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Dysregulated Macrophage-Inflammatory Protein-2 Expression Drives Illness in Bacterial Superinfection of Influenza

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Influenza virus infection is a leading cause of death and disability throughout the world. Influenza-infected hosts are vulnerable to secondary bacterial infection, however, and an ensuing bacterial pneumonia is actually the predominant cause of influenza-attributed deaths during pandemics. A number of mechanisms have been proposed by which influenza may predispose to superinfection with an unrelated or heterologous pathogen, but the subsequent interaction between the host, virus, and bacteria remains an understudied area. In this study, we develop and examine a novel model of heterologous pulmonary infection in which an otherwise subclinical Bordetella parapertussis infection synergizes with an influenza virus infection to yield a life-threatening secondary pneumonia. Despite a profound pulmonary inflammatory response and unaltered viral clearance, bacterial clearance was significantly impaired in heterologously infected mice. No deficits were observed in pulmonary or systemic adaptive immune responses or the viability or function of infiltrating inflammatory cells to explain this phenomenon, and we provide evidence that the onset of severe pulmonary inflammation actually precedes the increased bacterial burden, suggesting that exacerbated inflammation is independent of bacterial burden. To that end, neutralization of the ELR⁺ inflammatory chemokine MIP-2 (CXCL2/GRO-β) attenuated the inflammation, weight loss, and clinical presentation of heterologously infected mice without impacting bacterial burden. These data suggest that pulmonary inflammation, rather than pathogen burden, is the key threat during bacterial superinfection of influenza and that selective chemokine antagonists may be a novel therapeutic intervention in cases of bacterial superinfection of influenza. *The Journal of Immunology, 2010, 184: 2001–2013.

It has long been acknowledged that a substantial fraction of influenza-related death and disability results not from primary viral disease but from secondary infection with bacteria unrelated (heterologous) to the virus. In fact, recent analysis points to secondary bacterial pneumonia, not primary viral disease, as the predominant cause of death in the three 20th century influenza pandemics (5) and suggests that the remarkable lethality of the 1918 influenza strain may be due to its ability to drive a secondary bacterial pneumonia (6). Deaths to secondary bacterial infection commonly result from immunopathology, rather than bacterial infection per se, and once initiated, immunopathology in the form of sepsis and septic shock may progress independently of the bacterial burden itself. Specifically, even if hosts suffering from heterologous infections are treated with sterilizing antibiotics, they still frequently succumb to pathologic inflammation (7, 8). Several clinical trials using corticosteroids (9), G-CSF (10), hemofiltration (11), or neutralizing Abs against endotoxin or TNF-α (12) to modulate the inflammatory response have largely failed to prevent patients’ progression to septic shock, failed to reduce mortality, and raised concerns about the risks of nonspecifically suppressing an already infected host’s immune defenses.

A number of mechanisms have been proposed through which viral infection may predispose to bacterial infection. Viruses may act first by lysing epithelial cells and disrupting the respiratory epithelium, thus exposing the basement membrane and permitting bacterial attachment as shown during times of influenza epidemics (13–15) and in experimental models of disease (16). In epithelial cells that escape direct lysis, influenza and other viruses may then increase the expression of receptors to which bacteria adhere (16) and impair ciliary function, reducing early mucociliary clearance of bacteria (17, 18). Additionally, viral insult may impair macrophage responsiveness to bacterial TLR ligands by reducing NF-κB activity (19) and reduce macrophage antibacterial function (20).
Viral induction of type I IFNs may then compromise innate antibacterial immunity by inducing granulocyte apoptosis (21) and reducing the production of inflammatory chemokines (22). Finally, activation of the adaptive antiviral system may compromise host defense against subsequent bacterial challenge via an IFN-γ–dependent impairment of alveolar macrophage (AM) function (23, 24). Whether the life-threatening pneumonia seen in heterologously infected individuals simply represents an appropriate immune response to a magnified bacterial burden or is the result of a fundamental alteration in immunity brought on by two divergent immune insults remains to be elucidated.

In this study, we report on a novel model of severe secondary bacterial pneumonia in H1N1 influenza A-infected mice, with exacerbated weight loss, clinical presentation, and mortality following administration of an otherwise subclinical dose of *Bordetella parapertussis* bacteria. We report that viral infection does not alter bacterial adherence or the early events of bacterial clearance but does lead to significantly impaired bacterial clearance both 1 and 2 wk postinfection. Even before bacterial burden becomes exacerbated, heterologously infected mice are afflicted by a severe pulmonary inflammation driven by the neutrophil chemokine MIP-2, which is not seen in animals given either pathogen alone. We provide evidence that pulmonary neutrophilic inflammation, rather than pathogen burden, is the key determinant of illness during bacterial superinfection of influenza virus and that specific immunoneutralization of MIP-2 results in reduced weight loss and symptom scores, without reducing bacterial burden. In contrast, CXCR2 blockade resulted in severely impaired antibacterial immunity and worsened clinical performance. These results suggest a role for targeted antichemokine interventions in the treatment of heterologous pulmonary infection.

**Materials and Methods**

**Animals**

Female C57BL/6 mice (6–8 wk old) were purchased from Charles River Laboratories (Montreal, Quebec, Canada) and kept in specific pathogen-free conditions on a 12-h light-dark cycle with food and water provided ad libitum. Cages, food, and bedding were autoclaved. Animals were handled in class II biosafety cabinets by gloved, gowned, and masked staff. Experiments were approved by the McMaster University Animal Research Ethics Board. After initial experiments in which animals succumbed to infection, additional hydration and feeding measures were instituted to prevent mortality in subsequent experiments.

**Infectious agents**

*B. parapertussis* strain 12822 (American Type Culture Collection, Manassas, VA) has previously been sequenced (25), studied microbiologically (26), and studied in the murine respiratory tract (27). Bacteria were maintained on Bordet-Gengou agar (Difco, Oakville, Ontario, Canada) containing 15% horse blood (Lampire Biological Laboratories, Pipersville, PA). Liquid culture bacteria were grown at 37°C overnight on a shaking incubator in Bordet-Gengou broth (28). The H1N1 influenza A (A/FM/1/47) virus used in this study is a fully sequenced, plaque-purified preparation that is biologically characterized with respect to mouse lung infections (29).

