Inflammatory response to viral airway infections and secondary bacterial complications
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Sendai virus infection amplifies inflammatory response to LPS challenge
Chapter 7

Abstract

Viral upper respiratory tract infections usually induce a mild inflammatory response leading to common cold symptoms such as cough and sneezing. Although viral infections may induce pneumonia, bacterial complications are a much more common cause of pneumonia. The severe outcome of these superinfections is at least in part due to an exaggerated inflammatory response. In the present study mice infected for 3 days with replicating Sendai virus show an enhanced inflammatory response to LPS as reflected by increased pulmonary IL-6 levels and increased neutrophil numbers in bronchoalveolar lavage fluid. TNF-α, KC and IP-10 levels were not further increased after LPS challenge, indicating that these mediators are regulated in a different way than IL-6. Taken together, lower respiratory tract infection with Sendai virus in normal immunocompetent mice show an exaggerated inflammatory response to LPS. This exaggerated inflammatory response may contribute to the severe outcome of secondary infections with gram-negative bacteria.
**Introduction**

Viral upper respiratory tract infections usually cause typical 'common-cold' symptoms, such as sneezing and cough, sometimes accompanied by headache, nausea, myalgia and mild fever [1-3]. These symptoms correlate with an inflammatory response in the upper airways, which is associated with the recruitment and activation of inflammatory cells and the production of pro-inflammatory mediators like IL-6 and IL-8 [4-7]. Viral airway infections in healthy humans may lead to pneumonia, but superinfections with bacteria such as *Streptococcus pneumoniae*, *Staphylococcus aureus* or *Haemophilus influenzae* are a much more common cause of pneumonia [12-14]. Secondary bacterial challenges induce an exaggerated inflammatory response and may lead to premature death [15-17]. This enhanced inflammatory response is partly explained by increased colonization of bacteria to the basal membrane or the airway epithelial layer [18]. The enhanced inflammatory response may also be due to hyperresponsiveness to bacterial components such as LPS and staphylococcal enterotoxin B (SEB), suggesting that a viral infection enhances the inflammatory response to secondary stimuli such as LPS and SEB [19, 20]. In patients with chronically inflamed tissue, such as seen in asthma and chronic obstructive pulmonary disease (COPD), viral infections may synergize with the pre-existing inflammatory responses, which may lead to exacerbations of the disease [8-10].

Taken together, viral airway infections can amplify inflammatory responses to existing (chronic inflammation) and to occurring (secondary bacterial infection) inflammatory challenges. The mechanisms that lead to these enhanced inflammatory responses are still not known. Recently, our laboratory showed that cultured airway epithelial-like cells (NCI-H292) become highly responsive to TNF-α and LPS after infection with human parainfluenza virus (HPIV) type 4 [21, 22]. The exaggerated production of IL-6 and IL-8 was dependent on both transcriptional and posttranscriptional regulation. The exaggerated production of these pro-inflammatory mediators were not observed in NCI-H292 cells infected with UV-irradiated, i.e. replication deficient, HPIV.

In the present study, we investigated whether these exaggerated pro-inflammatory mediator responses occurred *in vivo*. We used a mouse-model for Sendai virus (murine parainfluenza type 1) infection to study responses to LPS as a secondary stimulus. UV-irradiated Sendai-
virus as well as mock infection as controls to identify the role of viral replication in virus-induced responsiveness to LPS.

Materials and methods

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.

Experimental infection protocol
Sendai virus (ATCC VR-105; Rockville, MD) was grown on LLC-MK2 cells (RIVM, Bilthoven, Netherlands). Virus was harvested by a freeze/thaw cycle, followed by centrifugation at 680 g 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 [23]. To obtain replication deficient Sendai-virus, 1 ml of the viral stock was inactivated in a petri-dish (5 cm diameter) at a distance of 1 cm under a 254 nm UV-lamp (Desaga MinUVIS, Heidelberg, Germany). A non-infected cell culture was used for preparation of the control inoculum (mock infection). None of the stocks were contaminated by other respiratory viruses, i.e. influenza A and 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 and control inoculum 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^{6} TCID_{50} Sendai virus, UV-inactivated Sendai virus or control inoculum in a final volume of 50 µl PBS. For LPS-hyperresponsiveness experiments 1 µg LPS (E. coli strain O55:B5, Fluka, Zwijndrecht, Netherlands) was administered intranasally under isoflurane anesthesia on day 3 after viral infection.

