Neutrophils in respiratory syncytial virus disease

Untangling the NET

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Publication date
2017

Document Version
Other version

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Chapter 9

General discussion and summary
General Discussion

Severe RSV-LRTD

Respiratory syncytial virus (RSV) remains one of the most important causes of childhood lower respiratory tract disease (LRTD) worldwide.\(^1\) Approximately 8-10% of infants infected with RSV develop RSV-LRTD warranting hospital admission and even mechanical ventilation.\(^2\) This poses a great burden on both hospital capacity, as well as the costs associated with hospital admissions.\(^3,4\) The disease spectrum of severe RSV-LRTD ranges from bronchiolitis to acute respiratory distress syndrome (ARDS).\(^2,5\) Treatment of severe RSV-LRTD is hampered by the lack of effective therapies and vaccines, and supportive care is the only available treatment to date. Further insights into the mechanisms of disease are highly needed to find new prophylactic and therapeutic strategies.

Neutrophils

Severe RSV-LRTD is characterised by profound neutrophilic inflammation in the lungs: up to 85% of broncho-alveolar lavage (BAL) cells are neutrophils during severe RSV-LRTD.\(^6-9\) Their dynamics, plasticity and the effect and role of their effector functions have, so far, been largely overlooked. This is remarkable, as neutrophils are thought to play an important role in host defence during viral respiratory infections, like influenza infection.\(^10\) Neutrophils are also implicated in the development of lung tissue damage in a variety of medical conditions including ARDS, transfusion-related acute lung injury, cystic fibrosis and COPD.\(^11-18\) For example, in ARDS activated neutrophils release granule proteins and reactive oxygen species (ROS), leading to endothelial-epithelial damage and disruption of the capillary-alveolar barrier causing increased capillary leak.\(^18\) Neutrophils are in a constant delicate balance between being protective and becoming detrimental to the host, and it is currently unknown to which side this balance tips during RSV disease. In this thesis we set out to increase our understanding into the role neutrophils play during severe RSV-LRTD.

As a first step into deciphering the complex immunologic pathways that dictate the outcome of RSV infection we explored two cognate host pneumovirus animal models (chapters 2 & 3). These two models exploit calves and mice infected with bovine (b)RSV and pneumonia virus of mice (PVM) respectively. Both viruses are phylogenetically closely related to human RSV\(^9\) and induce severe clinical disease in their natural hosts, calves and mice.\(^20,21\) With regard to our research questions, these models offer the advantage of profound neutrophilic inflammation after infection, as compared to heterologous animal models (e.g. human RSV infection in mice, sheep, and cotton rats).\(^22\) These observations were confirmed in chapter 2, chapter 7 and in chapter 3, where we found that 72% and 46% of BAL immune cells were neutrophils during peak disease in calves and mice, respectively. This is much
higher compared to heterologous animal models, with 3-14% BAL neutrophils during human (h)RSV infection in mice\textsuperscript{23,24}, <5% BAL neutrophils during hRSV infection of cotton rats\textsuperscript{25}, and the 18-27% BAL neutrophils during hRSV infection in calves (chapter 2). These values do depend on the time of sampling during infection and the viral strain used, but overall, the heterologous models never achieve similar values compared to the cognate host models. Neutrophil recruitment is in many aspects similar in humans, calves and mice and is initiated by chemo-attractive factors like IL-8 (or KC, the IL-8 equivalent in mice) released by epithelial cells.\textsuperscript{26} However, baseline (blood) neutrophil numbers are markedly different between species, ranging from 70% in humans to only 5-10% in mice (chapter 3). Recruitment is also influenced by viral factors, such as the RSV soluble G protein (sG). This viral protein resembles the human chemokine CX3CR-ligand1 (or fractalkine). sG influences leucocyte migration \textit{in vivo}\textsuperscript{27}, and inhibition of sG by monoclonal antibodies in mice reduces neutrophil recruitment.\textsuperscript{24,28} When these neutrophils arrive in the airways they are activated, as can be seen by profound NETosis during bRSV infection and elevated MPO-levels in BAL during PVM infection. This suggests that they are not just idle bystanders but are actively involved in host defence and perhaps also in collateral damage.