**Mouse infection and monitoring**

Isolflurane-anesthetized mice were infected intranasally with 2.5 × 10^7 PFU influenza virus in 35 µL PBS vehicle. Five days later, mice were given 5 × 10^7 CFU log-phase *B. parapertussis* in 35 µL PBS in the same manner. Mice were individually weighed before infection and throughout the course of experimentation, and health status was scored by a blinded investigator as reported previously (30).

**Tissue and cell collection and isolation**

At sacrifice, blood was collected by retro-orbital bleeding using heparin-coated capillary tubes (Fisher Scientific, Pittsburgh, PA). Total leukocytes were counted after lysing RBCs, and blood smears were prepared and stained with Hema 3 (Biochemical Sciences, Swedensboro, NJ) for differential cell counts by counting a minimum of 300 cells per slide. To isolate serum, animals were bled retro-orbitally with nonheparinized Pasteur pipets, and serum was obtained by incubating whole blood for 30 min at 37°C, followed by centrifugation. Samples were stored at −20°C until assayed.

Lungs were removed, tracheas were cannulated, and bronchoalveolar lavage (BAL) was obtained by lavaging twice with PBS (250 and 200 µl). Total cell counts were determined by hemocytometer. BAL supernatants were stored at −20°C until analysis of inflammatory mediators. Cytospins for differential cell counts were prepared by resuspending cell pellets in PBS, followed by cytocentrifugation at 10 × g for 2 min. Cells were stained by Hema 3 (Biochemical Sciences), and differentials were determined by counting at least 300 cells. Standard hemacytological criteria were used to classify mononuclear cells, neutrophils, and eosinophils. For histology, lung lobes were inflated at 20 cm H2O pressure with 10% formalin, embedded in paraffin, and 3-µm-thick sections were stained with H&E. Images were collected with Openlab software (version 3.03.0; Improvision, Guelph, Ontario, Canada) via a Leica camera and microscope (Leica Microsystems, Richmond Hill, Ontario, Canada). For determination of lung bacterial burdens and viral titers, lung lobes were collected in 1 ml PBS and then homogenized with a Polytorn PT 1200C (Kinematica, Littau-Lucerne, Switzerland).

**Bacterial burdens and viral titers**

Twenty-five microliters of serially diluted fresh homogenates in PBS was plated onto Bordet Gengou blood agar plates and grown for 3 d at 37°C to enumerate viable bacteria. Homogenates were stored at −80°C prior to determination of viral titers on Madin-Darby canine kidney cell monolayers. Madin-Darby canine kidney cells grown to confluence in 6-well tissue culture plates were rinsed twice with warm PBS to remove serum, and 200 µl serially diluted samples were added. Plates were rocked and incubated at 37°C for 30 min, after which 2 ml 0.65% agarose (BioShop Canada, Burlington, Ontario, Canada) in MEM supplemented with 1% t-glutamine, 1% penicillin and streptomycin, and 1 µg/ml trypsin was added to each well. Once the agarose solidified, plates were incubated at 37°C for 2 d. Cells were fixed using Carnoy’s fixative and plaques were enumerated with Giemsa stain.

**Measurement of type I IFN by plaque reduction assay**

IFN bioactivity was measured in BAL samples by plaque reduction assay as described previously (31). Briefly, BAL samples were assessed for their ability to protect primary IFN regulatory factor–3–deficient mouse embryonic fibroblasts (MEFs) (provided by Dr. K. Mossman, McMaster University, Hamilton, Ontario, Canada) from infection with a vesicular stomatitis virus expressing GFP under an endogenous viral promoter (VSV–GFP; provided by Dr. B. Lichty, McMaster University). The replication efficiency of VSV is decreased, because type I IFN present in BAL is able to produce an antiviral state in MEFs. IFN regulatory factor–3–deficient MEFs are incapable of producing IFN; however, they are capable of responding to exogenous IFN to produce an antiviral state.

Cells were removed from BAL samples by centrifugation, and monolayers of MEFs were incubated with serial dilutions of supernatants for 24 h in 24-well dishes. Subsequently, supernatants were removed, and MEFs were infected with 4 × 10^3 PFU VSV–GFP in serum-free α-MEM. Viral inocula were replaced with DMEM containing 1% methylcellulose following 40 min of incubation at 37°C. GFP fluorescence intensity was measured 24 h later on a Typhoon Trio (GE Healthcare, Piscataway, NJ) imager and quantified using ImageQuantTL (GE Healthcare) software.

**Flow cytometry**

Lungs were cut into ~2-mm pieces, shaken for 1 h at 37°C in 150 U/ml collagenase III (Invitrogen Life Technologies, Burlington, Ontario, Canada) in HBSS, pressed through nylon mesh, washed twice in HBSS, and counted using a hemocytometer. Cells (2 × 10^6) were plated in 96-well, round-bottom plates, blocked for 15 min on ice with 50 µl anti-CD16/CD32 (puriﬁed, clone 93; eBioscience, San Diego, CA), then stained for 1 h at room temperature with a PE-conjugated H2K^b^ tetramer loaded with inﬂuenza H1N1 (A/FM/1/47) HA2 (Magnuson Laboratory, Houston, TX). Cells were washed and resuspended in staining mixture for 30 min on ice. Abs used in this study include anti-CD11c (FITC, HL3; BD Biosciences, San Jose, CA), anti-CD11b (PE, M1/70; eBioscience), anti-CD4 (PE-610, RM4-5; Invitrogen, Carlsbad, CA), anti-CD69 (PerCP-Cy5.5, 1H.2F3; BD Biosciences), anti-NK1.1 (PE-Cy7, eBioscience), anti-CD4 (PE-610, RM4-5; Invitrogen, Carlsbad, CA), anti-CD69 (PerCP-Cy5.5, 1H.2F3; BD Biosciences), anti-NK1.1 (PE-Cy7, eBioscience), and anti-CD8 (Pacific Orange, 5H10; eBioscience), and anti-CD8 (Pacific Orange, 5H10; eBioscience). For flow cytometric determination of apoptosis, cells were

