MCP-1 Antibody Treatment Enhances Damage and Impedes Repair of the Alveolar Epithelium in Influenza Pneumonitis

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Recent studies have demonstrated an essential role of alveolar macrophages during influenza virus infection. Enhanced mortalities were observed in macrophage-depleted mice and pigs after influenza virus infection, but the basis for the enhanced pathogenesis is unclear. This study revealed that blocking macrophage recruitment into the lungs in a mouse model of influenza pneumonitis resulted in enhanced alveolar epithelial damage and apoptosis, as evaluated by histopathology, immunohistochemistry, Western blot, RT-PCR, and TUNEL assays. Abrogation of macrophage recruitment was achieved by treatment with monoclonal antibody against monocyte chemoattractant protein-1 (MCP-1) after sub-lethal challenge with mouse-adapted human influenza A/Aichi/2/68 virus. Interestingly, elevated levels of hepatocyte growth factor (HGF), a mitogen for alveolar epithelium, were detected in bronchoalveolar lavage samples and in lung homogenates of control untreated and nonimmune immunoglobulin (Ig)G-treated mice after infection compared with anti-MCP-1-treated infected mice. The lungs of control animals also displayed strongly positive HGF staining in alveolar macrophages as well as alveolar epithelial cell hyperplasia. Co-culture of influenza virus-infected alveolar epithelial cells with freshly isolated alveolar macrophages induced HGF production and phagocytic activity of macrophages. Recombinant HGF added to mouse lung explants after influenza virus infection resulted in enhanced BrdU labeling of alveolar type II epithelial cells, indicating their proliferation, in contrast with anti-HGF treatment showing significantly reduced epithelial regeneration. Our data indicate that inhibition of macrophage recruitment augmented alveolar epithelial damage and apoptosis during influenza pneumonitis, and that HGF produced by macrophages in response to influenza participates in the resolution of alveolar epithelium.

Keywords: alveolar epithelial regeneration; apoptosis; hepatocyte growth factor; influenza pneumonitis; Monocyte chemoattractant protein-1

Influenza virus is enveloped with a segmented negative-sense RNA genome, and belongs to the *Orthomyxoviridae* family. In recent years, frequent outbreaks of influenza caused by the H5N1 subtype have resulted in fatalities in humans, poultry, and other animal species worldwide (1, 2). The global circulation of H3N2 virus strains is significant, and there is evidence for genetic compatibility of reassortants derived from H5N1 and H3N2 viruses (3, 4). Recently, the novel swine-origin influenza

CLINICAL RELEVANCE

This study demonstrated that inhibition of macrophage recruitment augmented alveolar epithelial damage and apoptosis in influenza virus infection, suggesting the protective role of alveolar macrophages in the resolution of alveolar epithelium via hepatocyte growth factor (HGF) production. Our findings indicate the active role of HGF in the regeneration and resolution of alveolar epithelium after influenza virus infection.

A H1N1 virus is spread to many countries around the world, leading to a pandemic (5). The epithelial lining of the respiratory tract including nasal, tracheal, bronchial, and alveolar epithelia are targets for influenza virus replication (6–8). Influenza viruses also infect monocytes, macrophages, and other leukocytes (9, 10).

The accumulation of macrophages has been linked with immunopathology during influenza virus infection, as they produce proinflammatory cytokines (e.g., TNF- α , IL-1, IL-6, GM-CSF), chemokines (including IP-10, MIP-1 α , RANTES), and also stimulate expression of inducible nitric oxide synthase (9, 11–13). Chemokines are small peptide molecules with chemotactic and activating effects on leukocytes. Among chemokines, monocyte chemoattractant protein-1 (MCP-1) is the major chemoattractant responsible for the recruitment of macrophages, but also attracts neutrophils and T-lymphocytes (14–16). Mice lacking MCP-1 display decreased macrophage and neutrophil infiltration with increased viral load and elevated levels of TNF- α , IL-6, MIP-2, and IFN- γ , suggesting that MCP-1 contributes to an adequate protective immune response during influenza infection (17). Administration of MCP-1 also protects animals from challenge with lethal doses of *Salmonella typhimurium* and *Pseudomonas aeruginosa* (18). Several animal models with depleted macrophages demonstrate the essential role of macrophages in controlling influenza virus replication. Enhanced lethality occurs in macrophage-depleted mice after infection with genetically reassorted H1N1 virus containing hemagglutinin (HA) and neuraminidase (NA) of 1918 virus (19). Depletion of macrophages during H1N1 infection in pigs causes 40% lethality with decreased antibody titers and CD8+ lymphocytes expressing IFN- γ (20).

Alveolar macrophages are present in close proximity with alveolar epithelial cells, and communication between these cells is important for maintaining homeostasis within the alveoli. The alveolar lining is covered by alveolar type I and type II epithelial cells, which play essential roles in fluid balance and in secretion of surfactant proteins SP-A, SP-B, SP-C, and SP-D (21–24). Although enhanced lethality in macrophage-depleted animals is attributed to elevated virus titers, the fate of alveolar epithelial cells in macrophage-depleted animals after influenza virus infection is unclear. While previous studies implicate the

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role of macrophages in reducing virus titers, there are no studies on whether macrophages participate in the protection or resolution of alveolar epithelium. Macrophage lineage cells promote tissue repair by producing potential growth factors such as hepatocyte growth factor or HGF (25, 26). HGF is a mitogen for various types of epithelia, including bronchial and alveolar epithelial cells (27, 28). Recombinant HGF augments DNA synthesis of alveolar type II cells in acute lung injury, and also reduces pulmonary fibrosis during bleomycin-mediated lung injury (29). MCP-1 can induce HGF production in macrophages, and can also enhance their phagocytotic ability (30).

