Inflammatory response to viral airway infections and secondary bacterial complications
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Chapter 3

IL-12 plays a limited role in host defense against respiratory tract infection with Influenza A virus

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Abstract

T helper 1-driven immune responses have been implicated in protective immunity against viral infections. Interleukin (IL)-12 is a heterodimeric proinflammatory cytokine formed by a p35 and a p40 subunit that can induce differentiation of naïve T cells towards a T helper 1-response. To determine the role of IL-12 in respiratory tract infection with influenza, p35 gene deficient (p35−/−) and normal wild type mice were intranasally infected with influenza A virus. IL-12 p35−/− mice displayed a transiently enhanced rather than an impaired viral clearance, as indicated by a 10-fold reduction in viral loads on day 8 after infection. Although interferon-γ levels were significantly lower in the lungs of IL-12 p35−/− mice, their cellular immune responses were not altered, as reflected by similar T cell CD69 expression and influenza-specific T cell recruitment. These data strongly argue against a role for IL-12 in host defense against influenza A infection.
Introduction

Influenza A virus usually causes upper respiratory tract infection associated with fever, chills, cough, soar throat, headache, general malaise and sometimes nausea and myalgia. In addition, primary influenza infection may lead to pneumonia [1, 2]. Host defense against influenza virus is accomplished by an interplay between innate and adaptive immune responses. Adaptive immunity against respiratory tract infection with influenza virus is mediated through antigen presentation by macrophages and dendritic cells [3]. Virus-infected cells are able to produce several cytokines and chemokines that facilitate the adaptive immune response against influenza virus [4].

IL-12, is a heterodimeric cytokine, which consists of a p35 subunit and a p40 subunit (IL-12 p70). The expression of both subunits is independently regulated, whereby the p40 subunit is expressed in excess to the p35 subunit [6]. Therefore, most of the p40 subunit is present as monomer or homodimer [7, 8]. The p40 subunit can also dimerize with a p19 subunit to form IL-23 [9]. IL-12 has been shown to induce proliferation and differentiation of T cells towards interferon (IFN-γ) producing T helper (Th) 1 cells. IL-12 is also able to induce interferon (IFN)-γ production in natural killer (NK) cells. Like IL-12, IL-23 has been shown to induce proliferation of naive T cells and the production of IFN-γ [10]. IFN-γ is considered to be the most important antiviral mediator during influenza infection and induces several antiviral mechanisms, including inhibition of viral replication in virus infected cells [11].

Although T-cell mediated immune responses have been shown to be important in protective immunity against influenza virus [12], the role of IL-12 herein is less clear. Administration of recombinant IL-12 to influenza infected mice has been reported to enhance [13] or to delay [14] the clearance of influenza A. In many viral infections, including influenza, the endogenous production of IL-12 seems limited [15-19], and treatment with an anti-IL-12 antibody only modestly and transiently impaired the clearance of influenza virus from the lungs of mice [16]. Of note, in this sole study in which the role of endogenous IL-12 in host defense against influenza A was investigated an antibody raised against the p40 subunit was used to inhibit IL-12p70 activity [16]. In that investigation the extent of IL-12 neutralization was not evaluated. Moreover, since the p40 component of IL-12p70 is also part of IL-23, it is likely that this antibody also influenced the activity of endogenous IL-23 [20]. In the present
study we sought to determine the role of endogenous IL-12 in the host response to influenza A virus by making use of p35 gene deficient mice, animals in which IL-12 is the only cytokine that cannot be produced.

Materials and methods

Animals
All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center of the University of Amsterdam. IL-12 p35−/− mice on a C57Bl/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in the animal facility of the Academic Medical Center. Normal wildtype C57BL/6 mice were obtained from Harlan Sprague-Dawley (Horst, The Netherlands). Sex- and age-matched (8-week old) mice were used in all experiments.

Experimental virus infection
Influenza infection was induced as described previously [21]. In brief, influenza A/PR/8/34 (ATCC no. 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 (TCID50) of the viral stock [22]. 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, RSV A and B, rhinovirus, enterovirus, corona virus and adenovirus, as determined by PCR or cell culture. Viral stock was 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 TCID50 influenza (1400 viral copies) or control in a final volume of 50 μl PBS.