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further stained with biotinylated Annexin V (BD Biosciences) and streptavidin-Qdot800 (Invitrogen) and 7-aminoactinomycin D (7-AAD) (BD Biosciences), or with the in situ cell death detection kit according to the manufacturer’s protocol (Roche, Laval, Quebec, Canada). Cells were washed, and $3 \times 10^5$–$3 \times 10^6$ forward light scatter (FSC)/side scatter (SSC)-gated events were collected on samples on an LSRII flow cytometer for flow cytometric analysis, or a FACSVantage digital cell sorter for cytchemistry. Data were analyzed using FlowJo (Tree Star, Ashland, OR).

Myeloperoxidase assay

Myeloperoxidase (MPO) was measured as previously described in Ref. 30 using a modification of the technique described in Ref. 32. Briefly, 0.5 ml lung homogenate was mixed with 0.5 ml 5 mg/ml hexacyclotrimelethyammonium bromide (Sigma-Aldrich, Oakville, Ontario, Canada) in potassium phosphate buffer (pH 6.0) and centrifuged at 12,000 rpm for 2 min. Twenty microliters of supernatant was then added to 200 ml of the reaction mixture (16.7 mg o-dianisidine [Sigma-Aldrich], 90 ml H2O, 10 ml potassium-phosphate buffer, and 50 ml 1% H2O2) in a 96-well plate. ODs were obtained by spectrophotometer at 1-min intervals at 450 nm. MPO activity was expressed as units per gram lung tissue, where 1 U MPO was defined as the amount of enzyme capable of degrading 1 $\mu$mol H2O2/min at room temperature.

Cytokine measurements

Bead array-based analysis of cytokines was performed by Rules Based Medicine with the Rodent multi-Analyte Profile (Austin, TX). The least detectable dose was determined as the mean + 3 SDs of 20 blank readings. Results below the least detectable dose were assigned a value of 0. Means of four independent mice in each treatment group and time were visualized in a heat map using Excel (Microsoft, Seattle, WA). Individual scales were applied to account for the large numerical differences between the mediators. Levels of key cytokines were confirmed by Duoset ELISAs (R&D Systems, Minneapolis, MN) according to the manufacturer’s specifications.

MIP-2 neutralization and CXCR2 blockade

Anti–MIP-2 Abs were generated as previously described (33) and have previously been characterized in models of infectious disease (34). Briefly, rabbits were immunized with multiple intradermal injections of 20 pg rMIP-2 emulsified with CFA, then boosted 10 d later. The rabbit was bled 10 d later with antisera isolated and heat inactivated. Antiserum was passed over Protein A columns (Pierce Biotechnology, Fischer Canada, Nepean, Ontario, Canada), and purified Igs were quantified by Bradford assay (Bio-Rad, Hercules, CA), both according to the manufacturer’s instructions. To neutralize MIP-2 in vivo, mice were given an i.p. administration of 25 $\mu$g purified Ig once daily, commencing 3 d post-B. parapertussis infection, and continuing throughout the period of observation.

Goat anti-murine CXCR2 serum was generated as described in Ref. 34. Briefly, goats were immunized at multiple intradermal sites with a peptide encompassing the ligand-binding domain of the murine CXCR2 (Met-Gly-Glu-Phe-Lys-Val-Asp-Lys-Asn-Leu-Glu-Asp-Phe-Ser-Gly), described in Ref. 35 in CFA. To block CXCR2 ligand binding, 0.5 ml anti-CXCR2 immune serum was administered i.p. on the same schedule as for anti-MIP-2. Control mice were given a 0.5 ml daily i.p. administration of normal goat serum.

Ag-specific Ig ELISA

Influenza- and B. parapertussis-specific Abs were detected in the BAL or serum using a minor modification of the procedure described previously (31). Briefly, MaxiSorp plates (Nalge Nunc International, Rochester, NY) were coated overnight at 4˚C with a lysate from influenza-infected HELA cells or with a lysate from log-phase B. parapertussis bacteria. Coated wells were blocked with 1% casein in PBS for 2 h at room temperature. After washing, serum and BAL samples serially diluted in PBS were incubated overnight at 4˚C, washed, and developed with biotin-labeled anti-mouse IgG or IgA (Southern Biotechnology Associates, Birmingham, AL), respectively. Plates were washed and incubated with alkaline-phosphatase streptavidin for 1 h at room temperature. The color reaction was developed with p-Nitrophenyl phosphate tablets (Sigma-Aldrich) in Diethanolamine buffer.

Data analysis

Data are expressed as the means ± SEMs. All statistical analysis was performed using SigmaStat version 2.03 (Systat Software, Chicago, IL), with tests and results as indicated in figure legends. Differences were considered statistically significant at $p < 0.05$.

