Essential role of IL-6 in protection against H1N1 influenza virus by promoting neutrophil survival in the lung

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Influenza virus infection is considered a major worldwide public health problem. Seasonal infections with the most common influenza virus strains (e.g., H1N1) can usually be resolved, but they still cause a high rate of mortality. The factors that influence the outcome of the infection remain unclear. Here, we show that deficiency of interleukin (IL)-6 or IL-6 receptor is sufficient for normally sublethal doses of H1N1 influenza A virus to cause death in mice. IL-6 is necessary for resolution of influenza infection by protecting neutrophils from virus-induced death in the lung and by promoting neutrophil-mediated viral clearance. Loss of IL-6 results in persistence of the influenza virus in the lung leading to pronounced lung damage and, ultimately, death. Thus, we demonstrate that IL-6 is a vital innate immune cytokine in providing protection against influenza A infection. Genetic or environmental factors that impair IL-6 production or signaling could increase mortality to influenza virus infection.

INTRODUCTION

Influenza virus infection is a major worldwide public health problem. Without considering the highly pathogenic strains of influenza virus (e.g., H5N1), seasonal infections with the most common influenza virus strains (e.g., H1N1) cause significant mortality with >40,000 deaths per year and >250,000 hospitalizations in the United States alone. Although seasonal infections with the most common influenza virus strains can usually be resolved by the immune system without major sequelae, in some cases, the infection cannot be controlled, virus clearance is impaired, and death ensues.[1] It remains unclear why certain populations (other than elderly and infants) have serious difficulties in the resolution of influenza virus infection, and what factors influence the outcome of the infection. Thus, during the 2009 H1N1 pandemic, it was found that African Americans are more susceptible and had more severe complications than did Caucasians.[2,3]

Although adaptive immune response is essential for developing immunological memory, it is not the primary mechanism of defense during primary influenza infection.[4,5] Instead, the innate immune response is considered the major mechanism in the resolution of influenza infection.[6] Recent studies have given evidence that macrophages and neutrophils can provide protection against primary influenza infection. Macrophages contribute to elimination of the influenza virus through release of inflammatory cytokines and interferon (IFN)α/β, and depletion of macrophages has been shown to increase susceptibility to H3N2 virus infection in mice.[7] Although lung neutrophilia is often associated with lung pathology (e.g., acute lung injury),[8] a number of recent studies support a protective role for neutrophils in infection with the influenza virus by helping with virus clearance.[9,10] The elevated levels of proinflammatory cytokines (tumor necrosis factor-α, interleukin (IL)-1β, and IL-6) during influenza infection have also been primarily associated with increased lung pathology and worse outcome,[11] but it is uncertain whether some of these cytokines could also be protective during seasonal influenza infection.

IL-6 is produced by macrophages, dendritic cells, mast cells, and other innate immune cells, and consequently it has long

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been considered a marker of inflammation. The increased levels of IL-6 found in a number of diseases are mostly defined to be the result of ongoing inflammatory cell activation. However, in addition to immune cells, IL-6 can also be produced by non-immune cells such as epithelial cells, endothelial cells, keratinocytes, and fibroblasts among others, in response to specific stimuli.[12,13] A number of studies have shown that human bronchoepithelial cell lines can produce IL-6 in response to different exposures such as allergens or respiratory viruses.[14,15] We have recently demonstrated that mouse primary lung epithelial cells, but not lung resident immune cells, exhibit a constitutive expression of the *IL-6* gene before exposure to any environmental insult.[16] Thus, the presence of IL-6 may not necessarily correlate with the production of other inflammatory cytokines and it may not be just a marker of ongoing inflammation, but a direct player in the immune response. A number of studies have shown a role of IL-6 in the adaptive immune response, primarily on the differentiation fate of CD4 T cells,[17] but IL-6 can also modulate aspects of the innate immune response.[12,13]

Elevated levels of IL-6 in the lung and in serum have been found in patients infected with the influenza virus, including the 2009 H1N1 pandemic influenza.[18,19] However, it is unknown whether IL-6 in these patients contributes to the lung pathology caused by the virus or whether it is elevated as a protective mechanism and eliminating IL-6 could worsen the course of the infection. This study shows that, instead of being pathogenic, IL-6- and IL-6-mediated signals are essential for survival to a non-lethal dose of influenza H1N1 virus infection. Deficiency of IL-6 or IL-6R prevents clearance of the H1N1 virus in association with low numbers of neutrophils present in the lungs of infected mice. We also show that IL-6 provides survival signals to protect neutrophils from influenza virus-triggered apoptosis. Impaired virus clearance caused by the lack of IL-6 or IL-6R signals leads to emphysema-like destruction of the lung and, ultimately, death. Thus, IL-6 is a protective factor against primary infection with the influenza H1N1 virus by promoting the innate phase of the immune response and virus clearance.