In this study, we investigated the fate of the alveolar epithelium in a murine model of influenza pneumonitis after inhibition of macrophage recruitment into the lungs using anti-MCP-1 monoclonal antibody treatment. Histopathology, immunohistochemistry, Western blot, reverse transcription-polymerase chain reaction (RT-PCR), and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were performed to evaluate the extent of alveolar damage. HGF production in bronchoalveolar lavage fluid (BALF) and lung homogenate after infection and anti-MCP-1 treatment was investigated. In addition, cultured lung explants were treated with recombinant HGF to characterize the role of HGF in lung repair after influenza viral infection.

MATERIALS AND METHODS

Mice, Influenza Virus Infection, and Anti-MCP-1 Treatment

Female 4- to 6-week-old BALB/c mice were housed in micro-isolator cages in an animal BSL-2 laboratory facility. All animal protocols were approved by the Institutional Animal Care and Use Committee, National University of Singapore. Animals were divided into four groups, and anesthetized with 375 mg/kg Avertin. Animals were infected intranasally with 30 μ l of mouse-adapted human influenza virus A/Aichi/2/68Influenza virus at 10^4 TCID₅₀ (6). The anti-MCP-1 treatment group ($n = 15$) received three intraperitoneal injections of 15 μ g per dose of anti-MCP-1 monoclonal antibody (R&D Systems, Minneapolis, MN) at 12, 24, and 48 hours after infection. The control group ($n = 15$) received 15 μ g per dose of mouse nonimmune immunoglobulin (IgG) (R&D Systems) at similar time-points after infection. Another control group was infected but untreated ($n = 10$), while the last control group received normal uninfected mouse lung homogenate ($n = 10$). Animals were monitored daily for clinical signs of infection, including body weight loss, and all mice were killed on Day 4.

Determination of Virus Titers

The virus titers in the lung homogenate were assayed by infectivity in MDCK cells. TCID₅₀ was determined by a reduction in cytopathic effect (CPE) of 50%, and the log TCID₅₀ of virus per milliliter of homogenate was calculated.

Bronchoalveolar Lavage Fluid Analyses

For bronchoalveolar lavage fluid (BALF) sample collection, animals were anesthetized, the trachea was exposed, and the lungs were washed twice with 0.5 ml of cold phosphate-buffered saline (PBS). The recovery of the lavage fluid was approximately 90%. The BALF samples were centrifuged at $1,100 \times g$ for 10 minutes, and the supernatants were immediately frozen at -80°C until further use. The cell pellets were resuspended in PBS, and total cell counts were measured using a hemocytometer. For differential cell counts, the cells were processed onto microscopic slides using a Cytospin 2 cytocentrifuge (StatSpin, Westwood, MA), and subjected to modified Giemsa staining. Cells (500 per animal) were counted at a magnification of $\times 400$.

Measurement of Mouse Keratinocyte-Derived Chemokine, Leukotriene B₄, and HGF Levels by Enzyme-Linked Immunosorbent Assay

Serum concentrations of mouse keratinocyte-derived chemokine (KC) and leukotriene B₄ (LTB₄) were measured using double-ligand enzyme-linked

immunosorbent assay (ELISA) and competitive enzyme immunoassay (R&D Systems) according to the manufacturer's instructions. HGF levels in BALF and macrophage-LA-4 mixed culture supernatant were determined using a double-ligand ELISA kit (Institute of Immunology, Tokyo, Japan) according to the manufacturer's recommendations.

Determination of Myeloperoxidase Enzyme Activity

Myeloperoxidase (MPO) activity in the lung homogenate was assayed as described previously (31). Briefly, lung homogenate (20 μ l) was mixed with MPO assay solution (980 μ l). The latter was prepared fresh before use by mixing 107.6 ml of H₂O, 12 ml of 0.1 M sodium phosphate buffer (pH 7.0), 0.192 ml of guaiacol, and 0.4 ml of 0.1 M H₂O₂. The generation of tetraguaiacol was measured spectrophotometrically at 470 nm wavelength, and the change in optical density (Δ OD) per minute was calculated from the initial rate. The MPO activity was then calculated using the formula (units/ml = Δ OD/min $\times$ 45.1), and expressed as units per milligram of protein. One unit of the enzyme is defined as the amount that consumes 1 μ mol of H₂O₂ per minute.

Evaluation of Pathogenicity by Histopathology

All animals from each group were studied by histologic analyses of their lungs. Lung tissues were fixed in 4% formaldehyde in PBS, dehydrated, and embedded in paraffin. Paraffin sections of 4 μ m thickness were cut, dewaxed, and stained with hematoxylin-eosin for histopathologic evaluation under light microscopy. Histopathology slides were scored in a blinded manner on the basis of the following parameters: necrotizing bronchiolitis (damage of airway epithelial cells, presence of necrotic bodies, or denudation of the entire airway lining); inflammation in bronchioles (bronchioles filled with inflammatory cells, including macrophages, neutrophils, and lymphocytes); alveolitis (damaged alveolar epithelial cells with denudation or necrosis, and their presence in the alveoli); interstitial inflammation (inflammation in alveoli or thickening of alveolar interstitium); hemorrhage (presence of erythrocytes in the alveolar space due to capillary leakage or endothelial damage); and edema (presence of proteinaceous material in the alveolar space). The severity of damage was scored on a scale ranging from 0 to 4 (0 for none or very minor, 1 for mild, 2 for intermediate, 3 for moderately severe, and 4 for severe and widespread). The total lung surface was scored at $\times 400$ magnification, and each overall score was expressed as mean value $\pm$ SE.

