Inflammatory response to viral airway infections and secondary bacterial complications

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Chapter 10

Summary and discussion
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Summary

Viral infections of the upper respiratory tract usually induce common-cold symptoms, such as dry cough, soar throat and sneezing. Although primary infection with respiratory viruses may lead to pneumonia, complicating bacterial infections are a much more common cause of pneumonia. These bacterial complications are associated with excessive bacterial outgrowth, exaggerated inflammatory responses and premature death. Bacterial complications during or shortly after viral airway infections account for 40,000 death cases each year worldwide. Knowledge about the underlying mechanism of enhanced responses to secondary bacterial infections is limited, but essential to intervene adequately. The studies described in this thesis focused on innate immune responses to primary viral airway infections and secondary bacterial complications.

The inflammatory response to pathogens is initiated by recognition of pathogen-associated molecular patterns (PAMPs). Recognition of these PAMPs by Toll-like receptors (TLRs) leads to the production of pro-inflammatory cytokines and chemokines. Several ligands have been identified for TLR4, including the fusion protein of respiratory syncytial virus (RSV). The role of TLR4 during infection with Sendai virus, which expresses a similar fusion protein, was studied in chapter 2. TLR4 mutant mice responded in a similar fashion as wildtype mice as reflected by cytokine production, cellular influx and viral load. Hence, host defense against Sendai virus does not require TLR4, and is possibly mediated by other TLRs. The activation of TLR4 by viruses may be restricted to RSV.

Production of cytokines and chemokines coordinate the innate and adaptive immune system in order to eliminate the virus. Interleukin (IL)-12 is a heterodimeric cytokine and has been shown to induce macrophage and neutrophil activation as well as proliferation and differentiation of T cells towards T helper 1 phenotype. Studies into the role of IL-12 during influenza virus infection in mice are described in chapter 3. Deletion of the IL-12 p35 gene in mice resulted in enhanced viral clearance after influenza infection, despite the fact that bioactive IL-12 could not be detected during influenza infection. Our data indicate that low constitutive expression of IL-12 inhibits rather than accelerates viral clearance in vivo. IL-12 seems to play a limited role in host defense against influenza virus infection. Although IL-12 and IL-18 are not structurally related, their biological properties are quite similar. In contrast to IL-12, IL-18 was upregulated in the lungs during influenza virus infection in mice. Chapter 4 shows that the increased IL-18-levels largely depends on proteolytic cleavage of
inactive pro-IL-18. IL-18 deficiency resulted in enhanced viral clearance, which correlated with enhanced CD4+ T cell activation as reflected by increased CD69 expression. TNF-α release by CD4+ T cells was significantly reduced, whereas IFN-γ production was similar in wildtype mice and IL-18-deficient mice. In contrast, IL-12 deficient mice showed reduced IFN-γ levels after influenza virus infection. Moreover, T cell responses were not affected by a lack of IL-12. Taken together, both IL-12 and IL-18 inhibit viral clearance during influenza pneumonia. However, the underlying mechanism seem to differ for these two cytokines.

A mouse-model for secondary bacterial pneumonia shortly after recovery from influenza infection is described in chapter 5. Mice were intranasally inoculated with influenza virus and after full recovery, i.e. normalized bodyweight and complete viral clearance, infected with Streptococcus pneumoniae. The interval of 14 days was chosen to exclude direct interaction between bacteria and influenza. Mice recovered from influenza infection were highly susceptible to secondary pneumococcal pneumonia as reflected by a 10,000-fold increased bacterial outgrowth and 5 to 20-fold increased levels of pro-inflammatory cytokines and chemokines. Besides the excessive production of pro-inflammatory mediators, the expression of IL-10, an anti-inflammatory cytokine was also increased after pneumococcal infection in mice recovered from influenza infection. The role of IL-10 was further analysed by treating these mice with neutralizing antibodies directed against IL-10. Blockade of IL-10 resulted in reduced bacterial loads and diminished TNF-α production. Moreover, anti-IL-10 treatment improved survival after secondary bacterial pneumonia. These data indicate that excessive IL-10 production impairs host defense against secondary pneumococcal infection.