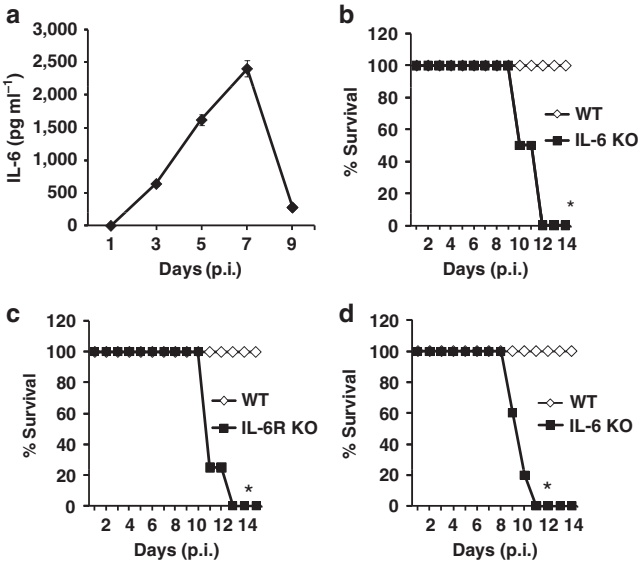

Figure 1 IL-6 and IL-6R are essential to survive infections with sublethal doses of the influenza H1N1 virus. (**a**) Wild-type mice ($n=4$) were infected with a sublethal dose of the H1N1 PR8 virus (3×10^3 EIU) intranasal (i.n.). IL-6 levels in bronchoalveolar lavage fluid (BALF) of infected mice were determined at the indicated periods of time p.i. by Luminex. Values represent mean±s.d. (**b**) Kaplan–Meier survival curve of wild-type mice (WT) and IL-6 KO mice ($n=4$) after i.n. infection with the H1N1 PR8 virus (3×10^3 EIU). (**c**) Kaplan–Meier survival curve of WT and IL-6R KO mice ($n=5$) infected with the H1N1 PR8 virus as described in panel **a**. (**d**) Kaplan–Meier survival curve of WT and IL-6 KO mice ($n=5$) infected with a sublethal dose of the A/California/7/2009 H1N1 influenza virus (3×10^3 EIU). * Denotes $P<0.05$. Statistical significance was determined by log-rank test (panels **b**–**d**). IL, interleukin.

RESULTS

IL-6 and IL-6R are required for survival to primary infection with the H1N1 influenza virus

As elevated IL-6 levels have been found in the lungs of patients during influenza virus infection, we examined the regulation of IL-6 production in the lungs of mice infected with H1N1 influenza. Similar to what has been found in humans, infection of wild-type mice with a sublethal dose of the influenza A/Puerto Rico 8/34 (PR8) H1N1 virus promoted the release of IL-6 into the lung airways (**Figure 1a**). However, IL-6 accumulation did not follow rapid kinetics as the levels were not high until day 3 post infection (p.i.), and remained high at least until day 7 p.i. (**Figure 1a**). To determine whether IL-6 could have a role in either the pathogenesis or the resolution of infection, we examined the response of wild-type and IL-6-deficient mice[20] to infection with sublethal doses of the PR8 H1N1 influenza A virus. Although wild-type mice recovered from the infection as expected, all IL-6-deficient mice died between days 10 and 12 p.i. (**Figure 1b**). To further demonstrate that IL-6 signaling is necessary for survival from a sublethal dose of the PR8 virus, we also infected the recently generated IL-6 receptor (IL-6R)-deficient mice[21] with the PR8 H1N1 influenza virus. Similar to IL-6 knockout (KO) mice, IL-6R KO mice succumbed to the sublethal dose of virus between days 10 and 12 p.i. (**Figure 1c**). In 2009, a new subtype of H1N1 influenza ("swine" flu) emerged that was a reassortment of swine, avian, and human H1N1 viruses. It led to a significant increase in influenza-related morbidity and mortality during the 2009 influenza pandemic.[22–24] Therefore, we examined the role of IL-6 during infection with a sublethal dose of the A/California/7/2009 H1N1 isolate of the 2009 pandemic H1N1 influenza virus.[25] As with the PR8 H1N1 subtype, virus infection in wild-type mice was followed by 100% recovery, whereas IL-6 KO mice again succumbed to the 2009 H1N1 virus between days 9 and 11 p.i. (**Figure 1d**). Taken together, these results demonstrate that IL-6 is a key cytokine for survival from primary infection with the H1N1 influenza virus.

H1N1 influenza virus infection in the absence of IL-6 leads to emphysema-like lung damage

Death due to influenza infection in humans is generally associated with the development of acute respiratory distress syndrome and respiratory failure.[26] To address whether mortality caused by H1N1 infection in IL-6-deficient mice could

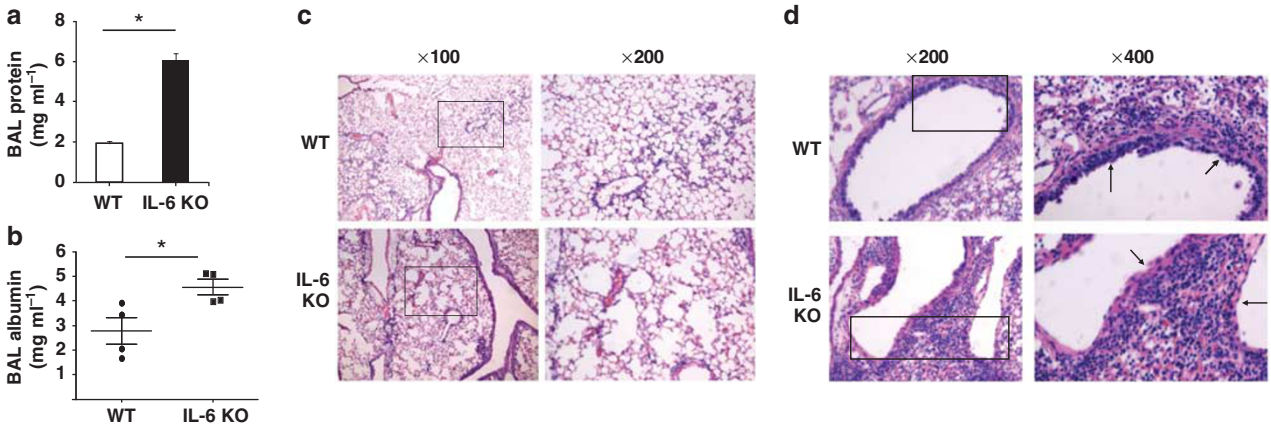

Figure 2 Severe lung damage in IL-6-deficient mice caused by H1N1 influenza virus infection. (**a** and **b**) Wild-type mice ($n=4$) and IL-6 KO mice ($n=4$) were infected with a sublethal dose of the H1N1 PR8 virus (3×10^3 EIU) intranasal (i.n.). Total protein (panel **a**) and albumin (panel **b**) concentrations in BALF were determined at day 9 p.i. (**c** and **d**) Histopathology of lungs from WT and IL-6 KO mice at day 9 p.i. with PR8 H1N1. In all, ×100 and ×200 (panel **c**) or ×200 and ×400 (panel **d**) magnifications of H&E-stained lung sections are shown. Arrows in panel **d** point to the epithelium of the airways. *$P<0.05$. Statistical significance was determined by Student's *t*-test (panels **a** and **b**). Results are representative of at least two independent experiments. BALF, bronchoalveolar lavage fluid; H&E, hematoxylin and eosin; IL, interleukin; p.i., post infection; WT, wild type.