Determination of viral outgrowth
Viral load was determined on day 4, 8, and 12 after viral infection using real-time quantitative PCR as described [21, 23]. On day 4, 8 and 12, mice (6-8 mice per group) 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). 100 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). Five 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 a sample was calculated using a standard curve of particle counted influenza virus included in every assay run. The following primers were used: 5'-GGACTGCAGCGTAGACGCTT-3' (forward); 5'- CATCCTGTTGTATATGAGGCCCAT-3' (reverse), 5'-CTCAGTTATTCTGCTGGTGCACTTGCC-3' (5'-FAM labelled probe).

Cytokine assays
Lung homogenates were lysed with an equal volume of lysisbuffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 2% (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 centrifugation at 680 g for 10 minutes. Supernatants were stored at -80°C until further use. Cytokine levels in total-lung-lysates were measured by enzyme-linked immunosorbent assays according to the manufacturers’ instructions (interleukin(IL)-6 from R&D Systems, Minneapolis, MN; interferon (IFN)-γ, IL-12 p40 and IL-12 p70 from Pharmingen, San Diego, CA). In addition, IL-12 p70 were measured by Cytometric Bead Assay (CBA) (BD Biosciences, San Jose, CA). The detection limits of the IL-12 p70 assays were 32 pg/ml (ELISA) and 20 pg/ml (CBA) resulting in detection limits of 320 pg/g and 200 pg/g lung tissue respectively.

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 (BALF, 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).
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Histological examination
The left lung was harvested on day 4 or day 8 after viral infection, fixed in 10% formalin and embedded in paraffin. 4 μm sections were stained with hematoxylin and eosin (H/E) and were analyzed by a pathologist.

Flow cytometry
Pulmonary cell suspensions were obtained by grinding the lung tissue through nylon sieves. Cells ($10^6$) were collected in 96-well U-bottomed plates in FACS staining buffer (PBS with 0.5% (w/v) bovine serum albumin). All samples were stained for 15 min at 4°C with anti-CD4-PE (clone RM4-5, PharMingen, San Diego, CA), anti-CD8-APC (clone 53-6.7, PharMingen, San Diego, CA) and anti-CD69-FITC monoclonal antibodies (clone H1.2F3, PharMingen, San Diego, CA). Alternatively, cells were stained with with CD8-APC, and a PE-labeled tetrameric complex of H-2Db molecules with the PR/8 influenza virus nucleoprotein epitope ASNENMETM (NP366-374; tetramers were kindly provided by T. Schumacher, Netherlands Cancer Institute, Amsterdam, The Netherlands). Splenic cell suspensions were obtained on day 21 after viral infection and stained with CD8-FITC (clone 53-6.7, PharMingen, San Diego, CA), CD44-PE (clone IM7, PharMingen, San Diego, CA) and APC-labeled PR-tetramers. FACS analysis was done on a FACSCalibur with Cell Quest software (BD Biosciences, San Jose, CA).

Statistical analysis
Data are expressed as mean ± SE. Comparison between the groups was conducted by using the Mann-Whitney U test. P < 0.05 was considered to represent a statistically significant difference.

Results

Expression of IL-12 p40 and IL-12 p70 during influenza virus infection
Pulmonary levels of IL-12 p40 and IL-12 p70 were measured in total lung-homogenates at several time-points to determine the expression of these mediators after influenza virus infection. Pulmonary levels of IL-12 p40 were elevated on days 8 and 12 after viral infection and had returned to control levels on day 21 postinfection (Figure 1). Using two different assays (ELISA and CBA), IL-12 p70 could not be detected in total lung lysates of influenza infected mice, indicating that IL-12 p70 concentrations did not exceed 200 pg/g lung tissue.
Role of IL-12 during influenza infection

![Graph showing IL-12 p40 expression over days 4, 8, 12, and 21 after intranasal inoculation.](image)

**Figure 1:** IL-12 p40 expression after influenza virus infection. Pulmonary IL-12 p40 levels in total-lung homogenates were measured in influenza-virus-infected mice (open bars) and control mice (filled bars) on day 4, 8, 12 and 21 after intranasal inoculation (6-8 mice per group). Data are expressed in pg/g lung tissue (mean ± SE). * p < 0.05 vs control mice.

