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Neutrophils Ameliorate Lung Injury and the Development of Severe Disease during Influenza Infection[1]

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The clinical response to influenza infection ranges from mild disease to severe pneumonia and it remains unclear whether the inflammatory response to infection is protective or pathogenic. We have defined a novel role for neutrophils in ameliorating lung injury during influenza infection, thereby limiting development of severe disease. Infection of neutrophil-depleted mice with influenza virus HKx31 (H3N2) led to rapid weight loss, pneumonia, and death. Neutropenia was associated with enhanced virus replication in the respiratory tract; however, viral titers were declining at the time of death, leading us to investigate other factors contributing to mortality. In addition to thymic atrophy, lymphopenia, and viremic spread, depletion of neutrophils led to exacerbated pulmonary inflammation, edema, and respiratory dysfunction. Thus, while it is well established that neutrophils contribute to lung injury in a range of pathological conditions, reduced numbers or impaired neutrophil function can facilitate progression of mild influenza to severe clinical disease. ***The Journal of Immunology,*** **2009, 183: 7441–7450.**

Influenza in its seasonal or pandemic forms is a leading cause of human illness and death throughout the world, accounting for more than 35,000 deaths annually in the United States alone. The clinical response to different virus strains ranges from mild disease to severe pneumonia, and there is great interest in whether the inflammation it induces is protective or pathogenic. Neutrophils are a characteristic and prominent feature of the early inflammatory response to influenza virus infection of humans, ferrets, and mice (1), but their role in host defense remains unclear. Recent studies using the reconstructed virulent 1918 Spanish influenza pandemic virus (H1N1) or highly pathogenic H5N1 viruses to infect mice showed that neutrophils (and macrophages) predominate in the airways early after infection (2, 3). Therapeutic blockade of the neutrophil-attracting chemokine MIP-2 was associated with reduced neutrophil recruitment and a milder lung pathology following infection with mouse-adapted A/PR/8/34 virus (PR8, H1N1), suggesting that dysregulated or excessive neutrophil responses might contribute to disease during severe influenza infection (4). In contrast, direct depletion studies using mAb RB6-8C5 (anti-Gr-1) enhanced the susceptibility of mice to infection with mouse-adapted influenza viruses (5, 6), human virus strains of intermediate virulence (7), or with a recombinant virus bearing the surface glycoproteins of the 1918 pandemic virus (2).

However, concerns arising from analysis of the specificity of RB6-8C5 have underscored this controversy. RB6-85C has been used extensively to deplete $Ly6G^+$ neutrophils in murine models of infection and inflammation (reviewed in Ref. 8), including influenza infection (2, 7, 9). While Ly6G is the major Ag detected by mAb RB6-8C5 (10), it is clear that this mAb also binds to the Ly6C Ag expressed by additional leukocyte populations, including subsets of monocytes, dendritic cells (DC),[3] and $CD8^+$ T cells (11), implicated in anti-viral host defense, confounding interpretation of cellular mechanisms. In particular, the well-documented cross-reactivity of mAb RB6-8C5 with $Ly6C^+CD8^+$ T cells (11–13) makes it difficult to elucidate the role of neutrophils during the latter phase of infection. To circumvent this issue, in the present study we have instead used a novel anti-Ly6G-specific mAb, 1A8, which has previously been shown to deplete blood neutrophils without affecting numbers of $Gr\text{-}1^+$ monocytes (14).

Herein, we demonstrate that mAb RB6-8C5 binds to multiple leukocyte subsets in the lungs of uninfected and influenza virus-infected mice. In contrast, mAb 1A8 bound specifically to $Ly6G^{high}$ neutrophils, enabling selective depletion studies to define the role of neutrophils during influenza virus infection in vivo. All neutropenic mice infected with influenza virus developed severe disease and succumbed to infection 7–8 days postinfection. While neutrophil depletion was associated with increased virus replication in the lungs, we also report intensified pulmonary immunopathology, edema, and dysregulated lung function. Thymic atrophy, lymphopenia, and spread of virus to additional organs were also associated with severe disease in neutropenic animals. Taken together, these data demonstrate the critical role that neutrophils play in containment and clearance of influenza virus and define both local (pulmonary) and systemic manifestations of disease regulated by neutrophils during influenza infection.

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[3] Abbreviations used in this paper: DC, dendritic cell; ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; cDC, conventional DC; DN, double negative; DP, double positive; i.n., intranasal(ly); MDCK, Madin-Darby canine kidney; pDC, plasmacytoid DC; PI, propidium iodide; SP, single positive.

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Materials and Methods

Mice and viruses

C57BL/6 (B6) mice were bred and housed in specific pathogen-free conditions at the Department of Microbiology and Immunology, University of Melbourne, Australia. Male 6- to 8-wk-old mice were used in all experiments. The influenza A virus strain used in this study was HKx31 (H3N2), a high-yielding reassortant of A/PR/8/34 (PR8) that bears the surface glycoproteins of A/Aichi/2/68 (H3N2). HKx31 was grown in 10-day embryonated hens eggs by standard procedures and titrated on Madin-Darby canine kidney (MDCK) cells as described (15).

Infection and treatment of mice

For total respiratory tract infection, mice were anesthetized and infected with 10^2 or 10^5 PFU of HKx31 via the intranasal (i.n.) route in 50 μl of PBS. Mice were weighed daily and assessed for visual signs of clinical disease, including inactivity, ruffled fur, labored respiration, and huddling behavior. Animals that lost ≥25% of their original body weight and/or displayed evidence of pneumonia were euthanized. All research complied with the University of Melbourne's Animal Experimentation Ethics guidelines and policies. At various times after infection, mice were euthanized and the lungs, nasal tissues, brain, heart, spleen, and kidneys were removed, homogenized in PBS, and clarified by centrifugation. Titers of infectious virus in tissue homogenates were determined by standard plaque assay on MDCK cells in the presence of trypsin.

For the depletion of neutrophils in vivo, purified anti-Ly6G rat mAb (1A8, a gift from Prof. T. Malek, Department of Microbiology and Immunology, University of Miami, Miami, FL) was administered to mice. A combination of i.p. (0.5 mg in 0.2 ml) and i.n. (0.2 mg in 0.05 ml) routes were administered to obtain >90% depletion in the blood and to maintain >90% depletion in the airways during influenza virus infection. Mice were treated 24 h before infection and every 48 h thereafter. Control animals received a similar dose of purified whole rat IgG (Jackson ImmunoResearch Laboratories). Depletion of neutrophils in the blood and airways was confirmed by differential leukocyte counts (data not shown).

