

GM-CSF in the Lung Protects against Lethal Influenza Infection

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Rationale: Alveolar macrophages contribute to host defenses against influenza in animal models. Enhancing alveolar macrophage function may contribute to protection against influenza.

Objectives: To determine if increased expression of granulocyte/macrophage colony-stimulating factor (GM-CSF) in the lung increases resistance to influenza.

Methods: Wild-type mice and transgenic mice that expressed GM-CSF in the lung were infected with influenza virus, and lung pathology, weight loss, and mortality were measured. We also administered GM-CSF to the lungs of wild-type mice that were infected with influenza virus.

Measurements and Main Results: Wild-type mice all died after infection with different strains of influenza virus, but all transgenic mice expressing GM-CSF in the lungs survived. The latter also had greatly reduced weight loss and lung injury, and showed histologic evidence of a rapid host inflammatory response that controlled infection. The resistance of transgenic mice to influenza was abrogated by elimination of alveolar phagocytes, but not by depletion of T cells, B cells, or neutrophils. Transgenic mice had far more alveolar macrophages than did wild-type mice, and they were more resistant to influenza-induced apoptosis. Delivery of intranasal GM-CSF to wild-type mice also conferred resistance to influenza.

Conclusions: GM-CSF confers resistance to influenza by enhancing innate immune mechanisms that depend on alveolar macrophages. Pulmonary delivery of this cytokine has the potential to reduce the morbidity and mortality due to influenza virus.

Keywords: GM-CSF; influenza; alveolar macrophages

Seasonal influenza causes an estimated three to five million cases and 250,000 to 500,000 deaths worldwide annually (<http://www.who.int/mediacentre/factsheets/fs211/en/>). Pandemic disease can result in substantial additional morbidity, mortality, and economic cost, as evidenced by the recent H1N1 swine influenza

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Strategies to protect against influenza have focused on development of antiviral drugs and enhancing the adaptive immune response through vaccination. Little information is available on harnessing innate immunity to protect against influenza.

What This Study Adds to the Field

We found that expression of granulocyte/macrophage colony-stimulating factor (GM-CSF) in the lungs of mice provided protection against infection with otherwise lethal doses of influenza virus strains. Protection depended on alveolar macrophages, but not on T cells or B cells.

pandemic. The tremendous human burden of influenza mandates improved methods to prevent and treat this infection.

Control and clearance of influenza infection are believed to hinge on adaptive immunity, mediated by B and T cells. B cells produce antibodies to influenza hemagglutinin and neuraminidase, which protect against homologous virus (1), and CD8⁺ cytolytic T cells clear influenza virus, limit viral replication, and protect against lethal challenge (2–4). Recent studies also suggest a protective role for CD4⁺ T cells (5–8), which can lyse infected target cells (6), provide help to B cells, and promote expansion of CD8⁺ cytolytic T cells (7). Based on the concept that adaptive immunity is central to protection against influenza, preventive strategies have focused primarily on development of vaccines. Unfortunately, vaccines have been variably effective, in part because of antigenic shift and drift in circulating viruses.

Recent studies have shown that innate immunity is also critical for resistance to influenza (9). Alveolar macrophages (AM) are the first line of host defense against respiratory microbes, and they contribute to clearance of influenza virus by Fc receptor-mediated phagocytosis (10). Depletion of AM markedly enhances disease severity due to influenza in murine and porcine experimental models (11, 12), but the mechanisms by which AM mediate protection are not well understood. We hypothesized that enhancing the functional capacities of AM would increase resistance to influenza infection.

Granulocyte/macrophage colony-stimulating factor (GM-CSF) contributes to maturation of mononuclear phagocytes and AM (13, 14). In patients with pulmonary alveolar proteinosis, circulating neutralizing antibodies against GM-CSF cause AM dysfunction (15), and AM from GM-CSF-deficient (GM^{-/-}) mice have impaired capacity for phagocytosis and cytokine production, and these functions were restored by GM-CSF (16). Studies in GM^{-/-} mice have shown that GM-CSF contributes

to immune responses during pneumonia due to *Pseudomonas aeruginosa* and *Pneumocystis carinii*, and administration of GM-CSF to septic patients reversed monocyte immunosuppression and improved their clinical course (17–19). DNA vaccination with a plasmid encoding influenza hemagglutinin, GM-CSF, and IL-12 reduced viral titers and increased neutralizing antibody titers (20), suggesting that GM-CSF may enhance adaptive immune responses. Also, during preparation of this manuscript, Huang and coworkers demonstrated that human recombinant GM-CSF can protect against lethal influenza infection in mice (21). To determine whether GM-CSF could up-regulate innate and adaptive immunity to influenza infection, we studied mice that transgenically express GM-CSF in the lung.

METHODS

Mice

Transgenic mice overexpressing GM-CSF in alveolar epithelial type II cells were generated from GM^{-/-} mice on a C57BL/6 background, by expression of a chimeric gene containing GM-CSF under the control of the human SP-C promoter (SPC-GM) (22). More details on the animals and all other aspects of the methods are provided in the online supplement.

Influenza A Virus Infection

Eight- to 12-week-old mice were intranasally inoculated with 50 μ l of PBS containing the H1N1 PR8 strain (Charles River, Wilmington, MA), the H1N1 mouse-adapted swine influenza strain, California/04/09 (23), or the H3N2 HK68 strain.

Intranasal Treatment with GM-CSF

Wild-type (WT) mice were treated intranasally daily with 1.34 mg/kg recombinant murine GM-CSF (Invitrogen, Carlsbad, CA) for 7 days, prior to infection with PR8 H1N1 influenza.

DNA Plasmids and *In Vivo* Transfection

DNA was obtained from a plasmid that expresses murine GM-CSF, mixed with *in vivo*-JetPEI (Genesee Scientific, San Diego, CA), and administered by retro-orbital injection.

Flow Cytometry

Cells were stained with monoclonal antibodies to murine CD11b (Mac-1a), CD11c (HL3), CD3e (145–2C11), CD4 (L3T4), CD8 α (53–6.7), CD8a (Ly-2), CD45 (Ly5), major histocompatibility complex class II (I-A/I-E), F4/80 (BM8), Fas (15A7), and Annexin V (all from eBioscience, San Diego, CA). All flow cytometry data were acquired on a BD FACS Calibur (BD, San Jose, CA) and analyzed using FlowJo software (TreeStar, Inc., Ashland, OR).

Depletion of Cell Subpopulations

CD8⁺, CD4⁺, and peripheral T cells were depleted with monoclonal antibodies to CD8a, CD4, and Thy-1.2, respectively. For B cell and neutrophil depletion, we used humanized anti-CD22 conjugated to N-acetyl- γ -calicheamicin dimethylhydrazide and anti-GR1, respectively. Clodronate-liposome was used to deplete alveolar phagocytes.