**Viral load**

To investigate the role of IL-12 on viral titers after influenza virus infection, we inoculated IL-12 p35+/- and wildtype mice and measured the viral load in the lungs over time. Viral load was 10-fold lower in IL-12 p35+/- mice than in wildtype mice on day 8 after infection (p = 0.05). Similar viral titers in wildtype mice and IL-12 p35+/- mice were found on days 4 and 12 after infection. Hence, these data indicate that IL-12 transiently impairs clearance of influenza A in vivo (Figure 2).

![Graph showing log viral load over days 4, 8, and 12 after inoculation.](image)

**Figure 2:** Viral load in the lungs of IL-12 p35+/- mice. Viral load was determined on day 4, 8 and 12 after influenza virus infection (7 mice per time-point) in IL-12 p35+/- mice (open bars) and wildtype mice (filled bars). Viral load is expressed as viral RNA copies per lung. In control mice influenza could not be detected either (4 mice per time-point, data not shown).

**Pulmonary cytokine levels**

To obtain insight in the role of IL-12 in cytokine release during influenza infection, IL-6, IFN-γ and IL-12 p40 concentrations were measured in total lung lysates of IL-12 p35+/- and wildtype mice, 4, 8 and 12 days after infection. IL-6 levels were similar in both mouse strains at all time-points (Figure 3). IFN-γ levels were significantly lower in IL-12 p35+/- mice than in wildtype mice on day 8 (p = 0.0058) and day 12 (p = 0.0064) after infection (Figure 3B). IL-12 p40 levels were significantly higher in IL-12 p35+/- mice than in wildtype mice on day 8 (p = 0.02) and day 12 (p=0.002) after infection (Figure 3C).
Figure 3: Cytokine expression in the lungs of IL-12 p35−/− mice. IL-6 (A), IFN-γ (B) and IL-12 p40 (C) levels in total-lung homogenates were measured in IL-12 p35−/− mice (open bars) and wildtype mice (filled bars) on day 4, 8 and 12 after influenza virus infection (7 mice per group). Data are expressed in pg/g lung tissue (mean ± SE). * p < 0.05 vs wildtype mice.

Cellular immune response to influenza virus infection

To investigate the role of IL-12 in the cellular immune response to influenza A we determined expression of CD69, an early activation-marker, on CD4+ and CD8+ T cells isolated from the lungs on day 8 after viral infection. Cell-surface expression of CD69 was similar on CD4+ and CD8+ T cells of IL-12 p35−/− mice and wildtype mice (Figure 4A and 4B), indicating that activation of T cells is not affected by IL-12 deficiency. We also investigated whether IL-12 regulates the recruitment of influenza-specific T cells into the lungs by using tetramer-staining. No differences were observed between IL-12 p35−/− mice and wildtype mice, indicating that IL-12 does not play a role in the cellular immune response to influenza virus (Figure 4C).

Figure 4: CD69 expression and influenza specific T-cell recruitment in IL-12 p35−/− mice. CD69 expression on lung-derived CD4+ (A) and CD8+ (B) T cells in IL-12 p35−/− mice (open bars) and wildtype mice (filled bars) on day 8 after influenza virus infection (6 mice per group). Influenza-specific T cell recruitment was determined by tetramer-staining (C) in IL-12 p35−/− mice (open bars) and wildtype mice (filled bars) on day 8 after viral infection (6 mice per group). All data are expressed as percentage of total number of CD4+ or CD8+ T cells in the lungs. Total leukocyte numbers in the lungs on day 8 after viral infection were similar in IL-12 p35−/− mice and wildtype mice (data not shown).
To exclude that IL-12 plays a regulatory role in memory T cell formation, we determined the total number of influenza-specific T cells in the spleen on day 21 after influenza infection. Again, no difference was observed in IL-12 p35−/− mice and wildtype mice (Figure 5A). To confirm a memory phenotype of these influenza-specific T cells in the spleen, we determined CD44 expression on these T cells. In both wildtype and IL-12 p35−/− mice, more than 95% of the influenza-specific T cells expressed CD44 on the cell-surface (Figure 5B). These data indicate that IL-12 does not regulate T cell activation or T cell memory formation during influenza virus infection in vivo.

