IMPLICATIONS OF HOST-MICROBE INTERACTIONS IN CHRONIC LUNG DISEASES

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INDEX

1 SUMMARY ........................................................................................................5

2 ZUSAMMENFASSUNG ......................................................................................7

3 GENERAL INTRODUCTION .............................................................................9

3.1 Mucosal surfaces ........................................................................................... 9

3.2 The innate immune system ...........................................................................9
  3.2.1 The role of the epithelium in homeostasis ...............................................9
  3.2.2 The pulmonary immune system in infection .........................................10
  3.2.3 Pulmonary dendritic cells: immune sentinels .......................................11
  3.2.4 IL-1β induction and signaling ...............................................................13
  3.2.5 IL-1β effector function and implication in diseases .........................14
  3.2.6 Induction and function of IL-17A ........................................................15

3.3 Chronic pulmonary diseases .......................................................................16
  3.3.1 COPD and asthma: similarities and differences ..................................16
  3.3.2 COPD pathology ..................................................................................17
  3.3.3 Lung function in COPD .........................................................................18
  3.3.4 Exacerbations of asthma and COPD .....................................................18

3.4 Microbial impacts on disease development and health .........................19
  3.4.1 Infections of the respiratory tract ..........................................................19
  3.4.2 Beneficial effects of commensal bacteria .............................................20

3.5 References ....................................................................................................22

4 RESULTS .........................................................................................................30

4.1 Dysregulation of allergic airway inflammation in the absence of microbial colonization ........................................................................................................30
  4.1.1 Abstract ..................................................................................................31
  4.1.2 Introduction ............................................................................................31
  4.1.3 Materials and Methods ..........................................................................33
  4.1.4 Results ...................................................................................................35
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1.5</td>
<td>Discussion</td>
<td>41</td>
</tr>
<tr>
<td>4.1.6</td>
<td>References</td>
<td>44</td>
</tr>
<tr>
<td>4.2</td>
<td>Targeting IL-1β and IL-17A driven inflammation during influenza-induced exacerbations of COPD</td>
<td>47</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Abstract</td>
<td>48</td>
</tr>
<tr>
<td>4.2.2</td>
<td>Introduction</td>
<td>48</td>
</tr>
<tr>
<td>4.2.3</td>
<td>Materials and Methods</td>
<td>50</td>
</tr>
<tr>
<td>4.2.4</td>
<td>Results</td>
<td>52</td>
</tr>
<tr>
<td>4.2.5</td>
<td>Discussion</td>
<td>62</td>
</tr>
<tr>
<td>4.2.6</td>
<td>References</td>
<td>65</td>
</tr>
<tr>
<td>4.3</td>
<td>Stromal cell influences upon viral-driven exacerbations of allergic airway inflammation</td>
<td>69</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Abstract</td>
<td>70</td>
</tr>
<tr>
<td>4.3.2</td>
<td>Introduction</td>
<td>70</td>
</tr>
<tr>
<td>4.3.3</td>
<td>Materials and Methods</td>
<td>72</td>
</tr>
<tr>
<td>4.3.4</td>
<td>Results and discussion</td>
<td>74</td>
</tr>
<tr>
<td>4.3.5</td>
<td>Conclusions</td>
<td>85</td>
</tr>
<tr>
<td>4.3.6</td>
<td>References</td>
<td>87</td>
</tr>
<tr>
<td>5</td>
<td>Concluding Remarks</td>
<td>93</td>
</tr>
<tr>
<td>5.1</td>
<td>References</td>
<td>96</td>
</tr>
<tr>
<td>6</td>
<td>Appendix</td>
<td>99</td>
</tr>
<tr>
<td>6.1</td>
<td>Abbreviations</td>
<td>99</td>
</tr>
<tr>
<td>6.2</td>
<td>Acknowledgments</td>
<td>101</td>
</tr>
</tbody>
</table>
1 SUMMARY

Asthma and chronic obstructive pulmonary disease (COPD) are chronic inflammatory disorders of the respiratory tract that currently affect 300 or 65 million people worldwide, respectively. Additional infections with pathogenic microbes can cause acute respiratory distress and exacerbate these chronic diseases, representing a major cause of disease-related morbidity and mortality. Notably, microorganisms do not only play detrimental roles, but can in fact be beneficial for health. For example, the commensal microbiota of the gut is crucial for the development and function of the host immune system in addition to providing important energy and nutrient sources by breakdown of dietary material. Notably, dysbiosis of the commensal microbiota has been reported within the gut and the lung of asthmatics. Particularly within the context of the lung, the mechanisms underlying a potential regulation of allergic responses by commensal bacteria has yet to be deciphered.

In the first part of this thesis we demonstrated that the presence of commensal bacteria is critical for the control of allergic airway responses in mice. We found that in the absence of exposure to any environmental microbes, mice developed an excessive allergic response characterized by increased T helper 2 (Th2) inflammation and exaggerated airway hyperresponsiveness. This was associated with a dysregulated cellular recruitment, resulting in increased numbers of basophils and decreased numbers of plasmacytoid dendritic cells and alveolar macrophages. Moreover, the number of conventional dendritic cells was reduced and their activation state altered, likely indicating a phenotype that is more prone to induce Th2 responses. In conclusion, we report a key role for commensal bacteria in the regulation of allergic responses and demonstrate the importance of the microbiota for an appropriate recruitment and activation of immune cells in the respiratory tract.

In the second part of this thesis we investigated mechanisms of viral-induced exacerbations in murine models of asthma and COPD. We found that interleukin-1β (IL-1β), a pleiotropic cytokine involved in the initiation and persistence of inflammatory responses, is a key mediator of lung dysfunction and neutrophilic inflammation in influenza-induced exacerbations of COPD. IL-1β driven neutrophilia was mediated by IL-17A in the initial phase of viral infection, but became independent of IL-17A later on. In line with this, treatment with an IL-1 receptor antagonist efficiently attenuated the recruitment of neutrophils to the airways at the
peak of viral replication, while blocking of IL-17A abrogated neutrophilia at early time points of the infection. Therefore, we propose IL-1β and IL-17A as valid therapeutic targets for the treatment of viral exacerbations of COPD.

Finally, epithelial cells are key players in asthma exacerbations and represent ideal targets for inhaled therapeutic treatments due to their location at the interface between the lung and the environment. To gain a better understanding of their contribution to exacerbations, we identified genes specifically regulated in structural airway cells during influenza-induced exacerbations of allergic airway inflammation. We found that 37 genes were uniquely regulated only in the context of exacerbations, and that out of these, eight have previously been associated with asthma or asthma exacerbations. Additional to these eight, we suggest that five genes, that have not yet been reported in the context of asthma or exacerbations, are worth pursuing according to their reported functions. Although further studies are necessary to unravel the mechanisms through which these genes influence asthma exacerbations, our work provides novel insight into potential targets for therapeutic approaches against viral exacerbations of disease.

In conclusion, the work outlined in this thesis provides new mechanistic insights into the regulation of allergic airway inflammation by the commensal microbiota and into viral-driven exacerbations of asthma and COPD. The work sheds light on disease mechanisms and highlights possible new target molecules for future therapeutic interventions.
ZUSAMMENFASSUNG


Im zweiten Teil dieser Arbeit untersuchten wir Mechanismen, die den von Viren ausgelösten akuten Krankheitsverschlechterungen, so genannten viralen
Zusammenfassung

Exazerbationen, von Asthma oder COPD zugrunde liegen. In einem Mausmodell von Influenzaviren induzierten COPD Exazerbationen wiesen wir nach, dass Interleukin-1β (IL-1β), ein pleiotropes Zytokin, das an der Auslösung und der Aufrechterhaltung von Entzündungen beteiligt ist, einen entscheidenden Beitrag zur Lungenfehlfunktion und zur neutrophilen Entzündung leistete. Die durch IL-1β ausgelöste Infiltration der Atemwege mit Neutrophilen wurde zu Beginn der Virusinfektion von IL-17A getrieben, im späteren Infektionsverlauf jedoch unabhängig von IL-17A. Dementsprechend konnte die Infiltration von Neutrophilen zum Zeitpunkt der maximalen Virenreplikation durch Verabreichung eines IL-1 Rezeptorantagonisten deutlich verringert werden, während das Neutralisieren von IL-17A den Influx von Neutrophilen zu Beginn der Infektion hemmte. Aufgrund dieser Ergebnisse empfehlen wir IL-1β und IL-17A als wirkungsvolle therapeutische Angriffspunkte für die Behandlung viraler Exazerbationen von COPD.


Zusammenfassend eröffnet diese Doktorarbeit neue Einblicke in die Kontrolle allergischer Atemwegsentzündungen durch die kommunale Flora sowie in durch Viren verursachte akute Verschlechterungen von Asthma und COPD. Unsere Ergebnisse bringen somit neues Licht in die zugrunde liegenden Mechanismen und zeigen neue Zielmoleküle für zukünftige therapeutische Strategien auf.
3 GENERAL INTRODUCTION

3.1 Mucosal surfaces

Mucosal surfaces account for up to 400\,m^2 of the surface area of our body and line the intestinal, genital, and respiratory tract. Being in direct contact with the external environment they contribute to vital functions such as uptake of nutrients and gas exchange, while at the same time they are the entry portal of a wide range of pathogens. As our body is not only exposed to dangerous pathogens, but also to a myriad of harmless environmental antigens, the tremendous challenge for the mucosal immune system is to discriminate between both. While innocuous non-pathogenic stimuli have to be immunologically tolerated or ignored in order to prevent unnecessary tissue destruction and organ dysfunction, pathogenic attacks have to be answered with a robust immune response in order to clear the invading organism. In both cases, dysregulation of the immune system can lead to inappropriate responses and have severe consequences. Indeed, mounting an immune response against a harmless antigen can initiate the development of chronic diseases such as asthma. But failure of an appropriate defense against a pathogen can cause severe tissue destruction and systemic infection, leading to organ failure and death. A tight regulation and control of immune responses at body surfaces is therefore crucial to maintain vital function and survival.

3.2 The innate immune system

3.2.1 The role of the epithelium in homeostasis

The immune system consists of two arms of defense that work in concert to fight invading pathogens. While innate immunity provides a comparatively non-specific, immediate first-line defense, the adaptive immunity requires some days to develop, but acts in an antigen specific manner and can provide lifelong memory against reinfection.

As the lung is constantly exposed to harmless antigens that should be immunologically ignored in order to retain homeostasis, the pulmonary environment is rather immunosuppressive. The epithelium plays a key role in physically and biologically preserving this suppressive milieu. By building tight and adherence
junctions between single epithelial cells it constitutes an impermeable barrier for inhaled particles and shields the body from entry of harmful and harmless antigens\(^3\). Secretory epithelial cells produce anti-microbial peptides\(^2\) and a mucus layer that covers the epithelium. Inhaled antigens are thus trapped by the mucus and subsequently expelled by ciliary transport\(^4\). Additionally, epithelial cells produce a variety of inhibitory molecules in steady state, such as CD200, IL-10, TGF-β, and mucin-1\(^2,5,6\). These keep lung resident immune cells such as alveolar macrophages and dendritic cells in a state of low responsiveness and thus maintain immune homeostasis.

### 3.2.2 The pulmonary immune system in infection

Once a pathogen is recognized by the innate immune system, several alert mechanisms are switched on and the suppressive pulmonary environment can change rapidly into a highly inflammatory one. The recognition of pathogens is mediated by pattern recognition receptors (PRRs) that are expressed by most cells of the innate immune system\(^7\). A wide range of PRRs can be found on airway epithelial cells, macrophages and dendritic cells, which are closely associated with mucosal tissues and are typically the first cells to encounter foreign antigens. PRRs recognize conserved pathogenic antigens, so-called pathogen associated molecular patterns (PAMP), and once triggered initiate an innate and adaptive response against the invading organism\(^7\). Epithelial cells stimulated through PRR ligation produce a wide range of proinflammatory mediators that orchestrate the ensuing immune response\(^8\). They elicit the recruitment and activation of additional innate immune cells, such as neutrophils and inflammatory monocytes, in order to defend the host against the pathogen. Neutrophils are one of the most crucial fighters against many infections and eliminate microorganisms by producing a myriad of effector molecules such as proteases, defensins, myeloperoxidase, elastases and reactive oxygen species\(^9,10\). They can release DNA neutrophil extracellular traps that contain anti-microbial proteins and thus trap and kill the pathogen\(^11\). However, given the broad and unspecific effector functions of neutrophils, they also cause damage of host cells and tissues and are thus involved in the pathology of several inflammatory disorders\(^12,13\).

Dendritic cells (DC) are similarly activated by PRR triggering and additionally by epithelial derived factors\(^14\). Activated DCs mature and migrate to the lung draining lymph node where they activate naïve antigen-specific T cells and differentiate them into effector cells, thus initiating an adaptive immune response\(^15\). Primed T cells
subsequently migrate to the lung where they exert their full effector function upon restimulation by local DCs. DCs integrate signals from invading pathogens and innate cells into a carefully tailored signal for T lymphocyte stimulation. They are therefore considered as a key bridge between the innate and the adaptive immune system.

3.2.3 Pulmonary dendritic cells: immune sentinels

In the murine lung 5 distinct subsets of dendritic cells have been described, depending on their location and their surface markers. They present a heterogeneous population of antigen-presenting cells that can be divided into two main subsets, plasmacytoid DCs (pDCs) and conventional DCs (cDCs).

Plasmacytoid DCs represent only a minor population and are localized in the conducting airways and the parenchyma (Fig 1). They can be characterized by the surface expression of PDCA-1, B220, Ly-6C, and Siglec-H. They express some PRRs, such as some Toll-like receptors (TLR), and can react with a rapid production of type I interferons following microbial stimuli. However, their contribution to immune responses is controversial and appears to vary depending on the type of ongoing inflammation. Evidence indicates that pDC depletion does not impact on viral clearance or the strength of the anti-viral CD8+ T cell response in influenza infections. But on the contrary, pDCs have been shown to be required for the induction of inhalation tolerance, and in this case their depletion resulted in the breaking of tolerance. In line with this, pDCs have been shown to have anti-inflammatory effects in allergen challenges in mice.

Conventional DCs are the major drivers of immune responses and can be found in the conducting airways as well as in the lung parenchyma (Fig 1). They express high levels of the integrin CD11c, the antigen-presenting molecule major histocompatibility complex II (MHCII), and can be further subdivided depending on their tissue localization. The lung parenchyma contains CD11b+ and CD11b− cDCs that can also access the alveolar lumen, while the conducting airways are populated by submucosal CD103+CD11b+ cDCs and intraepithelial CD103+CD11b− cDCs. Intraepithelial cDCs are positioned directly below the epithelium and protrude cellular extensions into the airway lumen that they utilize to sample continuously for inhaled antigens. Being in close proximity to the airway epithelium, pulmonary dendritic cells are in constant crosstalk with epithelial cells and receive regulatory and
proinflammatory signals according to the current inflammatory situation\textsuperscript{14, 21}. The importance of this epithelial-dendritic cell communication has been demonstrated by showing that stimulation of TLR4 on epithelial cells is sufficient to recruit and activate DCs\textsuperscript{22}. Furthermore, epithelial cell derived factors are key to the recruitment, differentiation and maturation of other DC subtypes during inflammation. Those newly recruited cDCs are derived from monocytes and are called inflammatory cDCs. They are highly active and can be distinguished by retained surface expression of the monocyte marker Ly-6C, and by the expression of CD11c, CD11b, and FcεRIα.

