The T Helper 17 Lineage in Pulmonary Diseases

cytokine analysis in local and systemic inflammation

Marthe Sentijna Paats

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cytokine analyses in lokale en systemische inflammatie

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Ik trok een streep:
Tot hier,
Nooit ga ik verder dan tot hier.
Toen ik verder ging
Trok ik een nieuwe streep.
De zon scheen
En overal zag ik mensen
Haastig en ernstig,
En iedereen trok een streep,
Iedereen ging verder.

Toon Tellegen
Dichtbundel “Over liefde en niets anders” (1997)

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A

AIMS AND OUTLINE OF THE THESIS

Already in 1986, Mosmann and Coffman introduced the concept of separate types of T helper cells, which was based on the distinct cytokine profiles that these key effector cells of the immune system produce when stimulated to differentiate. Cytokines are small proteins that are crucial in the induction of an inflammatory response in the body. Originally, two types of $CD4^+$ T lymphocytes were described: type 1 helper T cells (Th1 cells) and type 2 helper T cells (Th2 cells). Th1 cells produce large quantities of interferon (IFN)γ, mediate autoimmunity and are essential for the defense against intracellular pathogens such as in tuberculosis. Th2 cells produce mainly interleukin (IL)-4, play a central role in asthma pathogenesis and are important in clearing parasitic infections. In recent years, $CD4^+$ T cells were shown to produce proinflammatory cytokines that could not be classified according to this Th1-Th2 paradigm. IL-17 was the most prominent amongst these cytokines and $CD4^+$ T cells that preferentially produce IL-17 but not IFNγ or IL-4 were named Th17 cells. The discovery of this novel subset of T helper cells that produces IL-17 and IL-22 has since provided crucial new insights into immunoregulation, host defense and the pathogenesis of many conditions including autoimmune diseases. However, most of the evidence for an essential role for Th17 cells has been generated in mouse models and data on the contribution of Th17 cells in human diseases, including pulmonary disorders, remains limited. A better understanding of the cytokine networks involving the Th17 lineage in human pulmonary infection and inflammation will ultimately lead to the development of more effective treatment options in pulmonary diseases. Analysis of the local compartment, i.e. the lungs, in humans is, however, practically and ethically challenging, since procedures involving bronchoscopy are invasive, not without risks and unpleasant for patients. Because of this, studies on the Th17 lineage in human pulmonary diseases are also directed to other compartments in the body, in particular peripheral blood and sputum.

In this thesis we aimed to explore the involvement of the Th17 lineage in the context of innate and adaptive immune responses in different infectious and inflammatory pulmonary diseases.

To start, an extensive overview of the current knowledge on IL-17 and Th17 cells is given, focusing on the mucosal immunity of the lung (**Chapter 1**). In order to determine the presence and possible participation of the Th17 lineage in acute pulmonary infection, we studied patients with a community-acquired pneumonia (CAP) and followed these patients over time while the pneumonia resolved. In **Chapter 2** we quantified levels of soluble cytokines of both the innate and adaptive immune system in peripheral blood and bronchoalveolar lavages of CAP patients. Additionally, we analyzed local and systemic populations of $CD4^+$ T cells in CAP patients and healthy controls, to determine the proportions of cells that produced IL-17, IL-22 or both of these cytokines (**Chapter 3**).

In **Chapters 4-6** cystic fibrosis (CF) patients were studied. Although the gene defective in CF encodes a membrane protein that controls the transit of chloride ions across the plasma membrane of cells, CF is characterized by progressive lung damage due to massive airway inflammation. First, the incidence of viral pathogens in acute pulmonary exacerbations in adult cystic fibrosis patients was investigated (**Chapter 4**). Next, chronic infection and inflammation was studied in *Pseudomonas aeruginosa*-infected adult cystic fibrosis patients. In **Chapter 5**, cytokines associated with the Th17 lineage were quantified in sputa, nasal lavages and plasma. Furthermore, IL-17 and IL-22 positive T cells were studied in bronchial biopsies and peripheral blood of CF patients (**Chapter 6**).

Chapter 7 evaluates the systemic inflammatory status in COPD patients by providing a comprehensive cytokine analysis of circulating $CD4^+$ and $CD8^+$ T cells in COPD patients with different disease severity. In **Chapter 8** evidence for the involvement of the Th17 subset in sarcoidosis patients is demonstrated. A detailed description of the expression of IL-17 and IL-22, as well as other pro-inflammatory cytokines, in peripheral blood T cells was combined with studies on bronchoalveolar lavages and pulmonary mucosal biopsies of sarcoidosis patients.

In addition to quantification of soluble cytokine levels and T cell cytokine proportions, data from these cytokine analyses in patients with CAP, CF and COPD were also correlated with clinical parameters such as lung function measurements and markers of disease severity. Finally, the findings presented in this thesis are evaluated and the importance of the novel Th17 subset in the immunopathology of important respiratory disease conditions is put into clinical perspective in **Chapter 9**.

CHAPTER 1

Introduction

Adapted from:

M.S. Paats, P.Th.W. van Hal, C.C. Baan, H.C. Hoogsteden, M.M. van der Eerden and R.W. Hendriks (2012). **Interleukin-17 and T Helper 17 Cells in Mucosal Immunity of the Lung**, Lung Diseases - Selected State of the Art Reviews, Dr. Elvisegran Malcolm Irusen (Ed.), ISBN: 978-953-51-0180-2

1 Interleukin-17 and T helper 17 cells in mucosal immunity of the lung

In all mammals, including humans, the immune system is responsible for the protection against potentially hazardous pathogens, such as bacteria, viruses, parasites and fungi. In this remarkably effective defense system leukocytes, which mediate both innate and adaptive immune responses, play a central role.

The innate immune system comprises granulocytes (neutrophils, eosinophils and basophils), natural killer (NK) cells, mast cells and macrophages. These cells are the first line of defense and provide the immediate response against pathogens. Neutrophils and macrophages can eliminate a pathogen directly by phagocytosis. Moreover, their pattern-recognition receptors, recognizing structurally conserved molecules derived from microbes such as bacterial lipopolysaccharides, unmethylated CpG, or viral double-stranded RNA, allow them to respond to a wide variety of microbial invaders, e.g. by producing cytokines that activate T lymphocytes of the adaptive immune system.

Acquired or adaptive immunity is characterized by a slower but highly specific immune response. Three major cell types are involved in adaptive immunity: antigen presenting cells (APCs), T lymphocytes and B lymphocytes. Dendritic cells (DCs) are the most potent APCs. They act as messengers between the innate and the adaptive immune system by taking up, processing and presenting antigens to T lymphocytes. In response to presented antigens, T lymphocytes may react in different ways: $CD4^+$ T helper cells produce various cytokines that direct the immune response, whereas $CD8^+$ cytotoxic T cells produce toxic granules that induce death of infected cells. B cells are able to respond to pathogens by terminal differentiation into plasma cells after which they produce large quantities of antibodies. Modulation of B cell function and antibody production by $CD4^+$ T cells is an important step in coordinating immune responses. Upon activation, B cells can migrate to germinal centers, which are specialized structures in secondary lymphoid organs, where they interact with T cells and DCs. Costimulatory signals from T cells then facilitate selection of B cells with high affinity for immunoglobulins and control class switching of the immunoglobulin to IgG, IgA and IgE.

Following pathogen elimination, lymphocytes leave a lasting legacy of the antigens they have come across represented by memory cells. As a result, lymphocytes are able to mount a faster and stronger immune response in future encounters with the same antigen. Defective T cell function can increase susceptibility to infections, allergies and autoimmune diseases. T lymphocytes can however also be manipulated to either eradi-

cate tumor or control graft rejection after organ transplantation. Therefore, in addition to basic biological interest, knowledge on T cell biology is important to the understanding of the etiology of a wide variety of diseases and may improve current therapies.

During activation in a particular cytokine milieu, naïve $CD4^+$ T cells can differentiate into one of the several subsets of T helper (Th) cells. Already in 1986, Mosmann and Coffman introduced the concept of distinct types of T helper cells, which was based on the distinct cytokines profiles that T cells produce when they are stimulated to differentiate [1]. They described two types of Th lymphocytes, type 1 helper T cells (Th1 cells) and type 2 helper T cells (Th2 cells). Th1 cells produce large quantities of interferon (IFN)γ, induce delayed hypersensitivity reactions, activate macrophages, and are essential for the defense against intracellular pathogens. Th2 cells produce mainly interleukin (IL)-4 and are important in inducing IgE production, recruiting eosinophils to sites of inflammation, and helping to clear parasitic infections. Cytokines produced by cells of the innate immune system govern the differentiation of these T helper cells. IFNγ and IL-12 drive naive T cells into the Th1 pathway, whereas IL-4 initiates the differentiation of naive T cells into Th2 cells. At a molecular level, the differentiation of Th1 and Th2 cells requires specific transcription factors: T-bet for Th1 cells [2] and GATA3 for Th2 cells [3] (Figure 1). An additional T helper subset was recently identified which restrains excessive effector T cell responses and therefore accounts for the maintenance of immune homeostasis and prevention of immunopathology. These cells are called regulatory T (Treg) cells and are naturally present in the immune system as a functionally distinct $CD4^+$ T cell expressing the forkhead transcription factor FoxP3 and producing the cytokines IL-10 and transforming growth factor (TGF)-β. The differentiation of naïve T cells towards this lineage is driven by IL-2 and TGF-β [4] (Figure 1). T follicular helper (Tfh) cells are yet another $CD4^+$ T cell population [5-6]. They are important for the formation of germinal centers. Once these germinal centers are formed, Tfh cells are needed to maintain and regulate B cell differentiation into plasma cells and memory B cells. The signals that specifically instruct the differentiation of human Tfh cells remain unclear, but IL-12 and IL-21 seem to be required. Tfh cells express Bcl6 as their master transcription factor and may produce IL-21 and IL-4 [7-9] (Figure 1).

Interestingly, in recent years T cells were shown to produce proinflammatory cytokines that could not be classified according to this Th1-Th2 scheme. IL-17 is the most prominent amongst these cytokines, and T cells that preferentially produce IL-17 but not IFNγ or IL-4 were named Th17 cells. The discovery of this new subset of helper T cells that selectively produces IL-17 has provided better and exciting insights into immunoregulation, host defense and the pathogenesis of autoimmune diseases. In particular, it now appears that Th17 cells do not only play a key role in chronic inflammatory lung disorders, but also mediate protective immunity against various pathogens at respiratory mucosal sites.

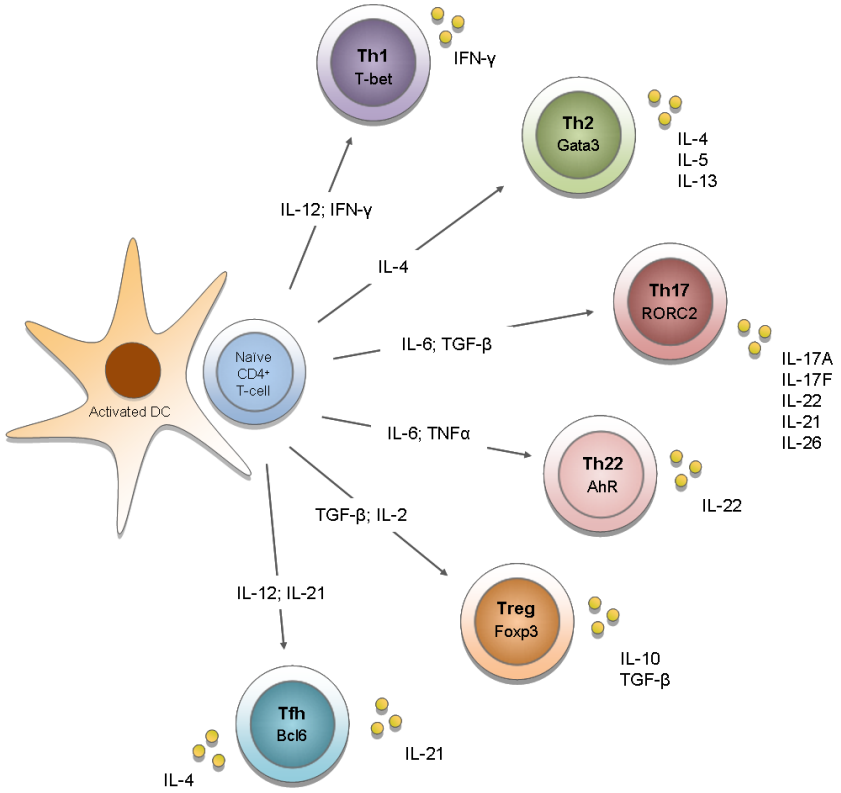

Figure 1. Overview of human CD4⁺ effector T cell differentiation. Upon activation in a particular cytokine milieu, naïve CD4⁺ T cells may differentiate into one of several lineages of T helper (Th) cells, including Th1, Th2, Th17, and Treg cells. These separate lineages are characterized by their distinct cytokine production pattern. The differentiation pathways are mainly based on the induction of transcription factors that serve as master regulators of specific lineages. However, cytokine production by Th cells seems to be more flexible than previously believed and recently new cells, such as Th22 and Tfh cells have been described. Whether these new subsets represent distinct lineages remains to be elucidated.

INTERLEUKIN 17 AND T HELPER 17 CELLS

IL-17 (also denoted IL-17A) was cloned in 1993 and initially called CTLA-8 [10]. In 1995, it was renamed as IL-17, its receptor was cloned and it was identified as a cytokine expressed by T cells, exerting effects on epithelial, endothelial, and fibroblast cells [11]. IL-17 has diverse biological functions, but the best characterized functions relate to its proinflammatory effects. Specifically, IL-17 recruits neutrophils via effects on granulopoiesis [12-13] and CXC chemokine induction, including CXCL8/IL-8 [14]. Furthermore, it acts on macrophages to promote their recruitment and survival and stimulates the production of proinflammatory cytokines and anti-microbial peptides, particularly β-defensins, from a variety of immune and non-immune cells [15-18]. By now we know that the IL-17 family includes 6 family members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. IL-17A and IL-17F are the most closely

related isoforms, sharing 55% homology with each other. Because of their structural and functional similarities and the fact that they are both produced by Th17 cells, IL-17A and IL-17F have been most thoroughly studied and characterized. Although it was known for more than 15 years that IL-17 is a product of activated $CD4^+$ T cells, it was not until 2005 that the Th17 cell was described as a distinct $CD4^+$ T-cell subset, critically responsible for the production of IL-17 in the context of autoimmunity [19].

Phenotype and differentiation of Th17 cells

IL-17A is the hallmark cytokine for Th17 cells. Nevertheless, these cells also produce other cytokines, such as IL-21, IL-22, tumor necrosis factor (TNF)-α, other members of the IL-17 family and, specifically in humans, IL-26 [20]. As with Th1 and Th2 cells, no single surface marker is specific for Th17 cells. However, human Th17 cells are thought to preferentially express CD161 on the cell surface [21]. Additionally, the selective expression of chemokine receptors in subsets of human memory T cells has been useful in defining lineages with different effector functions and migratory capacity [22]. It has been shown that human Th17 cells express the chemokine receptor CCR6 and its ligand CCL20 [20,23]. Coexpression of CCR4 and CCR6 further defines human T cells that produce IL-17 but not IFNγ [24]. In contrast, expression of CCR6 and CXCR3 identifies a more heterogeneous effector T cell population that produces both IFNγ and IL-17 [24]. These patterns of chemokine receptors appear to be biologically significant, as memory Th17 cells specific for *Candida albicans* are mainly $CCR6^+CCR4^+$ positive, whereas those that recognize *Mycobacterium tuberculosis* antigens are present in the $CCR6^+CXCR3^+$ subgroup producing both IFNγ and IL-17 [24-25].

The combination of cytokines that stimulate differentiation of Th17 cells has been subject of much debate. Initial studies on human T cell differentiation indicated that T cell activation in the presence of IL-1β, IL-6 and/or IL-23 was sufficient to induce Th17 cells, and that TGF-β inhibited this process [18,23,26-27]. In subsequent studies, however, TGF-β was reported to be important for the development of human IL-17 producing cells [28-30]. This discrepancy could be explained by more recent reports showing that the requirement for TGF-β in the differentiation process is indirect and relates to suppression of Th1 differentiation [18,31]. In the current view, the combination of IL-1β and IL-6 is essential for proper human Th17 cell differentiation whereas IL-23 is important for both expansion and survival of lineage-committed Th17 cells [23]. In addition to cytokine-driven Th17 lineage commitment, it has also been shown that prostaglandin E2 (PGE2), which is a mediator of tissue inflammation, directly promotes differentiation, expansion and proinflammatory function of human and mouse Th17 cells [32]. In humans, PGE2 induces up-regulation of the IL-23 and IL-1 receptors (IL-23R and IL-1R, respectively) and by synergism with IL-1β, IL-6 and IL-23 [33].

The observation that Th17 cells are a distinct lineage of cells with a unique cytokine and chemokine/chemokine receptor profile, led to the discovery of RORγt in mice [34]. RORγt

encodes the retinoid orphan nuclear receptor, and this transcription factor is required for the differentiation of Th17 cells. In the human system it has also been shown that forced over-expression of RORC2 (the human equivalent of RORγt) in human naïve T cells induces a Th17-like phenotype, by inducing IL-17A, IL-17F, IL-26 and CCR6 expression and down-regulating IFNγ secretion [28,35] (Figure 1). Activation of RORγt also causes expression of the IL23R, indicating that IL-23 acts on T cells that are already committed to the Th17 lineage. Exposure of developing Th17 cells to IL-23 not only enhances the expression of IL-17 but also induces IL-22 and suppresses IL-10 and IFNγ [36]. Yet, RORC2 alone can induce IL-17 production in only 20% of the T cell population [37] indicating that it acts in cooperation with other transcription factors for full commitment of precursors to the Th17 lineage. In addition to RORC2, the most specific and master transcription factor, at least four other transcription factors are linked to the human Th17 cell fate. These include signal transducer and activator of transcription-3 (STAT3), interferon regulatory factor-4 (IRF4), runt box transcription factor-1 (Runx1), and the aryl hydrocarbon receptor (AhR) [37]. Together they form a sophisticated network with positive and negative feedback loops. In addition, Th17 cells are inhibited by IL-2 (produced by Treg cells), IFNγ (produced by Th1 cells), and IL-4 (produced by Th2 cells) but also by other negative regulators such as retinoic acid [38].

Although this scheme of T helper cell differentiation might seem complex (Figure 1), it is in fact an oversimplification. Recent studies on T helper cell differentiation have revealed more plasticity in cytokine production than predicted by conventional models of T helper cell lineage commitment. Activated memory T cells preserve plasticity to alter their cytokine program according to the stimuli they receive. A cytokine restricted to one T helper subset can therefore be secreted by another subset under changing stimulation conditions. This feature is also observed in human Th17 cells [37]. Acquisition of IFNγ-producing potential by Th17 cells, particularly the simultaneous production of IFNγ and IL-17, is common [23,27] (see Figure 2). Additionally, Th17 cells can even stop producing IL-17 and become selective IFNγ producers resulting in a complete subset switch [39]. Although Th1 cells do not become IL-17 producers, under particular circumstances they can make IL-13 [40]. Th17 cells produce IL-22, but cells that make IL-22 and not IL-17 ("Th22 cells") have recently been identified as well [41-42]. Simultaneous production of IL-22 and IFNγ has also been described [39]. This plasticity even concerns master regulators: FoxP3 expression within Treg cells is heterogeneous and transient and former Treg cells have the capacity to produce proinflammatory cytokines such as IL-17 [43].

Moreover, also multiple master regulators can be expressed, such as Gata3 and FoxP3 in Tregs [44], or a combination of RORC2 and FoxP3 (mixed Th17-Treg) [39]. Therefore, expression of master regulators should not be simplified as mutually exclusive but rather as a gradient of transcription factors [39]. It remains to be shown whether there are preferential directions for plasticity or whether effector T cells can change in any direction from every

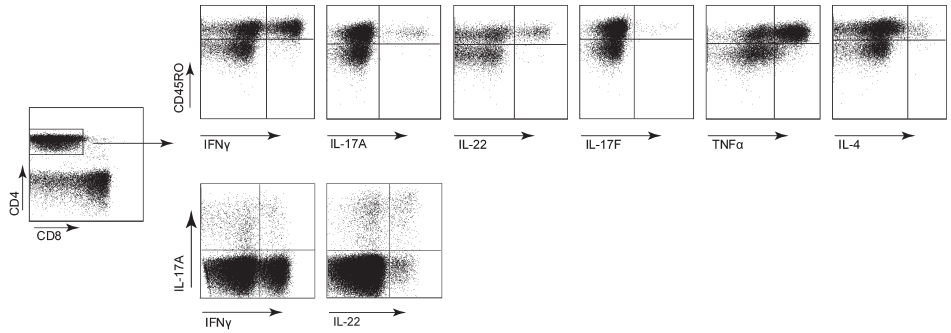

Figure 2. Flow cytometric analysis of cytokine production by CD4⁺ T helper cells. With the use of a technique called flow cytometry, it is possible to depict the cytokine producing potential of individual cells. In this experiment peripheral blood mononuclear cell (PBMC) suspensions were stained with monoclonal antibodies specific for CD3, CD4, CD8, and the indicated cytokines. Live CD4⁺ CD3⁺ T cells were gated and analyzed for the presence of the indicated cytokines in combination with the CD45R0 marker for memory T cells. Results are shown as dot plots and illustrate that CD4⁺ T helper cells are capable of producing all of the tested cytokines (top row, right upper quadrants). Moreover, CD4⁺ T helper cells have the potential to be simultaneously positive for IL-17 and IFNγ, and IL-17A and IL-22 respectively (bottom row, right upper quadrants).

starting point [43]. Plasticity could be an answer to the evolution of pathogens, allowing a proper response to new threats.

IL-17 producing cells other than Th17 cells

Th17 cells are not the exclusive producers of IL-17 nor is this production their only function. Other cell populations capable of producing IL-17 include both adaptive and innate immune cells.

Within the adaptive arm of the immune system, a subset of CD8⁺ cytotoxic T cells is also capable of producing IL-17. Studies have shown that these cells develop under conditions that are similar to those required by Th17 cells, but different from those required by IFNγ producing CD8⁺ T cells [45]. However, adaptive immune responses cannot explain the early IL-17-mediated immune responses that have crucial roles during stress responses and host defense. Early responses are induced within hours following tissue injury or exposure to pathogens [46-48], which is not enough time to allow for Th17 differentiation, indicating that innate immune cells play a crucial role in these early responses. The key feature of this innate IL-17 response is the early neutrophil recruitment. This results in a more efficient resolution of infection, in the maintenance of mucosal barrier integrity, but also in the potential induction of autoimmunity [46-49]. Recent studies have shown that γδ T cells are important innate-like IL-17-producing cells during infectious diseases and autoimmune inflammation [49-51]. Additionally, innate(-like) IL-17-producing cells described in literature include CD3⁺ invariant natural killer T (iNKT) cells, lymphoid tissue inducer (LTi)-like cells, natural killer (NK) cells and myeloid cells [52-54].

The γδ T cell subset is an innate-like immune cell population that has an important role at the mucosal barrier. These cells do not express the classical αβ T cell receptor (TCR)

but a γδ TCR instead. They bind to epitopes in much the same way as antibodies do and provide a rapidly available source of IL-17 [50]. Like γδ T cells, iNKT cells play a pivotal role in immunity as they provide a rapid response, with the capacity to critically amplify and regulate adaptive immune responses [55]. Initially, they have been divided into subsets that produce either IL-4 or IFNγ, but recently a new IL-17-producing subset that develops in the thymus has been described. This subset seems already committed to making IL-17 [56]. The LTi cell represents a primitive precursor of NK, NKT, and $CD4^+$ T cells. Specifically immature ($CD127^+$) NK cells are closely related to LTi cells [57]. LTi cells promote the formation of lymphoid organs and sustain primed $CD4^+$ T cell memory responses [57]. Thus, like IL-17 producing γδ T cells and NKT cells, LTi cells provide a rapidly available source of IL-17. Interestingly, it was recently recognized that innate lymphoid cells (ILCs) can be considered a family of non-T/non-B lymphocytes that includes not only NK and LTi cells, but also cells that produce IL-5, IL-13, IL-17 or IL-22. These ILC subsets are developmentally related and require cytokine signals through the common γ-chain of the IL-2 receptor. The distinct ILC subsets, which seem to have important roles in protective immunity analogous to helper T cell subsets, were recently reviewed by Spits and DiSanto [58]. Next to LTi and NK cells, other innate IL-17 producers have been postulated, including macrophages and neutrophils (reviewed by [53,59], however data is limited and further studies are needed to understand more of their role in mucosal tissue.

Interactions between Th17 and other cells of the immune system

Cells of the immune system modulate each other's function. Many cells may interact with Th17 cells including APCs, other T helper subsets, B cells and neutrophils (Figure 3). APCs play a central role in directing immune responses by secreting cytokines that polarize $CD4^+$ T cells into distinct lineages. Several studies support the hypothesis that changes in APC function probably precede inappropriate development and expansion of Th17 cells. For example, monocytes from inflamed joints of rheumatoid arthritis patients promote the development to Th17 cells but not Th1 or Th2 cells via a cell-contact-dependent mechanism [60]. Furthermore, it was found that monocyte-derived DCs from patients with multiple sclerosis secrete elevated levels of IL-23 when compared to healthy controls [61]. Additionally, in psoriasis DCs secrete IL-1β, IL-23 and CCL20, promoting both the development of Th17 cells and their migration to the skin [62]. However, the initial stimuli that polarize APCs to produce cytokines that promote Th17 cells are still unclear.

It has long been known that Th1 and Th2 cells antagonize each other's differentiation and function. Not surprisingly, IFNγ produced by Th1 cells and IL-4 produced by Th2 cells inhibit Th17 development [63]. For Treg cells and Th17 cells there appears to be an even closer developmental relationship because the differentiation of both of these cell types require transforming growth factor (TGF)β [64]. Additionally, Th17 differentiation is inhibited by Treg cells, via the production of IL-2. Th17 cells can also modulate B cell function as

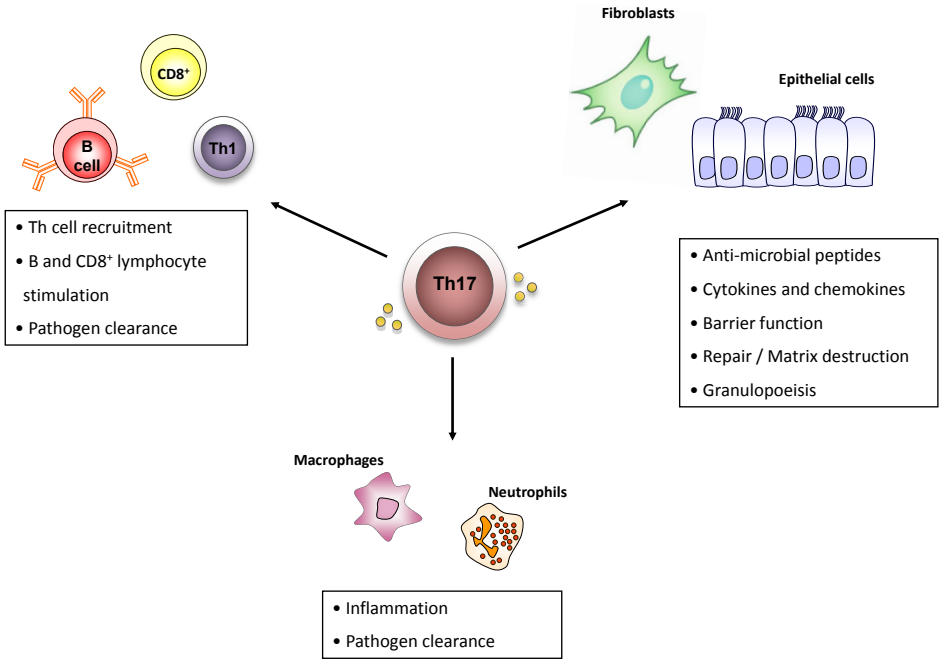

Figure 3. Th17 cells act on other immune cells and on cells of non-hematopoietic origin. Cytokines produced by Th17 cells have the ability to act on other cells. This allows for a crosstalk between immune and non-immune cells to provide protection and promote inflammation.

has been shown by their ability to promote antibody production (IgM, IgG and IgA but not IgE) [26].

There is growing evidence that T cells are involved in orchestrating sustained mobilization of neutrophils. In the lungs for instance, in a subpopulation of COPD patients there is an accumulation of $CD4^+$ and $CD8^+$ T cells, which is associated with the presence of neutrophils [65]. IL-17 seems to be an important mediator of linking activated T cells to accumulation of neutrophils, although solid data on T helper cells and neutrophils are lacking. In vitro work confirmed that IL-17 orchestrated neutrophilic influx by the production of CXCL8 (IL-8), CXCL1 (GRO-a), and granulocyte-macrophage colony stimulating factor (GM-CSF) in airway epithelial cells, smooth muscle cells, endothelial cells, and fibroblasts [66]. So, the importance of Th17 cells in neutrophilic inflammation lies in the ability of IL-17 to induce granulopoiesis, neutrophil chemotaxis, and the anti-apoptotic properties of G-CSF [15,17]. Accordingly, administration of IL-17A to the lung induces robust neutrophil recruitment [14], although – by contrast - chronic IL-17A/F overexpression resulted in enhanced lymphocyte and macrophage but not neutrophil numbers [67-68].

IL-17 IN LUNG DISEASES

Although Th17 cells have only recently been recognized as a distinct lineage of $CD4^+$ T cells, associations between IL-17 and human disease have been known for many years. Particularly disorders previously classified as typical Th1 disease, such as rheumatoid arthritis [69], inflammatory bowel disease [70], and psoriasis [71], are now considered to be primarily Th17-driven. For that reason (chronic) lung conditions previously believed to be Th1 cell disorders deserve special attention as Th17 cells might contribute to their pathogenesis. Moreover, immunity mediated by Th17 cells seems particularly important at epithelial and mucosal surfaces, as indicated by the distinct pattern of expression of Th17 subset-associated chemokine and cytokine receptors [17,72].

Because Th17 cells and IL-17 play a role in regulating neutrophilic and macrophage inflammation in the lung, a potential role in many different lung diseases including asthma and chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), pulmonary infectious diseases, sarcoidosis and other interstitial lung diseases and rejection after lung transplantation, seems legitimate. Asthmatics were shown to have elevated levels of IL-17A mRNA and protein levels in induced sputum and these levels were positively correlated with disease severity [73-75]. In COPD, recent studies showed increased expression of Th17 cytokines in bronchial mucosa and sputum [76-77]. Airway neutrophilia is a major feature of CF exacerbations and it is shown that sputum IL-17 is upregulated and correlates with *Pseudomonas aeruginosa* colonization [78]. In infection models in mice, there is considerable evidence that IL-17 and/or IL-23 are important in host responses against *Klebsiella pneumoniae* [79]. Furthermore, several studies have now linked IL-17 to fibrosis in the lung in mouse models of pulmonary fibrosis and idiopathic pulmonary fibrosis in humans [80-82]. Similarly, Th17 cells and IL-17 may be important regulators of the airway fibrotic response driving the development of bronchiolitis obliterans syndrome (BOS) upon lung transplantation.

For functional analysis of IL-17 producing cells in relation to other immune cells or epithelial cells, it is important to consider their anatomical localization. Obviously, bronchoscopy-guided or surgically guided biopsies allow histopathologically examination in situ, but are not frequently performed because they are invasive techniques. Bronchoalveolar lavage (BAL) is again not commonly performed except in lung transplantation and interstitial lung disease. For this reason sputum and nasopharyngeal washes are often studied. Blood and serum might be ideal to study because they are easily accessible. However, it is not always clear to what extend these compartments reflect what is happening in the lung. The methodology to study $IL\text{-}17^+$ T cells in biopsies or in serum and BAL represents only indirect evidence of Th17 cells. Flow cytometry does provide direct evidence as it can combine several parameters (Figure 2). In this way, identification of distinct $IL17^+$ T cells and even separate subpopulations is relatively simple.

COPD and asthma

COPD and asthma represent two classes of chronic obstructive lung disorders that may share some similar immunological disease mechanisms. COPD is marked by a progressive and irreversible airway obstruction and emphysematic changes in the lung. In asthma the airway obstruction is reversible and there is a marked airway hyperresponsiveness and airway inflammation. Recent studies on the immunological mechanisms of COPD and asthma pathogenesis point towards a role for IL-17 and Th17 cells in both diseases.

COPD

In COPD, chronic inhalation of toxic particles and gases causes destruction of lung parenchyma, activates epithelial cells, increases mucus production and stimulates migration of many inflammatory cells [83-84]. This results in an abnormal inflammatory response in the small airways and alveoli. It is believed that both the innate and the adaptive immune system are involved in this inflammatory process [84-85]. Progression of the disease is associated with the presence of lymphoid follicles, a histological hallmark of an adaptive immune response and termed bronchus-associated lymphoid tissue (BALT) collections. The presence of neutrophils, BALT collections, autoantibodies in the lungs, and also autoreactive T cells in the periphery, indicate $CD4^+$ T cell involvement in the pathogenesis of COPD [84,86-88]. A potential role for adaptive immune responses in COPD has also been suggested in studies that show expansion of lung T and B cells with oligoclonality in patients with COPD and in murine emphysema models [89-90]. To date, there are only few studies examining the expression of IL-17A and IL-17F in COPD. However, since neutrophilic inflammation (including elevated CXCL8 levels) is a common feature of COPD [91], and infiltrating $CD4^+$ T cells in COPD were previously considered to be Th1 cells, it is expected that Th17 cells play an important role in this disease.