Recovery of leukocytes from mice

Bronchoalveolar lavage (BAL) cells, heparinized blood, and nasal tissues cells (nasal cavity and nasal turbinates) were obtained as described (7). To obtain single-cell suspensions, lung and nasal tissues were finely minced with scissors, incubated for 30 min at 37°C with 2 mg/ml collagenase A (Roche Diagnostics), and passed through a wire mesh. Samples were treated with Tris-NH_4Cl (0.14 M NH_4Cl in 17 mM Tris, adjusted to pH 7.2) to lyse erythrocytes and washed in RPMI 1640 medium supplemented with 10% FCS (RF_{10}). Cell numbers and cell viability were assessed via trypan blue exclusion using a hemocytometer.

To obtain samples for the identification of conventional and plasmacytoid DCs (cDCs and pDCs, respectively), perfused whole lungs were digested for 20 min at room temperature with collagenase/DNase (1 mg/ml collagenase type II; Worthington Biochemicals; and 1 μg/ml grade II bovine pancreatic DNase I; Boehringer-Mannheim) as previously described (16). Samples were then treated for a further 5 min with EDTA to disrupt T cell-DC complexes. cDCs were identified as $CD11c^{high}$, MHC class II^{high} ($I\text{-}A^b$) (17), and pDCs as $CD11c^{int}$, PDCA-1^+ cells (18, 19).

Differential leukocyte counts and flow cytometry

BAL and blood samples were analyzed by differential leukocyte counts as described (7). For flow cytometry analysis, single-cell suspensions prepared from BAL, lung, and nasal tissues were incubated on ice for 20 min with supernatants from hybridoma 2.4G2 to block Fc receptors and then stained with appropriate combinations of FITC, PE, allophycocyanin, or biotinylated mAbs to Ly6G (1A8), Gr-1 (RB6-8C5), Ly6C (AL-21), CD45.2 (104), CD8a (53–6.7), CD4 (GK1.5), B220 (RA3-6B2), NK1.1 (PK136), CD3e (145-2C11), MHC class II ($I\text{-}A^b$, AF6-120.1), CD11c (HL3; all from BD Pharmingen), F4/80 (BM8, Caltag Laboratories), and mPDCA-1 (JF05-1C2.4.1; Miltenyi Biotec). To identify NKT cells, suspensions were stained with allophycocyanin-labeled-α-galactosylceramine loaded mCD1d tetramers (a gift from Dr. D. Godfrey, Department of Microbiology and Immunology, The University of Melbourne). Living cells were analyzed by the addition of propidium iodide (PI; 10 μg/ml) to each sample and cells were analyzed on a FACSCalibur flow cytometer. A minimum of 50,000 live cells (PI^-) were collected. Leukocyte populations were sorted using a MoFlo cell sorter (Dako).

Pulmonary histopathology

Mice were euthanized by i.p. anesthesia (ketamine and xylazine) overdose and then perfusion fixed via a tracheal cannula with 4% formaldehyde at 200 mm H_2O pressure. After 1 h the trachea was ligated, and the lungs were removed and immersed in 4% formaldehyde for a minimum of 24 h. After fixation of the lung tissue and processing in paraffin wax, sections (4 μm thick) were prepared and stained with H&E. Airway inflammation of H&E-stained lung sections was evaluated on a subjective scale of 0, 1, 2, 3, 4, or 5 (corresponding to no inflammation and very mild, mild, moderate, marked, and severe inflammation, respectively) on randomized, blinded sections by three independent readers (20). Tissues were graded for peribronchiolar inflammation (around three to five small airways per section) and alveolitis in multiple random fields per section.

Immunoperoxidase staining of paraffin-embedded lung sections was performed to examine the distribution of viral Ags. Sections were subjected to Ag retrieval by heat treatment (10 mM citrate buffer (pH 6.0)), and endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol. Sections were blocked for 1 h (protein block; Dako) and incubated with polyclonal goat anti-influenza A Ab (AbD Serotec). The goat Vectastain ABC kit (Vector Laboratories) was then used in conjunction with diaminobenzidine substrate (ImmPACT DAB; Vector Laboratories) according to the manufacturer's instructions. Sections were counterstained with hematoxylin (Sigma-Aldrich).

Lung sections were viewed on a Leica DMI3000 B microscope and photographed at ×10 magnification with a Leica DFC 490 camera running from the Leica Application software.

Assessment of lung function

Mice were anesthetized and connected to a computer-controlled small animal ventilator (flexiVent; SCIREQ). Mice were ventilated at rate of 300 breaths per minute. After 3 min of ventilation, the constant-phase model was used to measure airway resistance (R_N), tissue damping (G), and tissue elastance (H) using the flexiVent software. Only measurements with a coefficient of determination of ≥0.95 were used.

Assessment of lung edema

The lung wet-to-dry weight ratio was used as an index of lung water accumulation during influenza virus infection. After euthanasia of mice, the lungs were surgically dissected, blotted dry, and weighed immediately (wet weight). The lung tissue was then dried in an oven at 60°C for 72 h and reweighed as dry weight. The ratio of weight-to-dry was calculated for each animal to assess tissue edema as previously described (21). The concentration of protein in BAL supernatants were measured by adding Bradford protein dye. A standard curve using BSA was constructed and the OD was determined at 595 nm.

Statistical analysis

For the comparison of two sets of values, a Student's t test (two-tailed, two-sample equal variance) was used. When comparing three or more sets of values, a Kruskal-Wallis test (nonparametric) was used, followed by the Dunn's posttest. A p value of ≤0.05 was considered statistically significant.

Results

Comparison of Gr-1, Ly6C, and Ly6G expression on lung cell subsets from naive and influenza virus-infected mice

A recent study by Daley et al. described the use of a Ly6G-specific mAb, 1A8, as an alternative to mAb RB6-8C5 for the detection and depletion of neutrophils in mice (14). We first assessed the suitability of mAb 1A8 for detection of neutrophils recovered from the airways of influenza virus-infected mice. Staining with mAb 1A8 revealed a distinct population of $Ly6G^{high}$ cells in BAL of mice at day 3 postinfection (Fig. 1*A*). $CD45^+Ly6G^{high}$ BAL leukocytes sorted by FACS displayed morphological features characteristic of neutrophils (Fig. 1*B*) and, on this basis, >95% of $Ly6G^{high}$ leukocytes purified from BAL were classified as neutrophils (Fig. 1*C*).

mAb 1A8 has been shown to bind $Ly6G^{high}$ blood neutrophils, but not Gr-1^+ monocytes (14); however, expression of Ly6G Ag by additional cell types has not been well defined. As leukocyte markers, including Ly6C, are modulated during inflammation