Viral Quantitation

Viral burden was quantified by cellular pathologic changes in Madin-Darby Canine Kidney cells and by real-time polymerase chain reaction, using standard methods.

Histopathology

Lung tissue was fixed, stained, and evaluated by standard methods.

Measurement of Cytokine Concentrations

Cytokine concentrations were measured by enzyme-linked immunoassay in supernatants of mouse lung homogenates.

Phagocytosis Assays

Macrophage phagocytosis *in vivo* was evaluated by intranasal administration of fluorescein isothiocyanate (FITC)-labeled influenza A PR8 virus, as previously described (24). To quantify phagocytosis *in vitro*, AM from SPC-GM and WT mice were collected and cultured with 50 μ l of PBS containing 3.64×10^7 of 1.0 μ m yellow-green FluoSphere Carboxylate-Modified beads (Invitrogen). Thirty minutes later, AM were stained with F4/80 and analyzed by flow cytometry.

Evaluation of Apoptosis

AMs were collected by centrifugation of bronchoalveolar lavage fluid from mice infected with 5LD₅₀ of PR8 virus, and stained with allophycocyanin-anti-F4/80, with or without phycoerythrin-anti-Fas or FITC-annexin V, and results were analyzed by flow cytometry.

To evaluate apoptosis *in vitro*, AM were obtained from bronchoalveolar lavage fluid of naive mice and infected with influenza virus PR8 at a multiplicity of infection of 1 for 60 minutes at 37°C. Eighteen hours later, cells were harvested, stained with allophycocyanin-anti-F4/80 and FITC annexin V, and flow cytometry was performed.

Adoptive Transfer of Alveolar Cells

SPC-GM mice were depleted of alveolar phagocytes by treatment with clodronate-liposome, as outlined above. Three days later, bronchoalveolar lavage cells (99% AM) from naive SPC-GM mice were transferred intratracheally to alveolar phagocyte-depleted SPC-GM mice, which were infected intranasally with influenza virus 16 hours later.

RESULTS

GM-CSF Expression in the Lung Reduces Mortality from Influenza

To determine whether GM-CSF could protect against influenza, SPC-GM, GM^{-/-}, and WT mice were infected with 5 LD₅₀ of the influenza PR8 strain. All WT mice died after 8–11 days, and all GM^{-/-} mice died after 6–8 days. In contrast, all SPC-GM mice, which only express GM-CSF in the lung, survived (Figure 1A). WT and GM^{-/-} mice progressively lost weight, whereas SPC-GM mice lost less weight and soon recovered (Figure 1B). These results show that pulmonary expression of GM-CSF confers marked protection against lethal influenza infection.

PR8 is a laboratory influenza strain. To determine if SPC-GM mice were resistant to clinical influenza strains, we infected mice with lethal doses of the H3N2 strain, HK68, and a mouse-adapted H1N1 California/04/092009 swine influenza pandemic strain (23). All WT mice died, whereas all the SPC-GM mice survived, indicating that GM-CSF in the lung protects against clinical and laboratory influenza strains (Figure 1A).

Delivery of GM-CSF to the Lungs of WT Mice Protects against Influenza

The studies above suggest that GM-CSF produced in the lung protects against influenza. However, because SPC-GM mice have been genetically manipulated, genetic differences other than GM-CSF production may confer resistance to influenza. To exclude this possibility, we evaluated the effect of GM-CSF on WT mice. We coated a murine GM-CSF-expressing vector with polyethylenimine, which enhances DNA expression 400-fold and delivers it to the lung (25). All WT mice treated with this GM-CSF-expressing plasmid survived influenza infection, compared with no mice receiving the empty plasmid (Figure 1C).

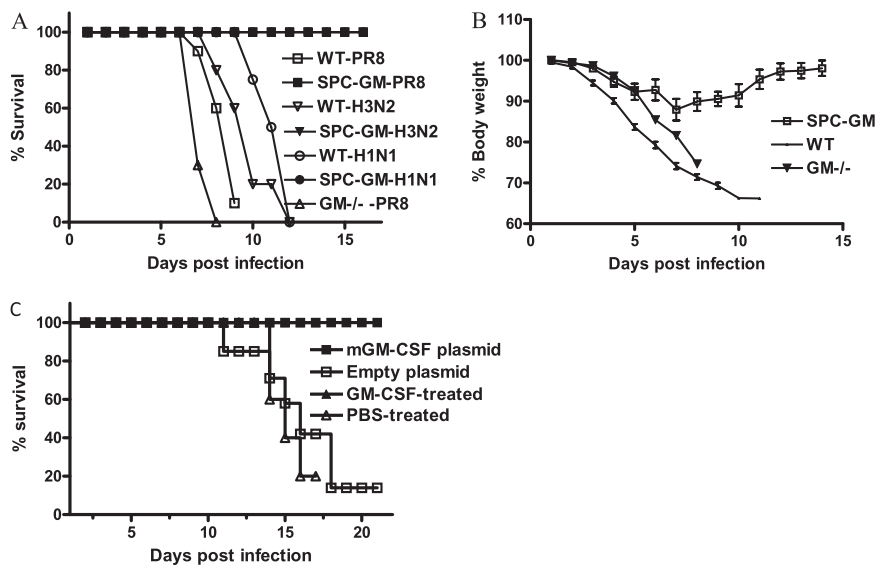


Figure 1. Pulmonary granulocyte/macrophage colony-stimulating factor (GM-CSF) expression protects against influenza. (A) Wild-type (WT), GM-CSF-deficient ($GM^{-/-}$), and SPC-GM mice that overexpress GM-CSF by alveolar epithelial type II cells and were generated from $GM^{-/-}$ mice on a C57BL/6 background, by expressing a chimeric gene containing GM-CSF under the control of the human SP-C promoter ($n = 10$ per group, result representative of three experiments) were infected with 5 LD₅₀ of influenza A virus PR8. WT and SPC-GM mice were infected with lethal doses of H3N2 HK68 ($n = 5$ per group) or mouse-adapted H1N1 swine influenza ($n = 4$ per group). All mice were followed until death or recovery. (B) WT, $GM^{-/-}$, and SPC-GM mice ($n = 8$ per group) were infected with 5 LD₅₀ of PR8, and weighed daily until death or recovery. Means \pm SE are shown. (C) WT mice were given a polyethylenimine-coated GM-CSF expression vector or a control empty vector ($n = 7$ –8 per group) by retro-orbital injection. Four to 6 weeks later, mice were infected with 2 LD₅₀ of PR8, and fol-

lowed until death or recovery ($P = 0.0008$, comparing treatment with the GM-CSF-expressing vector and empty vector). In an independent experiment, mice were treated with PBS ($n = 5$ per group) or rGM-CSF intranasally for 7 days ($n = 6$ per group), then infected 1 day later with 2 LD₅₀ of PR8 ($P = 0.008$, comparing rGM-CSF-treated and PBS-treated mice). Results are representative of two to five experiments.