Western Blot Analysis

Frozen lung tissues were homogenized in lysis buffer (10 mM Tris-HCl pH 7.5, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin), and protein concentration was determined using the DC protein assay kit (Bio-Rad, Hercules, CA). Protein (30 μ g) was solubilized in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, 0.01% bromophenol blue), separated on SDS-PAGE (12%), and transferred onto a nitrocellulose membrane at 100 mA for 2 hours. To check the efficiency of protein transfer, each membrane was stained with Ponceau S and blocked for 1 hour with 5% milk in 100 mM Tris-buffered saline containing 0.1% Tween 20 (TBST). Each membrane was incubated at 4°C overnight with 1:1,000 dilutions of anti-SP-C (Santa Cruz Biotechnology, Santa Cruz, CA), anti-T1- α , or anti- β -actin (Sigma, St. Louis, MO) antibodies. Each membrane was then washed thrice (5 min each) in TBST, and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:5,000; Santa Cruz Biotechnology) for 1 hour. After washing thrice (5 min each), each blot was developed with enhanced chemiluminescence reagents and exposed to X-ray film to visualize the protein bands. Densitometric analyses were performed using a Bio-Rad densitometer for all proteins, and the intensity of each target protein was expressed as a percentage of the β -actin band.

Semi-Quantitative RT-PCR

Total RNA was isolated from frozen lung tissues using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany), and reverse-transcribed into cDNA with the MMLV Reverse Transcription system (Promega, Madison, WI) using random primers (32). Aliquots of cDNA (1 μ l each) were amplified by PCR using SP-C, T1- α , HGF, and β -actin

primers (Table 1). The thermal cycling profile was 94°C for 3 minutes, followed by 34 cycles each at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for 10 minutes. For β -actin, PCR was performed for up to 22 cycles to ensure amplification in the exponential range. The RT-PCR products from each animal were quantified using a Bio-Rad densitometer, and each band intensity was expressed as a percentage of the corresponding β -actin amplicon.

Immunohistochemistry

Lung sections were deparaffinized in xylene, permeabilized with 0.5% Triton X-100 in PBS for 20 minutes, and blocked with 5% milk in PBS for 30 minutes. The sections were then incubated at 4°C overnight with 1:100 dilutions of primary antibodies (i.e., mouse anti-T1- α , anti-SP-C, anti-PCNA [Santa Cruz Biotechnology] or anti-HGF [R&D Systems]). After washing thrice with PBS for 5 minutes, the slides were incubated with 1:250 dilutions of secondary antibodies conjugated to Alexa 546 or 488 (Molecular Probes, Eugene, OR) at room temperature for 1 hour. The slides were washed thrice with PBS, mounted, and examined using an Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan). For quantitative analysis of type II epithelial hyperplasia, lung sections were double-stained with anti-SP-C and anti-PCNA. Cells staining positive for both antigens were considered as proliferating, and the ratio of double-positive cells to total SP-C-positive cells was ascertained. At least five fields from each lung section at $\times 400$ magnification were evaluated, and data were obtained from all animals per group. For detection of HGF, BALF cells were subjected to cytocentrifugation, fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100, incubated with anti-HGF antibody (R&D Systems), and mounted with mounting medium containing diaminodiphenylindole or DAPI (Vector Laboratories, Burlingame, CA).

Detection of Apoptosis by TUNEL Assay

TUNEL assay was performed on 4- μ m formalin-fixed slides using the In Situ Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions (33). For quantification of alveolar epithelial cells, at least five randomly selected fields at $\times 400$ magnification were selected per lung section in a blinded manner. Cells on the surface of the alveolar epithelium were counted, but those within the airspace or not clearly attached to the alveolar surface were excluded. To calculate the apoptosis index, the number of TUNEL-positive alveolar cells was expressed as the percentage of the total number of nuclei in the same field. Sections from all mice in the treatment and control groups were analyzed.

Isolation of Mouse Alveolar Macrophages

Animals were anesthetized and alveolar macrophages were harvested by repeated lung lavage with PBS (34). The lavaged cells were centrifuged at $1,000 \times g$ at 4°C for 10 minutes, and then resuspended in Dulbecco's modified Eagle's medium. Viable cells were counted by the trypan blue exclusion assay. Giemsa staining of cytospin preparations showed that more than 98% of cells were normal lung macrophages.

Preparation of Influenza Virus-Infected LA-4 Cells and *In Vitro* Assay for HGF Production

LA-4 mouse lung epithelial cells (American Type Culture Collection, Manassas, VA) were cultured overnight in F12K medium with 15% fetal bovine serum. Cells were infected with mouse-adapted influenza virus (at MOI of 4) in serum-free medium for 1 hour, replaced with the same medium, and further incubated at 37°C for 12 hours. Immunocytochemistry using polyclonal anti-influenza antibody (6) showed that at MOI of 4, 95% of cells stained positive for influenza viral antigen at 12 hours after infection. To determine whether macrophages produce HGF when exposed to infected epithelial cells, freshly isolated alveolar macrophages from mouse lungs were mixed with infected LA-4 cells at a ratio of 2:1, and incubated for 30 minutes in the presence or absence of anti-MCP-1 antibody (1 μ g/ml). Cells were washed thoroughly to remove unattached cells (mostly infected LA-4 cells), and further incubated for 24 hours. Cell culture supernatants were then collected to measure HGF levels by ELISA. Cells were lysed, total RNA was extracted, and semiquantitative RT-PCR was performed to determine HGF transcript levels. The amplified product from each animal was

TABLE 1. PRIMERS USED FOR SEMIQUANTITATIVE RT-PCR

Gene	Forward Primer	Reverse Primer
SP-C	5'-AGCAAAGAGGTCCTGATGGA-3'	5'-AATCGGACTCGGAACAGTA-3'
T1- α	5'-GCCAGTGTGTTCTGGGTTT-3'	5'-TGATTCCAACAGGGTGACT-3'
HGF	5'-GCCAGGTGACCTTTGCTTAA-3'	5'-TGAACGTAAAGCCCCTGTTC-3'
β -actin	5'-ACTGGGACGACATGGAGAA-3'	5'-TCTCAGCTGTGGTGGTAA-3'

Definition of abbreviations: HGF, hepatocyte growth factor; SP-C, surfactant protein C.

quantified by densitometry, and expressed as the percentage of the intensity of the corresponding β -actin amplicon.