be due to acute lung injury, we examined protein content in bronchoalveolar lavage fluid (BALF) as a parameter indicative of failed barrier function in the lung and lung damage. Increased levels of total protein (**Figure 2a**) and albumin (**Figure 2b**) were found in infected IL-6 KO mice compared with wild-type mice at the time IL-6-deficient mice were moribund (days 9–11 p.i.). To further assess lung damage during H1N1 infection, histological examination of lungs from infected mice was performed. No differences in the overall number of inflammatory cells in the lung were observed between wild-type and IL-6 KO mice. However, IL-6 KO mice were noted to have developed emphysema-like enlargement of the alveoli, a finding not present in wild-type mice (**Figure 2c**). This phenotype was seen in all examined IL-6 KO mice (**Supplementary Figure S1**), discounting a possible non-specific effect of the lung inflation. Furthermore, the enlarged alveoli-phenotype was also found in the lungs of virus-infected IL-6R KO mice and absent in wild-type controls (**Supplementary Figure S1**). This pattern of lung damage was not detected in IL-6 KO mice at day 7 p.i., despite marked inflammation in the lung compared with mice at days 10–11 p.i. (**Supplementary Figure S2**), suggesting that this phenotype was probably associated with chronic viral infection instead of acute inflammation. In addition to the damage of the lung parenchyma, most airways in IL-6 KO mice appeared to be denuded of their epithelial lining compared with wild-type mouse airways (**Figure 2d**). Thus, mortality caused by H1N1 infection in the absence of IL-6 is associated with capillary leak and emphysematous destruction of the lung parenchyma.

IL-6 deficiency impairs the presence of neutrophils in the lung and influenza virus clearance

To investigate the potential causes of the increased H1N1-induced lung damage in IL-6 KO mice, we first examined viral titers in the lung during infection. As expected, the peak of virus titer in wild-type mice occurred around day 3, with clearance of virus by day 9 p.i. (**Figure 3a**). Viral titers in wild-type and IL-6 KO mice were comparable at days 3 and 5 p.i., indicating that viral replication and infectivity were not affected by the absence of IL-6. However, viral titers in IL-6 KO mice remained high even at day 9 p.i., showing that these mice were unable to clear the virus effectively.

As CD8 T cells can contribute but are not essential for influenza virus clearance during primary infection,[27] we examined the presence of virus-specific CD8 T cells in the lung draining mediastinal lymph node. We found that IL-6 deficiency did not affect the expansion of virus-specific CD8 T cells in wild-type vs. IL-6 KO mice infected with influenza (**Figure 3b**). Similarly, expansion of CD4 T cells was not significantly altered in the absence of IL-6 (**Supplementary Figure S3a**). B cells have been shown to provide protection against primary influenza virus infection[28] but neither their cell numbers (**Supplementary Figure S3a**) nor influenza-specific IgG (**Supplementary Figure S3b**) were significantly different between wild-type and IL-6 KO mice. In addition, the levels in BAL of IFNγ, normally associated with a strong antiviral response, were not significantly different between IL-6 KO and wild-type mice (**Supplementary Figure 3c**). Lymphocyte infiltration into the airways was also not affected by the absence of IL-6 (data not shown). Thus, an impaired adaptive immune response does not seem to underlie the inability of IL-6 KO mice to clear influenza virus infection in the lung.

The innate immune response is a major mechanism for influenza virus clearance during primary infection. Macrophages support influenza virus clearance through release of inflammatory cytokines and IFNα/β, and depletion of macrophages has been shown to increase susceptibility to H3N2 virus infection.[7] Nevertheless, analysis of BAL from infected wild-type and IL-6 KO mice showed no statistically significant differences in the percentage of macrophages during the course of infection (**Figure 3c**), and only a slight increase in macrophage number