Previous studies indicated that upregulation of of indoleamine-2,3-dioxygenase (IDO) resulted in exaggerated IL-6 and IL-8 responses to LPS and TNF-α in human airway epithelial-like cells. Since influenza virus infection in mice induced IDO expression in the lungs on day 14 post-infection, it was hypothesized that increased biosynthesis of IDO enhanced the production of pro-inflammatory mediators during secondary bacterial pneumonia. As described in chapter 6, administration of methyltryptophan (MeTrp), an IDO-inhibitor, resulted in reduced bacterial loads after secondary pneumococcal infection. TNF-α and IL-10 levels in the lungs were significantly reduced after MeTrp-treatment, whereas IL-6 and KC levels were not affected. Although MeTrp treatment resulted in reduced bacterial loads and lower TNF-α levels in the lungs, inhibition of IDO did not prevent lethality during post-influenza pneumonia.
Previous *in vitro* studies indicated that the production of pro-inflammatory mediators by virus-infected epithelial cells is enhanced in response to secondary stimuli such as TNF-α and lipopolysaccharide (LPS). The inflammatory response to LPS early after viral infection was studied in chapter 7. Intranasal LPS challenge on day 3 after Sendai virus infection resulted in enhanced IL-6 production in the lungs and increased neutrophil numbers, whereas production of IP-10, KC and TNF-α was not further enhanced. These data point towards specifically enhanced inflammatory response shortly after viral airway infection in mice.

The studies described in chapter 5, 6 and 7 indicate that viral airway infection leads to an altered inflammatory response to bacteria or bacterial cell-wall components. The mouse-models used in these studies exclude direct interaction between virus and bacteria. Chapter 8 focused on the interaction between influenza virus and non-typable *Haemophilus influenzae* (NTHI). Binding experiments revealed that influenza virus binds to NTHI. Further analysis indicated that the interaction between influenza and NTHI reduced viral outgrowth after combined viral/bacterial infection *in vitro* and *in vivo*.

The classical way to explain the increased susceptibility to bacterial infections during and shortly after recovery from viral airway infection is by increased adherence to the airway mucosa leading to enhanced colonization by bacteria. Invasion of *S. pneumoniae* is mediated by phosphocholine-binding to the platelet-activating factor receptor (PAFR). The role of the PAFR during secondary bacterial pneumonia was studied in chapter 9. Secondary pneumococcal infection in PAFR-deficient mice resulted in reduced bacterial outgrowth in the lungs as well as decreased bacterial loads in the blood. Moreover, prolonged survival was observed for PAFR-deficient mice after secondary bacterial pneumonia. However, previous studies indicate that PAFR-deficient mice also show decreased bacterial loads and increased survival rates after primary pneumococcal pneumonia. Therefore, the contribution of the PAFR to the increased susceptibility to secondary infections is limited.

**Discussion**

Most of the data presented in this thesis were obtained from mouse studies. Viral airway infections with influenza and Sendai virus in mice are well established models to study innate as well as adaptive immune responses to these viruses. The role of specific receptors and soluble mediators can be studied by using mice with a targeted deletion of specific genes. This approach was used to study the role of IL-12 and IL-18 during influenza virus infection.
The role of TLR4 was studied using mice lacking a functional receptor due to a point-mutation in the *tlr4* gene. Despite the advantage of studying the role of specific proteins during viral infection *in vivo* by (functional) deletion of the gene, the use of mouse-models has its limitations as well. The host response to viruses may involve compensatory mechanisms masking the effect of deleted genes. The anatomical and histological differences between mice and humans may interfere with the course of infection as well. Indeed, influenza infection in mice leads to pneumonia, while influenza virus causes inflammation of the upper airways in humans. The immune response is also affected by the host-specificity of viruses. In our models we used either a mouse-adapted human influenza strain or Sendai virus, a murine homologue of human parainfluenza type 1. Although these viruses cause only mild inflammation in humans, mouse-models are still useful to study the host response to negative-stranded RNA viruses. After all, the receptors, soluble mediators and mechanisms involved in innate and adaptive immunity show considerable homology and analogy between mice and humans.

These mouse-models revealed an inhibitory role for IL-12 or IL-18 in viral clearance during influenza infection. However, the underlying mechanism leading to reduced viral clearance appears to be different for both cytokines. The reduced viral loads in IL-18-gene deficient mice correlated with enhanced T cell activation as reflected by CD69 expression, whereas CD69 expression on T cells remained unchanged in IL-12 p35 gene deficient mice. Although IL-18 was originally identified as interferon gamma-inducing factor (IGIF), the production of IFN-γ after influenza virus infection appeared to be independent of IL-18. However, IFN-γ levels were significantly lower in IL-12 p35 knockout mice, indicating that IL-12 enhances IFN-γ production during airway infection with influenza virus. Our data point towards an inhibitory role for both cytokines in host response to influenza virus. Further research is required to elucidate the function of reduced viral clearance by IL-12 and IL-18.