Results

Influenza infection impairs host defense against B. parapertussis

To model heterologous infection, mice were first infected intranasally with influenza virus and were then given an intranasal administration of B. parapertussis 5 d later. To ensure clarity, time points throughout this article are expressed as the number of days of infection (triangular icons). Heterologously infected mice lost more body weight at the peak of illness and showed reduced survival following bacterial challenge when compared with single-infection control mice; $n = 5$–10/group. C. Mice were given PBS (○) or influenza virus (●), then challenged with B. parapertussis 5 d later. Seven (left) and 14 (right) d postchallenge, bacterial burden was significantly greater in homogenized lungs from heterologously infected animals than from B. parapertussis-only ones; $n = 4$–5/group. Data are expressed as the means ± SEMs and are analyzed by two-way ANOVA for weights and bacterial burdens and by Mantel-Cox log-rank test for survival curves. *, †, and ‡ denote $p < 0.05$ compared with PBS + PBS, PBS + B. parapertussis, and influenza + PBS-exposed mice, respectively. Two symbols denote $p < 0.01$ and three denote $p < 0.001$. Data are representative of at least two independent experiments.
FIGURE 2. Heterologous infection alters aspects of the adaptive immune response without impairing protective immune memory. A, Mice were given sterile PBS or influenza virus intranasally and then 5 d later sterile PBS (○) or B. parapertussis (■). Influenza NP366–374-specific \( CD^8^+ \) T cells were enumerated following a 7-wk convalescence by flow cytometry in enzymatically digested lungs and found to be reduced in heterologously infected mice. B, Seven weeks postchallenge, influenza (top panels) and B. parapertussis-specific (bottom panels) Abs were determined by ELISA in the serum (left panels) and BAL (right panels) in mice given sterile PBS (gray symbols) or influenza virus (black symbols) intranasally and then 5 d later sterile PBS (squares) or B. parapertussis (triangles). Convalescent mice were rechallenged with influenza (top panels) or B. parapertussis, and pathogen burden (C) and BAL neutrophilia (D) were determined 5 d later for influenza and 7 d later for B. parapertussis, showing comparable protection against rechallenge in heterologous and single-infection mice; \( n=4–7/\)group. Data are expressed as the means ± SEMs and are analyzed by two-way ANOVA. *, †, ‡, and ‡‡ denote \( p < 0.05 \) compared with PBS + PBS, PBS + B. parapertussis, influenza + PBS, and influenza + B. parapertussis-exposed mice, respectively. Two symbols denote \( p < 0.01 \) and three denote \( p < 0.001 \). Data are representative of two independent experiments.
influenza infection (d 5), followed by the number of days of secondary bacterial infection (i.e., “day 5+3” denotes 5 d of influenza infection, followed by an additional 3 d of secondary bacterial infection). Mice infected with B. parapertussis alone did not become overtly ill and did not lose body weight when compared with control mice, whereas mice infected with influenza virus lost 22.1 ± 2.3% (n = 8) of their original body weight at the peak of illness and began recovering 8 d postinfluenza infection. Mice infected with both influenza virus and B. parapertussis lost significantly more body weight (30.4 ± 1.6%; n = 8) and had a more prolonged

Table I. Flow cytometric analysis of lymphocyte subsets in the lungs of mice given PBS or influenza virus on day 6, PBS or B. parapertussis on day 5, and then sacrificed 7 d later

<table>
<thead>
<tr>
<th></th>
<th>PBS + PBS</th>
<th>PBS + B. parapertussis</th>
<th>Influenza + PBS</th>
<th>Influenza + B. parapertussis</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells</td>
<td>3.8 ± 0.2</td>
<td>2.7 ± 0.5</td>
<td>4.1 ± 0.6</td>
<td>3.9 ± 0.4 (×10^6 cells/lung)</td>
</tr>
<tr>
<td>CD69*</td>
<td>7.7 ± 0.4</td>
<td>5.3 ± 1.2</td>
<td>8.2 ± 1.4</td>
<td>6.3 ± 0.6 (×10^5)</td>
</tr>
<tr>
<td>NK cells</td>
<td>2.1 ± 0.6</td>
<td>2.3 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>1.9 ± 0.3 (×10^6)</td>
</tr>
<tr>
<td>CD69*</td>
<td>5.2 ± 2.1</td>
<td>7.4 ± 0.3</td>
<td>2.5 ± 0.9</td>
<td>2.1 ± 1.3 (×10^6)^1</td>
</tr>
<tr>
<td>T cells</td>
<td>4.7 ± 0.6</td>
<td>4.0 ± 1.1</td>
<td>7.3 ± 3.9</td>
<td>12.9 ± 2.1 (×10^5)^3</td>
</tr>
<tr>
<td>CD4*</td>
<td>2.3 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>2.3 ± 0.9</td>
<td>3.5 ± 0.5 (×10^6)</td>
</tr>
<tr>
<td>CD25*</td>
<td>1.9 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>2.0 ± 0.5</td>
<td>3.0 ± 0.3 (×10^6)</td>
</tr>
<tr>
<td>CD69*</td>
<td>5.5 ± 0.8</td>
<td>4.6 ± 1.1</td>
<td>4.6 ± 2.0</td>
<td>10.0 ± 1.6 (×10^6)</td>
</tr>
<tr>
<td>CD8*</td>
<td>1.9 ± 0.4</td>
<td>1.3 ± 0.6</td>
<td>4.4 ± 2.3</td>
<td>7.3 ± 1.4 (×10^6)^11,11,11</td>
</tr>
<tr>
<td>CD25*</td>
<td>7.0 ± 3.8</td>
<td>5.3 ± 3.8</td>
<td>22.3 ± 10.4^*</td>
<td>36.3 ± 6.9 (×10^6)^11,11,11</td>
</tr>
<tr>
<td>CD69*</td>
<td>5.5 ± 1.2</td>
<td>3.0 ± 1.3</td>
<td>10.0 ± 5.5</td>
<td>13.8 ± 2.2 (×10^6)^3</td>
</tr>
<tr>
<td>Tetramer*</td>
<td>0.0 ± 1.0</td>
<td>0.1 ± 2.3</td>
<td>6.8 ± 6.0^*</td>
<td>14.9 ± 3.6 (×10^6)^11,11,11</td>
</tr>
</tbody>
</table>

Data are expressed as the means ± SEMs and are analyzed by two-way ANOVA. B cells were defined by FSC/SSC/CD45R profile, NK cells FSC/SSC/Nk1.1, and T cells by FSC/SSC/CD3; n = 4/group. *, †, and ‡ denote p < 0.05 compared with PBS + PBS, PBS + B. parapertussis, and influenza + PBS-exposed mice, respectively. Two symbols denote p < 0.01; three denote p < 0.001. Data are representative of two independent experiments.