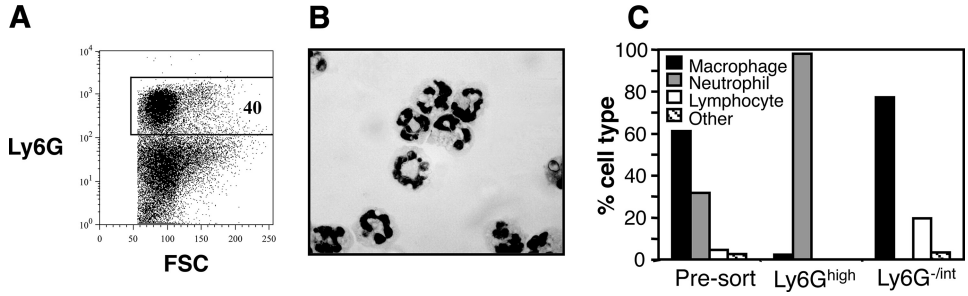

FIGURE 1. Identification of Ly6G^{high} neutrophils using mAb 1A8. Mice infected with 10^5 PFU of HKx31 were killed at day 3 postinfection and BAL was performed. *A*, BAL cells were stained with mAb 1A8 (anti-Ly6G) and analyzed by flow cytometry. A dot plot of CD45$^+$ BAL cells is shown with a gate to identify Ly6G^{high} cells for subsequent analysis. *B*, Morphological characteristics of Ly6G^{high} cells. Ly6G^{high} BAL cells were purified by FACS, centrifuged onto glass slides, and stained with Diff-Quick as described in *Materials and Methods*. *C*, Differential cell counts of BAL cell preparations before (Pre-sort) and after cell sorting based on levels of Ly6G expression (Ly6G^{high} or Ly6G$^{-/int}$). The percentage of macrophages, neutrophils, or lymphocytes in each sample was determined by nuclear morphology. Data are representative of >100 cells counted in four to eight independent fields.

(22–24), it was critical to characterize Ly6G expression on airway leukocytes derived from both naive and influenza virus-infected animals. We compared binding of mAb RB6-8C5 (anti-Gr1; Ly6C/G), mAb AL-21 (Ly6C), and mAb 1A8 (Ly6G) to leukocyte subsets from the lungs of uninfected mice (Fig. 2*A*) or from mice 3 days after infection with virus strain HKx31 (Fig. 2*B*). Gr-1 was expressed on CD8$^+$ cells, NK1.1$^+$ cells, and pDCs (mPDCA1$^+$, CD11c^{int}) from the lungs of uninfected mice and was up-regulated following virus challenge. Furthermore, while Gr-1 was low or absent on lung macrophages (CD11c^{high}, MHC class II^{int}), cDCs (CD11c^{high}, MHC class II^{high}), B220$^+$ cells, and NKT cells (TCRβ^+, CD1d-tetramer$^+$) from uninfected mice, it was up-regulated following influenza virus infection. F4/80$^+$ monocytes/macrophages from the blood of naive or virus-infected animals also expressed Gr-1 and Ly6C, but not Ly6G (data not shown). For each of the leukocyte subsets examined, expression of Gr-1 (as detected with mAb RB6-8C5) correlated with expression of Ly6C (as detected with mAb AL-21). In contrast, mAb 1A8 appears to bind exclusively Ly6G^{high} neutrophils (Fig. 1), and significant Ly6G expression was not detected on other leukocytes from naive or virus-infected animals.

Use of mAb 1A8 to deplete neutrophils during influenza virus infection of mice

Following i.n. challenge with influenza virus, neutrophils are recruited into the alveolar spaces from the marginated lung pool, circulating blood, and the bone marrow reserve via the pulmonary capillary microvasculature. As seen in Fig. 3*A*, neutrophils are virtually absent from the alveolar space of naive mice, but were rapidly recruited following i.n. infection with 10^2 PFU of influenza virus strain HKx31, peaking at day 3 postinfection and declining thereafter.

Next, mice were depleted of neutrophils before and during infection with 10^2 PFU of influenza virus strain HKx31 as described in *Materials and Methods*. Briefly, mice were treated with purified mAb 1A8 (0.5 mg i.p. and 0.2 mg i.n.) 1 day before infection and every second day thereafter. Control mice received an equivalent treatment of purified rat IgG. We confirmed systemic depletion of neutrophils using this treatment regime, as neutrophil numbers were reduced >90% in the blood of mice treated with anti-Ly6G compared with IgG-treated control animals (Fig. 3*B*). Moreover, neutrophil numbers were reduced >90% in BAL of mice treated with anti-Ly6G Abs at day 3 postinfection (Fig. 3*B*), the time of peak neutrophil recruitment to the airways that was recorded (Fig. 3*A*).

Mice treated with mAb 1A8 or control rat IgG were infected with 10^2 PFU of HKx31 and monitored daily for signs of disease (data not shown), changes in body weight, and survival. Uninfected mice receiving a similar regime of purified mAb 1A8 or rat IgG did not lose weight or display any physiological abnormalities over a 10-day monitoring period (data not shown), confirming that depletion of neutrophils in the absence of virus challenge did not lead to clinical illness. Following virus infection, IgG-treated control mice did not lose weight (Fig. 3*C*), did not present any visible signs of clinical disease (data not shown), and all animals survived infection (Fig. 3*D*) out to day 15 postinfection (data not shown). In contrast, virus infection after neutrophil depletion was associated with rapid and profound weight loss (Fig. 3*C*) and all mice succumbed to disease 7–8 days after infection (Fig. 3*D*). Neutropenic mice developed labored breathing, hunched posture, and huddling behavior indicative of acute respiratory distress syndrome (ARDS)-like respiratory failure. If neutrophil depletion was delayed until day 7 postinfection, we did not record any subsequent weight loss or signs of disease (data not shown), indicating that the protective effects of neutrophils were critical during the early phase of infection.

Neutrophils play an important role in moderating bacterial load during respiratory viral infections (25, 26), and secondary bacterial pneumonia is a common cause of death after influenza infection in humans. Therefore, it was important to ascertain bacterial counts in the lungs of virus-infected mice to ensure that severe disease observed in neutropenic animals was not due to overwhelming bacterial superinfection. Seven days after virus infection, BAL supernatants from control or neutropenic mice were plated onto nutrient agar and horse blood agar and, after incubation at 37°C for 3 days, the number of CFU was determined. CFU counts were low (0–500 CFU/ml of BAL for control and neutropenic mice that were (1) mock infected or (2) infected with 10^2 PFU of HKx31 and analyzed 7 days later) and not statistically different between any of the groups in two independent experiments, thereby excluding secondary bacterial infection as a major factor associated with disease.