Phagocytic Activity

Alveolar macrophages from uninfected animals were isolated, counted, and mixed with uninfected or virus-infected LA-4 cells at a ratio of 1:2. To assess phagocytic activity, co-cultured cells were incubated at 37°C for 1 hour and washed extensively with PBS to remove unattached cells. Cells were then fixed in 4% formaldehyde, and permeabilized with 0.05% Triton X-100. Ingested LA-4 cells were detected by staining with antibody against virus, while macrophages were stained with F4/80 antibody (Santa Cruz Biotechnology). Macrophages displaying viral immunostaining were considered positive for phagocytosis. Experiments were performed in duplicate, and a total of five randomly selected fields per well was observed in triplicate. Phagocytic index was calculated by measuring the percentage of positive cells out of total cells.

Culture of Lung Explants and Treatment with Recombinant HGF

Female BALB/c mice were anesthetized, the trachea was exposed, the lungs were cleared of blood by perfusion with cold PBS and immediately placed in BGJb medium (Gibco, Invitrogen, Carlsbad, CA) containing penicillin, streptomycin, and neomycin. The lungs were sliced into 1-mm-thick sections, and each slice (in 4 μ g/ml trypsin-EDTA) was infected with an influenza virus inoculum of 0.5×10^6 TCID₅₀ for 1 hour, placed on Transwell translucent polycarbonate culture dish inserts of 8.0 μ m pore size, 12 mm diameter (Costar, High Wycombe, UK), and cultured with serum-free BGJb medium. At 12 hours after infection, the wells were supplemented with 500 ng/ml of recombinant HGF (R&D Systems) or 10 μ g/ml of anti-HGF monoclonal antibody. BrdU (2 mM) was added to the medium 4 hours before lung explants were collected at 24, 48, and 72 hours after infection. The explants were fixed with 4% formaldehyde in PBS, and processed for histologic analysis as described above.

Evaluation of Alveolar Type II Cell Proliferation

Immunohistochemistry of the lung explants was performed to measure alveolar type II cell proliferation. Lung sections were processed for immunostaining, and incubated overnight at 4°C with anti-mouse BrdU (1:200), anti-SP-C (1:100), and anti-PCNA (1:100) antibodies. Secondary anti-mouse-Alexa-546 and anti-rabbit-Alexa-488 antibodies were used for detection, and slides were mounted with DAPI-containing medium. For quantitative analysis of type II epithelial proliferation, data were obtained from two independent experiments performed in triplicate at each time-point, and at least five fields from each section at $\times 400$ magnification were evaluated.

Statistical Analyses

Each result was expressed as mean $\pm$ SE. Statistical analyses were performed by ANOVA or Student's *t* test. Differences in animal weights were analyzed by the Mann-Whitney U test. A value of *P* < 0.05 was considered statistically significant.

RESULTS

Anti-MCP-1 Treatment Decreases Leukocyte Recruitment to Infected Lungs

To determine the cellular infiltrates into infected lungs after anti-MCP-1 treatment, BALF-associated cells were subjected

to Giemsa staining. Elevated numbers of total leukocytes including macrophages and neutrophils were noted after influenza virus infection. Infected animals injected with non-immune IgG also displayed increased cellular infiltration comparable with the infected group. In contrast, treatment with anti-MCP-1 antibody significantly reduced infiltration of both macrophages and neutrophils (Figure 1A).

Effect of Anti-MCP-1 Treatment on Animal Weights and Virus Titers

Compared with control uninfected mice, the weights of infected animals treated with anti-MCP-1 revealed a decreasing trend at all time-points. Significant weight loss was observed especially on Day 4 (Figure 1B). Animals treated with anti-MCP-1 experienced more than 20% weight loss, and two treated mice died on Day 4. However, no significant differences in virus titers were observed among untreated and antibody treatment groups (Figure 1C).

Anti-MCP-1 Treatment Reduces MPO Activity and Serum Mouse KC Levels

Given that anti-MCP-1 treatment reduced the total number of neutrophils in BALF, we further investigated whether blocking MCP-1 influenced the levels of mouse KC, the murine analog of human IL-8 which is a chemoattractant for neutrophils. Significant reduction of mouse KC in serum was observed (Figure 2A). Congruent with this finding, MPO levels were drastically reduced in lungs of anti-MCP-1-treated animals compared with the infected or IgG-treated groups (Figure 2B). The LTB₄ levels (in pg/ml) for the four groups were 20.5 ± 2.5 (anti-MCP-1 treatment), 22.8 ± 2.7 (IgG treatment), 21.5 ± 3.0 (infected), and 10 ± 1.6 (uninfected).

Histopathologic Analyses Reveal Differences in Pulmonary Damage

To investigate the histopathologic changes in influenza virus-infected animals after anti-MCP-1 treatment, histopathologic

Figure 1. (A) Effect of anti-monocyte chemoattractant protein (MCP)-1 treatment on leukocyte recruitment into murine lungs after infection with 10^4 TCID₅₀ of mouse-adapted human influenza A/Aichi/2/68 virus. Lung homogenates (LH) prepared from uninfected mice served as the uninfected control. Cellular infiltrates were evaluated in bronchoalveolar lavage fluid (BALF) samples collected on Day 4 after infection without and with treatment with anti-MCP-1 or nonimmune IgG. Total cell counts were significantly increased in virus-infected and nonimmune IgG-treated groups compared with uninfected animals. In contrast, both macrophage and neutrophil numbers were drastically diminished in the anti-MCP-1-treated group. Results are expressed as means $\pm$ SE, with $n = 5$ per group. * $P < 0.05$ versus LH group; ** $P < 0.05$ versus infected but untreated group. (B) Changes in body weights of BALB/c mice after infection and treatment with antibodies. Animal weights were recorded daily for 4 days, and expressed as means $\pm$ SE, with $n = 10$ for LH and virus-infected groups, and $n = 15$ for anti-MCP-1 and IgG treatment groups. (C) Determination of virus titers in mouse lung homogenates. MDCK cells were infected with serial 10-fold dilutions of lung homogenates from uninfected, virus-infected, anti-MCP-1-treated, and IgG-treated animals. The numbers on the y axis represent virus titers expressed as 10^4 TCID₅₀ per gram of total protein (means $\pm$ SE), with $n = 5$ for the virus-infected group and $n = 10$ for anti-MCP-1 and IgG treatment groups. The virus titers ($\sim 10^7$) of the three infected groups were similar.