FIGURE 3. Heterologous infection leads to severe pulmonary inflammation. Mice were given sterile PBS or influenza virus intranasally as indicated and then 5 d later sterile PBS (●) or B. parapertussis (●●), A, Representative flow cytometry plots showing an increased proportion of Gr-1 bright cells in the lung digest of heterologously infected animals on d 5+7, as quantified in B; n = 4/group. C, Representative cytospin preparations of Gr-1 bright cells isolated by FACS sorting from lung digest of mice on d 5+7, showing severe neutrophilic inflammation and an inflammatory exudate in the lumen in heterologously infected mice (original magnification ×400; >95% pure neutrophils by standard cytological criteria). D, Representative H&E-stained histological cross-sections from mouse lungs on d 5+7, showing severe neutrophilic inflammation and an inflammatory exudate in the lumen in heterologously infected mice (original magnification ×200 [images] and ×400 [insets]). E, BAL inflation on day showing increased total cells and neutrophils (PMNs) in heterologously infected mice on d 5+7 (top panel), and total cells and mononuclear cells (MNCs) on d 5+14 (bottom panel) compared with mice given B. parapertussis or influenza alone. Inset in the center bottom panel shows the same data on a smaller scale. Data are expressed as the means ± SEMs and are analyzed by two-way ANOVA; n = 3–4/group. *, †, and ‡ denote p < 0.05 compared with PBS + PBS, PBS + B. parapertussis, and influenza + PBS-exposed mice, respectively. Two symbols denote p < 0.01 and three denote p < 0.001. Data are representative of at least two independent experiments.
duration of symptoms (Fig. 1A and our unpublished data) than did mice given either pathogen alone. Moreover, 25% of heterologously infected mice succumbed to infection, whereas control and single-infection mice suffered no mortality (Fig. 1B). In all subsequent experiments, heterologous mice were provided with additional feeding and hydration measures to prevent mortality. Although
heterologous infection did not impact mice’s ability to completely clear influenza virus from the lungs by d 5+7 (12 d postinfluenza infection; our unpublished data), it did significantly impair B. parapertussis clearance when measured on d 5+7 (3.7 ± 2.6 × 10⁷ B. parapertussis CFU/ml in bacteria-only mice versus 2.6 ± 0.42 × 10⁶ CFU/ml in heterologously infected mice) and 5+14 (2.2 ± 1.2 × 10² versus 5.9 ± 1.2 × 10⁵ CFU/ml) (Fig. 1C). These findings confirm that influenza infection has a long-lasting deleterious impact on antibacterial host defense, as has previously been reported with respect to other bacteria (19). Of note, greater doses of B. parapertussis than these do not cause mortality, prolonged illness, or weight loss when administered to naive mice (our unpublished data), suggesting that increased bacterial burden was not the independent cause of the observed pathology.

**Heterologous infection impacts aspects of adaptive immune responses without impairing immune protection**

We questioned whether the deleterious effects of heterologous infection might extend beyond the acute phase of infection and impact the formation of immune memory. When mice were allowed to convalesce for 7 wk following infection, numbers of cells specific for influenza nucleoprotein residues 366–374 (NP366–374, influenza’s immunodominant CD8 T cell epitope) in the lungs were decreased in heterologously infected mice, as were influenza-specific IgG titers in the serum (which was principally due to a decrease in influenza-specific IgG3 [our unpublished data]) and IgA titers in the BAL (Fig. 2A, 2B). In contrast, B. parapertussis-specific Ab titers were unchanged between bacteria-only and heterologously infected mice in the serum and were undetectable in the BAL (Fig. 2B). Any alterations observed in Ag-specific immune responses did not appear to have functional consequences, because when convalescent mice were rechallenged with influenza virus or B. parapertussis, mice that had previously been given a heterologous infection were equivalently protected against bacterial or viral rechallenge compared with their single-infection controls, both in terms of pathogen clearance (Fig. 2C) and pulmonary inflammation (Fig. 2D). On rechallenge, the differences in bacterial burden between influenza- or heterologously infected mice compared with PBS- or B. parapertussis-treated mice was not statistically significant after allowing for the effects of prior B. parapertussis infection (p = 0.101). In similar fashion, the effect of prior Bordetella infection (either alone or as part of a heterologous infection) on bacterial burden following rechallenge was not significant after accounting for influenza’s effects (p = 0.058). Collectively these data suggested that although heterologous infection impacted aspects of the adaptive immune response, it did not compromise overall immune protection.

**Heterologous infection significantly worsens inflammatory responses in the murine lung**

To further examine the early host defense response in heterologous infection, mice were given an intranasal administration of PBS or influenza virus and then challenged 5 d later with PBS or B. parapertussis. Visual examination of heterologously infected lungs 7 d postchallenge (d 5+7) revealed discoloration and overt hemorrhage (our unpublished data). Examination by flow cytometry revealed a modest but significant increase in CD8 T cells in heterologously infected mice (Table I). We further observed a dramatic increase in the frequency of Gr-1bright (Ly-6Gbright) cells in the lungs of heterologously infected mice (52.0 ± 6.3%), which was not observed in control mice, or mice infected with B. parapertussis or influenza alone (9.8 ± 0.5, 9.6 ± 1.2, and 11.8 ± 0.6%, respectively) (Fig. 3A, 3B). Gr-1bright cells have been classified as neutrophils but may also represent populations of monocytes and/or dendritic cells. Therefore, we isolated these cells by FACS and found them to be >95% pure neutrophils by standard cytologic criteria (Fig. 3C).

