This information is current as of April 19, 2017.

Koenraad F. van der Sluijs, Leontine J. R. van Elden, Monique Nijhuis, Rob Schuurman, Jennie M. Pater, Sandrine Florquin, Michel Goldman, Henk M. Jansen, René Lutter and Tom van der Poll

*J Immunol* 2004; 172:7603-7609; doi: 10.4049/jimmunol.172.12.7603

http://www.jimmunol.org/content/172/12/7603

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
IL-10 Is an Important Mediator of the Enhanced Susceptibility to Pneumococcal Pneumonia after Influenza Infection

Koenraad F. van der Sluijs,*‡‡ Leontine J. R. van Elden,† Monique Nijhuis,† Rob Schuurman,† Jennie M. Pater,* Sandrine Florquin,§ Michel Goldman,† Henk M. Jansen,‡ René Lutter,‡‡ and Tom van der Poll†,*

Secondary pneumococcal pneumonia is a serious complication during and shortly after influenza infection. We established a mouse model to study postinfluenza pneumococcal pneumonia and evaluated the role of IL-10 in host defense against Streptococcus pneumoniae after recovery from influenza infection. C57BL/6 mice were intranasally inoculated with 10 median tissue culture infective doses of influenza A (A/PR8/34) or PBS (control) on day 0. By day 14 mice had regained their normal body weight and had cleared influenza virus from the lungs, as determined by real-time quantitative PCR. On day 14 after viral infection, mice received 10⁴ CFU of S. pneumoniae (serotype 3) intranasally. Mice recovered from influenza infection were highly susceptible to subsequent pneumococcal pneumonia, as reflected by a 100% lethality on day 3 after bacterial infection, whereas control mice showed 17% lethality on day 3 and 83% lethality on day 6 after pneumococcal infection. Furthermore, 1000-fold higher bacterial counts at 48 h after infection with S. pneumoniae and, particularly, 50-fold higher pulmonary levels of IL-10 were observed in influenza-recovered mice than in control mice. Treatment with an anti-IL-10 mAb 1 h before bacterial inoculation resulted in reduced bacterial outgrowth and markedly reduced lethality during secondary bacterial pneumonia compared with those in IgG1 control mice. In conclusion, mild self-limiting influenza A infection renders normal immunocompetent mice highly susceptible to pneumococcal pneumonia. This increased susceptibility to secondary bacterial pneumonia is at least in part caused by excessive IL-10 production and reduced neutrophil function in the lungs. The Journal of Immunology, 2004, 172: 7603–7609.

Influenza infections usually cause only mild symptoms, such as fever, headache, sore throat, sneezing, and nausea, accompanied by decreased activity and food intake (1). Although influenza alone may lead to pneumonia, secondary bacterial infections during and shortly after recovery from influenza infections are much more common causes of pneumonia (2). The excess mortality rates during the pandemics of 1918–1919 and 1957–1958 can mainly be attributed to secondary bacterial complications (3). Even today, secondary bacterial pneumonia causes at least 20,000 deaths each year in the U.S. (4). Bacteria such as Staphylococcus aureus and Haemophilus influenzae are known to cause postinfluenza pneumonia, but Streptococcus pneumoniae is the most prominent pathogen causing secondary bacterial pneumonia in recent decades (1). Primary infection with this pathogen is usually less severe than secondary infection (5).

The severity of secondary bacterial pneumonia during or shortly after influenza infection is determined by a complex interaction among virus, bacteria, and host. During combined viral/bacterial infections, the severity of the infection can increase due to enhanced virulence of the influenza virus facilitated by bacterial proteases (6). However, the host remains more susceptible to bacterial infections for several weeks after clearance of the influenza virus, which indicates that the enhanced susceptibility is not only due to an increased viral virulence (7). Influenza infection is known to increase adherence of and subsequent colonization with bacterial respiratory pathogens. Bacteria may adhere to the basal membrane after disruption of the airway epithelial layer by the cytopathic effect of the virus (8). It has also been suggested that the increased adherence is due to up-regulation of receptors involved in the attachment of these bacteria (9). Alternatively, influenza virus alters the innate immune response of the host to subsequent bacterial challenges as well. Several researchers showed that influenza-infected mice were more sensitive to bacterial components, such as staphylococcal enterotoxin B and LPS (10, 11). Cytokines such as IFN-γ, TNF-α, and IL-6 are synergistically up-regulated by staphylococcal enterotoxin B or LPS during influenza infections in mice. Influenza virus has also been reported to reduce neutrophil activity in mice, which results in decreased pulmonary clearance after secondary bacterial infection with S. pneumoniae (11). These data clearly indicate that influenza virus dramatically alters the innate immune response to bacterial infections. To date, little has been known about the mechanism by which influenza virus modulates the innate immune response to bacterial infections of the lungs.