Figure 2. Effect of anti-MCP-1 antibody treatment on levels of (A) serum mouse keratinocyte-derived chemokine (KC) and (B) lung MPO. Both mouse KC and myeloperoxidase (MPO) levels were significantly decreased in anti-MCP-1-treated infected animals. Data are represented as means $\pm$ SE, with $n = 5$ for uninfected and infected groups, and $n = 10$ for anti-MCP-1 and IgG treatment groups. * $P < 0.05$ versus LH group; ** $P < 0.05$ versus infected but untreated group.

analyses were evaluated using a scoring system. As shown in Table 2, significant alveolitis was evident in anti-MCP-1-treated animals compared with IgG-treated or nontreated infected mice. The alveolar spaces exhibited enlargement, with some denuded areas showing complete loss of epithelium. However, cellular infiltration was more prominent in the untreated group compared with the anti-MCP-1-treated group. Although alveolar epithelial damage was observed in both untreated and IgG-treated animals, hyperplasia of type II alveolar epithelial cells was also noted (Figure 3). Interstitial inflammation was less conspicuous in anti-MCP-1-treated and IgG-treated animals compared with untreated controls. No significant difference in inflammation in bronchioles, hemorrhage, or edema was noted among untreated and antibody-treated infected animals.

Pronounced Alveolar Epithelial Damage and Apoptosis after Anti-MCP-1 Treatment

The alveolar epithelium is a major target for influenza virus replication, which was confirmed by histopathologic evidence of remarkable alveolitis. To further evaluate the extent of alveolar epithelial damage, we specifically investigated T1- α as well as SP-C, which represent surface epithelial markers for alveolar type I and type II pneumocytes, respectively. Our results revealed considerable reduction in expression of these markers at both protein (Figures 4A and 4B) and transcriptional levels (Figures 4C and 4D) in anti-MCP-1-treated animals. Immunostaining for SP-C protein showed drastic decrease in SP-C staining intensities in anti-MCP-1-treated animals compared with the other three control groups. Staining for T1- α was continuous in the lungs of control uninfected mice. Despite enhanced cellular infiltration in untreated and IgG-treated infected animals, T1- α staining was intense. In contrast, the lungs of anti-

MCP-1-treated animals displayed discontinuous T1- α staining, indicating type I pneumocyte damage (Figure 4E). Alveolar type II epithelial hyperplasia was also determined by double immunostaining for PCNA and SP-C (Figure 5). The percentages of cells positive for both PCNA and SP-C were significantly elevated in untreated and IgG-treated infected animals, thus indicating alveolar type II epithelial proliferation.

By the TUNEL assay, animals treated with anti-MCP-1 demonstrated increase in apoptotic cells in the alveolar septae (Figures 6A and 6B). Although the total numbers of apoptotic alveolar epithelial cells in untreated and IgG-treated infected animals were comparatively fewer, their lungs displayed more apoptotic cells within the alveolar spaces, the majority of which were macrophages with engulfed infected cells. In contrast, alveolar spaces of anti-MCP-1-treated animals exhibited fewer infiltrated cells showing apoptosis.

Enhanced HGF Production in BALF and Lungs after Influenza Virus Infection

The recovery of BALF from all animals was approximately 90%, and HGF levels in BALF were measured by ELISA. HGF protein levels were significantly increased in untreated compared with anti-MCP-1-treated infected animals (Figure 7A). In agreement with this, HGF mRNA expression in lung tissue was significantly higher in control versus anti-MCP-1-treated animals (Figures 7B and 7C). Both HGF protein and mRNA levels were similar in the untreated and IgG-treated groups. Immunostaining for HGF in lung tissue and BALF cells revealed strongly positive signals mainly in alveolar macrophages, with occasional staining in alveolar epithelial cells. BALF cells also displayed HGF staining only in macrophages but not in neutrophils or lymphocytes (Figure 7D).

TABLE 2. SEMIQUANTITATIVE SCORING OF LUNG HISTOPATHOLOGY

Sample	Necrotizing Bronchiolitis	Inflammation in Bronchioles	Alveolitis	Interstitial Inflammation	Hemorrhage	Edema
LH	0.2 $\pm$ 0.4	1 $\pm$ 0.7	0	0.5 $\pm$ 0.5	0	0
Virus	1.2 $\pm$ 1.1	2.2 $\pm$ 1.0	1.2 $\pm$ 0.8	3 $\pm$ 0.9†	0.8 $\pm$ 0.9	1.1 $\pm$ 0.9
Virus + MCP-1	1.9 $\pm$ 1.0	1.9 $\pm$ 0.9	2.5 $\pm$ 0.7*	1.7 $\pm$ 0.9	0.8 $\pm$ 0.8	0.7 $\pm$ 0.7
Virus + IgG	2 $\pm$ 0.7	2.4 $\pm$ 0.8	1.6 $\pm$ 0.8	2.2 $\pm$ 1.0	0.6 $\pm$ 0.6	0.7 $\pm$ 0.6

Definition of abbreviations: IgG, immunoglobulin G; LH, lung homogenates; MCP-1, monocyte chemoattractant protein-1.

* $P < 0.05$ versus virus group and virus + IgG group.

† $P < 0.05$ versus virus + MCP-1 group.