Apart from these major subsets of pulmonary DCs, additional subtypes have been described varying from case-to-case depending on the conditions investigated. DC biology is further complicated as distinct subsets exert different functions\textsuperscript{15} that might depend on the kind of ongoing inflammation and the time point investigated. In influenza infections for example, CD11b\textsuperscript{−} cDCs are the main antigen presenting cells priming CD4\textsuperscript{+} and CD8\textsuperscript{−} T cell, while CD11b\textsuperscript{+} cDCs are poor in antigen presentation and thought to rather contribute by producing an array of inflammatory mediators\textsuperscript{16}. CD11b\textsuperscript{−} DCs have also been shown to efficiently cross-present inhaled innocuous antigen to CD8\textsuperscript{+} T cells\textsuperscript{23}. Furthermore, in secondary asthma challenges inflammatory like CD11b\textsuperscript{+} cDCs were sufficient to restore all characteristic features of asthma that were abrogated by depletion of CD11c\textsuperscript{+} cells\textsuperscript{24, 25}.

In one project of this thesis we investigated the phenotype of pulmonary DCs during the absence or presence of commensal bacteria in a mouse model of allergic airway inflammation. We distinguished the main DC subpopulations such as CD11b\textsuperscript{+} and CD11b\textsuperscript{−} cDCs as well as pDCs and characterized their state of activation.
General introduction

Figure 1: Dendritic cell subsets in the murine lung. Pulmonary dendritic cell subsets are differentiated according to their location and their expression of surface markers. Intraepithelial cDCs (CD103⁺ CD11b⁻) are located directly below the epithelium and protrude dendrites into the airways thus sampling the inhaled air for antigens. Conductive airways contain furthermore submucosal cDCs (CD103⁻ CD11b⁺). The lung parenchyma is populated by CD11b⁻ and CD11b⁺ cDCs that can also access the alveolar lumen, while plasmacytoid DCs can be found in airways and parenchyma. Upon infections, monocyte-derived inflammatory DCs are recruited to the airways and the parenchyma. Figure adapted from Holt Nat Rev Immunol 2008 vol. 8 (2) pp. 142-52.

3.2.4 IL-1β induction and signaling

Interleukin-1β (IL-1β) is a member of the IL-1 family and is mainly produced by monocytes, macrophages, and dendritic cells, in response to inflammatory agents and infections. It is transcribed as a proform and has to be cleaved to gain full effector function. Processing of proIL-1β is mediated by a multiprotein complex, the inflammasome. Inflammatory stimuli such as LPS, TNFα, or IL-1β itself, induce the expression of proIL-1β as well as of NLRP3, a component of the inflammasome. NLRP3 belongs to the Nod-like receptor family of PRRs and remains in an autorepressed state in the absence of inflammation. Upon activation by danger signals such as reactive oxygen species, extracellular ATP, decreased intracellular potassium concentrations, uric acid crystals, or lysosomal release of NLRP3...
activating contents, autorepression is released and NLRP3 oligomerizes. This leads to the binding of the adapter protein ASC, and the subsequent recruitment of inactive caspase-1. Caspase-1 clustering induces its own activation by autoprocessing and allows proteolytic cleavage of proIL-1β. In the last years inflammasome independent cleaving mechanisms of IL-1β have been discovered and serine proteases, elastase, matrix metalloproteases and caspase-8, 30 have been shown to be capable to process proIL-1β.

IL-1β exerts its function through the IL-1 receptor I (IL-1RI) which is constitutively expressed by most cells. Binding of IL-1β to IL-1RI allows the recruitment of the IL-1R accessory protein (IL-1RAcP), thus leading to the formation of a heterodimer competent for signaling. Signal transduction is accomplished by consecutive activation of MyD88 (myeloid differentiation primary response protein 88), IRAK4 (IL-1 receptor-associated kinase), and TRAF6 (TNFR associated factor 6) and results in the activation of the NF-kB (nuclear factor kappa B), the MAPK p38 (mitogen-activated protein kinase), and the JNK (c-Jun N-terminal kinase) pathway. IL-1β signaling is regulated by the expression of IL-1RII, a decoy receptor incompetent for signal transduction, and by the IL-1 receptor antagonist (IL-1Ra) that competes with IL-1β for receptor binding.

IL-1α is structurally related to IL-1β and signals through the IL-1RI as well, thus triggering the same biological activities as IL-1β. Nevertheless, IL-1α and IL-1β have different functional contributions during inflammation due to differences in gene expression regulation, in cellular expression patterns and in activation. Additionally, IL-1β is secreted and can have systemic effects while IL-1α remains mainly membrane-bound acting thus predominantly locally.

3.2.5 IL-1β effector function and implication in diseases

IL-1β is a pleiotropic cytokine implicated in the initiation and persistence of inflammatory responses. It acts on several cells of the immune system and enhances inflammation by inducing proinflammatory cytokine and chemokine production, promoting cell survival, enhancing phagocytosis, mediating degranulation, oxidative burst, and protease release. Furthermore, IL-1β promotes T cell survival and expansion, and is particularly important for the differentiation and maintenance of T helper 17 (Th17) cells. Due to its strong proinflammatory properties IL-1β has been shown to be protective in bacterial and viral infections, but it has also...
been linked to the pathology of several inflammatory disorders. Accordingly, inhibition of IL-1β signaling can reduce inflammation and thus disease severity, but it can at the same time increase the susceptibility to infections and impair pathogen clearance. Thus, influenza infected mice deficient for IL-1RI displayed overall reduced inflammation but exhibited increased mortality. However, in inflammatory disorders not involving a pathogenic infection, inhibition of IL-1 has been shown to efficiently reduce inflammation and disease severity without causing severe side effects. Consequently, several molecules targeting IL-1 signaling are either already approved for clinical use or undergoing clinical investigation. Therapy with the recombinant IL-1Ra antagonist, anakinra (Kineret™) is highly beneficial in patients with systemic onset juvenile idiopathic arthritis and adult onset Still’s disease, and it has been approved for rheumatoid arthritis treatment for years. Recent studies treating type 2 diabetes patients either with anakinra or an anti-IL-1β antibody resulted in improved glycemia scores and improved beta cell function. Moreover, a pilot study using anakinra in gout documented a rapid relief of inflammatory symptoms. As studies in mice indicated that IL-1 is also involved in the development of inflammation and emphysema in COPD, one project in this thesis addressed the role of IL-1β during viral infections of mice suffering from COPD. By studying IL-1β driven mechanisms of exacerbations we sought to elucidate whether IL-1β serves as a valid therapeutic target in this disease indication.

3.2.6 Induction and function of IL-17A

IL-17A is the signature cytokine of the T helper subset 17 (Th17) of CD4+ T cells. Th17 cells differ from other T helper subsets by the expression of the transcription factor RORγt. They require stimulation by combinations of IL-6, TGF-β, IL-21, IL-1β, and IL-23 for their differentiation, expansion and survival (Fig 2). Besides Th17 cells, γδ T cells are a major source of IL-17A, and NK cells, NKT cells, lymphoid tissue inducer cells, and some non-immune cells have been described to express IL-17A. For the induction of IL-17A in γδ T cells, stimulation by IL-1β and IL-23 is sufficient, as they constitutively express RORγt, IL-1RI, and IL-23R. γδ T cells thus constitute one of the primary sources of IL-17A early during infections, although their importance remains to be fully unraveled.

IL-17A plays a key role in the defense against bacterial and fungal infections by inducing the expression of various proinflammatory cytokines (e.g. TNFα, IL-1, IL-6, GM-CSF, G-CSF), chemokines (e.g. CXCL1, CXCL2, CXCL5, IL-8, CCL2, CCL7),
anti-microbial peptides (e.g. defensins), and matrix metalloproteases from fibroblasts, endothelial, and epithelial cells (Fig 2)\textsuperscript{58}. It stimulates granulopoiesis and induces the recruitment of neutrophils to inflammatory sites leading to the eradication of the invading microorganism\textsuperscript{58,64,65}. However, due its strong proinflammatory effects and the induction of neutrophil recruitment, IL-17A has also been associated with several autoimmune diseases such as multiple sclerosis, psoriasis, rheumatoid arthritis, inflammatory bowel disease, and allergic diseases such as contact hypersensitivity (Fig 2)\textsuperscript{66}. In line with this, inhibition of IL-17A has been shown to improve disease severity in rheumatoid arthritis or psoriasis in recent clinical trials\textsuperscript{67,68}.

Figure 2: The role of IL-17A during infection and inflammation. IL-17A is produced by several cell types including T cells, innate cells, and structural cells, in response to cytokines such as TGF-b, IL-6, IL-1\textbeta, IL-23, IL-21. It induces the expression of many cytokines and chemokines as well as anti-microbial peptides and matrix metalloproteinases. These mediators recruit neutrophils, thus contributing to host protection but also to tissue destruction and the development of autoimmune and allergic diseases. Figure adapted from Iwakura, Immunity 2011 vol.34(2) pp.149-62\textsuperscript{58}.

3.3 Chronic pulmonary diseases

3.3.1 COPD and asthma: similarities and differences

COPD and asthma are chronic inflammatory disorders of the respiratory tract. Both are heterogenic diseases whose development is driven by an interplay between genetic susceptibility and environmental influences. A key feature of both diseases is a limitation of the airflow that is caused by airway obstruction. Despite their similarity in some clinical symptoms, immunological mechanisms in COPD and asthma are markedly different. Airway obstruction is a progressive, irreversible process in COPD that affects mainly small airways, while in asthma it is reversible and primarily restricted to the conducting airways\textsuperscript{69}. COPD is characterized by a T helper 1 (Th1)
and CD8⁺ T cell mediated adaptive response and a mainly neutrophilic inflammation. Comparatively, asthma is most well recognized to be mediated by a T helper 2 (Th2) response and the associated accumulation of eosinophils. Nevertheless, in severe asthma the inflammation pattern changes and resembles more of that found in COPD, with increased neutrophilia, a mix of Th1 and Th2 cells, and an increase in CD8⁺ T cells.

### 3.3.2 COPD pathology

Emphysema and chronic bronchitis are key pathological features of COPD. They contribute to the development of irreversible airway obstruction resulting in airflow limitation and thus a progressive loss of lung function. Destruction of alveolar walls and alveolar attachments to small airways causes decreased lung elasticity, increased airway obstruction, and even airway collapse (Fig 3). Small airways are occluded by chronic inflammation and mucus hypersecretion, and the airway wall is thickened (Fig 3). Thus, airway obstruction is increased and airflow limitation further potentiated. It is also notable that the alveolar damage characteristic of COPD diminishes the surface area available for gas exchange, rendering the lungs less effective in this core function.

![Figure 3: Mechanisms of airflow limitation in COPD.](image)

Figure 3: Mechanisms of airflow limitation in COPD. While in a healthy person small airways are held open by alveolar attachments, disrupted alveolar attachments in COPD contribute to airway closure during expiration. Chronic inflammation and mucus hypersecretion obstruct small airways, thus potentiating airflow limitation. Figure adapted from Barnes PJ, Nat Rev Drug Discov 2002 vol. 1 (6) pp. 437-46.
3.3.3 Lung function in COPD

COPD is classified into four severity stages by the global initiative for COPD. They are defined by a decrease in lung function, more specifically by the reduction of the amount of air that can be exhaled in the first second of expiration, the forced expiratory volume in one second (FEV1). The FEV1 is affected by emphysema and inflammation, as they both impair exhalation, and it decreases with progressing COPD. The full volume capacity (FVC) describes the total amount of air exhaled after a full inspiration, and is not affected in mild to moderate COPD patients. In severe disease a decrease in FVC might occur though due to air being trapped in the lungs. Lung function can be further characterized by measuring pulmonary resistance and compliance. The airway resistance $R_n$ reflects only the constriction of the airways while the total pulmonary resistance $R$ assesses the resistance of the airways and the tissue. Both can be influenced by a variety of factors such as inflammation, loss of tissue elasticity, tissue destruction, airway collapse, muscle contraction, and fibrosis. Thus, the values of resistance are generally increased in COPD patients. While the resistance is mainly linked to airway obstruction, the compliance reflects the ease with which the lungs extent. As the lungs are highly elastic, they stretch to a certain extent during inhalation and recoil to their previous state upon exhalation. When lung tissue and thus elastic fibers are damaged like in emphysema, the lungs stretch easier and have a poor elastic recoil which results in an increased compliance in COPD patients.

To assess the described lung function parameters in mice, we used an invasive measurement technology. Following deep anaesthetization, the mice were tracheotomized and mechanically ventilated to ensure physiological gas exchange. Thus, resistance and compliance, but also FEV and FVC could be determined, the latter ones being assessed against a negative pressure reservoir to ensure complete expiration.

3.3.4 Exacerbations of asthma and COPD

Even though the disease pathology of COPD and asthma is distinct, individuals with either disease experience periods of acute symptom worsening, so-called exacerbations, that are primarily caused by bacterial or viral infections. Exacerbations are characterized by increased wheezing, coughing, and shortness of breath, and can result in an acute drop in lung function necessitating
hospitalization\textsuperscript{84-86}. They are thus the major cause for disease related morbidity, and in the case of COPD are also associated with mortality. The acute inflammation developing during exacerbations is mediated by an enhanced production of inflammatory cytokines and chemokines. These cause the recruitment of additional inflammatory cells, in COPD mainly neutrophils\textsuperscript{83} and in asthma neutrophils and eosinophils\textsuperscript{87, 88}. Additionally, excessive mucus production causes the formation of mucus plugs in the airways that cause further obstruction and impair respiratory function. Although a variety of molecules and cell subsets have been identified in patients during exacerbations, the specific mechanism driving exacerbations is still poorly understood. Due to safety reasons, and the often severe health status of patients undergoing an exacerbation, it is difficult to receive clinical samples for detailed studies. It is therefore crucial to investigate exacerbations of chronic lung diseases in animal models. A better understanding of disease mechanisms might then open new doors for potential therapeutic strategies.

In this thesis, we developed a murine model of viral-induced exacerbations of COPD. We were able to study IL-1β dependent mechanisms of influenza-induced exacerbations, and consequently the efficacy of potential therapeutic strategies based on the results found.

3.4 Microbial impacts on disease development and health

3.4.1 Infections of the respiratory tract

The lung is constantly exposed to pathogenic microorganisms, including bacteria, fungi, and viruses, that infect the respiratory tract in order to survive, multiply, and spread. When they are not efficiently controlled and cleared they can cause severe diseases and death, particularly in people with compromised immunity such as young children, elderly, or persons with chronic disorders. Indeed, acute respiratory infections are the leading cause of acute illnesses worldwide according to the world health organization\textsuperscript{89, 90} and they are the predominant cause of mortality in infants and young children\textsuperscript{91-93}. Respiratory infections can cause exacerbations in patients suffering from chronic diseases such as COPD or asthma and thus contribute significantly to disease related morbidity and mortality\textsuperscript{84-86}. Moreover, repeated respiratory infections in early childhood can cause a predisposition to the development of asthma later in life\textsuperscript{94, 95}. 
3.4.2 Beneficial effects of commensal bacteria

Our mucus membranes and the skin epithelium are colonized by microorganisms shortly after birth. In the lower intestine, the density of commensal bacteria reaches $10^{12}$ organisms per gram of intestinal contents and includes a broad variety of species. The lungs had been considered as sterile, however it is now clear that the lung is colonized by a persistent microbial flora even under healthy conditions. As the ‘healthy’ respiratory microbiome was first reported in 2010, most data to-date concerning commensal bacteria is based on investigations of the gut microflora.

The composition of the microflora differs in individuals and can be influenced by several factors such as diet, antibiotics, infections, inflammation, stress, hygiene and host genetics. Commensal bacteria of the gut perform many beneficial functions such as degradation of indigestible dietary substances, synthesis of vitamins, and protection against pathogenic colonization. Moreover, the microbiota is crucial for the development and function of the host immune system, and commensal bacteria have been shown to influence inflammation not only in the gut but also at peripheral sites including the lung. A new emerging idea suggests that changes in diet and associated changes in gut microbiota drive the increased incidence of inflammatory diseases in developed countries. This theory might replace or at least supplement the long proposed “hygiene hypothesis”, which suggests that reduced exposure to pathogens due to a higher hygiene standard is responsible for the increased risk of allergic diseases and asthma in developed countries.

Epidemological evidence for a “dietary hypothesis” comes for example from Japan, a country with a high degree of hygiene, but a very distinct diet, and a coinciding low number of asthmatics.