On histological examination of the lungs, mild and moderate perivascular and peribronchiolar inflammation was noted in mice infected with either B. parapertussis or influenza virus, respectively, whereas heterologously infected mice had a greater extent of both types of inflammation, along with extensive inflammatory cell infiltration into the alveoli and the conducting...
FIGURE 6. Increased chemokine expression in the lungs of heterologously infected mice. Mice were given an intranasal administration of PBS or influenza as indicated, challenged intranasally with PBS or *B. parapertussis* 5 d later, and sacrificed 1, 3, 7, and 14 d later. A, Heat map of bead array-based analysis of cytokines in lung homogenates of animals in treatments and time points as indicated. Blue cells denote low values for a given cytokine and red denote high ones, with gradations through black indicating intermediate values. Cytokines are sorted from greatest increase in expression between...
airways. These inflammatory exudates were replete with neutrophils on d 5+7 and mononuclear cells on d 5+14 (Fig. 3D and our unpublished data). Consistent with this, BAL inflammation was significantly greater in heterologously infected mice than in mice that received either infection alone. On d 5+7, heterologously infected mice had significantly elevated numbers of total cells as compared with B. parapertussis-infected, influenza-infected, or vehicle-only mice, with neutrophils accounting for the vast majority of this increase (Fig. 3E, top panels). An additional 7 d later, BAL inflammation remained elevated in heterologously infected mice as compared with B. parapertussis-infected, influenza-infected, or vehicle-treated mice, and this increase was composed principally of mononuclear cells (Fig. 3E).

**Increased inflammation precedes increased bacterial burden in heterologous infection**

To investigate whether the increased inflammation observed in heterologously infected hosts on d 5+7 was a response to an increased bacterial burden early in infection, mice were sacrificed 1 and 3 d postbacterial challenge. BAL inflammation was equivalent between heterologous and influenza-only mice on d 5+1, but on d 5+3, heterologously infected mice had a greater pulmonary inflammatory response with significantly increased numbers of neutrophils but not mononuclear cells (Fig. 4A). Importantly, bacterial burdens were unchanged at both of these time points, indicating that exacerbated inflammation is not the result of an increased bacterial burden (Fig. 4B). Equivalent bacterial burdens early postinfection also suggested that the increased bacterial burdens in heterologously infected animals on d 5+7 and d 5+14 are not the result of increased adherence or decreased mucociliary clearance early in infection. Increased inflammation did not appear to be a response to increased viral burdens either, because viral burdens were equivalent between influenza-only and heterologously infected mice at both time points (Fig. 4C), despite significant reductions in type I IFN production in the BAL at both time points (Fig. 4D).

**Increased recruitment, not decreased apoptosis, drives pulmonary inflammation in heterologously infected lungs**

Because type I IFNs play an important role in regulating inflammation through the induction of granulocyte apoptosis, we questioned whether reduced type I IFNs might result in decreased neutrophil apoptosis and consequent accumulation of neutrophils in the lungs of heterologously infected mice. No reductions were noted in the number of cells staining positive in the TUNEL reaction—a marker of late apoptosis at any time point examined (Fig. 5A). To exclude the possibility that necrotic neutrophils were accumulating in the lungs, neutrophil viability was assessed by 7-AAD exclusion; although a statistically significant increase was detected in neutrophil death on d 5+7, the increase was small (<1.5% compared with influenza-only mice and <1% compared with B. parapertussis-only infected mice [Fig. 5B]). Concordantly, MPO levels in lung homogenates on d 5+7 (Fig. 5C) reflected the numbers of neutrophils observed in those lungs (Fig. 3B), suggesting that the neutrophils in the lungs of heterologously infected mice were indeed viable and producing effector molecules.

Because reduced apoptosis did not appear to drive the accumulation of neutrophils in the lungs of heterologically infected mice, we questioned whether increased recruitment might instead. We conducted a bead array-based analysis of cytokine and chemokine expression in lung homogenates taken from all groups of animals on d 5+1, d 5+3, d 5+7, and d 5+14 (Fig. 6A and Supplemental Tables 1–4). Over this time course, a number of mediators were induced by influenza or B. parapertussis alone, and many were further elevated following heterologous infection. Most striking was the intense increase in the expression of the CXCR2 ligands MIP-2 (elevated by a factor of 33 with respect to influenza-only mice and a factor of 23 compared with B. parapertussis-only mice on d 5+7) and KC (10- and 3-fold increased, compared with influenza or B. parapertussis alone), both of which are potent neutrophil chemoattractants. We confirmed the expression of these mediators by ELISA (Fig. 6B) and noted that, in agreement with our bead array data, MIP-2 but not KC was increased at the d 5+3 time point, when inflammation in heterologically infected mice was first beginning to rise. We extended our analysis and found that both MIP-2 and KC were significantly elevated in the BAL of heterologically infected mice on d 5+7 (Fig. 6D). We did not detect systemic production of MIP-2 in the serum, and whereas B. parapertussis infection resulted in increased serum KC levels compared with naive animals, expression was not significantly different between heterologous mice and their B. parapertussis-only controls (our unpublished data). This pointed to locally produced MIP-2 as a potential cause for the profound neutrophilia in heterologously infected mice, and we therefore considered it as a therapeutic target.