In chapter 2 we describe both human and bovine RSV infection in calves, with the aim to evaluate hRSV infection in calves as a suitable new model to study hRSV disease. Although we found replication of hRSV in the upper and lower airways, accompanied by immune activation and inflammation, we could only detect minimal clinical upper airway disease symptoms, an absence of lower airway disease symptoms and mild neutrophilic infiltration of the lower airways. In contrast, we found severe clinical disease in both upper and lower airways in calves infected with bRSV, accompanied by profound neutrophil infiltration in the lower airways. Our results indicate that it is possible to infect calves with hRSV, but the magnitude of infection and neutrophilic infiltration is not enough to be able to study severe disease pathophysiology, and neutrophil biology in particular, in this model. The bRSV infected animals did develop severe disease which mimicked the disease progression as seen in paediatric patients and experimental infection in adults.\textsuperscript{29,30} It is important to note that bRSV elicits a natural immune response in calves, which can be utilized to study immuno-pathogenesis during RSV disease. Strikingly, like in humans, the bRSV infection model was accompanied by neutrophilic infiltration of the lungs, with approximately 72% of the immune cells present in the lumen of the lungs being neutrophils. Although higher compared to other animal models this is still lower compared to the 80-85% we and others have found in paediatric patients.\textsuperscript{6,7,9} This difference could be explained in several ways. For example the amount of viral inoculum used, time and method of sampling and the differences in immunogenicity of bRSV compared to hRSV. Furthermore, the life-saving mechanical ventilation in children allows sampling after the point of respiratory failure, whereas calves
are always sampled before respiratory failure. Another explanation could be the added effect of mechanical ventilation and induction of ventilator induced lung injury, which are known inducers of neutrophilic inflammation in the lungs.\textsuperscript{31-34} Still, altogether, the calf model is the only large animal model that approaches human disease in terms of neutrophilic inflammation, and as such offers unique opportunities for researchers. The calf model offers additional advantages, for example the extensive sampling possibilities of large animals, as it is possible to sample each animal multiple times, and on multiple consecutive days. This enables in-depth analysis with low animal numbers. Furthermore, maternal antibodies are transferred to the calf by colostrum (milk), instead of via the placenta, making it possible to study RSV disease in (colostrum deprived) animals without maternal antibodies. Also, due to the more comparable anatomy it is possible to study mucosal vaccination on a more detailed scale compared to mice. Animal models, and large animal models in particular, are accompanied with specific limitations. Because hRSV infection does not induce severe clinical disease in the studied bovine model, it is not possible to investigate, for example, the effect of hRSV-specific monoclonal antibodies on hRSV disease severity. Still, hRSV-specific antibodies which bind to epitopes with shared sequence homology between hRSV and bRSV could be investigated in the bRSV model, as the two most important bRSV surface proteins share 40\% (G protein) and 80\% (F protein) of their sequence with hRSV.\textsuperscript{35-36} Additionally, results from the bRSV infection cannot be directly extrapolated to humans, as the viruses are not identical. Nonetheless, as safety and efficacy of new treatment modalities need to be verified in animal models, and we think the bRSV model could be an appropriate model to use, in particular in research involving neutrophil biology.