Intranasal administration of recombinant mouse GM-CSF to WT mice also abrogated mortality from influenza (Figure 1C).

Effects of Pulmonary GM-CSF on Alveolar Injury, Viral Burden, and Lung Inflammation after Influenza Infection

To understand the mechanisms underlying the resistance of SPC-GM mice to influenza, we assessed the integrity of the alveolar barrier by measuring albumin levels in bronchoalveolar lavage fluid, which were similar in WT and SPC-GM mice before infection, but rose markedly in infected WT mice and were significantly higher than those in SPC-GM mice 5–6 days after infection (Figure 2A). SPC-GM mice also had a lower viral burden, as the TCID₅₀ in lung homogenates was reduced 23-fold in SPC-GM mice, compared with WT mice, 3 days after infection ($P = 0.03$, Figure 2B).

We next evaluated the pulmonary histopathology during influenza infection. One day after infection, inflammation was much more marked in lungs of SPC-GM mice than in WT mice, with cellular infiltrates composed predominantly of lymphocytes and macrophages (Figure 2C). By Day 3 after infection, SPC-GM mice showed a more diffuse inflammatory mononuclear cell infiltrate, whereas WT mice showed a diffuse neutrophilic infiltrate. By Day 6, inflammation was resolving in SPC-GM mice, while diffuse cellular infiltration persisted in WT mice, with almost no normal alveoli.

The histologic findings above suggest that SPC-GM mice mount a rapid immune response to influenza infection. We next measured concentrations of selected inflammatory cytokines in lung homogenates. Surprisingly, naïve SPC-GM mice had high levels of monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor (TNF)- α , compared with WT mice (Figure 2D). In SPC-GM mice, MCP-1 levels increased to slightly less than twice baseline levels, 6 days after influenza infection. In WT mice, MCP-1 levels rose markedly from very low levels to 32 ng/ml at Day 6. TNF- α levels did not change greatly in SPC-GM or WT mice during the first 3 days after influenza infection, and fell in SPC-GM mice to WT levels at Day 6. Interleukin (IL)-10 levels were much higher in SPC-GM than WT mice at Day 6 after infection, suggesting an enhanced anti-inflammatory response in SPC-GM mice. IFN- α is an important component of the innate response to influenza virus (26, 27), and IFN- γ is a major product of CD4⁺ cells that

mediate resistance to influenza (6). Levels of these cytokines were similar in SPC-GM and WT mice (data not shown).

In summary, the elevated baseline MCP-1 and TNF- α levels in lungs of SPC-GM mice, combined with the histologic findings and viral burden measurements, suggest that SPC-GM mice mount an early innate immune response that contributes to control of viral infection and reduces lung injury and mortality.

As an alternative means to determine the effect of GM-CSF on the pulmonary inflammatory response during influenza infection, we evaluated the effect of treating WT mice with GM-CSF, using flow cytometry to measure cellular surface markers of inflammation. CD200R and CD200 are an anti-inflammatory ligand and receptor pair that are normally expressed at high levels on AM and on lung epithelial cells, respectively, but are down-regulated during inflammation (28, 29). Baseline CD200R levels were greatly reduced in GM-CSF-treated WT mice and increased slightly during infection, whereas levels were much higher in untreated mice at baseline and during the first 2 days after infection, decreasing at 3 to 6 days after infection (Figure 3, and Figure E1A in the online supplement). The percentage of neutrophils, as measured by expression of Gr-1, had higher baseline values in GM-CSF-treated mice but decreased 2 to 3 days after infection, whereas PBS-treated mice had increased values after infection (Figure E1B), corresponding to the histologic findings of reduced neutrophil tissue infiltration in SPC-GM mice (Figure 2C). Expression of CCR2, the major receptor for MCP-1, increased to much higher levels during influenza infection in PBS-treated than in GM-CSF-treated WT mice (Figure E1C). MCP-1 levels in bronchoalveolar lavage fluid were also threefold higher in untreated than in GM-CSF-treated WT mice 6 days after infection (Figure E4), corresponding to the higher lung homogenate MCP-1 levels in WT than in SPC-GM mice (Figure 2D).

Resistance of SPC-GM Mice to Influenza Depends on Radiation-Sensitive Cells That Are Not Neutrophils or Lymphocytes

SPC-GM mice have increased lung volumes and high numbers of alveolar type II epithelial cells and macrophages (22). To determine if the resistance to influenza in SPC-GM mice was