**Critical role of MIP-2 in heterologous pulmonary inflammatory pathology**

To examine neutralization of MIP-2 as a therapeutic avenue in the treatment of the immunopathology that ensues following heterologous infection, mice were infected with influenza and challenged with B. parapertussis 5 d later. Commencing on d 5+3, a time point when heterologous mice can be differentiated from influenza-only mice on the basis of weight loss and/or pulmonary inflammation, we administered a daily i.p. injection of neutralizing anti–MIP-2 Abs or control IgG. Anti–MIP-2 treatment resulted in a significant improvement in animals’ clinical scores on d 5+7, as well as decreased weight loss over the course of treatment (Fig. 7A, 7B). Neutralization of MIP-2 had no impact on bacterial burden in these mice but did significantly reduce pulmonary neutrophilia (Fig. 7C, 7D), pointing to inflammation as a cause of heterologous infection-related illness, rather than simply a response to increased pathogen burdens. This beneficial impact did not appear to be mediated by reductions in TNF-α production, because anti–MIP-2–treated, heterologously infected mice had similar levels of TNF-α in their lungs on d 5+7 when compared with heterologously infected mice treated with control rabbit IgG (38 ± 10 versus 42 ± 6 pg/ml; n = 5 and 9, respectively). MIP-2 neutralization did not significantly attenuate pulmonary neutrophilia in mice infected with B. parapertussis or influenza alone (data not shown), which is unsurprising, given the modesty of pulmonary neutrophilia that exists in these animals on d 5+7. Because granulocyte chemotactic protein-2 and
KC (both of which are ELR + neutrophil chemokines that share the CXCR2 receptor with MIP-2) were also elevated in heterologously infected mice, we questioned whether blocking CXCR2, rather than just one ligand, might provide a more pronounced therapeutic effect. On the contrary, a blocking Ab against CXCR2 resulted in dramatically increased bacterial burdens and reduced weight recovery when administered on the same treatment regimen as the anti-MIP2 treatment (Fig. 8). In parallel experiments, administration of anti-CXCR2 to mice infected only with B. parapertussis resulted in similar significant impairments to bacterial clearance in the lungs (our unpublished data).

Discussion

During natural human influenza infection, bacterial pneumonia rather than viral disease is the predominant cause of death. Notably, the bacteria most commonly associated with these bacterial superinfections are not pathogens on their own but are instead common, commensal microbes, which suddenly become problematic when superimposed over viral infections (5). Although several mechanisms have been proposed by which a viral infection can predispose to bacterial infection, the mechanism by which a commensal bacterium becomes a threat remains an understudied area. The bacteria most commonly associated with natural cases of bacterial superinfection such as *Streptococcus pneumonia*, *Staphylococcus aureus*, and *Haemophilus influenzae* (5) are commensal in humans but not in specific pathogen-free mice. They are cleared quite quickly from the murine respiratory tract and induce substantial disease on their own, even without a concomitant viral infection, which makes them less than ideal models of this particular human pathology. To overcome this, we developed a model of heterologous pulmonary infection in which mice were infected with influenza virus, then challenged 5 d later with the Gram-negative bacterium *B. parapertussis*. *B. parapertussis* was chosen because at the dose given in this study, infection with *B. parapertussis* on its own is a subclinical event that causes no apparent illness or weight loss and only minimal inflammation. An Influenza-Bordetella coinfection model also has clinical relevance because this combination is observed in the clinic (36, 37). Moreover, the Bordetellae are challenging to culture from biological specimens and difficult to discriminate from other highly similar cocci by traditional microbiological techniques. Thus, Bordetella infections may indeed account for some of the secondary bacterial pneumonia seen in pandemics.

Lungs from mice heterologously infected with influenza virus and *B. parapertussis* were hemorrhagic on gross examination, whereas microscopic and flow cytometric examination revealed a BAL and tissue inflammation that was substantially greater than would be anticipated based on the sum of the Bordetella- and influenza-induced responses on their own. This inflammation was composed principally of neutrophils 7-d post-Bordetella infection and mononuclear cells an additional week later. Viral burdens were unaffected by heterologous infection, and although bacterial burdens were increased late in the course of infection, they were within the range of inoculums we have delivered to naive animals without inducing any significant pathology (our unpublished data). Furthermore, we have observed that a primary infection with live *B. parapertussis* protects against subsequent influenza virus challenge (our unpublished data), and others have similarly found that exposure to bacterial ligands such as FinH (38) or Cpg DNA (39, 40) or viral ligands such as poly(I:C) (41) protects against subsequent viral challenge. Whereas extracellular bacteria, bacterial ligands, and viral ligands are all potent activators of innate immune responses and may provide local protection against a later viral challenge, infection with live intracellular pathogens have been proposed to establish a disadvantageous environment for antibacterial host defense. Evidence suggests that viral infection results in lysis of epithelial cells, exposing the relatively “sticky” basement membrane to which bacteria may adhere, and in
upregulation of bacterial adhesion factors on surviving cells (16), so we questioned whether early changes in bacterial burdens might drive this pulmonary neutrophilia. In contrast, we found no evidence of an increased bacterial burden in the first 3 d post-infection and instead noticed that inflammation arose prior to differences in bacterial burdens.

We go on to show that severe pulmonary inflammation correlated with increased production of a wide range of mediators but specifically attribute it to an overproduction of the inflammatory chemokine MIP-2/CXCCL2/GRO-β. We demonstrate that therapeutic immunoneutralization of MIP-2, even beginning 3 d post-challenge, is an effective therapy in heterologously infected mice, which improves mice’s clinical presentation, weight recovery, and neutrophilia, without altering bacterial burdens. By showing that inflammatory immunopathology is the real threat to life in heterologously infected hosts, we recapitulate a notion that has been proposed in prior influenza pandemics and the present H1N1 swine influenza outbreak (42). Importantly, anti-MIP-2 was also given to influenza- or *B. parapertussis*-only mice without apparent deleterious effect. In contrast, blockade of the entire CXCR signaling axis with an antagonistic anti-CXCR2 resulted in dramatically impaired antibacterial immunity in bacteria-only and heterologous mice, as well as reduced weight recovery in heterologously infected ones. Others have made analogous findings. In a model of pulmonary *Pseudomonas aeruginosa* infection, CXCR2 blockade attenuated pulmonary neutrophilia but impaired bacterial clearance and reduced survival, whereas neutralization of MIP-2 significantly reduced neutrophilia, without compromising host defense (43).

In other models of severe lung pathology, more complete interference with the CXCR2 signaling axis has shown a positive effect; genetic deletion or immunoneutralization of CXCR2 or its ligands attenuates the pathology of ventilator-induced lung injury (VILI) in mice (44). VILI is an aseptic cause of acute respiratory distress syndrome (ARDS), a disease that kills 26–44% of those it afflicts (45, 46). VILI-minimizing strategies reduce mortality in ARDS by minimizing CXCR2 signaling, reducing neutrophil recruitment and neutrophilic injury (44, 47). In contrast, in cases of ARDS resulting from infectious causes such as heterologous pneumonia, the merits of preventing immunopathology must be weighed against the potentially deleterious effects of dampening the antiviral and antibacterial host defense responses. We find that although overexpression of MIP-2 may drive inflammatory immunopathology, clearly some signaling through CXCR2 is required for adequate antibacterial immunity.