Previous studies showed that neutrophils are unlikely to play a major role in viral clearance during RSV infection, as viral load were equal between neutrophil depleted and non-depleted mice.\textsuperscript{23} However, as mentioned above, these models are characterized by relatively low neutrophil inflammation. In order to determine an overall role of neutrophils during severe pneumovirus infections, such as human RSV-LRTD, we depleted PVM-infected mice of their neutrophils by using an antibody-mediated depletion strategy (\textbf{chapter 3}). Neutrophil depletion has been investigated before in influenza infected mice, with various effects ranging from improvement to deterioration, and it appears that the virus strains used, and timing of depletion, and the degree of depletion influences the outcome.\textsuperscript{10,37-39} In humans, neutrophil depletion, for example in patients receiving chemo-therapy, leaves the body prone to bacterial infections.\textsuperscript{40-42} However, these patients also frequently develop ARDS when they recover from their neutropenia\textsuperscript{43-44}, suggesting both a protective and a detrimental role for neutrophils, depending on the degree of neutrophil recruitment and activation. Strikingly, in \textbf{chapter 3}, while neutrophil depletion was successful, we did not detect any effect on clinical disease severity or viral load during acute PVM infection. We aimed to confirm whether or not this
observation was a result of either too strenuous viral pathology, as has been described for different influenza strains\textsuperscript{39}, or of mouse species susceptibility, as has been described during PVM infection in mice.\textsuperscript{45} Therefore, we investigated lower viral inocula and different mouse species (C57Bl6 and BALBc strains). However, we found similar results. Our findings of a complete absence of influence on disease severity left us puzzled. It either questions the importance of neutrophils during severe RSV disease or the PVM mouse model itself as a suitable RSV model to study neutrophil biology. We found evidence for the latter. The murine immune system is in many aspects similar to humans, but important differences do exist. For example, mouse neutrophils lack defensins and mouse blood contains only 10% neutrophils, while human blood contains 70% neutrophils.\textsuperscript{46} In chapter 3 we found another possible explanation: the almost complete absence of ‘neutrophil extracellular trap’ (NET) formation during PVM infection. NETs are clearly present during human and bovine RSV infection and cause airway obstruction, as we describe in chapter 5 and 6 of this thesis. Furthermore, mice neutrophils are capable of producing NETs as has been shown in other studies, thus this effect is not related to the inability of mouse neutrophils to produce NETs.\textsuperscript{47} A second possible explanation involves the genetic differences between RSV and PVM. We detected equal lung pathology in all animals (depleted and controls), even when using a low viral inoculation dose. This indicates that the lung pathology, as seen during PVM infection, is possibly caused by the virus itself. Consequently, PVM might be more virulent compared to human RSV. Hence, it seems that virus-induced (lung) pathology (opposed to host-induced pathology) is more severe during PVM infection and might have masked any effect of neutrophil depletion. The PVM mouse model has proven extremely useful as severe RSV disease model.\textsuperscript{21,22,48} Despite the profound neutrophilic inflammation induced by PVM, the absence of NET formation and the more virulent nature of the PVM virus, makes this mouse model less suited to extrapolate our findings to humans.

**Neutrophil extracellular traps**

One of the neutrophil effector functions which could have an impact on RSV disease is the formation of ‘neutrophil extracellular traps’ (NETs). NETs are extracellular networks of DNA-fibers studded with granule proteins and served to capture and neutralize a broad spectrum of pathogens, including viruses.\textsuperscript{49,50} Funchal and colleagues found NET production upon stimulation of neutrophils with the RSV F protein.\textsuperscript{51} NETs have both been implicated in anti-viral protection\textsuperscript{50}, as well as in collateral damage during viral infection.\textsuperscript{47} In chapter 5 we present an overview of the identity of these ‘traps’, and how they could play a role during (paediatric) respiratory diseases. Furthermore, we describe what is known about targeting NETs to prevent collateral damage. Many pathogens and diseases are accompanied by NET formation. Whether these NETs are beneficial, detrimental or both depends on the disease in question. Despite rapidly evolving evidence regarding NET formation and function during
disease, there is yet little knowledge regarding pro- or anti-NET treatments, leaving many questions unanswered.

Since little was known about the occurrence of NETs and the effect they have on RSV disease severity, we investigated their role in chapter 6. In this explorative study, we hypothesized that the presence of DNA-rich mucus might well be largely due to NET formation by the copious amounts of neutrophils present in the airways in patients with severe RSV disease. Indeed, we found evidence for robust NET formation during RSV disease in both humans and calves. Although in vitro experiments suggested that NETs perform an anti-viral function by trapping the viral particles, in vivo evidence showed that this was associated with airway obstruction with dense plugs consisting of NETs with trapped necrotic immune cells, neutrophils and sloughed epithelial cells. Sometimes containing trapped viral antigens, but more often (63% of the plugs, chapter 6) no trapped virus was detected, suggestive of an overshoot and thus an immuno-pathological role. NETs have been described as injurious in for example influenza infection and transfusion related acute lung injury.12,13,47 However, these findings were always related to the toxic functions the proteins covering NETs and the DNA of NETs itself could exert.12,52 One example is increased endothelial permeability after exposure of human endothelial cells to histones stuck on NETs in vitro. Blocking these histones with antibodies in vivo, reduced the associated increase in vascular permeability and increased survival.12 Our study was the first to show mechanical airway obstruction by NETs, a striking pathological phenomenon during severe RSV disease, which provides insight into our current understanding on how NETs contribute to disease. Thus, NETs may play an important role in RSV-LRTD disease pathogenesis. The key question arising from this study is whether it is safe to inhibit or dissolve NETs to prevent detrimental effects without exacerbating the disease against which they are formed.