Virus replication and spread in neutropenic mice following infection with HKx31

We next sought to define mechanisms underlying the severe disease observed in neutrophil-depleted mice infected with influenza virus at day 7 postinfection, the time at which mice developed pneumonia and succumbed to infection. First, a standard plaque assay was used to determined titers of infectious virus in the upper

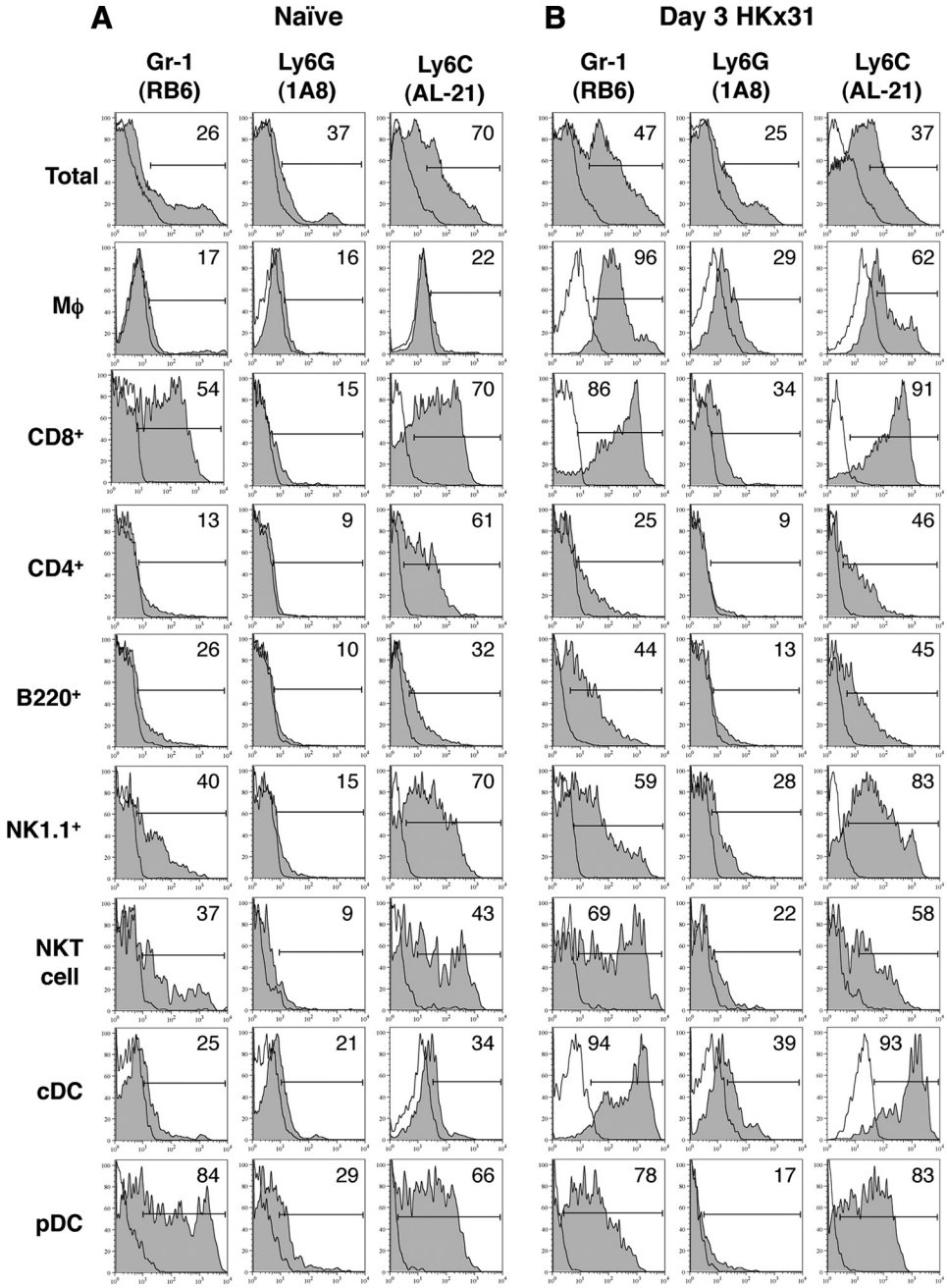

FIGURE 2. Expression of Gr-1, Ly6G, and Ly6C on lung leukocytes from naive and influenza virus-infected mice. Lung-cell suspensions were prepared from naive mice or from mice 3 days after infection with 10^5 PFU of HKx31. Binding of fluorescent-labeled anti-Gr-1 (mAb RB6-8C5: Ly6G and Ly6C), anti-Ly6G (mAb 1A8), and Ly6C (mAb AL-21) to lung leukocytes prepared from (*A*) naive animals or (*B*) virus-infected mice are shown as gray histograms. Appropriate isotype controls are shown as white histograms. Data show binding of mAbs to pooled lung cell suspensions prepared from five mice per group. Leukocyte subsets were identified as follows: macrophages (Mϕ; CD11c^{high}, MHC class II^{int}), CD8$^+$ cells (CD8$^+$), CD4$^+$ cells, B220$^+$ cells, NK1.1$^+$ cells, NKT cells (NK1.1$^+$, CD1d-tetramer$^+$), cDCs (CD11c^{high}, MHC class II^{high}), and pDCs (PDCA-1$^+$, CD11c^{int}). A gate was set to include 5% of cells in the isotype control, and the percentages of cells staining positive for expression of Gr-1, Ly6G, or Ly6C are shown relative to this in each panel. Data are representative of at least two independent experiments.

(nasal tissues) and lower (lung) respiratory tract. Virus replication was significantly enhanced in lung and nasal tissues of 1A8-treated mice compared with IgG-treated controls at day 3 and day 7 postinfection (Fig. 4*A*) and, in neutropenic mice, immunoperoxidase staining at day 7 postinfection indicated extensive infection of epithelial cells lining the bronchioles and small airways in the lungs (Fig. 4*B*). In contrast, very occasional cells of the bronchiolar epithelium stained positively for viral Ag in the lungs of IgG-treated control mice, with no evidence of infection observed in the alveolar compartments.

Extrapulmonary spread is unusual for laboratory-adapted virus strains such as HKx31 (27, 28); however, we consistently detected infectious virus in homogenates prepared from brain ($n = 5$ out of 5, 4 out of 5, and 5 out of 5 mice in three independent assays), heart ($n = 5$ out of 5, 5 out of 5, and 5 out of 5 mice in 3 independent assays), and kidneys ($n = 4$ out of 5 and 3 out of 5 mice in two independent assays) of neutropenic animals infected with HKx31 (Fig. 4*C*). In contrast, virus titers were below detection in all tissue homogenates prepared from IgG-treated control animals in three independent assays. Despite the recovery of infectious virus, no obvious lesions were observed in brain, heart, or kidney, although hearts recovered from neutropenic mice at day 7 postinfection were visibly smaller and significantly reduced in wet weight compared with IgG-treated controls (Expt. 1: 1A8 = 110.2 ± 11.6 mg compared with IgG = 140.0 ± 11.0 mg, $p < 0.01$ and Expt. 2: 1A8 = 113.6 ± 2.8 mg compared with IgG = 149.6 ± 15.8 mg, $p < 0.01$; $n = 5$ mice per group); however, no differences were noted in levels of cardiac troponin I, a marker of myocardial injury (29, 30), in serum from neutropenic or control mice at day 7 postinfection (data not shown).