Figure 3. Effect of anti-MCP-1 treatment on histopathologic changes in lungs at Day 4 after infection. Animals administered with uninfected LH displayed normal architecture of airway (BR) and alveolar epithelia (AV) without inflammation (A, B). Mice infected with influenza virus (C, D) and treated with IgG (G, H) exhibited increased cellular infiltration with mild damage of both airway and alveolar epithelia. Prominent hyperplasia of alveolar epithelial cells was also observed in both infected and IgG-treated animals (arrows). Enhanced alveolar and airway epithelial damage with enlarged airspaces and some denuded epithelial lining (arrowheads) were noted in infected animals treated with anti-MCP-1 (E, F). Tissue sections were stained with hematoxylin and eosin, and examined at magnifications of $\times 100$ and $\times 400$ in the left and right columns, respectively. Semiquantitative histopathology scoring was performed ($n = 5$ for LH and infected groups and $n = 10$ for anti-MCP-1 and IgG treatment groups), with the scores shown in Table 2.

Co-Culture of Infected Cells with Alveolar Macrophages Augments the Production of HGF

To investigate whether macrophages produce HGF when exposed to influenza virus-infected epithelial cells, we conducted *in vitro* co-culture studies using freshly isolated mouse alveolar macrophages with influenza virus-infected LA-4 cells. HGF levels in culture supernatants were significantly elevated when macrophages were incubated with infected LA-4 cells compared with uninfected cells (Figure 8A). Accordingly, HGF mRNA expression was also induced in macrophages co-cultured with infected epithelial cells (Figures 8B and 8C). Further addition of anti-MCP-1 to the co-culture did not inhibit HGF production. Uninfected macrophages and LA-4 cells alone or in co-culture showed no induction of HGF expression.

Anti-MCP-1 Treatment Does Not Affect the Phagocytic Activity of Alveolar Macrophages

To explore whether MCP-1 plays a role in the phagocytosis of influenza virus-infected cells, freshly isolated macrophages were incubated with uninfected or infected LA-4 cells in the presence or absence of anti-MCP-1 (1 $\mu\text{g}/\text{ml}$). Cells were then washed and stained with anti-influenza and anti-F4/80 antibodies. An increase in phagocytic index (22.6 ± 0.7) was observed when macrophages were incubated with infected epithelial cells. However, phagocytic activity (24.9 ± 1.2)

was not significantly altered in the presence of anti-MCP-1 antibody.

Treatment with Recombinant HGF Stimulates Alveolar Epithelial Cell Proliferation in Murine Lung Explants

To interrogate the role of HGF in alveolar epithelial repair, lung explants were treated with recombinant HGF or anti-HGF monoclonal antibody after infection. BrdU labeling assay exhibited significantly higher numbers of alveolar type II epithelial cells positive for both BrdU and SP-C in recombinant HGF-treated lung explants compared with anti-HGF-treated or untreated infected cultures (Figure 9). Alveolar type II epithelial cell proliferation was approximately 2.7-fold higher in HGF-treated infected explants compared with uninfected controls at 48 and 72 hours after infection. These findings clearly indicate the involvement of HGF in the mechanism of alveolar epithelial regeneration during influenza virus infection.

DISCUSSION

Recent studies indicate that alveolar macrophages may play a protective role during influenza virus infection. Although cytokines produced by macrophages are implicated in the pathogenesis of severe influenza virus infections (11–13), macrophage-depleted animals infected with influenza virus suffer high

Figure 4. Evaluation of alveolar epithelial damage by Western blot, RT-PCR, and immunohistochemistry. Alveolar epithelial markers including T1- α (for type I epithelium) and surfactant protein (SP)-C (for type II epithelium) were used to evaluate alveolar epithelial damage. (A) Western blot analyses depicting T1- α and SP-C expression in lung homogenates of (1) uninfected, (2) virus-infected, (3) anti-MCP-1, and (4) IgG treatment groups. Anti-MCP-1-treated animals showed significantly reduced expression of SP-C and T1- α , with β -actin (42 kD) serving as a loading control. (B) Densitometric analyses of Western blot bands, each expressed as a percentage of the corresponding control β -actin band (with the LH group assigned as 100%). (C) Semiquantitative RT-PCR assay for mRNA expression of T1- α and SP-C. (D) Densitometric analyses of RT-PCR amplicons, each expressed as a percentage of the corresponding control β -actin amplicon (with the LH group assigned as 100%). Representative results are shown for uninfected and infected groups ($n = 5$ each), and for anti-MCP-1 and IgG treatment groups ($n = 10$ each). Asterisk denotes statistical significance at $P < 0.05$ versus LH group. (E) Immunostaining for detection of T1- α and SP-C in lung sections counterstained with DAPI nuclear dye. Uninfected lung section shows continuous T1- α staining (arrows) covering 95% of the alveoli. Strong SP-C signals (arrowheads) are depicted in uninfected and IgG-treated groups. However, discontinuous T1- α staining (*) and minimal SP-C staining (white arrow) were observed in anti-MCP-1-treated animals.

Figure 5. Quantitative analysis for alveolar type II cell hyperplasia by staining lung sections with SP-C and PCNA. Out of the total SP-C-positive cells, the number and percentage of cells positive for both PCNA and SP-C were determined for each group. Results are expressed as mean percentages $\pm$ SE, with $n = 5$ for uninfected and infected groups, and $n = 10$ for anti-MCP-1 and IgG treatment groups. * $P < 0.05$ versus LH group; ** $P < 0.05$ versus infected group.

mortalities compared with nondepleted controls (19, 20). Alveolar macrophages exist in close proximity with alveolar epithelium, which is a major target for influenza virus replication. Although elevated viral titers are thought to contribute to enhanced lethality in macrophage-depleted animals, the fate of the alveolar epithelium is unclear. In this study, we provide evidence of enhanced alveolar epithelial damage and apoptosis after blocking macrophage recruitment by treatment with anti-MCP-1 antibody in a mouse model of influenza pneumonitis. Control mice sub-lethally infected with influenza virus, and control infected mice treated with nonimmune IgG, displayed hyperplasia of alveolar type II cells and enhanced HGF production in BALF. In contrast, no epithelial proliferation and decreased HGF levels were observed in anti-MCP-1-treated

infected animals. Furthermore, HGF expression was induced in alveolar macrophages incubated with influenza virus-infected LA-4 cells, and recombinant HGF treatment of infected lung explants stimulated alveolar type II epithelial cell proliferation, thus suggesting the potential role of macrophages in the resolution of alveolar epithelium.