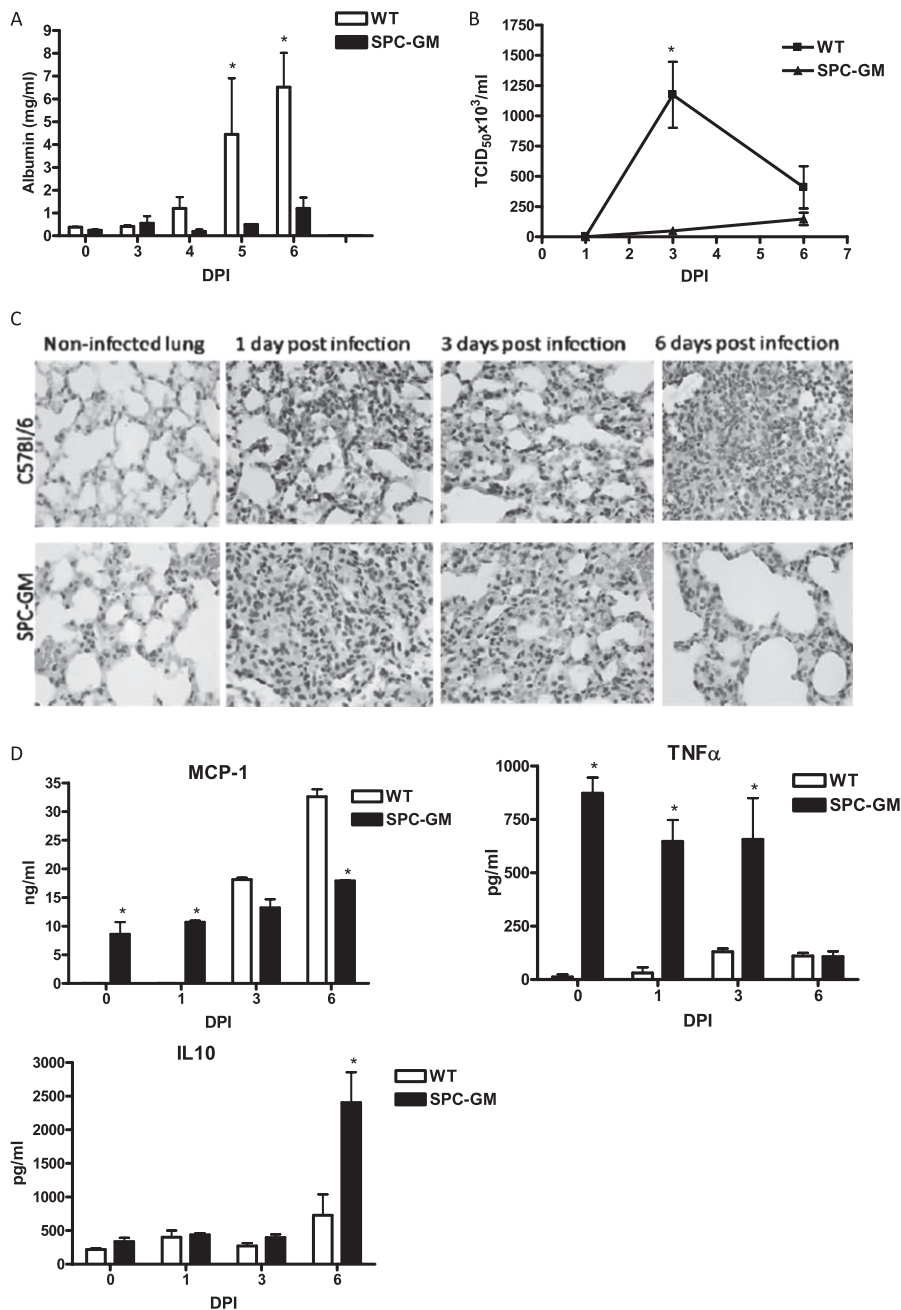


Figure 2. Pulmonary GM-CSF expression reduces lung injury and viral burden, and elicits early inflammation. (A) SPC-GM and WT mice ($n = 20$ per group) were infected with $5 LD_{50}$ of influenza virus PR8. Three to 6 days after infection (DPI), four mice in each group were killed daily, and albumin levels in bronchoalveolar lavage fluid were measured by enzyme-linked immunoassay. Means \pm SEs are shown. $*P = 0.01$, comparing SPC-GM and WT mice. (B) SPC-GM and WT mice ($n = 12$ per group) were infected with $5 LD_{50}$ of PR8. One, 3, and 6 days after infection, four mice in each group were killed, and viral loads were determined by measuring the $TCID_{50}$, as outlined in the methods. $*P = 0.03$, comparing SPC-GM and WT mice. (C) Histology of the lungs of SPC-GM and WT mice, both uninfected and 1–6 days after infection with PR8. Representative sections, stained with hematoxylin and eosin, are shown, at $\times 40$ magnification. (D) Cytokine levels were measured by enzyme-linked immunoassay in lung homogenates from SPC-GM and WT mice, before and after infection with PR8 ($n = 3$ per time point). Means \pm SEs are shown. $*P < 0.01$, comparing SPC-GM and WT mice.

mediated by radiation-resistant structural lung features or by radiation-sensitive myeloid cells, we exposed 10 SPC-GM mice to 50% of a lethal dose of whole-body irradiation (450 rads), and then infected 5 mice with $10 LD_{50}$ of influenza A virus PR8. All irradiated infected mice died (data not shown), suggesting that resistance depends on myeloid cells. Death was not due to irradiation, as the five uninfected mice remained well.

GM-CSF enhances development and maturation of neutrophils and macrophages. The latter can act as innate effectors or become dendritic cells that present antigens to T cells. To identify the cell populations that confer resistance to influenza in SPC-GM mice, we depleted different cell types. Removal of neutrophils from SPC-GM mice with anti-GR1 did not alter resistance to influenza (Figure 4A). Depletion of $CD4^+$ or $CD8^+$ T cells reduced the survival rate of SPC-GM mice to 80–90%, and depletion of both T cell subsets lowered survival to 70% (Figure 4B). Depletion of all T cells with anti-CD90.2, which removed

$CD4^+CD8^+$ cells and $\gamma\delta$ T cells, reduced survival to 80% (Figure 4C). Depletion of B-cells with anti-CD22/cal did not reduce survival after influenza infection (Figure 4D). Therefore, neutrophils, T cells, and B cells do not mediate the marked resistance of SPC-GM mice to influenza.

Resistance to Influenza of SPC-GM Mice Requires Alveolar Phagocytes

To determine if AM mediate resistance to influenza in SPC-GM mice, we administered intranasal clodronate liposomes or phosphate-buffered saline (PBS) liposomes, before influenza infection. Clodronate is taken up by phagocytes, causing apoptosis and death (30). Clodronate-treated mice all died after influenza infection, but all PBS liposome-treated mice survived (Figure 5A). Clodronate depleted 65–84% of the AM from SPC-GM mice (Figure E2), but did not affect the distribution

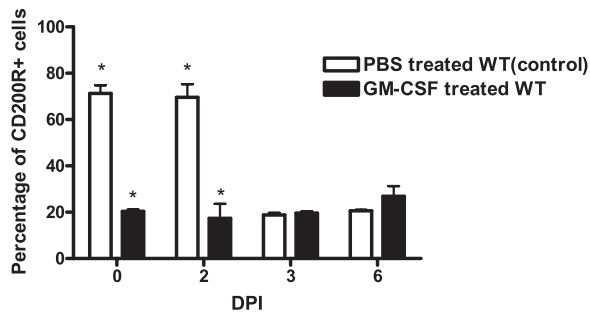


Figure 3. Expression of CD200R in bronchoalveolar lavage cells. WT mice were treated with 1.34 mg/kg of recombinant murine GM-CSF or with PBS for 7 days, prior to infection with PR8 H1N1 influenza. Mice were killed ($n = 3$ per time point), and bronchoalveolar lavage cells were stained with anti-CD200R. Mean values and SEs for the percentages of CD200R⁺ cells are shown. * $P < 0.001$.

of macrophages or dendritic cells in lung digests (data not shown), demonstrating that AM are essential for the resistance of SPC-GM mice to lethal influenza.

To determine when AM were needed to protect against influenza in SPC-GM mice, we administered clodronate 1 to 6 days after infection. Treatment with clodronate 1 to 2 days after infection caused 100% mortality, but treatment after 4 days caused only 20% mortality, and all SPC-GM mice treated after 6 days survived (Figure 5B). Therefore, AM were most critical in the first 3 days after infection.