Table 1: Cells in BAL fluid

<table>
<thead>
<tr>
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<th>t = 4</th>
<th>t = 8</th>
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<tbody>
<tr>
<td></td>
<td>wildtype</td>
<td>IL-12 p35−/−</td>
</tr>
<tr>
<td>Total cell count</td>
<td>348 ± 51</td>
<td>478 ± 67</td>
</tr>
<tr>
<td>Macrophages</td>
<td>180 ± 27</td>
<td>218 ± 38</td>
</tr>
<tr>
<td>Polymorphonuclear cells</td>
<td>167 ± 28</td>
<td>258 ± 36</td>
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<tr>
<td>Lymphocytes</td>
<td>ND</td>
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Mice (n = 6 per group) received Influenza A i.n. on day 0. Bronchoalveolar lavage fluid was obtained on day 4 and day 8 after viral infection. All data are mean ± SE. *p<.05 compared to wildtype mice. ND, not detectable, i.e. < 0.5% of total cell counts.
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Cells in BALF

To evaluate the role of IL-12 in cell recruitment to the lungs during influenza infection, BALF was obtained on day 4 and day 8 after infection. On day 4, total cell counts and differentials were similar in IL-12 p35⁻/⁻ mice and wildtype mice (Table 1). On day 8, BALF of IL-12 p35⁻/⁻ mice contained less leukocytes than BALF of wildtype mice (p < 0.05, table 1). The relative decreased leukocyte numbers in IL-12 p35⁻/⁻ mice concerned both macrophages (p = 0.10) and neutrophils (p = 0.06).

Figure 6: Histopathology of the lungs of IL-12 p35⁻/⁻ mice. Increased lung inflammation during influenza pneumonia. Histopathological analysis of the lungs of IL-12 p35⁻/⁻ mice (B and D) and wildtype mice (A and C). Lungs were isolated on day 4 (A and B) and day 8 (C and D) after viral infection and prepared for histopathological analysis. Original magnification: 100x. Slides are representative for 6 mice per group.

Histopathology

To identify differences in inflammation between wildtype mice and IL-12 p35⁻/⁻ mice, histopathological analysis of H/E stained lung-slides from days 4 and 8 after infection was performed. Influenza virus infection was associated with mild interstitial inflammation, endothelialitis and bronchitis on day 4 after infection (Figure 6). The interstitial inflammation,
endothelialitis and bronchitis were more pronounced on day 8 after viral infection and accompanied by pleuritis, focal necrosis together with apoptotic granulocytes (Figure 6). Differences between wildtype mice and IL-12 p35\textsuperscript{−/−} mice were not observed.

**Discussion**

Cellular immunity plays an important role in viral clearance. Host defense against viral infections are predominantly mediated by Th1 immune responses [4, 12]. Antigen-presentation by dendritic cells leads to the activation of naive T cells to proliferate and differentiate into T helper cells [24]. Cytokines play a decisive role in the formation of either Th1 or Th2 responses. IL-12 has been implicated in the formation of Th1 lymphocytes and is therefore may play an important role in host defense against viral infections [4, 10]. In the present study we investigated the role of IL-12 during influenza virus infection by making use of IL-12 p35\textsuperscript{−/−} mice, in which the production of IL-12 is selectively eliminated. Our data demonstrate that IL-12 does not contribute to innate and adaptive immunity against influenza A. If anything, IL-12 deficiency was associated with lower rather than higher viral loads at 8 days after infection.