Although direct evidence for the role of IL-17 and Th17 cells in COPD remains largely absent, the importance of IL-17 in stimulating chemokine production and the role of neutrophils and macrophages in promoting COPD pathogenesis have led to interest in a potential connection [86]. Another possible link derives from the ability of IL-17 to drive matrix metalloproteinases (MMP)9 production, a protein which is involved in the breakdown of extracellular matrix, as is observed in emphysema [92]. It is also known that IL-17-mediated signalling induces target cells to produce various inflammatory mediators such as TNF-α, IL-6 and IL-1β. Interestingly, increased levels of IL-6 and TNFα are found in sputum and BAL fluid and have been associated with disease severity in patients with COPD [93]. TNFα promotes CXCL8 expression from airway epithelial cells. Elevated levels of serum TNFα have also been linked to exacerbations in COPD patients [94]. In addition TNFα production by mast cells is increased due to IL-17A, leading to neutrophil infiltration in the airways [95]. Furthermore, IL-17 is capable of increasing mucin production from airway epithelial cells [92] and excessive mucus production is one of the characteristic of

COPD. Recently it was shown that patients with stable COPD exhibited elevated numbers of IL-22- and IL-23-positive cells in the bronchial epithelium and IL-17-positive cells in the submucosa when compared to healthy controls [76-77,96]. Additionally, airway smooth muscle cells from COPD patients express IL-17RA and respond to IL-17 by inducing CXCL8 production [97]. In contrast to these findings, the levels of IL-17 in sputum from patients with COPD do not differ from control subjects [98]. In addition to human studies, mice exposed to cigarette smoke exhibit enhanced IL-17 production [99-100]. Experiments on murine lung epithelial cells have also shown that overexpression of IL-17A induces a COPD-like lung inflammation [67]. Taken together, these findings indicate a role for Th17 cells in COPD, but it is still unclear whether and how these cells contribute to disease pathogenesis or progression. Moreover, to what extend Th1 and Th17-mediated immune responses affect airway obstruction, emphysematic changes, inflammation or COPD exacerbations are questions that need to be addressed.

Asthma

Asthma is usually characterized by concurrent airway inflammation, cytokine production, and airway hyperresponsiveness to relevant antigens and a specific trigger. The central role of the Th2 subset in the disease, inducing airway eosinophilia and bronchial hyperresponsiveness, is well accepted. Individually, the Th2 cytokines can explain many of the salient features of asthma, including IgE induction in B cells (IL-4), airway eosinophilia (IL-5) goblet cell hyperplasia (IL-4, IL-13) and bronchial hyperreactivity (IL-13 acting on bronchial smooth muscle cells) [101]. However, some individuals with asthma display airway neutrophilia rather than eosinophilia [102]. It appears that in those patients with asthma in which inflammation is nonatopic, non-IgE-dependent, and noneosinophilic, airway neutrophilia is correlated with asthma severity. This suggests a major role for neutrophils, at least in this subset of patients with asthma [103]. Neutrophilic inflammation has also been described in sudden-onset fatal asthma and neutrophil numbers are highly elevated in status asthmaticus [104]. These observations suggest a role for these cells in severe and fatal asthma [105]. With the involvement of neutrophils, several studies tried to find an association between Th17 lymphocytes and asthma.

Asthmatics have elevated levels of IL-17A mRNA and protein in breath condensate, sputum, BAL, and airway biopsies [73-74,106]. Furthermore, increased IL-17A and IL-17F levels are positively correlated to disease severity, suggesting an important role for IL-17A and IL-17F in severe asthma [75]. Indeed, elevated IL-17A levels also correlate to increased neutrophilic inflammation, a characteristic of severe and steroid-resistant asthma [73]. One could also hypothesize that IL-17 may have opposite pathophysiological roles in different disease stages, as would be supported by findings in an asthma mouse model, indicating that IL-17A is required for induction of disease but negatively regulates established asthma [107]. IL-17F may also play an important role in the development of asthma, as a polymorphism

in IL-17F which results in a loss-of-function mutation, is inversely related to asthma risk [108]. In these studies however, the cellular source of IL-17 remained unknown, but recent studies attributed the production of IL-17 primarily to $CD4^+$ T cells [79,106]. A novel subset of Th2 memory cells that co-express the key Th2 and Th17 transcription factors, GATA3 and RORCT, respectively, and coproduce Th2 and Th17 cytokines was recently described [109]. Interestingly, the number of IL-17^+ Th2 cells was significantly increased in peripheral blood of atopic asthma patients. Compared with classical Th17 or Th2 cells, these IL-17^+ Th2 cells had an increased capacity to induce influx of inflammatory leukocytes, and therefore are thought to represent key pathogenic cells promoting exacerbation of allergic asthma.

Pulmonary infections

There is considerable evidence that IL-17 and other Th17 cytokines are important in pulmonary host responses to infection by a variety of different bacteria, fungi and protozoa, and viruses. Also in infection, the major function of IL-17 appears to be to promote chemokine and pro-inflammatory cytokine production and consequent recruitment and activation of neutrophils and macrophages. Additionally, Th17 cytokines can control the infection by induction of anti-microbial peptides during the early immune responses at mucosal sites. Upon stimulation with various microbial agents, activated DCs secrete cytokines which determine the type of adaptive immunity that develops, i.e., whether the immune response is skewed toward Th1 or Th17 cells. Nevertheless, Th17 responses do not always seem to have a protective effect in mucosal infections. Current studies suggest that limited and correctly timed Th17 responses are protective, when appropriately balanced with concurrent Th1 immunity, but that uncontrolled Th17 cell activity could lead to a counterproductive level of organ inflammation [79].

Human studies on the role of IL-17 and Th17 cells in pulmonary infections are limited. The best human "model" demonstrating the role of IL-17 and Th17 cells in clearing pulmonary infections is Job's syndrome or the hyper-IgE syndrome. This syndrome is caused by loss-of-function mutations in STAT3, resulting in the inability of naïve T cells to differentiate into Th17 cells. These patients manifest chronic, recurrent and severe bacterial and fungal infections [110]. Although other factors such as disturbed neutrophil chemotaxis are also involved in hyper-IgE syndrome [111], the Th17 cell deficiency is prominent. This therefore suggests an essential role for Th17 cells in the host immune system.

Bacteria

The host response to bacteria is largely triggered by Toll like receptor (TLR) ligands stimulating the production of inflammatory mediators, such as the pro-inflammatory cytokines IL-1β, IL-6 and TNFα, and the recruitment of phagocytic cells to the lung [112]. Several components of the innate immune system have been identified as key mediators of bacterial clearance such as neutrophils and macrophages. The role of Th17 cells in

bacterial pneumonia is less clear. However, HIV patients with depleted $CD4^+$ T cells are more susceptible to bacterial infections in the lung [113], indicating a role for T cells in bacterial pneumonia.

One of the best studied bacterial pathogens in pulmonary host defense is *Klebsiella pneumoniae*. *Klebsiella pneumoniae* is a virulent Gram-negative pathogen that can cause pneumonia. In mice infected with this organism, TLR4 activation in the lung leads to production of IL-23 by DCs, which then stimulates $CD4^+$, $CD8^+$ and even γδ T cells to release IL-17 [47]. Interestingly, both IL17A and IL17F are induced in a dose-dependent fashion [72,114]. Accordingly, the protective effects of IL-17 in host defense against bacterial pathogens were shown in studies that compared the susceptibility of IL-17R-deficient and control mice to *K. pneumoniae* infection [115]. After intranasal infection, IL-17R-deficient mice were more susceptible to lung infection with *K. pneumoniae* [114-115]. The increased bacteraemia and mortality observed in these mice were associated with delayed neutrophil recruitment and reduced expression levels of CXCL1, CXCL2, and G-CSF in the lung 12–24 hrs after infection. Related experiments demonstrated the essential role of IL-23 in triggering IL-17 production during this infection. Also IL-23-deficient mice are highly susceptible to *K. pneumoniae* and do not upregulate IL-17 in response to infection, whereas IL-17 production readily occurs after infection in control mice [114]. Furthermore, administration of recombinant IL-17 restores the early chemokine response, enhances local production of TNFα and IL-1β, and reduces the bacterial burden in IL-23-deficient mice after *K. pneumoniae* infection [114-115]. Together, these findings demonstrate that IL-17 produced in an IL-23-dependent fashion is essential for early recruitment of neutrophils and other inflammatory cells to provide immunity to *K. pneumoniae* infection. In these studies it was also shown that in addition to IL-17 also IL-22 is measurable during infection. In contrast to gene deletion of IL-17, which results in a substantial reduction of CXCL1 and G-CSF in response to bacterial challenge, antibody neutralization of IL-22 causes an even more profound defect in mucosal immunity that leads to rapid dissemination of bacteria from the lung to the spleen [72]. The loss of mucosal immunity was not associated with defects in G-CSF or CXCL1 but with loss of barrier function and anti-microbial protein expressed in lung epithelium. Thus while IL-17 production by Th17 cells is critically important in host defense against *K. pneumoniae* infection in the airway because of its role in neutrophil recruitment and activation, IL-22 acts by augmenting the barrier defense against pathogens by triggering the production of anti-microbial peptides and enhancing healing of the epithelium should it be breached [72]. Importantly, not only Th17 responses are necessary for optimal protective immunity to *K. pneumonia*. Also IL-12-driven Th1 responses, resulting in efficient IFNγ production, contribute to the optimal bacterial clearance in a mouse model of *K. pneumonia* [114].

Following these initial studies with *K. pneumoniae*, the importance of IL-23 and IL-17 in host defense has been further established for a growing list of pathogens. Similar to *K. pneumoniae*, in mice infected with *Mycoplasma pneumoniae*, infiltration of the lungs

by neutrophils is dependent on IL-23–induced upregulation of IL-17 [116]. Additionally, accumulating evidence suggests that another Gram-negative extracellular respiratory pathogen, *Bordetella pertussis* which causes the whooping cough, may bias the host response towards the production of Th17 cytokines by preferentially inhibiting IL-12 and inducing IL-23 [117]. The above-referenced studies clearly demonstrate a protective role for Th17 effector cytokines in host defense against primary challenges with specific extracellular Gram-negative pathogens. Th17 response may also play a role in controlling primary infection with intracellular pathogens such as *Mycobacterium tuberculosis*, although a much more limited one when compared with extracellular bacterial pathogens. It was shown that although Th17 cells are not critical to the primary response to *M. tuberculosis*, Th17 activation is clearly involved in response to vaccination against tuberculosis [118]. In addition to this Th17-mediated vaccine-induced immunity to *M. tuberculosis*, Th17 cytokine responses have also been implicated in vaccine-induced immunity against *B. pertussis* [119] and *Streptococcus pneumoniae* [120]. This indicates that the host Th17 effector cytokines have evolved as protective immune mechanisms against extracellular bacteria but are dispensable for primary protection against most intracellular pathogens that require a Th1 pathway for protection, such as in tuberculosis infection.

Pseudomonas aeruginosa is another Gram-negative pathogen. Although not as virulent as *K. pneumoniae*, *P. aeruginosa* is a highly adaptable pathogen that causes both acute and chronic pulmonary infections. Chronic colonization and infection in the lung is associated with pre-existing airway disease such as CF. CF is a disease characterized by the excessive production of aberrantly hydrated mucus in the airways, resulting from mutations in the ion channel cystic fibrosis transmembrane conductance regulator (CFTR). This increased mucus production, blocks normal ciliary function and thereby enhances recurrent pulmonary infections. During pulmonary exacerbation, CF patients exhibit airway neutrophilia and elevated levels of IL-23 and both IL-17A and IL-17F in bronchoalveolar lavage fluid and sputum [121]. Recently it was shown that $CD4^+$ Th17 cells are prominently featured in the airway walls of CF patients but that NKT cells and γδ T cells are also sources of IL-17 in patients with CF [122]. In the latter study, $IL\text{-}17^+$ cells were correlated with CF and non-CF bronchiectasis, but not with the presence of *P. aeruginosa*. It has been shown that clearance of *P. aeruginosa* is dependent on Th17 responses [78].

Fungi, viruses and other opportunistic pathogens

Several reports from mouse and human studies have shown that Th17 cells are important for clearing opportunistic infections such as *Cryptococcus neoformans*, *Pneumocystis jirovecii* and *Candida albicans*. E.g. patients with Job's syndrome are extremely susceptible to mucocutaneous fungal infections caused by Candida species. It has been suggested that Th17 cytokines, particularly IL-17, contribute to tissue pathology in invasive *Aspergillus* infection in the lung particularly in the setting of NADPH oxidase deficiency [123]. In

respiratory tract models of fungal infections using *P. jirovecci*, induction of IL-23 and IL-17 following pathogen challenge is protective, since IL-23KO mice or neutralization of the IL-23/IL-17 axis resulted in impaired clearance of the pathogen [124].

Human viruses can induce IL-17 responses, as shown for herpes simplex virus [125] and respiratory syncytial virus [126]. Human rhinovirus infections are associated with exacerbations of asthma and COPD and IL-17 was shown to function synergistically with human rhinovirus to induce IL-8 from epithelial cells. This may contribute to the recruitment of neutrophils, immature DCs and memory T cells to the lung contributing to severe inflammatory profiles seen during viral exacerbations of airway disease [127].

Taken together, there is accumulating evidence for the involvement of IL-17 in bacterial, fungal and viral infection in the respiratory system in the mouse, whereas in human the role of IL-17 or Th17 cells is largely unexplored.

Sarcoidosis, pulmonary fibrosis and other interstitial lung diseases

Interstitial lung diseases (ILD) refer to a very heterogeneous group of lung diseases affecting the lung parenchyma. The exact nature of the initiating event and the subsequent cascade of mechanistic proceedings are most likely different in every single ILD. Multiple factors are likely to be involved but it is now clear that the immune system plays a major part in the pathogenesis of ILD. A similarity in every ILD is the interaction of growth factors, cytokines, and other mediators with cells that reside in the lung which seem to form part of the cascade of events that have been identified in the pathogenesis.

Recent data point to a potential role of IL-17 and Th17 cells in a number of ILD. E.g. Wegener granulomatosis [128], Langerhans histiocytosis [129], and hypersensitivity pneumonitis [130-131] have been reported to be linked to IL-17. Pulmonary IL-17 producing γδ T cells have also been detected in response to bleomycin-induced tissue damage, a model for induced pulmonary fibrosis [80]. Conversely, a particular subset of γδ T cells secreting IL-17 has been shown to contribute to hyperinflammatory granulomatous disease and fatal lung tissue damage during pulmonary aspergillosis [123]. Recently, also sarcoidosis was suggested as a Th1/Th17 multisystem disorder [132]. Sarcoidosis is a systemic inflammatory disease characterized by non-caseating granulomas in various organs with pulmonary involvement in over 90% of patients [133]. These granulomas are compact, organized collections of macrophages and epithelioid cells, surrounded by and infiltrated with T lymphocytes, but the pathological processes that result in granulomatous inflammation are largely unknown. The accumulation in the lung of apparently oligoclonal IFNγ-producing T helper cells in sarcoidosis indicated an antigen-driven Th1 response [134-135]. A recent study demonstrated the presence of IL-17 positive $CD4^+$ T cells in sarcoid lung tissue and the ability of these cells to respond to the chemotactic stimulus CCL20. Moreover IL-17A was expressed by macrophages infiltrating sarcoid tissue. Because IL-17A has also been implicated in the formation of a mycobacterial infection-induced

granuloma in the lung [136], it is conceivable that Th17 cells are involved in the pathogenesis of sarcoidosis.

IL-17 IN TRANSPLANTATION

Organ transplantation is currently a valid treatment option for selected patients with end-stage disease. Graft rejection is still the most severe complication following organ transplantation. In lung transplantation, episodes of acute rejection (AR) tend to lead to chronic rejection, which is the main cause of late graft loss and poor long-term survival [137-138]. The diagnosis of AR is based on clinical findings and/or histological confirmation in transbronchial biopsies [139]. It has been shown that in addition to the frequency and severity of AR, also other risk factors such as ischemia-reperfusion injury [137], gastro-oesophageal reflux [140], CMV pneumonitis and other infections [141] are associated with an increased risk of chronic rejection. Chronic graft rejection, clinically known as BOS is defined as a progressive decline in lung function with other underlying conditions being absent [142]. More than 50% of the patients surviving five years after lung transplantation suffer from BOS [143].

Classically, graft rejection has been shown to be mediated by $CD4^+$ and $CD8^+$ T cells [144]. Th1 cells were associated with graft rejection, whereas Th2 cells were considered to protect against rejection [145]. Evidence is accumulating for an important role of IL-17 in allograft rejection, both in rodent models and humans. Prior to the first description of Th17 cells, IL-17 was implicated in the process of allograft rejection. Blocking IL-17 function in a rat cardiac allograft transplantation model increased graft survival significantly [146]. Around that same period, a number of reports highlighted the importance of IL-17 in the context of renal transplantation. Already in 1998 it was shown that IL-17 was detectable by immunofluorescent staining of acutely rejecting human renal transplant biopsies, but not in healthy kidneys or pre-transplant biopsies [147]. Moreover, elevated IL-17 mRNA and protein levels could be detected in renal biopsy specimens and urinary sediment from patients found to have subclinical rejection when compared with control samples without any evidence of rejections [148]. Additionally, elevated IL-17 mRNA and protein levels were detectable as early as the second post-operative day in a rat renal allograft model and its appearance is followed by the local production of pro-inflammatory molecules known to be induced by IL-17 [149].

It is important to keep in mind that transplantation procedures themselves may have a direct effect on the cytokine profile within the graft. Following an organ harvest the ischemia-reperfusion injury results in the release of a number of inflammatory mediators. These mediators include some of the cytokines that are important in T cell differentiation such as TGFβ [150]. A recent study demonstrated that factors released by human endothelial cells as a consequence of ischemia-reperfusion injury could enhance the production of

both IL-17 and IFNγ by $CD4^+$ T cells [151]. These findings indicate that perioperative factors might result in increased Th17 activity within the graft.

IL-17 in lung transplantation

In lung transplantation, IL-17 has been implicated in ischemia reperfusion injury, acute rejection, infection and BOS [152-154]. At day 28 after lung transplantation, IL-17 mRNA levels were found to be elevated in the bronchoalveolar lavage (BAL) fluid from patients with acute rejection when compared with those without rejection. This difference disappeared at longer follow up [139]. These increased IL-17 levels were associated with increased numbers of both BAL lymphocytes and neutrophils and correlated with the severity of rejection [139]. However, such a correlation with severity of rejection could not be confirmed in another study even though this study did show increased numbers of IL-17 positive cells in endobronchial biopsies early after lung transplantation [155]. These apparently conflicting results may be explained by differences in the time of sampling, suggesting that early events after transplantation may be critical for inducing IL-17 production or that patient selection is crucial [156]. Additionally, patient heterogeneity may also cause conflicting results, e.g. by including both unilateral and bilateral transplant patients or by not discriminating between primary lung diseases.

Protein levels of IL-6 and IL-1β and mRNA levels for TGF-β, IL-17, IL-23 and IL-8 in BAL fluid were increased in lung transplant recipients with BOS when compared to controls [157]. CXCL8, a potent chemoattractant for neutrophils, has previously been associated with BOS, but it was unclear whether the presence of neutrophils was just a marker of general inflammation or a key mediator of obliterative bronchiolitis [158]. Since IL-17 promotes neutrophil chemotaxis, the presence of neutrophils has been suggested to be secondary to a Th17-mediated alloimmune or autoimmune response [156]. In a mouse model increased levels of IL-6 and IL-17 also correlated with tracheal obliteration, and blockade of IL-6 decreased both allograft fibrosis and IL-17 transcripts [159]. Increased neutrophilic inflammation of the airways with upregulation of IL-8 is common in the BAL of BOS patients. However, there are also many of these patients without considerable BAL neutrophilia despite the fact that they seem to be in an identical clinical condition with progressive decline in lung function, compatible with BOS. This may indicate the existence of different phenotypes within BOS with possible different treatment strategies. BAL neutrophilia might therefore be an important tool to select patients who might benefit from azithromycin treatment, since it has been demonstrated that azithromycin significantly reduces airway neutrophilia and CXCL8 in patients with BOS [160].

Th17 cell responses may also trigger BOS by facilitating autoimmune responses, because autoantibodies against collagen type V have been described to be involved in lung allograft rejection [161]. Immunohistochemical analysis indicated that collagen V becomes exposed in the lung matrix after ischemia-reperfusion injury in rat lung isografts and

allografts [154], and that collagen V peptides are released in the BAL [162]. Additionally, in humans it has been shown that pre-transplant patients who exhibit collagen V reactivity have an increased incidence of early graft dysfunction following lung transplantation [152].

CONCLUSIONS

We still have much to learn about the phenotype, function and regulation of human Th17 cells. It is however clear that IL-17 and other Th17 associated cytokines play a central role in regulating diverse immune responses. With their potential to induce a pronounced neutrophilic inflammation, which is a common feature of many pulmonary inflammatory conditions, Th17 cells are subject of great research interest in the field of pulmonary medicine.

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CHAPTER 2

Local and systemic cytokine profiles in non-severe and severe community-acquired pneumonia

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ABSTRACT

Local inflammatory responses in community-acquired pneumonia (CAP) remain insufficiently elucidated, especially in patients with non-severe CAP. In this study we determined local and systemic cytokine responses in CAP patients and correlated these with disease severity and other clinical parameters.

Levels of interleukin (IL)-6, IL-8, IL-10, IL-1β, tumor necrosis factor (TNF)α, interferon (IFN)γ, IL-22, IL-17A and IL-4 were determined in bronchoalveolar lavage (BAL) fluid and serum of 20 CAP patients upon admission and 10 healthy individuals. Systemic cytokine levels were also measured on days 7 and 30.

In BAL fluid of CAP patients, levels of IL-6, IL-8, and IFNγ were significantly increased compared with healthy individuals, but no correlations with disease severity were found. Systemic levels of IL-6, IL-10 and IFNγ were significantly higher in severe CAP patients than in non-severe CAP patients and healthy individuals. Moreover, these cytokines showed a significant correlation with the pneumonia severity index (PSI). In the total group of CAP patients systemic IL-8 and IL-22 levels were also increased compared with healthy individuals.

We therefore conclude that IL-6, IL-10 and IFNγ are important cytokines in CAP, although differences in disease severity upon admission are only reflected by systemic levels of these cytokines.

INTRODUCTION

Community-acquired pneumonia (CAP) continues to be a common and serious illness. Major gaps remain in the understanding of its pathogenesis: it is not clear why some individuals can easily control bacterial challenges and remain healthy, whereas others develop pneumonia. Several risk factors to calculate the probability of morbidity and mortality among CAP patients are known and described in prediction rules such as the pneumonia severity index (PSI) [1].

The clinical course of CAP is determined by inflammatory responses evoked by the causative pathogen. Studies in mice have shown that survival is associated with a strong inflammatory response early in the course of infection and rapid bacterial clearance [2]. In both mice and humans, regulation of this inflammatory response in pneumonia is dependent on complex interactions between immune cells and pro- and anti-inflammatory cytokines [2-4].

Several cytokines have been studied in relation to severity, aetiology and outcome of CAP [4-15]. Although the number of cytokines identified in the immunopathogenesis of CAP has grown considerably over the years, studies remain focused on well-known cytokines of the innate immune response, including interleukin (IL)-6, IL-10, IL-8, IL-1β, and tumour necrosis factor (TNF)α. But also IL-17A and IL-22, which belong to the novel T helper (Th) 17 subset, have been implicated in CAP [2-3]. Furthermore, interferon (IFN)γ is an important cytokine in both innate and adaptive immunity to respiratory pathogens [16], and IL-4 might be important in the immune response against *Mycoplasma pneumonia* [17]. Further characterization of local and systemic cytokine responses in CAP patients may increase our understanding of the host defence, with the goal of providing prognostic tools for clinicians or identifying potential therapeutic targets.

To date, most studies have focused on systemic inflammatory responses by measuring cytokines in peripheral blood of CAP patients. Those few reports in which local pulmonary cytokines were investigated generally included severe CAP patients on ICU wards [12,14,18] or patients with treatment failure [4]. Meanwhile, information on local cytokine responses in non-severe CAP patients is very limited. We hypothesised that the inflammatory response in CAP is compartmentalized and that disease severity correlates better with the local inflammatory response than with the systemic response. In this study we therefore determined both local and systemic cytokine responses in patients with non-severe and severe CAP, and correlated these with severity scores and other clinical parameters.

MATERIALS AND METHODS

See the Supplementary data for an extended version of the methods.

Study design

A prospective study was performed in twenty CAP patients between January 2009 and May 2011. Patients admitted through the emergency wards of the Erasmus MC and Sint Franciscus Gasthuis, which are both teaching hospitals, were enrolled in the study. The medical ethics committee of both hospitals approved the study. Written informed consent was obtained from the patient or closest relatives.

Inclusion and exclusion criteria are described in the supplementary data. Ten healthy volunteers matched for age, sex and smoking status and without history of cardiac or pulmonary disease, malignancy or autoimmune disease served as the control group.

Selection of antibiotic treatment was based on national guidelines [19]. The pneumonia severity index (PSI) was determined upon admission and patients were classified as non-severe CAP (PSI classes 1-3), or severe CAP (PSI classes 4 or 5).

Obtaining and processing of BAL and blood samples

After written informed consent and within 24 hours after admission, bronchoalveolar lavage (BAL) fluids were collected with a flexible fibre-optic bronchoscope (Olympus) according to recommended guidelines [20]. Venous blood samples were collected directly prior to the BAL procedure. At day 7 and 30 after admission, additional venous blood samples were collected. Methods of processing are described in the supplementary data.

Cytokine measurements

Levels of IL-6, IL-8, IL-10, IL-1β, TNFα, IFNγ, IL-22, IL-17A and IL-4 were determined by enzyme-linked immunosorbent assay (ELISA), using commercially available assays. Details and detection limits are provided in the supplementary data.

Statistical analysis

Data are shown as mean values (±SD) in cases of normally distributed data or median values with percentiles if not normally distributed. Cytokine levels were not normally distributed and therefore nonparametric tests were used to compare groups (Kruskal-Wallis test for across group comparison of three or more groups, Mann-Whitney U-test for pair-wise analyses). Normally distributed data were analyzed by unpaired t-tests. Correlations were calculated using Spearman's Rank correlation coefficient. Data analysis was performed using Statistical Package for Social Sciences (SPSS) 15.0 and Prism 5.01 (GraphPad). Statistical significance was taken as a p-value <0.05.

RESULTS

Clinical characteristics of study population

Twenty CAP patients and ten healthy individuals matched for age, gender and smoking status were included in this study. Clinical characteristics at baseline of the study population are shown in Table 1.

Thirteen patients (65%) had significant comorbidity (chronic obstructive pulmonary disease, heart disease, neurological disorder, chronic renal disease and diabetes mellitus). Based on the PSI scores upon admission, 10 patients were allocated to the non-severe CAP patient group, and 10 to the severe CAP patient group. In the total patient group,

Table 1. General characteristics of the study population

	CAP (total) n=20	Non-severe CAP (PSI 1-3) n=10	Severe CAP (PSI 4-5) n=10	Healthy controls n=10
Mean (SD) age in years	60.6±19	54.5±18.4	66.6±15.8	54.8±5.7
Sex, no. (%)				
Male	13 (65)	6 (60)	7 (70)	6 (60)
Female	7 (35)	4 (40)	3 (30)	4 (40)
Smoking, no. (%)	8 (40)	4 (40)	4 (40)	4 (40)
Comorbidity, no. (%)				n/a
COPD	5 (25)	4 (40)	1 (10)	
Heart disease	6 (30)	1 (10)	5 (50)	
Neurological disorder	4 (20)	1 (10)	3 (30)	
Chronic renal disease	1 (5)	-	1 (10)	
Diabetes mellitus	2 (10)	1 (10)	1 (10)	
PSI class, no. (%)				n/a
I	3 (15)	3 (30)	-	
II	5 (25)	5 (50)	-	
III	2 (10)	2 (20)	-	
IV	6 (30)	-	6 (60)	
V	4 (20)	-	4 (40)	
Mechanical ventilation, no. (%)	4 (20)	-	4 (40)	n/a
Mortality, no. (%)	2 (10)	-	2 (20)	n/a
Bacteriaemia, no. (%)	5 (25)	1 (10)	4 (40)	n/a
Prior antibiotic use, no. (%)	5 (25)	3 (30)	2 (20)	n/a
ICS	5 (25)	4 (40%)	1 (10)	n/a
Microbiological species, no. (%)	14 (70)	7 (70)	7 (70)	n/a
Streptococcus pneumoniae	7 (35)	3 (30)	4 (40)	
Stenotrophomonas maltophilia	1 (5)	1 (10)	-	
Pseudomonas aeruginosa	1 (5)	1 (10)	-	
Streptococcus pyogenes	1 (5)	-	1 (10)	
Staphylococcus aureus	1 (5)	1 (10)	-	
Haemophilus influenzae	1 (5)	-	1 (10)	
H1N1	1 (5)	-	1 (10)	
Adenovirus	1 (5)	1 (10)	-	
Unknown	6 (30)	3 (30)	3 (30)	

CAP: community-acquired pneumonia. SD: standard deviation. COPD: chronic obstructive pulmonary disease. PSI: pneumonia severity index. ICS: inhaled corticosteroids. n/a: not applicable.

5 patients (25%) were on statin therapy and 5 patients (25%) reported taking inhalation corticosteroids (ICS). Time from onset of symptoms to hospital admission ranged from 2 to 144 hours, with a median of 48 hours. Eight patients (40%) were included within a time frame of 48 hours and the other 12 (60%) at more than 48 hours from onset of symptoms. Severe CAP patients had a shorter period of time between onset of symptoms and hospital admission compared with the non-severe CAP patients (medians 30 and 48 hours respectively, p=0.01). Six patients (30%) reported taking antibiotics prior to hospital admission. Mean C-reactive protein (CRP) level of the patients upon admission was 227±170 mg/l. No differences in CRP levels were found between the non-severe and severe CAP patients. Furthermore, no significant correlation between CRP concentration and PSI was found. The two patients (5%) who died were both severe CAP patients.

In 14 patients (70%) a microorganism was identified (Table 1). There was no difference in incidence of pathogens found between non-severe and severe CAP patients. The most common pathogen was *S. pneumoniae*, present in seven patients (35%). A viral pathogen was found in 2 patients (10%).

Cytokine levels in BAL fluid

Levels of IL-6, IL-8, IL-1β and IFNγ were detectable in BAL fluid of all CAP patients. BAL fluid levels of IL-10, and IL-22 were only detectable in part of the CAP patients (five and six severe CAP patients, respectively). TNFα was only detectable in one non-severe and three severe CAP patients. In healthy individuals IL-10, TNFα and IL-22 were all below the detection levels. IL-17A and IL-4 were not detectable in BAL fluid of patients or healthy individuals.

When comparing the total group of CAP patients with healthy individuals, we found that IL-6, IL-8 and IFNγ levels in BAL fluid of CAP patients were significantly higher (Figure 1). A separate analysis of the non-severe and severe CAP patient groups showed that in both groups IL-6 was significantly increased, compared with healthy individuals (Figure 1). Levels of IFNγ were also significantly increased in severe CAP patients compared with healthy individuals, but not compared with non-severe CAP patients (Figure 1). For IL-8, IL-10 and IL-22 trends were observed towards increased levels in severe patients versus non-severe patients and healthy individuals, but significance was not reached (Figure 1).

None of the cytokines detectable in BAL fluid of patients upon admission correlated with PSI (data not shown). Furthermore, cytokine levels in BAL fluid of CAP patients upon admission showed no significant correlations with the causative pathogen found or with time from onset of symptoms to hospital admission. Finally, no correlations with cytokine levels in BAL fluid were observed with other clinical parameters, including mechanical ventilation, mortality, presence of bacteraemia, prior use of antibiotics, use of statin therapy, smoking habits or CRP levels upon admission. IFNγ levels in BAL fluid of CAP patients were however significantly lower in ICS users compared with non-users (p=0.02).

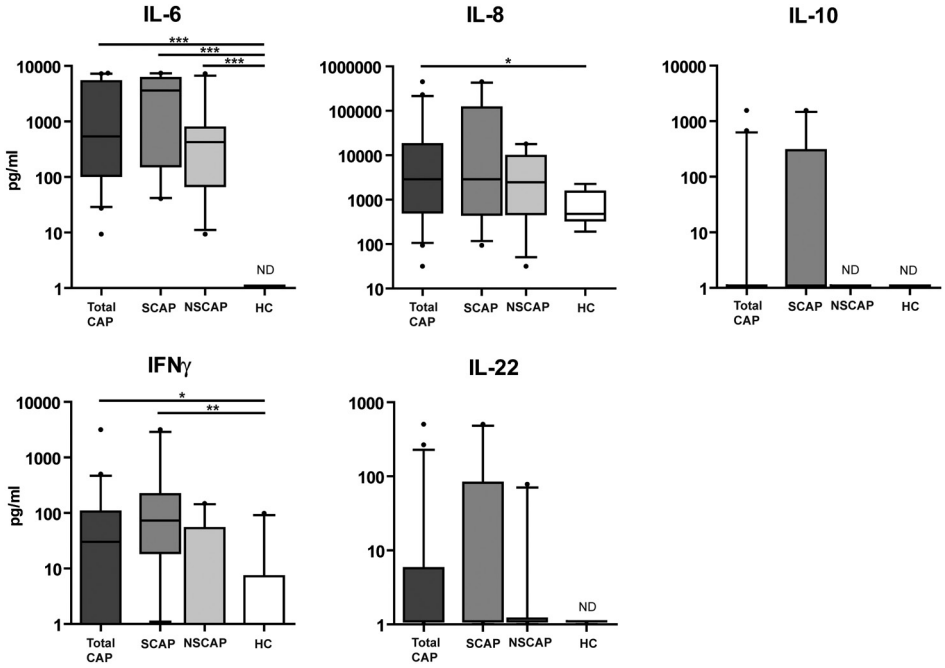

Figure 1. BAL fluid cytokine levels upon admission in CAP patients and in healthy individuals. CAP patients were classified as non-severe CAP (NSCAP, PSI classes 1-3) or as severe CAP (SCAP, PSI classes 4 or 5). Data are shown as box and whisker plots with 10th and 90th percentiles. Bold lines represent median values. Differences between groups were first tested with Kruskall Wallis tests and when significant, pair wise tested with the Mann Whitney U test. *: p<0.05; **: p<0.01; ***: p<0.001. CAP: community-acquired pneumonia. SCAP: severe CAP. NSCAP: non-severe CAP. HC: healthy control. IL: interleukin. IFN: interferon. N.D.: not detectable.