Host defense against pneumococcal pneumonia is coordinated by the action of proinflammatory and anti-inflammatory cytokines (12, 13). In the current study we present a mouse model to study host defense against S. pneumoniae after recovery from influenza infection. We show that the enhanced susceptibility of mice recovered from influenza infection to secondary pneumococcal pneumonia is accompanied by an exaggerated production of proinflammatory and anti-inflammatory cytokines. The finding of strikingly elevated pulmonary IL-10 concentrations in mice with postinfluenza pneumonia compared with mice with primary S. pneumoniae pneumonia and our previous finding of a detrimental role for endogenous IL-10 during primary pneumococcal pneumonia (14) prompted us to determine the contribution of exaggerated...
IL-10 production during secondary bacterial pneumonia to the enhanced susceptibility to *S. pneumoniae* of mice recovered from influenza.

### Materials and Methods

#### Mice

Pathogen-free, 8-wk-old, female C57BL/6 mice were obtained from Harlan Sprague Dawley (Horst, The Netherlands) and maintained at biosafety level 2. All experiments were approved by the animal care and use committee of Academic Medical Center, University of Amsterdam.

#### Experimental infection protocol

Influenza A/PR/8/34 (ATCC VR-95; American Type Culture Collection, Manassas, VA) was grown on LLC-MK2 cells (RIVM, Bilthoven, The Netherlands). Virus was harvested by a freeze/thaw cycle, followed by centrifugation at 680 × g for 10 min. Supernatants were stored in aliquots at −80°C. Titration was performed in LLC-MK2 cells to calculate the median tissue culture infectious dose (TCID₅₀) of the viral stock (15). A noninfected cell culture was used for preparation of the control inoculum. None of the stocks was contaminated by other respiratory viruses, i.e., influenza B; human parainfluenza types 1, 2, 3, 4A, and 4B; Sendai virus; RSV A and B; rhinovirus; enterovirus; and adenovirus, as determined by PCR or cell culture. Viral stock and control stock were diluted just before use in PBS (pH 7.4). Mice were anesthetized by inhalation of isoflurane (Abbott Laboratories, U.K.) and 7 MTris–7 MTrisSequenecylated with 10 TCID₅₀ of influenza (1400 viral copies) or control inoculum in a final volume of 50 μl of PBS. Pneumococcal pneumonia was induced 14 days after inoculation with influenza or control suspension according to previously described methods (14, 16, 17). In brief, *S. pneumoniae* serotype 3 (ATCC 6303) was cultured for 16 h at 37°C in 5% CO₂ in Todd Hewitt broth. This suspension was diluted 100 times in fresh medium and grown for 5 h in midlogarithmic phase. Bacteria were harvested by centrifugation at 2750 × g for 10 min at 4°C, and the pellet was resuspended in 0.5 ml of sterile PBS. Total cell numbers were counted using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Miami, FL). Differential cell counts were performed on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, IL).

#### Bronchoalveolar lavage (BAL)

The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). BAL was performed by instillation of two 0.5-ml aliquots of sterile saline into the right lung. The retrieved BAL fluid (−0.8 ml) was spun at 260 × g for 10 min at 4°C, and the pellet was resuspended in 0.5 ml of sterile PBS. Total cell numbers were counted using a 22 Coulter Particle Count and Size Analyzer (Beckman Coulter, Miami, FL). Differential cell counts were performed on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, IL).

#### Myeloperoxidase (MPO) activity measurements

MPO activity was measured as described previously (22). BAL was 5-fold diluted in potassium-phosphate buffer (pH 6.0) supplemented with 0.5% hexadecyl-trimethyl-ammonium bromide and 10 mM EDTA. MPO activity was determined by measuring the H₂O₂-dependent oxidation of 3,3′-5,5-tetramethylbenzidine at 37°C. The reaction was stopped by adding glacial acetic acid (0.2 M) to the reaction mixture. The amount of converted 3,3′-5,5-tetramethylbenzidine was determined by measuring the OD at 655 nm. MPO activity is expressed in units per milliliter of BAL fluid. One unit is defined as the amount of MPO required to yield one OD₅₅₀ unit per minute. MPO activity measurements in BAL fluid represents the activation status of polymorphonuclear cells present in the lung.