Certain chronic diseases are associated with changes in the composition and diversity of the gut microbiota. Notably, infants susceptible for asthma have already a different gut microbiota with the age of three weeks compared to non-susceptible newborns. Recent studies found that also the respiratory microbiota differs in patients with chronic diseases such as asthma and COPD. Whether those changes in the respiratory microflora contribute to the development of diseases as well or whether they are rather a by-product of diseases remains to be elucidated.

Influences of commensals on disease development can be studied in germ-free mice that lack the commensal microflora and have never been exposed to any
microorganism. One part of this thesis accomplished in collaboration with the group of Prof. Harris (EPFL, Switzerland) addresses the question whether the microbiota impacts on the development of allergic airway inflammation and the mechanisms underlying its influence.
3.5 References


4 Results

4.1 Dysregulation of allergic airway inflammation in the absence of microbial colonization

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4.1.1 Abstract

The increasing incidence of allergic diseases in developed countries has been associated with reduced exposure to microorganisms and changes in the commensal flora. Although epidemiological studies have indicated that dysbiosis of the microbiota can influence allergic responses, experimental evidence is still missing and underlying mechanisms are unknown. In this study, we report that germ-free (GF) mice that lack any exposure to environmental microorganisms exhibit an increased allergic airway inflammation, marked by enhanced airway hyperresponsiveness and aggravated T helper 2 (Th2) inflammation, compared to specific pathogen free (SPF) mice. This increased inflammation can be reversed by recolonization with a complex microflora of SPF mice. We further report that the exaggerated inflammation is associated with an impaired regulation of inflammatory and regulatory cells in the lung. Thus, numbers of lung plasmacytoid dendritic cells (pDC), conventional dendritic cells (cDC), and alveolar macrophages were reduced, the number of basophil in the airways was increased, and the maturation state of pulmonary cDCs was altered in the absence of the microbiota. In conclusion, our data demonstrates that the presence of commensal bacteria is critical for normal cell recruitment, maturation, and regulation of allergic airway inflammation.

4.1.2 Introduction

The prevalence of allergic diseases has increased notably in developed countries over the last decades. It was hypothesized that this augmented risk results from the reduced exposure to pathogens due to an improved hygiene status. Variations in the exposure to environmental microorganisms early in life have indeed been linked to enhancement of, or protection from, allergic diseases. Moreover, changes in the gut microbiota have been associated with chronic diseases such as type 1 diabetes, obesity, experimental autoimmune encephalomyelitis, and also with the development of allergic diseases. In addition, epidemiological studies have shown dysbiosis in bacterial communities within the gut and also the lung of asthmatics. Commensal bacteria colonize all human body surfaces like mucus membranes and the skin starting from birth. The greatest density of colonizing bacteria can be found in the intestine, with more than 10^{12} organisms per gram of intestinal content, and a diversity of at least 1000 different species. The composition of the microbiota differs in individuals and is influenced by a variety of factors such as maternal transfer.
during birth\textsuperscript{12}, antibiotics, infections, hygiene, and diet\textsuperscript{13}. Only recently it has been recognized that also the respiratory tract is colonized by bacteria\textsuperscript{10}.

Most research has focused on the commensal bacteria in the gut and it is widely accepted that the gut microbiota shapes immune responses in the digestive tract\textsuperscript{13-16}. For many years it has been suggested that immune responses at mucosal sites, such as the gut and the lung, are linked and might influence each other, although underlying mechanisms were yet to be defined\textsuperscript{17}. Recent data demonstrates that the gut microbiota can indeed influence inflammation at peripheral sites such as the lung\textsuperscript{18}. Thus, probiotic or prebiotic treatment might not only be beneficial in gastrointestinal disorders\textsuperscript{14}, but also in lung diseases.

To better understand how the microbiota impacts on allergic diseases we compared allergic airway inflammation in germ-free (GF), recolonized (Recol), and specific pathogen free (SPF) mice. GF mice are born and housed under sterile conditions, thus lacking any exposure to microorganisms while recolonized mice are former GF mice that were recolonized with a complex SPF microflora.

This project was accomplished in collaboration with Tina Herbst from the group of Nicola Harris. Following her findings that the microbiota did indeed impact on the development of allergic airway hyperresponsiveness, we assessed various cell types of the respiratory immune system in detail in order to elucidate mechanisms underlying this commensal imprinting. We specifically focused on dendritic cell (DC) responses, as they have been shown to be influenced by commensal bacteria\textsuperscript{19}. As an altered commensal composition was observed in gut\textsuperscript{7-9} and lung\textsuperscript{10} of asthmatics, and as dendritic cells are known to be key to allergic airway inflammation\textsuperscript{20, 21}, one can hypothesis that dendritic cells that are not appropriately educated by the commensal flora would have a major influence in allergic inflammation.

In this study, we indeed found that the commensal flora influences dendritic cell numbers and function. We noted that in the absence of a microflora, dendritic cell subpopulation proportions and activation states were reduced. Moreover, macrophage numbers were reduced, whilst basophil recruitment was enhanced. Our data shows for the first time that the commensal flora plays a key role in regulating cells involved in pulmonary inflammation.
4.1.3 Materials and Methods

Mice
Germ-free C57BL/6 mice (originally obtained from Harlan) were kindly provided by the Institute of Laboratory Animal Science, University of Zurich, or from the Clean Animal Facility, University of Bern, Switzerland. GF mice were housed in flexible isolators until the day of sacrifice and their germ-free status was controlled by culturing fecal samples and swabs of the inner wall of the isolators under aerobic and anaerobic conditions. In addition, gram stains and DNA stains were performed of fecal samples collected immediately before export from the isolators. Specific pathogen free (SPF) C57BL/6 mice (originally obtained from The Jackson Laboratory) were housed in a SPF facility and fed the same food as GF mice. Recolonization of 5-6 week old GF mice was established by housing a SPF mouse in the same cage for at least 3 weeks prior to the first immunization. Mice were between 9-12 weeks of age when the experiments were performed. All animal experiments were accomplished according to institutional guidelines and to Swiss federal and cantonal laws on animal protection.

Protocol for experimental allergic airway inflammation (see Fig 1A)
For immunization, mice received 100 μg ovalbumin (OVA) (Sigma-Aldrich) in 200 μl 2% aluminum hydroxide (SERVA Electrophoresis GmbH) intraperitoneal. They were subsequently challenged intranasally with 100 μg OVA in 50 μl PBS on day 9 and 10 post immunization. Control mice were either immunized with aluminum hydroxide only and challenged with OVA in PBS or were untreated. Both control groups showed comparable results. Cell subsets and cytokines in lung and bronchoalveolar lavage (BAL) were assessed 4 days after the last OVA challenge. Total cell numbers in the BAL were determined and differential cell counts were performed on cytospins stained with QuickDiff (Dade Behring) according to standard criteria.

Airway function
Airway hyperresponsiveness (AHR) was assessed by unrestrained plethysmography (Buxco Electronics) 3 days after the last OVA challenge. The main parameter of airflow obstruction, enhanced pause (Penh), was determined by exposure of mice to PBS as baseline control, followed by increasing concentrations of methacholine-chloride.
Histology
Lungs were inflated with 1 ml of 10% formalin and embedded into paraffin. Sections (4 µm) were stained with hematoxylin and eosin (H&E) or periodic acid Schiff reaction (PAS) using standardized protocols. Stained sections were analyzed using an Axioskop 2 plus microscope equipped with AxioCam HRc (Zeiss).

Flow cytometry
To analyze cytokine production, BAL cells were restimulated in vitro with PMA, ionomycin, and monensin (Sigma–Aldrich) for 3 h at 37 °C. Cells were subsequently stained with anti-CD4 PerCP, fixed with 2% paraformaldehyde, permeabilized with saponin buffer and stained with antibodies against IL-4, IL-5, IL-10, and IFNγ. For the analysis of antigen presenting cell subsets lung tissue was digested using collagenase IV (BioConcept). Antigen presenting cells were characterized by antibodies against CD11c APC-Cy7, CD11b PerCP-Cy5.5, F4/80 APC (eBioscience), I-A/I-E Alexa700, PDCA-1 Alexa647, CD45R/B220 FITC, and CD40 PE, CD80 PE, Ox40L PE, CD86 Biotin, CD137 Biotin, ICOSL Biotin, for activation markers using Streptavidin PE-Cy7 as detection antibody. T regulatory cells of lung and BAL were determined using antibodies against CD4 PerCP, CD25 PE (eBioscience), and Foxp3 APC (eBioscience). Basophils were identified with antibodies against CD49b PE (BD Biosciences) and IgE FITC. All antibodies were purchased from Biolegend if not indicated otherwise. Stained cells were acquired on a FACS Calibur or FACS LSR II (BD Biosciences) and the data was analyzed using FlowJo software (TreeStar).

Statistical analysis
Statistical differences were calculated by performing a Student’s t test (unpaired, two-tailed). P values of less than 0.05 were considered significant and were depicted as *p<0.05; **p<0.01; ***p<0.001.
4.1.4 Results

Allergic airway inflammation is exaggerated in the absence of the commensal microbiota.

To address whether commensal bacteria impact on allergic airway disease, we induced a Th2 inflammation against ovalbumin (OVA) in germ-free (GF) mice, specific pathogen free (SPF) mice, and GF mice recolonized with a complex SPF microbiota (Recol). Mice were immunized with OVA, using alum as adjuvant, and intranasally challenged with OVA antigen according to Fig 1A. Airway hyperresponsiveness (AHR) was assessed by unrestrained whole body plethysmography, an indirect measurement of lung function that indicates airway obstructions. As expected, antigen challenged SPF mice responded with an increased AHR to the bronchoconstrictor methacholine compared to control SPF mice (Fig 1B). OVA challenged GF mice exhibited a further aggravated AHR compared to their SPF counterparts which reflects a more severe allergic response (Fig 1B).

Reduced airflow in the absence of commensals correlated with increased goblet cell hyperplasia and higher peribronchial and perivascular cell infiltrates as determined by histological sections (Fig 1C). Moreover, GF mice exhibited an overall exaggerated inflammatory Th2 response characterized by a striking increase in total cell numbers (Fig 1D), eosinophils (Fig 1E), and lymphocytes (Fig 1F), and significantly enhanced production of the key Th2 effector cytokines IL-4 (Fig 1G) and IL-5 (Fig 1H) in CD4⁺ T cells of the airways. The T helper 1 (Th1) cytokine IFNγ (Fig 1I) and the regulatory cytokine IL-10 (data not shown) were not altered in the absence of commensals, thus indicating that the exaggerated Th2 response is not the result of a dysregulated Th1 or T regulatory response.

Recolonization of GF mice was sufficient to reverse the enhanced Th2 inflammation, and cell numbers and Th2 cytokine production were comparable to those found in SPF mice (Fig 1D-I).
Figure 1: Commensal bacteria attenuate allergic airway inflammation. (A) Experimental protocol of ovalbumin (OVA) induced allergic airway inflammation. (B) Airway hyperresponsiveness (AHR) upon exposure to increasing concentrations of methacholine was assessed in specific pathogen free (SPF) and germ-free (GF) mice by whole body plethysmography (n=5-9). (C) Histological sections of lungs from control or OVA treated GF or SPF mice were stained with H&E or PAS. Enhanced cellular infiltration and mucus production in GF compared to SPF mice following OVA challenge was observed. Results are representative of two independent experiments (n=4-6). Error bars represent standard error of the mean. (D) Total cell numbers and total numbers of (E) eosinophils and (F) lymphocytes
were determined by cytospins in the BAL of SPF, recolonized (Recol), and GF mice. Results are pooled from three experiments (controls: n=6, OVA: n=10-14) and are representative of 5 independent experiments. (G-I) Percentage of (G) IL-4, (H) IL-5, (I) IFNγ produced by CD4+ T cells in the bronchoalveolar lavage (BAL) was determined by flow cytometry following unspecific in vitro restimulation. Results are pooled from two experiments (n=7-10) and are representative of 4 independent experiments. Mean ± standard error of the mean (s.e.m.) is shown. Figure adapted from Herbst, Sichelstiel, et al., Am J Respir Crit Care Med. 2011 Jul 15;184(2):198-205.

Commensal bacteria alter the number and activation state of lung dendritic cells.

Several different antigen-presenting cells can shape the induction and effector function of an adaptive T cell response, acting either locally or upon migration to the draining lymph nodes. In order to decipher the impact of the microbiota on distinct antigen-presenting populations, we utilized flow cytometry analyzes of single cell suspensions from lung tissue.

To distinguish macrophages from dendritic cells we utilized the macrophage marker F4/80. Alveolar macrophages were determined from the F4/80 positive population by autofluorescence and expression of CD11c, while interstitial macrophages were distinguished by expression of F4/80 but lack of CD11c (Fig 2A).

Conventional DCs were defined as F4/80−CD11c+ cells and subdivided by their expression levels of CD11b and major histocompatibility complex (MHC) II into three subsets, CD11b+MHCIIhi, CD11b+MHCIIint, CD11b−cDCs (Fig 2A, 3A). Plasmacytoid DCs were characterized by expression of PDCA1 on B220+ cells, and further differentiated by the lack of CD11b (Fig 2B) and MHCII (data not shown), as well as by low granularity.


Regulation of allergic inflammation by the microbiota

A

SPF control

F4/80 cells

FSC

CD11c

CD11c

Alveolar Macrophages
(F4/80+ CD11c+ APC+)

Interstitial Macrophages
(F4/80+ CD11c)

CD11b+ MHCII+ cDCs

CD11b+ MHCII+ cDCs

CD11b+ cDCs

SPF OVA

F4/80 cells

FSC

CD11c

CD11c

Alveolar Macrophages
(F4/80+ CD11c+ APC+)

Interstitial Macrophages
(F4/80+ CD11c)

CD11b+ MHCII+ cDCs

CD11b+ MHCII+ cDCs

CD11b+ cDCs

B

SPF control

B220+ cells

FSC

CD11c

CD11b

PDCA-1

PDCA-1

pDCs

SPF OVA

PDCA-1

pDCs

PDCA-1

pDCs
Figure 2: Differentiation of dendritic cell and macrophage subsets in the lung. Subsets of dendritic cells and macrophages were determined by flow cytometry in lung tissue of control mice and mice suffering from OVA-induced allergic airway inflammation. (A) Macrophages were differentiated from conventional dendritic cells (cDC) by expression of F4/80. They were subsequently divided into CD11c⁺autofluorescent⁺ (AF) alveolar macrophages and CD11c⁻ interstitial macrophages. Conventional dendritic cells were determined from the F4/80 negative population by expression of high levels of CD11c. They were further subdivided according to their expression levels of CD11b and MHCII into CD11b⁺MHCII⁺, CD11b⁺MHCII⁻, and CD11b⁻ cDCs. (B) Plasmacytoid dendritic cells (pDC) were defined by expression of PDCA-1 on B220⁺ live cells, followed by gating on CD11b⁻ cells with low granularity and low expression of MHCII (data not shown). Figure adapted from Herbst, Sichelstiel, et al., Am J Respir Crit Care Med. 2011 Jul 15;184(2):198-205.

Upon exposure to OVA we observed reduced numbers and an overall decreased activation of all cDC subsets in GF mice (Fig 3A-D). In detail, all cDC subsets exhibited decreased expression of CD40, CD86, and/or CD80 in the absence of commensals (Fig 3B-D). CD137 and Ox40L levels were also reduced in CD11b⁺MHCII⁺ cDCs (Fig 3B), but remained unaltered in the CD11b⁺MHCII⁻ (Fig 3C) population. CD137 expression was not affected and Ox40L was not detectable in CD11b⁻ cDCs (Fig 3D). Notably, ICOSL expression was either unaffected by the absence of commensals, as observed for CD11b⁺MHCII⁺ cDCs (Fig 3B), or even increased, as found for CD11b⁺MHCII⁻ (Fig 3C) and CD11b⁻ cDCs (Fig 3D). Although still controversial, ICOSL has been implicated in promoting Th2 responses.