Determination of viral outgrowth
Viral load was determined on day 1, 3, 5 and 8 after viral infection using real-time quantitative PCR as described [24]. 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 H_{2}O;
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 Sendai virus (virus particles were counted by electron microscopy), included in every assay. The following primers were used: 5'-AGTACGATCGCAGTCCACCAT-3' (forward); 5'-CGACAGGGCATCTCCAGAA-3' (reverse), 5'-AGGGGAATTGCCCCACTTGAGCCAC-3' (5'-FAM labelled probe).

*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 cytopsin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, UK).

*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 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 and IP-10 measurements were obtained from R&D systems (Abingdon, UK).
Statistical analysis
All data are expressed as mean ± SE, unless stated otherwise. Differences between groups were analysed by Mann-Whitney U test. p < 0.05 was considered to represent a statistically significant difference.

Results

Viral load in lungs over time
Viral load was measured using real-time quantitative PCR. Viral replication was observed between day 1 and day 5 after viral infection after which viral load decreased (Figure 1). In mice inoculated with UV-inactivated Sendai virus a 100-fold lower number of viral copies was detected on day 1 after infection. Replication was still observed between day 1 and 5 after infection, indicating that UV-inactivation of Sendai virus occurred with approximately 99% efficiency. Sendai virus was not detected in the lungs of mice that received a mock infection.

Cell influx in BAL fluid
Sendai virus infection resulted in an influx of inflammatory cells into the lungs (Figure 2). Neutrophil numbers were increased in BAL-fluid on day 3 after viral infection, were maximal on day 5 after viral infection and started to decline thereafter. Lymphocyte numbers and non-lymphocyte mononuclear cells were significantly increased on day 5 and day 8 after viral infection. These cellular infiltrates were not present in mice inoculated with either control inoculum or UV-inactivated virus. These data indicate that although the UV-inactivated virus still has a limited capacity to replicate, the amount of replicating virus is not sufficient to induce influx of inflammatory cells into the lungs.
Sendai virus infection amplifies responses to LPS

**Figure 2**: Cell differentials in BALF after Sendai virus infection.
Bronchoalveolar lavage was performed on day 1, 3, 5 and 8 after infection with Sendai virus (filled bars) or UV-inactivated virus (open bars). Total cell counts, neutrophils, macrophages and lymphocytes are expressed as mean ± SE (x 10^3, 5-8 mice per group).

**Cytokine and chemokine production in the lungs**
Cytokine and chemokine levels in total-lung-homogenates were measured on day 1, 3, 5 and 8 after infection. Both IL-6 and IP-10 levels were increased in total-lung-homogenates on day 3, 5 and 8 after Sendai virus infection (Figure 3A and B). Production of KC was only elevated on day 3 after infection (Figure 3D). Sendai virus infection resulted in slightly increased TNF-α levels on day 3, 5 and 8 (Figure 3C). None of these mediators were elevated in total-lung-homogenates of mice exposed to UV-inactivated virus.

**Exaggerated IL-6 responses upon stimulation with LPS on day 3 after viral infection**
LPS was administered on day 3 after Sendai virus infection. Cytokine and chemokine levels were measured in total-lung-homogenates 2 hours after LPS administration (Figure 4). LPS challenge resulted in IL-6 production in both Sendai-virus infected mice and mice treated with UV-inactivated virus. IL-6 was synergistically produced after LPS administration in Sendai virus-infected mice (Figure 4A). Intranasal administration of LPS did not further
increase IP-10 production in Sendai-virus infected mice, whereas mice treated with UV-inactivated virus showed an increase in IP-10 levels in the lungs (Figure 4B).

![Graphs of cytokine levels](image)

**Figure 3**: Pulmonary cytokine and chemokine levels in Sendai virus-infected mice. IL-6, IP-10, TNF-α and KC levels in the lungs were measured on day 1, 3, 5 and 8 after infection with Sendai virus (filled bars) or UV-inactivated virus (open bars). IL-6 and IP-10 levels are expressed in ng/g lung tissue (mean ± SE, 8 mice per group).