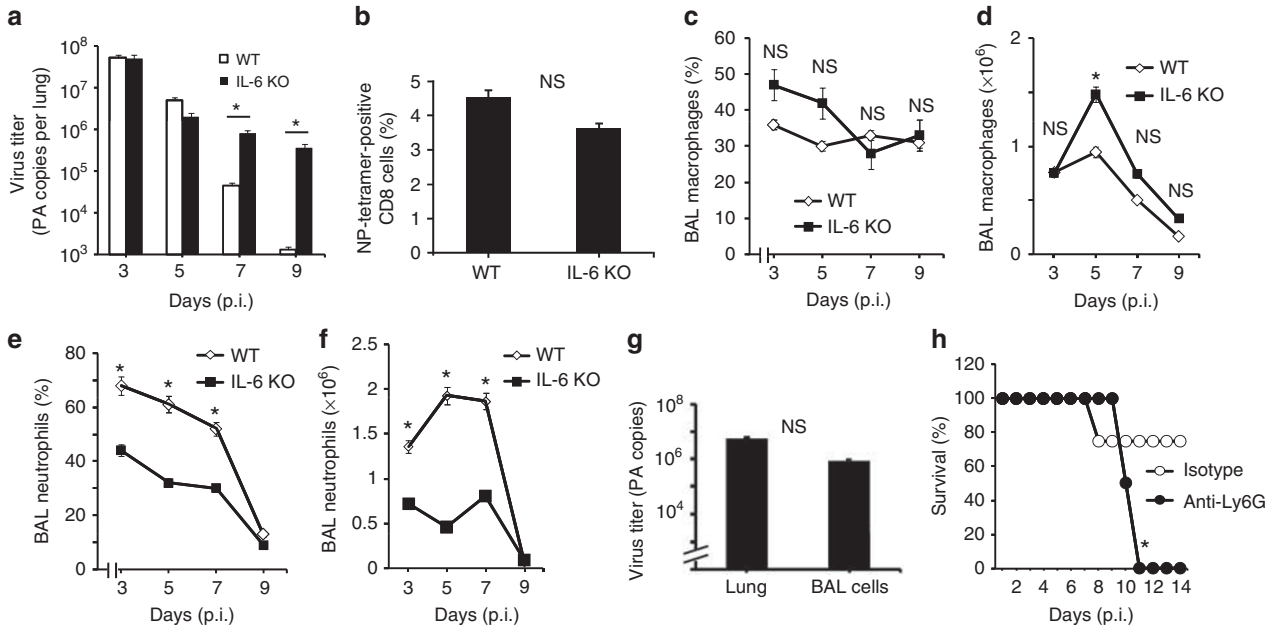

Figure 3 IL-6 deficiency results in insufficient neutrophils in the lung during influenza H1N1 virus infection and inability to clear the virus. (**a**) Virus titers determined by the number of influenza polymerase A (PA) RNA copies by real-time RT-PCR in total lung of WT and IL-6 KO mice ($n=5$) at different days p.i. with the H1N1 PR8 virus (3×10^3 EIU). (**b**) Percentage of CD8 T cells that were positive for nucleoprotein (NP) tetramers within the mediastinal lymph node from WT and IL-6 KO mice ($n=4$) 9 days p.i., as determined by flow cytometry analysis. (**c**–**f**) Percentage (panels **c** and **e**) and total number (panels **d** and **f**) of macrophages (panels **c** and **d**) and neutrophils (panels **e** and **f**) in BAL from WT and IL-6 KO mice ($n=5$) at different days p.i. with the H1N1 PR8 virus. (**g**) Virus titers determined by the number of influenza PA RNA copies in the total lung and in cells from BAL of WT mice ($n=4$) 5 days p.i. with PR8 virus. (**h**) Kaplan–Meier survival curve of WT mice ($n=5$) infected with the H1N1 PR8 virus that received an intraperitoneal administration (500 μg per mouse) of an anti-Ly6G Ab or an isotype control 1 day before infection and another administration at day 3 p.i. $^*P<0.05$. NS denotes not significant. Statistical significance was determined by two-way ANOVA (panels **a**, **c**–**f**), Student's *t*-test (panels **b** and **g**), or log-rank test (panel **h**). Results are representative of two to four independent experiments. ANOVA, analysis of variance; BAL, bronchoalveolar lavage; IL, interleukin; KO, knockout; p.i., post infection; RT-PCR, reverse transcription-PCR; WT, wild type.

in IL-6 KO mice at day 5 p.i. (**Figure 3d**). In contrast, there was a significant decrease in the percentage (**Figure 3e**) and total number (**Figure 3f**) of neutrophils in IL-6-deficient mice compared with wild-type mice as early as day 3 p.i. Thus, IL-6 deficiency selectively impairs the presence of neutrophils in the lung during influenza virus infection. The greatest difference in the number of neutrophils in the lungs of IL-6 KO vs. wild-type mice coincided with the most active period of virus clearance in wild-type mice at days 5–7 p.i.

Although accumulation of neutrophils in the lung is often associated with lung pathology (e.g., acute lung injury),[29] recent studies supported a protective role for neutrophils in infection with the influenza virus. Thus, depletion of neutrophils has been shown to be fatal in a model of H3N2 influenza virus infection and to increase severity of disease caused by PR8 virus infection.[10,30] Although neutrophils do not support virus replication, they have the ability to take up virus and they contribute to influenza virus clearance.[31,32] Analysis of H1N1 PR8 in BAL cells from mice 3 days p.i., when the majority of cells are neutrophils, indeed revealed virus copy numbers almost as high as those observed in the infected whole-lung tissue (**Figure 3g**). No difference in virus copy number in BAL cells was observed between wild-type and IL-6 KO mice at this time of infection (**Supplementary Figure S4**). To determine the importance of neutrophils in primary infection with a sublethal dose of the H1N1 influenza virus, neutrophils from wild-type mice were depleted with an antibody that recognizes the neutrophil-specific antigen Ly6G. Similar to IL-6 and IL-6R KO mice, anti-Ly6G-treated mice succumbed to the virus between days 10 and 12 p.i. (**Figure 3h**). These results demonstrate that neutrophils are indeed critical for the resolution of primary influenza A virus infection. Thus, impaired H1N1 virus clearance in the absence of IL-6 is likely due to insufficient neutrophil numbers in the lung during infection.

IL-6 is dispensable for neutrophil release and recruitment to the lung during influenza virus infection

To determine whether IL-6 deficiency could interfere with normal bone marrow generation of neutrophils causing mice to be neutropenic, we examined neutrophil levels in both blood and bone marrow from uninfected wild-type and IL-6 KO mice. No differences in neutrophil levels in bone marrow (**Figure 4a**) or peripheral blood (**Figure 4b**) were observed. To determine whether the absence of IL-6 could cause a decrease in neutrophil recruitment during viral infection, BALF from infected wild-type and IL-6 KO mice was collected at day 3 p.i. and levels of chemokines that mediate neutrophil recruitment were measured. No significant differences in BALF levels of MIP-2 or KC (two critical murine neutrophil chemoattractants) between wild-type and IL-6 KO mice were detected at day 3 p.i.