Taken together, reduced cell numbers, decreased activation, and enhanced ICOSL expression might indicate a Th2-biasing contribution of cDCs to the lung environment. Although the functional role of each cDC subset during allergic airway inflammation remains to be elucidated, our data showed that the microbiota can impact on their number and phenotype.
Regulation of allergic inflammation by the microbiota

Figure 3: Commensal bacteria impact on the number and activation of lung conventional dendritic cells in allergic airway inflammation. Dendritic cell subsets were determined as depicted in Fig 2A and Fig 3A. (A) Representative flow cytometry plots indicate the distinction into (B) CD11b^+MHCII^hi cDCs, (C) CD11b^+MHCII^int cells and (D) CD11b^-cDCs of SPF or GF mice suffering from allergic airway inflammation. (B-D) Total cell numbers and activation state assessed by flow cytometry were determined for each cDC subset. Ox40L was not detected on CD11b^-cDCs. Activation markers are shown as geometric mean of fluorescence intensity ± s.e.m., and are normalized against isotype control antibodies or fluorescence minus one controls. Total numbers are shown as mean ± s.e.m. Presented data shows results of one experiment (controls: n=3-5, OVA: n=6) and is representative of two independent experiments. Figure adapted from Herbst, Sichelstiel, et al., Am J Respir Crit Care Med. 2011 Jul 15;184(2):198-205^22.

Dysregulated numbers of regulatory immune cells in the lung in the absence of commensal bacteria.

Several innate and adaptive immune cells can impact on Th2-mediated allergic airway inflammation. Basophils have been shown to be efficient in the production of IL-4 and have therefore been suggested to contribute to allergic responses^28. In contrast, pulmonary macrophages^23, regulatory T cells^29, and plasmacytoid DCs^30 have been associated with a regulatory function during allergic airway inflammation. In line with these findings, we found increased frequencies in basophils (Fig 4A,B), but decreased frequencies in alveolar macrophages (Fig 4C) and pDCs (Fig 4E), in the exaggerated Th2 inflammation that developed in the absence of commensals. Frequencies of regulatory T cells (data not shown) or interstitial macrophages (Fig 4D) were not influenced by the microbiota.
Figure 4: Commensal bacteria impact on basophils, alveolar macrophages, and plasmacytoid dendritic cells in allergic airway inflammation. The frequency of basophils, macrophages, and pDCs was assessed by flow cytometry in BAL and/or lung. Alveolar macrophages, interstitial macrophages, and pDCs were characterized according to Fig 3A,B. Basophils were distinguished by expression of CD49b and IgE and small size. The frequency of basophils was determined in (A) BAL (controls: n.d. = not detected, OVA: n=13-16) and (B) lung (controls: n=5-8, OVA: n=14-17). Results are pooled from three experiments and are representative of three independent experiments. Mean ± s.e.m. is shown. The frequency (%) of (C) alveolar macrophages, (D) interstitial macrophages, and (E) pDCs was determined in lung tissue. (C-E) Data presented shows results of one experiment (controls: n=5, OVA: n=5) and is representative of two independent experiments. Mean ± s.e.m. is shown. Adapted from Herbst, Sichelstiel, et al., Am J Respir Crit Care Med. 2011 Jul 15;184(2):198-205.

4.1.5 Discussion

Our data provide the first experimental evidence for a functional impact of the microbiota on allergic airway inflammation. We found that allergic airway hyperresponsiveness and Th2 inflammation were enhanced in the absence of commensal bacteria. Moreover, recolonization of germ-free mice with a complex SPF microbiota prior to OVA sensitization was sufficient to protect against the exaggerated inflammation. This promotes the idea that susceptibility to allergic diseases could be regulated by changing or reconstituting the microflora. The recolonization of germ-free mice could be considered within the framework of bacterial colonization of neonates that come from the sterile environment of the womb, and then encounter commensal bacteria from the moment of birth. Our data
Regulation of allergic inflammation by the microbiota

might therefore explain how exposure to environmental antigens early in life, during the time of microbial colonization, could have a significant impact on the development of allergic diseases.

To elucidate the mechanisms underlying this commensal driven regulation of allergic airway inflammation, we investigated the impact of the microflora on distinct cell populations of the respiratory tract. We found that the numbers of cells associated with a regulatory function, such as alveolar macrophages\(^3\) and pDCs\(^3\), were drastically reduced in the absence of commensal bacteria (Fig 4C,E). As plasmacytoid DCs have previously been reported to exert anti-inflammatory effects in allergen challenged mice\(^3\), it is plausible that the observed reduction in pDCs aggravated the Th2 inflammation. In contrast, basophils, that have been suggested to contribute to Th2 responses by expression of IL-4\(^28,32,33\), were augmented in the absence of the microflora (Fig 4A,B). Germ-free mice displayed a decreased number and a dampened activation state of CD11b\(^+\)MHCII\(^{hi}\), CD11b\(^+\)MHC\(^{int}\), and CD11b\(^-\)cDCs (Fig 3B-D). These semi-mature cDCs might present a phenotype that is more prone to induce Th2 responses and might thus promote allergic airway inflammation. The increased expression of ICOSL on CD11b\(^+\)MHCII\(^{int}\) and CD11b\(^-\)cDCs (Fig 3C,D) is supportive of this hypothesis, as ICOS-ICOSL interactions have been proposed to promote Th2 differentiation and expansion\(^27\).

In conclusion, our data demonstrates that commensal bacteria impact on the numbers of pulmonary DCs, macrophages and basophils, and regulate cDC activation. Any of these changes alone could be responsible for the increased allergic airway inflammation observed in germ-free mice. Nevertheless, it is likely that they act in concert to mediate the exaggerated Th2 response. Such an education of immune cells by commensal bacteria might be important for the regulation of mucosal immunity and might thus play a key role in maintaining the balance between immune homeostasis and inflammation. Indeed, host-microbe dysbiosis has already been linked to chronic autoinflammatory diseases such as experimental autoimmune encephalomyelitis\(^5\), type 1 diabetes\(^3\), obesity\(^4\), and rheumatoid arthritis\(^34\). Notably, as for asthma, the prevalence for these diseases has increased in developed countries over the last years\(^1\). For decades, the higher degree of hygiene in western countries has been considered a driving factor in the development of allergies and asthma. This "hygiene hypothesis" is based on the idea that a reduced exposure to pathogens early in life impacts on the appropriate education of the immune system and results in Th2-prone inflammation. The revelation that the microbiota is essential for full
immunological development and function\textsuperscript{13, 15, 16} has led to the formulation of an additional “diet hypothesis”\textsuperscript{16}. It suggests that different diets promote certain commensal bacteria species that subsequently have distinct effects on the immune system. Data supporting this hypothesis came from a study by Maslowski et al.\textsuperscript{18} who investigated experimental models of colitis and arthritis in the absence of commensal bacteria. They found that the exaggerated inflammation that developed in germ-free mice could be attenuated upon treatment with acetate, a breakdown product of fibers fermented by certain commensal bacteria. These findings indicate that treatment with probiotics and prebiotics might have a beneficial effect on the immunity.

The modulation of the immune system by the microbiota is likely to be very complex and might be mediated by different mechanisms in different diseases. In asthmatic patients not only the composition of the gut\textsuperscript{7-9} but also the respiratory flora is altered\textsuperscript{10}. It remains to be elucidated whether the observed changes in the respiratory microbiome are rather causal or consequential of the disease. However, it has to be considered that in responses in the lung the respiratory microbiome might be the key player. Indeed, Nembrini, Sichelstiel, et al. demonstrated that intranasal administration of \textit{Escherichia coli} efficiently attenuates the development of allergic airway inflammation\textsuperscript{35}.

Many questions still remain to be answered for a better understanding of the interactions between the commensal flora and the immune system. More detailed mechanistic insights into this symbiotic relationship are likely to offer new possibilities for the therapeutic management of chronic inflammatory diseases such as asthma.
4.1.6 References


4.2 Targeting IL-1β and IL-17A driven inflammation during influenza-induced exacerbations of COPD

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4.2.1 Abstract

For patients with chronic obstructive pulmonary disease (COPD), exacerbations are life-threatening events causing acute respiratory distress that can even lead to hospitalization and death. Although a great deal of effort has been put into research of exacerbations and potential treatment options, the exact underlying mechanisms are yet to be deciphered and no therapy that effectively targets the excessive inflammation is available. In this study, we report interleukin-1β (IL-1β) and interleukin-17A (IL-17A) as key mediators of neutrophilic inflammation in influenza-induced exacerbations of COPD. Using a mouse model of disease, our data shows a key role for IL-1β in mediating lung dysfunction, and in driving neutrophilic inflammation during the whole phase of viral infection. We further report a novel role for IL-17A as a mediator of IL-1β induced neutrophilia at early time points during influenza-induced exacerbations. Blocking of IL-17A or IL-1 signaling resulted in a significant abrogation of neutrophil recruitment to the airways in the initial phase of infection or at the peak of viral replication, respectively. Therefore, we propose IL-17A and IL-1β as valid targets for therapeutic treatment of viral exacerbations of COPD.

4.2.2 Introduction

Chronic obstructive pulmonary disease (COPD) is currently ranked the 4th leading cause of death worldwide by the World Health Organization, and its incidence is increasing. The main risk factor of COPD is exposure to tobacco smoke which triggers a cascade of inflammatory pathways leading to disease induction in susceptible people. Major hallmarks of the disease pathology are the development of emphysema and chronic bronchitis that lead to a progressive and irreversible airflow limitation resulting in a continuous decline of lung function. COPD severity has been associated with acute periods of disease worsening, so-called exacerbations, a key factor in COPD morbidity and mortality. By causing acute respiratory distress, they impact on the quality of patient’s health and are responsible for most hospital stays related to the disease.

Exacerbations are primarily caused by respiratory viral or bacterial infections. Amongst those, viral-induced ones account for about half of the cases and are associated with more severe acute exacerbations and prolonged recovery time. The most common viral pathogen in exacerbated patients is rhinovirus, followed by influenza virus, RSV and coronavirus. Due to targeted vaccination of high risk
groups, influenza infections occur less frequently in COPD patients of westernized countries\textsuperscript{11}. However, they continue to be the predominant cause of viral exacerbations in Hong Kong\textsuperscript{13} and Singapore\textsuperscript{14}.

COPD exacerbations have been linked to excessive inflammatory responses, including enhanced recruitment of inflammatory cells\textsuperscript{15} and upregulation of a variety of proinflammatory mediators\textsuperscript{16, 17}. Nevertheless, the underlying mechanisms and the most effective therapeutic strategies are still poorly understood and first-line therapy still predominantly relies on corticosteroids and bronchodilators\textsuperscript{18}, which are limited in their efficacy\textsuperscript{17, 19}. Thus, the study of cellular and molecular mechanisms leading to exacerbations is key for the identification of urgently required therapeutic targets. One of the proinflammatory cytokines that has been associated with COPD is IL-1β, a major player in initiation and persistence of inflammation. In animal models mimicking features of COPD, IL-1 has been shown to be key to the induction of emphysema and inflammation\textsuperscript{20-27}. Furthermore, its expression is significantly enhanced in COPD patients during acute episodes of exacerbations\textsuperscript{17, 20, 28, 29}. Unraveling the role of IL-1β in viral exacerbations might therefore not only result in an overall better understanding of mechanisms of exacerbations, but also indicate whether it qualifies as a valid therapeutic target. A promising candidate for therapeutic inhibition of IL-1β signaling is one of its endogenous inhibitors, the interleukin-1 receptor antagonist (IL-1Ra) anakinra (Kineret\textregistered, Amgen), which has been used effectively in treatment of rheumatoid arthritis.

In order to investigate the role of IL-1β during COPD exacerbations we utilized a model of LPS and elastase induced COPD followed by infection with influenza in wild type or IL-1β deficient mice. We found that IL-1β was a key driver of pulmonary inflammation, primarily concerning recruitment of neutrophils and lung dysfunction. IL-1β driven neutrophilia was mediated by IL-17A in the initial phase of viral infection, but became independent of IL-17A during the peak phase of viral replication. Treatment with the IL-1Ra, anakinra, proved efficient in reducing neutrophilic inflammation at the peak of viral replication while blocking of IL-17A abrogated neutrophilia in the early phase of viral infection. Taken together our data indicate that blockade of IL-1β and IL-17A are valid therapeutic approaches for treatment of virus-induced COPD exacerbations.
4.2.3 Materials and Methods

Ethics statement
All animal experiments were performed according to institutional guidelines and Swiss federal and cantonal laws on animal protection. Animal experiments were approved by the following ethical committee: Service de la consommation et des affaires vétérinaires, Affaires vétérinaires, Canton de Vaud, Switzerland (permit numbers 2283 and 2216).

Mice
C57BL/6 or BALB/c mice were between 8-12 weeks of age and were purchased from Charles River Laboratories. IL-1β deficient mice on C57BL/6 background were received from Prof. Iwakura, Tokyo University of Science, Japan, and bred in house.

LPS/elastase exposure and viral infection
Mice were exposed intranasally to a mixture of 7 µg LPS from E. coli O26:B6 (Sigma-Aldrich) and 1.2 U porcine pancreatic elastase (EPC) in a total volume of 100 µl, and treated once per week for four consecutive weeks (Fig 1A). Two weeks after the last LPS/elastase challenge, mice were infected with 250 PFU influenza A virus, strain PR8 (A/Puerto Rico8/34, H1N1, Viropur). The virus was administered intranasally in a total volume of 50 µl PBS; control mice received only PBS.

Assessment of pulmonary resistance
Total lung resistance was measured using the whole body restrained plethysmograph system flexiVent from Scireq. Mice were anesthetized by intramuscular injection of 100 mg/kg ketamine (Ketasol-100, Graeub) and intraperitoneal injection of 50 mg/kg pentobarbital (Esconarkon, Streuli Pharma). Subsequently, mice were tracheotomized and mechanically ventilated at a rate of 450 breaths/min and a tidal volume of 10 ml/kg bodyweight.

Flow cytometry
Single cell suspensions from lung and trachea were obtained by digestion with 2 mg/ml Collagenase IV (Invitrogen) and 50 U/ml DNasel (Roche). Neutrophils and monocytes in lung and bronchoalveolar lavage fluid were distinguished by staining with CD11c APC-Cy7, CD11b PerCP-Cy5.5, Ly-6G Biotin, Ly-6C Pacific Blue, Streptavidin PE-Cy7. Neutrophils were defined as CD11c<sup>-</sup>CD11b<sup>+</sup>Ly-6C<sup>+</sup>Ly-6G<sup>+</sup> and inflammatory monocytes as CD11c<sup>-</sup>CD11b<sup>+</sup>Ly-6C<sup>+</sup>Ly-6G<sup>low-intermediate</sup>.
To analyze cytokine production, cells from lung digests were stimulated with 10^7 M PMA, 1 µg/ml ionomycin and 2x10^-6 M monensin for 4h at 37°C (indicated chemicals were purchased from Sigma-Aldrich). Subsequently, cells were surface stained with CD4 PerCP-Cy5.5, CD8b FITC, γδ TCR Biotin, CD3 Pacific Blue, Streptavidin PE-Cy7, fixed with BD lysis buffer (BD Biosciences), and stained intracellularly with IL-17A Alexa700. All antibodies were purchased from Biolegend. Stained cells were acquired on a BD FACS CANTO or BD FACS LSRII and analyzed by using FlowJo software (Tree Star).

**Antibodies for in vivo studies**

For neutralization of IL-17A, mice were treated with 250 µg of anti-IL-17A (clone 17F3) or the corresponding isotype control antibody (clone MPOC-21) from BioXCell. Antibodies were administered intraperitoneally one day before viral infection and two days post infection.

