Inflammatory response to viral airway infections and secondary bacterial complications
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IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection

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Secondary pneumococcal pneumonia is a serious complication during and shortly after influenza infection. We established a mouse-model to study post-influenza 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 TCID$_{50}$ Influenza A (A/PR/8/34) or PBS (control) on day 0. By day 14 mice had regained their normal bodyweight and had cleared influenza virus from the lungs, as determined by real-time quantitative PCR. At day 14 after viral infection, mice received $10^4$ CFU S. pneumoniae (serotype 3) intranasally. Mice recovered from influenza infection were highly susceptible to subsequent pneumococcal pneumonia as reflected by a 100% lethality at day 3 after bacterial infection, whereas control mice showed 17% lethality at day 3 and 83% lethality at day 6 after pneumococcal infection. Furthermore, 1000-fold higher bacterial counts at 48 hours 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 one hour before bacterial inoculation resulted in reduced bacterial outgrowth and markedly reduced lethality during secondary bacterial pneumonia compared to IgG1-control treated mice. In conclusion, mild self-limiting Influenza A infection renders normal immunocompetent mice highly susceptible for pneumococcal pneumonia. This increased susceptibility for secondary bacterial pneumonia is, at least in part, caused by excessive IL-10 production and reduced neutrophil function in the lungs.
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Introduction

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 contributed to secondary bacterial complications [3]. Even nowadays, secondary bacterial pneumonia causes at least 20,000 deaths each year in the U.S. only [4]. Bacteria such as Staphylococcus aureus and Haemophilus influenzae are known to cause post-influenza 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 between virus, bacteria and the 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 upregulation 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 authors showed that influenza-infected mice were more sensitive to bacterial components, such as staphylococcal enterotoxin B (SEB) and lipopolysaccharide (LPS) [10, 11]. Cytokines like IFN-γ, TNF-α and IL-6 are synergistically upregulated by SEB or LPS during influenza infections in mice. Influenza virus has also been described 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 alters the innate immune response to bacterial infections dramatically. So far, little is known about the mechanism by which influenza virus modulates the innate immune response to bacterial infections of the lungs.
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Host defense against pneumococcal pneumonia is coordinated by the action of pro-inflammatory and anti-inflammatory cytokines [12, 13]. In the current study, we present a mouse-model to study host defense against \textit{S. pneumoniae} after recovery from influenza infection. We show that the enhanced susceptibility of mice recovered from influenza infection for secondary pneumococcal pneumonia is accompanied by an exaggerated production of pro-inflammatory and anti-inflammatory cytokines. The finding of strikingly elevated pulmonary IL-10 concentrations in mice with post-influenza pneumonia compared with mice with primary \textit{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 \textit{S. pneumoniae} of mice recovered from influenza.

\textbf{Materials and methods}

\textit{Mice}

Pathogen-free 8 week-old female C57Bl/6 mice were obtained from Harlan-Sprague Dawley Inc. (Horst, Netherlands) and maintained at biosafety-level 2. All experiments were approved by the Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam.

\textit{Experimental infection protocol}

Influenza A/PR/8/34 (ATCC VR-95; Rockville, MD) was grown on LLC-MK2 cells (RIVM, Bilthoven, Netherlands). Virus was harvested by a freeze/thaw cycle, followed by centrifugation at 680g for 10 minutes. Supernatants were stored in aliquots at -80°C. Titration was performed in LLC-MK2 cells to calculate the median tissue culture infective dose (TCID$_{50}$) of the viral stock [15]. A non-infected cell culture was used for preparation of the control inoculum. None of the stocks were contaminated by other respiratory viruses, i.e. influenza B, human parainfluenza type 1, 2, 3, 4A and 4B, Sendai virus, RSV A and B, rhinovirus, enterovirus, corona virus and adenovirus, as determined by PCR or cell culture. Viral stock and control stock were diluted just before use in phosphate-buffered saline (PBS, pH 7.4). Mice were anesthetized by inhalation of isoflurane (Abbott Laboratories, Kent, UK) and intranasally inoculated with 10 TCID$_{50}$ influenza (1400 viral copies) or control inoculum in a final volume of 50 μl PBS. Pneumococcal pneumonia was induced 14 days after inoculation with influenza or control suspension according to previously described methods.
IL-10 impairs host defense during post-influenza pneumonia [14, 16, 17]. In brief, *S. pneumoniae* serotype 3 (ATCC 6303; Rockville, MD) was cultured for 16 hours at 37°C in 5% CO₂ in Todd Hewitt broth. This suspension was diluted 100 times in fresh medium and grown for 5 hours to midlogarithmic phase. Bacteria were harvested by centrifugation at 2750 g for 10 minutes at 4°C and washed twice with ice-cold saline. After the second wash, the bacteria were resuspended in saline and diluted to a concentration of 2 x 10⁵ colony forming units (CFU) per ml, which was verified by plating out 10-fold dilutions onto blood-agar plates. Mice were anesthetized by inhalation with isoflurane and were inoculated with 50 μl of the bacterial suspension (10⁴ CFU *S. pneumoniae*). Control mice received 10⁴ CFU *S. pneumoniae* as well. Bodyweight was measured daily during the course of the first 14 days of the study (influenza vs control) and 48 hours after *S. pneumoniae* infection. For experiments using neutralizing IL-10 monoclonal antibodies, mice were injected with JES5-2A5 (IgG1, 1 mg per mouse) or LO-DNP (control IgG1, 1 mg per mouse) intraperitoneally, 1 hour before inoculation of the bacteria. JES5-2A5 has been used in several previous studies to inhibit IL-10 in mice in vivo [14, 18-20].