#### Cytokine and chemokine measurements

Lung homogenates were lysed with an equal volume of lysis buffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% (v/v) Triton X-100, 20 ng/ml pepstatin A, 20 ng/ml leupeptin, and 20 ng/ml aprotinin, pH 7.4) and incubated for 30 min on ice, followed by centrifugation at 680 × g for 10 min. Supernatants were stored at −80°C until further use. Cytokines and chemokines in total lung lysates were measured by ELISA according to the manufacturer’s protocol. Reagents for IL-6, IL-10, KC, and TNF-α measurements were obtained from R&D Systems (Abingdon, U.K.); IFN-γ reagents were obtained from BD PharMingen (San Diego, CA).

#### Statistical analysis

All data are expressed as the mean ± SE unless stated otherwise. Differences between groups were analyzed by Mann-Whitney U test. Survival was analyzed with Kaplan-Meier analysis using a log-rank test; p < 0.05 was considered to represent a statistically significant difference.

#### Results

##### Body weight loss after influenza infection and secondary bacterial pneumonia

In previous studies influenza infection has been shown to induce loss of body weight (23). Therefore, body weight can be used as a marker to follow the course of influenza infections in mice. In this study influenza infection led to a transient weight loss starting on day 5 after viral exposure (Fig. 1A). Body weight reached its minimum on day 9 and returned to the baseline level on day 13 after exposure. On day 14 both control mice and influenza-infected mice received 10⁴ CFU of *S. pneumoniae*. Body weight decreased again after induction of pneumococcal pneumonia in mice recovered from influenza infection (Fig. 1A). The body weight of control mice did not change (p < 0.0001 vs mice with postinfluenza pneumonia).

### Determination of viral outgrowth

Viral load was determined on days 4, 8, 12, and 14 after viral infection and 48 h after pneumococcal infection (i.e., 16 days after viral infection) using real-time quantitative PCR as previously described (21). Mice (eight mice per time point) were anesthetized using 0.3 ml of fentanyl citrate (0.079 mg/ml), fluanisone (2.5 mg/ml), midazolam (1.25 mg/ml) in H₂O (7.0 ml/kg i.p.) and were sacrificed by bleeding out the vena cava inferior. Lungs were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Lung homogenates (100 μl) were treated with 1 ml of TRIzol reagent to extract RNA. RNA was resuspended in 10 μl of diethylyprocarbonate-treated water. cDNA synthesis was performed using 1 μl of the random suspension and a random hexamer cDNA synthesis kit (Applied, Foster City, CA). Five microtitors of 25 μl of cDNA suspension was used for amplification in a quantitative real-time PCTABI PRISM 7700 Sequence Detector System; Applied Biosystems, Foster City, CA). The viral load present in each sample was calculated using a standard curve of particle-counted influenza virus (virus particles were counted by electron microscopy), included in every assay. The following primers were used: 5′-GGACTGCGACGTGAGGT-3′ (forward), 5′-CATCCTGGTTGTATAT-3′ (reverse), and 5′-CTCAGGTATTCTGCTGTCAC-3′ (5′-FAM-labeled probe).

### Determination of bacterial outgrowth

Serial 10-fold dilutions of the lung homogenates in sterile saline and 10-μl volumes were plated out onto blood-agar plates. Plates were incubated at 37°C at 5% CO₂, and CFUs were counted after 16 h.
FIGURE 1. Body weight and viral load in the lungs after influenza infection in mice. The body weight (A) of virus-infected mice (▲) and control mice (■) was measured daily after intranasal inoculation (eight mice per group). Viral load was determined at several time points after influenza infection (six to eight mice per time point). Viral load is expressed as viral RNA copies per lung (B). Influenza virus was below the detection level (B.D.) on days 14 and 16 (i.e., 48 h after bacterial infection) after viral infection. In control mice influenza also could not be detected (four mice per time point; data not shown).