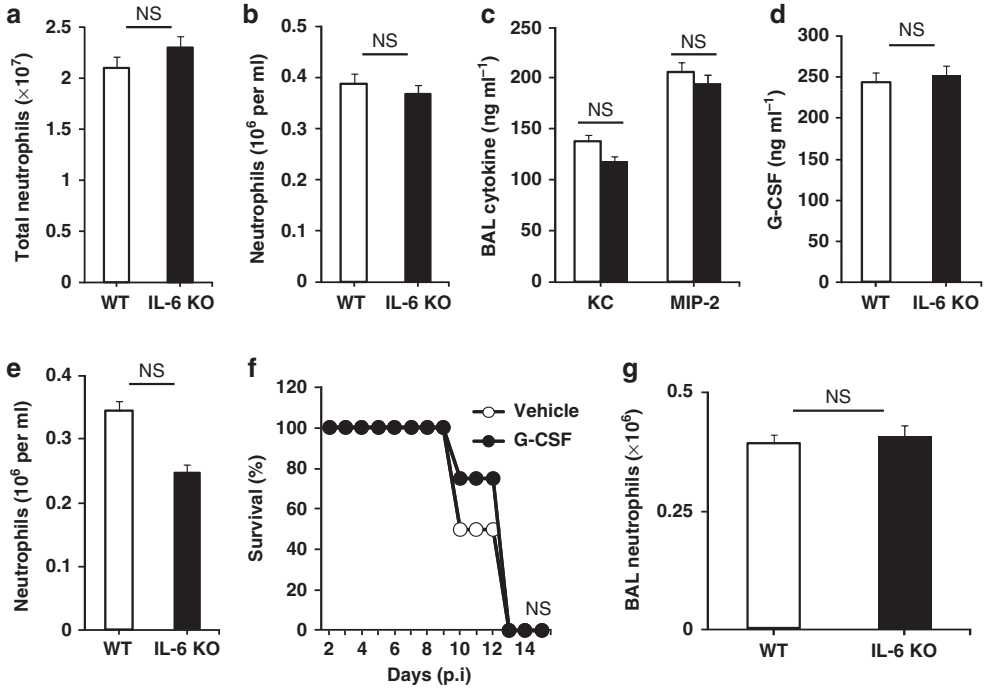

Figure 4 IL-6 deficiency does not interfere with neutrophil release or recruitment to the lung. (**a**) Neutrophil numbers in total bone marrow from non-infected WT and IL-6 KO mice ($n=3$). (**b**) Frequency of neutrophils (cell number per ml) in peripheral blood of non-infected WT and IL-6 KO mice ($n=3$). (**c**) Levels of KC and MIP-2, in BALF from WT and IL-6 KO mice ($n=5$) 3 days p.i. with the H1N1 PR8 virus. (**d**) Levels of G-CSF in BALF from WT and IL-6 KO mice ($n=5$) 3 days p.i. with the H1N1 PR8 virus. (**e**) Frequency of neutrophils (cell number per ml) in peripheral blood of WT and IL-6 KO mice ($n=4$) 3 days p.i. with the H1N1 PR8 virus. (**f**) Survival curve of IL-6 KO mice ($n=4$) administered i.p. with G-CSF (5 μg per mouse) or PBS control a day before infection and at day 3 p.i. (**g**) Neutrophils in BAL from WT and IL-6 KO mice ($n=3$) 24λh after administration of nebulized LPS. *$P<0.05$, NS denotes "not significant". Statistical significance was determined by Student's *t*-test (panels **a**–**e** and **g**), or log-rank test (panel **f**). Results are representative of at least two independent experiments. BALF, bronchoalveolar lavage fluid; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; i.p., intraperitoneal; KO, knockout; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; p.i., post infection; WT, wild type.

(**Figure 4c**) or at days 1 or 5 p.i. (data not shown). Granulocyte colony-stimulating factor (G-CSF) has been shown to induce the release of neutrophils from the bone marrow to the circulation during "stress granulopoiesis" in response to lung infection.[33] G-CSF levels in BALF were comparable between IL-6 KO and wild-type mice day 3 p.i. (**Figure 4d**), as well as at days 1 and 5 p.i. (data not shown). Furthermore, levels of circulating neutrophils in IL-6 KO mice 3 days p.i. did not differ from those found in infected wild-type mice (**Figure 4e**). To further address the potential role of G-CSF in influenza pathogenesis in the absence of IL-6, we supplemented IL-6 KO mice with G-CSF during infection. Treatment of IL-6 KO mice with G-CSF did not improve survival in these mice (**Figure 4f**), indicating that impaired G-CSF production is not the cause of increased mortality, and that enhanced neutrophil release from the bone marrow does not rescue these mice. Taken together, these results show that the effects of IL-6 deficiency during influenza infection are not due to impaired release of neutrophils from the bone marrow. We also examined neutrophil accumulation to the lungs after exposure to nebulized lipopolysaccharide, known to induce rapid recruitment of neutrophils to the lung.[34] No difference in neutrophil recruitment to the lungs was observed between wild-type and IL-6 KO mice after lipopolysaccharide administration (**Figure 4g**). Thus, IL-6 deficiency does not seem to interfere with neutrophil release from the bone marrow or recruitment to the lung, but interferes with the accumulation of neutrophils in the lung, suggesting an effect in neutrophil survival during influenza infection.