**Determination of viral outgrowth**

Viral load was determined on day 4, 8, 12 and 14 after viral infection and 48 hours after pneumococcal infection (i.e. 16 days after viral infection) using real-time quantitative PCR as described [21]. Mice (8 mice per time-point) were anesthetized using 0.3 ml FFM (fentanyl citrate 0.079 mg/ml, fluanisone 2.5 mg/ml, midazolam 1.25 mg/ml in H₂O; of this mixture 7.0 ml/kg intraperitoneally) and 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). Hundred μl of lung homogenates were treated with 1 ml Trizol reagent to extract RNA. RNA was resuspended in 10 μl DEPC-treated water. cDNA synthesis was performed using 1 μl of the RNA-suspension and a random hexamer cDNA synthesis kit (Applera, Foster City, CA). 5 μl out of 25 μl cDNA-suspension was used for amplification in a quantitative real-time PCR reaction (ABI PRISM 7700 Sequence Detector System). 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'GGACTGCAGCTGAGACGCT-3' (forward); 5'-CATCCTGTTGTATATGAGGCCCAT-3' (reverse) and 5'-CTCAGTTATTTCTGCTGGTGCACTTGCC-3' (5'-FAM labelled probe).
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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 hours.

Histopathological analysis
Lungs for histological examination were harvested 48 hours after pneumococcal infection, fixed in 10% formalin and embedded in paraffin. Four μm sections were stained with hematoxylin and eosin and analyzed by a pathologist who was blinded from the groups.

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 (approximately 0.8 mL) was spun at 260g for 10 min at 4°C and the pellet was resuspended in 0.5 mL sterile PBS. Total cell numbers were counted using a Z2 Coulter Particle Count and Size Analyzer (Beckman-Coulter Inc., Miami, FL). Differential cell counts were done on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, UK).

Myeloperoxidase activity measurements
MPO activity was measured as described previously [22]. Bronchoalveolar lavage was 5-fold diluted in potassium-phosphate buffer (pH 6.0) supplemented with 0.5% hexadecyl-trimethyl-ammoniumbromide (HETAB) and 10 mM EDTA. MPO activity was determined by measuring the H₂O₂-dependent oxidation of 3,3'-5,5'-tetramethylbenzidine (TMB) at 37°C. The reaction was stopped by adding glacial acetic acid (0.2 M) to the reaction mixture. The amount of converted TMB was determined by measuring the OD at 655 nm. MPO-activity is expressed in U/mL BAL fluid. 1U is defined as the amount of MPO required to yield one OD₆₅₅-unit per minute. MPO activity measurements in BAL fluid represents the activation status of polymorphonuclear cells (PMNs) present in the lung.

Cytokine and chemokine measurements
Lung homogenates were lysed with an equal volume of lysisbuffer (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, 20 ng/ml Aprotinin, pH 7.4) and incubated for 30 minutes on ice, followed by
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centrifugation at 680 g for 10 minutes. Supernatants were stored at -80°C until further use. Cytokines and chemokines in total lung lysates were measured by enzyme-linked immunosorbent assay (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, UK); IFN-γ reagents were obtained from Pharmingen (San Diego, CA).

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

Results

Bodyweight loss after influenza infection and secondary bacterial pneumonia
In previous studies, influenza infection has been shown to induce bodyweight-loss [23]. Therefore, bodyweight 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 (figure 1A). Bodyweight reached its minimum on day 9 and returned to baseline level on day 13 after exposure. On day 14 both control mice and influenza-infected mice received $10^4$ CFU S. pneumoniae. Bodyweight decreased again after induction of pneumococcal pneumonia in mice recovered from influenza infection (figure 1A). Bodyweight of control mice did not change (p < 0.0001 vs mice with post-influenza pneumonia).