As an alternative means to demonstrate the essential role of AM in resistance to influenza in SPC-GM mice, SPC-GM mice were first treated with clodronate to deplete alveolar phagocytes. Clodronate-treated SPC-GM and WT mice served as recipients. Three days later, recipient mice received PBS intratracheally or AM from naïve SPC-GM donor mice, and were challenged with a lethal dose of PR8 virus 16 hours later. All clodronate-treated SPC-GM mice that received naïve AM survived, whereas 80% of PBS-treated mice died (Figure 5C). In contrast, AM did not prevent death in WT mice (Figure 5D).

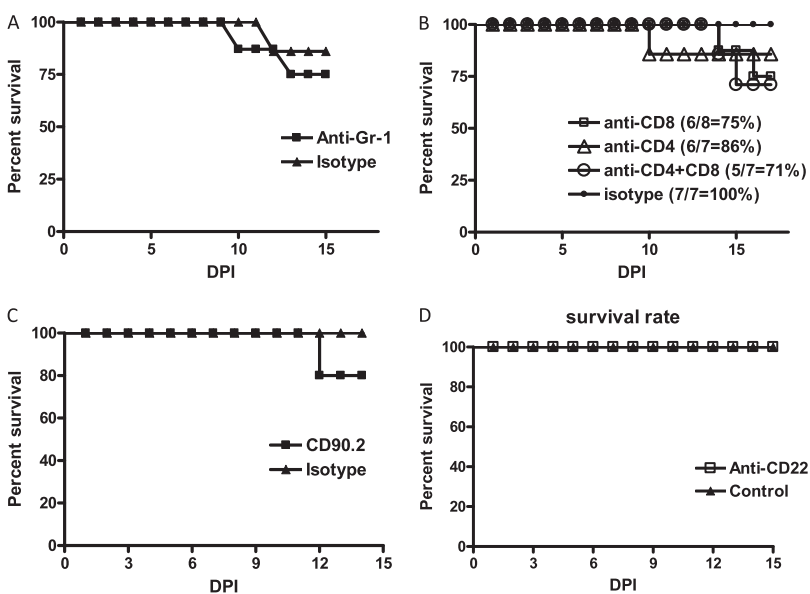


Figure 4. Resistance to influenza of SPC-GM mice does not require neutrophils, T cells, or B cells. (A) SPC-GM mice were treated intraperitoneally with anti-Gr1 or isotype control IgG ($n = 7$ per group) at Days $-1, 0, 3,$ and 6 after infection with 5 LD_{50} of influenza virus PR8, and followed until death or recovery. Anti-Gr1 reduced the percentages of neutrophils from 15% to 1% in blood. A representative result of two experiments is shown. (B) CD4⁺ and/or CD8⁺ T cells were depleted with monoclonal antibodies ($n = 5-7$ per group), at $-3, 0,$ and 3 days after infection with 5 LD_{50} of PR8, and followed until death or recovery. Monoclonal antibodies reduced the percentages of CD4⁺ and CD8⁺ cells in the mediastinal lymph nodes from 45% to 0.2%, and from 24% to 2%, respectively. Survival rates did not differ significantly in all groups. (C) SPC-GM mice were treated intraperitoneally with anti-CD90.2 or isotype control IgG ($n = 7$ per group) at Days $-1, 0, 3,$ and 6 after infection with 5 LD_{50} of influenza virus PR8 on Day 0 and followed until death or recovery. (D) SPC-GM mice were treated with N-acetyl- γ -calicheamicin dimethylhydrazide, conjugated either to anti-CD22 or isotype control

antibody ($n = 5$ per group) at Days -5 and 0 after infection with 5 LD_{50} of PR8, and followed until death or recovery. A representative result of two experiments is shown.

AM from SPC-GM Mice Show Reduced Apoptosis after Influenza Infection

AM are critical to clear infected cells and prevent tissue inflammation, and macrophage apoptosis during influenza infection causes increased mortality and tissue destruction (31). Using immunolabeling and flow cytometry, we found that the percentage of Annexin V⁺ apoptotic F4/80⁺ AM was 3.5-fold higher in WT than in SPC-GM mice, 2 days after influenza infection (Figure 6A, and Figure E3A). Expression of Fas on AM was four- to sevenfold higher in WT than in SPC-GM mice after influenza infection (Figure 6B, and Figure E3B). The reduced apoptosis in SPC-GM mice could be due in part to the reduced viral burden. To control for this factor, we infected AM from SPC-GM and WT mice with equal numbers of influenza PR8 virus *in vitro*. The percentage of Annexin V⁺, apoptotic cells was 2.2-fold higher in AM from WT mice (Figure 6C).

AM from SPC-GM Mice Do Not Have Increased Phagocytic Capacity

To assess whether resistance of SPC-GM mice to influenza is due to increased virus uptake by AM *in vivo*, we administered FITC-labeled PR8 virus intranasally to SPC-GM and WT mice, and measured the percentages of AM that contained virus, as well as the phagocytic index (percentage of virus-containing AM \times mean fluorescence intensity). AM from SPC-GM and WT mice had similar phagocytic activity (Figure 7A). When AM were exposed to fluorophore-labeled beads *in vitro*, the phagocytic activity of AM from SPC-GM and WT mice was also similar (Figure 7B).

DISCUSSION

Most studies of the immune response to influenza have focused on the importance of antibody production by B cells and cytolytic activity of CD8⁺ T cells in mediating protection against infection. The current study delineates a novel means of conferring marked resistance to influenza through enhancing innate immune mechanisms that depend on AM. We found that SPC-GM mice that overexpress GM-CSF only in the lungs are highly