Complete viral clearance within 14 days after influenza infection
Viral load was determined by real-time quantitative PCR at several time points after viral infection to follow viral load in time. Replication of the virus was observed between days 1 and 4 after virus infection (Fig. 1B). Viral load in the lungs peaked between days 4 and 8 after influenza infection. On day 14 after viral infection, influenza virus could not be detected in lung homogenates, indicating that the virus had been cleared from the lungs. Although several bacteria are known to increase the virulence of the influenza virus, *Streptococcus pneumoniae* was not able to induce viral outgrowth on day 16 after viral infection, which confirms that the influenza virus had been completely cleared from the lungs on day 14 after viral infection.

Increased lethality after pneumococcal infection in mice recovered from influenza infection
Lethality was monitored at least twice a day after pneumococcal infection in mice (12 mice/group) previously infected with influenza or control mice. Mice recovered from influenza infection appeared to be highly susceptible to secondary bacterial pneumonia, as reflected by increased lethality after pneumococcal infection with $10^4$ CFU of *S. pneumoniae* ($p < 0.0001$ vs control mice; Fig. 2). Pneumococcal pneumonia resulted in 100% lethality in mice recovered from influenza infection by day 3 and, in comparison, 17% lethality in mice with primary pneumococcal infection. Lethality in mice with primary pneumococcal infection increased up to 83% after 6 days (Fig. 2); mortality in the remaining mice did not occur thereafter.

Increased bacterial outgrowth in mice recovered from influenza infection
To further assess the host defense against secondary pneumococcal infection, we determined bacterial outgrowth in the lungs. After 48 h, just before the first deaths occurred, mice recovered from influenza infection showed a 1000-fold higher bacterial outgrowth compared with mice with primary pneumococcal infection ($p = 0.0002$ vs control mice; Fig. 3).

Histopathological analysis
Forty-eight hours after pneumococcal infection, lungs were harvested to prepare H&E-stained lung slides for histopathological examination. Primary pneumococcal infection resulted in interstitial inflammation, bronchiolitis, endothelialitis, and pleuritis, as reflected by granulocyte infiltrates present in ~20% of the lung (Fig. 4). Mice recovered from influenza infection with secondary pneumococcal pneumonia showed severe interstitial inflammation, bronchiolitis, endothelialitis, and pleuritis in the entire lung. In contrast to mice with primary pneumococcal infection, the infiltrates in the lungs of mice recovered from influenza consisted of not only granulocytes, but also lymphocytes. These features of severe pneumonia are obviously the result of secondary infection with *S. pneumoniae*, as no significant pathological findings were observed on day 12 after primary influenza virus infection (data not shown). These observations indicate that pneumococcal infection in mice induces more severe pulmonary inflammation after recovery from influenza infection.

FIGURE 2. Previous exposure to influenza results in enhanced lethality due to *S. pneumoniae* infection. Survival after pneumococcal infection in mice previously infected with influenza (▲) vs control mice (■). All mice (12 mice/group) received $10^4$ CFU of *S. pneumoniae* on day 14 after viral infection and were monitored at least twice a day after pneumococcal infection.

FIGURE 3. Enhanced outgrowth of pneumococci during postinfluenza pneumonia. Bacterial outgrowth in the lungs after pneumococcal infection in mice previously infected with influenza (▲) and control mice (■). All mice (eight mice per group) received $10^4$ CFU of *S. pneumoniae* on day 14 after viral infection and were sacrificed 48 h later. Horizontal lines represent medians for each group. Note that one mouse in the control group had cleared the bacteria at 48 h after infection (not shown in graph).
**FIGURE 4.** Increased lung inflammation during postinfluenza pneumonia. Histopathological analysis of the lungs of mice infected with influenza (C and D) or sham-infected control mice (A and B) was performed. All mice received 10⁷ CFU of *S. pneumoniae* on day 14 after viral infection. Lungs were isolated 48 h after pneumococcal infection and prepared for histopathological analysis. A 100-fold magnification (A and B) was used to compare the area of inflamed tissue. A 100-fold magnification (B and D) was used to identify infiltrating cells in the pulmonary compartment. Slides are representative for six mice per group.