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 day 1 and day 4 after virus infection (figure 1B). Viral load in the lungs peaked between day 4 and day 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 virulence of the influenza virus, S. pneumoniae was not able to induce viral outgrowth on day 16 after viral infection, which
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confirms that the influenza virus had been completely cleared from the lungs on day 14 after viral infection.

![Graph showing bodyweight and viral load in the lungs after influenza infection in mice.](image)

**Figure 1:** Bodyweight and viral load in the lungs after influenza infection in mice. Bodyweight (A) of virus-infected mice (triangles) and control mice (squares) was measured daily after intranasal inoculation (8 mice per group). Viral load was determined on several time-points after influenza-infection (6-8 mice per time-point). Viral load is expressed as viral RNA copies per lung (B). Influenza virus was below detection level (B.D.) on day 14 and day 16 (i.e. 48 hours after bacterial infection) after viral infection. In control mice influenza could not be detected either (4 mice per time-point, data not shown).

*Increased lethality after pneumococcal infection in mice recovered from influenza*

Lethality was monitored at least twice a day after pneumococcal infection in mice (12 mice per 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 *S. pneumoniae* ($p < 0.0001$ vs control mice; figure 2).

![Graph showing survival after pneumococcal infection in mice](image)

**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 (triangles) versus control mice (squares). All mice (12 mice per group) received $10^4$ CFU *S. pneumoniae* on day 14 after viral infection and were monitored at least twice a day after pneumococcal infection.
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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 (figure 2); mortality in the remaining mice did not occur thereafter. Increased up to 83% after 6 days (figure 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 the bacterial outgrowth in the lungs. After 48 hours, just before the first deaths occurred, mice recovered from influenza infection showed a 1000-fold higher bacterial outgrowth compared to mice with primary pneumococcal infection (p = 0.0002 vs control mice, figure 3).

![Figure 3: Enhanced outgrowth of pneumococci during post-influenza pneumonia. Bacterial outgrowth in the lungs after pneumococcal infection in mice previously infected with influenza (triangles) and control mice (squares). All mice (8 mice per group) received 10^4 CFU S. pneumoniae on day 14 after viral infection and were sacrificed 48 hours later. Horizontal lines represent medians for each group. Note that one mouse in control group had cleared the bacteria at 48 hours after infection (not shown in graph).](image)

**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 about 20% of the lung (figure 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*, since 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.
Increased lung inflammation during post-influenza pneumonia. Histopathological analysis of the lungs of mice infected with influenza (C and D) or sham-infected control mice (A and B). All mice received $10^4$ CFU *S. pneumoniae* on day 14 after viral infection. Lungs were isolated 48 hours after pneumococcal infection and prepared for histopathological analysis. A 10-fold magnification (A and C) 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 6 mice per group.

**Leukocyte influx in BAL fluid**

To further assess innate immunity to *S. pneumoniae* after recovery from influenza infection, bronchoalveolar lavage was performed to identify leukocyte influx into the lungs. Mice recovered from influenza infection demonstrated a substantial influx of leukocytes 48 hours after infection with *S. pneumoniae*, whereas primary pneumococcal infection as well as primary influenza infection causes only moderate recruitment of leukocytes to the lungs (Table 1). The increase in leukocyte numbers in BAL fluid after secondary pneumococcal pneumonia was mainly due to the recruitment of granulocytes (Table 1). 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 1).
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Table 1: Leukocytes in bronchoalveolar lavage fluid.

<table>
<thead>
<tr>
<th>Cells (x 10^3)</th>
<th>PBS + saline</th>
<th>PBS + S. pneumoniae</th>
<th>Influenza + saline</th>
<th>Influenza + S. pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td>total cell count</td>
<td>116 ± 22</td>
<td>240 ± 55*</td>
<td>315 ± 43*</td>
<td>2231 ± 416**</td>
</tr>
<tr>
<td>granulocytes</td>
<td>2.1 ± 0.7</td>
<td>39 ± 23*</td>
<td>16 ± 6*</td>
<td>1734 ± 340**</td>
</tr>
<tr>
<td>macrophages</td>
<td>105 ± 21</td>
<td>199 ± 39*</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>

Mice (n = 6 per group) received PBS or Influenza A i.n. at day 0, followed by S. pneumoniae i.n. or saline at day 14. Bronchoalveolar lavage fluid was obtained 48h after i.n. administration of S. pneumoniae or saline. All data are mean ± SE. * p<.05 compared to PBS and saline-treated mice. † p<.05 compared to mice with primary pneumococcal infection. ‡ p<.05 compared to saline-treated mice recovered from influenza infection.