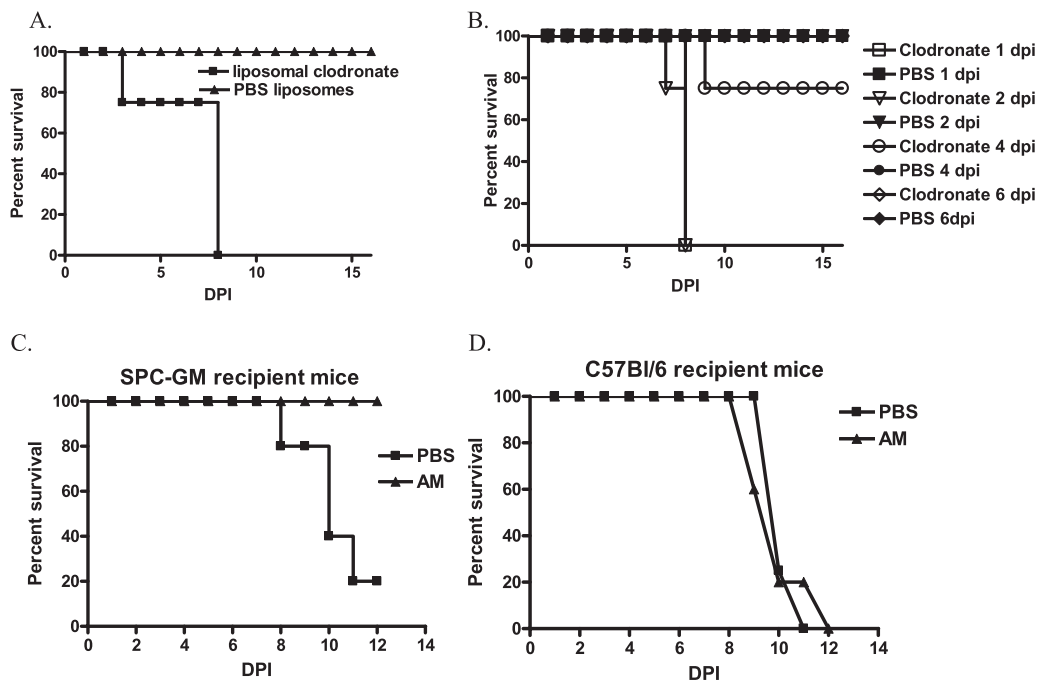


Figure 5. Alveolar macrophages (AM) are required for resistance of SPC-GM mice to influenza. (A) SPC-GM mice were given clodronate-liposomes or PBS-liposomes ($n = 5$ per group). Eighteen to 24 hours later, mice were infected with 5 LD₅₀ of influenza virus PR8, and followed until death or recovery. (B) SPC-GM mice were given clodronate-liposomes or PBS-liposomes 1–6 days after infection with 5 LD₅₀ of PR8 ($n = 3$ –5 per group), and followed until death or recovery. (C) SPC-GM mice were treated with clodronate-liposome to deplete alveolar phagocytes. Three days later, bronchoalveolar lavage cells (99% AM) from naïve SPC-GM mice were collected. Clodronate-treated SPC-GM mice each received 2×10^6 of these AM or PBS intratracheally, and were infected with PR8 16 hours later. A representative of two experiments with identical results is shown. (D)

WT mice ($n = 5$ per group) received 2×10^6 bronchoalveolar lavage cells (99% AM) from naïve SPC-GM mice or PBS intratracheally, and were infected with PR8 16 hours later. A representative result of two experiments is shown.

resistant to infection with laboratory and clinical influenza strains, including the pandemic swine H1N1 strain. SPC-GM mice had no mortality, and markedly reduced lung injury and alveolar damage after influenza infection (Figures 1 and 2). Resistance to influenza was unaffected by depletion of neutrophils, T cells, and B cells, but was completely abrogated by elimination of alveolar phagocytes and reconstituted by intratracheal transfer of naïve AM (Figures 4 and 5). AM from SPC-GM mice were more resistant to influenza-induced apoptosis (Figure 6). Furthermore, SPC-GM mice showed increased baseline lung levels of MCP-1 and TNF- α , and earlier histologic evidence of mononuclear cell infiltrates after influenza infection, indicating development of a more rapid host inflammatory response that reduced the viral burden (Figure 2). Similar to our findings in SPC-GM mice, delivery of GM-CSF to the lungs of WT mice increased lung MCP-1 and TNF- α levels, reduced the viral burden (Figure E4) and conferred resistance to influenza (Figure 1C), emphasizing the therapeutic potential of these findings.

Current strategies to combat influenza focus on vaccines and antiviral agents. Vaccines are designed to elicit antibody responses against hemagglutinin and neuraminidase antigens in viruses that circulated in the preceding influenza seasons. However, antigenic drift and the emergence of new strains require formulation of modified vaccines, which may be produced too slowly to protect the population at risk. In addition, stockpiling of current vaccines may be ineffective against future outbreaks. The efficacy of antiviral agents has also been reduced by the rapid spread of drug-resistant influenza strains. We found that GM-CSF provides an alternative strategy to ameliorate disease due to influenza. Human recombinant GM-CSF was recently shown to protect against lethal influenza infection in mice (21). We extended these findings to additional influenza strains, including the recent pandemic swine H1N1 strain. Intravenous and subcutaneous GM-CSF is used to treat neutropenia and bone marrow suppression, and is well tolerated. Intranasal or aerosol delivery should minimize toxicity while maximizing

effects on AM. By stimulating innate responses that do not require recognition of strain-specific antigens, GM-CSF should be effective against a broad range of influenza strains, which are unlikely to develop resistance to its effects. If additional studies show that GM-CSF is effective in animal models when administered after influenza infection, GM-CSF-based strategies may offer a promising approach to reduce morbidity and mortality from influenza.

MCP-1 and TNF- α can exhibit dichotomous effects during influenza infection. MCP-1 contributes to protection against influenza, as MCP-1 gene-deleted mice show greater weight loss and higher viral burdens than infected WT mice (32), and neutralization of MCP-1 causes increased alveolar epithelial cell damage (33). However, highly pathogenic H5N1 influenza strains elicit greater MCP-1 production by human macrophages than H1N1 strains (34), and inhibiting MCP-1 production reduces inflammation during influenza infection (35), suggesting that uncontrolled MCP-1 production contributes to lung injury. In the case of TNF- α , elevated levels are typical of infection with highly pathogenic H5N1 influenza strains in animal models (35, 36), and inhibition of TNF- α reduces disease severity (37). On the other hand, TNF- α markedly reduces influenza virus replication in lung epithelial cells (38), and induction of pulmonary TNF- α before influenza infection reduces mortality, viral titers, and lung inflammation (39), demonstrating that early TNF- α production contributes to protection. In the current study, SPC-GM mice had elevated baseline lung MCP-1 and TNF- α levels without histologic evidence of inflammation, and these levels remained relatively stable during influenza infection, associated with a reduced viral burden and resolution of lung inflammation. In contrast, in WT mice, MCP-1 levels rose markedly to > 30 ng/ml during infection, associated with severe lung injury and death. When WT mice were treated with intranasal GM-CSF prior to influenza infection, they also developed high baseline MCP-1 and TNF- α levels that changed little during infection, accompanied by reduced viral titers, compared with