Subsets

Neutrophils are often perceived as a single group of identical effector cells acting only in the innate realm. However, there is strong evidence that even these ‘basic’ immune cells can be divided into different subsets, each with its own distinct functions.53-56 For example, in a transplantation mouse model, specific neutrophils develop in response to G-CSF treatment and modulate adaptive immune responses by inhibiting IFN-γ production in T-cells, thereby ameliorating graft-versus-host disease.57,58 Furthermore, it is known that bacterial infections induce ‘suppressive neutrophils’, which can dampen the immune response and inhibit T-cells.59 Neutrophil subsets capable of regulating adaptive immune responses have also been described in other clinical conditions.59

In chapter 4 we describe the results of an observational study in paediatric patients with severe viral respiratory infections admitted to the paediatric intensive care unit. We
aimed to investigate the occurrence of different neutrophil subsets. Interestingly, suppressive neutrophils were absent during severe respiratory viral infections, but present during severe respiratory viral infections with a bacterial co-infection. Furthermore, viral infections were accompanied by highly activated young progenitor neutrophils, which developed during admission. Whether the absence of regulating suppressor neutrophils and the high abundance of young activated neutrophils could cause collateral damage during viral infections remains to be determined. The finding of different subsets of neutrophils offer an interesting new view on possible immune-modulation therapy. If induction of different subsets could modulate the immune response in a particular direction (e.g. pro- or anti-inflammatory state), we could steer the immune response into a protective direction, or deviate the response from a state that causes collateral damage (e.g. NET formation during severe RSV disease). The potential to steer neutrophil subset formation has been shown by Fridlender et al., where TGF-β blockade resulted in a shift in subset formation. Apart from these new insights into the behaviour of neutrophils during viral infections, the striking difference in the blood neutrophil phenotype distribution between patients with and without bacterial co-infection offers an interesting perspective on diagnostic tests to determine if patients have bacterial co-infection. Accurate and fast diagnosis is of critical importance to prevent overtreatment and antibiotic resistance. Cross-validation and testing in larger cohorts, with patients exhibiting different disease severities are mandatory to validate and determine accuracy of this new diagnostic approach. Future research should focus on the effects that each specific subset exerts (e.g. NETosis) during RSV disease, which could guide new therapies stimulating or inhibiting specific subsets during disease.

While neutrophils are the dominant cell type within the airways during RSV infection, this does not rule out important roles for other immune cells, including alveolar macrophages, monocytes, eosinophils, dendritic cells, cytotoxic T-cells and antibody producing B-cells. Alveolar macrophages are known contribute to enhance type 1 IFNs production during RSV infection, thereby recruiting monocytes which contribute to viral clearance and are thus essential to initiate (part of) the host anti-viral response. The eventual resolution of the RSV infection is mediated through activation of the adaptive immune system. RSV specific cytotoxic T-cells and RSV specific antibodies produced by B-cells are essential to clear the virus and offer (although incomplete and short-lived) protection against RSV re-infection. Taken together it is highly unlikely, if not completely excluded, that a single cell type is responsible for RSV induced immunopathology. Interestingly, more often than not are neutrophils interlinked with these immune cells. Not only can neutrophils carry and present viral antigens in the bone marrow and lymph nodes to induce CD8+ memory T cells, they also deposit a CXCL12 trail during migration, which can direct cytotoxic T cells towards the infection. NKT cell activation is regulated via neutrophil-derived IL-17, and neutrophil-
derived IL-18 is necessary for NK cell IFN-γ production. Furthermore, they regulate dendritic cell recruitment in a CCL3 dependent manner. Neutrophils also induce macrophage apoptosis through TRAIL production. These data highlight the importance of neutrophils as controllers, rather than just as a simple effector cells. Unravelling their regulating functions together with effector functions will be imperative to be able to safely alter both number and function of neutrophils during RSV disease.