Systemic cytokine levels

When comparing the total group of CAP patients upon admission with healthy individuals, we found that the concentrations of IL-6, IL-8, IL-10 and IL-22 in serum were significantly increased in patients (Figure 2). TNFα, IL-17A and IL-4 could not be detected in the serum of patients or healthy individuals. For IL-1β, seven CAP patients (35%) had low but detectable serum levels (median 0 pg/ml; 10th and 90th percentiles 0-4.3 pg/ml, data not shown). Serum levels of IL-6, IL-10 and IFNγ upon admission were significantly higher in severe CAP patients than in non-severe patients and healthy individuals (Figure 2). In addition, IL-6 in non-severe CAP patients was significantly increased compared with healthy individuals. IL-8 and IL-22 levels were similar in non-severe and severe CAP, but IL-22 levels of both patient groups were significantly higher than in healthy individuals (Figure 2).

We also investigated changes of serum cytokine levels over time. Seven days after admission, IL-6 and IL-10 already normalized to levels similar to those of healthy individuals. Whereas IL-22 reached normal levels after 30 days, IL-8 remained elevated at day 30 after admission compared with healthy individuals (Figure 3).

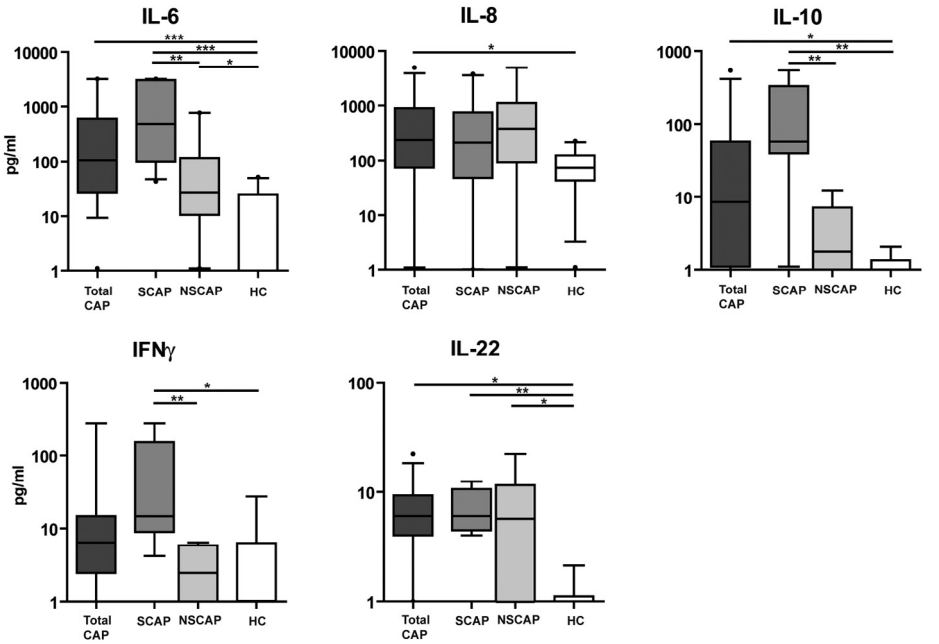

Figure 2. Serum cytokine levels upon admission in CAP patients and in healthy individuals. CAP patients were classified as non-severe CAP (NSCAP, PSI classes 1-3) or as severe CAP (SCAP, PSI classes 4 or 5). Data are shown as box and whisker plots with 10th and 90th percentiles. Bold lines represent median values. Differences between groups were first tested with Kruskall Wallis tests and when significant, pair wise tested with the Mann Whitney U test.*: p<0.05; **: p<0.01; ***: p<0.001. CAP: community-acquired pneumonia. SCAP: severe CAP. NSCAP: non-severe CAP. HC: healthy control. IL: interleukin. IFN: interferon. N.D.: not detectable.

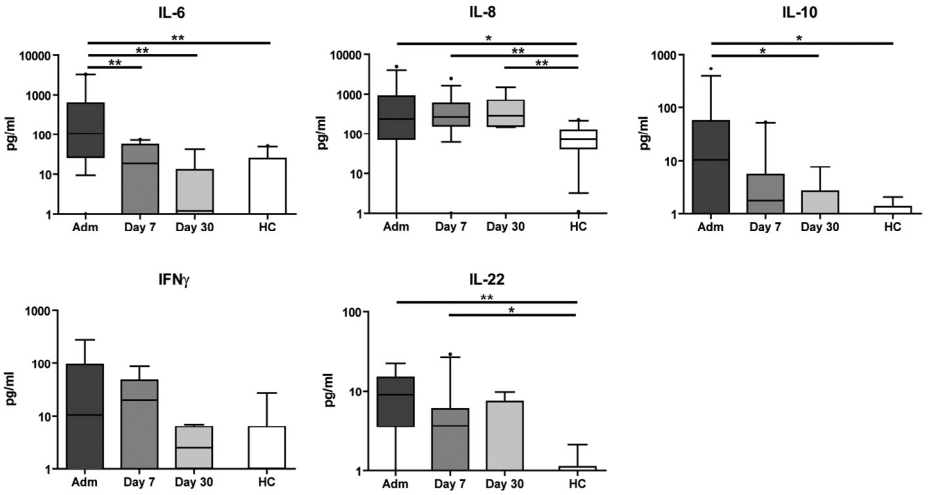

Figure 3. Serum cytokine levels upon admission, at day 7 and day 30 after admission in CAP patients and in healthy individuals. Data are shown as box and whisker plots with 10th and 90th percentiles. Bold lines represent median values. Differences in serum levels in CAP patients over time were tested as paired data with Wilcoxon signed rank test. Differences between different time points in CAP patients and healthy individuals were first tested with Kruskall Wallis tests and when significant, pair wise tested with the Mann Whitney U test. *: p<0.05; **: p<0.01; ***: p<0.001. CAP: community-acquired pneumonia. Adm: Admission. HC: healthy control. IL: interleukin. IFN: interferon.

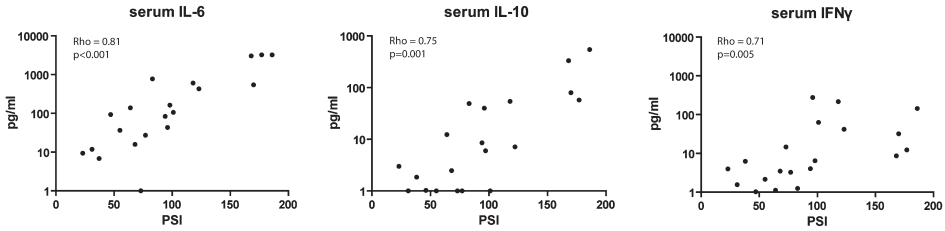

Figure 4. Correlations of serum cytokine levels with pneumonia severity index upon admission. PSI: pneumonia severity index. IL: interleukin

When correlating serum cytokine levels with clinical parameters, we found several correlations. First, in contrast to our analyses in BAL, serum concentrations of IL-6, IL-10 and IFNγ upon admission showed strong positive correlations with PSI (Figure 4). Furthermore, patients with bacteraemia had significantly higher serum levels of IL-6 and IL-10, compared with non-bacteraemic patients (p=0.005 and p=0.007, respectively, data not shown). Those four patients who required mechanical ventilation had higher serum IL-10 levels upon admission than patients who did not (p=0.02). The two patients (5%) who died within 30 days of hospital admission also had higher serum IL-10 levels, compared with surviving patients upon admission (p=0.03). Although we could identify a causative pathogen in 70% of patients, we did not find correlations between serum cytokine levels and pathogens. In addition, the reported usage of antibiotics prior to hospital admission or the time of onset between symptoms and admission to the hospital had no detectable effect on serum cytokine levels. Statin therapy, which may induce systemic inhibition of pro-inflammatory cytokines [21], and ICS usage did not have any detectable effect on serum cytokine levels in our cohort. Finally, no correlations between serum levels of any of the cytokines tested, including IL-6 and CRP levels upon admission, were found.

Correlations between BAL fluid and serum cytokines

In CAP patients, IL-6, IL-8 and IL-1β levels in BAL fluid were significantly higher than those in serum (p=0.0019, p=<0.0001 and p=0.0007 respectively). In contrast, IL-10 levels were higher in the serum than in BAL fluid (p=0.03), although levels were low in both compartments.

In CAP patients, a positive correlation was found between IL-6 levels in serum and BAL fluid (rho=0.58, p=0.003, data not shown). None of the other cytokines tested, showed a correlation between serum and BAL fluid.

DISCUSSION

The host inflammatory response in CAP is largely compartmentalized to the affected lung [6-7]. Nevertheless, local pulmonary cytokine responses remain insufficiently eluci-

dated, especially in patients with non-severe CAP. To our knowledge, this is the first study investigating local pulmonary and systemic cytokine profiles in both non-severe and severe CAP patients directly upon admission to the hospital. Our most important finding is that although inflammatory cytokine responses in CAP are higher in the lungs than in peripheral blood, disease severity only correlated with systemic IL-6, IL-10 and INFγ levels and not with any of the local cytokines tested. This study showed that in BAL fluid, levels of IL-6, IL-8 and IFNγ were significantly elevated in CAP patients compared with healthy individuals. In serum, IL-6, IL-8, IL-10 and IL-22 levels, but not IFNγ, were significantly increased compared with healthy individuals. However, of these cytokines IL-6, IL-10 and IFNγ in serum could differentiate between non-severe and severe CAP. Furthermore, levels of IL-6 in serum and BAL fluid were correlated. Finally, important inflammatory cytokines like TNFα and IL-17A were undetectable in BAL fluid or serum of CAP patients.

IL-6, IL-8 and IL-10 are three of the most studied cytokines in CAP. In line with previous studies, we found significantly increased IL-6 and IL-8 levels in BAL fluid in CAP patients [4,6-7,14,18]. IL-10 was not detectable in BAL fluid of healthy individuals or non-severe CAP patients and only in five out of ten severe CAP patients. Two groups have previously reported low but detectable IL-10 levels in BAL fluid of CAP patients [4,12]. Our inability to detect IL-10 in part of our patients could be due to differences in detection limits, study design or study population. Lee *et al.* studied only severe CAP patients on mechanical ventilation [12] and in Moret *et al*. there is a delay in sampling compared to our study, because BAL samples were analyzed in patients with treatment failure at 72 hours after start of antibiotic treatment [4].

Whereas serum levels of IL-6 and IL-8, IL-10 and IL-22 were significantly higher in patients compared with healthy individuals, serum IFNγ levels were only significantly higher in severe CAP patients. In contrast to BAL fluid cytokine levels, serum levels of IL-6 and IL-10 and IFNγ, proved to be good tools to discriminate between non-severe and severe CAP. Hereby, IL-6 and IL-10 acted as acute phase responders, since at day 7 levels decreased to values similar to those found in healthy individuals, consistent with previous reports [5,8,10].

The observed strong local and systemic induction of IL-6 emphasizes the importance of this cytokine in the inflammatory response in pneumonia. Furthermore, the correlation between IL-6 in BAL fluid and serum suggests that IL-6 produced in the lung contributes at least in part to serum levels of this cytokine [7,11]. Systemic IL-6 might therefore be a valuable biomarker to define severity of disease and act as a prognostic indicator in CAP patients [22-24]. Interestingly, in contrast to systemic IL-6, IL-10 and IFNγ concentrations, CRP levels could not differentiate between non-severe and severe CAP patients. Adding systemic IL-6 and/or IL-6 to IL-10 ratio measurements [25] to existing prognostic scales such as the PSI, might therefore improve mortality prediction in CAP patients.

To our knowledge, no previous studies of CAP patients have included IFNγ measurements in BAL fluid. We found that (i) IFNγ levels in BAL fluid were significantly elevated

in severe CAP patients compared with healthy individuals, (ii) systemic levels were significantly increased in severe CAP patients compared with non-severe CAP patients or healthy individuals, and (iii) systemic levels correlated with PSI. Although many cells have the capacity to produce IFNγ, it is the hallmark cytokine of Th1 cells. *In vitro* experiments with *S. pneumoniae* demonstrated the importance of Th1 cytokine production in early phases of disease [26]. The Th2 cytokine IL-4 and the Th17 cytokine IL-17A were not detectable in BAL fluid or in serum of CAP patients in early or late phases of disease. IL-22 is an IL-10 family cytokine member and can be produced by Th17 cells [27]. Importantly, in an experimental model of Gram-negative pneumonia, it has been shown that IL-22 can augment epithelial antimicrobial activity, thereby providing a crucial role in mucosal host defence in mice [2]. In BAL fluid, 60% of severe CAP patients had detectable but not significantly elevated levels of IL-22. Our finding that in serum of patients, both with non-severe and severe CAP, levels of IL-22 were significantly elevated supports the importance of this cytokine in the host response in human pneumonia.

Several factors can potentially influence inflammatory responses in patients with CAP. Previous studies showed that high doses of ICS may affect the immune system [28]. In our study population, we found lower concentrations of IFNγ in the BAL fluid of ICS users, compared with non-users. Because most ICS users were non-severe CAP patients (Table 1), it is possible that the lower levels of IFNγ in BAL fluid of non-severe CAP patients, is in fact due to ICS use. Similar to other studies [4,13], we found a large scatter in cytokine concentrations in both BAL fluid and serum. One explanation could be that the type and magnitude of cytokine secretion varies between different causative pathogens [29-30], although in our cohort we did not detect a relation between bacterial species and cytokine levels.

The present study has several limitations that should be considered. First, the number of patients included was small, although comparable to other studies of local inflammatory responses in CAP [6,14]. Nevertheless, we were able to classify the study cohort into both non-severe and severe CAP patients and to determine both local and systemic cytokine concentrations in all patients. Another limitation relates to uncontrolled factors present before patients entered our study. Patients were admitted to the hospital at different disease stages and some of them had already started antibiotic treatment. Although we did not find a relationship between time of onset of symptoms or prior antibiotic use and cytokine levels, we cannot completely exclude the possibility of modulation of the inflammatory response and cytokine expression by these factors.

In conclusion, our study provides a comprehensive analysis of cytokine profiles in CAP. We show that systemic levels of IL6, IL-10 and IFNγ can discriminate between non-severe and severe CAP patients. Levels of IL-6, IL-8 and IFNγ in BAL fluid were significantly higher in patients than in healthy individuals, but did not correlate with disease severity. We also found a correlation between IL-6 levels in BAL fluid and serum of patients. These

results show the importance of the systemic inflammatory response in CAP and further emphasize the importance of IL-6, but also of IFNγ in the local and systemic inflammatory response in patients with CAP. Future studies should show whether measurements of these cytokines are valuable to improve prognosis predictions.

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SUPPLEMENTARY DATA

Material and methods

Study design

Patients who fulfilled the following criteria were enrolled in the study: 1) age 18 years or over; 2) clinical presentation of an acute illness with one or more of the following symptoms suggesting CAP: presence of fever (t ≥ 38°C), dyspnoea, coughing (with or without expectoration of sputum), chest pain; and 3) presence of new consolidation(s) on the chest radiograph. Patients were excluded from the study if one of the following criteria applied: nursing home residency or hospital admission in the previous 15 days, presence of immunosuppression (e.g. HIV infection, systemic immunosuppressive agents including oral corticosteroids and chemotherapy), presence of a systemic autoimmune disease, pulmonary tuberculosis, bronchiectasis, malignancy, or the presence of obstruction pneumonia. Ten healthy volunteers matched for age, sex and smoking status and without a history of cardiac or pulmonary disease, malignancy or autoimmune disease served as the control group.

Upon admission, we collected data on age, gender, smoking habits, comorbidities, clinical signs and symptoms, chest radiography, biochemical analysis, microbiological findings, previous antibiotic treatment and time between pneumonia onset and inclusion. Selection of antibiotic treatment was based on national guidelines [1]. The pneumonia severity index (PSI) was also determined upon admission and patients were classified as non-severe CAP patients (PSI classes 1-3), or as severe CAP patients (PSI classes 4 or 5).

Obtaining and processing of BAL and blood samples

After written informed consent and within 24 hours after admission, bronchoalveolar lavage (BAL) fluids were collected with a flexible fibre-optic bronchoscope (Olympus) according to recommended guidelines [2]. The bronchoscope was introduced into the bronchus corresponding to the most abnormal area on the chest radiography. In patients with diffuse pulmonary infiltrates, the middle lobe or lingular division was chosen. Three aliquots of 20 ml sterile saline were instilled and subsequently gently aspirated. BAL fluids were filtered through a 100µm cell strainer (BD Biosciences), collected on ice and immediately centrifuged at 450x*g* for 10 minutes at 4°C. Supernatant was stored at −80°C and analysed in one batch.

Venous blood samples were collected directly prior to the BAL procedure. At days 7 and 30 after admission, another venous blood sample was collected from the patients. Blood samples were centrifuged at 1200x*g* for 10 minutes at 4°C. Serum was taken, stored at −80°C and then analysed in a single batch.

Measurement of cytokines in BAL fluid and serum

Cytokine levels in BAL fluids and serum of IL-6, IL-8, IL-10, IL-1β, TNFα, IFNγ, IL-22, IL-17A and IL-4 were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available assays (IL-8 OptEIA Set, BD Biosciences; all other cytokines Ready-Set-Go kits, eBiosciences). The limits of detection in pg/ml were: 2.0 for IL-6, 0.8 for IL-8, 2.0 for IL-10, 4.0 for IL-1β, 4.0 for TNFα, 4.0 for IFNγ, 8.0 for IL-22, 4.0 for IL-17A and 2.0 for IL-4.

Microbiological studies

Prior to initiation of antibiotic therapy, two sets of blood cultures were taken from each patient. In patients with a productive cough sputum samples were obtained upon admission for Gram staining and culture. Furthermore, urine samples were taken for antigen detection of *Streptococcus pneumoniae* and *Legionella pneumophila* serogroup 1. Gram stainings and cultures were performed on BAL specimens and, if clinical symptoms suggested, pharyngeal swabs were taken for identification of viral pathogens.

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CHAPTER 3

T helper 17 cells are involved in the local and systemic inflammatory response in community-acquired pneumonia

Submitted for publication, pending revisions

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ABSTRACT

Background Recent findings in mouse models suggest that T helper (Th)17 cells, characterized by production of interleukin (IL)-17A and IL-22, are involved in the immunopathogenesis of pneumonia.

Objective In this study we aimed to identify the involvement of Th17 cells in human community-acquired pneumonia (CAP).

Design Within 24 hours of admission, T cells from peripheral blood (n=39) and bronchoalveolar lavage (BAL, n=20) of CAP patients and of 10 healthy individuals were analyzed by intracellular flow cytometry for the production of various cytokines, including IL-17A and IL-22. Peripheral blood T cells were also analyzed 7 and 30 days after admission. Th17 cytokine profiles were correlated with pneumonia severity index (PSI) and microbial etiology.

Results In the BAL of CAP patients, proportions of IL-17A and IL-22 single positive, as well as IL-17A/IL-22 double positive $CD4^+$ T cells were significantly increased compared with healthy individuals. Significantly increased proportions of IL-17A/IL-22 double positive $CD4^+$ T cells in BAL were found in non-severe and severe CAP patients, as well as in pneumococcal and non-pneumococcal CAP. In the peripheral blood of CAP patients upon admission, we found significantly increased proportions of IL-17A/IL-22 double positive $CD4^+$ T cells. One week after admission, the proportions of these double positive cells were still significantly increased in CAP patients compared with healthy individuals.

Conclusions These data indicate that Th17 cells are engaged in the local and systemic immune response in human pneumonia. Especially, IL-17A/IL-22 double positive Th17 cells seem to be involved in the immunopathogenesis of CAP.

INTRODUCTION

Community-acquired pneumonia (CAP) is a common and serious illness despite the use of pathogen-directed or empirical broad-spectrum antimicrobial therapy [1]. Major gaps remain in the understanding of its pathogenesis: it is not clear why some individuals can easily control bacterial challenges and remain healthy, whereas others develop pneumonia.

The immunopathology of CAP is characterized by a strong local and systemic acute phase response. Several components of the innate immune system, such as neutrophils and macrophages, have been identified as key mediators of bacterial clearance in the lungs [2-3]. The role of T cells in the pathogenesis of pneumonia is, however, less evident. HIV patients with depleted $CD4^+$ T cells are at increased risk for developing lung infections, suggesting that these cells are involved in the immune defense against pneumonia [4].

Recently, it has been demonstrated that the novel T helper (Th)17 subset expressing the signature cytokines interleukin (IL)-17A and IL-22, has a protective role in host defense against different respiratory pathogens in the mouse [5]. IL-17A is thought to promote chemokine and pro-inflammatory cytokine production and consequent recruitment and activation of neutrophils [6]. IL-22 controls tissue responses during inflammation, for instance by increasing lung epithelial proliferation and transepithelial resistance to injury [7-8]. Several groups showed that IL-17A represents an important component in *Streptococcus pneumoniae* immunity in mice [9-11]. In mouse models of *Klebsiella pneumoniae* infection, both IL-17A and IL-22 production were required for optimal bacterial clearance [7,12-13]. Th17 responses are also involved in murine host defense against other bacterial pathogens including *Mycoplasma pneumoniae* [14], *Bordetella pertussis* [15] and *Pseudomonas aeruginosa* [16], as well as respiratory viruses [17-18]. Human studies on Th17 cells in pneumonia are currently lacking, except for the demonstration of a role for Th17 cells in clearing pulmonary infections in the hyper IgE-syndrome. In this disease, caused by mutations in STAT3, Th17 cells are lost and patients manifest chronic, recurrent and severe bacterial and fungal infections [19].

In conclusion, whereas there is accumulating evidence for the involvement of Th17 cells in bacterial and viral respiratory infection in mice, in humans Th17 cells are largely unexplored. In this study, we therefore aimed to investigate the presence of Th17 cells both locally and systemically in patients with CAP and to correlate these findings with disease severity and causative pathogen.

METHODS

Subjects

A prospective study was performed between January 2009 and May 2011. Thirty-nine CAP patients admitted through the emergency ward of the Erasmus MC and the Sint Franciscus Gasthuis, both teaching hospitals, were enrolled in the study. The medical ethics committees of both hospitals approved of the study. Written informed consent was obtained from patients or closest relatives.

Patients who fulfilled the following criteria were included: 1) age ≥18 years; 2) clinical presentation of an acute illness with one or more of the following symptoms suggesting CAP: presence of fever (t ≥38°C), dyspnoea, coughing (with or without expectoration of sputum), chest pain; and 3) presence of new consolidation(s) on the chest radiograph. Patients were excluded if one of the following criteria applied: nursing home residency or hospital admission in the previous 15 days, presence of immunosuppression (e.g. HIV infection, systemic immunosuppressive agents including oral corticosteroids and chemotherapy), presence of a systemic autoimmune disease, pulmonary tuberculosis, bronchiectasis, malignancy, or obstruction pneumonia. Ten healthy volunteers matched for age, sex and smoking status and without a history of cardiac or pulmonary disease, malignancy or autoimmune disease served as controls.

Cell collection and isolation

After written informed consent and within 24 hours after hospital admission, venous blood samples were collected (n=39). In a subset of patients (n=20) and in all healthy individuals (n=10) a bronchoalveolar lavage (BAL) was performed with a flexible fibre-optic bronchoscope (Olympus) according to recommended guidelines [20]. The bronchoscope was introduced into the bronchus corresponding to the most abnormal area on the chest radiography. In patients with diffuse pulmonary infiltrates and healthy individuals, the middle lobe or lingular division was chosen. Three aliquots of 20 ml saline were instilled and subsequently gently aspirated. Venous blood samples were collected directly prior to the BAL procedure. At day 7 and 30 after admission, additional venous blood samples were collected.

BAL and peripheral blood mononuclear cells were isolated and stored as described previously [21].

Flow cytometry analysis

For cytokine analysis, cells were stimulated with ionomycine (Sigma) and PMA (Sigma) in the presence of Brefeldin A (ebiosciences) and incubated for 4 hours at 37°C. Cells were stained as described previously [22] and measured on a LSRII Flowcytometer (BD Biosciences). Data were analyzed using FlowJo (BD) software. Isotype controls and fluo-

rescence minus one (FMO) controls were used to set gates. Flow cytometry experiments of all samples were performed in a single batch in order to eliminate the influence of day-to-day variation.

Microbiological studies

Prior to initiation of antibiotic therapy, two sets of blood cultures were taken from each patient. In patients with a productive cough, sputum samples were obtained upon admission for Gram staining and culture. Furthermore, urine samples were taken for antigen detection of *S. pneumoniae* and *Legionella pneumophila* serogroup 1. Gram stainings and cultures were performed on BAL specimens and, if clinical symptoms suggested, pharyngeal swabs were taken for identification of viral pathogens.

Statistical analysis

Data are shown as mean (SD) in case of normally distributed data or median with interquartile ranges (IQR) or percentiles when not normally distributed. Most data were not normally distributed and therefore nonparametric tests were used to compare groups (Kruskal-Wallis test for across group comparison of three or more groups, Mann-Whitney U-test for pair-wised analyses). Normally distributed data were analyzed by unpaired t-tests. Data analysis was performed using Statistical Package for Social Sciences (SPSS) 17.0 and Prism 5.01 (GraphPad). For statistical significance $p < 0.05$.

RESULTS

Clinical characteristics of study population

Venous blood samples were drawn from all 39 CAP patients included in the study; in 20 patients also a BAL was performed. No significant differences in clinical parameters were present between the total patient group and the subset of patients undergoing BAL procedure (Table 1). Based on pneumonia severity index (PSI) scores, patients were allocated to the non-severe (PSI class I-III, n=22[56%]) or severe (PSI class IV-V, n=17[44%]) CAP group. In ten patients (26%) *S. pneumoniae* was isolated. Thirteen patients (33%) had a known non-pneumococcal bacterial (n=10) or viral (n=3) etiology. In 16 patients (41%) no causative pathogen could be identified (Table 1). Patients with a pneumococcal pneumonia were not more severely ill than patients with a bacterial non-pneumococcal etiology (Supplementary Table 1). For the distribution of identified pathogens: see Supplementary Table 2.

As a control group, we included ten healthy individuals matched for age (54.8±5.7 years), gender (6 males) and smoking status (4 smokers).

Table 1. Baseline characteristics of CAP patients

	PBMC n = 39	**BAL** n = 20
Mean age±SD, years	55,6±18.5	60.6±19.0
Sex, no. (%)		
Male	25 (64)	13 (65)
Female	14 (36)	7 (35)
Smoking, no. (%)	15 (38)	8 (40)
Comorbidity, no. (%)		
COPD	10 (26)	5 (25)
Heart disease	8 (21)	6 (30)
Neurological disorder	6 (15)	4 (20)
Chronic renal disease	1 (3)	1 (5)
Diabetes mellitus	6 (15)	2 (10)
Pneumonia Severity Index, no. (%)		
Low I	10 (26)	3 (15)
Low II	7 (18)	5 (25)
Low III	5 (13)	2 (10)
Moderate IV	12 (31)	6 (30)
High V	5 (13)	4 (20)
Mechanical ventilation, no. (%)	6 (15)	4 (20)
Mortality, no. (%)	2 (5)	2 (10)
Bacteriaemia, no. (%)	6 (15)	5 (25)
Microbiological species, no. (%)		
Streptococcus pneumoniae	10 (26)	7 (35)
Other bacterial	10 (26)	5 (25)
Viral	3 (7.7)	2 (10)
Unknown	16 (41)	6 (30)

CAP: community-acquired pneumonia. PBMC: peripheral blood mononuclear cells. BAL: bronchoalveolar lavage. SD: standard deviation. COPD: chronic obstructive pulmonary disease.

Comparison of cytokine expression between CAP patients and healthy individuals

First, we quantified $CD4^+$, $CD8^+$ and γδ T cells in peripheral blood and BAL of all study subjects using flow cytometry. No significant differences were found in proportions of these lymphocyte subsets between CAP patients and healthy individuals (Supplementary Table 3). Absolute numbers of $CD4^+$ T cells in peripheral blood were similar in CAP patients upon admission compared with day 7 after admission (Supplementary Figure 1).

Next, we activated total peripheral blood mononuclear cell fractions and BAL cell suspensions and determined proportions of intracellular IL-17A and IL-22 single and double positive $CD4^+$ T cells (Figure 1A), as well as proportions of IFNγ (Th1) and IL-4 (Th2) positive $CD4^+$ T cells in peripheral blood (data not shown).

Based on PSI scores upon admission, CAP patients were distributed over a non-severe (n=22) and a severe patient group (n=17). In peripheral blood, no differences in the proportions of IL-17A and IL-22 single positive $CD4^+$ T cells were found between CAP patients and healthy individuals. The proportions of IL-17A/IL-22 double positive $CD4^+$ T cells were however significantly higher in the total CAP patient group and in severe CAP

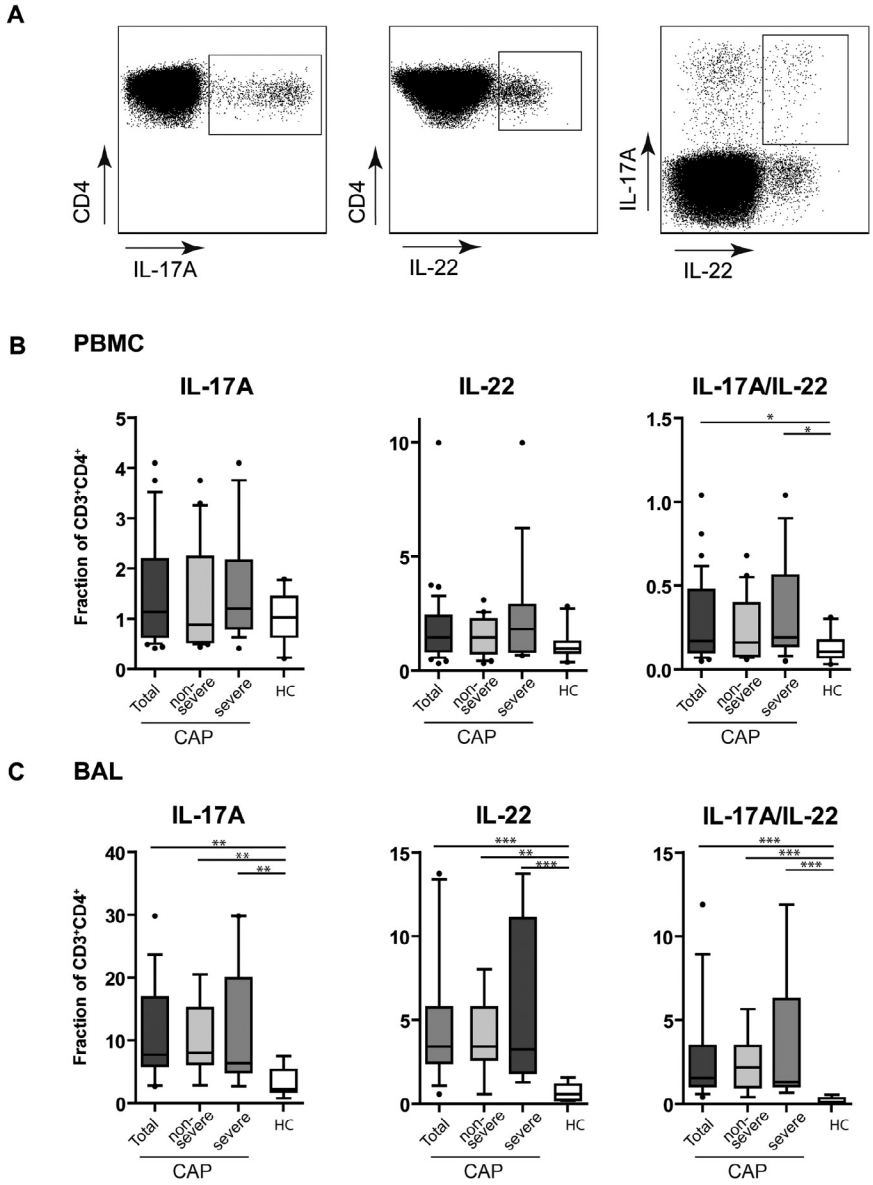

Figure 1. Cytokine expression by CD4⁺ T cells in peripheral blood and BAL of CAP patients and healthy individuals. CAP patients were subdivided as non-severe CAP (PSI class I-III) or as severe CAP (PSI class IV-V). **A**. Flow cytometric characterisation of CD4⁺ T cells. CD4⁺ T cells were analyzed for IL-17A expression (left panel), IL-22 expression (middle panel) and double expression of IL-17A and IL-22 (right panel). PBMC were gated from live CD56⁻ cells, BAL cells from live cells. Results are shown as dot plots. **B.** Proportions of total CD4⁺ T cells positive for the indicated cytokines in PBMC from the total group of CAP patients (n=39), non-severe (n=22) and severe (n=17) CAP patients, versus healthy individuals (n=10). **C.** Proportions of total CD4⁺ T cells positive for the indicated cytokines in BAL from the total group of CAP patients (n=20), severe (n=10) and non-severe (n=10) CAP patients versus healthy individuals (n=10). Data are shown as box and whisker plots with 10th and 90th percentiles. Bold lines represent median values. Differences between CAP groups and HC were tested with the Mann Whitney U test. *: p<0.05; **: p<0.01; ***: p<0.001. CAP: community-acquired pneumonia. HC: healthy control. PBMC: peripheral blood mononuclear cells. BAL: bronchoalveolar lavage.

patients compared with healthy individuals (Figure 1B). In BAL of CAP patients, we found significantly increased proportions of IL-17A and IL-22 single and double positive $CD4^+$ T cells (both non-severe and severe patients, when compared with healthy individuals; Figure 1C). No significant differences were found in the proportions of IFNγ or IL-4 single positive or IL-17A/IFNγ double positive $CD4^+$ T cells in blood between CAP patients and healthy individuals (data not shown).