LPS challenge resulted in a 50-fold increase in TNF-α levels in the lungs. No difference was observed between Sendai virus infected mice and mice treated with UV-inactivated virus (Figure 4C). KC levels in the lungs were increased after LPS challenge in both Sendai virus-infected mice and mice treated with UV-inactivated virus. Sendai virus did not enhance KC production in the lungs in response to LPS (Figure 4D).

*Cell influx in BALF after LPS challenge in Sendai infected mice*

LPS was administered on day 3 after Sendai virus infection. Sendai virus infection resulted in an increase in neutrophils, whereas macrophages were not elevated on day 3 after Sendai infection (Figure 2). Similar results were observed 2 hours after NaCl treatment in Sendai virus-infected mice. LPS challenge resulted in a small increase in the number of macrophages for both groups (Figure 5). Neutrophil recruitment was induced by intranasal challenge with LPS in control mice (not shown) and mice treated with UV-inactivated virus.
Figure 4: Cytokine and chemokine production after LPS challenge in Sendai-infected mice. Mice were intranasally challenged with NaCl or LPS on day 3 after infection with Sendai virus (filled bars) or UV-inactivated virus (open bars). IL-6, IP-10, TNF-α and KC levels were measured 2 hours after LPS or NaCl challenge. Cytokine and chemokine levels are expressed in ng/g lung tissue (mean ± SE, 6 mice per group). * p < 0.05 vs mice treated with UV-inactivated virus. ** p < 0.05 vs NaCl-treated mice with similar viral exposure. *** p < 0.05 vs mice treated with UV-inactivated virus and vs NaCl-treated mice with similar viral exposure.

LPS challenge in Sendai-virus infected mice further, and significantly, increased neutrophil numbers in BALF. Macrophage numbers were similar in NaCl- and LPS-treated mice for both groups (Figure 5).

Figure 5: Cell influx in BALF after LPS challenge in Sendai virus infected mice. Mice were challenged with LPS on day 3 after infection with Sendai virus (filled bars) or Uv inactivated virus (open bars). Macrophages and neutrophils are expressed as mean ± SE (x 10⁵, 5-6 mice per group). * p < 0.05 vs mice treated with UV inactivated virus. ** p < 0.05 vs NaCl-treated mice with similar viral exposure. *** p < 0.05 vs mice treated with UV inactivated virus and vs NaCl-treated mice with similar viral exposure.

Discussion

Parainfluenza virus infection is associated with typical common-cold symptoms such as cough, sneezing, soar throat and mild fever [1-3], which is associated with the production of inflammatory mediators. Viral infections predispose the host to a more pronounced pro-inflammatory mediator response to inflammatory stimuli such as LPS [20, 25]. In the present
study we show that IL-6 is synergistically upregulated after intranasal LPS treatment in Sendai-virus infected mice but not in control mice or mice exposed to UV-inactivated virus. This enhanced response to LPS was accompanied by increased numbers of neutrophils in BAL fluid.

IL-6, IP-10 and KC were produced in the lungs after Sendai virus infection, whereas control mice and mice inoculated with UV-inactivated virus displayed low levels of these cytokines in the lungs. TNF-α levels were modestly increased in Sendai-infected mice. The production of these mediators correlates with viral load in the lungs. Replication of the virus was observed in mice inoculated with live virus as well as UV inactivated virus. However, viral loads were 100-fold higher in mice infected with live virus. Together, these data indicate that production of inflammatory mediators depends on either viral load and/or the extent of viral replication.