**Leukocyte influx in BAL fluid**

To further assess innate immunity to *S. pneumoniae* after recovery from influenza infection, BAL was performed to identify leukocyte influx into the lungs. Mice recovered from influenza infection demonstrated a substantial influx of leukocytes 48 h after infection with *S. pneumoniae*, whereas primary pneumococcal infection as well as primary influenza infection caused only moderate recruitment of leukocytes to the lungs (Table I). The increase in leukocyte numbers in BAL fluid after secondary pneumococcal pneumonia was mainly due to the recruitment of granulocytes (Table I). Remarkably, mice with secondary pneumococcal pneumonia showed a significantly lower number of lymphocytes in BAL fluid than saline-treated mice recovered from primary influenza infection (Table I). Although granulocyte numbers were significantly higher after secondary pneumococcal pneumonia, MPO activity in BAL fluid appeared to be similar in mice with primary and secondary pneumococcal infections (Fig. 5). These data indicate that granulocyte activity is relatively reduced during secondary pneumococcal pneumonia.

**Increased cytokine and chemokine levels in lung homogenates of mice recovered from influenza**

Pneumococcal infection elicits a number of inflammatory responses within the lungs. These responses include the production of proinflammatory cytokines (TNF-α, IL-6, and IFN-γ) and chemokines (KC). Proinflammatory cytokine and chemokine levels appeared to be 3- to 10-fold higher in lung homogenates of mice with postinfluenza pneumococcal pneumonia compared with mice with primary pneumococcal infection (all *p* < 0.05; Fig. 6). Interestingly, mice recovered from influenza showed 20-fold higher concentrations of IL-10 in lung homogenates than mice not previously exposed to influenza. Pulmonary cytokine levels were similar in control mice and influenza-infected mice on days 14 and 16 after infection (Fig. 6).

**Anti-IL-10 reduces bacterial outgrowth**

IL-10 has been found to impair host defense during primary pneumococcal pneumonia (14). In light of the markedly elevated IL-10 concentrations in lungs of mice with postinfluenza pneumococcal pneumonia, we considered it of interest to determine the contribution of IL-10 in the reduced antibacterial defense of these mice. For this, mice recovered from influenza infection were treated with a neutralizing mAb against IL-10 1 h before pneumococcal inoculation. Forty-eight hours after pneumococcal infection, bacterial outgrowth was significantly lower in anti-IL-10-treated mice compared with control IgG1-treated mice (*p* = 0.02; Fig. 7). Thus, the high IL-10 levels after infection with *S. pneumoniae* in mice recovered from influenza impaired bacterial clearance.

**Cytokine response after treatment with anti-IL-10**

Cytokine levels in lung homogenates were measured to determine the effect of anti-IL-10 on the induction of proinflammatory mediators (Fig. 8). IL-6 and KC production were similar in anti-IL-10-treated mice and IgG1 control-treated mice. TNF-α and IFN-γ levels were lower in anti-IL-10-treated mice than in control mice (both *p* < 0.05).

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**Table I. Leukocytes in BAL fluid**

<table>
<thead>
<tr>
<th>Cells (×10³)</th>
<th>PBS + Saline</th>
<th>PBS + <em>S. pneumoniae</em></th>
<th>Influenza + Saline</th>
<th>Influenza + <em>S. pneumoniae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count</td>
<td>116 ± 22</td>
<td>240 ± 55[^b^]</td>
<td>315 ± 43</td>
<td>223 ± 416</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>2.1 ± 0.7</td>
<td>39 ± 23[^b^]</td>
<td>16 ± 6</td>
<td>1734 ± 340</td>
</tr>
<tr>
<td>Macrophages</td>
<td>105 ± 21</td>
<td>199 ± 39[^b^]</td>
<td>224 ± 31</td>
<td>476 ± 88</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2.3 ± 0.8</td>
<td>1.9 ± 0.7</td>
<td>75 ± 16</td>
<td>27 ± 10</td>
</tr>
</tbody>
</table>

[^a^] Mice (*n* = 6 per group) received PBS or influenza A intranasally at day 0, followed by *S. pneumoniae* intranasally or saline at day 14. BAL fluid was obtained 48 h after intranasal administration of *S. pneumoniae* or saline. All data are mean ± SE.

[^b^] *p* < 0.05 compared to PBS and saline-treated mice.

[^c^] *p* < 0.05 compared to mice with primary pneumococcal infection.

[^d^] *p* < 0.05 compared to saline-treated mice recovered from influenza infection.
FIGURE 5. MPO activity in BAL fluid. MPO activity in BAL fluid was measured for control mice, primary influenza-infected mice, primary S. pneumoniae-infected mice, and secondary S. pneumoniae-infected mice on day 16 after viral inoculation, i.e., 48 h after pneumococcal infection. Mice (six mice per group) received 10 TCID₅₀ of influenza or PBS on day 0 and 10⁷ CFU of S. pneumoniae or saline on day 14 after viral infection. Data are expressed as units per milliliter (mean ± SE). *, p < 0.05 vs control mice and primary influenza-infected mice.