We next characterized virus isolates recovered from the heart and brain for comparison with isolates from the lung to determine whether systemic spread was associated with virus mutation. Three independent plaques were picked from lung, heart, and brain

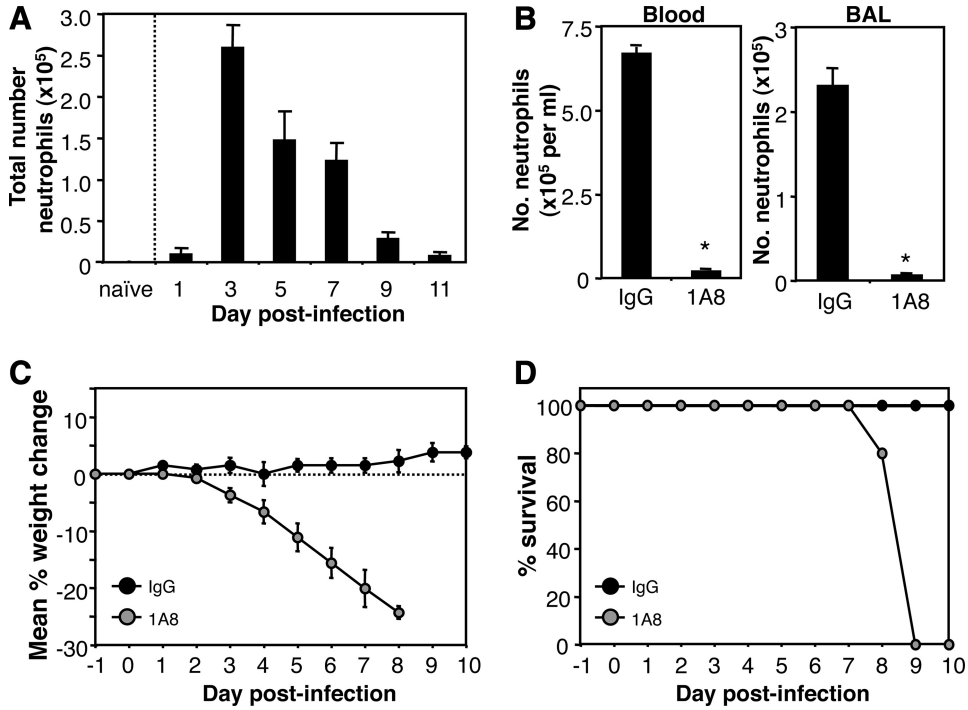

FIGURE 3. Neutrophil recruitment and depletion following inoculation of mice with influenza virus. *A*, Groups of five mice were infected with 10^2 PFU of virus strain HKx31 via the i.n. route, and, at the times indicated, mice were killed and BAL was performed. Total numbers of BAL neutrophils from virus-infected mice are shown. Cell numbers in the BAL fluids from naive mice are included for comparison. Neutrophils were identified as $CD45^+Gr\text{-}1^{high}$ cells by flow cytometry. A minimum of 50,000 living cells (PI^-) were collected and analyzed from each mouse. *B–D*, Groups of five B6 mice were depleted of neutrophils via i.n. and i.p. administration of purified anti-Ly6G (1A8) 24 h prior to infection with 10^2 PFU of HKx31 and every second day thereafter. Control groups received an equivalent concentration of rat IgG. *B*, At day 3 postinfection, cytospin analysis was used to confirm depletion of neutrophils in the blood and BAL fluid of animals treated with purified anti-Ly6G. Data are representative of >100 cells counted in four to eight independent fields. *C*, Weight loss and (*D*) survival curves of control (IgG) or neutrophil-depleted mice (1A8) infected with HKx31. Mice were weighed daily, and results are expressed as the mean percentage of weight change of each group (±SEM), compared with original body weight. Animals that had lost ≥25% of their original body weight and/or presented with evidence of pneumonia were killed. Data shown are from one experiment and are representative of two or more independent experiments.

homogenates of neutropenic mice and from the lungs of IgG-treated controls at day 7 postinfection, amplified once in eggs, and their properties were examined in more detail. As mutations in HA, NA, PB1, and PB2 genes have been associated with virulence (reviewed in Ref. 31), we first sequenced the full genomic regions encoding each of these genes from all 12 plaque isolates. No common mutations were observed in viruses isolated from extrapulmonary sites (brain and heart) when compared with those isolated from the lungs of either neutropenic or IgG-treated animals (data not shown). Second, no significant differences were noted in plaque size or phenotype between any of the 12 isolates when compared with plaques isolated from the original allantoic fluid used to inoculate mice ($n = 10$ plaques per group, data not shown). Finally, i.n. inoculation of untreated mice with 10^2 PFU of each of the three isolates recovered from brain homogenates of neutropenic mice was not associated with significant weight loss or any signs of clinical disease. Thus, the presence of virus at extrapulmonary sites does not appear to be associated with selection of mouse-virulent mutants but rather indicates a critical role for neutrophils in restricting infectious virus to the respiratory tract during influenza infection.

Systemic responses in neutropenic mice infected with HKx31

In addition to enhanced virus replication and spread, additional factors have been reported to contribute to morbidity and mortality during influenza virus infection. The severe clinical disease observed following infection with virulent avian influenza virus strains has been associated with lymphopenia in mice (32, 33) and humans (34). Consistent with these findings, blood leukocyte and lymphocyte counts from 1A8-treated mice were significantly lower than those from IgG-treated mice at day 7 postinfection (Fig. 5*A*). Dysregulated cytokine responses have also been associated with severe morbidity (34, 35); however, serum levels of IFN-γ, TNF-α, IL-10, and IL-12 did not differ significantly between virus-infected neutropenic mice and their corresponding IgG-treated controls at day 7 postinfection in two independent experiments (data not shown). In one of two experiments serum IL-6 levels showed a modest, but significant, elevation when compared with IgG-treated controls (Expt. 1: 1A8 = 71.7 ± 38.2 pg/ml compared with IgG = 29.8 ± 13.7 pg/ml, $p < 0.05$ and Expt. 2: 1A8 = 109.2 ± 85.4 pg/ml compared with IgG = 45.3 ± 20.1 pg/ml, $p = 0.14$; $n = 5$ mice per group).