MCP-1 belongs to the family of CC chemokines with pleiotropic activities. It is produced by many different cell types, and is a major chemoattractant for leukocytes. Hyperoxia-mediated lung injury and septic peritonitis models reveal that treatment with anti-MCP-1 antibody reduces leukocyte infiltration in the lungs and peritoneum, respectively (35, 36). Here we provide evidence that anti-MCP-1 treatment significantly reduced infiltration of macrophages into the lungs after influenza virus infection. Given that elevated MCP-1 levels were detected by 12 hours (data not shown), anti-MCP-1 antibody treatment was commenced 12 hours after infection and continued until 48 hours to achieve significant reduction in macrophage infiltration. Blocking MCP-1 also reduced the neutrophil population and MPO level in bronchoalveolar lavage. Similarly, mice lacking the MCP-1 gene display diminished pulmonary infiltration of both macrophages and neutrophils after influenza virus infection (17). Moreover, after anti-MCP-1 treatment, reduction of serum murine KC (a chemokine similar to cytokine-induced neutrophil chemoattractant-1 or CINC-1), but not of the lipid mediator leukotriene B₄, suggest that mouse KC may influence the recruitment of neutrophils during influenza virus infection. These results concur with previous studies elucidating the role of mouse KC and MIP-2 in neutrophil recruitment in influenza virus infection (37). In addition, MCP-1 facilitates the pulmonary recruitment of CD8⁺ T cells that are crucial in host defense against viral infection (20). Hence, reduced MCP-1-dependent CD8⁺ T cell recruitment may also contribute to the observed pathologic effects.

Compared with infected but untreated control animals, infected mice treated with anti-MCP-1 experienced significant weight loss on Day 4 after infection, with two animals dying on Day 4. These observations are compatible with studies on macrophage depletion in pigs and mice that report increased mortality upon challenge with sub-lethal influenza virus doses.

Figure 6. Detection of apoptosis by TUNEL assay. (A) Representative micrographs depicting lungs of (i) uninfected, (ii) virus-infected, (iii) anti-MCP-1 treatment, and (iv) IgG treatment groups. Arrows indicate apoptotic epithelial cells, while arrowheads show TUNEL-positive macrophages at $\times 400$ magnification. (B) Quantification of apoptosis was determined by the number of positively stained nuclei within the alveolar epithelial lining. The apoptosis index represents the average percentage of TUNEL-positive alveolar epithelial cells, and the results are expressed as means $\pm$ SE, with $n = 5$ for LH and infected groups, and $n = 10$ for anti-MCP-1 and IgG treatment groups. * $P < 0.05$ versus LH group; ** $P < 0.05$ versus infected but untreated group.

Figure 7. Effect of anti-MCP-1 treatment on HGF production in BALF and lungs. (A) HGF levels in BALF measured by ELISA ($n = 5$ per group). (B) Expression of HGF mRNA in lungs analyzed by RT-PCR exemplifying HGF-specific fragments amplified from total RNAs of lungs of three representative mice per group. (C) Densitometry analysis, with each HGF RT-PCR amplicon expressed as a percentage of the corresponding β -actin control amplicon. Data are represented as means $\pm$ SE, with $n = 5$ for uninfected and infected groups, and $n = 10$ for anti-MCP-1 and IgG treatment groups. * $P < 0.05$ versus LH; ** $P < 0.05$ versus infected group. (D) Immunostaining for HGF detection in lung sections and BALF cells counterstained with DAPI nuclear dye. (i) Uninfected lung section shows occasional staining in alveolar epithelial cells (arrowheads). Strong HGF signals are apparent in macrophages (arrows) of (ii) infected lung sections and (iii) BALF cells. (iv) Distinct HGF staining in macrophages (arrows) but absent in neutrophils (open arrows) in BALF.

Depletion of macrophages in mice before infection with recombinant influenza virus containing the HA and NA genes of highly virulent 1918 virus (1918 HA/NA:TX91 strain) leads to decreased levels of cytokines (including TNF- α , IFN- β , IFN- γ , MIP-1 α , and MIP-2), but culminates in elevated viral load, systemic viral spread into the brain, and enhanced lethality. However, depletion of macrophages 3 days after infection does not have any impact on the disease outcome. Similar findings with reduced TNF- α and IFN- γ , increased viral load and lethality are noted in another study of macrophage-depleted pigs infected with human H1N1 virus (A/New Caledonia/20/99). Notwithstanding that previous studies document enhanced virus titers in macrophage-depleted animals (19, 20), we observed no significant change in virus titers after anti-MCP-1 treatment. This may be explained by initial clearance of the virus by resident macrophages, since anti-MCP-1 treatment commenced only at 12 hours after infection,

whereas macrophages were depleted before infection in other studies.

We previously demonstrated that influenza virus replicates efficiently in both alveolar type I and type II epithelial cells, with the severity of infection being concomitant with loss of these cells (6). Depletion of macrophages may further enhance the susceptibility of alveolar epithelium to virus infection. Our results clearly indicate enhanced alveolar wall disruption with enlarged alveolar spaces in anti-MCP-1-treated animals. Decreased lung expression patterns of SP-C and T1- α at both protein and mRNA levels in anti-MCP-1-treated animals imply augmented susceptibility of epithelium to virus infection in the absence of macrophages. Congruent with these findings, significantly more pronounced apoptosis of alveolar epithelium was observed in anti-MCP-1-treated animals. Although macrophages and neutrophils constitute part of the innate immune system and act as the first-line defense against influenza virus,