Anti-IL-10 improves survival during secondary pneumococcal infection

To assess whether the increased IL-10 levels adversely influenced survival during postinfluenza pneumonia, lethality was monitored in mice (12 mice/group) treated with the anti-IL-10 Ab or the IgG1 control Ab (Fig. 9). Anti-IL-10 appeared to be protective against S. pneumoniae-induced lethality in mice recovered from influenza infection, as reflected by prolonged survival and increased survival rates compared with control Ab-treated mice (p = 0.0005).

Discussion

Secondary bacterial infections are serious complications during and shortly after respiratory tract infections with influenza virus. Several mechanisms have been proposed to explain the enhanced susceptibility to secondary bacterial infections after viral infection. Many studies focused on the virus-induced damage of the respiratory epithelium as a factor in the enhanced susceptibility to secondary bacterial infections (8, 24–26). Other investigations have pointed to a decreased cellular immune response to bacteria, reflected by reduced chemotaxis and phagocytic capacity (27–29). We show in this study that pneumococcal pneumonia in mice recovered from influenza infection is associated with profoundly elevated IL-10 concentrations in the lungs, and that these IL-10 levels contribute to the increased susceptibility to secondary bacterial pneumonia.

Our present finding that influenza virus infection renders mice more susceptible to pneumonia caused by S. pneumoniae confirms earlier reports of bacterial complications following influenza infection (9, 11). In the earlier investigations mice were infected with S. pneumoniae on day 7 after inoculation with influenza virus. Our model for secondary bacterial pneumonia was designed to exclude direct interactions between influenza virus and S. pneumoniae. In particular, mice received the bacterial inoculum on day 14 after viral exposure, i.e., at the point the virus was completely cleared from the lungs. By then, these mice had clinically recovered from influenza infection, as reflected by normal bodyweight. This time point was also chosen because clinical data indicate that 2 wk is a common interval between influenza infection and the occurrence of secondary bacterial complications (1, 2). Our data clearly indicate that mice remain highly susceptible to secondary bacterial infections after recovery from airway infection from influenza virus. Indeed, mice with postinfluenza pneumococcal pneumonia showed an increased lethality and an enhanced bacterial outgrowth in the lungs compared with mice with primary pneumococcal pneumonia.

Mice with S. pneumoniae pneumonia preceded by influenza infection displayed an enhanced inflammatory response compared with mice with primary pneumococcal infection, as indicated by histopathology, cell influx in BAL fluid, and higher cytokine and chemokine levels in lung homogenates. Possibly this exaggerated inflammation was the consequence of the much higher bacterial load, providing a stronger proinflammatory stimulus in these postinfluenza mice. In support of this idea are previous findings by Dallaire et al. (30), demonstrating that pulmonary cytokine levels...
correlate closely with the extent of bacterial outgrowth during pneumococcal pneumonia. Alternatively, colonization of S. pneumoniae may be due to the damage of the epithelial layer caused by an exaggerated inflammatory response. However, our data do not support this latter explanation, as neutralization of IL-10, an anti-inflammatory cytokine, appears to be protective.

Mice recovered from influenza infection displayed much higher bacterial counts after infection with S. pneumoniae, yet had 50-fold higher neutrophil numbers in BAL fluid compared with mice with primary pneumococcal pneumonia. Probably the increased neutrophil numbers were the result of the higher bacterial load, providing a more potent proinflammatory stimulus. Indeed, in murine pneumococcal pneumonia the extent of inflammation is closely correlated with the number of bacteria (30). Moreover, our data indicate that granulocyte function was significantly reduced during secondary pneumococcal pneumonia, as reflected by similar MPO activities in BAL fluid obtained in mice with primary and postinfluenza bacterial pneumonia (despite much higher neutrophil numbers in the latter group). Similar results were obtained by LeVine et al., who showed that MPO release by isolated bronchoalveolar lavage neutrophils was significantly lower after pneumococcal infection in mice previously exposed to influenza (−7 days) than in control mice (11). Considering that IL-10 potently inhibits neutrophil functions, including degranulation (31–33), we consider it highly likely that the elevated IL-10 levels in mice with postinfluenza pneumonia are at least in part responsible for the relatively reduced neutrophil function.