Proportions of IL-17A and IL-22 single and double positive $CD4^+$ T cells were significantly higher in BAL when compared to peripheral blood (all $p<0.001$; Figure 1). However, no correlations were found between IL-17A and IL-22 positive $CD4^+$ T cells in blood and BAL of CAP patients (data not shown).

We also correlated Th17 responses in blood and BAL with other clinical parameters. Proportions of IL-17A/IL-22 double positive $CD4^+$ T cells in peripheral blood upon admission of CAP patients admitted to the ICU were significantly higher compared to the non-ICU patient group (Figure 2). No other correlations were found between Th17 responses and parameters of disease severity including PSI score, need for mechanical ventilation, extent of pulmonary consolidations on chest X-ray, bacteraemia, hypoxemia, CRP levels or mortality. The time of onset of symptoms before hospital admission, the use of antibiotics previous to hospital admission or the length of hospital admission also did not correlate with Th17 responses in blood or BAL of CAP patients.

In addition to $CD4^+$ T cells, IL-17A and IL-22 can also be produced by other T cell subsets, including γδ and $CD8^+$ T cells [23]. The proportions of IL-$17A^+$ or IL-22^+ γδ T cells in peripheral blood and BAL were very low and did not differ between CAP patients and healthy individuals. Likewise, the proportions of cytokine positive $CD8^+$ T cells in peripheral blood were not significantly increased in patients compared to healthy individuals. In BAL, the proportions of IL-17A and IL-22 single and double positive $CD8^+$ T cells were significantly elevated in patients when compared to healthy individuals, but percentages were 5-10 fold lower than those in the $CD4^+$ T cell compartment (data not shown).

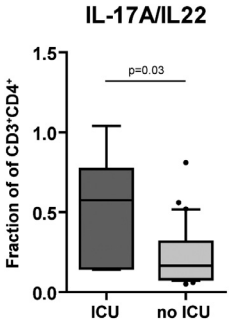

Figure 2. Proportions of IL-$17A^+$IL-22^+ double positive $CD4^+$ T cells upon admission in peripheral blood of CAP patients admitted to the ICU (n=6) and non-ICU (n=33) patients. ICU: intensive care unit.

Correlation of Th17 cells with microbiological aetiology

In the total CAP patient group, 10 (26%) patients had *S. pneumoniae* isolated as the causative pathogen. Next, we compared patients with a pneumococcal pneumonia with patients with a known bacterial causative pathogen other than *S. pneumoniae* (Supplementary Table 1). Cytokine profiles of peripheral blood CD4⁺ T cells were not significantly different between pneumococcal pneumonia, bacterial non-pneumococcal pneumonia, and healthy individuals (Figure 3A). By contrast, both patients with pneumococcal pneumonia and patients with a bacterial non-pneumococcal pathogen isolated, had significantly increased proportions of IL-17A and IL-22 single and double positive CD4⁺ T cells in BAL, compared with healthy individuals (Figure 3B). Consistent with the heterogeneity of pathogens involved, non-pneumococcal patients showed a large range in the proportions of cytokine expressing CD4⁺ T cells, both in peripheral blood and in BAL.

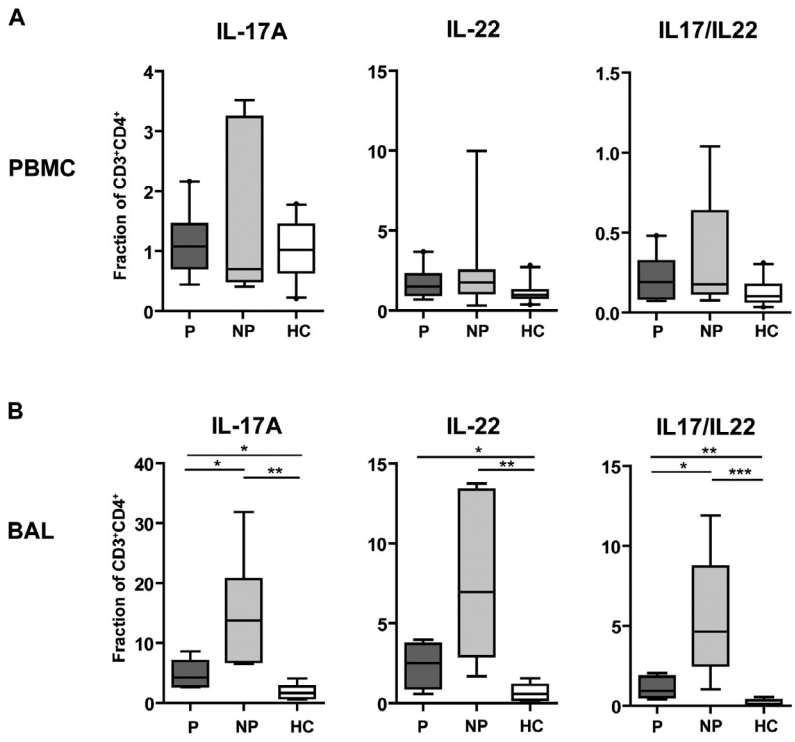

Figure 3. Cytokine expression by CD4⁺ T cells in peripheral blood and BAL of CAP patients with pneumococcal pneumonia, bacterial non-pneumococcal pneumonia and healthy individuals. CAP patients were classified as having pneumococcal pneumonia (P) when *Streptococcus pneumoniae* was identified as the pathogenic microorganism. Non-pneumococcal pneumonia (NP) patients had a bacterial pathogen isolated other that *S. pneumoniae*. **A.** Proportions of total CD4⁺ T cells positive for the indicated cytokines in PBMC from P (n=10), NP (n=10) and HC (n=10). **B.** Proportions of total CD4⁺ T cells positive for the indicated cytokines BAL from P (n=7), NP (n=5) and HC (n=10). Data are shown as box and whisker plots with 10th and 90th percentiles. Bold lines represent median values. Differences between groups were tested with the Mann Whitney U test. *: p<0.05; **: p<0.01; ***: p<0.001. HC: healthy control. PBMC: peripheral blood mononuclear cells. BAL: bronchoalveolar lavage.

Systemic Th17 cytokine responses over time in CAP

Peripheral blood samples were drawn from CAP patients upon admission and at day 7 and 30 after admission. To investigate systemic Th17 responses over time, we focused on proportions of IL-17A$^+$IL-22$^+$ CD4$^+$ T cells, which upon admission were significantly higher in CAP patients than in healthy controls (Figure 1B). At day 7 these proportions were still significantly higher than those found in healthy individuals and showed a small but not significant increase compared with values upon admission (Figure 4A). At day 30 proportions of IL-17A$^+$IL-22$^+$ CD4$^+$ T cells in CAP patients were similar to those in healthy individuals (Figure 4A).

Similar to the total group of CAP patients, also the group of severe CAP patients had significantly elevated proportions of IL-17A$^+$IL-22$^+$ CD4$^+$ T cells upon admission and at day 7, when compared with healthy individuals (Figure 4B). In non-severe CAP patients, the proportions of these cells were not significantly different from values in healthy individuals at any time point (Figure 4B).

When comparing pneumococcal and bacterial non-pneumococcal CAP patients with healthy individuals at different time points, we found that patients with pneumococcal pneumonia only had significantly higher proportions of IL-17A$^+$IL-22$^+$ CD4$^+$ T cells in their peripheral blood on day 7 after admission (Figure 4C).

DISCUSSION

In this study we aimed to identify the involvement of Th17 cells in the immunopathogenesis of CAP. The most important findings are that, in the BAL of CAP patients, proportions of IL-17A and IL-22 single and double positive CD4$^+$ T cells were significantly increased compared with healthy individuals. Significantly increased proportions of IL-17A$^+$IL-22$^+$ CD4$^+$ T cells in BAL were found in non-severe and severe CAP patients, as well as in pneumococcal and non-pneumococcal CAP. Upon admission, we found no differences in the proportions of IL-17A or IL-22 single positive CD4$^+$ T cells in peripheral blood between CAP patients and healthy individuals. The proportions of IL-17A$^+$IL-22$^+$ CD4$^+$ T cells were however significantly elevated in the blood of CAP patients and were still significantly increased one week after admission. Moreover, proportions of IL-17A/IL-22 double positive CD4$^+$ T cells upon admission in peripheral blood of CAP patients admitted to the ICU were significantly higher than those in the non-ICU patient group.

While Th17 responses to pneumonia are well established in mice [7,9-18], this is the first study demonstrating *in vivo* Th17 involvement as a distinct feature of CAP in humans. Our findings in peripheral blood point to an isolated Th17 response, since we did not find increased proportions of circulating IFNγ$^+$ (Th1) or IL-4$^+$ (Th2) T helper cells. An important difference between our study and the other studies on T cell cytokine responses in human

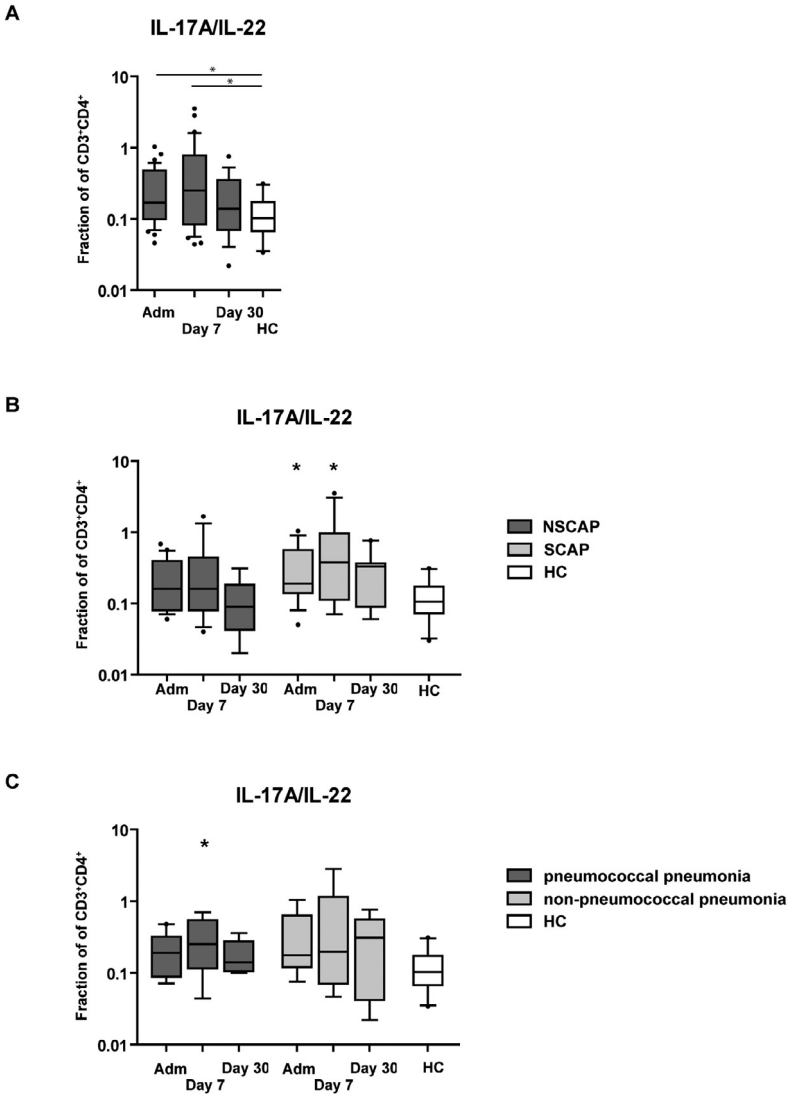

Figure 4. Proportions of IL-17A$^+$IL-22$^+$ double positive CD4$^+$ T cells in peripheral blood of CAP patients over time. **A.** Proportions of IL-17A$^+$IL-22$^+$ CD4$^+$ T cells in peripheral blood of CAP patients (n=39) upon admission, at day 7 and day 30 after admission and in healthy individuals (n=10). **B.** Proportions of IL-17A$^+$IL-22$^+$ CD4$^+$ T cells in peripheral blood of non-severe (n=22) and severe CAP patients (n=17) upon admission, at day 7 and day 30 after admission and in healthy individuals. **C.** Proportions of IL-17A$^+$IL-22$^+$ CD4$^+$ T cells in peripheral blood of CAP patients with pneumococcal pneumonia (n=10), bacterial non-pneumococcal pneumonia (n=10) upon admission, at day 7 and day 30 after admission and in healthy individuals. Data are shown as box and whisker plots with 10th and 90th percentiles. Bold lines represent median values. *: Significantly different from HC ($p<0.05$). HC: healthy control. NSCAP: non-severe community-acquired pneumonia. SCAP: severe community-acquired pneumonia.

pneumonia is the fact that we studied patients with a heterogeneous microbial aetiology. This could explain that, contradictory to our findings, previous studies showed Th1 or Th2 responses against certain respiratory pathogens [10,17,24-26]. Kemp *et al*. showed a Th1

response at the onset of pneumococcal infection [24]. However, their study population also included patients without pneumonia. Furthermore, in another study human monocytes were *in vitro* infected with pneumococci, which resulted in both Th1 and Th17 responses [10]. In pandemic 2009 H1N1 influenza elevated plasma levels of both Th17 and Th1 related cytokines were observed, but the cellular source of these cytokines was not identified [17,26]. The three patients in our study in whom a viral pathogen was isolated showed a wide range in cytokine expression, but IL-17A and IL-22 single and double positive $CD4^+$ T cells were in all cases above the median values of healthy individuals. A Th2 response was reported in Mycoplasma pneumonia [25]. However, since we included only two patients with *M. pneumoniae* we were not able to confirm these results.

It is conceivable that Th17 cytokines contribute to the vigorous recruitment and activation of neutrophils, which is one of the most important components of the innate immune response against bacterial infection. Particularly, since IL-17A has the ability to induce the expression of various proinflammatory mediators, such as IL-1, IL-6, TNF-α, CXCL8, G-CSF and GM-CSF by epithelial and stromal cells, leading to recruitment and activation of neutrophils [27]. In agreement with this notion, in a subgroup of patients where peripheral blood neutrophil counts were available we observed a positive correlation between the absolute numbers of circulating neutrophils and the proportions of IL-17^+ $CD4^+$ T cells in BAL (p=0,016, r=0,73; M.P. *et al*, unpublished).

In vitro differentiation of Th17 cells leads to cell populations with IL-17A or IL-22 single or double expression, a phenomenon also observed *in vivo* in the skin and lung [8,28-29]. Co-expression of IL-17A and IL-22 is particularly of interest, as we observed high proportions of IL-17A/IL-22 double-producing T helper cells in both BAL and peripheral blood of CAP patients. IL-17A and IL-22 can act synergistically to augment the expression of genes involved in defence against microbial pathogens, such as β-defensin-2 in keratinocytes [28]. Additionally, in bleomycin-induced lung inflammation IL-17A can regulate the expression, proinflammatory properties, and tissue-protective functions of IL-22, thereby determining the functional consequences of IL-22 expression in the lung [29]. Because of the protective effects of IL-17A and IL-22 in mouse models of pneumonia [7,9,11], our finding of increased proportions of IL-$17A^+$IL-22^+ $CD4^+$ T cells in CAP supports the hypothesis that Th17 cells are necessary for a vital beneficial host response in human pneumonia.

Our results show increased IL-17A and IL-22 single positive $CD4^+$ T cells in BAL, but not in peripheral blood of CAP patients. Furthermore, CAP patients had higher proportions of IL-17A and, to a lesser extend, of IL-22 $CD4^+$ T cell in BAL than in peripheral blood, indicating that these cells preferentially home to or are generated at the site of infection, i.e. the lungs.

Several factors have to be considered for the interpretation of our results. First of all, the number of patients included in this study is small, although comparable to other studies of local inflammatory responses [30-31]. Furthermore, like in other observational patient

studies, there are uncontrolled factors present before patients enter the study. We cannot completely exclude the possibility that e.g. differences in time of onset of symptoms and hospital admission causes modulation of inflammatory responses. Inferred from mouse models, respiratory inflammatory responses depend on the causative pathogen. We included CAP patients with different microbial aetiology. It would therefore be interesting to analyze larger cohorts of patients with viral or non-pneumococcal pneumonia to investigate if Th17 responses are different from those found in pneumococcal pneumonia. Nevertheless, by studying a cohort of CAP patients independently of microbial aetiology, results can be easily translated to daily clinical practice.

In conclusion, our data indicate that $CD4^+$ T cells with a Th17 profile are engaged in local and systemic immune responses in CAP. Especially, our novel finding of significantly increased IL-$17A^+$IL-22^+ Th17 cells in BAL and peripheral blood supports their role in host defence in CAP.

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SUPPLEMENTARY DATA

Supplementary Table 1. Clinical characteristics of CAP patients with pneumococcal versus non-pneumococcal bacterial etiology

	Pneumococ		Bacterial Non-pneumoncoc	
	PBMC (n=10)	**BAL (n=7)**	**PBMC (n=10)**	**BAL (n=5)**
Mean (SD) age (years)	61±17	65±16	50±19	60±22
Male sex, no. (%)	6 (60)	3 (43)	7 (70)	3 (60)
Smoking, no. (%)	6 (60)	3 (43)	5 (50)	3 (60)
Comorbidity, no. (%)				
COPD	2 (20)	2 (29)	4 (40)	2 (40)
Heart disease	2 (20)	1 (14)	3 (30)	2 (40)
Neurological disease	1 (10)	1 (14)	2 (20)	2 (40)
Chronic renal disease	0 (0)	0 (0)	0 (0)	0 (0)
Diabetes mellitus	2 (20)	1 (14)	0 (0)	0 (0)
PSI, no. (%)				
Low I	3 (30)	2 (29)	3 (30)	0 (0)
Low II	2 (20)	1 (14)	2 (20)	2 (40)
Low III	0 (0)	0 (0)	1 (10)	1 (20)
Moderate IV	4 (40)	3 (43)	2 (20)	1 (20)
High V	1 (10)	1 (14)	2 (20)	1 (20)
Mean (SD) CRP (mg/l)	265±156	306±171	225±167	310±159
Chest consolidation(s), no. (%)				
Unilobar, unilateral	10 (100)	7 (100)	7 (70)	4 (80)
Multilobar, unilateral	0 (0)	0 (0)	3 (30)	1 (20)
Bilateral	0 (0)	0 (0)	0 (0)	0 (0)
Hypoxemia (sat<90%), no. (%)	4 (40)	2 (29)	3 (30)	1 (20)
ICU admission, no. (%)	1 (10)	1 (14)	3 (30)	1 (20)
Mechanical ventilation, no. (%)	1 (10)	1 (14)	2 (20)	1 (20)
Bacteriaemia, no. (%)	4 (40)	3 (43)	1 (10)	1 (20)
Mean (SD) LOS (days)	7±5	8±4	10±10	11±9
Mortality, no. (%)	1 (10)	1 (14)	1 (10)	1 (20)

PBMC: peripheral blood mononuclear cells. BAL: bronchoalveolar lavage. SD: standard deviation. COPD: chronic obstructive pulmonary disease. PSI: pneumonia severity index. CRP: C-reactive protein. Sat: saturation. ICU: intensive care unit. LOS: length of stay.

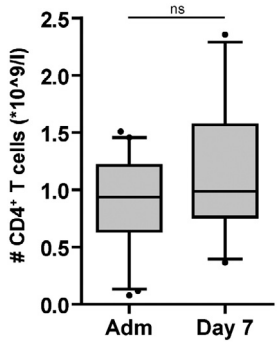
Supplementary Table 2. Distribution of isolated pathogens in CAP patients

	PBMC n=39	**BAL** n=20
Bacterial pathogen, no. (%)	**20 (51)**	**12 (60)**
Streptococcus pneumoniae	10 (26)	7 (35)
Klebsiella pneumoniae	2 (5)	0 (0)
Mycoplasma pneumoniae	2 (5)	0 (0)
Haemophilus influenzae	1 (3)	1 (5)
Stenotrophomonas maltophilia	1 (3)	1 (5)
Pseudomonas aeruginosa	1 (3)	1 (5)
Streptococcus pyogenes	2 (5)	1 (5)
Staphylococcus aureus	1 (3)	1 (5)
Viral pathogen, no. (%)	**3 (8)**	**2 (10)**
H1N1	2 (5)	1 (5)
Adenovirus	1 (3)	1 (5)
Unknown, no. (%)	**16 (41)**	**6 (30)**

Supplementary Table 3. Proportions of lymphocyte subsets

	PBMC		BAL cells	
	CAP	HC	CAP	HC
Subjects (n)	39	10	20	10
$CD4^+$ (% of $CD3^+$)	63.5 (52.9-74.4)	56.2 (45.0-75.0)	43.7 (31.7-58.6)	41.1 (33.5-67.0)
$CD8^+$ (% of $CD3^+$)	27.4 (18.2-33.63)	31.9 (18.5-40.7)	38.6 (25.6-47.6)	45.5 (27.6-51.7)
γδ T cells (% of $CD3^+$)	2.6 (2.0-5.6)	2.7 (1.0-4.0)	0.9 (0.3-2.5)	0.5 (0.3-2.1)

Values are expressed as median (IQR). Lymphocyte subsets are presented as proportions of viable $CD3^+$ cells. There were no significant differences between CAP and HC.



Supplementary Figure 1. Absolute number of $CD4^+$ T cells in peripheral blood of CAP patients upon admission (Adm) and on day 7 after admission.

CHAPTER 4

Incidence of viral respiratory pathogens causing exacerbations in adult cystic fibrosis patients

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ABSTRACT

Respiratory infections caused by respiratory viruses are common in paediatric cystic fibrosis (CF) patients and are associated with increased morbidity. There is only little data on the incidence of viral respiratory pathogens causing exacerbations in the adult CF patient population.

In this observational pilot study we show, by using molecular as well as conventional techniques for viral isolation, that during one year a viral pathogen could be isolated in 8/24 (33%) adult CF patients that presented with a pulmonary exacerbation.

This result shows that there is a considerable incidence of viral pathogens in pulmonary exacerbations in adult CF patients. Newly identified viruses such as pandemic influenza A/H1N1, human metapneumovirus, human bocavirus and human coronavirus NL63 were not detected in our population, except for one human coronavirus NL63.

INTRODUCTION

Respiratory viruses are thought to produce serious complaints in infants, immunocompromised patients and the elderly. Speculation about the growing predominance of viruses in respiratory infections emerged with severe acute respiratory syndrome (SARS), avian influenza A (H5N1) and the 2009 pandemic influenza A (H1N1). Over the last decade, new viruses like human metapneumovirus, human bocavirus and human coronavirus NL63 were identified.

The influence of viral infections in exacerbations of cystic fibrosis (CF) in the paediatric population has been extensively studied [1-5]. These studies show that many acute pulmonary exacerbations in CF children are associated with respiratory viruses, leading to decrease in lung function and disease progression. In adult CF patients this relationship remains less clear because of a lack of studies in this specific population. Also, incidence numbers reported may be an underestimation because of the use of conventional techniques in identifying respiratory viruses only. Information about viral incidence was most often obtained by using standard tissue culture, immunofluorescence, or serological assays only, which have a low sensitivity in identifying viral infections [6]. A study in a paediatric CF cohort showed that detection of respiratory viruses increased when using molecular techniques such as real-time polymerase chain reaction (RT-PCR) for viral isolation. In this study, in 46 % of young CF patients with a pulmonary exacerbation a viral pathogen was detected, which points to an important role of viral pathogens in the pathogenesis of exacerbations of CF [3].

We hypothesized that, by using molecular techniques for viral identification, the incidence of viral pathogens at the start of a pulmonary exacerbation in an adult CF population would be similar to that reported in paediatric cohorts.

MATERIALS AND METHODS

Patients were included from January to December 2010. At time of study enrolment nasal washes and spontaneous sputum, representative for the lower respiratory tract, were collected for bacterial and viral identification, according to standard diagnostic and handling procedures of the Erasmus Medical Center.

We performed a prospective observational pilot study in adult (≥ 18 years old) CF patients who presented with a pulmonary exacerbation in the Erasmus Medical Center, which is a CF centre with a total adult population of 125 patients. There were no exclusion criteria. Since this was an observational pilot study, there was no control group.

Pulmonary function test data (vital capacity; VC, and forced expiratory volume in one second; FEV1) were collected upon study enrolment, at day 21 and after three months during clinical stability after exacerbation.

Pulmonary exacerbation was defined by the presence of at least three new clinical findings (including increased cough, sputum production, fever, weight loss, school or work absenteeism, increased work of breathing, decreased exercise tolerance, or a deterioration in the chest exam, chest radiograph, FEV1 or haemoglobin saturation) [7].

This study was approved by the medical ethics committee of the Erasmus Medical Center Rotterdam, the Netherlands. Written informed consent was obtained from each patient before enrolment in the study.

Viral isolates

All samples were screened for the presence of viral respiratory pathogens by a real-time reverse transcriptase (RT-) PCR with primers and probes used in a routine setting of Erasmus MC's molecular viral diagnostics, described in Table 1.

Nasal washes and sputum were pre-treated to prevent inhibition in subsequent (RT)-PCRs. Pre-treatment consisted of a seven times dilution step in DMEM (Lonza Biowhittaker, Vervier, Belgium), including 7,5% NaHCO3 (Lonza Biowhittaker, Vervier, Belgium), 1M HEPES (Lonza Biowhittaker, Vervier, Belgium), 10% penicillin/streptomycin (Greiner

Bio-one, Alphen a/d Rijn), 5% amphotericine-B (Bristol-Meyers Squibb, Woerden, The Netherlands) and 40% fetal bovine serum (Lonza Biowhittaker, Vervier, Belgium). After centrifugation, 5 min at 3,000 rpm (Hettich, Rotina 380, Beun de Ronde, Abcoude, The Netherlands), supernatant was stored at −80°C.

RNA was extracted using MagnaPureLC (Roche Diagnostics, Almere, The Netherlands) and total nucleic acid isolation kit with an input and output volume of 200 µl and 100 µl, respectively. The extraction was internally controlled by the addition of a known concentration of Phocine Distemper Virus. For RNA viruses, 20 µl extracted RNA was amplified in a 50µl final volume, containing 12.5 µl 4x TaqMan® Fast Virus 1-Step Master Mix (Lifetechnologies, Nieuwerkerk a/d Ijssel, The Netherlands), 0.5 µl (1U/ul) Uracil-N-Glycosylase (Lifetechnologies, Nieuwerkerk a/d IJssel, The Netherlands), and 1 µl of primers and probe mixture (table 1). RT-PCR temperature profile was 5 min 50°C, 20 sec 95°C, 40 cycli of 3 sec 95°C and 30 sec 60°C. For DNA viruses, 20 µl extracted DNA was amplified in a 50 µl final volume, containing 25 µl TaqMan® Universal PCR Master Mix (Lifetechnologies, Nieuwerkerk a/d IJssel, The Netherlands) and 1 µl of primers and probe mixture (table 1). PCR temperature profile was 2 min 50°C, 5 min 95°C, 40 cycli of 20 sec 95°C and 1 min 60°C. Five duplex reactions were performed combining influenza A virus and internal control PDV, influenza B virus and HCoV-OC43, HMPV and PIV-2, HCoV-229E and PIV-4, HCoV-NL63 and PIV-1, the remaining pathogens were detected using a singleplex (RT-) PCR format. Amplification was performed in a LC480 (Roche Applied Science, Almere,

Table 1. Methods of viral isolates.

Viral pathogen	Oligo name	Sequence 5'-3'	Conc*	Reference
Influenza A virus	Infa_fwd	CTTCTRACCGAGGTCGAAACGTA	45	In house
	Infa_probe1	FAM-TCAGGCCCCCTCAAAGCCGAGA-BHQ-1	5	
	Infa_probe2	FAM-TCAGGCCCCCTCAAAGCCGAAA-BHQ-1	5	
	Infa_rev	TCTTGTCTTTAGCCAYTCCATGAG	45	
Influenza B virus	Infb-NSfwdB	GRACAACATGACCACAACACAAAT	30	In house
	Infb-NSprobeB	DF-CGGGAGCAACCAATGCCACCATAAA-BHQ-2	5	
	Infb-NSrevB	CACTCCARAATTCCTGCTTCAAA	45	
RSV-A	RSVA-fwd	AGATCAACTTCTGTCATCCAGCAA	45	In house
	RSVA-probe	DF-CACCATCCAACGGAGCACAGGAGAT-BHQ-2	5	
	RSVA-rev	TTCTGCACATCATAATTAGGAGTATCAAT	30	
RSV-B	RSVB-fwd	AAGATGCAAATCATAAATTCACAGGA	20	[9]
	RSVB-probe	DF-TTCCCTTCCTAACCTGGACATAGCATATAACATACCT-BHQ-2	5	
	RSVB-rev	TGATATCCAGCATCTTTCAGTATCTTTATA	35	
Rhinovirus	rhino fwd 45	CGAAGAGTCTACTGTGCTCACCTT	15	Voermans et al., in preparation
	rhino fwd 5	GAAGATCCTATTGCGCTTAGCTGT	45	
	rhino fwd Ag	GGTGTGAAGAGCCCCGTGTG	15	
	rhino fwd Bg	GGTGTGAAGACTCGCATGTGC	15	
	rhino rev	GTAGTCGGTCCCATCCC	45	
	rhinoprobe 4	FAM-TCCTCCGGCCCCTGAATGYGG-BHQ-1	15	
Enterovirus	entero-fwd-D	GACATGGTGYGAAGAGTCTATTGA	45	Voermans et al., in preparation
	entero-probe-TM	CGGAACCGACTACTTTGGGTGTCCGTGTTTC	5	
	entero-rev-TM	GATTGTCACCATAAGCAGCCA	30	
HMPV	hmpv-fwd-TM	CATATAAGCATGCTATATTAAAAGAGTCTC	50	Adapted from [10]
	hmpv-probe-2	FAM-TGTAATGATGAGGGTGTCACTGCGGTTG-BHQ-1	10	
	hmpv-probe-TM	FAM-TGCAATGATGAGGGTGTCACTGCGGTTG-BHQ-1	10	
	hmpv-rev-TM	CCTATTTCTGCAGCATATTTGTAATCAG	20	
HCoV-229E	229E-fwd	CGCAAGAATTCAGAACCAGAG	10	In house
	229E-probe	DF-CCACACTTCAATCAAAAGCTCCCAAATG-BHQ-2	5	
	229E-rev	GGGAGTCAGGTTCTTCAACAA	15	
HCoV-OC43	OC43TM_fwd1	CGATGAGGCTATTCCGACTAGGT	30	In house
	OC43TM_prb1	FAM-TCCGCCTGGCACGGTACTCCCT-BHQ-1	5	
	OC43TM_rev1	CCTTCCTGAGCCTTCAATATAGTAACC	45	
HCoV-NL63	NL63-fwd	GAAGCGTGTTCCTACCAGAGA	30	In house
	NL63-probe	DF-AAATGTTATTCAGTGCTTTGGTCCTCGTGA-BHQ-2	5	
	NL63-rev	GAATCCCCCATATTGTGATTAAA	45	

Table 1. (*continued*)

Viral pathogen	Oligo name	Sequence 5'-3'	Conc*	Reference
Parainfluenza-1 virus	PARA-1-FWD	TGATTTAAACCCGGTAATTTCTCAT	15	In house
	PARA-1-probe	FAM-ACGACAACAGGAAATC-MGB	5	
	PARA-1-REV	CCTTGTTCCTGCAGCTATTACAGA	15	
Parainfluenza-2 virus	piv2HNfwdA	CTGCATCGCTYTTTTACAGGATC	30	In house
	piv2HNrevA	CTTGTTGCATTRCATGGCAT	30	
	piv2probeA	FAM-TCATTGAGGCTCAATGGGTACCGTCCTATC-BHQ-1	10	
Parainfluenza-3 virus	PARA-3-FWD	GGACCAGGGATATACTAYAAA	45	In house
	PARA-3-probe	FAM-ATCTGYAACACAACTGGRTGTCCYGGGAA-BHQ-1	5	
	PARA-3-REV	TTGACCATCCTYCTRTCTGAA	45	
Parainfluenza-4 virus	PARA-4-FWD	ATGGTGGGAGAYATTGCAAA	30	In house
	PARA-4-probe	FAM-ATATAGCYAATGTCGGAATGAGYGCGTTCTTT-BHQ-1	10	
	PARA-4-REV	CCAAGCCGAACTTAAGYGTAA	30	
Adeno virus	Adenoquant1	GCCACGGTGGGGTTTCTAAACTT	10	[11]
	Adenoquant2	DF-GCCCCAGTGGTCTTACATGCACATC-BHQ-2	45	
	Adenoquant-probe	TGCACCAGACCCGGGCTCAGGTACTCCGA	5	
Boca virus	Boca-TM-fwd	GCCCGATCCGACACAGTG	45	In house
	Boca-TM-probe	FAM-AGAGAGGCTCGGGCTCATATCATCAGGAA-BHQ-1	5	
	Boca-TM-rev	TGCAAGACGATAGGTGGCTG	45	
PDV	PDV fwd	CGGGTGCCTTTTACAAGAAC	30	Primers from [12] and probe from[13]
	PDV rev	TTCTTTCCTCAACCTCGTCC	7.5	
	PDV-CY5-MGB	Cy5-ATGCAAGGGCCAATT-MGB	10	

*DF = dragon fly orange, BHQ-1= black hole quencher, BHQ-2 = black hole quencher-2, MGB=minor groove binding protein;*Conc=concentration (pmol/50ul rx)

The Netherlands) using Fit point analysis module. Quality assurance was performed using the free MedlabQC software. Criterion for a successful RT-PCR run was

Ct-values of both internal control and positive (RT-)PCR control should be within 3xSD of mean [8-13].