In order to study secondary bacterial complications, we extended the primary influenza infection model by intranasal inoculation of *Streptococcus pneumoniae*. The mouse-model for secondary bacterial pneumonia was designed to study the impaired host defense against bacteria shortly after recovery from influenza infection. The time-interval of 14 days is in line with the occurrence of secondary bacterial pneumonia in humans, i.e. 4-10 days after recovery from influenza infection. The advantage of this model is complete eradication of the virus when the mice were inoculated with *S. pneumoniae*, thus excluding direct interactions.
between influenza and bacteria. Secondary infection resulted in enhanced bacterial outgrowth and increased production of pro- and anti-inflammatory cytokines. The enhanced production of these mediators correlated with reduced neutrophil function, indicating that the anti-inflammatory cytokine IL-10 may play a critical role during secondary bacterial pneumonia. Indeed, neutralization of IL-10 improved the outcome of secondary pneumococcal infection. IL-10 is therefore an important target in the early treatment of post-influenza pneumococcal pneumonia. The clinical use of neutralizing antibodies against IL-10 is likely hampered by the expenses of this treatment. Alternative ways to reduce IL-10 levels are probably more important targets to treat secondary bacterial infections. In this thesis we show that IL-10 production is at least in part mediated by IDO. Inhibition of IDO resulted in reduced bacterial loads, pulmonary TNF-α levels and neutrophil numbers. However, inhibition of IDO did not prevent lethality in mice with post-influenza pneumonia. Likely, IDO regulates the expression of other mediators as well. These data indicate that the efficacy of IDO inhibitors in the treatment of secondary bacterial infections is poor. Still, temporary inhibition of IDO or inhibition of IDO in combination with other treatments may be useful. The proinflammatory mediators IL-6 and KC were not affected by inhibition of IDO. In contrast, Sendai virus infection potentiated IL-6 production after LPS challenge, whereas production of IP-10, KC and TNF-α were not affected. KC and TNF-α levels were only modestly increased after viral infection, whereas IP-10 was strongly induced by Sendai. Although IP-10 was induced after LPS challenge in control mice, LPS challenge did not further increase IP-10 levels in the lung. IP-10 has been described to be induced by IRF-3. Activation of IRF-3 requires the adapter molecule TRIF, which associates with either TLR3 and TLR4. Since TLR4 is not involved in host defense against Sendai virus (chapter 2), it could be suggested that TLR3 plays an important role in recognition of Sendai virus. Indeed, TLR3 has been implicated in the recognition of double-stranded RNA, which is produced by negative-stranded RNA viruses during replication. Whether IDO mediates the exaggerated IL-6 response after LPS challenge in Sendai-infected mice is not yet determined. Nevertheless, our data indicate that cross-talk between several pathways exists, leading to enhanced production of IL-6 after LPS stimulation in Sendai virus infected mice.

Both LPS and *S. pneumoniae* induce an exaggerated inflammatory response during or shortly after recovery from viral airway infection. These inflammatory responses contribute to the severity of secondary bacterial infections. Both models exclude direct interaction between the two pathogens involved in secondary pneumococcal pneumonia. Interaction between viruses
and bacteria may influence the course of infection. In this thesis we show that NTHI binds influenza virus. This interaction diminished viral outgrowth in airway epithelial cells. The reduction in viral load was even more pronounced in a mouse-model for combined viral/bacterial infection. These data point towards a protective role for NTHI during influenza virus infection. From our data it is not clear whether NTHI prevents viral entry or replication in airway epithelial cells. Phagocytosis of NTHI may play an important role as well. Indeed, NTHI is cleared from the lungs within 24 hours after NTHI or combined infection. Since NTHI is a commensal in the upper airways, it could be suggested that NTHI, and probably also other inhabitants of the nasopharynx, serve as a scavenger for viral pathogens. Despite this protective effect, influenza virus infection in the lower respiratory tract may induce colonization of NTHI leading to combined viral/bacterial pneumonia. Enhanced colonization of bacteria is the classical dogma to explain secondary bacterial pneumonia. Destruction of the airway epithelium leads to increased adherence of bacteria to the airway submucosa. Alternatively, increased colonization may occur due to upregulation of specific receptors. The platelet-activating factor receptor (PAFR) has been suggested to play a role in the increased colonization of *S. pneumoniae*. Indeed, PAFR gene deficient mice showed reduced bacterial loads and prolonged survival after secondary pneumococcal pneumonia. However, these differences were also observed for primary pneumococcal pneumonia. In conclusion, expression of the PAFR does not contribute to the enhanced susceptibility to secondary bacterial pneumonia. Previous studies with PAFR antagonists did not improve the outcome of post-influenza pneumococcal pneumonia. Although PAFR expression does not contribute to the enhanced susceptibility to secondary bacterial pneumonia, the PAFR might be a useful target to treat either primary or secondary pneumococcal pneumonia.

Overall, the increased susceptibility to secondary bacterial pneumonia during viral airway infection depends on several factors, including increased adherence to the airway (sub)mucosa, increased virulence of both pathogens and enhanced pro- and anti-inflammatory responses. All these aspects should be taken into account in order to develop strategies to prevent or treat secondary bacterial complications during or shortly after viral airway infection. Further research is required to identify the key mediators or key mechanisms involved in secondary bacterial pneumonia.