Although the lung levels of IL-12p70 did not exceed 200 pg per gram lung tissue, IL-12 p35\textsuperscript{−/−} mice temporarily had lower viral loads and displayed significantly lower IFN-γ concentrations than wild type mice after influenza A infection. Previous studies support our finding of marginal IL-12 production during influenza. Indeed, influenza-infected macrophages did not produce significant amounts of IL-12 [15, 19] and influenza infection in mice resulted in IL-12 concentrations in lungs that were barely higher than in lungs of control mice [16, 18]. Our data expand the results of a previous study in which administration with an anti-p40 antibody (in theory inhibiting both IL-12 and IL-23 activity) modestly reduced local IFN-γ release during airway infection with influenza A [16]. The fact that very low concentrations of IL-12 p70 can contribute to IFN-γ production has also been demonstrated in other experimental conditions such as whole blood cultures stimulated with endotoxin or bacteria [25, 26].

In light of the important role for IL-12 in IFN-γ production, a logic assumption would have been that IL-12 p35\textsuperscript{−/−} mice display a reduced resistance against influenza A. However, the evidence for this assumption is not readily available in the literature. To the best of our
knowledge only one study directly evaluated the role of endogenous IL-12 in host defense against influenza. In this investigation, Monteiro et al. [16] used an antibody directed against the p40 subunit to inhibit IL-12 activity in influenza infected mice; the authors did not report to what extent IL-12 activity was inhibited by the antibody. In addition, it is quite likely that the antibody also interfered with the activity of IL-23, a novel cytokine that shares the p40 subunit with IL-12p70 and further consists of a p19 subunit [27]; this cytokine had yet to be discovered at the time Monteiro et al. published their study. Importantly, IL-23 can also direct Th1 responses and has been claimed to be more important in cellular immunity than IL-12 [28]. Anti-p40 treatment was associated with modestly elevated influenza titers in lungs at three days after infection when compared with mice treated with rat immunoglobulin, whereas viral loads were similar in both experimental groups at day 5 and day 7 after infection [16].

Our current finding that IL-12 p35\(^{-}\) mice had lower rather than higher influenza titers at day 8 after infection strongly argues against a role for IL-12 in the protective immune response against this common virus. This notion is supported by an earlier study showing that administration of recombinant IL-12 to influenza infected mice delayed the clearance of influenza A [14], although this was not confirmed in another investigation [13]. Nonetheless, our data together with the results obtained by Monteiro et al. [16] indicate that further research is required to identify the role of IL-23 during influenza A infection.

Wildtype mice and IL-12 p35\(^{-}\) mice displayed a similar cellular immune response against influenza A infection, as reflected by similar expression of CD69 and influenza-specific T cell receptors on the surface of recruited lymphocytes when compared with wild type mice. Yet cell counts in BALF indicate that the recruitment of neutrophils and to a lesser extent macrophages was reduced in IL-12 p35\(^{-}\) mice. These reduced cell numbers on day 8 after infection could be the consequence of IL-12 deficiency, since IL-12 has been implicated in recruitment of macrophages [29-32] and neutrophils [33], via induction of other inflammatory mediators. Alternatively, diminished cell numbers in IL-12 p35\(^{-}\) mice could have been caused by reduced viral load, providing a less potent proinflammatory stimulus. Homodimeric p40 can function as a chemotactic factor for macrophages [34, 35], which has been shown to play an important role during respiratory tract infection with Sendai virus in mice [36]. Here we found elevated p40 concentrations in lungs of IL-12 p35\(^{-}\) mice on day 8 and day 12 after infection. Although we did not measure the amount of monomeric and homodimeric p40, previous studies indicate that excess amounts of p40 consists for up to one-third of homodimeric p40-p40. Likely, the excess amount of p40 produced in IL-12 p35\(^{-}\) mice is, in
part, present in a homodimeric form. Additional research is warranted to determine the contribution of homodimeric p40 in host defense against influenza A.

In conclusion, we here demonstrate that the host response to influenza A is only modestly altered in IL-12 p35−/− mice, animals that are totally and selectively deficient for IL-12. This finding contrasts with numerous reports on strongly enhanced susceptibility of IL-12 deficient mice to infections by a variety of other pathogens, in particular mycobacteria and parasites (37). Our data strongly suggest that endogenous IL-12 is not important for protective immunity against respiratory tract infection with influenza A.

Acknowledgment

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References

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Role of IL-12 during influenza infection