**Targeting neutrophils: clinical implications**

From the various studies in this thesis and previous reports it becomes clear that it remains extremely difficult to capture the precise role of neutrophils during RSV-LRTD. It is likely that neutrophils have multi-functional roles (e.g. neutrophil subsets) in disease and in (recovering) health. These roles seem to be time-dependent and are possibly also age-dependent. However, their ability to cause collateral tissue damage is prominent, and forms a valuable target for intervention. In part III of this thesis we describe two completely different approaches to prevent severe RSV disease and to limit disease-related complications such as respiratory failure due to airway obstruction.

The best example of detrimental host induced effects is the profound NET formation within the airway lumen, thus increasing the viscosity of mucus present in the lumen, resulting in airway obstruction and atelectasis (chapter 6). To further establish the role of NETs in airway obstruction during RSV-LRTD, we administered dornase alfa (human recombinant DNase) to calves with bRSV-LRTD (chapter 7). Dornase alfa degrades the DNA backbone of NETs and might thus reduce the viscosity of the airway plugs, as others have shown. In our study, therapeutic treatment with dornase alfa resulted in reduced total NET content in the lungs and, more importantly, it reduced the extent of airway obstruction. This was in contrast to results from studies in children with mild RSV infection, where dornase alfa treatment did not result in noticeable differences in disease severity. However, these studies were done in patients with mild disease, opposed to severe disease in our calf model. This study proposes a pathophysiological mechanism involving NETs as the basis for the use of dornase alfa to alleviate airway obstruction during severe RSV-LRTD. An important potential side-effect of NET-lysis could be renew dissemination of captured virus particles. However, in our study we did not detect enhanced viral dissemination in the dornase alfa treated group, when compared to the control group (chapter 7). Taken together, these preliminary results indicate that dornase alfa treatment effectively degrades NETs in the airways and alleviates airway obstruction without enhancing viral dissemination. New randomized controlled trials in calves and humans are needed to study the clinical efficacy of dornase alfa treatment in specific patient populations (e.g. severe RSV-LRTD).
The studies in this thesis, in part, support a detrimental role for neutrophils, and specifically for neutrophil effector functions (e.g. NET formation), during severe RSV disease. As neutrophils are implicated in many diseases, ranging from inflammatory conditions (e.g. ARDS)\textsuperscript{17} to auto-immune diseases (e.g. SLE, rheumatoid arthritis)\textsuperscript{73-74}, it seems strange that, to date, no specific anti-neutrophilic compounds are utilized in clinical practise. The major explanation for this absence is the absolute necessity for properly functioning neutrophils. Simply removing or depleting all neutrophils will thus result in severe danger to the host. On the contrary, as we and others have shown, too many neutrophils or too much activation (chapter 6 and 7) will result in detrimental sequelae. It is this ‘double edged sword’ character of neutrophils that pose us with this difficult problem. However, attempting to target neutrophil activation (by for example anti-inflammatory drugs, cytokine-inhibitors) or reducing (or disabling) specific neutrophil effector functions (e.g. inhibition of NET formation by PAD4 inhibitors) might result in favourable disease outcomes during different diseases, including RSV. N-\alpha-benzoyl-N5-(chloro-iminoethyl)-L-ornithine amide, or Cl-amidine, is such a PAD4-inhibitor, and prophylactic treatment with Cl-amidine reduced NET formation and improved survival in a mouse sepsis model.\textsuperscript{75} Cl-amidine also proved therapeutically effective in MPO-ANCA vasculitis in mice.\textsuperscript{76} Other therapeutics (derivatives of Cl-amidine) have been developed and will have to prove their use in future (human) studies.\textsuperscript{77,78}