IL-6 protects neutrophils from influenza virus-induced death

Histological analysis of the airways of lungs from wild-type mice 5 days p.i. showed the presence of neutrophils in the airways (**Figure 5a**). Interestingly, few viable neutrophils were present in the airways of infected IL-6 KO mice (**Figure 5a**). Instead, there was an accumulation of pyknotic nuclei characteristic of neutrophil death. To determine whether IL-6 provides survival signals to neutrophils in the setting of influenza viral infection, neutrophils were isolated from uninfected wild-type mice, incubated *in vitro* with the PR8 influenza virus in the presence or absence of IL-6, and cell survival was assessed. The influenza virus enhanced neutrophil death at 12 h and few cells were recovered intact after 24 h (**Figure 5b**). However, the presence of IL-6 abrogated virus-induced death of neutrophils (**Figure 5b**). A slight decrease in spontaneous neutrophil death by IL-6 was also observed (**Supplementary Figure S5**). The effect of IL-6 in preventing neutrophil death caused by H1N1 infection was further delineated by analysis of lactate dehydrogenase (LDH) in culture supernatants as a parameter of cell lysis.

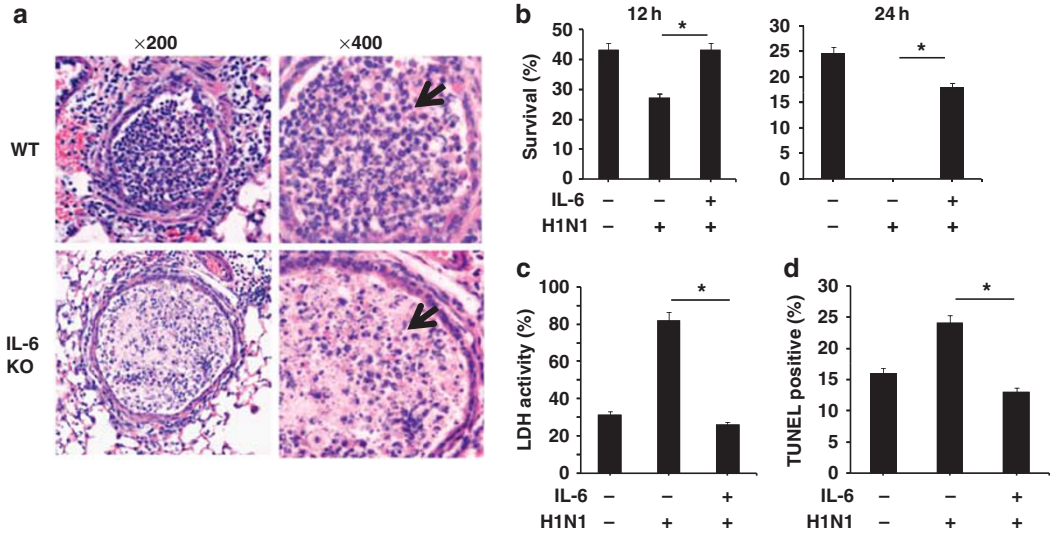

Figure 5 IL-6 protects neutrophils from H1N1 virus-mediated apoptosis. (**a**) Histopathology of lungs (H&E staining) from WT and IL-6 KO mice at day 5 p.i. with the PR8 H1N1 virus. In all, ×200 and ×400 magnifications of airways are shown. Arrows point to well-defined polymorphonuclear neutrophil in WT airways, and apoptotic nucleus in IL-6 KO airways. (**b**) Neutrophils from WT mice were cultured in the presence or absence of the PR8 H1N1 virus at 1:10 neutrophil:virus (EIU) ratio with or without IL-6 (20 ng ml^{-1}). After 12 and 24 h, live cells were counted by trypan blue staining. Values (mean±s.d., $n=3$) represent the frequency of live cells recovered relative to the initial number. (**c**) LDH activity in supernatants from neutrophils cultured as described in panel **b**. (**d**) Neutrophils were cultured as described in panel **b** and apoptosis was determined by TUNEL assay and flow cytometry analysis. Values show the percentage of TUNEL-positive cells (mean±s.d., $n=3$). *$P<0.05$. Statistical significance was determined by Student's *t*-test. Results are representative of 2–3 independent experiments. H&E, hematoxylin and eosin; IL, interleukin; KO, knockout; LDH, lactate dehydrogenase; p.i., post infection; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; WT, wild type.

High levels of LDH were detected in the supernatants of neutrophils incubated with the PR8 H1N1 virus alone (**Figure 5c**), but not in neutrophils incubated with the PR8 H1N1 virus in the presence of IL-6 (**Figure 5c**). Thus, the H1N1 virus promotes neutrophil death, but this effect can be prevented if IL-6 is present. To determine whether virus-induced neutrophil cell death occurred through apoptosis, TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) analysis was performed. The PR8 H1N1 virus increased the number of apoptotic neutrophils (**Figure 5d**), but this effect was abrogated with the addition of IL-6. Thus, the presence of IL-6 can provide survival signals to protect neutrophils from acute apoptosis triggered by influenza infection in the lung.

Downregulation of Mcl-1 and Bcl-X_L in neutrophils by the H1N1 virus is prevented by IL-6

