ann Arbor, MI).[22] All mice were housed in specific pathogen-free conditions within the University of Michigan Animal Care Facility. Animal studies were reviewed and approved by the University Committee on Use and Care of Animals at the University of Michigan.

Virus infection _in vivo_. Influenza A/Puerto Rico/8/34 virus was used for all of experiments. For intranasal infection, mice were anesthetized by intraperitoneal injection of ketamine and xylazine and then infected by intranasal administration of $2 \times 50\%$ lethal dose (LD$_{50}$: 40 pfu) of virus in 30 μl of saline.

Bronchoalveolar lavage and lung sampling. Mice were killed by CO$_2$ inhalation. The trachea was exposed and cannulated with a 22G intravenous catheter. BAL was performed with 1.5 ml phosphate-buffered saline (PBS) (three aliquot 500 μl of PBS), and then pulmonary circulation was rinsed by 1 ml PBS. Right lungs were harvested to determine the virus titer and stored at $-80\,^{\circ}$C. Left lungs were harvested for RNA extraction, immediately snap-frozen in liquid nitrogen. After a collection of leukocytes in BAL fluids, cytospin (1000 r.p.m. $\times$ 5 min) preparations were made from each sample and stained with modified Wright stain. Differential cell counts of

neutrophils and monocytes and macrophages were obtained for at least 400 cells counts in each sample at a magnification of × 1,000.

Histopathological examination. Lungs and trachea were removed from killed animals and inflated at 20cm H_2O with 4% paraformaldehyde through trachea, and fixed for paraffin embedding. All lungs were sectioned and stained with hematoxylin and eosin. Quantitative analysis of tissue injury was measured using the lung injury scoring system as described.[24] Lung injury scoring system parameters include neutrophils in the alveolar space (A), neutrophils in the interstitial space (B), hyaline membranes (C), proteinaceous debris filling the airspaces (D), and alveolar septal thickening (E). At least 20 random regions were scored 0–2 independently at a magnification of × 400 in a blinded fashion. The final lung injury score per each lungs was calculated as below: score = ((20 × A) + (14 × B) + (7 × C) + (7 × D) + (2 × E))/(number of fields × 100).

Virus titer. Madin-Darby canine kidney (MDCK) cells were plated at 1×10^6 cells in a flat bottomed six-well plate 24 h before infection. Supernatant from right lung homogenates serially diluted were used to infect the MDCK cells at 37 °C for 1 h. The cells were subsequently overlaid with Dulbecco's Modified Eagle Medium mixed 0.4% agarose (Sigma-Aldrich, St Louis, MO) in the presence of 0.5 μg ml⁻¹ Trypsin (Sigma-Aldrich) and incubated for 96 h. The plaques were visualized by staining the cells with crystal violet.

Total lung leukocyte. Lung leukocytes were prepared as described in our previous study.[46] Briefly, lungs were homogenized in 15 ml of digestion buffer (RPMI1640 medium supplemented with 5% fetal calf serum and containing 1 mg ml⁻¹ collagenase (Roche Diagnostics, Mannheim, Germany) and 30 μg ml⁻¹ DNase I (Sigma-Aldrich)) per lung and enzymatically digested for 60 min at 37 °C. Tissue fragments were removed by passing the mesh. Total lung cell pellet was resuspended in 4 ml of 40% (v/v) Percoll (Pharmacia, Uppsala, Sweden) and layered onto 4 ml of 80% (v/v) Percoll. After centrifugation at 2,000 r.p.m. for 20 min at room temperature, the cells were collected.