In chapter 8 we focus on the natural repertoire of human antibodies against the RSV G protein. The G protein is associated with infectivity in vitro and in vivo and can be expressed as a membrane bound and a soluble form.\textsuperscript{79,80} The G protein contains a motif (the CX3C-motif) which can block the humane immune receptor CX3CR1, thus preventing induction of immune-cell recruitment, including neutrophils.\textsuperscript{27} Targeting this molecule could therefore reduce the amount of inflammation in response to RSV infection. Besides immune-modulating properties, antibodies could also aid in the direct neutralization of the virus in for example complement dependent or a complement independent manner. The latter could be achieved by the inhibition of virus-host cell binding. In the past, antibody therapies have focussed on the RSV F protein, due to its homology between different RSV strains.\textsuperscript{19} Currently, only palivizumab (a monoclonal antibody against human RSV F protein) is registered for prophylactic administration in children.\textsuperscript{81} Unfortunately this antibody does not provide therapeutic protection.\textsuperscript{82} Furthermore, prophylactic treatment with palivizumab reduced hospital admission with 55\%.\textsuperscript{83} However, the remaining 45\% is still admitted, which leaves considerable room for improvement. In chapter 8 we show that individuals (day-care providers) who regularly encounter RSV have very high levels of RSV-reactive antibodies (0.35% of the total memory B cells), which is much higher compared to other studies in adults who found < 0.01% RSV G reactive B cells.\textsuperscript{84} Their response is not only targeting the RSV F protein but also the RSV G protein, confirming the immunogenicity of the G protein. Interestingly, we
found that the majority of the most potent RSV binding B cell clones recognized regions in the conserved domain of the G protein and these regions correlated with cross reactivity between the two strains of RSV (strain A and B). Furthermore, we show that antibodies binding within the CX3C-motif can inhibit RSV infection in primary human airway epithelial cell cultures. So far, we do not know if these monoclonal antibodies function equally effective in vivo. However, if they are effective, they may very rapidly prevent viral entry into the host cells, and thus limit further infection and subsequent virus induced damage. A second function of these monoclonal antibodies could be the suppression of CX3CR1 mediated leukocyte chemotaxis, thereby reducing the amount of neutrophils recruited to sites of injury. This, in turn, could prevent large numbers of toxic activated neutrophils in the airways, which might reduce collateral damage. These findings have great implications for future RSV treatment and prophylaxis.

**Future directions**

It remains important to unravel the hosts’ response(s) to RSV. Future research should therefore focus on the basic pathophysiology of RSV disease, for example by assessing which functions different neutrophil subsets exert during RSV disease in vivo. Furthermore, this thesis provides new insides in possible novel treatment modalities, which should be studied in more detail before they can be assessed in a clinical setting. It will be important to investigate whether targeting NETs in calves and humans will result in decreased clinical disease. Furthermore, it would be interesting to see if anti-CX3CR1 specific human antibodies have clinical effect in vivo, and if they act in an immune-modulating way, preventing both viral entry and host immune cell recruitment.

It might seem logical to observe RSV infection in a human host as a complex network of virus-host interactions, where both viral- and host-factors dictate the outcome of the disease. However, most research to date focuses on either viral factors (e.g. development of anti-virals) or host-factors (e.g. anti-inflammatory drugs). Although both approaches resulted in a series of important discoveries, it is now time to look at RSV disease from a broader perspective by combining and implementing all available knowledge. For example, anti-virals show potent inhibition of viral replication in experimental human RSV infection and this is accompanied by a decrease in upper airway symptoms. One of these anti-viral compounds has also been tested in the bRSV calf model with similar results, highlighting the potential of this model. However, these medications can only be administered upon presentation at the hospital or at the primary care centre, and take 1-4 days to reduce viral loads to baseline. As a consequence these patients might still proceed to respiratory failure before the medication sorts effect. There are several studies in animals which implement a combination of two interventions. Two studies implemented an anti-viral (Ribavirin) with...
an immunomodulatory compound (anti-MIP-1α mAb or a leukotriene receptor antagonist) during PVM infection in mice and the authors show that each single treatment did not result in clinical benefit, but the combined treatment resulted in dramatically improved survival.\textsuperscript{88,89} Another example of combination therapy in the RSV field is the simultaneous administration of two RSV G protein specific monoclonal Abs, again showing no individual benefit, while decreasing lung inflammation after combination therapy.\textsuperscript{28} Therefore, I would plea that we should regard RSV as a complex disease consisting of a whole team of players inducing injury. We should not try to substitute single players, but we should target the complete team.