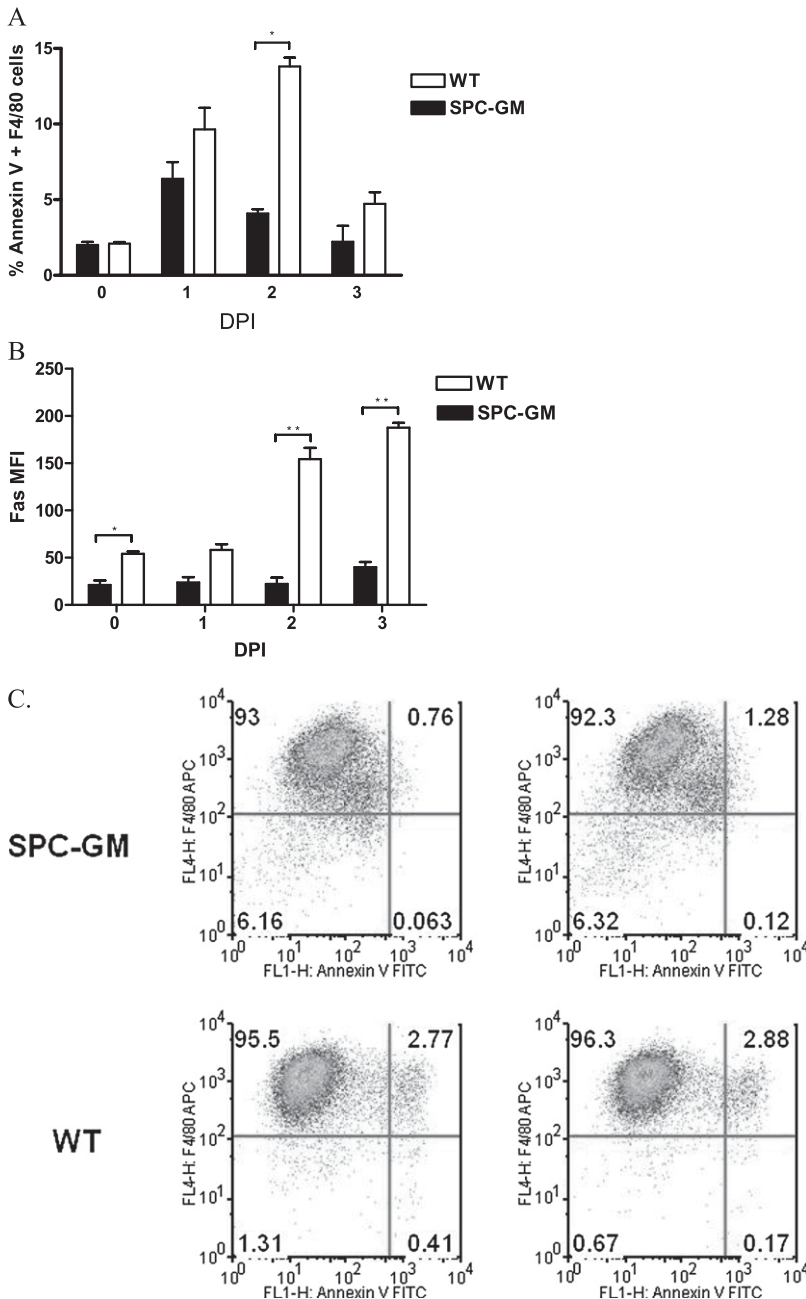


Figure 6. AM of SPC-GM mice are more resistant to apoptosis than those of WT mice. SPC-GM and WT mice were infected with 5 LD50 of influenza virus PR8. Bronchoalveolar lavage cells were collected at 0–3 days after infection (DPI) and stained with anti-F4/80 and annexin V or anti-Fas. (A) Mean values and SEs for the percentages of Annexin V⁺ cells ($n = 5–6$ per time point) are shown. $*P < 0.05$, comparing SPC-GM and WT mice. (B) We gated on F4/80⁺ cells and measured the mean fluorescence intensity (MFI) of Fas. Mean values and SEs for the net MFI of Fas ($n = 5–6$ per time point) are shown. $*P < 0.05$, $**P < 0.01$, comparing SPC-GM and WT mice. (C) *In vitro* analysis of apoptosis. AM from naïve SPC-GM and WT mice ($n = 4$ per group) were incubated with influenza virus PR8, as detailed in METHODS. Cells were stained with anti-F4/80, followed by FITC–Annexin V, and analyzed by flow cytometry. A representative result of two experiments is shown.

PBS-treated mice (Figure E4). In combination with published studies cited above, our findings suggest that MCP-1 and TNF- α contribute to the potent early innate immune response to influenza in SPC-GM mice, which controls infection and prevents uncontrolled cytokine release, thereby limiting tissue injury. SPC-GM mice also produced more IL-10 at later stages of infection (Figure 2D), which is important in limiting pulmonary inflammation (40).

Adaptive immunity is crucial for protection against influenza infection in animal models and in humans, with substantial published evidence supporting the role of antibodies and CD8⁺ T cells in mediating these effects (1–4). However, recent studies indicate that AM are also essential for effective defense against influenza. Depletion of AM increased the viral burden and markedly increased mortality from H1N1 influenza in pigs (11), H3N2 influenza in mice (41) and H1N1 influenza bearing the hemagglutinin and neuraminidase of the 1918 pandemic strain (12). AM are the major source of IFN- α during pulmonary

infection with RNA viruses (41), and can phagocytose influenza virus through Fc receptor–mediated phagocytosis (10) and through opsonization with surfactant protein A (42). In addition, AM can phagocytose necrotic and apoptotic epithelial cells that are generated during infection, preventing release of cellular debris and proteinases that would otherwise stimulate cytokine production, lung injury, and reduced vascular integrity (31). Influenza virus induces apoptosis of macrophages (43), and CCL5–CCR5 signaling reduces macrophage apoptosis, enhancing viral clearance and reducing lung inflammation and death (31).

GM-CSF is essential for differentiation of AM, and mice with a deleted GM-CSF gene have defective phagocytosis of adenovirus, reduced bacterial killing, Toll-like receptor–mediated signaling and TNF- α production, and impaired capacity to metabolize surfactant (24, 44–46). GM-CSF also affects lung structure, as SPC-GM mice have enhanced proliferation and hyperplasia of alveolar epithelial cells (22), reduced apoptosis of alveolar epithelial cells and improved alveolar barrier function after exposure