Severe respiratory disease, including influenza infection, has also been associated with selective destruction of double-positive (DP; $CD4^+CD8^+$) thymic lymphocytes (27, 36, 37). At day 7 postinfection, the wet weight of thymi recovered from HKx31-infected neutropenic mice was significantly lower than that from the corresponding IgG-treated control animals (data not shown) or from uninfected mice. Total cell numbers and, in particular, the numbers of DP lymphocytes were markedly reduced in the thymi of virus-infected, neutropenic mice (Fig. 5*B*). Significant increases were observed in populations of single-positive (SP) $CD4^+$ and SP $CD8^+$ thymic lymphocytes populations (Fig. 5*B*) in IgG control animals compared with neutropenic mice; however, overall cell

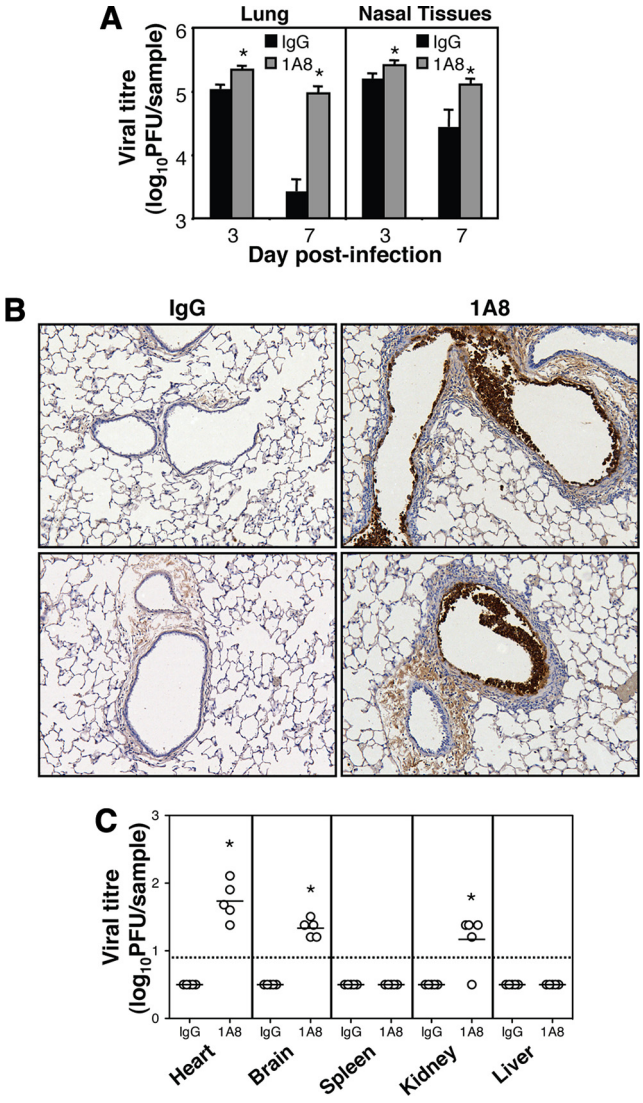

FIGURE 4. Depletion of neutrophils is associated with enhanced virus replication and spread in influenza virus-infected mice. Groups of five B6 mice were depleted of neutrophils via i.n. and i.p. administration of purified anti-Ly6G (1A8) 24 h prior to infection with 10^2 PFU of HKx31 and every second day thereafter. Control groups received an equivalent concentration of rat IgG. Mice were killed and analyzed at day 7 postinfection. *A*, Lungs and nasal tissues were homogenized and virus titers determined using a standard plaque assay on MDCK cells. Bars represent mean viral titer ± 1 SD. ∗, Viral titers from neutropenic mice (1A8) that were significantly higher than those from control (IgG) animals ($p < 0.01$, Student's *t* test). *B*, Distribution of viral Ag in the lungs of neutrophil-depleted or control mice. Representative images are shown at ×10 magnification. *C*, Systemic isolation of infectious virus. Homogenates were prepared from hearts, brains, kidneys, liver, and spleen of influenza virus-infected mice, and titers of infectious virus were determined using a standard plaque assay. Data are shown for individual animals and are representative of three independent experiments. The detection limit for the plaque assays is indicated as a dotted line. ∗, Viral titers from neutropenic mice (1A8) that were significantly higher than those from control (IgG) animals ($p < 0.01$, Student's *t* test).

counts were significantly reduced. In data not shown, flow cytometry was used to demonstrate that Ly6G is not expressed on DP, SP $CD4^+$, SP $CD8^+$, or double negative (DN) thymic lymphocytes, indicating that the reduction in DP cells is not the result of direct depletion following treatment of mice with purified mAb 1A8.

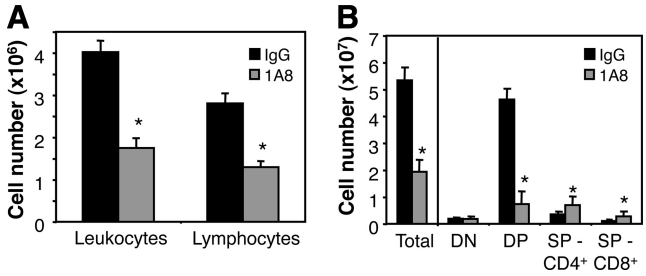

FIGURE 5. Lymphopenia and thymic atrophy are associated with the severe disease in neutropenic mice infected with HKx31. Groups of five B6 mice were treated with purified anti-Ly6G (1A8) or control IgG and killed and analyzed at day 7 postinfection. *A*, Viable cell counts were performed on whole blood to determine total leukocyte numbers and, following cytospin analysis, the number of blood lymphocytes. ∗, Cell numbers from neutropenic mice (1A8) that were significantly lower than those from control (IgG) animals ($p < 0.01$, Student's *t* test). *B*, Numbers of total thymocytes, DN ($CD4^-CD8^-$), DP ($CD4^+CD8^+$), and SP ($CD4^+$ or $CD8^+$) thymocytes. ∗, Cell numbers from neutropenic mice (1A8) that were significantly higher or lower than those from control (IgG) animals ($p < 0.05$, Student's *t* test).

Inflammatory responses in the airways of neutropenic mice infected with HKx31

The magnitude and nature of the cellular infiltrate in the airways and/or the production of local immunomodulators could be critical factors contributing to morbidity and mortality during influenza virus infection. Surprisingly, despite marked differences in morbidity, we did not observe significant differences in levels of IL-6, MCP-1, IFN-γ, IL-10, and IL-12 in BAL fluids recovered from neutrophil-depleted mice when compared with IgG-treated controls at day 7 postinfection (data not shown), although levels of TNF-α were higher in BAL fluids from 1A8-treated mice (Expt. 1: 1A8 = 60.6 ± 15.4 pg/ml compared with IgG = 17.3 ± 5.1 pg/ml, $p < 0.05$ and Expt. 2: 1A8 = 58.7 ± 31.5 pg/ml compared with IgG = 22.8 ± 9.3 pg/ml, $p < 0.05$; $n = 5$ mice per group). We did, however, observe marked differences in pulmonary histology between neutrophil-depleted and control animals at day 7 postinfection (Fig. 6). Lung sections from infected, neutrophil-depleted mice were characterized by epithelial destruction in the respiratory airways, extensive perivascular and peribronchiolar infiltrates, and cellular inflammation extended into the tissue and airspaces of the surrounding alveolar parenchyma. When observed under higher magnification, macrophages and lymphocytes were the predominant cell types infiltrating the alveolar spaces of neutrophil-depleted mice. Notably, the degree of both peribronchiolar inflammation and alveolitis was markedly diminished in IgG-treated controls.