**Conclusion**

This thesis increases our understanding of neutrophil biology during severe RSV-LRTD. Specifically, we found evidence for exaggerated NET formation during RSV-LRTD, leading to airway obstruction. Targeting NETs via dornase alfa administration reduced lung NETs and alleviated airway obstruction. Furthermore, viral LRTD is characterized by different neutrophil subsets with a distinct phenotype compared to subsets from bacterial LRTD. However, a definitive causal role for neutrophils and NETs in disease severity during severe RSV-LRTD could not be established. Future research should address the impact of NETs on disease severity, explore different anti-neutrophilic strategies and address the effects of different neutrophil subsets during RSV-LRTD. Finally, these results need to be incorporated into the evolving knowledge of RSV in general, and should lead to combined therapeutic approaches to target or prevent severe RSV-LRTD.
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Summary

Respiratory syncytial virus (RSV) is one of the most important causes of childhood pneumonia and bronchiolitis worldwide. The disease is accompanied by prominent neutrophilic inflammation of the airways. However, the precise role of these immune cells during the disease is largely unknown. The central focus of this thesis is the neutrophil, with the overall aim to increase our understanding of the role of this important innate immune cell in the pathogenesis of RSV lower respiratory tract disease (RSV-LRTD).

Modelling Respiratory Syncytial Virus Infection

Animal models provide an essential bridge to investigate the many outstanding research questions regarding pathophysiology of RSV disease. However, all animal models present with specific limitations, with one of the most important one in the RSV research field, the lack of a suitable host-specific infection model.

In part I we go more into detail about two host-specific RSV animal models. In chapter 2 we describe the bovine calf model as a suitable host-specific pre-clinical animal model for RSV. We determined clinical and pathophysiological disease outcome in both bovine-RSV and human-RSV infection in young calves. We found prominent clinical upper and lower respiratory tract symptoms in calves infected with bovine RSV, accompanied with viral replication and (histo)pathology in lower airways and alveoli. In contrast, infection of calves with human RSV resulted in only upper respiratory tract symptoms. Despite the absence of lower airway symptoms we could detect viral replication in the lower airways, accompanied by the activation of the immune system (e.g. neutrophilic infiltration and neutrophil extracellular trap formation in the lower airways), albeit at a lower magnitude when compared to bovine-RSV infected animals. This makes the cognate host-specific bRSV model better suitable to investigate neutrophil biology during severe RSV infection. We conclude that the bovine and human RSV model, together, mimic many aspects of human-RSV infection in young infants, making the RSV calf model a suitable model to explore RSV related research questions.

In chapter 3 we explore another RSV animal model; the pneumonia virus of mice (PVM) model. In this study we sought to unravel the role neutrophils play during acute PVM infection in C57BL6 and BALBc mice, by utilizing an antibody-mediated depletion method. We hypothesized that depletion of neutrophils would reduce neutrophil-mediated lung damage and improve disease outcome in this model. However, despite adequate neutrophil depletion, we did not find any clinical or histopathological improvement in the depleted group. Furthermore, to our surprise we could not detect, the for human/bovine RSV characteristic, mucus plugs obstructing the airways. In calves and humans these plugs are rich of neutrophil extracellular traps (NETs). PVM infection did induce potent neutrophilic infiltration, as shown by large amounts of neutrophils present in the broncho-alveolar lavage fluid of these mice.
But these neutrophils, although they are capable to produce NETS, do no form NETs in the PVM model, like their bovine and human counterparts. We conclude that the PVM-model lacks activation of essential neutrophil effector functions needed to investigate the role of neutrophils during pneumovirus infections, this might be the explanation why we could not find any difference between neutrophil depleted and non-depleted mice in this RSV model.