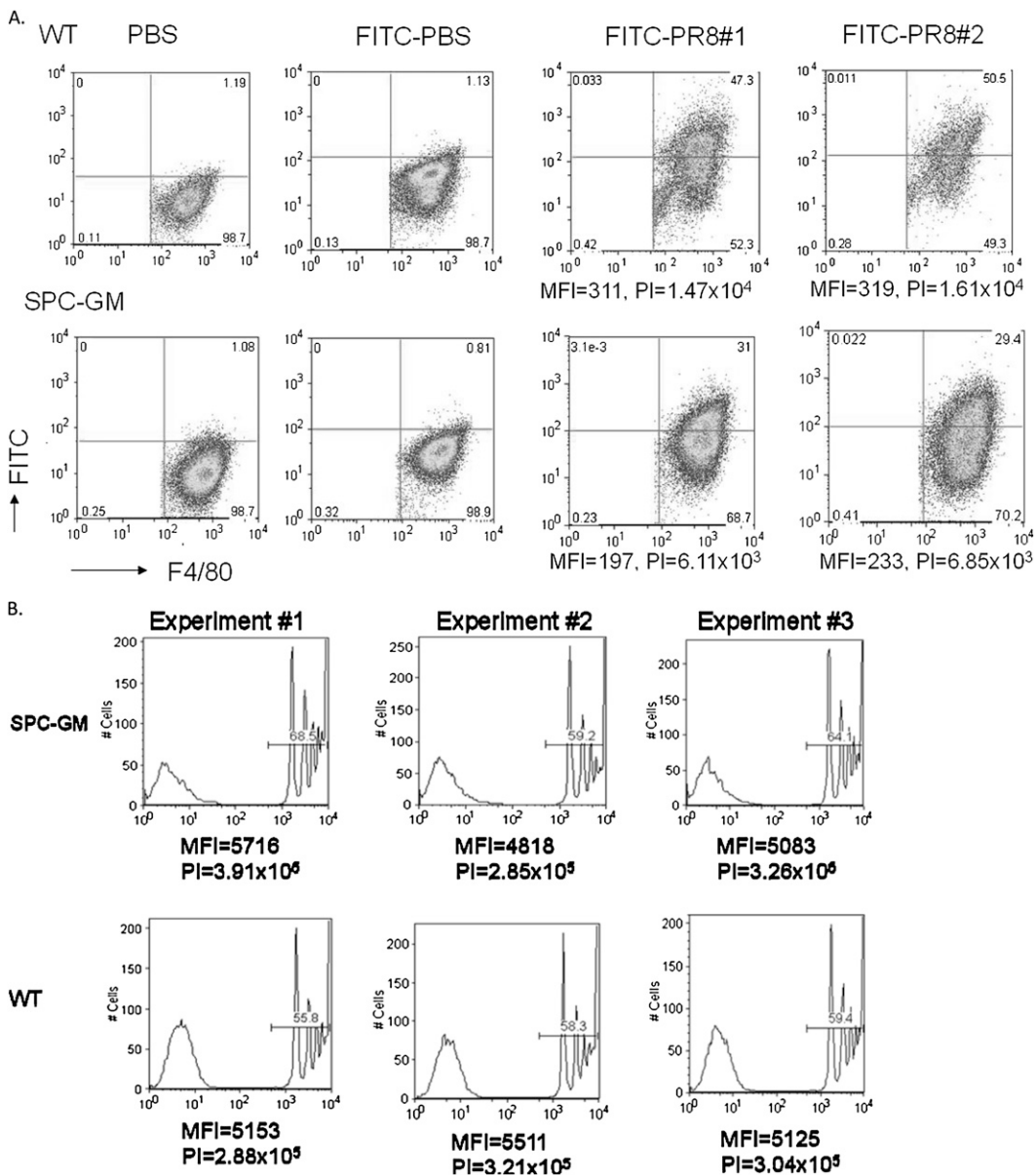


Figure 7. Phagocytic capacity of AM from SPC-GM and WT mice. (A) Mice were intranasally inoculated with FITC-labeled PR8 ($n = 2$), FITC stock in PBS ($n = 1$), or PBS alone ($n = 1$). Two hours later, AM from bronchoalveolar lavage were stained with allophycocyanin anti-F4/80, and subjected to flow cytometry. We show the MFI of FITC-labeled PR8 in F4/80⁺ cells, and the phagocytic index (PI), which is the percentage of FITC⁺ F4/80 cells multiplied by the MFI of FITC-labeled PR8 in F4/80⁺ cells. (B) AM from SPC-GM and WT mice ($n = 3$ per group) were cultured with yellow-green FluoSphere Carboxylate-Modified beads. Thirty minutes later, AMs were stained with anti-F4/80 and analyzed by flow cytometry. MFI and PI were calculated, as in Panel A.

to hyperoxia (47). We found that the resistance of SPC-GM mice to influenza was abrogated by administration of clodronate and restored by transfer of AM, indicating that AM are necessary to confer this resistance. Our results show that GM-CSF markedly increases resistance to influenza by increasing the number of AM. SPC-GM mice have 5 to 10 times more AM than WT mice (22), and this difference was amplified during the critical first 3 days after influenza infection, when apoptosis was greatly reduced in AM from SPC-GM mice, compared with WT animals. Levels of Fas were increased on AM from WT mice, consistent with previous findings that macrophage apoptosis during influenza infection is mediated through the Fas/FasL extrinsic pathway (48). GM-CSF induces expression of the anti-apoptotic proteins, *mcl-1* and *bcl-2*, in hematopoietic cells (49, 50), and AM from SPC-GM mice are likely to be relatively resistant to apoptosis. Although the phagocytic capacity of individual AM from SPC-GM and WT mice was similar, the greatly increased numbers of AM in SPC-GM mice should enhance uptake of influenza virus and ingestion and clearance of dead and dying epithelial cells, reducing lung injury. Although GM-CSF modulates differentiation of dendritic cells (14), these

effects were not central to the capacity of GM-CSF to confer resistance to influenza, as depletion of T cells and B cells did not abrogate influenza resistance.

There are three limitations to our study. First, because we used a model of high-dose lethal influenza, it is uncertain if GM-CSF confers similar benefit in nonlethal influenza that better mimic human disease. Second, we evaluated three influenza strains, and it is possible that GM-CSF may be less effective for some clinical influenza isolates. Third, we used experimental systems in which high GM-CSF levels were present in the lung prior to influenza infection. Because treatment of influenza with GM-CSF in the clinical setting is much more feasible and cost-effective when delivered after development of symptoms, it is critical to determine if treatment after infection is beneficial. This is difficult in a mouse model because mice require anesthesia for administration of intranasal GM-CSF, and influenza-infected mice tolerate anesthesia poorly. Nevertheless, in a pilot study, delivery of GM-CSF to WT mice after lethal IAV infection reduced mortality from 100% to 70% (Figure E5). Work is underway to determine the optimal therapeutic window to administer GM-CSF after infection. It will be important to study

larger number of animals and to evaluate clinically relevant routes of GM-CSF delivery, such as subcutaneous injection and inhalation. It may also be useful to consider use of water-in-fluorocarbon emulsions that are better suited for drug delivery to edematous alveoli.

In summary, we identified a novel means to confer marked resistance to influenza through enhancing innate immune mechanisms that depend on AM. We found that mice expressing GM-CSF in the lung were highly resistant to influenza virus infection, and that this was mediated through AM, which showed increased resistance to apoptosis. If additional work determines that delivery of GM-CSF to the lungs after the onset of symptoms improves the outcome of influenza infection, this strategy has the potential to represent a new therapeutic approach to reduce morbidity and mortality from influenza in humans.

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