Validation procedure of (RT-)PCR procedures for all targets were performed according to International Standards Organization guidelines 15189 (http://www.iso.org/iso/search.htm), including: nt Blast of primer and probe sequences in the NCBI sequence database and a subsequent check on lack of mutations with a significant impact on (RT-)PCR amplification [8], (RT-)PCR efficiency of more than 90% on selected virus stocks, lack of cross-reactivity with other respiratory pathogens, estimated lower limit of detection below 500 virus particles per mL based on (RT-)PCR efficiency and proper detection of virus dilutions with Ct-values above 32, clinical validation using both conventional

techniques (direct immunofluoresence and virus culture) as well as (RT-)PCR technique to control for false negative results.

Bacterial isolates

All patients in this study underwent standard sputum culture at time of study enrolment. Sputum culture was examined by conventional microbiological techniques for the presence of aerobic and anaerobic pathogens, including Pseudomonas aeruginosa and Burkholderia cepacia.

Statistical analysis

Data are shown as mean values with standard deviations (SD). Mann-Whitney U tests were used for comparisons between two groups. Paired data were tested using the paired Wilcoxon rank test.

RESULTS

We included 24 patients according to the strict criteria of a pulmonary exacerbation. Patient inclusion was equally distributed over the year (January: 1, March: 2, April: 4, May: 2, June: 2, July: 3, September: 5, October: 3, and December: 2). No patient refused participation in the study. In eight patients (33%) a viral pathogen could be detected (both from sputum and nasal wash). In three patients, more than one virus was isolated. Ten viruses were isolated by using (RT-)PCR (adenovirus n=2, parainfluenza type A n=1, rhinovirus n=3, enterovirus n=3, and human coronavirus NL63 n=1) and in one patient a dual infection was detected by using (RT-)PCR (adenovirus and rhinovirus). Two viruses were identified by the use of immunofluorescence (one was identified rhinovirus and one unclassified picornavirus). Both were also detected by PCR (both rhinovirus). In all eight patients, a bacterial pathogen was also detected in the sputum sample (six patients with Pseudomonas aeruginosa, one with Burkholderia cepacia, and one dual infection with Psudomonas aeruginosa and Staphylococcus aureus). In all patients negative for respiratory viruses, a bacterial pathogen was isolated (Table 2).

In the total patient group, FEV1 values (both absolute and %predicted) improved significantly after antibiotic treatment (p=0.01 and p=0,007, respectively). The number of patients with a viral pathogen isolated was too small to perform reliable calculations about the influence of clinical parameters, but average absolute changes in FEV1 between admission and day 21 were not significantly different between patients in whom a viral pathogen was detected compared to patients with only bacterial pathogens (p= 0.24).

Table 2. Patient characteristics

	Total	Viral infection	Non-viral infection
Number of patients	24	8	16
Sex (M/F)	11/13	2/6	9/7
Admission/outpatient	21/3	6/2	15/1
Mean age in years (range)	29,7 (21-49)	26.4 (22-35)	31.5 (21-49)
Mutation			
Delta F508 (homozygous)	17	6	11
Delta F508 (heterozygous)	2	0	2
Other	3	2	1
Unknown	2	0	2
Colonization			
Pseudomonas aeruginosa	16	6	10
Staphylococcus aureus	1	0	1
Stenotrophomonas maltophilia	1	0	1
Burkholderia species	1	1	0
Combination	5	1	4
Mean FEV1 (%predicted) day 1 of exacerbation (SD)	49.9 (23.0)	61.6 (23.8)	43.6 (19.9)
Mean FEV1 (%predicted) day 21 of exacerbation (SD)	54.9 (23.4)	69.4 (22.9)	47.2 (19.7)

DISCUSSION

In this study we show that there is a considerable incidence of viral pathogens causing CF pulmonary exacerbations in an adult CF population (29%). Furthermore, viral infections did not result in significant FEV1 changes compared to non-viral exacerbations. New viruses like 2009 pandemic influenza A/H1N1, human metapneumovirus, human bocavirus and human coronavirus NL63 were not detected, except for one human metapneumovirus.

We included patients with a pulmonary exacerbation during one calendar year to exclude seasonal effects. A study on the impact of influenza virus demonstrated that CF pulmonary exacerbations increase significantly during the winter and that they are highly associated with the influenza season. These findings suggested that a substantial proportion of CF morbidity may be preventable by influenza vaccination or chemoprophylaxis [14]. All our patients received annually influenza vaccination as part of standard of care. This could have contributed to a lower viral incidence and the absence of isolates with influenza A and B viruses.

A recent study showed that in 47.2% of adult CF patients a viral pathogen could be identified [15]. However, in this study symptoms of respiratory viral illness were used as an inclusion criterion, so an exacerbation according to strict criteria was not used. Two other studies also included CF patients with influenza-like symptoms to investigate whether 2009 pandemic influenza A/H1N1 infection was present [16-17]. These studies showed that

presence of the influenza A H1N1/09 strain did not result in a significant decrease in FEV1 and did not influence clinical course, compared to influenza A H1N1/09 negative adult CF patients. Although using different inclusion criteria, clinical consequences of these reports were comparable to the results of our study.

Whitaker et al. used PCR methods to investigate the prevalence of viral infection in acute exacerbations of adult patients with CF. Viral diagnostics were performed on throat swabs alone and did not include sputum analysis. In 8% of the CF patients with an exacerbation a viral pathogen was detected. The low incidence in this study can be explained by the use of throat swabs only and the fact that also in this study, acute exacerbation was used as inclusion criteria in contrast to using respiratory viral symptoms as in the studies described before [18].

We were not able to detect differences between results from nasal washes or spontaneous sputum. However, because of the limited number of viral isolates in this study, no firm conclusions can be made about effectiveness of viral diagnostics in sputum. Jones et al. showed that sputum, in comparison to nasal swabs, is a suitable specimen for analysis by PCR for the rapid diagnosis of common respiratory viral infections in adult CF patients [15].

Because this was a pilot study, we did not select a control group. This is a limitation of the study and to the power of conclusions that can be drawn from this study.

In summary, our results show that there is a considerable incidence of viral pathogens in CF pulmonary exacerbations in an adult population (33%). New viruses such as 2009 pandemic influenza A/H1N1, human metapneumovirus, human bocavirus and human coronavirus NL63 were not detected, except for one human coronavirus NL63. Further studies with larger cohorts and controlled by adult CF patients during a phase of clinical stability and healthy controls should be conducted to further elucidate the role of viral pathogens in the pathogenesis of pulmonary exacerbations in adult CF patients.

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CHAPTER 5

Interleukin-6 and interleukin-10 in nasal lavages and plasma correlate with clinical parameters in adult cystic fibrosis patients

Submitted for publication

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ABSTRACT

Background Persistent local and systemic inflammation plays a dominant role in cystic fibrosis (CF). In this study we hypothesised that the inflammatory response in CF is increased in acute pulmonary exacerbations compared with stable clinical conditions and that the intensity of the inflammatory response correlates with disease severity.

Methods To test this hypothesis, we followed a cohort of *Pseudomonas aeruginosa*-infected adult CF patients (n=16) over time and measured cytokine levels in stable disease as well as in pulmonary exacerbation. Cytokines reflecting innate and adaptive immune activation were determined both locally, in sputum and nasal lavages, and systemically in plasma by means of enzyme-linked immunosorbent assays (ELISA) and Multiplex assays.

Results Levels of interleukin (IL)-6 and IL-10 in nasal lavages of CF patients were significantly increased in exacerbation compared with stable disease. Cytokine levels in sputum and plasma of CF patients did not discriminate between exacerbations and stable phases of disease. In contrast, systemic IL-6 levels significantly correlated with CRP levels and FEV1 (%predicted), independently of disease status. Systemic IL-10 levels also correlated significantly with CRP and FEV1 (%predicted), but only in exacerbation.

Conclusions In conclusion, determination of IL-6 and IL-10 in nasal lavages may provide a minimally invasive tool in the assessment of an exacerbation in CF. Furthermore, systemic IL-6 levels might be a valuable marker to improve prognosis predictions in adult CF patients.

INTRODUCTION

Cystic fibrosis (CF) is the most frequent lethal genetic disorder in Caucasian populations and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1]. It is a systemic disorder in which multiple organ systems are affected, but pulmonary disease with progressive deterioration in lung function is the major cause of morbidity and mortality in CF patients. The CFTR defect affects a multitude of cellular functions, including transmembrane transport of Cl^-, and signalling pathways responsible for the transcription of inflammatory mediators [2-3]. This predisposes CF patients to chronic respiratory infections, resulting in progressive tissue damage due to airway inflammation [4-5]. Airway inflammation in CF, however, begins already in early infancy [6-7] and, although clearly associated with infection, there is still some uncertainty about whether CF lungs are innately primed for a pro-inflammatory response. Recurrent and persistent lung infections with bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* are common from infancy and the incidence of infection increases with age [8]. In adulthood, most CF patients are chronically infected with *Ps. aeruginosa* [9], and this chronic infection results in a neutrophil-dominated lower airway inflammation and progression of obstructive lung disease and bronchiectasis [4-5].

The inflammatory activity reported in CF is not restricted to acute pulmonary exacerbations of CF, but has also been demonstrated during phases of clinical stability [10-12]. Inflammatory mediators as markers of the host response to infection may reflect the intensity of the lung injury and may relate to changes in clinical status (pulmonary exacerbation or clinical stability). Because airway inflammation plays a crucial role in progressive lung damage in CF, it is important to find reliable markers of its severity. Bronchoalveolar lavage (BAL) probably reflects the airway inflammation most accurately, but bronchoscopy with BAL is invasive and unpleasant for patients. Surrogate markers, collected by minimally or noninvasive procedures, would therefore be of great assistance [13].

Thus far, it has been demonstrated by Nixon *et al*. that serum interleukin (IL-)6 concentrations in CF patients at the time of exacerbation were greater than in healthy control subjects [12]. However, no differences between serum IL-6 concentrations in the patients in exacerbation and those defined as clinically stable were found [12]. Additionally, this study demonstrated a relationship between serum IL-6 and CRP levels in the total group of CF patients, but no distinction in clinical status was made [12]. Sputum levels of IL-6 were detectable but did not change with clinical status of the patients [12]. IL-10 levels in sputum and serum, studied by Colombo *et al*. [14], were generally undetectable in both compartments and showed no correlation with the forced expiratory volume in one second (FEV1) [14]. Reports on cytokine levels in nasal lavages are limited and no study has compared levels in exacerbation with those in stable disease.

We hypothesized that the inflammatory response in CF is increased in patients in acute exacerbations compared with those in stable disease. Additionally, we hypothesized that the intensity of this inflammatory response correlates with disease severity as defined by pulmonary function testing (FEV1 %predicted) and C-reactive protein (CRP) levels. To test this hypothesis, we followed a cohort of *Ps. aeruginosa*-infected adult CF patients over time and measured cytokine levels in stable disease as well as in acute pulmonary exacerbation. Cytokines reflecting innate and adaptive immune activity were determined both locally, in sputum and nasal lavages, and systemically in plasma.

METHODS

Study design

A prospective study was performed in CF patients with chronic *Ps. aeruginosa* infection between January and December 2010. All patients admitted for intravenous antibiotic therapy during acute respiratory exacerbation to the Erasmus MC, a CF centre with a total adult population of 120 patients, were enrolled in the study. The medical ethics committee of the Erasmus MC approved of the study. Written informed consent was obtained from each patient.

A pulmonary exacerbation was defined as a deterioration in symptoms perceived by the patients, and included an increase in cough, sputum production and dyspnoea, a decline in FEV1 compared with previous best, weight loss, and fever ($t \geq 38°C$). Chronic *Ps. aeruginosa* infection was diagnosed if the organism was isolated in at least 3 consecutive sputum samples within a 6-month period. Exclusion criteria were known immunodeficiency or autoimmune disease, the use of systemic corticosteroids, and the presence of allergic bronchopulmonary aspergillosis (ABPA) or asthma. In all patients intravenous antibiotics were administered for 21 days. Selection of antibiotics was based on the sensitivity of the cultured bacteria and consisted in all patients of two antibiotics with different mechanisms of action.

Upon admission, we collected venous blood and sputum samples and performed nasal lavages in all patients. Additionally, spirometry and routine biochemical analysis (including CRP) were done. Measurements were repeated 3 months after hospital admission when patients were clinically stable, which was defined as no need for intravenous antibiotics for at least 6 weeks prior to measurements.

Collection of sputa, nasal lavages and plasma

Sputum samples were collected by spontaneous coughing, and were stored at 4°C for a maximum of 2 hours until processing. Samples were processed using Sputolysin (Calbiochem). Briefly, 1 ml of 10% Sputolysin was added per 1 mg of sputum, and incubated

for 15 minutes at 37°C while vigorously shaking. Subsequently, samples were centrifuged at 600x*g* for 10 minutes at 4°C, and supernatants were aliquoted. Nasal secretions were collected by nasal lavage as previously described [15] and stored at 4°C for a maximum of 2 hours until processed. Nasal lavages were centrifuged at 1200x*g* for 10 minutes at 4°C and supernatants were aliquoted. To obtain plasma, venous blood was collected into EDTA containing vials and centrifuged at 1200x*g* for 10 minutes at 4°C. All samples were stored at −80°C until cytokine analysis.

Cytokine level measurements

Levels of IL-6, IL-10, IL-8, IL-1β, TNFα, IL-17A, IL-22, IFNγ, IL-4, IL-5 and IL-2 in sputum samples and nasal lavages were determined using Multiplex assays (FlowCytomix, eBioscience) according to manufacturer's instructions. Levels of IL-6, IL-10, IL-8, IL-1β, TNFα, IL-17A, IL-22, and IL-4 in plasma were assessed by enzyme-linked immunosorbent assay (ELISA) using commercially available assays (IL-8 OptEIA Set, BD Biosciences; all other cytokines Ready-Set-Go kits, eBiosciences). Specific sensitivity levels can be found in the manufacturers' manuals.

Statistical analysis

Data are shown as mean values (±SD) in cases of normally distributed data or median values with interquartile ranges (IQR) when not normally distributed. Cytokine levels were not normally distributed and therefore nonparametric tests were used to make comparisons between groups (Kruskal-Wallis test for across group comparison of three or more groups, Mann-Whitney U-test for pair-wised analyses). Paired data were tested using the paired Wilcoxon rank test. Correlations were calculated by using Spearman's Rank correlation coefficient. Data analysis was performed using Statistical Package for Social Sciences (SPSS) 15.0 and Prism 5.01 (GraphPad). Statistical significance was taken as a p-value <0.05.

RESULTS

Clinical characteristics of the study population

Sixteen CF patients with chronic *Ps. aeruginosa* infection were included in this study. No patients who met the inclusion criteria refused participation. All patients were studied at the start of acute pulmonary exacerbation and three months later (range 74-122 days) when clinically stable. Clinical characteristics of the study population are shown in Table 1.

All patients used azithromycine as maintenance therapy. In all CF patients CRP levels were raised when in acute pulmonary exacerbation compared with stable disease, but no statistical significance was reached. FEV1 values decreased when in pulmonary exacerba-

Table 1. Patient characteristics

N	16
Male sex	10
Age, years	31±9
Mutations Delta F508 (homozygous) Delta F508 (heterozygous) Other unknown	10 1 3 2
Kolonisation *Ps. aeroginosa* (only) *Ps. aeruginosa* and *S. aureus*	13 3
Azithromycin use	16
ICS use	8
CRP, mg/l Stable CF Exacerbation CF	22±25 41±43
FEV1, %pred Stable CF Exacerbation CF	53±24 50±26

Data are represented as numbers or mean±SD. *Ps. aeruginosa*: *Pseudomonas aeruginosa*. *S. aureus*: *Staphylococcus aureus*. ICS: inhaled corticosteroids. FEV1, %pred: forced expiratory volume in 1 second, percent predicted

tion but this was also not a significant difference. Nine patients had a history of nasal polyps.

Local cytokine levels

In sputa of CF patients, levels of IL-10, IL-8, IL-1β, TNFα, IL-22 and IL-4 were detectable but no significant differences were found between clinically stable patients and patients in exacerbation (Table 2). In nasal lavages, only IL-6, IL-10, IL-8 and IL-1β levels could be detected. IL-6 and IL-10 levels in nasal lavages were significantly higher in patients in acute pulmonary exacerbation compared with the same patients in stable disease (Table 2). A pair-wised analysis of CF patients in our study population without nasal polyps (n=7) also revealed a significant raise in IL-6 and IL-10 levels in exacerbation compared with stable disease (Figure 1). Levels of IFNγ, IL-5, IL-2 and IL-17A were not detectable in sputa or nasal lavages.

For all cytokines except for IL-6, sputum levels were higher than levels found in nasal lavages of CF patients (Table 2).

Systemic cytokine levels

The concentrations of IL-6, IL-10, IL-8, IL-1β and IL-22 in plasma of CF patients were above detection levels but no significant differences were found between CF patients in stable disease and in exacerbation (Table 2).

IFNγ, IL-5 and IL-2 were not detectable in sputa and nasal lavages of CF patients and were therefore not determined in plasma. We did measure IL-17A in plasma of CF patients but concentrations were below detection levels.

Local versus systemic cytokine levels

Levels of most cytokines were higher in local compartments than systemically. IL-10, IL-8, IL-1β, TNFα and IL-4 levels were significantly higher in sputum compared with plasma, both in clinically stable patients and in patients in exacerbation (Table 2). In contrast, IL-6 was not detectable in sputum of CF patients regardless of clinical status, but could be detected in the plasma of all patients (Table 2).

Table 2. Cytokine levels in sputa, nasal lavages and plasma of CF patients when clinically stable and when in acute pulmonary exacerbation

	Sputum (pg/ml)		Nasal lavage (pg/ml)		Plasma (pg/ml)	
	Stable	**Exacerbation**	**Stable**	**Exacerbation**	**Stable**	**Exacerbation**
IL-6	b.d	b.d	5,0 (1-10)	6,0 (3-62)*	4,6 (2,4-7,5)	3,6 (1,6-13,8)
IL-10	401 (218-1542)	446 (164-952)	2,5 (0-4)	5,0 (1-11)*	1,7 (0,5-2,7)	2,2 (0,4-4,3)
IL-8	1685 (1127-2541)	1757 (1172-4107)	712 (351-1474)	1465 (142-4175)	0 (0-2,0)	0 (0-11,1)
IL-1β	6025 (3221-9375)	9165 (868-11114)	120 (10-298)	69 (20-711)	3,6 (0-6,8)	6,3 (3,1-10,2)
TNFα	408 (105-895)	465 (64-869)	b.d.	b.d.	b.d.	b.d.
IL-22	133 (0-235)	144 (0-251)	b.d.	b.d.	58,2 (22,8-110,2)	72,5 (13,3-128,6)
IL-4	175 (0-212)	154 (66-375)	b.d.	b.d.	b.d.	b.d.

Data are represented as median values (IQR). IL: interleukin. TNF: tumor necrosis factor. * p<0.05, clinically stable compared with exacerbation CF (Mann-Whitney U-test). b.d.: below detection. All n=16 except for sputum (n=14), nasal lavages (n=13) and plasma (n=15) in stable disease.

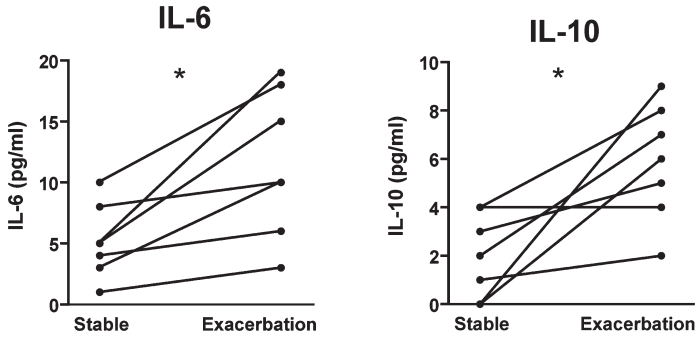

Figure 1. IL-6 and IL-10 levels in nasal lavages of CF patients without nasal polyps when clinically stable and when in acute pulmonary exacerbation. IL-6: p=0.02; IL-10 p=0.03 (paired Wilcoxon test). Median values in pg/ml: IL-6 stable disease 5.0, exacerbation 10.0; IL-10 stable disease 2.0, exacerbation 6.0.

Levels of IL-8 and IL-1β in nasal lavages exceeded systemic levels (Table 2). IL-22 was below detection in nasal lavages but notable levels were found in both sputum and plasma of all patients.

Correlations between cytokine levels and clinical parameters

When correlating plasma cytokine levels of CF patients with markers of disease severity, we found significant associations with systemic levels of IL-6 and IL-10. FEV1 (%predicted) showed a strong inverse correlation with plasma IL-6 levels, independently of clinical status (Figure 2A). For plasma IL-10 levels a significant inverse correlation with FEV1 (%predicted) was also found, but only in CF patients in pulmonary exacerbation (Figure 2B).

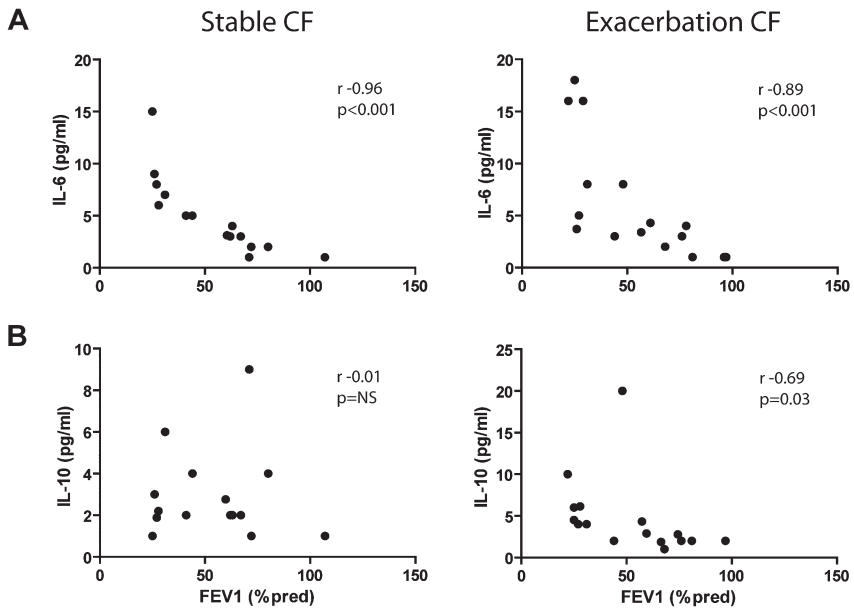

Figure 2. Correlations between plasma IL-6 (A) and IL-10 (B) and FEV1 (%predicted) in CF patients when clinically stable (n=15) and when in acute pulmonary exacerbation (n=16).

For CRP levels, we also found significant associations with plasma IL-6 and IL-10 levels: plasma IL-6 significantly correlated with CRP in both stable disease and exacerbation (Figure 3A), whereas plasma IL-10 levels only correlated significantly with CRP in patients when in exacerbation (Figure 3B). All other cytokines that were detectable in plasma did not correlate with either FEV1 or CRP (data not shown).

Thus, in contrast to IL-10, plasma IL-6 levels significantly correlated with markers of disease severity independently of clinical status. Cytokine levels in sputa and nasal lavages did not show any significant correlations with either FEV1 or CRP levels (data not shown).

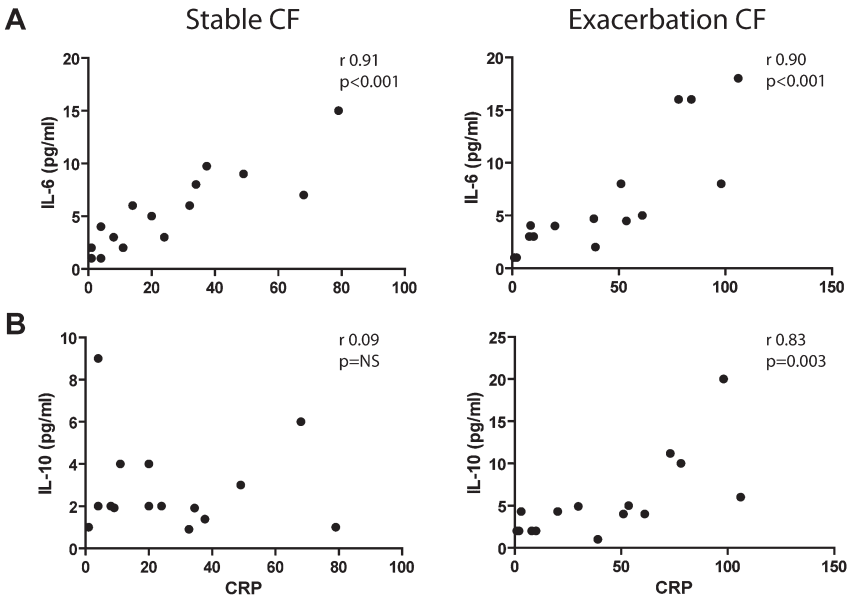

Figure 3. Relationship between plasma IL-6 (A) and IL-10 (B) and CRP in CF patients when clinically stable (n=15) and when in acute pulmonary exacerbation (n=16).

DISCUSSION

Previous studies have shown that inflammatory activity in CF is present during acute pulmonary exacerbations, as well as in phases of clinical stability [10-12]. To our knowledge, this is the first study investigating inflammatory responses simultaneously in sputa, nasal lavages and plasma of *Ps. aeruginosa*-infected adult CF patients both when in exacerbation and when clinically stable. We studied a large panel of cytokines which are of potential interest for CF. Our most important findings are that IL-6 and IL-10 levels in nasal lavages of CF patients were significantly higher in exacerbation compared with stable disease. Moreover, although cytokine levels in sputum and plasma of CF patients were unable to discriminate between exacerbations and stable phases of disease, systemic IL-6 levels did significantly correlate with CRP levels and FEV1 (%predicted), independently of disease status. Systemic IL-10 levels also correlated with CRP and FEV1 (%predicted), but only in exacerbation. Additionally, we found that cytokine levels were generally higher in sputum and nasal lavages than in plasma, supporting the concept of an inflammatory response that is compartmentalized to the airways.

The cytokines IL-6 and IL-10 are major regulators of the host inflammatory response. In our study, we found elevated levels of IL-6 and IL-10 in nasal lavages of CF patients in exacerbation compared with patients in stable disease. There are only few reports about cytokine levels in nasal lavages of CF patients. However, it has been demonstrated that

the neutrophilic inflammation which is prominent in CF lower airways, is also present in the nasal airways of CF patients [16-17]. Noah *et al*. measured IL-6 and IL-10 levels in nasal lavages of very young children with CF and found similar levels in these patients when compared with controls [17]. However, in this study no distinction was made between CF patients in exacerbation or newly diagnosed infants, and control subjects were all children with an indication for bronchoscopy [17]. Since nasal polyps have been shown to influence cytokine levels [18], we performed a subanalysis in CF patients without nasal polyps. Also in this paired analysis, patients in exacerbation had increased levels of IL-6 and IL-10 compared with stable clinical conditions.

In line with previous reports [14,19], we were unable to detect IL-6 in sputa of CF patients. This was observed independent of clinical status. Some groups have been able to detect IL-6 in sputum samples of CF patients, albeit in very low concentrations, but findings are contradictory. Eickmeier *et al*. found higher IL-6 levels in induced sputum samples of clinically stable CF patients when compared with healthy individuals [11], whereas another group reported lower levels of IL-6 in sputa of children with CF compared with healthy adult individuals [20]. Nixon *et al*. did not detect differences in IL-6 levels in patients in exacerbation versus stable disease but did find a relationship between sputum IL-6 levels and FEV1 (%predicted) which was present during acute exacerbations only [12].

Systemic levels of IL-6 in our study were low but detectable and although no significant differences were found between patients in exacerbation and patients in stable disease, we did find a significant inverse correlation with FEV1 (%predicted) and a significant positive correlation with CRP. This suggests that systemic inflammation is an independent risk factor for disease progression in CF. Furthermore, the close correlations of systemic IL-6 and IL-10 with CRP levels, supports the use of these cytokines as circulating markers of inflammation in CF. Colombo *et al*. also measured systemic IL-6 levels in CF children but could not detect biological activity [14]. Our results are supported by Nixon *et al*. who reported that systemic IL-6 levels were similar in exacerbation versus stable disease and significantly correlated with CRP levels [12]. Moreover, in a larger cohort of adult CF patients in stable clinical conditions, Ngan *et al*. found that plasma IL-6 levels correlated significantly with FEV1 (%predicted) [21].

Data on IL-10 levels in sputum or serum and clinical status of CF patients are limited. Two groups have previously studied sputum IL-10 levels but no comparisons between exacerbation and stable disease have been made [14,20]. Serum IL-10 levels were only reported in one study in which they were below detection level [14].

The cellular immune response in CF patients is thought to be mainly characterized by an accumulation of neutrophils [4,22] as a result of the production and release of IL-8 [10]. The high local levels of IL-8 found in the present study are consistent with findings by others [6-7,23]. Recently, it has been shown that IL-17A, which is the main product of the novel T helper 17 (Th17) subset, is elevated in the sputum and BAL of CF patients in exacerbation [24-25].

Moreover, increased amounts of IL-17A$^+$ cells were present in endobronchial biopsies and lower airways of CF patients compared with controls [26-27]. In our study, however, IL-17A was not detectable in any compartment tested, including sputum of patients in acute exacerbation. We did find considerable levels of IL-22, another product of Th17 cells, in sputum and plasma of CF patients in exacerbation or stable disease. Systemic IL-22 levels found in our CF patient cohort (Table 2) were higher than those we previously found in healthy individuals (below 10 pg/ml, M.P., unpublished data). Interestingly, another study reported elevated IL-22 production in the lymphoid tissues of CF patients [28]. Furthermore, it has been shown that IL-22 can induce anti-microbial proteins and maintain epithelial barriers in lung infection [28]. Taken together, these data indicate the possible importance of IL-22 in CF inflammation.

The main limitation of this study, although comparable to other studies investigating cytokines in CF [10-12], is the small number of patients included. Therefore, our observations need to be interpreted with caution and verified in a larger group of patients. Furthermore, the results presented in this study were obtained with spontaneously expectorated sputum. The collection of spontaneous sputum is non-invasive, but not all CF patients produce sputum on a consistent basis. In contrast, nasal lavages are minimally invasive and could therefore be performed in all CF patients regardless of age or clinical status. Azithromycin and inhaled corticosteroids (ICS) have known immunomodulatory effects [29-30]. In our study, all patients used azithromycin as maintenance therapy and continued the usage when in exacerbation. The conclusions of our study are therefore not likely to be influenced by this. In addition, we verified that our results were not influenced by ICS usage: no correlations were found between ICS use and cytokine levels (data not shown). Our patient cohort was not homogenous as three patients were co-infected with *S. aureus*, but this makes our study group more representative of the CF patients in clinical practice.

In conclusion, our study provides a comprehensive analysis of cytokine profiles in local and systemic compartments in adult *Ps. aeruginosa*-infected CF patients. We showed that levels of IL-6 and IL-10 in nasal lavages may provide a minimally invasive tool in the assessment of an exacerbation in CF patients. Furthermore, because of the relationship between plasma IL-6 concentrations and lung function and CRP levels, independent of clinical status, systemic IL-6 might be a valuable marker to improve prognosis predictions in adult CF patients. In addition, systemic IL-6 might also be useful to define pro-inflammatory status and potentially identify a subpopulation of CF patients that might benefit from anti-inflammatory therapy.

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CHAPTER 6

Compartmentalization of interleukin-22 producing cells in adult *Pseudomonas aeruginosa*-infected cystic fibrosis patients

Manuscript in preparation

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INTRODUCTION

Cystic fibrosis (CF) is the most frequent lethal genetic disorder in Caucasian populations and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene[1]. It is a systemic disorder in which multiple organ systems are affected, but pulmonary disease with progressive deterioration in lung function is the major cause of morbidity and mortality in CF patients. Recurrent and persistent lung infections with bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* are common, and in adulthood most CF patients are chronically infected with the latter [1].