In primary S. pneumoniae infection of the airways, endogenously produced IL-10 impairs host defense (14). Similarly, IL-10 hampered an adequate immune response during murine Klebsiella pneumoniae pneumonia (34). These data led us to hypothesize that the strongly elevated pulmonary levels of IL-10 in mice with postinfluenza pneumococcal pneumonia were important for the impairment of defense against S. pneumoniae. Our current findings provide evidence for this hypothesis, i.e., anti-IL-10 treatment directly before infection with S. pneumoniae reduced the outgrowth of pneumococci in lungs and increased survival, suggesting a direct link between elevated IL-10 levels and enhanced susceptibility to secondary bacterial infection. Interestingly, Steinhauser et al. (35) used an approach identical with that taken in the current study to establish that enhanced IL-10 production contributed significantly to the diminished lung antibacterial defense against Pseudomonas aeruginosa in mice with abdominal sepsis induced by cecal ligation and puncture. Together these data point to an immunosuppressive effect of endogenous IL-10 in the respiratory tract for highly different conditions (abdominal sepsis and influenza infection of the airways).

Proinflammatory mediators such as IL-6 and KC were not influenced by anti-IL-10 treatment, whereas TNF-α and IFN-γ concentrations even were decreased in anti-IL-10-treated mice. These findings contrast with earlier findings in primary pneumococcal pneumonia, where anti-IL-10 treatment resulted in increased TNF-α and IFN-γ concentrations in the lungs (14). Although IL-10 is expected to inhibit the production of proinflammatory cytokines, it is conceivable that the reduced bacterial load in the lungs of mice administered anti-IL-10 resulted in an attenuated proinflammatory stimulus and masked the potentiating effect of anti-IL-10 on the production of these mediators. In line with this, Dallaire et al. (30) have shown that proinflammatory cytokine levels in lungs of mice with pneumococcal pneumonia closely correlate with the bacterial load. Likewise, in a model of Listeria monocytogenes infection, anti-IL-10 treatment reduced bacterial outgrowth and concurrently inhibited the expression of proinflammatory cytokine gene expression in the liver (36).
Of note, some difference existed in the mortality curves of mice with postinfluenza pneumococcal pneumonia that were not treated with anti-IL-10, as shown in Figs. 2 and 9. Although only in the latter experiment were mice treated with control IgG, we consider it likely that this difference is the result of biovariability related to the fact that these experiments were performed at an interval of several months using different “shipments” of mice. In addition, the model described in this manuscript uses two subsequent infections, and both infections, without doubt, have a certain variability in inoculum size and infection efficiency, leading to subtle differences in pathology. However, we emphasize that all experiments were performed in an appropriately controlled way, i.e., the groups to be compared were always infected at the same time using exactly the same inoculum.

Primary influenza virus infection leads to recruitment of lymphocytes and macrophages, which are both able to release IL-10 (32). Macrophages, at least in part, may be responsible for the exaggerated IL-10 production, as macrophage numbers were further increased after secondary infection with S. pneumoniae, whereas lymphocyte numbers were significantly reduced. However, a 2-fold increase in macrophage numbers cannot fully account for a 50-fold increase in IL-10 production, unless these macrophages have become hyper-responsive to secondary bacterial challenges. An alternative explanation for this discrepancy is that other cell types produce high amounts of IL-10 as well. In this context, lymphocytes cannot be excluded as a prominent source of IL-10, as lymphocyte numbers are still higher after secondary infection with S. pneumoniae than after primary infection with S. pneumoniae. Further research is required to identify the cellular source of IL-10 and to unravel the mechanism by which influenza virus predisposes to an exaggerated IL-10 production upon infection with S. pneumoniae.

It is well established that influenza infection renders the host more susceptible to secondary pneumococcal pneumonia. We demonstrate in this study that IL-10 is an important mediator of an immunosuppressive state, predisposing to a fulminating course of respiratory tract infection with S. pneumoniae, from which the host still suffers after clinical recovery and complete clearance of the virus. Further research is warranted to determine whether neutralization of IL-10 provides an additional approach to early therapy of postinfluenza pneumococcal pneumonia.

Acknowledgments

We thank Joost Daalhuisen and Ingvild Kop for technical assistance during the animal experiments, and Teus van den Ham for assistance during the preparation and titration of the viral stocks.

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