Accumulation of reactive oxygen species (ROS) in the mitochondria in response to stimuli is known to promote neutrophil death.[35] We examined whether exposure of neutrophils to influenza virus could cause increased production of mitochondrial ROS using MitoSOX staining and flow cytometry analysis. Incubation of neutrophils with the PR8 H1N1 virus clearly increased mitochondrial ROS production (**Figure 6a**), but the presence of IL-6 had no effect on virus-induced mitochondrial ROS (**Figure 6a**). IL6-induced gp130/signal transducer and activator of transcription 3 signaling has been associated with chemokine production and trafficking of neutrophils, but not neutrophil apoptosis during acute inflammation.[36] Similarly, no effect of IL-6 on phosphorylation of signal transducer and activator of transcription 3 in neutrophils during exposure to influenza virus could be detected (data not shown). The balance of anti-apoptotic and pro-apoptotic members of the Bcl2 family has an essential role in determining cell survival or cell death. Mcl-1 is the most abundant anti-apoptotic member of the family in neutrophils,[37] and most stimuli that regulate neutrophil survival or death also modulate Mcl-1 expression. Mcl-1 has been shown to be critical for survival of neutrophils *in vitro* and *in vivo*.[38] Therefore, we examined the effect of the PR8 H1N1 virus and IL-6 on Mcl-1 levels in neutrophils. Exposure of neutrophils to the H1N1 virus caused a marked decrease in the levels of Mcl-1 (**Figure 6b**). Interestingly, the addition of IL-6 restored Mcl-1 levels (**Figure 6b**). In accordance with previous studies,[39] Bcl2 expression could not be detected in neutrophils under any conditions (data not shown). In contrast, other anti-apoptotic Bcl2 family member Bcl-X_L was found to be expressed in resting neutrophils, but was almost undetectable in cells exposed to H1N1 virus (**Figure 6b**). However, the presence of IL-6 also prevented the loss of Bcl-X_L caused by the PR8 H1N1 virus in neutrophils (**Figure 6b**). Thus, IL-6 prevents H1N1 virus-induced death of neutrophils by maintaining high levels of essential anti-apoptotic molecules in these cells.

DISCUSSION

Frequently, increased levels of proinflammatory cytokines in the lung are associated with exacerbated lung pathology (e.g., acute lung injury). We show that, similar to what is seen in humans, infection with the H1N1 influenza virus in mice causes a dramatic increase in the levels of pulmonary IL-6 and that these

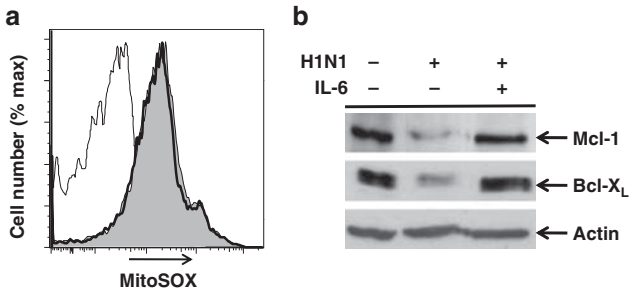

Figure 6 IL-6 prevents downregulation of anti-apoptotic molecules by influenza virus in neutrophils. (**a**) Neutrophils from WT mice were cultured in the presence or absence of the PR8 H1N1 virus at 1:10 neutrophil:virus (EIU) ratio with or without IL-6 (20 ng ml^{-1}). After 18 h, mitochondrial ROS was examined by staining with MitoSox-Red dye and flow cytometry analysis. Histogram profiles for neutrophils cultured in medium (thin line), with H1N1 virus (thick line) or H1N1 virus and IL-6 (filled) are shown. (**b**) Western blot analysis for Mcl-1 and Bcl-X_L in neutrophils from WT mice treated as described in panel **a**. Actin was used as loading control. IL, interleukin; ROS, reactive oxygen species; WT, wild type.

levels remain high during the period of virus clearance. Instead of being pathogenic, our studies here show that this increase in IL-6 levels is protective as IL-6 is essential for H1N1 influenza virus clearance and survival from infection. The emphysema-like lung damage that IL-6-deficient mice develop during H1N1 infection further supports a non-pathogenic, protective role of IL-6 in influenza infection. The role of IL-6 in influenza infection may be dependent on the influenza virus strain and the pathology caused by the virus. Previous studies have shown that IL-6 does not contribute to the pathogenesis of avian H5N1 influenza infection, which is primarily mediated by cytokine storm.[40,41] In addition, it does not seem to affect primary infection with a poorly pathogenic H3N2 strain (e.g., H17) that has minimal effect on lung function and host weight.[42] The effect of IL-6 on influenza infection differs from other classical pro-inflammatory cytokines as it is required for viral clearance. In contrast, loss of tumor necrosis factor-α does not impact viral clearance, whereas mice deficient in tumor necrosis factor-α and IL-1 receptors succumb to H1N1 infection similar to wild-type mice.[43,44] INFs are another class of cytokines considered vital in the antiviral response. Type I INFs promote expression of antiviral genes whereas IFNγ is mainly known for its immunmodulatory activity and is highly upregulated during influenza infection. However, inbred mouse strains are unable to express the antiviral *Mx1* gene after INF stimulation and therefore mice deficient in receptors for both IFN classes clear influenza virus similar to wild-type mice.[45] As C57BL/6 mice used here also lack a functional *Mx1* gene, the protective role of IL-6 is likely independent of IFN induction.

The new finding on the protective role of IL-6 against H1N1 influenza is highly relevant for seasonal influenza virus infection in humans as they indicate that levels of IL-6 may determine the outcome of infection. In light of broadly distributed polymorphisms in *IL-6* and *IL-6R* genes in the human population, these results suggest that genetic variations may affect influenza virus susceptibility and outcome. Interestingly, such polymorphisms in both *IL-6* and *IL-6R* genes have recently been found to associate with susceptibility to respiratory tract infection.[46]