To block IL-1β signaling mice received 200 µg of the interleukin-1 receptor antagonist (IL-1Ra) anakinra (Kineret®, Amgen) twice daily while control mice received only PBS. Anakinra was kindly provided by Prof. Alexander So (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland) and Mme Ghislaine Aubel (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland).

**Quantitative real-time PCR**

Total RNA was isolated from lung and trachea with TRI reagent (Molecular Research) and transcribed into cDNA by the iScript cDNA Synthesis kit (Bio-Rad). Quantitative real-time PCR was performed according to the manufacturer’s instructions utilizing the SsoAdvanced SYBR Green Supermix from Bio-Rad. Expression was determined by comparative delta-threshold cycle method using GAPDH as a comparator. The following primers were used: GAPDH forward 5'-GGG TGT GAA CCA CGA GAA AT-3', GAPDH reverse 5'-CCT TCC ACA ATG CCA AAG TT-3'; CXCL1 forward 5'-GCC TAT CGC CAA TGA GAA AT-3'; CXCL1 reverse 5'-ATT CTT GAG TGT GGC TAT GA-3'; CXCL2 forward 5'-AGT CAA CTG TTT CCC AGA G-3'; CXCL2 reverse 5'-GCC TTT CCA CGA TTT CCC AGA G-3'; CXCL5 forward 5'-AGC ATC TAG CTG AAG CTG CCC C-3'; CXCL5 reverse 5'-CCG TAG GGC ACT GTG GAC CTG-3'; IL-6 forward 5'-TTC CAT CCA GTT GCC TTC TTG-3'; IL-6 reverse 5'-TCA TTT CCA CTA GTC CCC AGA G-3'; TNFα forward 5'-GCC AGG AGG GAG AAC AGA AAC-3'; TNFα reverse 5'-GCC AGG GAG TGA AAG GGA CAG-3'; IL-17A forward 5'-ACC CTG GAC TCT CCA CCG CAA-3', IL-17A reverse
5'-'GGT GGT CCA GCT TTC CCT CCG-3'; influenza matrix protein forward 5'-GGA CTG CAG CGT AGA CGC TT-3', influenza matrix protein reverse 5'-CAT CCT GTT GTA TAT GAG GCC CAT-3'.

ELISA
To determine cytokine levels whole lung and trachea were collected and stored in protease inhibitor solution (Roche) at -20°C until use. Tissue homogenate was prepared using a TissueLyser (Qiagen) and IL-1β protein was determined using the mouse IL-1β ELISA kit Ready-SET-Go! from eBioscience by following the manufacturer's instructions.

Statistical analysis
Statistical significant differences were assessed using the Student's t test (two tailed, unpaired). P-values below 0.05 were considered significant and were depicted with p≤0.05 (*), p≤0.005 (**), p≤0.0005 (**). Standard error of the mean was applied.

4.2.4 Results
Influenza-induced exacerbations are characterized by neutrophilic inflammation and increased pulmonary resistance.
COPD is a heterogeneous disease in humans but core features of its pathology can be reproduced in mice by repetitive exposure to lipopolysaccharide (LPS) and elastase\textsuperscript{30}. LPS is a bacterial endotoxin present in tobacco smoke\textsuperscript{31, 32}, the predominant risk factor of COPD. It has been shown to cause inflammation, and particularly when co-administered with elastase chronic emphysema-like changes develop in mouse lungs\textsuperscript{30}. Repeated co-application of LPS and elastase induced sustained pulmonary inflammation and emphysema which remained above baseline levels for at least 2 months (data not shown). As such, we exposed mice once a week for 4 consecutive weeks to a mixture of 7 µg LPS and 1.2 U porcine pancreatic elastase via the intranasal route, as depicted in Figure 1A.

To study viral-induced exacerbations, mice were infected with influenza virus two weeks after the last LPS/elastase challenge (Fig 1A), when the acute inflammation caused by LPS/elastase exposure had subsided. The peak of viral replication was reached 5 days after the infection (Fig 1B) and was followed by a rapid decline in viral titers (Fig 1B) until complete viral clearance at day 9 post infection (data not shown). Invasive measurements of pulmonary resistance revealed a viral-induced
acute exacerbation of airway dysfunction in mice after viral infection (Fig 1C). Pulmonary resistance can be influenced by a variety of factors, of which the severity of inflammation is likely to play one of the key roles during exacerbations. In line with this we detected a strong inflammatory response upon infection with influenza virus associated with an augmented absolute number of cells infiltrating into the airways and the lung (Fig 1D). Those were primarily neutrophils (Fig 1E) and inflammatory monocytes (Fig 1F). The peak of neutrophilic inflammation was reached at day 5 post infection and neutrophil numbers declined afterwards (Fig 1E), thereby directly correlating with the kinetics of viral replication (Fig 1B). In line with this we observed augmented expression of the proinflammatory cytokines IL-6 and TNFα peaking at day 3 or 5 post infection, respectively, and subsiding at day 7 after the infection (Fig 1G).

Acute pulmonary dysfunction, neutrophilic inflammation and enhanced levels of proinflammatory cytokines such as IL-6 and TNFα have all been observed during exacerbations of COPD patients, indicating that the viral-induced pathology in our mouse model reflects that seen in humans.
**Figure 1: Influenza infection induced exacerbation of established disease in LPS/elastase exposed mice.** (A) Experimental protocol of influenza-induced exacerbation of LPS/elastase exposed mice. Control mice (indicated as day 0 in the following) were pre-exposed to LPS/elastase as their infected counterparts, but challenged only with PBS instead of influenza virus. (B) Viral load of whole lung and trachea was determined by quantitative real-time PCR at the indicated time points post infection or in non-infected mice (day 0) as control, respectively. Expression of influenza matrix protein was normalized to GAPDH. (C) Airway and tissue resistance was assessed by invasive plethysmography. (D) Absolute number of cells in the airways and lung was determined, and (E) the proportion of neutrophils and (F) inflammatory monocytes recruited to airways and lungs upon infection (day 3-7) or PBS challenge (day 0) was analyzed by flow cytometry. (G) Expression of IL-6 and TNFα was assessed by real-time PCR and normalized to GAPDH. (B-G) Experiments were performed in BALB/c mice and results are representative for at least two independent experiments (n=4-5). Error bars represent standard error of the mean (s.e.m.); i.n. (intranasal).
IL-1β contributes to lung dysfunction and pulmonary inflammation during influenza infection of LPS/elastase treated mice.

IL-1 has previously been shown to be a driving factor in the development of emphysema and inflammation in animal models of COPD\textsuperscript{20-27}. As we observed an increase of IL-1β protein in the lungs of LPS/elastase treated mice upon influenza infection (Fig 2A), we hypothesized that it also promotes innate immune responses and influences pulmonary function during exacerbations. Consequently, we exposed IL-1β deficient and C57BL/6 wild type mice to LPS/elastase and infected them with influenza virus as described above.

Of note, viral replication was not altered in IL-1β deficient mice (Fig 2B), demonstrating that any effects seen in the absence of IL-1β were not due to differences in the infection rate. C57BL/6 wild type mice exhibited a smaller change in pulmonary resistance in response to viral infection (Fig 2C) in comparison to BALB/c mice (Fig 1C), with a slight increase at day 1 post infection (Fig 2C). Nevertheless, pulmonary resistance in mice lacking IL-1β was significantly reduced already after LPS/elastase exposure alone (Fig 2C), thus supporting the described role for IL-1β in the development of COPD. Furthermore, pulmonary resistance in IL-1β deficient mice was also completely unaffected by the viral challenge (Fig 2C). Assessing the inflammatory response, we found a decreased number of neutrophils in non-infected IL-1β deficient mice upon exposure to LPS/elastase (Fig 2D,E). Similarly, the recruitment of neutrophils to the airways and lung following viral infection was also significantly abrogated in the absence of IL-1β (Fig 2D,E). We observed significantly lower frequencies and absolute numbers of neutrophils during the whole phase of viral replication including the peak of neutrophil infiltration and viral replication at day 5 post infection (Fig 2D,E). Nevertheless, control of the virus was unaffected as displayed by unaltered viral titers (Fig 2B).
Figure 2: IL-1β mediated pulmonary resistance and neutrophilic inflammation during influenza-induced exacerbations of COPD. Exacerbation of COPD in C57BL/6 mice was induced as depicted in Fig 1A. (A) IL-1β protein in whole lung and trachea following influenza infection (day 1-5) or PBS challenge (day 0) was assessed by ELISA. (B) Viral load in whole lung and trachea of wild type or IL-1β deficient animals was determined by quantitative real-time PCR and normalized to GAPDH. (C) Airway and tissue resistance was measured by invasive plethysmography at indicated time points after infection. (D) The proportion of neutrophils in airways and lung was determined by flow cytometry and (E) total numbers of neutrophils were calculated. (D,E) Data are pooled from two independent experiments (n=4-5). (A-E) All data are representative of at least two independent experiments (n=4-5) and mean ± s.e.m. is shown. Filled circles indicate wild type, open circles IL-1β deficient mice.

Considering its strong impact particularly upon neutrophils, we sought to address the mechanisms through which IL-1β mediated this neutrophilic inflammation. Expression of the main neutrophil chemoattractants CXCL1, CXCL2, and CXCL5 was induced upon influenza infection, but was unaffected by IL-1β (Fig 3A). Given there is substantial redundancy in neutrophil chemoattractants we thus next looked upstream at the proinflammatory cytokines IL-17A, IL-6, and TNFα, which can all stimulate neutrophilic inflammation by inducing chemotactic, growth, or survival factors. As expression of IL-6 and TNFα were induced in our mouse model upon influenza infection (Fig 1G), we hypothesized that IL-1β drove the observed inflammation by altering their production. The peak expression of IL-6 and TNFα upon influenza infection was reached faster in C57BL/6 wild type mice (Fig 3B,C) than in BALB/c (Fig 1G), at day 1 and 3, respectively, and thus correlated with the earlier response in pulmonary resistance observed in the C57BL/6 strain (Fig 2C).
However, lack of IL-1β did not influence IL-6 expression and in fact increased TNFα (Fig 3B,C). We therefore focused on IL-17A, a proinflammatory cytokine that has been shown to be elevated in COPD patients\textsuperscript{35, 36} and whose induction partially depends on IL-1β\textsuperscript{37-39}. We found significantly reduced protein levels of IL-17A in lung homogenate in the absence of IL-1β, in both non-infected as well as influenza-infected LPS/elastase exposed mice (Fig 3D). IL-17A production was significantly reduced in CD4\textsuperscript{+} T cells as well as in γδ T cells (Fig 3E) in IL-1β deficient mice, while produced in strikingly high amounts in their wild type counterparts (Fig 3E).

Taken together our data showed that besides contributing to lung dysfunction, IL-1β played a key role in driving neutrophilic inflammation during influenza-induced exacerbations, an effect that was tightly linked to IL-17A expression.
IL-1β and IL-17A in COPD exacerbations

**Figure 3:** IL-1β did not influence the expression of the neutrophil chemoattractants CXCL1, CXCL2, and CXCL5, but mediated the expression of IL-17A. (A) Expression of CXCL1, CXCL2, and CXCL5 in whole lung and trachea of C57BL/6 wild type and IL-1β deficient mice was assessed by quantitative real-time PCR and normalized to GAPDH. Data are pooled from two independent experiments (n=4-5). (B) Expression of IL-6, (C) TNFα, and (D) IL-17A was assessed by quantitative real-time PCR and normalized to GAPDH. (E) Proportion of IL-17A positive CD4⁺ T cells or γδ T cells was determined by flow cytometry after unspecific restimulation in vitro. All data are representative of at least two independent experiments (n=4-5) and mean ± s.e.m. is shown. Filled circles indicate wild type, open circles IL-1β deficient mice.

**Initial neutrophil recruitment during exacerbations is mediated by IL-17A and can be abrogated by treatment with neutralizing antibodies.**

To assess whether IL-17A was indeed a mediator of IL-1β driven neutrophilia we neutralized IL-17A during influenza infection of LPS/Elastase treated BALB/c mice. Mice received either an IL-17A neutralizing antibody or an isotype control antibody one day before and two days after the viral infection (Fig 4A). IL-17A neutralization did not impact on the control of viral replication, as viral burden was comparable to
IL-1β and IL-17A in COPD exacerbations

the isotype control treated animals (Fig 4B). We found that influenza-induced neutrophil recruitment to the airways and lung was indeed entirely attenuated 24 h after the infection in absence of IL-17A (Fig 4C,D). However, neutrophils infiltrated into the lung and airways during the later stages of infection (day 3 and 5 respectively) to finally reach the same frequencies as in mice treated with the isotype control (Fig 4D); thus indicating that IL-17A was only required for the initial but not for the later recruitment of neutrophils.

Hence, IL-1β driven neutrophilia during influenza infection of LPS/elastase exposed mice was mediated by IL-17A in the early phase of infection, but became independent of IL-17A during the ongoing phase of viral replication.

Figure 4: IL-17A mediated neutrophilic inflammation during the initial phase of influenza-induced exacerbation. (A) BALB/c mice were treated with anti-IL-17A (α-IL-17A) or isotype control antibody one day prior and two days after infection with influenza virus (day 1-5 post infection) or PBS challenge (day 0). (B) Viral load was measured by quantitative real-time PCR and normalized to GAPDH. (C,D) Proportion of neutrophils in airways and lung were assessed by flow cytometry. (C) Two representative FACS plots of airway neutrophilia of one mouse of each group at day 1 after infection are shown as well as (D) the plotted data. Data are representative of two independent experiments (n=4-5), error bars indicate s.e.m; i.n. (intranasal); p.i. (post infection); ctrl (control). Filled circles indicate anti-IL-17A treated, open circles isotype control treated mice.
Treatment with the human recombinant IL-1Ra anakinra impairs neutrophil recruitment at the peak of viral replication.

Our data showed that a constitutive lack of IL-1β substantially impaired neutrophil infiltration into the airways and lung during influenza-induced exacerbations of LPS/elastase treated mice. Hence, we sought to assess whether it is sufficient to block IL-1β signaling only during the course of infection, an important determinant regarding a potential therapeutic intervention. Accordingly, the recombinant IL-1Ra anakinra or PBS was administered twice daily, starting two days prior to the viral infection (Fig 5A). Mice receiving anakinra displayed an impaired control of viral infection early on leading to a higher viral burden at day 3 post infection, but viral titers rapidly declined afterwards to levels similar to non-treated mice at day 7 post infection (Fig 5B), and virus was completely cleared at day 9 post infection (data not shown). Treatment with anakinra was efficient in reducing neutrophil frequencies in the airways at day 5 post infection, the peak of neutrophilic infiltration and viral replication (Fig 5C).

Figure 5: Treatment with anakinra reduced neutrophil recruitment to the airways at the peak of viral-induced inflammation. (A) BALB/c mice received anakinra or PBS twice every day, starting two days prior to the viral infection and until mice were sacrificed. (B) Viral load was determined by quantitative real-time PCR and normalized to GAPDH. (C) Proportion of neutrophils recruited to the airways following influenza infection (day 1-5) or PBS challenge (day 0) was assessed by flow cytometry. Two representative FACS plots from one mouse of each group at day 5 after infection are shown as well as the plotted data. Data are representative of two independent experiments (n=4-5), error bars indicate s.e.m; i.n.
IL-1β and IL-17A in COPD exacerbations

(intranasal); p.i. (post infection); ctrl (control). Filled circles indicate treatment with anakinra, open circles PBS controls.

In conclusion our data demonstrated that IL-1β caused neutrophilic inflammation during influenza-induced exacerbation of COPD in mice throughout the entire phase of viral replication. Neutrophil recruitment was mediated by IL-17A in the first 24 h following viral challenge and could therefore be efficiently blocked in the early phase of infection by neutralizing IL-17A. During the peak of inflammation and viral replication IL-1β driven neutrophilia was independent of IL-17A, but could be significantly reduced by treatment with the IL-1Ra anakinra (Fig 6).