Vascular leakage, pulmonary edema, and respiratory dysfunction in neutropenic mice infected with influenza virus

In addition to exacerbated pulmonary immunopathology, neutrophil depletion was associated with increased protein levels in BAL fluids at day 7 postinfection, indicative of vascular leakage and edema (Fig. 7*A*). Lungs of neutropenic animals were also visibly enlarged, and the wet-to-dry weight ratio, a measure of extravascular lung water (38), was significantly increased in neutropenic animals infected with HKx31 (Fig. 7*B*). These overt manifestations of pulmonary injury were reflected in a marked deterioration of lung function measured by oscillatory mechanics. In IgG-treated mice we observed no significant detectable difference in lung function (Fig. 7*C*). In contrast, depleted animals demonstrated increased central airway resistance (R_N), tissue damping (G), and

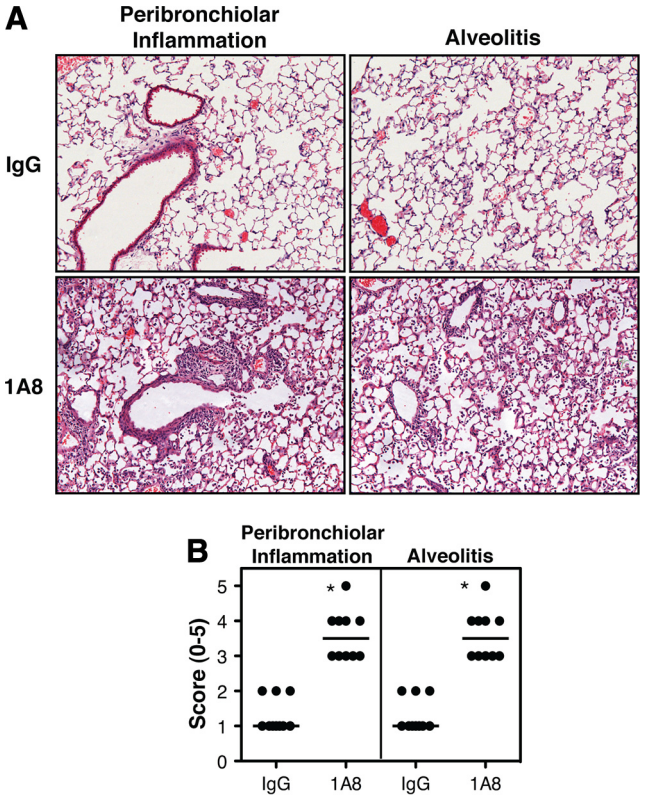

FIGURE 6. Severe clinical disease in neutropenic mice infected with HKx31 is associated with enhanced pulmonary inflammation. Groups of five B6 mice were treated with purified anti-Ly6G (1A8) or control IgG and killed and analyzed at day 7 postinfection. *A*, Lung sections from neutropenic and control mice following influenza virus infection. Shown are representative images of peribronchiolar inflammation and alveolitis in lung sections from IgG- and 1A8-treated mice following H&E staining. Images are shown at ×10 magnification. *B*, Corresponding histopathological scores for peribronchiolar inflammation and alveolitis. Lung sections were scored blind for alveolitis and peribronchiolar inflammation from 0 to 5 as described in *Materials and Methods*. Data shown represent scores from individual mice (as indicated by circles) and median values (as indicated by bar) obtained from one of three independent readers. For each reader, peribronchiolar inflammation and alveolitis were significantly higher in neutrophil-depleted mice (1A8) compared with IgG-treated control mice ($p < 0.01$, Student's *t* test).

tissue elastance (*H*), all consistent with increased lung size on resection, increased lung weight, and more severe histopathology.

Discussion

Influenza is a viral respiratory disease that induces a spectrum of clinical illness in humans, ranging from mild upper respiratory tract infection to severe bronchiolitis and pneumonia. While the mouse serves as a convenient model to study factors that may modulate disease severity, previous attempts to elucidate the role of neutrophils have been limited by reliance on the use of mAb RB6-8C5 to deplete $Ly6G^{high}$ neutrophils in vivo (2, 7, 9, 26). Herein, we demonstrate that mAb 1A8 can be used to identify and deplete $Ly6G^{high}$ neutrophils from mice during influenza virus infection. In contrast to control mice, neutrophil-depleted mice rapidly developed severe clinical disease and succumbed to infection. Infection of neutropenic mice was characterized by enhanced virus replication in the airways, pulmonary inflammation, and lung edema, as well as evidence of extrapulmonary virus spread, lymphopenia, and thymic atrophy at the time of death. Taken together, these data define the critical role of neutrophils in modulating disease severity during influenza virus infection.

Neutropenic mice infected with 10^2 PFU of HKx31 showed 100% mortality, characterized by the development of severe pulmonary inflammation, edema, and respiratory failure 5–7 days after infection. It was interesting that viral titers in the lungs of neutropenic mice were declining at day 7 postinfection, the time at which these mice succumbed to disease. Such observations are not without precedent, as mice infected with virulent H5N1 (39) or mouse-adapted A/PR/8/34 viruses (4) died at times after the peak of virus replication in the lung. Such observations suggest that inflammatory injury, rather than uncontrolled virus replication, might be an important determinant of the fatal outcome. In humans, severe influenza infections can lead to lung lesions with histopathological features of ARDS (40), and high mortality, inflammatory cell infiltration, and edematous lungs are characteristic features in the mouse model of ARDS induced with H5N1 viral infection (39). HKx31, a virus strain that does not cause clinical disease in control animals following inoculation with 10^2 PFU per mouse, was shown to induce severe morbidity and mortality in neutropenic mice, and animals presented with many hallmark features of ARDS, including pulmonary inflammation, vascular leakage, and lung edema (Fig. 6). Taken together, these findings point to a critical role for neutrophils both in containing and limiting early viral replication but also in ameliorating pathogenic effects on vascular permeability and pulmonary inflammation in the lung during influenza virus infections.

Neutrophil sequestration and activation in the lung are generally associated with inflammatory lung injury during acute lung injury, ARDS, and septic shock (reviewed in Ref. 41, 42). In such conditions, it has been proposed that activated neutrophils release free radicals, inflammatory mediators, and proteases that can damage lung tissue, leading to flooding of alveoli by plasma liquid and proteins. Conditions such as ARDS have, however, also been reported in severely neutropenic patients (43, 44) where alternative mechanisms, such as alveolar macrophage dysfunction (45), have been proposed to contribute to damage of the lung alveolar-capillary barrier. In experimental mouse models of airway inflammation, neutrophil depletion is often associated with attenuation of lung injury (reviewed in Ref. 46), although severe neutropenia did not modulate pulmonary edema associated with hyperoxic damage (47–49), and PMA has been shown to induce lung injury in a neutrophil-independent manner (50, 51). Moreover, lung injury was actually enhanced following infection of neutropenic guinea pigs with *Pseudomonas aeruginosa* (52), a finding similar to that which we report in the present study.