Together with macrophages, neutrophils are one of the major innate immune cell subsets appearing early in the lung after influenza virus infection. Neutrophils provide a first line of defense through phagocytosis of microbial pathogens and release of ROS and antimicrobial enzymes. A relatively recently discovered activity of neutrophils is the formation of neutrophil extracellular traps for which neutrophils release DNA fibers along with various antimicrobial proteins that attach to bacteria and other pathogens.[47] Despite their antimicrobial function, the sustained presence of neutrophil extracellular traps in the airways has been associated with lung tissue damage and worse disease during influenza infection.[48] However, another study demonstrated that mice that are unable to form neutrophil extracellular traps during influenza infection have similar pathology to wild-type mice.[49] In addition, depletion of neutrophils enhances weight loss in influenza infection suggesting they exert a more protective function.[7,48] Our data further support the critical role that neutrophils have in controlling influenza A virus infection. Furthermore, our study shows for the first time that the influenza virus can trigger the apoptosis of neutrophils. Although it has been shown that influenza virus can infect and initiate replication in a neutrophil population *in vivo*,[50] neutrophils do not seem capable of producing infectious virus.[51] Thus, inducing cell death in neutrophils is possibly an evolutionary adaptation of the virus to eliminate cells that do not support virus replication and can be detrimental for the virus (i.e., through virus clearance). However, we also show that release of IL-6 provides protection against virus by preventing downregulation of key anti-apoptotic molecules in neutrophils. Thus, the host also seems to have adapted to overcome influenza virus infection by providing IL-6-survival signals to neutrophils and enhancing virus clearance by these cells. It is well known that a large fraction of the healthy African-American population has what has been defined as a "benign ethnic neutropenia" that, although considered idiopathic at this point, is associated with the presence of several specific genetic polymorphisms.[52] Interestingly, African Americans appeared to be more severely affected by the 2009 influenza pandemic.[3] Thus, the IL-6/neutrophil axis may be of critical importance in determining susceptibility to influenza virus infection and subsequent outcome.

METHODS

Mice and *in vivo* treatments. Wild-type C57BL/6 mice were from Jackson Laboratories (Bar Harbor, ME). Null IL-6 KO mice[20] and IL-6R KO mice[21] have been published previously. Mouse procedures were approved by the University of Vermont Institutional Animal Care and Use Committee. Mice aged 10–12 weeks were used in all the experiments.

Mice were infected intranasally with sublethal doses (3×10^3 EIU) of the Puerto Rico A/PR/8/34 H1N1 (PR8) influenza A virus or the A/California/7/2009 H1N1. Mice were monitored daily for weight loss and other clinical signs of illness. Animals that lost >25% of their body weight at the day of infection or had become grossly moribund were killed. Mice were injected intraperitoneally with 500 μg of rat isotype

control antibody or anti-Ly6G antibody (BioXcell, West Lebanon, NH; clone 1A8) for depletion of neutrophils. Mice were injected intraperitoneally with G-CSF (5 μg) or phosphate-buffered saline for bone marrow release of neutrophils. Mice were treated with lipopolysaccharide as published previously.[34]

BAL collection and analysis of cell count, cell differential, and cytokines. In all, 1 ml cold phosphate-buffered saline was instilled into the lungs through the trachea and aspirated back as described previously.[53] Cells were centrifuged and counted on an Advia Cell Counter (Siemens, Tarrytown, NY). Cells (5×10^4) were cytospun and stained with Hema-3 (Biochemical Sciences, Swedesboro, NJ). In all, 100 cells per high power field were counted and classified as macrophages, neutrophils, or lymphocytes by cell morphology and staining. Neutrophils in blood and bone marrow were examined by the Advia Cell Counter. Cytokines and/or chemokines in BALF on indicated days after influenza infection were examined using Luminex Mouse cytokine/chemokine panel I (Millipore, Billerica, MA) according to the manufacturer's protocol. Total BALF protein was determined by standard Bradford assay (Bio-Rad, Hercules, CA) as per manufacturer's instructions. The concentration of albumin in BALF was determined by enzyme-linked immunosorbent assay (Bethyl Laboratories, Montgomery, TX).

Purification of Neutrophils. Purification of neutrophils from bone marrow was performed as published previously.[33] Cells were infected with the PR8 virus at a 10:1 virus (EIU) to neutrophil number ratio, with or without 20 ng ml^{-1} of mouse IL-6 (R&D Biosystems, Minneapolis, MN).

LDH assay. Supernatants from neutrophils in culture (24 h) were collected at the indicated periods of time and assayed for LDH activity using the Cytotox 96 LDH assay kit (Promega, Fitchburg, WI) as recommended by the manufacturer. % LDH activity in the culture supernatant was calculated relative to the total LDH activity present in the cell pellet, as recommended by the manufacturer.

TUNEL assay. Apoptosis of neutrophils cultured *in vitro* in the presence or absence of the virus for 20 h was examined by TUNEL assay as we previously described[54] using the Pharmingen TUNEL kit (BD Biosciences, San Diego, CA). Fluorescein isothiocyanate-dUTP incorporation was examined by flow cytometry analysis on a LSRII flow cytometer (BD Biosciences).

Histopathology. The lungs were slowly inflated by instilling 1 ml of formalin intratracheally. The trachea was tightened, the lungs fixed in formalin for 24 h at 4°C and paraffin embedded. Lung tissue sections were stained for hematoxylin and eosin according to routine procedures. Images were obtained by the Olympus BX50 light microscope (Olympus, Center Valley, PA) with an Optronics Magnafire digital camera (Optronics, Goleta, CA).

Lung viral titers. The number of viral RNA copies per lung was determined by quantitative reverse transcription-PCR. RNA was extracted from whole lung tissue using the RNeasy Kit (Qiagen, Germantown, MD) following the manufacturer's instructions and 2 μg of RNA was reverse transcribed into cDNA using random hexamer primers and Superscript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Viral titers were determined by real-time reverse transcription-PCR for PR8 virus acid polymerase (*PA*) gene. This method has been previously described to be equivalent to pfu determination.[55]

Western blot analysis. Western blot analysis was performed as published previously.[54] Anti-Mcl-1, anti-Bcl-X_L (Cell Signaling, Danvers, MA), and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used.

Statistical analysis. Survival curves were analyzed using the log-rank test. Time courses were analyzed using two-way analysis of variance; all other analysis was performed using the standard Student's *t*-test.

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

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DISCLOSURE

The authors declared no conflict of interest.

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