Sendai virus-infected mice displayed an exaggerated IL-6 production upon LPS stimulation compared to control mice. In contrast, IP-10, KC and TNF-α production upon stimulation with LPS were not enhanced in Sendai virus infected mice. This exaggerated IL-6 response can be explained in several mutually non-exclusive ways. Recently, our laboratory showed that HPIV type 4 infection in NCI-H292 airway epithelial-like cells resulted in an exaggerated IL-6 and IL-8 response upon stimulation with TNF-α and LPS [21, 22]. This enhanced IL-6 and IL-8 production was dependent on both transcriptional and posttranscriptional regulation. Stabilization of IL-6 and IL-8 mRNA by HPIV infection resulted in excessive IL-6 and IL-8 protein synthesis after secondary stimulation. Similarly, synergism between IFN-γ on IL-6 and IL-8 responses by airway epithelial cells depends on reduced mRNA degradation [26]. This mechanism of increased mRNA stability may account for the enhanced IL-6 production during Sendai virus infection in vivo. Of note, Sendai virus has been described as an epitheliotropic virus. Although production of IP-10, KC and TNF-α is not further enhanced by LPS stimulation, stabilisation of these messengers cannot be excluded. In the present study we show that Sendai virus appears to be a potent stimulus of IP-10 production. However, tools to prove stabilization of mRNA in vivo are currently not available. The ability to determine mRNA degradation rates is mainly hampered by increased mRNA levels from of inflammatory cells recruited to the pulmonary compartment.
Inflammatory cells recruited to the lungs may also contribute to the enhanced IL-6 production. Neutrophilic influx was observed on day 3, 5 and 8 after infection. Strikingly, synergistic IL-6 responses were observed on day 3 after viral infection. Neutrophil recruitment was further increased after LPS stimulation on day 3 after viral infection. Likely, neutrophils were already present in the submucosa of the airways and easily recruited to the alveolar space after LPS challenge. Still, LPS challenge in mice treated with UV-inactivated virus resulted in neutrophil recruitment as well. This enhanced neutrophil influx did not correlate with the production of either KC or IP-10, an ELR+ and an ELR- CXC chemokine respectively. Especially ELR+ CXC chemokines have been implicated in neutrophil recruitment [27]. Other chemokines, not included in this study, may have played a critical role in neutrophil recruitment. In addition, the expression of adhesion molecules as well as epithelial permeability may have contributed to increased neutrophil numbers after LPS stimulation in Sendai-virus infected mice.

During viral infections, resident cells, such as epithelial cells and alveolar macrophages, may show enhanced responses to LPS. The signalling pathways required for IL-6 production after Sendai virus infection or LPS stimulation are likely different. Cross-talk between these pathways may exist, resulting in enhanced IL-6 production after LPS stimulation in Sendai virus-infected mice. LPS activates Toll-like receptor (TLR) 4 and requires either MyD88 or TRIF for downstream signalling [28, 29]. Recently, our laboratory showed that the early host response to Sendai virus does not involve TLR4 [24]. Paramyxoviridae like Sendai virus produce double-stranded RNA during viral replication [30]. Since double-stranded RNA has been shown to activate TLR3 [31], which requires TRIF for downstream signalling [32], it could be suggested that Sendai virus induces cytokine production in a TLR3 and TRIF-dependent fashion. Remarkably, IP-10 production was not enhanced after LPS stimulation on day 3 after viral infection. LPS and Sendai virus may require the same signalling pathway to induce IP-10. LPS did not further increase IP-10 production, neither synergistically nor additively, which is explained by TRIF-dependent induction of IP-10 by LPS. However, it cannot be excluded that the high IP-10 levels present in the lung on day 3 after viral infection masked the LPS-induced IP-10 production. LPS challenge resulted in TNF-α and KC production in the lungs, whereas Sendai virus alone induced relatively modest amounts of these mediators. No synergistic effects were observed after LPS treatment in Sendai infected mice. Further research is warranted to address the role of TRIF in LPS and Sendai virus-induced cytokine production.
In conclusion, Sendai virus infection in mice modifies the inflammatory response to secondary challenges with LPS. These enhanced inflammatory responses may, at least in part, explain the increased susceptibility to secondary infections with gram-negative bacteria. Similarly, our group recently showed that mice previously infected with influenza virus became highly susceptible to *Streptococcus pneumoniae*, a gram-positive bacterium [17]. It should be noted that *S. pneumoniae* was inoculated after complete viral clearance, i.e. 14 days after viral infection. Additional mechanisms may play a critical role in post-influenza pneumonia as well. In both cases, secondary immune challenge results in excessive production of inflammatory mediators. Whether the underlying mechanism involves inhibition of labile mRNA degradation or an alternative pathway remains to be elucidated.

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**References**

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