Figure 6: Neutrophilic inflammation during influenza-induced exacerbation of COPD is mediated by IL-1β and IL-17A. (A) In the initial phase of viral replication, at 24 h following the infection, IL-1β-induced IL-17A caused the recruitment of neutrophils to the airways and lung. (B) At the peak of viral replication, day 5 post infection, neutrophilia became independent of IL-17A, but was still mediated by IL-1β. Blocking of IL-17A or IL-1β abrogated neutrophilic inflammation in the early phase of the infection or at the peak of viral replication, respectively.
4.2.5 Discussion

In addition to the permanent stable disease burden, COPD patients suffer from episodes of acute symptom worsening causing a rapid decline in respiratory function that can necessitate hospitalization and even lead to death. Indeed, a meta-analysis study estimated a case-fatality rate of 15.6% following hospitalization due to an exacerbation. As there is a clear need to understand the mechanisms driving exacerbations and responsiveness to therapy, we examined viral-induced exacerbations in mice. In our model (Fig 1A), mice developed a strong inflammatory response characterized by a neutrophilic infiltrate into the airways and lung (Fig 1E), enhanced expression of proinflammatory cytokines such as TNFα and IL-6 (Fig 1G), and an impairment in lung function (Fig 1C). Decline in lung function and neutrophil accumulation are characteristic of exacerbations in humans, and elevated levels of TNFα and IL-6 have similarly been measured in the sputum of patients undergoing an exacerbation. Thus, our mouse model reflects key pathological characteristics of COPD exacerbations in humans.

In this study we specifically focused on the role of the proinflammatory cytokine IL-1β. IL-1 signaling has been shown to be essential for the recruitment of neutrophils in other mouse models mimicking the pathology of stable COPD, such as exposure to cigarette smoke or to elastase alone. Building upon these studies we found that during influenza-induced exacerbations, pulmonary accumulation of neutrophils was also driven by IL-1β (Fig 2D,E). Our data is in line with results from a study of Botelho et al. who investigated IL-1 in a mouse model of acute cigarette smoke exposure. They found that interleukin-1 receptor (IL-1R) deficiency led to a reduction of neutrophils following infection with influenza and that this effect was independent of IL-1α. One could therefore speculate that IL-1β may play a critical role in neutrophil recruitment in their model as well.

In addition, our data showed that IL-1β driven neutrophil recruitment during the first 24 h of infection was mediated by IL-17A, a cytokine that plays a central role in amplifying inflammatory cascades by inducing a variety of chemokines and cytokines. We found that in the absence of IL-1β the expression of IL-17A was completely abrogated upon LPS/elastase exposure as well as during exacerbations (Fig 3D), thus demonstrating that IL-1β is required for the induction of IL-17A. IL-17A expression was induced by LPS/elastase exposure alone, and levels were maintained throughout the viral-induced exacerbation (Fig 3D,E); however, while
IL-1β levels increased during the later phase of infection (Fig 2A), IL-17A expression surprisingly remained unaltered and even decreased at the peak of viral replication (Fig 3D). It is therefore likely that during ongoing viral exacerbations additional cytokines regulating the IL-17A response are induced and overcome the impact of IL-1β. Nevertheless, we could efficiently block the recruitment of neutrophils at early time points following the influenza infection by neutralizing IL-17A (Fig 4C,D). During the peak of viral replication and thus a more severe state of inflammation, IL-17A neutralization could not prevent neutrophil influx (Fig 4D). This might be due to an induction of redundant cytokines during the progression of the infection, which could overcome the effect of IL-17A. Therefore, treatment with anti-IL-17A seems to be favorable in the early phases of exacerbations while blocking IL-1β might be more advantageous during the ongoing infection. Whether these findings translate into a clinical setting remains to be investigated.

Keeping in mind that neutrophil recruitment is elevated in the vast majority of cases of COPD exacerbations regardless of their etiology, and furthermore that the increase of neutrophils in the sputum correlates with exacerbation severity, attenuating neutrophilia could be beneficial in exacerbated patients. Current treatment relies mainly on corticosteroids and bronchodilators that have been shown to reduce the frequency of exacerbations, but have no positive effect on an ongoing episode of exacerbation. Indeed, no reduction in the inflammatory response including neutrophil influx in mice and cytokine expression in humans could be achieved by treatment with steroids during COPD exacerbations. In contrast, corticosteroids have been shown to actually support neutrophil survival. Moreover, treatment with corticosteroids, efficient in reducing IL-1β levels in stable COPD, did not affect the amount of IL-1β protein in exhaled breath condensate during exacerbations. As blocking of either IL-17A (Fig 4D) or IL-1β (Fig 5C) efficiently reduced neutrophilic inflammation during viral exacerbation, these two molecules present as potential targets for therapeutic treatment.

Considering such a clinically applied neutralization of IL-17A or IL-1β, one has to keep in mind that altering proinflammatory immune responses always harbors the risk of interfering with the control of the acute infection and of encountering new pathogens. To shorten the duration of intervention and thereby reducing the risk of prolonged or secondary infections, we treated mice directly before and during the viral infection with anti-IL-17A (Fig 4A) or IL-1Ra (Fig 5A). This was sufficient to abrogate neutrophil recruitment at the indicated time points. Furthermore,
neutralizing IL-17A did not promote elevated viral replication (Fig 4B). Treatment with IL-1Ra, and thereby blocking both IL-1α and IL-1β, led to increased viral titers in the initial phase of infection (Fig 5B), whereas the absence of IL-1β alone did not affect viral replication (Fig 2B). It is therefore likely that in our model the initial control of the virus might be mediated rather by IL-1α than by IL-1β. This is in line with data from Botelho et al., who found similarly elevated viral titers upon neutralization of IL-1α as in complete absence of IL-1R. Our data thus suggest that targeting specifically IL-1β and not its receptor would be favorable in a therapeutic application. However, as treatment with anakinra did not interfere with final viral clearance it still could qualify as a valid therapeutic option with the additional advantage of being already available for clinical use.

Taken together our data demonstrated that blocking of IL-17A or IL-1β signaling during influenza-induced exacerbations efficiently diminished neutrophilic infiltration at specific time points during viral replication and could therefore be beneficial for COPD patients. Whether those mechanisms apply also for other viral pathogens remains to be elucidated, but is plausible given the common early inflammatory pathways induced by respiratory viral infections.
4.2.6 References


4.3 Stromal cell influences upon viral-driven exacerbations of allergic airway inflammation

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4.3.1 Abstract

Asthma is a highly prevalent chronic respiratory disease. Although moderate asthma is often well controlled, many patients still experience repeated episodes of acute exacerbations, which account for a significant part of asthma associated costs and morbidity. Approximately 80% of asthma exacerbations are caused by infections with respiratory viruses such as rhinovirus, influenza virus, coronavirus, or respiratory syncytial virus (RSV). In the last years our understanding of the role the epithelium plays in asthma and asthma exacerbations has evolved from being a pure physical barrier into an immunologically relevant component that could play a key role in mediating disease pathology. In-depth studies of mechanisms underlying this epithelial cell mediated orchestration of inflammation might reveal novel opportunities for therapeutic intervention. As there is a disconcerting lack of effective therapies for exacerbations, new treatment strategies are urgently required. To-date, no in vivo data has been generated to determine whether exacerbations are associated with a unique pattern of epithelial cell gene expression; such an approach is important for the discovery of novel therapeutic targets. Therefore, we performed a gene array analysis of structural airway cells during an influenza-induced exacerbation of house dust mite (HDM) driven allergic airway inflammation in mice. We report the specific regulation of 37 genes, out of which 12 hold potential for being important components in the disease and possible therapeutic targets.

4.3.2 Introduction

Asthma is a chronic inflammatory disease of the conducting airways that currently affects about 300 million people worldwide, and its incidence is increasing. It is a heterogenous disease characterized by reversible airway obstruction, airway hyperresponsiveness (AHR), mucus hypersecretion, and a chronic inflammation involving eosinophils and CD4+ T helper (Th) 2 cells. Severe forms of the disease are also prevalent, are dominated by neutrophil infiltrates and current treatments are ineffective. Even though no cure for asthma exists, effective treatments are widely available for mild and moderate forms of the disease. Nevertheless, patients still experience repeated episodes of acute disease worsening. Such exacerbations are a major cause of disease related morbidity and can even require hospitalization. Approximately 80% of exacerbations are caused by infections with respiratory viruses, such as rhinovirus, influenza virus, coronavirus, or RSV, with rhinovirus accounting for most of the cases. Exacerbations are characterized by an acute
airflow obstruction that is caused by smooth muscle contraction, formation of mucus plugs, and enhanced inflammation. As current therapeutics are poorly effective in the treatment of exacerbations, a better understanding of the cellular and soluble mediators involved is important for improved disease management.

Epithelial cells form the interface between the host and the environment, and provide a physical and biological first-line of defense against pathogens and noxious agents. They sense environmental antigens via a broad range of pattern recognition receptors (PRR) and respond by secretion of inflammatory mediators that orchestrate the ensuing immune response. Thus, epithelial cells contribute to host defense, innate immunity, and immune regulation. In asthmatic patients, epithelial cells are thought to be dysfunctional and to produce increased amounts of certain growth factors, cytokines, and chemokines. This causes the recruitment and activation of innate immune cells that drive the chronic inflammation. In addition, epithelial tight junctions are disrupted in chronic asthmatics leading to an increased permeability of the epithelium, which allows enhanced access for allergens and pathogens. Although the dysregulated epithelium is thought to produce exaggerated levels of certain cytokines, it has been shown that interferon production is impaired in asthmatics, leading to an impaired early anti-viral response, and consequently enhanced susceptibility to viral infections. Viral infections of epithelial cells, from non-asthmatic tissue, have been shown to lead to enhanced mucus production and to induce the release of proinflammatory mediators such as IL-1, IL-6, CXCL8, GM-CSF, CXCL10, CCL5, which can trigger neutrophilic, eosinophilic, and lymphocytic inflammation. These findings indicate that the dysregulated epithelium in asthmatics not only increases the susceptibility to viral infections, but also drives the inflammation underlying viral exacerbations.

Epithelial cells are thus one of the key players in asthma exacerbations and could yield important targets for therapeutic interventions. We therefore sought to assess the gene expression pattern of epithelial cells isolated from mice undergoing influenza-induced exacerbations of house dust mite (HDM) driven allergic airway inflammation. By this, we aimed to unravel whether exacerbations are associated with a specific gene expression pattern differing from that of respiratory viral infections or allergic airway inflammation, alone. Such uniquely regulated genes would provide ideal therapeutic targets as they might play a key role in driving the exaggerated inflammation observed during asthma exacerbations, but are likely to be dispensable for effective anti-viral responses.
4.3.3 Materials and Methods

Mice
BALB/c mice were obtained from Charles River Laboratories and used between 8-12 weeks of age. All experiments were performed according to institutional guidelines and swiss federal and cantonal laws.

Induction of allergic airway inflammation
Mice were intranasally exposed to 15 µg purified house dust mite (HDM) extract (Greer Laboratories, lot 140153) in a total volume of 30 µl, three times per week (Mondays, Wednesdays, Fridays) for three consecutive weeks (Fig 1A). The administered HDM extract contained 33.6 U endotoxin per mg total protein content and 56.7 µg Der p1/mg protein. Control mice received PBS only. Three days after the last HDM challenge, HDM or PBS treated mice were either infected with 50 PFU influenza A virus, strain PR8 (A/Puerto Rico8/34, H1N1, Viropur), in PBS, or received only PBS. The virus was administered via the intranasal route in a total volume of 30 µl PBS.

Measurement of airway hyperresponsiveness
Airway hyperresponsiveness was determined by restrained invasive plethysmography using the flexiVent system from Scireq. Airway resistance Rn was assessed in response to increasing doses of methacholine (3-12 mg/ml, Sigma). For measurements, mice were anaesthetized by intraperitoneal injection of 50 mg/kg pentobarbital (Esconarkon, Streuli Pharma) and intramuscular injection of 100 mg/kg ketamine (Ketasol-100, Graeub), tracheotomized and mechanically ventilated at a tidal volume of 10 ml/kg bodyweight and a rate of 450 breaths/min.

Quantification of airway inflammation
Airways and lungs were flushed with PBS to recover the bronchoalveolar lavage (BAL) fluid. Cytospins were performed from recovered cells and stained with Diff-Quik (Dade Behring). Differential cell counts were scored according to standard criteria.

Cell sorting
Epithelial and structural airway cells were isolated by fluorescence activated cell sorting (FACS). Briefly, trachea and conducting airways were microdissected, digested with collagenase IV (Invitrogen), and single cell suspensions were stained
with CD45 Alexa700, Ter-119 PE-Cy5, T1α PE, CD31 PE-Cy7, and propidium iodide (PI), all purchased from Biolegend. Structural cells were defined as CD45^Ter119^- cells, and from these, epithelial cells were further distinguished by positive staining for T1α and absence of the endothelial marker CD31. All cells were sorted into TRI reagent (Molecular Research) using a FACS Aria (BD Biosciences) and stored at negative 80 °C until further use.

**RNA isolation**
Total RNA was extracted using the miRNeasy Mini kit from Qiagen according to the manufacturer's instructions. To further purify the RNA and to remove residual DNA contamination the protocol for RNA clean-up and concentration of the RNeasy Micro kit (Qiagen) was followed. The RNA quality was checked by the Lausanne Genomics Technologies Facility of the University of Lausanne, Switzerland, using an Agilent 2100 Bioanalyzer.

**Gene expression array**
Three individual RNA samples of each experimental group were pooled for the microarray analysis. Gene expression profiles were determined using the whole-transcript array GeneChip Mouse Gene 1.0 ST from affymetrix. The microarray and data analysis were performed by the Lausanne Genomics Technologies Facility of the University of Lausanne, Switzerland. Probesets with an average normalized expression value lower than 5.0 were excluded from the data analysis to filter out background signals and only expression changes of at least 4-fold were included in the analysis. To distinguish overlaps and differences of the regulated genes, the expression pattern of each experimental group was first compared to the baseline gene expression, constituted by the PBS only control, and then to the other samples.

**Statistical analysis**
Statistical significance was assessed by two tailed, unpaired Student’s $t$ test. $P$-values below 0.05 were considered significant and $P$-values were depicted with $p \leq 0.05$ (*), $p \leq 0.005$ (**), $p \leq 0.0005$ (***)}. Standard error of the mean was applied.
4.3.4 Results and discussion

Influenza-induced exacerbations of HDM-driven allergic airway inflammation are characterized by increased Th2 inflammation.

Asthma is a heterogeneous disease in humans but core features of its pathology can be reproduced in mice by exposure to HDM extract, one of the most common environmental aeroallergens. It has previously been established that repetitive intranasal challenges with HDM extract induces allergic airway pathology marked by a Th2-mediated chronic airway inflammation, eosinophilia, airway hyperresponsiveness, and airway remodeling. Thus, we established a model of HDM-induced allergic airway inflammation that involved exposing mice three times per week to 15 µg HDM extract via the intranasal route, as depicted in Figure 1A. Airway hyperresponsiveness was assessed by restrained invasive plethysmography in response to methacholine. As expected, HDM exposed mice exhibited an increase in airway resistance compared to PBS treated mice (Fig 1B), and an enhanced airway inflammation characterized by the infiltration of eosinophils (Fig 1C) and lymphocytes (Fig 1D) 4 days after the last HDM challenge. We next expanded the model to allow us to study viral exacerbations of asthma. Specifically, mice pre-exposed to HDM were infected with influenza virus, a common respiratory virus known to cause exacerbations in asthma patients. In order to directly study the inflammatory response following viral infection of ‘asthmatic’ mice, mice were infected with influenza A strain PR8, 3 days after the last HDM challenge (Fig 1A). We observed a significant increase in the inflammatory response at day 1 post infection, reflected by an elevated infiltration of eosinophils (Fig 1E), neutrophils (Fig 1F), and lymphocytes (Fig 1G). Eosinophilia and neutrophilia declined rapidly afterwards to levels similar to uninfected ‘asthmatic’ mice (Fig 1E,F), while lymphocytic inflammation was maintained (Fig 1G).