Murine PM and AEC isolation and culture. Murine PMs and type II AECs were isolated using the method described previously.[47] Briefly, PMs were isolated from dispersed lung homogenates by adherence purification and plated at a density 5×10^5 cells per ml⁻¹. For the isolation of murine AECs, the pulmonary vasculature was perfused. The lungs were filled via the trachea with 1.5 ml dispase (Worthington, Lakewood, NJ), then 1.5 ml of low-melting point agarose and finally placed in ice cold PBS. The lungs were submerged in dispase for 45 min at 24 °C before the lung tissue was teased from the airways and minced in Dulbecco's Modified Eagle Medium with 0.01% DNase. After swirling for 15 min, followed by passage through a series of nylon filters, the cell suspension was collected by centrifugation and incubated with biotinylated Abs (anti-CD32 and anti-CD45; BD Pharmingen, San Diego, CA). After incubation with streptavidin-coated magnetic particles, myeloid cells were removed using a magnetic tube separator. Mesenchymal cells were removed by overnight adherence in a Petri dish and the resulting non-adherent cells were plated on plastic dishes coated with fibronectin. Previous work has shown that the day 3 time point has > 90% pure AECs.[47] These cells were treated with influenza virus at a multiplicity of infection of 10–0.1, LPS (1 μg ml⁻¹) (Sigma-Aldrich), TLR-3 ligand: poly(I:C) (25 μg ml⁻¹), TLR-7 ligand: R843 (25 μg ml⁻¹) (Invivogen, San Diego, CA) and recombinant IL-36α (rIL-36α; R&D Systems Minneapolis, MN) (100 ng ml⁻¹). For blockade of caspase-1 and caspase-3/7, inhibitor of Caspase-1 and Caspase-3/7 (Cayman Chemical, Ann Arbor, MI), which were soluble in dimethyl sulfoxide (add vehicle control), was added to cultures 1 h before stimulation with medium and influenza virus. Final concentrations were 20 μM (caspase-1 inhibitor) and 100 μM (caspase-3/7 inhibitor).

Isolation of microparticles and EXs _in vivo_ and _in vitro_. We used a standard three-step differential centrifugation method to isolate MPs

and EXs _in vivo_ and _in vitro_ samples, as previously described.[22] BAL fluid and CM was centrifuged to remove cells and large debris at 3,000 r.p.m. for 10 min and stored these samples at − 80 °C overnight. Each sample supernatant was centrifuged at 17,000 r.p.m. for 45 min to obtain the MPs. After collecting the supernatant, the pellet containing the MPs fraction was suspended in 100 μl of lysis buffer or PBS. For the final step, each supernatant was centrifuged at 28,000 r.p.m. for 90 min to isolate EXs. The pellet was suspended in 100 μl of lysis buffer or PBS. In some experiments, MPs were washed twice by Dulbecco's Modified Eagle Medium and suspended 100 μl of Dulbecco's Modified Eagle Medium and cultured with MDCK cells. MPs, EXs, and the post-spin fluid in which MPs and EXs were removed by ultracentrifugation were stored in − 80 °C.

Flow cytometry analysis. Cells, isolated from whole lung or MPs and EXs as described above, were stained with the following FITC- or PE- or PerCP-Cy5.5 labeling antibodies to CD3, CD4, CD8, NK1.1, γδTCR, CD69, and Annexin V (BD Pharmigen, San Jose, CA) and analyzed by Attune Acoustic Focusing Cytometer (Thermo Scientific-Applied Biosystems, Foster City, CA). Isotype control was used for all the samples. MPs and EXs were defined as Annexin V-positive cells. To measure the size of MPs and EXs, we used calibration beads from 0.1 to 3 μm in diameter, as previously described.[48,49]

Determination of cytokine production by enzyme-linked immunosorbent assay. IL-36α, secreted in fluid or contained in MPs and EXs, was measured by sandwich ELISA protocol. Other cytokines/chemokines (TNF-α, IL-6, IFN-γ, IL-17, KC and IP-10; R&D systems) and albumin (Albumin Quantification Kit: Bethyl Laboratories, Montgomery, TX) were quantified using a modified double-ligand method as described.

RNA isolation and real-time PCR. RNA was isolated and real-time quantitative RT–PCR was performed by AB Step One plus Real-Time PCR System (Thermo Scientific-Applied Biosystems). Predesigned primer and probes of targeted molecules and β-actin as a control were purchased from Integrated DNA Technologies (Coralville, IA). Quantification of β-actin and target genes in each sample set was performed by the standard curve method.

Western blotting. Cells were digested by RIPA buffer (Sigma-Aldrich) plus protease inhibitors and gels were subjected to electrophoresis as previously described.[47] Membranes were incubated with primary anti-IL-36α antibody (R&D systems; diluted 1:250), anti-Influenza virus M2 antigen antibody (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1: 1,000), or β-actin (Sigma-Aldrich; diluted 1:5,000), blots were incubated with a secondary antibody linked to HRP, and the signals were developed with an ECL (SuperSignal West Pico Substrate; Pierce Biotechnology, Rockford, IL).

Statistical analysis. Descriptive statics, such as means and standard deviations, were collected. The difference in survival rates was evaluated by the log rank test (Mantel-Cox). Two sets of values were evaluated by the Student's _t_-test, and more than three sets of value were evaluated by ANOVA, followed by the Turkey's multiple comparison test. Data analysis was conducted using GraphPad prism 6 (GraphPad Software, La Jolla, CA). A _P_-value of < 0.05 was considered satirically significance.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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