Our data suggest that the severe clinical disease observed in neutropenic mice is likely to related to dysregulated pulmonary inflammation and lung dysfunction; however, additional factors are likely to contribute to disease severity. Body and heart weights were significantly reduced in neutropenic mice compared with control animals, perhaps reflecting anorexia and reduced water intake due to severe viral infection. Myocarditis has been associated with influenza infection in humans (53) and during experimental influenza of mice with mouse-adapted (54, 55) or highly pathogenic H5N1 viruses (56), but in our studies cardiac troponin I, a serum marker of myocardial injury, was not elevated at the time of severe disease (data not shown). Thymic atrophy is a common feature of severe infection, including influenza infection (27, 37, 57), and generally reflects specific depletion of $CD4^+CD8^+$ cortical thymocytes. Several mechanisms may contribute to thymocyte apoptosis; for example, immature $CD4^+CD8^+$ thymocytes are particularly sensitive to induction of apoptosis by glucocorticosteroids that are produced as part of the host's stress response to

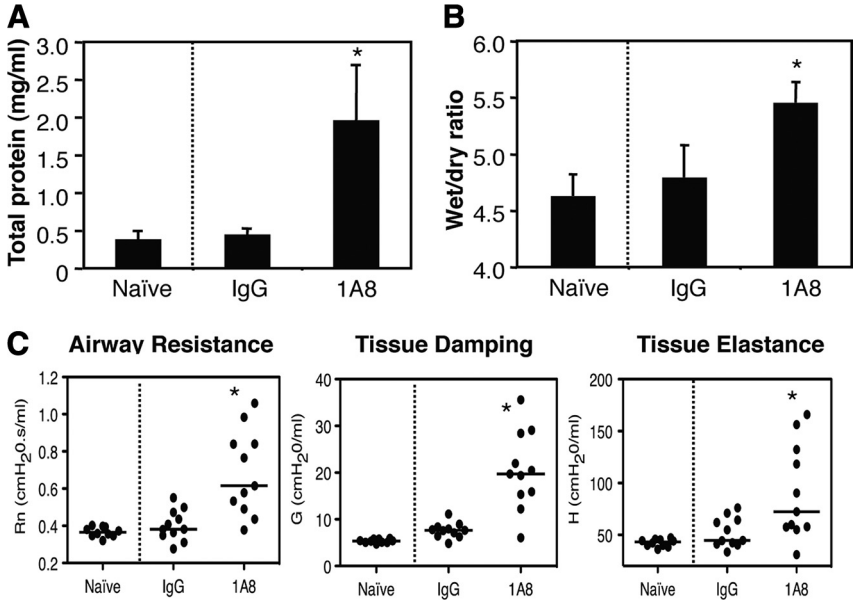

FIGURE 7. Vacular leak, pulmonary edema, and respiratory dysfunction in neutropenic mice infected with influenza virus. *A*, Total protein concentrations were determined in BAL supernatants from naive mice and from IgG- and 1A8-treated mice at day 7 postinfection. ∗, Neutropenic mice were significantly different from IgG-treated control mice ($p < 0.01$, Student's *t* test). *B*, Lung wet-to-dry ratios as an assessment of lung edema. Data are representative of at least two independent experiments. ∗, Neutropenic mice were significantly different to IgG-treated control mice ($p < 0.01$, Student's *t* test). *C*, Mice were anesthetized and connected to a computer-controlled small animal ventilator (flexiVent) and ventilated at 300 breaths per minute. The constant-phase model was used to measure central airway resistance (R_N), tissue damping (G), and tissue elastance (H). Data from two independent experiments were pooled, and data are shown for individual mice (as indicated by circles) as well as the mean values (as indicated by bar). ∗, Neutrophil-depleted mice were significantly different to IgG-treated control animals ($p < 0.05$, Kruskal-Wallis test). Naive animals were included in each experiment for comparison.

infection (58). It is possible that viral infection could further potentiate apoptotic death of thymocytes.

Multiorgan spread and systemic virus replication are common features of pathogenic human and avian H5N1 (59, 60) or avian/equine H7N7 (60) infections of mice, but they are rare complications following inoculation with other human strains, even with the virulent 1918 H1N1 pandemic virus (61). In general terms, seasonal human influenza viruses remain confined to the respiratory tract in the mouse model, as the protease enzymes required to cleave viral HA0 are common in the lungs and trachea but not in other tissues. Recovery of infectious HKx31 virus from extrapulmonary sites, including the brain, heart, and kidneys, points to hematogenous transport of virus from the infected lung in free form or in association with erythrocytes and/or leukocytes rather than virus replication per se at these sites. Although specific mutations have been associated with mouse adaptation and multiorgan spread of human isolates (62, 63), we found no conserved changes between isolates of HKx31 from the heart and brain when compared with those from the lungs. Given the well-documented ability of influenza viruses to agglutinate erythrocytes and mammalian cells (64), thrombus formation, cellular injury, and ischemia reported in patients with severe influenza infections (65, 66) could also be important pathological sequelae if hemagglutination were to occur in the microvasculature of neutropenic animals.

The exact mechanisms underlying the protective role of neutrophils during influenza virus infection are yet to be defined, although it is likely that many of the antiviral effectors could potentially contribute to lung injury and disease if not tightly regulated. Neutrophils adhere to virus-infected epithelial cells (67) and virions (68) to mediate phagocytosis and/or stimulate activation signals, including production of reactive oxygen species. Neutrophils exposed to influenza virus undergo activation of the respiratory burst, followed by depression of cell function when subsequently exposed to secondary stimuli (69), although innate defense proteins of the collectin family have been shown to protect neutrophils from these depressive effects (70). Neutrophils themselves have been reported as both nonpermissive (6) and susceptible to influenza virus infection (71, 72) and to respond to virus exposure via TLR7- and/or TLR8-mediated production of proinflammatory cytokines (71). Neutrophil granules, including azurophil/primary, secondary, and secretory granules, contain an array of molecules with potential for antiviral activity, including α and β defensins and pentraxins, which have been shown to exert antiviral activities against a range of viruses, including influenza (73–75).

Collectively, our studies point to a crucial and early role for neutrophils in limiting the extent of influenza replication and disease in the lung. Moreover, we define a novel role for neutrophils in ameliorating vascular permeability and pulmonary inflammation and limiting extrapulmonary spread of the virus associated with severe disease. Given that neutrophils are known agents that can be associated with lung damage, caution must be exercised when using therapies designed to limit neutrophil-mediated immunopathology in the context of influenza virus infection, as they could inadvertently exacerbate infection and its sequelae. Elucidating the specific mechanisms by which neutrophils mediate antiviral activity and/or modulate lung inflammation might enable development of therapies that retain antiviral function but limit neutrophil- or inflammation-associated lung damage.

Disclosures

The authors have no financial conflicts of interest.

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