At present, there is accumulating evidence that the T helper (Th)17 cell population which mainly produces interleukin (IL)-17A and IL-22, is involved in CF pathogenesis [2-3] and *Ps. aeruginosa* lung infection [4]. IL-17A promotes chemokine and proinflammatory cytokine production and consequent recruitment and activation of neutrophils [5]. IL-22 controls tissue responses during inflammation by increasing lung epithelial proliferation by inducing epithelial production of antibacterial peptides and by increasing transepithelial resistance to injury [6]. In addition to $CD4^+$ T cells also other immune cell subsets may express IL-17A and IL-22, including $CD8^+$ T cells, gammadelta T cells, activated NKT cells [6] and the recently identified population of innate lymphoid cells (ILCs) [7]. Importantly, the expression of the IL-22 receptor is mainly confined to epithelial cells [6].

In CF patients, IL-17A was shown to be markedly elevated in bronchoalveolar lavage fluid (BALF) and sputum of children and adults during infective exacerbations [3,8]. Furthermore, IL-17A was a prominent feature in the airway walls of CF patients [9-10]. Human studies on IL-22 in CF are rare but in a single report IL-22 responses were shown to be significantly elevated in lung lymphoid tissue [11]. Although CF is a systemic disorder, data on systemic Th17 responses is limited. In one study increased levels of IL-17A in the serum of clinically stable adult CF patients have been reported [12], but data on $IL\text{-}22^+$ T cells is missing. It is important to define these adaptive T cell responses in CF because antibody responses against *Ps. aeruginosa*, which are likely T cell dependent, have been correlated with worse pulmonary function [13].

MATERIALS AND METHODS

Study design

Two cohorts of CF patients were included through the CF clinic of the Erasmus MC, a CF centre with a total adult population of 105 patients. The first cohort underwent bronchoscopy with endobronchial biopsies (Table 1). Mucosal biopsies were taken from nine stable adult CF patients and fifteen age and sex matched healthy individuals. All CF patients were clinically stable at time of bronchoscopy. Healthy individuals had no

Table 1. Characteristics of *Pseudomonas aeruginosa*-infected adult CF patients and healthy individuals undergoing bronchoscopy with mucosal biopsies

	CF	Controls
N	9	15
Sex (M/F)	5/4	8/7
Mean age (range)	30 (24-43)	27 (19-39)
Smoking	0/9	0/15
CFTR mutation	5	n/a
Delta F508 (homozygous)	2	
Delta F508 (heterozygous)	1	
Other	1	
unknown		
Colonization		
Ps. aeruginosa	2	
Ps. aeruginosa & *S. aureus*	6	
Ps. aeruginosa & *B. cepacia*	1	n/a

n/a: not applicable

history of pulmonary disease and had no known autoimmune disease, atopic diseases or allergies. Details of the protocol for flexible bronchoscopy and endobronchial biopsies have been reported elsewhere [14].

A second cohort of CF patients was prospectively included between January and December 2010 (Table 2). Sixteen CF patients with chronic *Ps. aeruginosa* infection were enrolled in the study when admitted for intravenous antibiotic therapy during acute respiratory exacerbation. Written informed consent was obtained from all study subjects and the medical ethics committee of the Erasmus MC approved of the study.

A pulmonary exacerbation was defined as a deterioration in symptoms perceived by the patients, and included an increase in cough, sputum production and dyspnoea, a decline in forced expiratory volume in 1 second (FEV1) compared with previous best, weight loss, and fever ($t \geq 38°C$). Chronic *Ps. aeruginosa* infection was diagnosed if the organism

Table 2. Characteristics of subjects in peripheral blood group

	Stable CF	Exacerbation CF	Controls
N	16	16	10
Sex (male/female)	10/6	10/6	5/5
Mean age (range)	31 (21-49)	31 (21-49)	31 (21-44)
Smoking	0/16	0/16	0/10
CFTR mutation			
Delta F508 (homozygous)	10	10	n/a
Delta F508 (heterozygous)	1	1	n/a
Other	3	3	n/a
unknown	2	2	n/a
Colonization			
Ps. aeruginosa	13	13	n/a
Ps. aeruginosa & *S. aureus*	3	3	

Data are represented as medians with ranges. *: p<0.05; **: P<0.01. n/a: not applicable

was isolated in at least 3 consecutive sputum samples within a 6-month period. Exclusion criteria were known immunodeficiency or autoimmune disease, the use of systemic corticosteroids, and the presence of bronchopulmonary aspergillosis or asthma. In all CF patients intravenous antibiotics were administered for 21 days. Selection of antibiotics was based on the sensitivity of the cultured bacteria and consisted in all patients of two antibiotics with different mechanisms of action.

Venous blood samples were collected upon admission and 3 months after hospital admission when patients were clinically stable, which was defined as no need for intravenous antibiotics for at least 6 weeks prior to measurements.

Sample collection and analysis

Peripheral blood mononuclear cells (PBMC) were isolated using standard Ficoll-Paque (GE Healthcare) density gradient and resuspended in IMDM medium (BioWhittaker) containing 10% FCS, β-mercaptoethanol and gentamycin. For cytokine analysis, cells were stimulated with ionomycine (Sigma) and PMA (Sigma) in the presence of Brefeldin A (ebiosciences) and incubated at 37°C. After 4 hours cells were collected, centrifuged for 7 min at 400g, resuspended in FACS buffer (PBS supplemented with 0.25% BSA and 0.5 mM EDTA and 0.05% NaN3) and stained with conjugated monoclonal antibodies as described previously [14] to detect membrane markers. To detect intracellular cytokines, cells were subsequently fixed with 2% paraformaldehyde (PFA, Merck) and permeabilized with 0.5% saponin solution (Sigma). Cells were measured on a LSRII Flowcytometer (BD Biosciences). Data were analyzed using FlowJo (BD) software. Isotype controls and fluorescence minus one (FMO) controls were used to set gates. Flow cytometry experiments of all samples were performed in a single batch in order to eliminate the influence of day-to-day variation.

Statistical analysis

Data are shown as median values and individual data as symbols. Cytokine levels were not normally distributed and therefore nonparametric tests were used to make comparisons between groups (Kruskal-Wallis test for across group comparison of three or more groups, Mann-Whitney U-test for pair-wised analyses). Data analysis was performed using Statistical Package for Social Sciences (SPSS) 15.0 and Prism 5.01 (GraphPad). Statistical significance was taken as a p-value <0.05.

RESULTS & DISCUSSION

We hypothesized that IL-17A$^+$ and IL-22$^+$ T cells are involved in CF immunopathogenesis, both in local and in systemic inflammation in advanced CF lung disease. To test

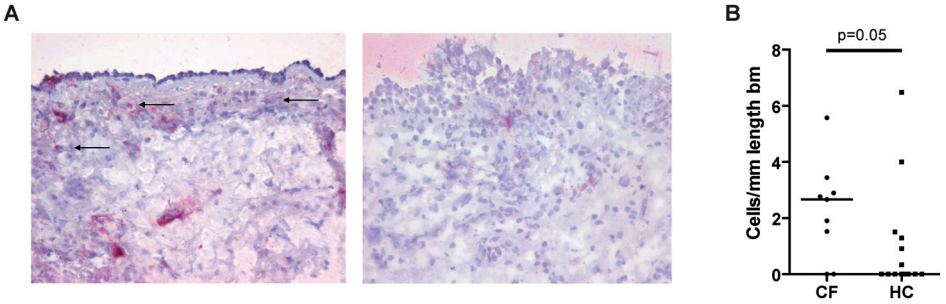

Figure 1. Increased number of IL-17A$^+$ cells in the subepithelial layer of CF lung mucosal biopsies. **A**. IL-17A staining (in red) of submucosal frozen sections from a CF patient (left) and healthy individual (right). **B**. Quantifications of the number of IL-17A$^+$ cells in the subepithelium. Lines indicate median values.

this hypothesis, we first analyzed lung mucosal biopsies of 9 clinically stable adult *Ps. aeruginosa*-infected CF patients and 15 age and sex matched healthy individuals (for subject characteristics see Table 1). Of all study subjects at least one biopsy was of sufficient quality to be analyzed. Immunohistochemical stainings of IL-17A$^+$ and IL-22$^+$ cells were performed as described previously [14]. IL-17A-expressing cells were particularly found in the subepithelium and numbers were higher in CF patients compared with healthy individuals (Figure 1), consistent with previous reports by other groups [9-10]. Mucosal biopsies of CF patients also contained more IL-22$^+$ cells than those of healthy controls (Figure 2). Remarkably, we noticed that these IL-22$^+$ cells were particularly located in the epithelium (Figure 2). The IL-22$^+$ cells in the bronchial mucosal biopsies of our cohort of CF patients seem to comprise a heterogeneous population of cells including immune cells and cells with an epithelial morphology (Figure 2). Further stainings are necessary to define the identity of these epithelium-associated IL-22$^+$ cells.

Because systemic inflammation is present in CF patients, not only when in pulmonary exacerbation but also when clinically stable [16], we analyzed peripheral blood T cells for IL-17A and IL-22 expression. For this, a cohort of sixteen *Ps. aeruginosa*-infected adult CF patients was followed over time and peripheral blood was taken when patients were in stable clinical conditions as well as when in exacerbation. Ten healthy age and sex matched individuals served as controls (for subject characteristics see Table 2). T cell subsets were analyzed by intracellular flow cytometry for the production of IL-17A and IL-22. Proportions of IL-17$^+$ CD3$^+$ T cells in peripheral blood of CF patients were similar to those found in healthy individuals (data not shown). Also in a subanalysis of different T cell subsets, αβ, γδ or NK T cells, no differences in IL-17 positive cells were found between CF patients and healthy individuals (data not shown). Remarkably, proportions of IL-22$^+$ CD3$^+$ T cells in CF patients were significantly lower compared with healthy individuals, both in stable disease and in exacerbation (p=0.007 and p=0,02, respectively). Subanalysis of circulating IL-22 positive T cell subsets revealed that both CD4$^+$ T cell and γδ T cell populations contained lower proportions of IL-22, independently of clinical status (Figure 3). Proportions of NKT

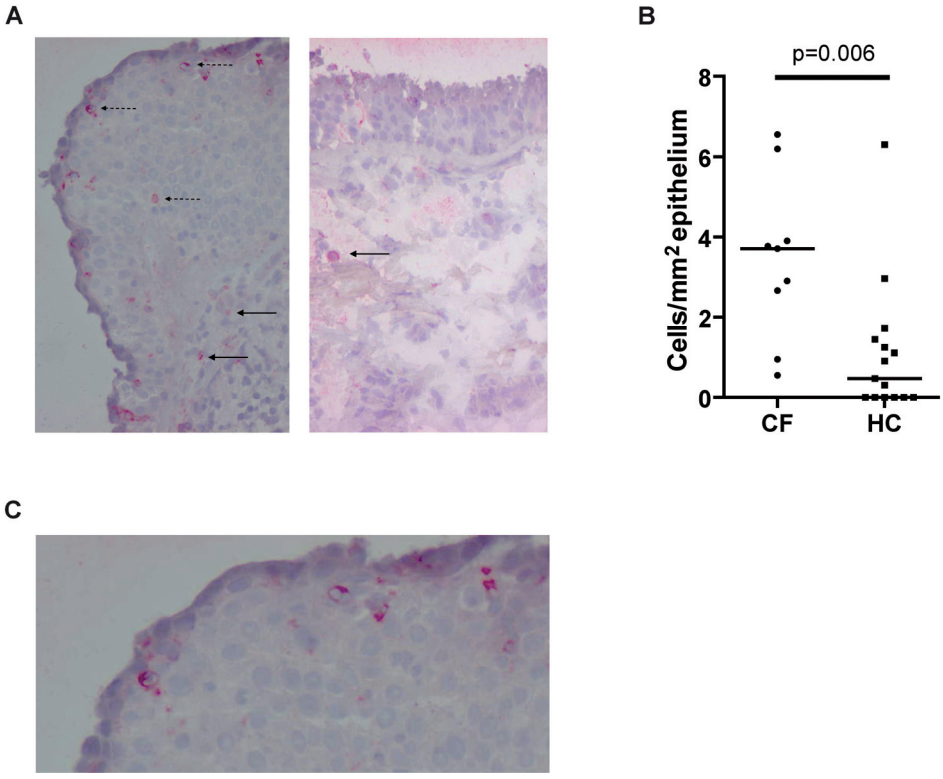

Figure 2. Increased number of IL-22$^+$ cells in the epithelial layer of CF lung mucosal biopsies. **A**. IL-22 staining (in red) of submucosal frozen sections from a CF patient (left) and healthy individual (right). Arrows indicate IL-22$^+$ cells in the epithelium (dashed arrows) or in the submucosa. **B**. Quantifications of the number of IL-22$^+$ cells in the epithelium. Lines represent median values. **C**. Close up of the epithelial layer of a bronchial mucosal biopsy from a CF patient with IL-22 staining (in red).

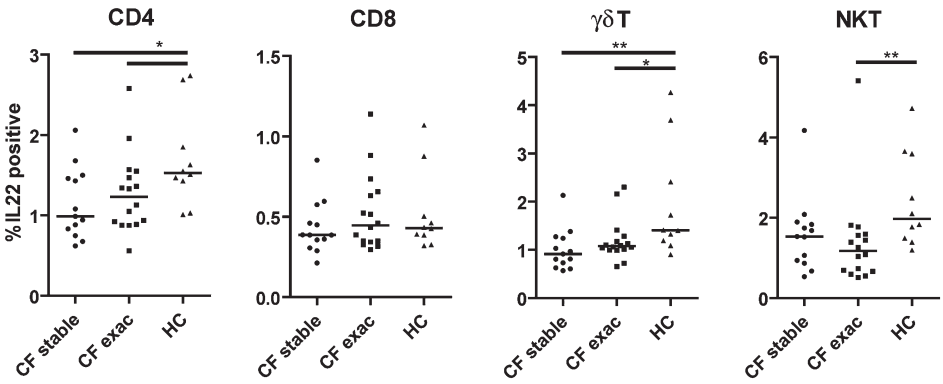

Figure 3. Proportions of IL-22$^+$ T cell subsets in peripheral blood from CF patients in stable disease and exacerbation (exac), and healthy individuals. *: p<0.05; **: p<0.01. HC: healthy control.

cells expressing IL-22 in peripheral blood of CF patients were only significantly lower than those of healthy individuals when in exacerbation (Figure 3). Proportions of IL-22$^+$ CD8$^+$ T cells were low and did not differ between CF patients and controls (Figure 3).

CONCLUDING REMARKS

In conclusion, numbers of IL-22$^+$ cells are increased in bronchial biopsies of *Ps. aeruginosa*-infected adult CF patients, pointing at a possible role for IL-22 in the local inflammatory response in advanced CF. Remarkably, positivity for IL-22 was particularly high in epithelial cells, which are normally not known to produce IL-22. A recent report in patients with Sjögren's syndrome showed that in chronically inflamed salivary glands, ductal epithelial cells were positive for IL-22 [15]. In addition, this study showed that the degree of IL-22 expression was correlated with the grade of inflammation of the salivary glands [15]. This finding indicates that chronic inflammation might be a possible trigger for epithelial cells to start producing this cytokine. The strong IL-22 staining in epithelial epithelial cells in our cohort of CF patients might therefore be related to the chronic infection with *Ps. aeruginosa*.

By contrast, the proportions of IL-22$^+$ T cells in peripheral blood of CF patients were reduced, indicating that there is compartmentalization of IL-22$^+$ cells. The decrease in circulating IL-22$^+$ T cells may, however, not only reflect homing of these cells to the lung but perhaps also to the intestine, since increased inflammation in the gut of CF patients has been well recognized [17]. The decreased proportions of IL-22$^+$ T cells in the peripheral blood of CF patients were mainly due to reduced expression of IL-22 by CD4$^+$ T cells and γδ T cells. Interestingly, CD4$^+$ and γδ T cells are present in both lung and gut mucosa and are important cytokine producing subsets in mucosal immunity [18].

Our results strengthen the concept of the important role of IL-22 in local CF inflammation and further research should focus on the identification of the identity of IL-22$^+$ cells in the lung and the parallels between local and systemic cytokine production in CF.

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CHAPTER 7

Systemic CD4$^+$ and CD8$^+$ T cell cytokine profiles correlate with GOLD stage in stable COPD

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is associated with pulmonary and systemic inflammation. Both $CD4^+$ and $CD8^+$ T lymphocytes play a key role in COPD pathogenesis, but cytokine profiles in circulating T lymphocytes have not been well characterized. Here we report the analysis of peripheral blood T cells from 30 stable COPD patients and 10 healthy never-smokers for IFNγ, IL-4, TNFα and the T helper 17 cytokines IL-17A, IL-17F and IL-22 by intracellular flow cytometry. We found significantly increased proportions of $IFN\gamma^+$ and $TNF\alpha^+$ $CD8^+$ T cells in COPD patients, when compared with healthy controls. This was most evident in patients with less severe disease. In contrast, expression profiles in circulating $CD4^+$ T cells were similar in COPD patients and healthy controls for all cytokines tested, except for IL-17F. COPD patients with more severely reduced diffusing capacity had lower proportions of $IL\text{-}17A^+$ $CD4^+$ T cells. Proportions of $IL\text{-}22^+$ cells in the $CD4^+$ memory T cell population were significantly increased in active smokers, when compared with past smokers. Collectively, this comprehensive cytokine analysis of circulating T cells in COPD patients revealed a correlation for $CD8^+$ T cells between GOLD stage and IFNγ or TNFα expression, but not for $CD4^+$ T cells.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality throughout the world [1]. Tobacco smoking is the primary risk factor but also other noxious particles can cause a poorly reversible airflow limitation, which is the principal feature of COPD. Chronic inhalation of toxic particles and gases causes destruction of lung parenchyma, activates epithelial cells, increases mucus production and stimulates migration of many inflammatory cells [2-3]. This results in an abnormal inflammatory response in the small airways and alveoli in which many inflammatory cells are involved including neutrophils, macrophages and T lymphocytes [3-4].

Different T cell subsets are identified in the pathogenesis of COPD. $CD8^+$ T cells predominate over $CD4^+$ T cells in the airways and lung parenchyma [5]. Several reports have shown that increased bronchus obstruction correlates with increased IFNγ production by lung $CD8^+$ T cells [5-7] and their increased expression of cytotoxic molecules [8], however also IL-4 production has been reported [9]. $CD4^+$ T cells that accumulate in the airways and lungs of patients also mainly produce IFNγ and therefore have a T helper 1 (Th1) phenotype [10]. However, recent studies have shown increased expression of T helper 17 (Th17) cytokines in bronchial mucosa and sputum of patients, indicating a role for this novel T helper subtype in COPD [11-13].

Besides airway inflammation the presence of systemic inflammation has been suggested by multiple studies showing increased oxidative stress, the activation of circulating neutrophils and lymphocytes, and altered circulating levels of inflammatory mediators and acute phase proteins including TNF-α and its receptors, IL-6 and IL-8, and C-reactive protein (CRP) [14-16]. Nevertheless, the cytokine profile of peripheral blood T cells from COPD patients has not been as well characterized as that from T cells in the lung. To our knowledge, the Th17 subset in the peripheral blood of stable COPD patients has not yet been studied.

The aim of this study was to determine intracellular cytokine profiles of circulating T cells in patients with stable COPD, analysing both $CD4^+$ and $CD8^+$ T cells, thereby providing a detailed characterization of a wide variety of cytokines including those produced by the Th1, Th2 and novel Th17 subsets. We correlated the cytokine profiles with GOLD classification, lung function parameters and smoking status.

METHODS

Subjects

The study population consisted of 30 stable COPD patients (FEV1/FVC<70%) and 10 healthy individuals with normal lung function and no history of pulmonary disease and

smoking. All subjects had no known autoimmune disease, atopic diseases or allergies. In addition, none of the COPD patients had chronic bacterial colonization of the airways or recurrent lower respiratory tract infections (4 or more infections per year) as defined by the Dutch national guidelines [17]. COPD patients did not have an exacerbation episode in the 4 weeks prior to inclusion. They were all treated with inhaled bronchodilatators and inhalation corticosteroids (ICS), but did not receive oral steroid therapy or antibiotic treatment. ICS doses ranged from 50 mcg once a day to 400 mcg 4 times daily. Patients were categorized into Global Initiative for chronic Obstructive Lung Diseases (GOLD) stages II-IV based on pulmonary function tests [18-19]. Past smokers were defined as not having smoked in the previous year. Peripheral blood was drawn from all subjects after written informed consent. The study was approved by the Medical Ethical Committee of the Erasmus Medical Centre Rotterdam.

Pulmonary function tests

All subjects underwent pulmonary function testing according to American Thoracic Society (ATS) Spirometry Standards (2005). Forced vital capacity (FVC) and forced expiratory volume in one second (FEV1) were measured to determine airflow limitation (FEV1/FVC <70%; FEV1 <80% of predicted value). Diffusing capacity for carbon monoxide (TLCO) and the transfer coefficient for carbon monoxide corrected for the alveolar volume (KCOc) was also determined. Diffusing capacity parameters were expressed as percentages of reference values.

Cell isolation from peripheral blood and flow cytometry analysis

Peripheral blood mononuclear cells (PBMC) were isolated using standard Ficoll-Paque (GE Healthcare) density gradient and resuspended in IMDM medium (BioWhittaker) containing 10% FCS, β-mercaptoethanol and gentamycin. For cytokine analysis, cells were stimulated with ionomycine (Sigma) and PMA (Sigma) in the presence of Brefeldin A (ebiosciences) and incubated at 37°C. After 4 hours cells were collected, centrifuged for 7 min at 400g, resuspended in FACS buffer (PBS supplemented with 0.25% BSA and 0.5 mM EDTA and 0.05% NaN3) and stained with conjugated monoclonal antibodies to detect membrane markers. To detect intracellular cytokines, cells were subsequently fixed with 2% paraformaldehyde fixation (PFA, Merck) and permeabilized with 0.5% saponin solution (Sigma). For intracellular FoxP3 detection the Foxp3 Staining Buffer Set (ebiosciences) was used. The following antibodies were applied: anti-CD3 APC-AF780, anti-CD8 APC, anti-CD45RO eFluor 650NC, anti-IL-17A AF647, anti-IL-17F PE, anti-IFNγ PerCP-Cy5.5, anti-TNFα PerCP-Cy5.5, anti-IL-4 Pe-Cy7, anti-FoxP3 APC (all ebiosciences), anti-CD4 FITC, anti-CD45RO PE, anti-CD25 Pe-Cy7 (all BD biosciences), anti-CD45RA PE-TxR (Invitrogen) and anti-IL-22 APC (R&D systems). Cells were measured on a LS-RII Flowcytometer (BD Biosciences); data were analysed using FlowJo (BD) software.

Isotype controls and fluorescence minus one (FMO) controls were used to set gates. To present the data we used proportions of cells. The absolute number of the cells depends on the number of gated events and on absolute numbers of lymphocytes. To present the proportion is more common in literature and seems to be more objective in comparative studies. Two separate experiments were performed using the same subject samples showing very similar results.

Statistical analysis

Parametric data were expressed as mean values with 95% confidence interval (CI); nonparametric data were described as median values with interquartile range (IQR). Differences between two groups were evaluated by using the Mann-Whitney U test. Across group comparison of three groups was done by the non-parametric Kruskal-Wallis test. When this was significant, a pair wised Mann-Whitney U test was performed to identify differences between groups. Correlations were assessed by Spearman's rank correlation coefficients. Data analysis was performed using Prism 5.01 (GraphPad) and Statistical Package for Social Sciences (SPSS) 17.0. A p value <0.05 was considered significant.

RESULTS

Clinical characteristics of study population

Clinical characteristics of the study population are shown in Table 1. Smoking history and all tested lung function parameters were significantly different in the COPD patients when compared to the healthy controls (all $p<0.0001$). Within the COPD group, there was no statistical difference in age or cigarette smoke exposure between moderate, severe and very severe COPD patients.

Characterization of T cells

We quantified the proportions of $CD4^+$ and $CD8^+$ T cells in peripheral blood (Table 2). CD4:CD8 ratios in live PBMC were not significantly different between COPD patients and healthy controls. In addition, we analysed the subpopulations of naïve ($CD45RA^+CD45RO^-$) and memory ($CD45RA^-CD45RO^+$) $CD4^+$ and $CD8^+$ T cells. With increasing age the proportions of memory T cells increased in all study subjects (data not shown); no statistical differences were found between COPD patients and healthy controls (Table 2).

Next, we analysed cytokine profiles for IFNγ, IL-17A, IL-22, IL-17F, TNFα and IL-4 upon activation in vitro. In $CD4^+$ T cells, cytokine production was almost completely restricted to the $CD45RO^+$ memory T cell compartment (Fig. 1). Although the memory fraction of $CD8^+$ T cells was the main cytokine producer, also naïve $CD8^+$ cells manifested TNFα and to a lesser extent IFNγ proportions (Fig. 1).

Table 1. Subject characteristics

	COPD patients (Total)	COPD GOLD II	COPD GOLD III	COPD GOLD IV	Healthy controls
Subjects (n)	30	10	11	9	10
Age (years)	63 (61-67)#[1]	64 (57-71)	66 (59-73)	61 (56-65)	52 (46-55)*
Sex ratio (male/female)	18/12	6/4	9/2	3/6	6/4
Smoking (packyears)	43 (33-52)#	50 (27-72)	41 (27-54)	37 (16-57)	0 (0-0)†
Current smoking (yes/no)	9/21	5/5	4/7	0/9	0/10
FEV1/FVC (%)	39 (33-44)#	46 (42-51)‡	43 (33-53)§	25 (18-32)	78 (74-83)†
FEV1 (% pred)	43 (37-49)#	57 (51-63)‡ǁ	44 (37-50)§	26 (19-29)	109 (104-114)†
TLCO (% pred)	58 (50-67)#	70 (51-89)	57 (49-67)	46 (28-63)**	112 (101-122)*
KCOc (% pred)	73 (63-83)#	84 (63-105)	76 (61-92)	55 (36-75)**	106 (100-113)**††

[1]Values are expressed as mean (95% CI). FEV1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity; TLCO: diffusing capacity of carbon monoxide (data available for 27 patients); KCOc: transfer coefficient for carbon monoxide corrected for the alveolar volume (data available for 27 patients). #: p<0.0001 versus healthy subjects; *: p<0.01 versus all GOLD stages; †: p<0.001 versus all GOLD stages; ‡: p<0.01 versus GOLD IV; §: p<0.05 versus GOLD IV; ǁ: p<0.001 versus GOLD III; **: p<0.05 versus GOLD II; ††: p<0.05 versus GOLD III and GOLD IV.

Table 2. Proportions of lymphocyte subsets

	COPD patients	Healthy controls
Subjects (n)	30	10
$CD3^+$ (% of MNC)	45 (36-57)	58 (46-67)*
$CD4^+$ (% of $CD3^+$)	60 (44-68)	63 (57-79)
$CD8^+$ (% of $CD3^+$)	35 (26-44)	28 (17-38)
CD4:CD8 ratio	1.7 (1.2-2.7)	2.3 (1.5-4.7)
$CD45RO^+$ (% of $CD3^+CD4^+$)	48 (33-63)	44 (24-62)
$CD45RO^+$ (% of $CD3^+CD8^+$)	19 (9.4-26.7)	17 (9.5-27.8)
$FoxP3^+$ Treg (% of $CD3^+CD4^+$)	4.3 (3.3-5.6)	3.8 (2.8-4.4)

Values are expressed as median (IQR). Lymphocyte subsets are presented as proportions of viable cells. MNC: mononuclear cells; Treg: regulatory T cell. *: p<0.05 versus COPD patients

Comparison of cytokine expression between COPD patients and healthy controls

Proportions of $IFN\gamma^+$ and $TNF\alpha^+$ $CD8^+$ T cells were significantly increased in COPD patients when compared with healthy controls (p<0.05 and p<0.01, respectively). Proportions of $IL\text{-}4^+CD8^+$ cells were low but we observed a trend towards higher proportions of $IL\text{-}4^+$ $CD8^+$ T cells in COPD patients when compared with healthy controls, although significance was not reached. Proportions of IL-17A, IL-22 and IL-17F positive $CD8^+$ cells were negligible and showed no differences between the two groups (Fig. 2A).

Proportions of $CD4^+$ T cells positive for IL-17A, IL-17F and IL-22 were higher than proportions of $CD8^+$ T cells positive for these cytokines. In contrast to the cytokine production by circulating $CD8^+$ T cells, proportions of $IFN\gamma^+$ and $TNF\alpha^+$ $CD4^+$ T cells were not

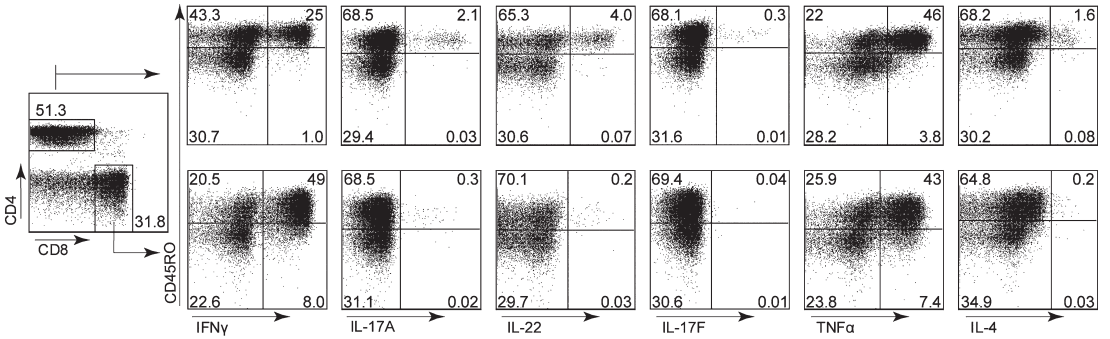

Figure 1. Flow cytometric characterisation of T cells. Peripheral blood mononuclear cell (PBMC) suspensions were stained with monoclonal antibodies specific for CD3, CD4, CD8, CD45RO and the indicated cytokines. Live CD3⁺ cells were gated and analysed for CD4/CD8 expression. CD4⁺ (top panels) and CD8⁺ (bottom panels) T cells were analyzed for the CD45RO memory T cell marker and the indicated cytokines. Results are shown as dot plots; percentages of cells within the quadrants are given.

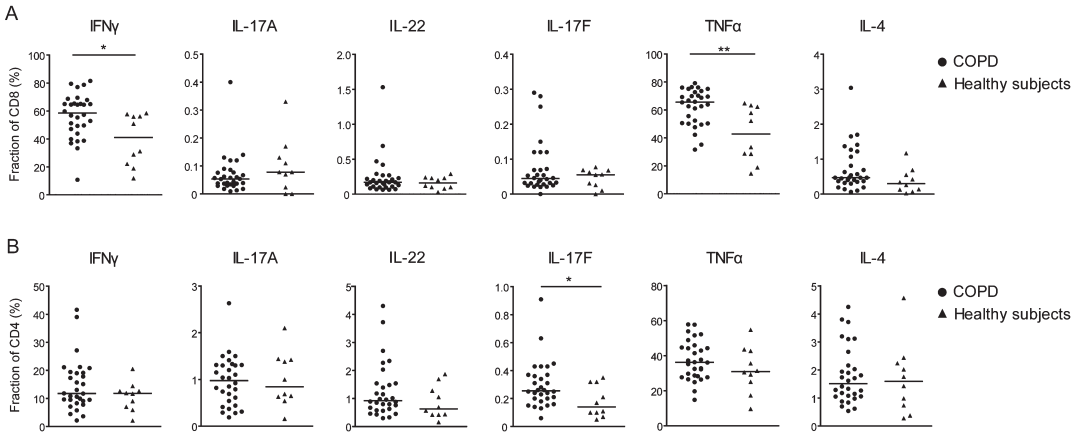

Figure 2. Cytokine expression by CD8⁺ T and CD4⁺ T cells. **A.** Proportions of total CD8⁺ T cells positive for the indicated cytokines in COPD patients versus healthy controls. **B.** Proportions of total CD4⁺ T cells positive for the indicated cytokines in COPD patients versus healthy controls Values are expressed as mean with SEM. *: p<0.05; **: p<0.01.

increased in COPD patients, when compared with healthy subjects (Fig. 2B). Proportions of $CD4^+$ T cells expressing the most prominent Th17 cytokines, IL-17A and IL-22 did not show significant differences between COPD patients and healthy controls. Although they comprise a small fraction of circulating $CD4^+$ T cells, $IL\text{-}17F^+$ T cells were increased in COPD patients, compared with healthy controls (Fig. 2B).

Correlations between cytokine expression and lung function parameters

IFNγ and TNFα proportions by $CD8^+$ T cells were higher in COPD patients when compared to healthy subjects. To examine a possible correlation with bronchus obstruction, we examined the differences within the COPD population with different stages of disease. For both cytokines there was a significant correlation present, patients in GOLD stage IV had lower proportions of $IFN\gamma^+CD8^+$ and $TNF\alpha^+CD8^+$ T cells when compared with stage II patients (Fig. 3). Also a reduced diffusing capacity was significantly correlated with lower proportions of $IFN\gamma^+CD8^+$ and $TNF\alpha^+CD8^+$ T cells (Fig. 4A,B). The low but detectable proportions of IL-17A, IL-17F, IL-22 and IL-4 by $CD8^+$ T cells did not correlate with either bronchus obstruction (FEV1 or GOLD stage) or lung diffusing capacity (TLCO).