The observed increase in eosinophils and neutrophils during the viral exacerbation is in line with findings from the clinic that describe eosinophils as the predominant cell type in stable asthma that can be further increased during exacerbations, while associating neutrophils with severe steroid-resistant subtypes and pathogen-induced exacerbations of asthma. Thus, the viral-induced pathology in our mouse model carries key features seen in humans.
Figure 1: Influenza-induced exacerbation of allergic airway inflammation in mice. (A) Experimental protocol of house dust mite (HDM) extract driven allergic airway inflammation and subsequent infection with influenza virus A, strain PR8. Four experimental groups were investigated: PBS (non-infected: received only PBS), PR8 (infected: treated with PBS for three weeks, infected with influenza virus), HDM (allergic airway inflammation: sensitized against HDM, challenged with PBS), HDM-PR8 (influenza-induced exacerbation of allergic airway inflammation: sensitized against HDM, infected with influenza virus). (B) Airway resistance was assessed in the HDM and PBS groups in response to methacholine (MetCh) 4 days following the last HDM challenge. Results (n=2-3) are representative for 2 independent experiments. Total numbers of (C) eosinophils and (D) lymphocytes in the bronchoalveolar lavage (BAL) of HDM and PBS mice were determined by cytospins 4 days after the last HDM challenge. Results (n=3-4) are representative for 4 independent experiments. Total numbers of (E) eosinophils, (F) neutrophils, and (G) lymphocytes, were determined in the BAL of exacerbated mice at day 1 and 4 post infection. Results (n=4) are representative for 3 independent experiments. Error bars indicate s.e.m.; i.n. (intranasal), p.i. (post infection).
Regulated genes in asthma exacerbations

Being positioned at the interface of the lung tissue and the environment, pulmonary epithelial cells are one of the first cells to encounter antigens. They are furthermore the target cells for infection and replication of respiratory viruses, including influenza. We hypothesize that epithelial cell responses will be key mediators of exacerbated inflammation, and are likely to be regulated in the first hours following an infection. Given the exaggerated infiltration of inflammatory cells occurred rapidly with the first days post infection, we proceeded to characterize the gene expression profile of structural cells at 24 h post infection.

Differentially regulated genes in structural airway cells during viral infection of HDM-induced allergic airway inflammation.

In order to assess the contribution of the epithelium to viral-induced exacerbations, we compared the gene expression profiles of allergic mice infected with influenza virus (HDM-PR8), to allergic, non-infected (HDM), and to non-allergic, influenza-infected, mice (PR8) (Fig 1A). For this, the trachea and conducting airways were microdissected and target cell populations were isolated by fluorescence activated cell sorting (FACS). Epithelial cells were distinguished by positive staining for the epithelial marker T1α and by exclusion of hematopoetic cells (CD45+), erythrocytes (Ter-119+), and endothelial cells (CD31+), according to the gating strategy shown in Fig 2A. As we recovered only low numbers of epithelial cells from the FACS, we did not obtain sufficient RNA material to perform a microarray, as revealed by RNA quantity and quality analyses (Fig 2B). Therefore, we decided to proceed by isolating all structural airway cells (CD45−TER-119−). We found that the RNA profile of structural cells displayed the two peaks of ribosomal 18S and 28S RNA, indicating that we could recover more RNA than from epithelial cells. However, the samples were contaminated with DNA, indicated by a high fluorescence baseline in the bioanalyzer graph (Fig 2C), and contained some dead cells or degraded RNA, as indicated by a peak at low nucleotide numbers (Fig 2C). Therefore, we performed an additional purification step, which improved RNA quality (Fig. 2D), and allowed us to assess the expression profile of structural airway cells in a microarray.
Figure 2: Isolation of epithelial versus structural cells by cell sorting and validation of RNA quality. (A) In order to isolate epithelial or structural cells from the lungs, the trachea and conducting airways were microdissected and target cell populations were separated by cell sorting. Dead cells were excluded by PI staining, and structural cells were subsequently distinguished by lack of CD45 and Ter-119. From these, epithelial cells were defined by expression of the epithelial marker T1α and lack of the endothelial cell marker CD31. (B) RNA bioanalyzer profile of sorted epithelial cells. Only a small peak for the 18S, and no peak for the 28S ribosomal RNA (rRNA) was detected, indicating an insufficient amount of RNA. (C,D) RNA profile of sorted structural cells (C) before and (D) after an additional purification step. (D) Two sharp and well-defined peaks for the ribosomal 18S and 28S RNA, reduced DNA contamination, and absence of dead cells indicated an improved RNA quality after purification. Nt (nucleotides); FU (fluorescence units); rRNA (ribosomal RNA).
To identify genes that were differentially regulated compared to the baseline gene expression, the transcriptional profile of each treatment group was compared to a control group that was treated with PBS alone. Only differences with a fold change of at least 4 were considered in the data analysis. In a next step, we determined which of the genes differently regulated compared to the PBS baseline were uniquely expressed in, or overlapped between, the experimental groups HDM, PR8, HDM-PR8. This Venn analysis revealed that only 37 genes were differentially expressed during exacerbations, with 31 genes being up- (Fig 3A) and 6 genes being down-regulated (Fig 3B). In the HDM group, 600 genes were specifically up- (Fig 3A) and 224 down-regulated (Fig 3B), while upon influenza infection alone, 45 genes were specifically up- (Fig 3A) and 55 down-regulated (Fig 3B).

**Figure 3:** Gene expression pattern of structural cells from influenza infected, allergic, and exacerbated mice. The Venn diagram displays numbers of commonly and uniquely (A) up-regulated and (B) down-regulated genes in influenza infected (PR8), allergic (HDM), and exacerbated (HDM-PR8) mice. Only genes with a fold change of at least 4 were included. The gene expression profile of each group was first compared to the baseline gene expression (PBS only group), and differentially regulated genes were subsequently compared between all treatment groups. Genes focused on in detail are indicated in the exacerbated group.

Table 1 and 2 list all genes that were differentially regulated during influenza-induced exacerbation of HDM-driven allergic airway inflammation. Within this list of genes, novel therapeutic targets could be identified, and will be the focus of future studies. Within the context of the current work, we have focused upon a subset of genes that we consider likely to be of particular interest concerning exacerbations.
Table 1: Differentially regulated genes during exacerbations that have previously been associated with asthma or asthma exacerbations. FC (fold change), GO annotation (Gene ontology annotation).

<table>
<thead>
<tr>
<th>Up-regulated genes</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>FC</th>
<th>Involvement in asthma &amp; asthma exacerbations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adamts9</td>
<td></td>
<td>A disintegrin-like and metalloproteinase (reprolysin type) with thrombospondin type 1 motif, 9</td>
<td>4.2</td>
<td>Family members are increased in asthma patients; might contribute to fibrosis and thus to airway remodeling&lt;sup&gt;29, 30&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chi3l4</td>
<td></td>
<td>Chitinase 3-like 4</td>
<td>15.7</td>
<td>Associated with allergic airway inflammation in mice; family member Chi3l1 elevated in asthma exacerbation in humans&lt;sup&gt;31, 32&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cleo4n</td>
<td></td>
<td>C-type lectin domain family 4, member n</td>
<td>4.1</td>
<td>Crucial for development of allergic airway inflammation in mice&lt;sup&gt;33&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hp</td>
<td></td>
<td>Haptoglobin</td>
<td>4.7</td>
<td>Elevated in asthma and asthma exacerbations in humans&lt;sup&gt;34, 35&lt;/sup&gt;</td>
</tr>
<tr>
<td>Itln1</td>
<td></td>
<td>Intelectin-1 (galactofuranose binding)</td>
<td>4.7</td>
<td>Elevated in airway epithelium of asthma patients&lt;sup&gt;36&lt;/sup&gt;</td>
</tr>
<tr>
<td>Muc5ac</td>
<td></td>
<td>Mucin 5, subtypes A and C, tracheobronchial/gastric</td>
<td>5.6</td>
<td>Elevated in asthma and in viral-induced asthma exacerbations in humans&lt;sup&gt;37, 38&lt;/sup&gt;</td>
</tr>
<tr>
<td>Muc5b</td>
<td></td>
<td>Mucin 5, subtype B, tracheobronchial</td>
<td>5.2</td>
<td>Increased in fatal asthma&lt;sup&gt;37&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Down-regulated genes</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>FC</th>
<th>Involvement in asthma &amp; asthma exacerbations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ptpn3</td>
<td></td>
<td>Protein tyrosine phosphatase, non-receptor type 3</td>
<td>-4.1</td>
<td>Family members involved in allergic airway inflammation in mice&lt;sup&gt;39&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Regulated genes previously associated with asthma exacerbations.**

Our dataset included only two genes that have previously been associated with asthma and asthma exacerbations, specifically: Haptoglobin (Hp), and mucin (Muc) 5AC (Table 1, Fig 4A). Notably, mucin (Muc) 5B, which forms together with Muc5AC the major respiratory mucin<sup>40</sup>, was also up-regulated in the analysis. Mucus hypersecretion causes airway obstruction and is thus a key factor in asthma pathology, especially during acute exacerbations. Muc5AC and Muc5B have both been reported to be increased in fatal asthma<sup>37</sup>, and elevated Muc5AC production has been observed in a human model of rhinoviral-induced asthma exacerbations<sup>38</sup>. Muc5AC expression was also increased in bacterial exacerbation studies <i>in vitro</i><sup>41</sup> and in a mouse model of fungal-driven HDM exacerbation<sup>42</sup>. As these data indicate that Muc5AC might be a crucial player in mucus hyperproduction in infectious exacerbations of all etiologies, Muc5AC represents a very promising therapeutic target. Muc5B has not been described in exacerbations to-date, but given its up-regulation in our study it might qualify as an additional therapeutic target.

Haptoglobin is an acute phase protein scavenging hemoglobin in the event of intra- or extravascular hemolysis<sup>43</sup>, and it has been associated with anti-inflammatory
Regulated genes in asthma exacerbations

Haptoglobin is elevated in the bronchoalveolar lavage of asthma patients\textsuperscript{35}, and increased serum concentrations have been reported in asthmatic children undergoing an acute exacerbation\textsuperscript{34}. However, considering that haptoglobin reduces oxidative damage by binding hemoglobin\textsuperscript{45} and that it has recently been reported to protect proteins from unfolding\textsuperscript{46}, its inhibition might have severe side effects.

Beside Muc5AC and Haptoglobin, we found that the chitinase 3-like 4 (Chi3l4) protein was up-regulated. Chitinase-like proteins lack the enzymatic activity of chitinases, but still contain the carbohydrate binding domains\textsuperscript{47}. They are therefore likely to bind pathogen-derived chitin or other polysaccharides\textsuperscript{47}, but might not degrade them. Chitinase-like proteins can be induced by fungal or parasitic infection and by T helper 2 (Th2) cytokines, and have been reported to be chemotactic for eosinophils, T cells, and polymorphonuclear leukocytes\textsuperscript{48}. Thus, they might promote exaggerated Th2 inflammation without being capable of removing the inflammatory stimuli. While increased production of Chi3l4 has only been described in a mouse model of allergic airway disease thus far\textsuperscript{32}, the family member Chi3l1 has been associated with asthma exacerbations\textsuperscript{31}. Thus, one can speculate about a potential involvement of Chi3l4 in mediating exaggerated inflammation in response to pathogens as well.

Regulated genes previously associated with stable asthma.

Our array revealed an up-regulation of a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 9 (Adamts9), c-type lectin domain family 4 (Clec4n), intelectin-1 (Itin1), Muc5B, and a down-regulation of protein tyrosine phosphatase non-receptor type 3 (Ptpn3) (Table 1, Fig 4A), proteins who themselves or whose families have been linked to asthma but not yet to exacerbations. Inteletcin-1 has been described to be elevated in the airway epithelium of asthmatic patients\textsuperscript{36} and silencing of inteletcin-1 and -2 was indeed effective in attenuating allergic airway inflammation in mice\textsuperscript{49}. Given its involvement in the recognition of molecular patterns such as furanosides from bacterial and fungal cell walls\textsuperscript{50}, inteletcin-1 is likely to impact on exacerbations as well. Similarly, Clec4n is also a pattern recognition receptor (PRR) that has recently been linked to asthma\textsuperscript{51}. Clec4n, also known as Dectin-2, belongs to the c-type lectin receptor family and is involved in the recognition of fungi\textsuperscript{52}. It is commonly expressed on myeloid immune cells and mucosal epithelial cells\textsuperscript{52}. Clec4n recognizes glycans in HDM extract\textsuperscript{33}, and has been reported to be crucial for the development of HDM-induced eosinophilic and neutrophilic inflammation in mice as well as for Th2 cytokine generation in the lungs and lymph nodes\textsuperscript{51}. Overall, inteletcin-1 and Clec4n are likely to be involved in
Regulated genes in asthma exacerbations

Exacerbations and inhibiting their function might dampen the exaggerated inflammatory response. Adamts9 is a member of the ADAM/ADAMTS proteinase family, which is closely related to the family of matrix metalloproteinases. Its members Adam8, Adam10, and Adam33, are increased in asthma patients as well as in murine models of asthma, and the expression of Adam 33 correlates with the severity of asthma. For Adamts9 very few studies have been reported, but it has been linked to transforming growth factor β (TGF-β) mediated collagen deposition, which is a hallmark of fibrosis. As TGF-β dependent fibrosis also contributes to the pathology of asthma, Adamts9 might be an interesting target to control airway remodeling in chronic asthma. Its role in the acute inflammation during exacerbations remains to be determined.

Finally, tyrosine-specific phosphatases such as Pttn3 are key regulatory components in signal transduction pathways, and several family members have been reported to be involved in allergic airway inflammation. The global inhibition of protein tyrosine phosphatases (PTP) reduced the development of asthma in mice. However, inhibition of single PTPs during allergic asthma led to diverse results indicating a regulatory function for some PTPs, but an inflammatory role for others. Thus, deficiency in Shp2 (=Pttn11) attenuated allergic responses while lack of Shp1 (=Pttn6) resulted in the spontaneous development of a Th2 like inflammation including key pathological features of asthma. The role of Pttn3 during exacerbations has therefore to be defined in detail. Nevertheless, as we observed a down-regulation of Pttn3 during exacerbations, it is likely that Pttn3 confers rather anti-inflammatory effects.
Regulated genes in asthma exacerbations

Figure 4: Regulated genes of particular interest concerning exacerbations. (A) Some genes that were differentially regulated upon influenza infection of mice suffering from allergic airway inflammation have already been associated with stable asthma or asthma exacerbations while (B) others have not yet been reported in this context. (A) Adamts9 can regulate TGF-β mediated collagen deposition; Muc5AC and Muc5B represent the major respiratory mucins; Ptpn3 is a phosphatase involved in signaling pathways; Chi3l4 might be activated by pathogen-derived chitin without being able to degrade it; Haptoglobin binds hemoglobin and prevents thus oxidative damage. (B) Myo9a and Syne2 have been associated with epithelial repair or wound healing, respectively; Pex1 is important for the assembly of peroxisomes which produce plasmalogens, that have anti-oxidant function; Asb7 negatively regulates cytokine signaling; Saa3 is likely to drive neutrophil recruitment. A green frame indicates up-regulated genes, a red frame down-regulated ones.
Regulated genes that have not yet been linked to asthma or asthma exacerbations.

In addition to the genes described above, our array revealed the differential regulation of 22 genes that have not yet been reported in the context of asthma or asthma exacerbations (Table 2). Amongst those, myosin IXa (Myo9a) has been associated with epithelial repair by regulating collective epithelial migration\(^57\) and synaptic nuclear envelope (Syne) 2 has been shown to be involved in processes of wound healing\(^58\) (Fig 4B). Ankyrin repeat and SOCS box-containing 7 (Asb7), peroxisomal biogenesis factor (Pex) 1, and Serum amyloid A (Saa) 3, have all been linked to the regulation of inflammation (Fig 4B). As not only pro-inflammatory responses, but also aberrant epithelial injury and repair processes have been suggested to contribute to asthma pathology\(^59\), all of those genes could also promote the inflammation during exacerbations.