**Neutrophils and the Pathogenesis of Respiratory Syncytial Virus Infection**

In the part II of this thesis we investigate the occurrence of neutrophil subsets and one of the effector functions of neutrophils; neutrophil extracellular trap formation during RSV infection. In chapter 4 we investigated if viral respiratory infections are accompanied by different neutrophil subsets and if these subsets could have either a suppressive phenotype or an activated phenotype. We could not find suppressive neutrophils in infants infected with virus alone, in contrast to infants with both viral and bacterial co-infection. Furthermore, we could detected a new neutrophil subset, consisting of very young progenitor neutrophils with an activated phenotype. We hypothesize that the presence of the latter subset during viral respiratory infections and the absence of suppressive neutrophils could both be important in vivo. In addition, the (absence of) suppressive neutrophils could serve as a potential diagnostic marker to discriminate viral infections from viral infections with a bacterial co-infection.

In chapter 5 we summarise what is known about NET formation during (paediatric) respiratory diseases. NETs are produced during many (infectious) diseases. They exhibit both protective and detrimental functions, which depend on the causative agent. Since RSV infection is accompanied by prominent neutrophil recruitment and activation, it therefore seemed interesting to investigate the role NETs play during acute severe RSV disease.

We expanded on this question in chapter 6. Here, we first investigated whether RSV was able to induce NET formation by human neutrophils in vitro, and if these NETs could trap viral particles and aid in the anti-viral response to RSV. We found RSV dependent NET formation. These NETs could trap viral particles and thus prevent infection of epithelial cells. Next, we found prominent NET formation in the airways of both humans and calves infected with RSV. NETs might increase the viscosity of the mucus, making the mucus more prone to plug-formation. Strikingly, these NETs were mainly produced in the lumen of the airways, leading to NET-rich mucus plugs obstructing the smaller airways. Interestingly, some of these plugs indeed contained viral antigens, indicative of viral trapping. However, most airway obstruction plugs did not contain viral particles and only contributed to airway obstruction. These results are indicative of a detrimental role for NETs during severe RSV disease.
Therapeutic Interventions

In part III of this thesis we describe several potential intervention strategies to treat severe RSV infection. Chapter 7 describes the effect of local dornase alfa treatment in bovine RSV infected calves. We found significant NET degradation during dornase alfa treatment compared to the control group. This resulted in improved mucus clearance, less airway obstruction and improved ventilation. Furthermore, this treatment was accompanied by decreased histopathological damage. Together, these results support the notion that treatment of NET-rich mucusplugs could alleviate airway obstruction.

Chapter 8 focusses on therapeutic monoclonal antibodies targeting the RSV G protein. This protein is, together with the RSV F protein, responsible for RSV attachment and fusion with target host cells, but also influences neutrophil recruitment. In this manuscript, we describe the build-up and functional characteristics of a panel of G-specific antibodies retrieved from humans. We found that these antibodies target specific epitopes in and around the conserved region of the G protein. Interestingly, antibodies binding the cysteine noose are capable of blocking viral attachment to primary human airway epithelial cells. A process likely to involve inhibition of the CX3CR1-G protein interaction.

Major findings in this thesis

- Calves can be productively infected with both human and bovine RSV in an experimental setting.
- Pneumonia Virus of Mice induced lung damage in mice is independent of neutrophil driven inflammation
- Mice lack neutrophil extracellular trap formation during PVM infection.
- Viral respiratory infections are characterized by the absence of suppressive neutrophil subsets, whereas viral respiratory infection with bacterial co-infection is characterised by this suppressive neutrophil subset.
- Neutrophil extracellular trap formation plays a prominent role during many (paediatric) respiratory diseases.
- RSV induces neutrophil extracellular trap formation which contributes to viral capture, but at the same time causes airway obstruction.
- Dornase alfa treatment lowers the amount of NETs in the airways and reduces the degree of airway obstruction during acute severe bovine RSV infection in calves.
- Human monoclonal antibodies targeting the RSV G protein perform various potent anti-viral functions.