Taken together, the disparity between our findings with MIP-2 neutralization and CXCR2 blockade suggest important pathological and protective roles for CXCR2 ligands in pulmonary host defense. Clinically, BAL levels of the CXCR ligands IL-8 and KC correlate with the severity of pulmonary neutrophilia in ARDS and pneumonia (47). In this regard, we show that KC is highly expressed in heterologously infected mice, as is another CXCR2 ligand, granulocyte chemotactic protein-2. When MIP-2 is neutralized, these and other CXCR2 ligands, as well as CCR ligands (which have evolved in mice to compensate for a species-wide deficiency in CXCR1), may partially overcome the loss of MIP-2 and allow coordination of a response, which is effective for host defense, but does not cause immunopathology. In our model of heterologous infection, the bacterial pathogen used does not cause major disease on its own. Whether neutralization of MIP-2 will provide benefit in models of heterologous infection that use more virulent pathogens such as *S. pneumoniae* remains to be seen; there is admittedly a risk that attenuating pulmonary neutrophilia might unacceptably impair host defense. Although we cannot wholly discount this possibility, in a mouse model of heterologous infection, Ab-mediated neutrophil depletion did not damage the antipneumococcal response any further than influenza infection already had (48).

The question, “What drives MIP-2 overexpression only in heterologously infected mice?” remains an important one. The pulmonary defenses must concern themselves not only with the prevention of infection, but also with the avoidance of inflammatory disease, which would compromise gas exchange. Consequently, a number of mechanisms have evolved that maintain a high degree of ignorance and tolerance to foreign invaders. To shift the “immune rheostat” (reviewed in Ref. 49) toward a pathological state of inflammation like that observed in our hands, these mechanisms must be overcome. Recent work by Shahgian et al. (22) may provide a clue to explain this phenomenon. They showed that mice with a genetic deficiency in the IFN-α receptor produce more KC and MIP-2 and mount more robust inflammatory responses during viral-bacterial superinfection than do wild-type controls (22). In that regard, one of our most important findings is that heterologously infected mice have reduced pulmonary IFN titers compared with influenza-only mice. Because early reductions in type I IFNs were noted in heterologously infected animals, this may have permitted the later increases in MIP-2 expression. One notable difference between Shahgian’s study and ours was that in their model, acute influenza infection attenuated inflammatory responses to bacterial challenge, rather than exacerbating it, so the administration of MIP-2 and KC, rather than their removal, was used as a therapy. Although Didierlaurent et al. (19) have shown a similar phenomenon, which begins weeks and continues for months after the resolution of influenza infection, to our knowledge, reduced lung inflammation is not a feature of acute bacterial superinfections of influenza in humans. In such cases, a surfeit of inflammation, rather than a deficit, is commonly noted and is implicated in disease pathogenesis.

That such an intense and prolonged inflammatory response is incapable of clearing the pulmonary bacterial load is an intriguing observation. Although neutrophils are required for *B. parapertussis* clearance (27), it is important to note that influenza infection may impair neutrophil function to the extent that even partial depletion of neutrophils from influenza-infected mice does not further damage antibacterial host defense (48). In addition, AMs are second only to respiratory epithelial cells as a target for influenza, and direct viral infection may further compromise their antibacterial function, as has been reported in other models (20, 24). Moreover, the presence of neutrophils, whether functional or not, in the influenza-infected airway at the time of bacterial challenge is a key difference between viral-only and heterologously infected mice. These cells must be cleared by AMs, which also play a crucial role in the early pulmonary antibacterial response. Efferocytosis has been shown to compromise their antibacterial function by inducing the release and autocrine activity of PGE2 (50), so having to clear the influenza-associated neutrophilia may place these macrophages at an immediate disadvantage when next tasked with clearing a bacterial infection. A self-reinforcing vicious circle may then arise, in which ever-increasing recruitment actually impairs resident antibacterial host defense cells, until ultimately the adaptive immune system arrives to clear the pathogen. That the formation of immune memory was not substantially impacted by heterologous infection is consistent with the finding that survivors of the 1918 influenza pandemic, many of whom presumably suffered from bacterial superinfections of their influenza, demonstrate robust B cell responses against influenza Ags even some 80 y later (51). The key issue seems to be that the host must survive the initial pneumonia for long enough to arm and use the adaptive arm of immunity.

It has been commonly stated that the Spanish influenza virus, which swept the globe in 1918–1919, was remarkably lethal. Elegant
work by McAuley et al. (6) has recently called that notion into question. Their experiments suggest not the 1918 virus’ intrinsic lethality, but its ability to drive a fatal secondary bacterial infection as its most remarkable feature (6). Indeed, secondary bacterial pneumonia, not only viral primary disease, causes the majority of deaths during influenza pandemics (5), and viral-bacterial confections lead to worse symptoms than single-agent ones even during seasonal influenza (52). Thus, it may be that a given influenza pandemic is only as lethal as its ability to drive bacterial superinfection and that a given superinfection is only as lethal as its ability to drive the inflammatory immunopathology, which actually kills. Through prophylactic or early therapeutic interventions against the causative pathogens, we may limit the extent of the inflammation and improve outcomes. Beyond a certain threshold (in time or in magnitude of infection), however, strategies that directly alleviate inflammation may warrant consideration. In this regard, it remains of interest to examine whether blocking mediators other than MIP-2 or removing the cells that produce them, such as CD8+ T cells, will prove beneficial. The relative merits of such strategies, however, have to be weighed against potential detrimental effects on the host defense response.

References


Disclosures

The authors have no financial conflicts of interest.