In more detail, Pex1 is important for the assembly of peroxisomes\(^60\) and contributes thereby to the generation of plasmalogens, phospholipids critical for normal lung function. Being a component of surfactant and having anti-oxidant functions, plasmalogens might also play a role in the immune defense\(^61\). Asb family members are negative regulators of cytokine signaling by mediating ubiquitin-driven proteolysis and are thus implicated in diverse biological processes including the regulation of proliferation and differentiation\(^62\). Asb3 has for example been shown to act as an inhibitor of TNFR mediated responses\(^63\). One can speculate that Asb7 might be involved in shaping the cytokine response during exacerbations and it might therefore be an interesting therapeutic target. Saa protein has been shown to induce glucocorticosteroid resistant lung inflammation in mice, marked by the induction of the chemokines CXCL1 and CXCL2, and an influx of neutrophils\(^64\). As glucocorticoidsteroids are still one of the first-line treatments during exacerbations\(^65\), targeting Saa might be of special value to reduce neutrophilic inflammation in steroid resistant exacerbations.
Table 2: Differentially regulated genes during exacerbations that have not yet been described in the context of asthma. FC (fold change), GO annotation (Gene ontology annotation).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>FC</th>
<th>Biological function / involvement in diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Up-regulated genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asb7</td>
<td>Ankyrin repeat and SOCS box-containing 7</td>
<td>5.1</td>
<td>Negative regulator of cytokine signaling(^6^)</td>
</tr>
<tr>
<td>Chd7</td>
<td>Chromodomain helicase DNA binding protein 7</td>
<td>7.0</td>
<td>Regulator of chromatin remodeling and gene expression; involved in CHARGE syndrome(^6^)</td>
</tr>
<tr>
<td>Fam18b</td>
<td>Family with sequence similarity 18, member B</td>
<td>4.2</td>
<td>Integral to membrane, unknown function (GO annotation)</td>
</tr>
<tr>
<td>Gm7285</td>
<td>Predicted gene 7285</td>
<td>4.8</td>
<td>Unknown</td>
</tr>
<tr>
<td>Gpatch8</td>
<td>G patch domain containing 8</td>
<td>4.9</td>
<td>Gpatch8 mutation associated with hyperuricemia(^6^)</td>
</tr>
<tr>
<td>Hmcn1</td>
<td>Hemicentin 1</td>
<td>4.9</td>
<td>Extracellular matrix protein; required during mammalian development(^60,69)</td>
</tr>
<tr>
<td>Iars</td>
<td>Isoleucine-tRNA synthetase</td>
<td>4.8</td>
<td>tRNA aminoacylation for protein translation (GO annotation)</td>
</tr>
<tr>
<td>Myo9a</td>
<td>Myosin IXa</td>
<td>6.2</td>
<td>Might sustain collective migration of epithelial cells(^7^)</td>
</tr>
<tr>
<td>Ociad2</td>
<td>OCIA domain containing 2</td>
<td>4.1</td>
<td>Associated with epithelial cancer (adenocarcinoma) in the lung(^7^)</td>
</tr>
<tr>
<td>Pex1</td>
<td>Peroxisomal biogenesis factor 1</td>
<td>4.7</td>
<td>ATPase important for assembly of peroxisomes(^60)</td>
</tr>
<tr>
<td>Prkaa2</td>
<td>Protein kinase, AMP-activated, alpha 2 catalytic subunit</td>
<td>6.0</td>
<td>Family member involved in mediating anti-inflammatory effects of metformin(^71)</td>
</tr>
<tr>
<td>Saa3</td>
<td>Serum amyloid A 3</td>
<td>5.1</td>
<td>Induces CXCL1, CXCL2 and neutrophilia in murine lung(^66)</td>
</tr>
<tr>
<td>Snh1</td>
<td>Small nucleolar RNA host gene (non-protein coding) 1</td>
<td>4.9</td>
<td>Unknown</td>
</tr>
<tr>
<td>Snora70</td>
<td>Small nucleolar RNA, H/ACA box 70</td>
<td>4.6</td>
<td>Small nucleolar ribonucleoprotein complex (GO annotation)</td>
</tr>
<tr>
<td>Stard13</td>
<td>STAR-related lipid transfer domain containing 13</td>
<td>4.4</td>
<td>Potential tumor suppressor gene; GTPase activator activity(^72)</td>
</tr>
<tr>
<td>Syn2</td>
<td>Synaptic nuclear envelope 2</td>
<td>5.5</td>
<td>Cytoplasmic-nuclear coupling; impacts on wound healing(^6^)</td>
</tr>
<tr>
<td>Taf1d</td>
<td>TATA box binding protein (Tbp)-associated factor, RNA polymerase I, D</td>
<td>4.0</td>
<td>Regulation of transcription (GO annotation)</td>
</tr>
<tr>
<td>U46068</td>
<td>cDNA sequence U46068</td>
<td>5.3</td>
<td>Lipid binding; extracellular location and cellular component (GO annotation)</td>
</tr>
<tr>
<td><strong>Down-regulated genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1324046</td>
<td>Expressed sequence A1324046</td>
<td>-4.9</td>
<td>Unknown</td>
</tr>
<tr>
<td>Plekhf1</td>
<td>Pleckstrin homology domain containing, family F (with FYVE domain) member 1</td>
<td>-4.1</td>
<td>May induce caspase-independent apoptosis(^73)</td>
</tr>
<tr>
<td>Tmem212</td>
<td>Transmembrane protein 212</td>
<td>-4.9</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
By comparing our results to a study investigating the expression pattern of epithelial cells from asthma patients that were infected with rhinovirus *in vitro*\(^7^4\), we found no overlap in differentially regulated genes. Regarding the different systems investigated, mouse versus human, *in vivo* versus *in vitro* infection, rhinovirus versus influenza virus, this might not be very surprising. It indicates that we urgently need more *in vivo* data in order to validate novel targets for therapeutic intervention.

### 4.3.5 Conclusions

Our understanding of mechanisms that drive viral-induced exacerbations of asthma has increased in the last years\(^3\). Nevertheless, asthma exacerbations are still poorly controlled and in-depth studies of underlying molecular pathways are required to provide new potential targets for therapeutic approaches\(^6,15\). As epithelial cells are likely key players in asthma exacerbations, identifying epithelial genes uniquely regulated during exacerbations has the potential to improve our understanding of immune pathways involved. Moreover, their physical location at the direct interface between the environment and lung tissue makes them particularly amenable to inhaled therapeutic agents. Up to date, only *in vitro* expression profile studies are available, such as data from viral-infected epithelial cells isolated from asthmatic patients\(^7^4\), but no *in vivo* data has been published; a key point considering the importance of an appropriate tissue environment for representative gene expression\(^7^5\).

Our study reports for the first time *in vivo* changes in the expression pattern of structural cells during viral-induced exacerbations. We found an increased expression of genes that have already been linked to asthma exacerbations in a direct or indirect way, Muc5AC, Haptoglobin, and Chi3l4, and of genes directly or indirectly associated with stable asthma, such as Adamts9, Clec4n, Itln1, Muc5B, and Ptpn3 (Table 1, Fig 4A). Moreover, we identified changes in the expression of 22 genes that to our knowledge have not yet been reported in the context of asthma and exacerbations (Table 2). According to available literature, we suggest that the roles of Asb7, Myo9a, Pex1, Saa3 and Syne2 (Fig 4B), are worth to pursue during exacerbations and that especially Saa3 might be of importance in steroid resistant exacerbations. Although further studies are necessary to unravel the role of these genes during influenza-induced exacerbations of allergic airway inflammation, any one of them might provide a potential target for therapeutic intervention. As none of these genes was regulated during viral infections alone, influencing their expression
during exacerbations might not interfere with the anti-viral response, an important aspect regarding a clinical application.
4.3.6 References


Regulated genes in asthma exacerbations


70. Ishiyama, T. et al. OCIA domain containing 2 is highly expressed in adenocarcinoma mixed subtype with bronchioloalveolar carcinoma component and is associated with better prognosis. *Cancer science* 98, 50-57 (2007).


5 CONCLUDING REMARKS

In this thesis we investigated interactions between microbes and their hosts in the context of chronic respiratory diseases. Being directly exposed to the outside environment, the lungs are constantly challenged by pathogenic and non-pathogenic microorganisms. While some of them perform beneficial functions, such as vitamin generation\(^1\), others cause infections and can be particularly dangerous for persons suffering from chronic diseases, such as chronic obstructive pulmonary disease (COPD) or asthma\(^2\text{-}^4\).

COPD and asthma are both highly prevalent disorders of the respiratory tract that are associated with substantial morbidity and mortality. Current therapy still relies predominantly on corticosteroids and bronchodilators\(^5\text{-}^6\) that relieve symptoms and improve disease outcome to some extent, but do not provide a cure. A better understanding of molecular and cellular mechanisms underlying these diseases might provide novel treatment strategies to regulate disease development and to improve disease management.

In recent years, a positive effect has been confirmed for the use of pro- and prebiotics in gastrointestinal disorders\(^7\), and treatment with *Escherichia coli*\(^8\) or probiotics\(^9\) has been shown to attenuate allergic airway inflammation in preclinical models. Furthermore, an immunostimulative extract from common bacterial pathogens of the upper respiratory tract is efficient in reducing frequencies and symptoms of recurrent respiratory tract infections in children\(^10\text{-}^11\). These findings indicate that microbes, or their components, can have a positive impact on disease outcome and development. Indeed, it is widely recognized that the commensal bacteria of the gut are important for the appropriate development and regulation of the intestinal immune system\(^1\text{-}^12\), and dysbiosis has been associated with several chronic disorders, such as inflammatory bowel disease, obesity, and also asthma\(^13\text{-}^14\). Nevertheless, experimental evidence proving a direct impact of the microbiota on allergic airway disease was still missing and underlying mechanisms were unknown. Therefore, we investigated the development of allergic airway inflammation in the absence of commensal bacteria.

We found that germ-free mice exhibited an overall exaggerated allergic airway inflammation characterized by increased airway hyperresponsiveness, elevated Th2 inflammation, and increased accumulation of eosinophils. This excessive response
could be reversed by colonization with a complete specific pathogen free microflora, indicating that changes in the composition of the commensal flora might regulate the susceptibility to allergic diseases. Detailed studies of immune cells involved revealed a dysregulation of dendritic cells, macrophages, and basophils, in the absence of the microbiota. Germ-free mice displayed increased numbers of basophils, cells that have been suggested to contribute to Th2 inflammation by IL-4 production. On the contrary, the numbers of plasmacytoid DCs and alveolar macrophages, which have both been associated with anti-inflammatory functions\textsuperscript{15, 16}, were decreased. Notably, we observed also decreased numbers and activation states of CD11b\textsuperscript{+}MHCII\textsuperscript{hi}, CD11b\textsuperscript{+}MHC\textsuperscript{int}, and CD11b\textsuperscript{-} conventional dendritic cells. These semi-mature cDCs could potentially be a driving force in the promotion of Th2 responses thus leading to exaggerated allergic airway inflammation. Taken together we report that the presence of the commensal microbiota is essential for the regulation of cellular recruitment and maturation, and thus for the control of allergic airway inflammation. In order to apply our findings within the context of therapeutics, several questions have still to be addressed such as: whether it is the commensal bacteria of the lung or the gut, or both, that confer protection; which species are important for the observed immune regulation; and to what extent it is feasible and safe to alter an existing microbiota in humans.

Compared to this immunoregulatory function that commensal bacteria exert on allergic airway inflammation, microorganisms can also cause infections and aggravate chronic pulmonary diseases. Patients suffering from asthma or COPD experience repeated episodes of acute disease exacerbations due to infections with respiratory viruses\textsuperscript{17-19}. These can cause major respiratory distress and thus impact on the quality of an individuals health. Severe exacerbations can also lead to hospitalization\textsuperscript{2, 20}, and especially in the case of COPD, result in death\textsuperscript{3, 4}. However, no therapy is available that effectively targets the excessive inflammation characteristic of exacerbations, and the underlying mechanisms still remain elusive\textsuperscript{21-23}. We therefore assessed molecular and cellular processes of influenza-induced exacerbations of COPD and asthma.

We found a key role for IL-1β in driving neutrophilic pulmonary inflammation and lung dysfunction in COPD exacerbations. IL-1β driven neutrophilia was mediated by IL-17A in the first 24 h of infection, and could efficiently be abrogated at this early time point by neutralizing IL-17A. Furthermore, blocking of IL-1 signaling with the
IL-1R antagonist anakinra efficiently attenuated neutrophilic inflammation at the peak of viral infection. This finding is of particular importance as anakinra is a clinically proven drug, so that a potential off-label application could be realized rapidly. In conclusion, we propose IL-17A and IL-1β as valid therapeutic targets for the treatment of viral-induced exacerbations of COPD.

The epithelium is a key player in orchestrating inflammation during asthma and exacerbations. We sought therefore to assess whether viral exacerbations are associated with a specific regulation of epithelial genes. We found that 37 genes were indeed uniquely regulated in structural cells from allergic mice 24 h after an infection with influenza virus. Thirteen of these genes are of special interest, regarding their described functions or previous association with asthma and asthma exacerbations. Specifically, Haptoglobin, Clec4n, and Muc5AC have been associated, in a direct or indirect way, with asthma exacerbations in humans, and Adamts9, Clec4n, Itlln1, Muc5B, Ptpn3, or their families, have been linked to stable asthma in mice or humans. Of the latter, Clec4n and Intelecin1 are both pattern recognition receptors sensing fungal and/or bacterial antigens and are thus likely to influence inflammation during exacerbations as well.

For all other genes we found to be regulated specifically under an exacerbation setting, no role in asthma or asthma exacerbations have been reported. However, Asb7, Myo9, Pex1, Saa3, and Syne2 are of particular interest regarding their roles in inflammatory or repair processes. Further studies are necessary to investigate the specific contribution of these genes to viral-driven exacerbations and to conclude whether they might be of value regarding therapeutic interventions.

In conclusion, our mechanistic findings in asthma development and in exacerbations of asthma and COPD suggest several new approaches for clinical treatment. While the importance of the proposed epithelial targets during asthma exacerbations require further investigations, our work on COPD exacerbations provides evidence for an improved disease outcome using treatment with anakinra, a drug already available for clinical use, and it reports IL-17A as novel therapeutic target, for which neutralizing antibodies are already in late stage clinical development. Finally, our studies of the commensal bacterial flora and germ-free mice highlight a novel pathway that could be considered for managing asthma development and severity.
Concluding remarks

5.1 References


## Appendix

### 6.1 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAI</td>
<td>Allergic airway inflammation</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional dendritic cell</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FEV</td>
<td>Forced expiratory volume</td>
</tr>
<tr>
<td>FVC</td>
<td>Full volume capacity</td>
</tr>
<tr>
<td>GF</td>
<td>Germ-free</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HDM</td>
<td>House dust mite</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>I.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>I.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>IL-1 receptor antagonist</td>
</tr>
<tr>
<td>IL-1RAcP</td>
<td>IL-1R accessory protein</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin-1 receptor</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 receptor associated kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Muc-1</td>
<td>Mucin 1</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T</td>
</tr>
<tr>
<td>NLRP</td>
<td>Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid Schiff</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>Penh</td>
<td>Enhanced pause</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>R</td>
<td>Pulmonary resistance</td>
</tr>
<tr>
<td>Recol</td>
<td>Recolonized</td>
</tr>
<tr>
<td>Rn</td>
<td>Airway resistance</td>
</tr>
<tr>
<td>RORγt</td>
<td>Retinoic acid-related orphan receptor γt</td>
</tr>
<tr>
<td>S.e.m.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen free</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper 2</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper 17</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
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