Although, apart from IL-17F, proportions of cytokines produced by $CD4^+$ T cells did not differ between COPD patients and healthy subjects, we correlated cytokine production with lung function parameters within the COPD patient group. None of the cytokines tested showed a significant correlation with either FEV1 or diffusing capacity except for IL-17A. A reduced diffusing capacity in COPD patients was significantly correlated with lower proportions of IL-17A expressing $CD4^+$ T cells. This correlation was present for both TLCO and KCOc (Shown for TLCO in Fig. 4C).

As it is conceivable that regulatory T cells (Tregs) suppress $CD4^+$ T cells particularly in more advanced COPD, we analyzed the population of circulating Tregs. We found no

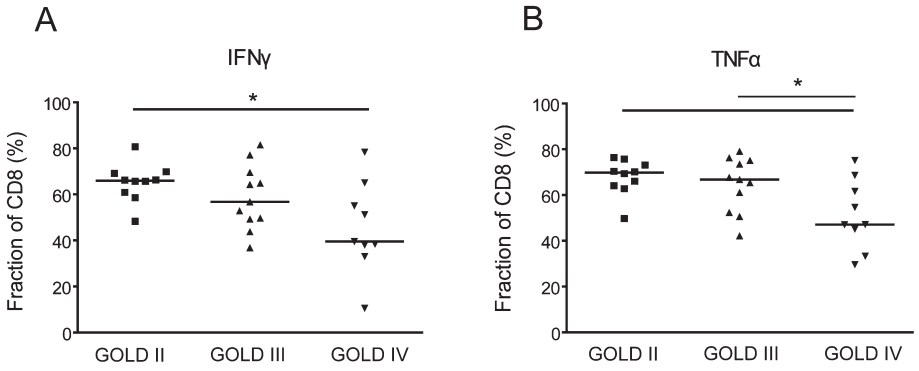

Figure 3. Proportions of $IFN\gamma^+$ and $TNF\alpha^+$ $CD8^+$ T cells in COPD patients with different GOLD stages. Results are shown as dot plots with median values. **A.** Kruskal-Wallis p=0.02. **B.** Kruskal-Wallis p=0.01. Subsequent pair wised Mann Whitney U test *: $p<0.05$.

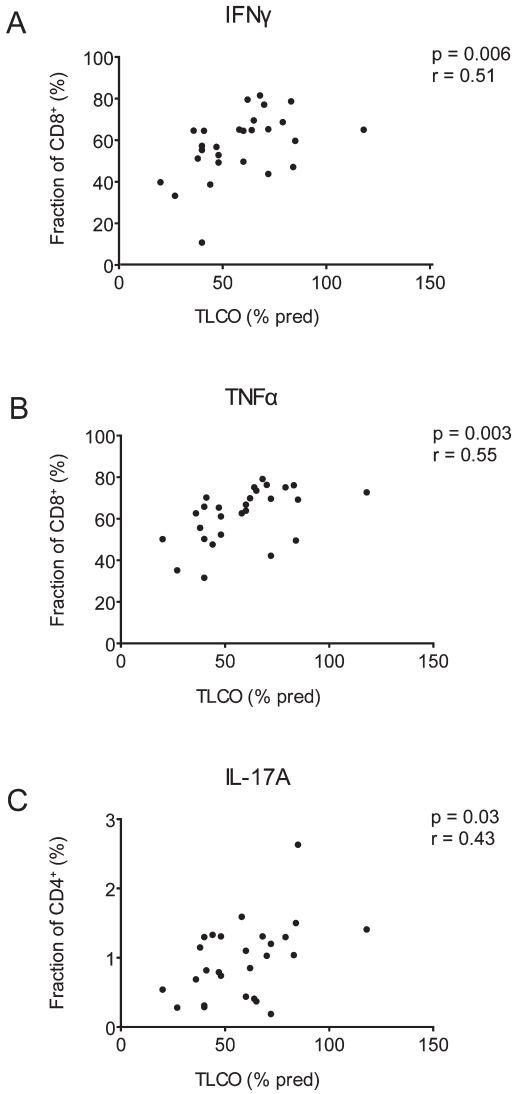

Figure 4. Correlation of cytokine proportions by $CD8^+$ and $CD4^+$ T cells with diffusing capacity. **A,B.** Correlations of proportions of $IFN\gamma^+$ and $TNF\alpha^+$ $CD8^+$ T cells with diffusing capacity (TLCO, n=27). **C.** Correlations of proportions of IL-$17A^+$ $CD4^+$ T cells with diffusing capacity (TLCO, n=27). *: $p<0.05$.

differences in the proportions of $FoxP3^+CD4^+$ Tregs between COPD patients and healthy controls (Table 2). Likewise, we did not find differences in IL-2R (CD25) or IL-7R (CD127) expression on Tregs between the two groups. Within the COPD population proportions of Tregs did not correlate with either bronchus obstruction or diffusing capacity (data not shown).

Effects of smoking on cytokine expression

We also examined the potential effect of current smoking on cytokine profiles in COPD patients. Comparisons of current and past smoker COPD cohorts did not reveal significant differences in cytokine production by $CD4^+$ or $CD8^+$ T cells. Only for $CD4^+$ T cells we observed a trend in higher proportions of IL-22 in current smokers when compared to past smokers (data not shown). Since it was demonstrated that the vast majority of IL-22 producing cells in PBMC were $CD4^+$ T cells with the CD45RO memory phenotype [20], we performed a subanalysis for IL-22 in the $CD45RO^+$ memory T cell compartment. Interestingly, $CD45RO^+CD4^+$ T cells of currently smoking COPD patients showed increased proportions of IL-22 when compared to COPD patients that have quit smoking (Fig. 5).

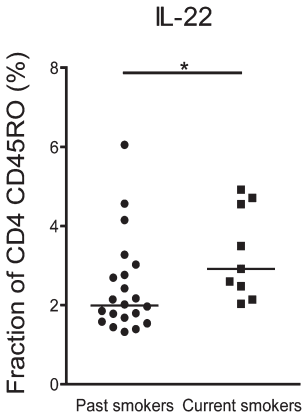

Figure 5. The effect of smoking status on IL-22 expression by $CD4^+$ memory T cells. Proportions of memory $CD4^+$ T cells positive IL-22 in former smoking versus current smoking COPD patients. Results are shown as dot plots with median values. *: $p<0.05$.

DISCUSSION

In the present study we determined the cytokine profiles of circulating T cells in patients with stable COPD and correlated these with GOLD classification, lung function parameters and smoking status. Our results show that proportions of $IFN\gamma^+$ and $TNF\alpha^+$ $CD8^+$ T cells were increased in COPD patients, when compared to healthy controls and that within the COPD population there was a significant correlation between the proportions of these two cytokines and GOLD stage. We also found that proportions of $CD4^+$ T cells positive for IL-17A and IL-22 cells were much higher than proportions of $CD8^+$ T cells positive for these cytokines. COPD patients with more severely reduced diffusing capacity had lower proportions of $IL\text{-}17A^+CD4^+$ T cells. Moreover, we show that within the COPD patient population the proportions of $IL22^+CD4^+$ memory T cells are higher in current smokers when compared with past smokers.

Many studies in COPD have focused on the role of the immune system in the lungs [3-4]. T lymphocytes are shown to be extensively present in the alveolar walls of COPD patients and the extent of intrapulmonary lymphocytic infiltrations is correlated with disease severity [2,4,10,21]. It has long been established that $CD8^+$ cells play a major role in the pathogenesis of COPD and that these cells are abundantly present in the airways of patients [3-4,10]. It has also been shown that increased COPD disease severity is characterized by an increased cytotoxic potential or cytokine production capacity of $CD8^+$ T cells in the lung [4,8,10]. Besides the local inflammation, it is now clear that also systemic inflammation is of great importance in the pathogenesis of COPD [22]. The intensity of this systemic inflammation increases during exacerbations of COPD [21]. However, in contrast with the pulmonary inflammatory component, only little is known about the characteristics and functions of circulating lymphocytes in COPD, and reported findings are often conflicting. In particular, reports on proportions of $IFN\gamma^+$ $CD8^+$ T cells are confusing. Our finding of increased proportions of $IFN\gamma^+CD8^+$ T cells in the circulation of COPD patients is in line with a number of other studies including a large study in COPD patients with varying disease severity [23-25]. Moreover this result is consistent with several reports investigating the local inflammation in the lung showing increased IFNγ production by $CD8^+$ T cells [5-6]. In contrast to our finding, Reyes et al. reported that $CD8^+$ T cells from COPD patients less frequently produced IFNγ compared to control subjects [26]. Two other studies could not detect differences in IFNγ production by circulating $CD8^+$ T cells of COPD patients compared with specimens from healthy subjects [7,27]. All of these studies were conducted in stable COPD patients, however disease severity differed greatly.

TNFα is a potent inflammatory cytokine that enhances neutrophil chemotaxis and migration by inducing the expression of the chemokine CXCL8 (IL-8), which is essential for neutrophil recruitment in COPD. Elevated levels of TNFα and soluble TNFα receptors in the serum and sputum of COPD patients suggest an important role for TNFα in this disease [28]. Yet, reports on TNFα production by T cells in the circulation of COPD patients remain limited and conflicting. Our finding of increased TNFα expression by $CD8^+$ T cells is supported by Hodge et al.[23], however another group reported a decrease in COPD patients, when compared with healthy controls [27]. Several studies have attempted to correlate lung function parameters to IFNγ and TNFα production by T cells in the circulation [25,27,29], however the results are still ambiguous. In our cohort, patients with more severe disease had lower proportions of both $IFN\gamma^+CD8^+$ and $TNF\alpha^+CD8^+$ T cells when compared to patients with mild disease. To the best of our knowledge we are the first to report this correlation in proportions of cytokine positive $CD8^+$ T cells and GOLD stage in the circulation of COPD patients. Our finding does not necessarily imply that progression of COPD to GOLD IV stage results in low proportions of IFNγ or TNFα expressing circulating $CD8^+$ T cells or conversely that patients with relatively low numbers of IFNγ or TNFα expressing circulating $CD8^+$ T cells have an increased susceptibility to progress to GOLD stage IV. In

any case, our findings show the importance of analysing the peripheral compartment in the context of COPD heterogeneity [30].

The role of circulating $CD4^+$ cells in the pathogenesis of COPD is not well known. We demonstrated that circulating memory ($CD45RO^+$), but not naïve ($CD45RO^-$) $CD4^+$ T cells had the capacity to produce cytokines including those of the IL-17 family, upon short stimulation *in vitro*. For none of the cytokines tested, except for IL-17F, we detected differences in the proportions of cytokine-expressing $CD4^+$ T cells between the total COPD patient group and healthy controls. Similar to studies on cytokine production by $CD8^+$ T cells, reports on $CD4^+$ T cells are conflicting. Zhu et al. found no differences in the cytokine profiles of $CD4^+$ T cells [25], however the group of Majori reported an increased percentage of $CD4^+IFN\gamma^+$ T cells in the circulation of COPD patients [7]. To our knowledge this is the first study to determine systemic Th17 cytokines in COPD. Th17 cells produce pro-inflammatory cytokines such as IL-17A and IL-22, which can recruit neutrophils to the site of inflammation. Although proportions of the major Th17 cytokines produced by $CD4^+$ memory T cells did not differ between patients and healthy controls, a correlation with lung function was found within the COPD population. More advanced COPD patients with severely reduced diffusing capacity had lower proportions of $IL\text{-}17A^+CD4^+$ memory T cells. This could imply that with worsening of emphysema the capacity of IL-17A production by circulating $CD4^+$ memory T cells is reduced. Di Stefano et al. investigated the role of Th17 cells in the bronchial mucosa of patients with COPD [11]. In this study there was a significant increase of the number of $IL\text{-}17A^+$ cells in the bronchial mucosa of mild/moderate and severe COPD patients compared to control non-smokers. They suggested that Th17-related cytokines might be involved in the activation of T cells and endothelial cells reported in patients with COPD. In addition, another study performed by Doe et al, reported an increase in $IL\text{-}17A^+$ cells in the submucosa of COPD patients compared with non-smoking control subjects as well as an increase in sputum IL-17A levels in COPD compared with asthmatic subjects [12]. Further studies, in which peripheral blood and lung tissue are analysed in parallel, should help to elucidate the relation between Th17 cells in the two compartments in the context of COPD.

Previous studies showed that high doses of ICS may affect the immune system, e.g. by suppressing T cell function [25]. In our study, all COPD patients received ICS which minimizes confounding by these agents. Although we did not have a control group of COPD patients not using ICS, we verified that our results were not influenced by the dosage of ICS. No correlations were found between ICS doses and FEV1, TLCO, T lymphocyte counts or cytokine production, except for proportions of $IL17F^+CD4^+$ T cells, which showed that a higher dose of ICS correlated with lower IL-17F proportions ($p<0.05$, data not shown).

We have also demonstrated a significant correlation of current cigarette smoking on IL-22 proportions by memory T helper cells in COPD patients, but not on any of the other cytokines tested. Interestingly, cigarette smoke contains many chemicals includ-

ing dioxins which have various toxic effects, most of which are mediated by the aryl hydrocarbon receptor (AHR) [31]. AHR, also called the dioxin receptor, was recently shown to specifically control IL-22 production [32]. It is therefore conceivable that IL-22 production is promoted by direct effects on T cells of AHR agonists present in cigarette smoke. This is supported by recent findings in the mouse [33] and in human [34], demonstrating that addition of cigarette smoke extract to T cells undergoing Th17 differentiation augments the percentage of IL-22 producing cells via an AHR dependent mechanism. Future studies should also examine a group of smokers with normal lung function to see if these results can be confirmed in this control group.

In our study, the diagnosis of airway obstruction was made based on a FEV1/FVC<0.70 as was proposed by the GOLD committee in 2001 [19]. However, more recently the GOLD committee recognized that using a fixed value may lead to potential overdiagnosis in the elderly and promoted the use of the lower limit of normal (LLN) instead of a fixed criterion [35]. We verified that there was no misclassification of patients in our study, in all COPD patients in our cohort FEV1/FVC ratio was below LLN.

We recognize that the observational nature of our study and the relatively small cohort sizes are important limitations to our study. Studies in larger cohorts would be valuable to confirm our results. Another limitation in our study is the observation that patient characteristics revealed an age difference between the COPD patients and the healthy controls (Table 1). However, proportions of lymphocyte subpopulations did not differ significantly between these two groups.

In conclusion, our study provides a comprehensive analysis of circulating $CD4^+$ and $CD8^+$ lymphocyte cytokine patterns in stable COPD, which is important since the role of systemic inflammation in the pathogenesis of COPD is not yet clear. Our main findings were that in the peripheral blood of COPD patients the proportions of IFNγ and TNFα producing $CD8^+$ T cells were increased when compared with healthy controls and that this was most evident in patients with less severe disease. These results further support the assumption that a systemic inflammation is present in patients with stable COPD. Furthermore, we show a new and interesting correlation between smoking and the proportions of IL-22 expressing $CD4^+$ memory T cells in peripheral blood. Future studies should also focus on the parallels in systemic and local airway cytokine production in COPD.

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CHAPTER 8

Increased IL-17A expression in granulomas and in circulating memory T cells in sarcoidosis

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ABSTRACT

Objective Sarcoidosis is a systemic inflammatory disorder characterized by granulomas. Although the etiology is unknown, sarcoidosis is thought to be mediated by T-helper (Th)1 lymphocytes. Recently, IL-17A has been implicated in granuloma formation in various diseases, including tuberculosis. Therefore, we hypothesized that Th17 cells play role in sarcoidosis, paralleling recent findings in autoimmune diseases such as rheumatoid arthritis.

Methods T cells were investigated by intracellular flow cytometry and immunohistochemistry, in blood, bronchoalveolar lavages (BAL) and bronchial mucosal biopsies from a cohort of newly diagnosed sarcoidosis patients and healthy controls.

Results Circulating memory $CD4^+$ T cell populations of sarcoidosis patients contained significantly increased proportions of IL-$17A^+$ cells, when compared with healthy controls. Interestingly, proportions of IL-17A/IFNγ and IL-17A/IL-4 double-producing cells were significantly increased in blood of sarcoidosis patients and were present in substantial numbers in BAL. In granuloma-containing, but not in non-granulomatous sarcoidosis biopsies, we found significantly increased numbers of IL-$17A^+$ T cells, located in and around granulomas throughout the lamina propria. IL-22^+ T cells were increased in the subepithelial layer.

Conclusions Enhanced IL-17A expression in granulomas and the presence of IL-$17A^+$, IL-$17A^+$IFNγ$^+$ and IL-$17A^+$IL-4^+memory T helper cells in the circulation and BAL indicate Th17 cell involvement in granuloma induction or maintenance in sarcoidosis. Therefore, neutralization of IL-17A activity may be a novel strategy to treat sarcoidosis.

INTRODUCTION

Sarcoidosis is a systemic inflammatory disease characterized by the presence of non-caseating granulomas in various organs with pulmonary involvement in over 90% of patients [1]. These granulomas are compact, organized collections of macrophages and epithelioid cells, surrounded by and infiltrated with $CD45RO^+$ memory T lymphocytes. Besides granulomas, pulmonary alveolitis and peripheral blood lymphopenia are typically present in sarcoidosis [1]. The pathological processes that result in granulomatous inflammation are largely unknown. Nevertheless, the findings of $CD4^+$ T cell accumulation, oligoclonal $TCR\alpha\beta^+$ expansions and production of IFNγ and T helper (Th)1-promoting cytokines, including interleukin (IL)-12, chemokines and chemokine receptors at sites of inflammation provide evidence for a pathological antigen-driven Th1 response [2-3].

Recently, the proinflammatory cytokine IL-17A has been implicated in the pathogenesis of various granulomatous diseases, in particular in the formation of a mycobacterial infection-induced granuloma in the lung [4] Although IL-17A can be produced by various cell types, it is the main cytokine produced by the novel subset of Th17 cells, which is distinct from the Th1 and Th2 subset. Th17 cells were shown to be crucially involved in many autoimmune diseases, including rheumatoid arthritis (RA), inflammatory bowel disease, multiple sclerosis, autoimmune uveitis and in allergic lung disease [5-8]. Th17 cells have the capacity to confer protection against extracellular bacterial and fungal pathogens such as Klebsiella pneumonia, Citrobacter rodentium and Candida albicans, although accumulating evidence demonstrates that Th17 cells also provide protective effects during infection with more traditional intracellular pathogens [4,9]. Interestingly, there are many reports of sarcoidosis coexisting with or mimicking rheumatic diseases such as systemic lupus erythematosus, rheumatoid arthritis, and ankylosing spondylitis [10]. In this context, the observation that IL-17A is highly expressed in synovium of RA patients and that the cellular source is mainly $CD4^+$ cells is particularly important [11-14]. Differentiation and maintenance of Th17 cells in human is critically dependent on IL-1β, IL-6, TGF-β and IL-23 [15]. The finding in sarcoidosis of increased IL-12p40 (which is not only part of the Th1-inducing cytokine IL-12 but also of the Th17-inducing cytokine IL-23), together with increased IL-1β mRNA expression in lymph nodes [16-18], may therefore also point to a role of Th17 cells.

Human Th17 cells comprise heterogeneous subsets. Next to IL-17A, these cells produce various other proinflammatory cytokines, including IL-17F, IL-22 and in some conditions IFNγ. IL-22 is a cytokine involved in mucosal immunity against extracellular pathogens and can also be produced independently of IL-17A, as was recently found in the context of psoriasis [19-21]. Also IL-17A/IFNγ double producing cells have been described [22-24], which could possibly be more pathogenic, since e.g. these cells preferentially cross the blood-brain barrier in patients with multiple sclerosis.

Recently, sarcoidosis was suggested as a Th1/Th17 multisystem disorder [25], based on the presence of IL-17 positive CD4 T-cells in sarcoid lung tissue and their ability to respond to the chemotactic stimulus CCL20. Moreover IL-17A was expressed by macrophages infiltrating sarcoid tissue. However, the involvement of IL-22 or the recently identified pathogenic populations of IL-17A/IFNγ and IL-17A/IL-4 double-producing Th cells [26-27] in sarcoidosis pathogenesis remains unknown.

Therefore, in this report we studied the presence of double-producing Th cells. Moreover we investigated whether there would be granuloma-dependent differences in the presence of IL-22 and IL-17A positive cells in granuloma-containing compared with non-granulomatous lung mucosa biopsies obtained from sarcoidosis patients. Because of the heterogeneous cytokine profile of the Th17 subset, we analyzed peripheral blood, BAL cells and lung mucosal biopsies of newly diagnosed immunosuppressive drugs free stage 1 or stage 2 sarcoidosis patients. Flow cytometric (FACS) analyses were performed for the expression of the main Th17 cytokines IL-17A, IL-17F and IL-22, along with IFNγ, TNFα and IL-4 and immunohistochemistry for IL-17A and IL-22.

MATERIALS AND METHODS

See supplementary data for more details regarding bronchoscopy procedure, peripheral blood mononuclear cell (PBMC) processing, flow cytometric analysis and immunohistochemical staining of lung mucosa biopsies.

Patients and healthy control subjects

After informed consent (according to the Declaration of Helsinki) 33 patients and 33 healthy volunteers underwent fibre-optic bronchoscopy. The protocol was approved by the Medical Ethical Committee of the Erasmus MC Rotterdam. The patient group consisted of 15 males and 18 females with newly diagnosed sarcoidosis (mean age: 37.3 y). The diagnosis of sarcoidosis was made conform the guidelines of the ATS/ERS/WASOG statement on sarcoidosis [1]. All patients were newly diagnosed with stage 1 (19 patients) or stage 2 (14 patients) sarcoidosis. None of the patients were on corticosteroid or immunosuppressive drugs at the time of diagnosis and sampling. Healthy volunteers had a male/female ratio of 13/20 and the mean age was 24.2 y. Bronchoalveolar lavage (BAL) was performed with a flexible fibre-optic bronchoscope (Olympus) according to standard procedures (see Supplementary data).

Immunohistochemical analysis of lung mucosa biopsies and cytospins

Immunohistochemical stainings are detailed in the Supplementary data. All biopsy sections were stained in one session to reduce inter-staining-variation and analysed in a

blinded fashion by two different researchers. Sections from lung mucosal biopsies fulfilled the following criteria: intact epithelium (32 out of 48 biopsies), a subepithelial mucosa of 100μm depth (38 out of 48 biopsies) and a good overall morphological quality. Cell numbers were expressed as cells per mm length basal membrane. The entire lamina propria region was analysed in all 48 biopsies and cell numbers were expressed as cells per square mm.

Statistical analysis

For statistical evaluations the Kruskal-Wallis 1-way ANOVA and the Mann-Whitney U-test were performed. A p-value <0.05 indicated significant differences. Associations between cells were assessed with Pearson rank correlations.

RESULTS

Increased Th17 profile in circulating memory CD4$^+$ T cells from sarcoidosis patients

To investigate the involvement of Th17 cells in the pathogenesis of sarcoidosis, we analyzed peripheral blood samples of nine recently diagnosed sarcoidosis patients and ten healthy controls by flow cytometry. Surface stainings for CD3, CD4, CD8 and CD45RO revealed a specific CD4$^+$ T cell lymphopenia in the sarcoidosis patients (Supplementary Figure S1A). The proportions of CD3$^+$ T cells in the mononuclear cell fractions were significantly reduced (p=0.02) in sarcoidosis patients (median: 45%; range: 8-56%), when compared with healthy controls (median: 58%; range: 35-74%; Supplementary Figure S1A). Likewise, the proportions of CD4$^+$ T cells of the total CD3$^+$ populations were significantly reduced (p=0.001) in sarcoidosis patients (median: 33%; range 11-61%; and healthy controls: median: 63%; range 51-90%) (Supplementary Figure S1B). The proportions of CD8$^+$ T cells in the mononuclear cell fractions were not different between sarcoidosis patients and healthy controls (Supplementary Figure S1A).

We did not detect significant differences in the fractions of antigen-experienced memory CD45RO$^+$ CD4$^+$ T cells between sarcoidosis patients (median: 51%; range: 31-92%) and healthy controls (median: 41%; range: 14-64%) (Figure 1A). We used intracellular flow cytometry to determine the expression profiles for IL-17A, IL-17F, IL-22, IFNγ, IL-4 and TNFα in total mononuclear cell fractions upon 4 hours of stimulation with PMA and ionomycin (See Supplementary Figure S1C for gating strategy). Intracellular expression of these cytokines was almost completely confined to the CD45RO$^+$ memory T cell fractions (data not shown). Importantly, the proportions of IL-17A$^+$ cells within the CD45RO$^+$CD4$^+$ memory T cell population were significantly higher (p=0.009) in sarcoidosis patients (median: 3.7%; range: 3.0 - 6.6%) than in healthy controls (median: 2.4%; range: 1.4-5.8%; Figure 1B). We

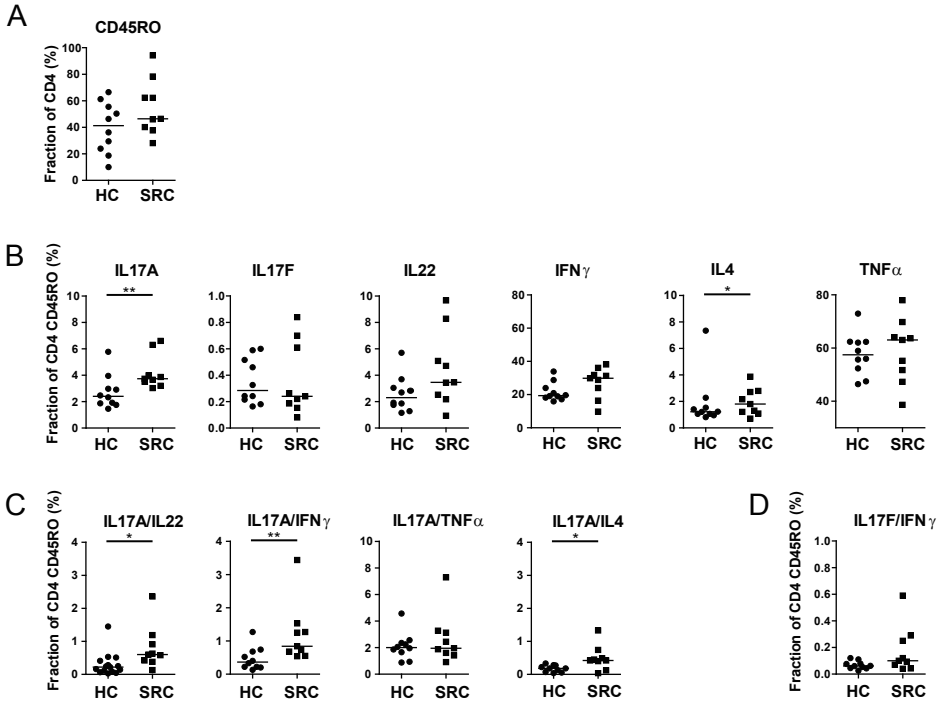

Figure 1. Cytokine profiles of circulating memory T helper cells in sarcoidosis patients and healthy controls. **(A)** Proportions of antigen-experienced memory CD45RO$^+$ cells within the populations of CD4$^+$ T cells in PBMC fractions. **(B)** Proportions of CD45RO$^+$CD4$^+$CD3$^+$ cells expressing the indicated cytokines, as determined by intracellular flow cytometry **(C, D)** Proportions of CD45RO$^+$CD4$^+$CD3$^+$ cells that were double positive for the indicated cytokines, as determined by intracellular flow cytometry. Symbols represent individual healthy controls (HC, ●) and sarcoidosis patients (SRC, ■) and lines indicate median values. * p<0.05; ** p < 0.01.

did not detect differences between patients and healthy controls for the other two Th17 cytokines tested, IL-17F and IL-22 (Figure 1B). The frequencies of IFNγ-expressing cells tended to be higher in sarcoidosis patients (median: 30% of CD45RO$^+$CD4$^+$T cells, range: 10-38%), when compared with healthy controls (median: 19%; range: 16-34%), but this difference did not reach significance (Figure 1B). Also, the proportions of IL-4$^+$ cells were significantly higher (p=0.05) in sarcoidosis patients (median: 1.8% of CD45RO$^+$CD4$^+$ T cells, range: 0.7-3.8%; healthy controls: median: 1.2%, range: 0.8-2.3%; Figure 1B). No differences were observed for TNFα between sarcoidosis patients and healthy controls (Figure 1B).

Because IL-17A/IL22 and IL-17A/IFNγ double-producing cells have been described, whereby particularly IL-17A$^+$IFNγ$^+$ cells might be more pathogenic [21-22], we next analyzed coexpression of Th17 cytokines. We observed significantly higher proportions of CD45RO$^+$CD4$^+$ T cells expressing IL-17A, together with IL-22, IFNγ or IL-4 in sarcoidosis patients than in healthy controls (Figure 1C). No differences were observed for the frequencies of IL17A/TNFα or IL-17A/IL-17F (Figure 1C and data not shown) or IL17F/IFNγ double producers (Figure 1D).

Taken together, these findings show that in recently diagnosed sarcoidosis patients the peripheral blood memory T helper cell compartment contained increased proportions of IL-17$^+$ T cells, indicating enhanced Th17 differentiation. Moreover, the increased frequencies of IL-17A/ IL-22 and particularly IL-17A/IFNγ double producing T cells would indicate an active state of the disease. The increased frequency of IL-4$^+$ single and IL-17A$^+$IL-4$^+$ double positive memory T helper cells point to a possible involvement of IL-4 in sarcoidosis, as previously suggested by Hauber et al. [28].

Increased IL-17A, but not IL-22, in BAL memory CD4+ T cells from sarcoidosis patients

The majority of CD4$^+$ T cells in the alveolar space of sarcoidosis patients were CD45RO$^+$ memory T cells, as determined by analysis of BAL cells (median: 94%; range 91%-96%; Figure 2A). Stimulation of BAL cells by PMA/ionomycin, similar to PBMC, indicated the abundant presence of cells expressing IL-17A (median: 25%; range: 11-33%), IFNγ (median 54%; range: 33-70%), and TNFα (median: 60%; range: 33-72%) within the

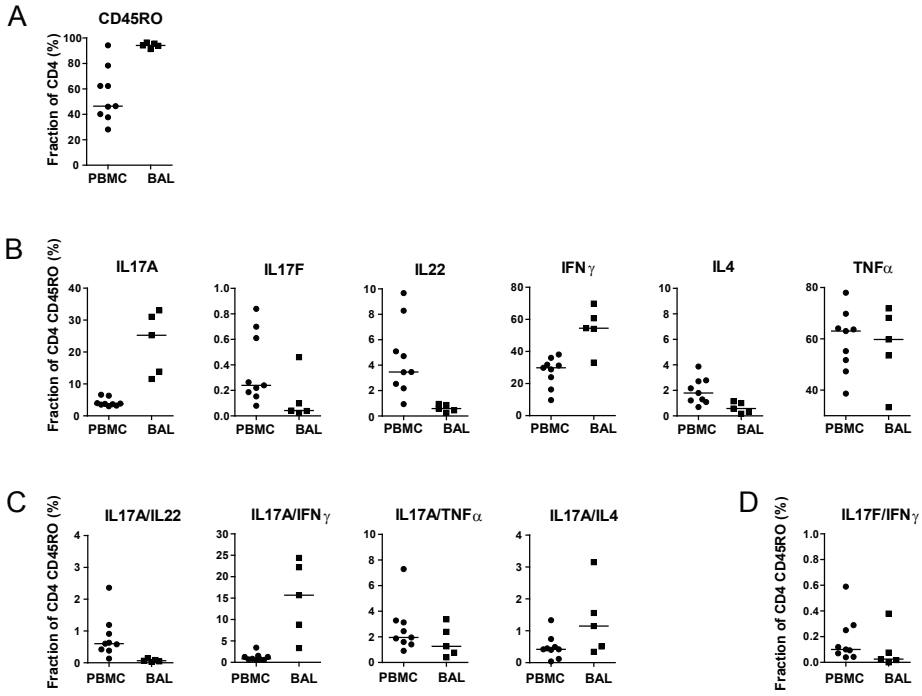

Figure 2. Cytokine profiles of circulating and BAL memory T helper cells in sarcoidosis patients. **(A)** Proportions of antigen-experienced memory CD45RO$^+$ cells within the populations of CD4$^+$ T cells in PBMC and BAL. **(B)** Proportions of CD45RO$^+$CD4$^+$CD3$^+$ cells expressing the indicated cytokines, as determined by intracellular flow cytometry. **(C, D)** Proportions of CD45RO$^+$CD4$^+$CD3$^+$ cells that were double positive for the indicated cytokines, as determined by intracellular flow cytometry. Symbols represent PBMC (●) and BAL (■) from individual patients and lines indicate median values.

$CD45RO^+CD3^+CD4^+$memory helper T cell population. IL-$17F^+$, IL-22^+ or IL-4^+ T helper cells were present a very low frequencies (Figure 2B). Importantly, large proportions of IL-$17A^+$ memory T helper cells in BAL were also IFNγ^+. The frequency of IL-17A/IFNγ double producing memory T helper cells was therefore remarkably higher in BAL (~15%) than in peripheral blood (~1%) of sarcoidosis patients (Figure 2C).

IL-17A/IL-4 double-producing cells were present at low but detectable proportions, representing a novel subpopulation (Figure 2C). By contrast, IL-17^+IL-22^+ cells were almost completely absent and proportions of IL-17A/TNFα or IL-17A/IL-17F double producers were present in the same ranges in BAL and peripheral blood from sarcoidosis patients (Figure 2C and data not shown). IL17F/IFNγ double producers were virtually absent in BAL of sarcoidosis patients (Figure 2D). The proportions of IL-$17A^+$, IL-$17A^+$IFNγ^+ and IL-$17A^+$IL-4^+ memory T helper cells in BAL varied between patients and did not show a positive correlation with the T cell alveolitis in BAL (data not shown).