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 infection (figure 5). These data indicate that granulocyte activity is relatively reduced during secondary pneumococcal pneumonia.

Figure 5: Myeloperoxidase activity in bronchialveolar lavage 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 hours after pneumococcal infection. Mice (6 mice per group) received 10 TCID_{50} influenza or PBS on day 0 and 10^6 CFU S. pneumoniae or saline on day 14 after viral infection. Data are expressed in U/mL (mean ± SE). * p < 0.05 vs control mice and primary influenza infected mice.

Increased cytokine and chemokine levels in lung homogenates of influenza-recovered mice
Pneumococcal infection elicits a number of inflammatory responses within the lungs. These responses include the production of pro-inflammatory cytokines (TNF-α, IL-6, IFN-γ) and chemokines (KC). Pro-inflammatory cytokine and chemokine levels appeared to be 3 to 10-fold higher in lung homogenates of mice with post-influenza pneumococcal pneumonia compared to mice with primary pneumococcal infection (all p < 0.05; figure 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 day 14 and day 16 after infection (figure 6).

**Figure 6:** Lung cytokine and chemokine concentrations on day 14 and day 16 after primary viral infection and 48 hours after primary and post-influenza pneumococcal pneumonia (+ S. pneu). Cytokine and chemokine levels in total-lung homogenates were measured for mice previously infected with influenza virus (open bars) and control mice (filled bars). Mice (8 mice per group) received $10^4$ CFU *S. pneumoniae* on day 14 after viral infection. Pulmonary levels of TNF-α, IFN-γ, IL-6, KC and IL-10 are expressed in ng/g lung tissue (mean ± SE). *p < 0.05 vs control mice with primary pneumococcal pneumonia.

**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 post-influenza 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 monoclonal antibody against IL-10 one hour before pneumococcal inoculation. Forty-eight hours after pneumococcal infection, bacterial outgrowth was significantly lower in anti-IL-10-treated mice compared to IgG1-control-
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treated mice (p = 0.02; figure 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 pro-inflammatory mediators (figure 8). IL-6 and KC production appeared to be 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).

Anti-IL-10 improves survival during secondary pneumococcal infection

To assess whether the increased IL-10 levels adversely influenced survival during post-influenza pneumonia, lethality was monitored in mice (12 mice per group) treated with the anti-IL-10 antibody or the IgG1 control antibody (figure 9). Anti-IL-10 appeared to be protective against *S. pneumoniae* induced lethality in mice recovered from influenza infection as reflected by a prolonged survival and increased survival rates compared to control antibody-treated mice (p = 0.0005).
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 here show 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 time-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 since clinical data indicate that two weeks 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
IL-10 impairs host defense during post-influenza pneumonia secondary bacterial infections after recovery from airway infection from influenza virus. Indeed, mice with post-influenza pneumococcal pneumonia showed an increased lethality and an enhanced bacterial outgrowth in the lungs compared to mice with primary pneumococcal pneumonia.

Mice with *S. pneumoniae* pneumonia preceded by influenza infection displayed an enhanced inflammatory response when compared to mice with primary pneumococcal infection, as indicated by histopathology, cell influx in BAL fluid and by higher cytokine and chemokine levels in lung homogenates. Likely, this exaggerated inflammation was the consequence of the much higher bacterial load, providing a stronger pro-inflammatory stimulus in these post-influenza mice. In support of this notion are previous findings by Dallaire et al., demonstrating that pulmonary cytokine levels correlate closely with the extent of bacterial outgrowth during pneumococcal pneumonia [30]. 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, since 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. Likely, 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 (in spite of 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 post-influenza 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 the enhanced susceptibility to secondary bacterial infection. Interestingly, Steinhauser et al. used an approach identical to the approach 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 [35]. 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).

Pro-inflammatory mediators like 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 with 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, Dallaire et al. have shown that proinflammatory cytokine levels in lungs of mice with pneumococcal pneumonia closely correlate with the bacterial load [27]. 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 shown in Figures 2 and 9. Although only in the latter experiment mice were treated with control IgG, we consider it likely that this difference is the result of biovariability related to the fact that these experiments were done with an interval of several months using different “shipments” of
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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 would like to emphasize that all experiments were done 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, since 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 hyperresponsive 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, since 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 here demonstrate that IL-10 is an important mediator of an immunosuppressive state, predisposing to a fulminant 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 post-influenza pneumococcal pneumonia.

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References


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