Figure 8. HGF expression in freshly isolated mouse alveolar macrophages incubated with influenza virus-infected LA-4 cells. (A) HGF levels in the cell culture supernatants were determined by ELISA. (B) RT-PCR analysis of HGF mRNA expression. HGF-specific fragments (*upper panel*) together with β -actin fragments (*lower panel*) were amplified from total RNAs of lungs and subjected to agarose gel electrophoresis. Lanes represent (1) macrophages, (2) LA-4 cells, (3) macrophages and uninfected LA-4 cells, (4) macrophages and LA-4 cells at 24 hours after infection, and (5) macrophages and infected LA-4 cells treated with anti-MCP-1 antibody. (C) Amplicons were subjected to densitometry, and the HGF readings expressed as percentages of the corresponding β -actin amplicons. Data are represented as means $\pm$ SE of two independent experiments. * $P < 0.05$ compared with cultured macrophages.

alveolar epithelial cells also produce soluble factors such as surfactant proteins that play important roles in host defense. Thus, SP-A, SP-B, and SP-D are generated by alveolar type II and airway epithelial cells, while SP-C is exclusively synthesized by type II cells. Also known as collectins, SP-A and SP-D facilitate the elimination of invading pathogens by enhancing phagocytic activity of macrophages (23, 38, 39). SP-A-deficient mice exhibit an exaggerated inflammatory response to influenza virus infection (40). Type I epithelial cells also mediate in defense by producing apolipoprotein E and transferrin, which reduce oxidative stress (41). Our data offer strong evidence that enhanced alveolar epithelial damage can contribute to influenza pathogenesis after interruption of macrophage recruitment. The loss of alveolar epithelium causes failure of gas exchange, fluid imbalance, and inadequate respiration, thereby ultimately culminating in death. Our results concur with studies documenting enhanced alveolar epithelial apoptosis in autopsy samples of patients who succumbed to H5N1 infection (42, 43). The mechanism of lung epithelial apoptosis in influenza pneumonia has been attributed to macrophage-expressed TNF-related apoptosis-inducing ligand or TRAIL (44).

Interestingly, in control infected and IgG-treated animals, apoptosis of alveolar epithelium was observed concomitantly with hyperplasia of alveolar type II cells. The latter cells are considered to be pulmonary stem cells involved in the lung repair process for normal alveolar epithelial regeneration after injury. Macrophage lineage cells produce HGF, which is a mitogen for alveolar type II cells (27, 28). Anti-MCP-1

treatment aggravates lung injury in a pneumonia model, whereas treatment with MCP-1 reduces lung injury due to enhanced HGF production by macrophages. We next asked whether HGF is induced during influenza virus infection. The elevation of HGF protein levels in BALF and of HGF transcripts in the lungs of control infected and nonimmune IgG-treated animals, which displayed epithelial cellular hyperplasia, implies the potential role of HGF in epithelial proliferation. Alveolar macrophages exhibited strongly positive staining for HGF, indicating that these cells are a major source of HGF production in the lungs after influenza infection. Furthermore, anti-MCP-1 treatment resulted in significantly diminished HGF levels, further affirming that macrophages are important producers of HGF. In concordance with these findings, we observed significant induction of HGF levels in alveolar macrophages together with their enhanced phagocytic activity when co-cultured with virus-infected LA-4 cells. Addition of MCP-1 induces HGF production by macrophages incubated with aged neutrophils. MCP-1 treatment stimulates HGF production, thus promoting tissue repair during acute bacterial pneumonia (45). In contrast, MCP-1-deficient mice show delayed re-epithelialization and collagen synthesis (46). However, our *in vitro* study revealed that anti-MCP-1 treatment did not affect HGF secretion and phagocytic activity by alveolar macrophages during interaction with infected cells, indicating that there may be other factors involved in stimulating HGF production by macrophages. Lung explants cultured in the presence of recombinant HGF elicited mitogenic activity of

Figure 9. Analysis of alveolar type II epithelial proliferation in murine lung explants exposed to recombinant mouse HGF or anti-HGF monoclonal antibody after influenza virus infection. (A) Immunostaining for detection of SP-C and BrdU in lung sections (at $\times 400$ magnification) of (i) uninfected, (ii) infected, (iii) infected and recombinant HGF-treated, and (iv) infected and anti-HGF-treated lung explants. Double staining (arrows) represent alveolar type II cells undergoing proliferation. BrdU-positive cells were also observed in bronchiolar epithelium of HGF-treated lung explants (open arrow). (B) Quantification of type II epithelial cell proliferation by staining sections of lung explants with SP-C and BrdU. Out of the total SP-C-positive cells, the number and percentage of cells positive for both BrdU and SP-C were determined. Two independent experiments were performed in triplicate at each time-point, and results are expressed as mean percentages $\pm$ SE. * $P < 0.05$ versus uninfected.

alveolar type II epithelial cells compared with explants treated with anti-HGF. Increased BrdU-positive labeling index indicative of DNA synthesis was observed within 24 hours, but more significant stimulation was observed at 48 and 72 hours. These results concur with previous reports documenting the mitogenic effect of HGF in alveolar type II epithelial cells (27, 28). Moreover, HGF is known to possess antifibrotic activity via the up-regulation of Smad7 expression in epithelial cells (47). The addition of anti-HGF blocks the endogenous HGF produced by alveolar macrophages, implying that macrophages are major producers of HGF in the lung. These findings indicate the active role of HGF in the regeneration and resolution of alveolar epithelium after influenza virus infection. Interestingly, Clara cells of the airway epithelium expressing CC10 (Clara cell-specific antigen) also exhibited strongly positive BrdU staining (data not shown), which warrants further investigation. In conclusion, our study demonstrated that inhibition of macrophage recruitment augmented alveolar epithelial damage and apoptosis in influenza virus infection, suggesting the protective role of alveolar macrophages in the resolution of alveolar epithelium via HGF production. Type II pneumocytes serve as local stem cells to regenerate damaged alveolar epithelium

via a mechanism that is partially dependent on MCP-1 signaling, which is involved in pulmonary epithelial repair processes.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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