In summary, the observation of high proportions in BAL of IL-$17A^+$ memory T helper cells, and particularly of IL-17A/IFNγ double-producing cells, which are thought to be more pathogenic [22], clearly point to the involvement of Th17 cells in sarcoid inflammation.

Increased IFNγ and TNF-α, but not IL-17A in $CD4^-$ T cells in sarcoidosis

Little is known about $CD8^+$ and γδ T cells (the $CD4^-CD3^+$ T-cell population) in sarcoidosis, although the general observation is that these cells show a similar or a less pronounced cytokine pattern as $CD4^+$ T cells do [29-30]. Here, we observed that a substantial fraction of circulating $CD8^+$ and γδ T cells produced IFNγ, whereby the proportions were significantly higher ($p<0.01$) in sarcoidosis patients compared with healthy controls (Supplementary Figure S2). Frequencies of IL-17A, IL-17F, IL-4 or TNFα expressing $CD8^+$ and γδ cells T cells were not different between sarcoidosis patients and healthy controls. When we measured IL-22 expression in circulating $CD3^+CD4^-$ cells, we found fewer IL-22^+ cells in sarcoidosis patients when compared with healthy controls (Supplementary Figure S2). BAL $CD8^+$ and γδ T cell populations of sarcoidosis patients contained significant proportions of IL-22^+, IFNγ^+ and TNFα^+ cells and limited proportions of cells expressing IL-17A, IL-17F or IL-4.

Taken together, these findings show that IL-17A is not a prominent cytokine produced by $CD8^+$ and γδ T cells in sarcoidosis. Nevertheless, a substantial fraction of $CD8^+$ and γδ T cells in the BAL produced IL-22.

Increased IL-$17A^+$ cells in sarcoidosis lung biopsies containing granulomas

The presence of Th17 cytokines in memory T helper cells in the BAL, representing the alveolar space of the lungs, prompted us to analyze T cell cytokine expression in lung mucosal tissue of sarcoidosis patients.

We investigated the presence of IL-17$^+$ cells in lung mucosal biopsies from 27 sarcoidosis patients and 22 healthy controls (Figure 3). From the 27 biopsies from sarcoidosis patients ten contained clear granulomas (Figure 3C) and 17 were non-granulomatous, showing diffuse cellular infiltrates only (Figure 3B). IL-17A-expressing cells were particularly found in sarcoidosis lung biopsies containing granulomas. Low magnifications of immunohistochemical stainings revealed that IL-17$^+$ cells were specifically present in areas of inflammatory cells surrounding granulomas, as well as within the granulomas (Figure 3C). In addition, we observed diffuse IL-17A staining within the granuloma areas (Figure 3C). IL-17$^+$ cells were not detected in the epithelium. Quantification of IL-17-expressing cells in biopsies from healthy controls and non-granulomatous and granuloma-containing biopsies from sarcoidosis patients showed that in all three groups the subepithelial area contained very few IL-17$^+$ cells, although a trend of higher numbers was observed in granuloma-containing biopsies (p=0.059, Kruskal Wallis test; Figure 3D). Importantly, in the entire lamina propria, the numbers of IL-17$^+$ cells were significantly increased in granuloma-containing biopsies (median: 44 cells/mm^2, range: 0-505 cells/mm^2), when compared with biopsies from healthy controls or non-granulomatous biopsies (median values: 0 cells/mm^2 and ranges of 0-107 and 0-314 cells/mm^2, respectively; p=0.001, Kruskal Wallis test; Figure 3D). By immunohistochemical double stainings for and CD3, the IL-17$^+$ cells were characterized as T cells (Figure 3E).

In summary, we observed increased numbers of IL-17A$^+$ T cells in sarcoidosis in association with granulomas. Together with the presence of diffuse IL-17A staining in the granulomas, these findings suggest a role for IL-17A in granuloma formation or maintenance.

Increased subepithelial IL-22$^+$ T cells in sarcoidosis lung biopsies containing granulomas

Finally, we investigated the presence of IL-22$^+$ cells in the epithelium, subepithelium and lamina propria (Figure 4). The epithelium could be evaluated in 32 biopsies. IL-22$^+$ cells were detected in nine of them, but no significant differences were found between sarcoidosis patients and healthy controls. Importantly, granuloma-containing biopsies showed significantly more IL-22$^+$ cells in the subepithelial area (median: 15 cells/mm basal membrane, range: 5-27 cells/mm; Figure 4B and 4C) than non-granulomatous biopsies (5 cells/mm, range: 0-13 cells/mm; p=0.004, Mann-Whitney U test) or healthy control biopsies (7 cells/mm, range: 0-14 cells/mm; Figure 4A and 4C). When we quantified the numbers of IL-22$^+$ cells in the total lamina propria areas, we did not detect significant differences between the three groups of biopsies (Figure 4D). Analyses of alveolar biopsies demonstrated the incidental presence of IL-22$^+$ cells in healthy controls as well as in sarcoidosis patients, without significant differences between these groups. Immunohistochemical double stainings showed that IL-22$^+$ cells were mainly CD3$^+$ T cells, as illustrated in Figure 4E. In those biopsies where IL-17$^+$ cells were detected in the lamina propria, we

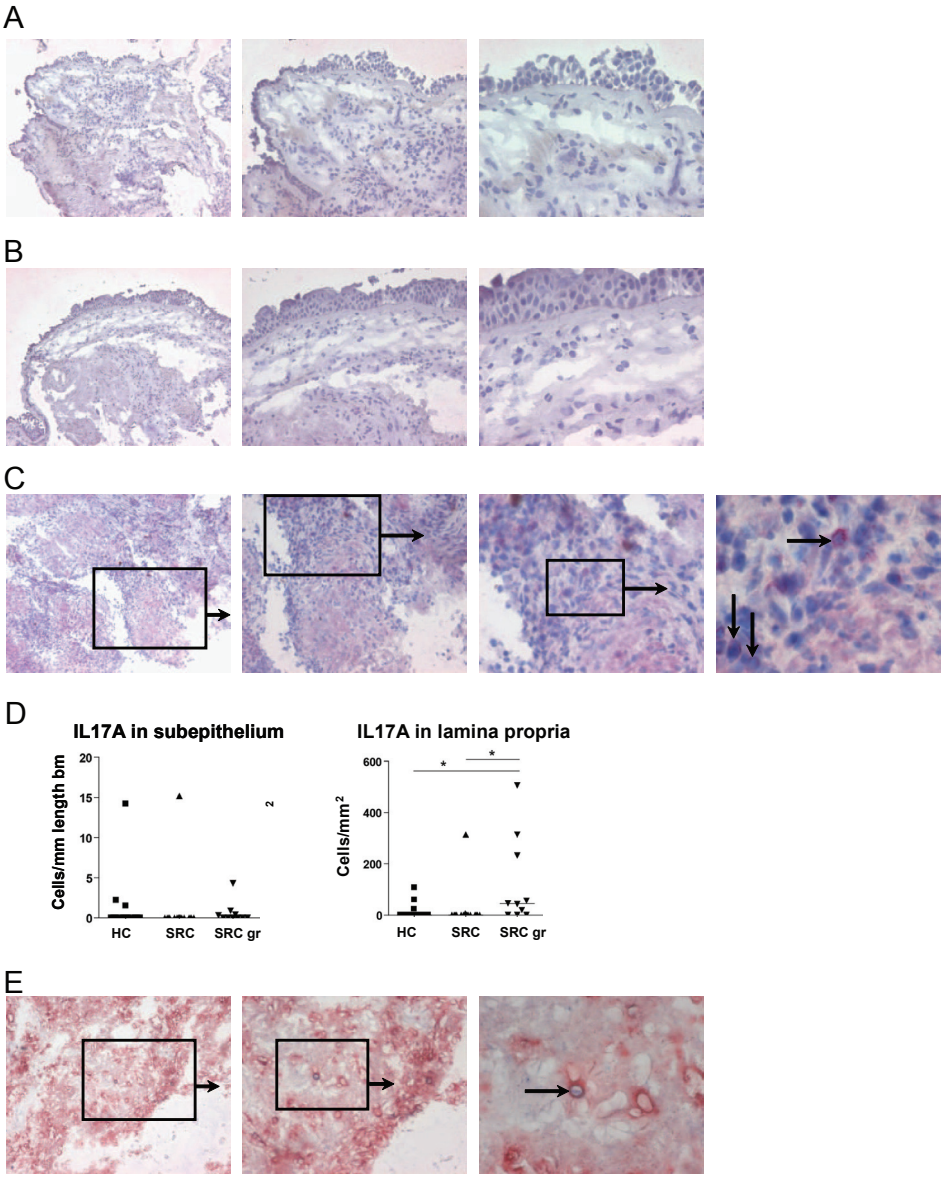

Figure 3. Increased numbers of IL-17A$^+$ cells in sarcoidosis lung biopsies containing granulomas. **(A,B,C)** Hematoxylin nucleus staining and IL-17A staining of lung mucosal frozen sections from a healthy control biopsy **(A)**, a non-granulomatous sarcoidosis biopsy **(B)** and a granuloma-containing sarcoidosis biopsy **(C)** at 100x, 200x and 400x magnification. IL-17A$^+$ cells as well as diffuse IL-17A staining are observed in red. **(C)** The high magnification photograph (1000x, *far right*) illustrates cytoplasmic staining with anti-IL-17A antibodies (arrows). **(D)** Quantifications of the numbers of IL-17$^+$ cells in the subepithelium (*left*) and entire lamina propria area (*right*) in the indicated groups. Symbols represent biopsies from individual healthy controls (HC, ■), sarcoidosis patients without (SRC, ▲) and sarcoidosis patients with granuloma (SRC gr, ▼); lines indicate median values. * p<0.05. **(E)** Co-localization of an IL-17$^+$ (in blue) and CD3$^+$ (in red) T cell in a lung mucosal biopsy.

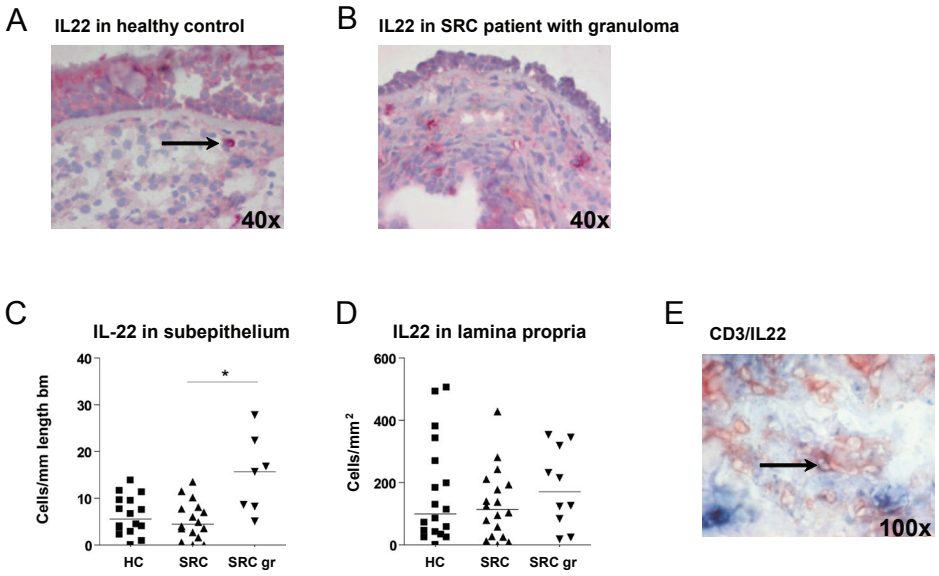

Figure 4. Increased numbers of IL-22$^+$ cells in sarcoidosis lung biopsies containing granulomas. HE and IL-22 staining (in red) of submucosal frozen sections from a healthy control **(A)** and a granuloma-containing sarcoidosis biopsy **(B)**. Quantifications of the numbers of IL-22$^+$ cells in the subepithelium **(C)** and in the entire lamina propria area **(D)** in the indicated groups. Symbols represent biopsies from individual healthy controls (HC, ■), sarcoidosis patients without (SRC, ▲) and sarcoidosis patients with granuloma (SRC gr, ▼); lines indicate median values. * p<0.05. **(E)** Co-localization of an IL-22$^+$ (in blue) and CD3$^+$ (in red) T cell in a lung mucosal biopsy.

found an association with the presence of IL-22$^+$ cells in the subepithelial area (r=0.185; p=0.05, Pearson test, see Supplementary Figure S3).

In summary, our findings show that sarcoidosis patients have a significant increase in IL-22-producing cells in the subepithelial area in the lung. Thus, their main localisation is different from IL-17-producing cells, which are mainly localized around and within granulomas.

8

DISCUSSION

The pathological mechanisms that control granulomatous inflammation in sarcoidosis are only poorly understood, but it is clear that cytokines play an important role in granuloma formation. To date, sarcoidosis pathogenesis has mainly been related to increased Th1 cytokines. In this report, we provide several lines of evidence for the involvement of pro-inflammatory Th17-lineage cytokines. First, we found increased proportions of circulating IL-17A$^+$ memory T helper cells. Second, IL-17A$^+$ cells, and in particular IL-17A/IFNγ and IL-17A/IL-4 double-producing cells, which are normally very rare, were also present in BAL samples of sarcoidosis patients. Third, we observed an increase in IL-17A-expressing

T cells in the lamina propria of the lung in sarcoidosis patients, specifically in granuloma-containing biopsies, where IL-17A$^+$ cells were present around and inside granulomas. Fourth, we identified an increase in IL-22$^+$ cells, in particular in subepithelial regions in granuloma-containing biopsies. To the best of our knowledge, we show for the first time differential distribution of IL-17A$^+$ and IL-22$^+$ T cells in local granulomas, BAL and the circulation in sarcoidosis. Our data particularly point at a possible role for IL-17A/IFNγ and IL-17A/IL-4 double-producing CD4$^+$ T cells, while we did not find evidence for increased IL-17A production by other T cell subsets, such as γδ or CD8$^+$ T cells.

Until now sarcoidosis was considered a Th1-mediated multi-system disease and there is convincing evidence reported for the role of Th1 cells in the pathogenesis of sarcoidosis over the last few years [1]. Thus, Th17 cells are clearly not the only effector cells capable of inducing or regulating granuloma pathogenesis. This would parallel findings in autoimmune uveitis and in experimental autoimmune encephalomyelitis, in which both Th1 and Th17 cells can drive tissue damage [6,31]. Cooperation of IL-17A and IFNγ is particularly of interest, since we observed high frequencies of IL-17A$^+$IFNγ$^+$ memory T helper cells in blood and BAL of sarcoidosis patients. Furthermore, it has recently been reported that IL-17A/IFNγ double producing CD4$^+$ T cells can become single IFNγ$^+$ cells or single IL-17A producing cells [22]. IL-17A$^+$IFNγ$^+$ cells are thought to be more pathogenic and have also been identified in Crohn's disease [32] and in coronary atherosclerosis [33].

Also our identification of IL-17A/IL-4 double-producing cells in sarcoidosis is of interest. Very few of these cells are present in the circulating memory T cell populations in healthy individuals, but their proportions were reported to be significantly increased in patients with chronic asthma [34]. In this regard, it is very well possible that these cells contribute to IL-4-induced pro-fibrotic features, such as fibroblast growth and collagen production, that are often observed in later stages of sarcoidosis [35]. In contrast to the IL-17A$^+$IFNγ$^+$ and IL-17A$^+$IL-4$^+$ memory T helper cells present in the circulation as well as BAL of sarcoidosis patients, we detected only few IL-17A$^+$IL-22$^+$ cells. On the contrary, our findings of (i) high proportions of IL-17A$^+$ and very low proportions of IL-22$^+$ T helper cells in BAL and (ii) different locations of IL-17A$^+$ and IL-22$^+$ T helper cells in mucosal biopsies of sarcoidosis patients support the hypothesis that IL-22 can be produced in a IL-17 independent fashion by Th22 cells [19,21].

IL-17A has previously been implicated in various conditions characterized by granuloma formation. In a *Mycobacterium bovis* infection model, IL-17A expression was detected early after pulmonary infection and IL-17A-deficient mice showed impaired granuloma formation [36]. In humans living in regions with high prevalence of *Mycobacterium tuberculosis* infection, peripheral blood contains high frequencies of IL-17A$^+$ and IL-22$^+$ memory T helper cells, which may have protective properties against tuberculosis [37]. In mouse models and in humans with active pulmonary tuberculosis both IL-17A- and IL-22-producing CD4$^+$ T cells and IL-17A$^+$ γδ T cells were shown to contribute to the anti-mycobacterial

immune response in human [7,38]. Lung injury in a mouse model for chronic granulomatous disease with lethal aspergillosis was shown to involve unrestrained γδ T cell reactivity and dominant production of IL-17A [39]. In Langerhans cell histiocytosis, which is accompanied by aggressive chronic granuloma formation, yet another cell population, dendritic cells, was shown to synthesize IL-17A [6]. An IL-17A-dependent pathway for dendritic cell fusion was identified, which was potentiated by IFNγ and led to giant cell formation. In this context, interesting parallels between Langerhans cell histiocytosis and sarcoidosis further include the presence of multinucleated giant cells [2,6,40].

The etiology of sarcoidosis is still unknown. Epidemiological and histopathological data have been suggestive for occupational airborne antigens or infectious antigens underlying this disease, but until now attempts to link sarcoidosis to a causative pathogen are difficult and remain controversial. It is therefore presently unclear which mechanisms would initiate a Th17 response in sarcoidosis. It is conceivable that the involvement of Th17 cells in sarcoidosis points to an autoimmune process that is comparable to various other IL-17A-driven autoimmunity disorders, such as autoimmune uveitis, rheumatoid arthritis, inflammatory bowel disease or psoriasis. Future studies are required to determine putative genetic components that enhance IL-17A synthesis, e.g. IL-23R polymorphisms, which have also been associated with autoimmunity. As we observed diffuse IL-17A staining, it is very well possible that Th17 effector cells within granulomas are required to achieve local IL-17A concentrations that can activate various myeloid cell populations. Such a model would parallel the proposed role of local Th17 cells in the bone marrow, which may regulate myeloid development [41]. An alternative explanation for the observed diffuse IL-17A staining in granuloma may be that granuloma cells are a source for IL-17A, analogous to Langerhans cells in Langerhans cell histiocytosis [40].

In summary, we provide evidence for the involvement of the Th17 lineage in sarcoidosis: IL-17A-expressing T cells were present in and around the granuloma and IL-22-expressing T cells were found in the subepithelial lamina propria in mucosal biopsies of sarcoidosis patients. This was accompanied by the presence of IL-17A$^+$, IL-17A$^+$IFNγ$^+$ and IL-17A$^+$IL-4$^+$ memory T helper cells in BAL and by a significant increase in the proportions of these cells in the circulation. Therefore, IL-17A and IL-22 represent targets that may have clinical value in the treatment of sarcoidosis. In this context, it is promising that clinical trials of the fully human antibody, AIN457, in RA, psoriasis and noninfectious uveitis, show that targeting IL-17A interrupts inflammation and reduces disease activity [42].

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SUPPLEMENTARY DATA

MATERIALS AND METHODS

Bronchoscopy

BAL was performed with a flexible fibre-optic bronchoscope (Olympus) placed in the right middle lobe in wedge position. Four aliquots of 50 ml saline were instilled en subsequently gently aspirated. BALF was collected in siliconized bottles to prevent cell adherence. BAL fluid was filtered through a 100µm cell strainer (BD Biosciences) and centrifuged for 7 min, at 450g, at 4°C. The supernatant was removed and the BALF cells were diluted in PBS (1x 10^e6 BALF cells/ml). Cytospins (approximately 50,000 BALF cells) were prepared (3 min at 400g (Cytofuge, Nordic immunological laboratories, Tilburg, The Netherlands), air dried overnight and stored at −80°C until use.

Mucosal biopsies were taken at the right middle lobe, processed in PBS and Tissue Tek, O.C.T. compound, (Sakura Finetek Europe) and afterwards directly frozen in Tissue Tek, O.C.T. compound and stored at −80°C.

Peripheral blood mononuclear cell processing

Peripheral blood mononuclear cells (PBMC) were isolated using standard procedures. Briefly, the blood was diluted 1:1 with RPMI 1640, (Gibco) and centrifuged over a Ficoll-Paque (GE Healthcare) density gradient for 20 min, 1200g, at room temperature. The mononuclear cells at the interface were collected and washed in RPMI 1640 (Gibco). Cells were counted and subsequently frozen in 1 ml RPMI 1640 (Gibco), 10% Fetal Calf Serum (FCS, Sigma), 10% Dimethyl sulphoxide Hybri-Max (DMSO, Sigma) in a cryovial using a 5100 Cryo 1°C Freezing Container (Nalgene) to −80°C. Afterwards the cells were stored at −150°C.

Flow cytometry

PBMCs were quickly defrosted at 37°C. 5 ml RPMI 1640 (Gibco) with 20% FCS (Sigma) was added and the cells were centrifuged for 7 min at 450g at 4°C. Cells were resuspended in IMDM medium (BioWhittaker) containing 10% heat-inactivated FCS (Sigma), 5 x 10^e −5 M 2-ME, 4 x 10^e −3 M ultraglutamine (Lonza) and 55 µg/ml gentamycin (Gibco) into a final concentration of 1x10^e6 cells/ml. Cells were stimulated with ionomycine (Sigma), phorbol 12- myristate 13- acetate (PMA, Sigma) and golgiplug (BD biosciences) and incubated at 37°C. After 4 hours cells were collected, centrifuged for 7 min at 450g at 4°C and resuspended in FACS buffer (PBS supplemented with 0.25% Bovine serum albumine (BSA), 0.5mM ethylenediaminetetraacetic acid (EDTA) and 0.05% NaN3) supplemented with 1% heat inactivated human serum and subsequently stained with the following antibodies for extracellular staining: CD3, CD4, CD8 (all BD biosciences) and CD45RO FITC

(eBiosciences). For intracellular staining were antibodies used against IL-17A, IL-17F, IL-22 (R&D), IFNγ, IL-4 and TNFα (eBiosciences). Fixable Aqua Dead Cell Stain kit for 405 nm (Invitrogen, Molecular Probes) was used as a live dead marker. Cells were measured on a Flowcytometer LSRII (BD Biosciences).

Flow cytometric analysis

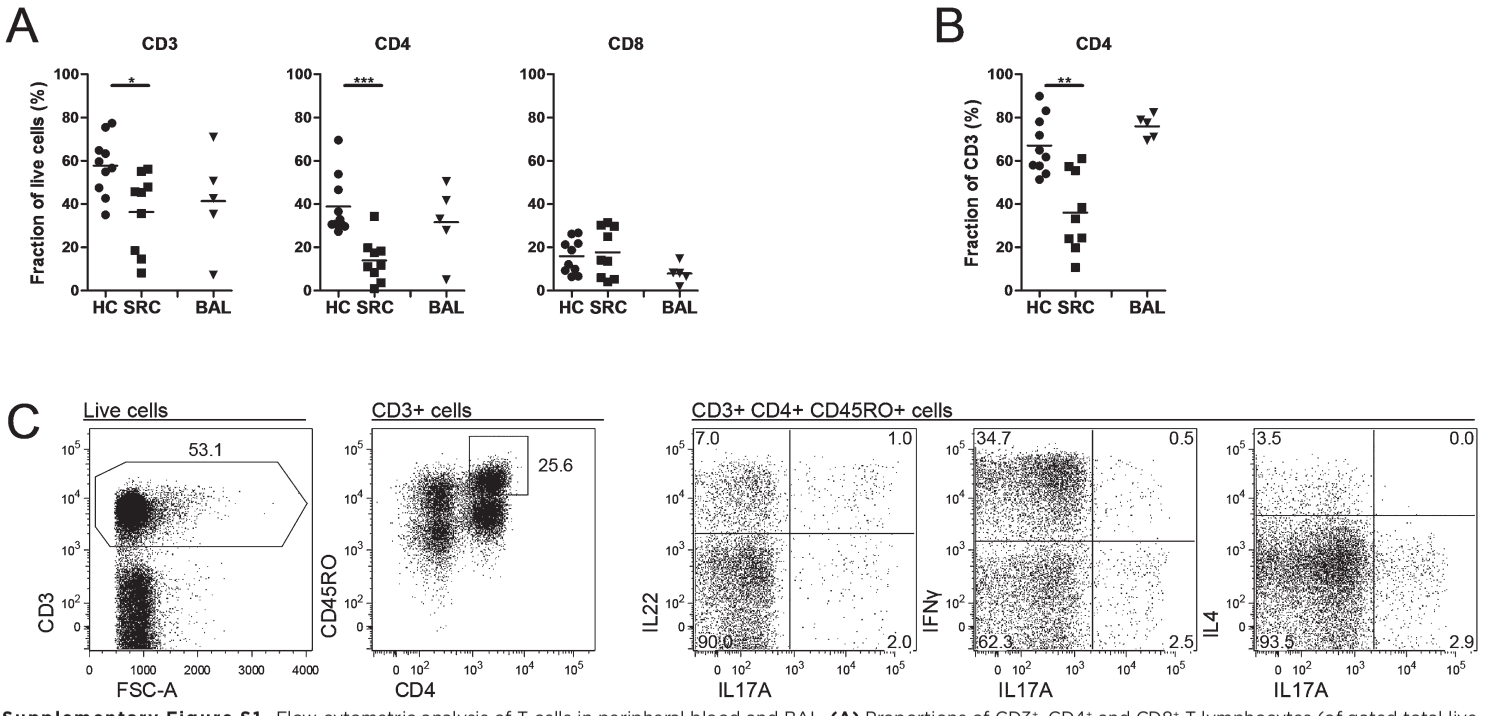
PBMC fractions were stained with the following monoclonal antibodies: anti-CD3 APC-AF780, anti-CD8 APC, anti-CD45RO eFluor650, anti-IL-17A AF647, anti-IL-17F PE, anti-IFNγ PerCP-Cy5.5, anti-TNF-α PerCP-Cy5.5, anti-IL-4 Pe-Cy7 (all ebiosciences), anti-CD4 FITC, anti-CD45RO PE (both BD biosciences), anti-CD45RA PE-TxR (Invitrogen) and anti-IL-22 APC (R&D systems). Fixable Aqua Dead Cell Stain kit for 405 nm (Invitrogen, Molecular Probes) was used as a live-dead marker. Cells were measured on a LSRII Flow-cytometer (BD Biosciences).

Immunohistochemical staining of lung mucosa biopsies

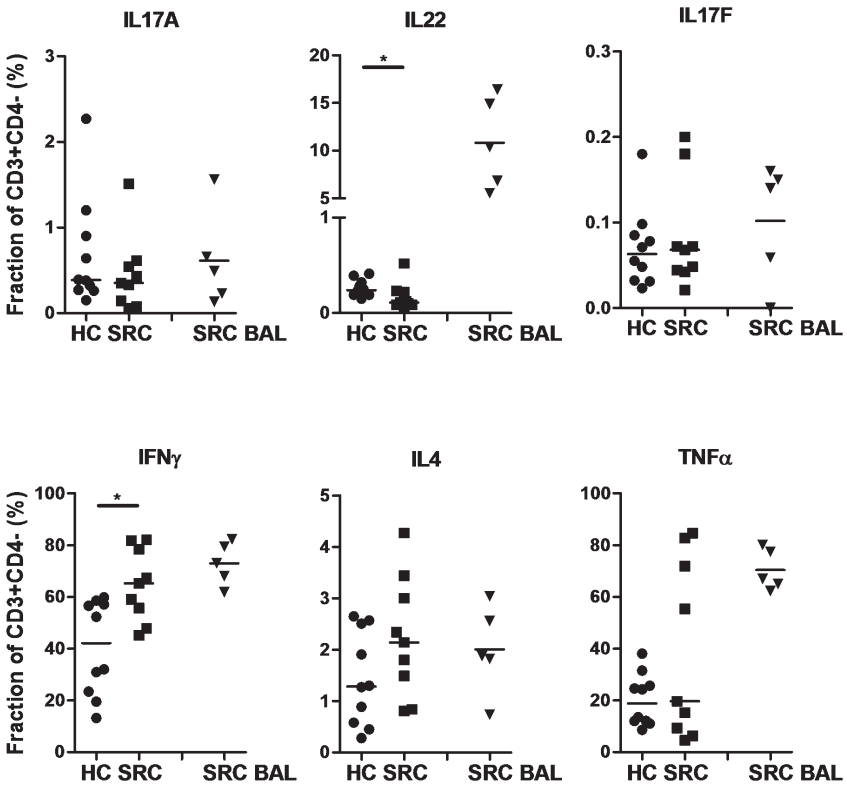
Immunohistochemical single stainings were performed in a half automatic stainer (Sequenza) as previously described (1). Aceton fixed slides were blocked in diluted normal goat serum (CLB, Amsterdam, the Netherlands) and were stained with primary monoclonal Abs against human IL-17A (eBioscience) IL-22, IL-23r (R&D) rinsed with PBS and incubated for 30 min with diluted long chain biotin conjugated goat anti mouse (Biogenix). Rinsed again and followed by alkaline phosphatase conjugated streptavidin for 30 minutes. After rinsing, slides were incubated with New Fuchsin substrate. Finally, the sections were counterstained with Gills triple strength haematoxylin and mounted in glycerin gelatin.

Double stainings were performed to elucidate the cellular source of IL-17A and IL-22. Frozen sections were fixed in acetone for 10 minutes and rinsed in PBS. Endogenous peroxidase was blocked with 0.1% sodium azide and 0.01% hydrogen peroxide in PBS for 30 minutes. Sections were then rinsed in PBS for 10 minutes and incubated with 10% normal goat serum (CLB, Amsterdam, the Netherlands) and 10% normal rabbit serum (CLB). After this, the slides were incubated with mouse anti-human IL-17A (eBioscience) IL-22, (R&D) for 60 minutes at room temperature. The sections were then rinsed again in PBS for 5 minutes and incubated for 30 minutes with diluted long chain biotin conjugated goat anti mouse (Biogenix) rinsed successively in PBS and incubated with alkaline phosphatase conjugated streptavidin (Biogenix) for 30 minutes. Additionally the slides were incubated with the 10% normal mouse serum (CLB) for 10 minutes and incubated with Fitc conjugated mouse anti-human CD11c(SHCL-3; Becton Dickinson), CD3, CD14, CD15, CD16 (eBioscience, Immunosource Belgium), CD68 (Kim-7 Caltag, Invitrogen Breda, The Netherlands) for 60 minutes. Slides were rinsed with PBS for 5 minutes and incubated with Peroxidase-conjugated goat anti-Fitc (1:100 Rockland, Gilbertsville, USA) for 30 min-

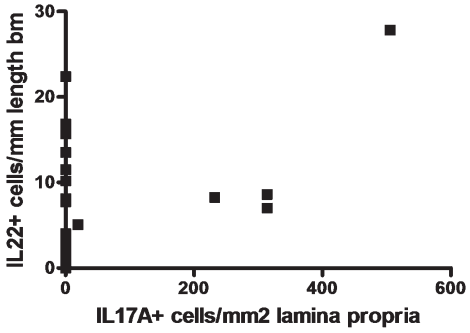
utes. Slides were again rinsed with PBS for 5 minutes and incubated with TRIS buffer (0.1 mol/L, pH 8.5) for 5 minutes, followed by incubation for 30 minutes in Fast Blue substrate containing levamisole to block endogenous alkaline phosphatase. Finally, slides were rinsed with sodium acetate (0.1 mol/L, pH 4.6) for 5 minutes and incubated with amino-ethylcarbazole 0.05% in sodium acetate 0.1 mol/L (pH 4.6) and 0.01% peroxide (Sigma, Zwijndrecht, The Netherlands) substrate for 30 minutes and sections were rinsed with distilled water and mounted in Kaisers, glycerin-gelatin (Merck, Amsterdam The Netherlands). Control staining was performed with an irrelevant mAb of the same subclass.



Supplementary Figure S1. Flow cytometric analysis of T cells in peripheral blood and BAL. **(A)** Proportions of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes (of gated total live cells) in PBMC fractions of healthy controls (HC, ●) and sarcoidosis patients (SRC, ■), as well as in BAL of sarcoidosis patients (BAL, ▼), as determined by flow cytometry. Lines indicate median values. * $p<0.05$; *** $p<0.001$. **(B)**. Proportions of total $CD4^+$ cells (of total $CD3^+$ lymphocytes) in PBMC fractions of healthy controls (HC, ●) and sarcoidosis patients (SRC, ■), as well as in BAL of sarcoidosis patients (BAL, ▼) as determined by flow cytometry. Lines indicate median values. ** $p<0.01$; **(C)**. Gating strategy. Live cells were analyzed for CD3 and FSC and $CD3^+$ cells were gated and subsequently analyzed for CD4 and CD45RO expression. $CD3^+CD4^+CD45RO^+$ cells were gated and analyzed for intracellular IL-17A expression together with IL-22, IFNγ and IL-4, respectively.



Supplementary Figure S2. Cytokine profiles $CD4^-$T cells in peripheral blood and BAL. Proportions of $CD4^-CD3^+$ cells, containing $CD8^+$ and $\gamma\delta^+$ T cells, positive for the indicated cytokines in PBMC fractions of healthy controls (HC, ●), sarcoidosis patients (SRC, ■) and BAL of sarcoidosis patients (BAL, ▾) as determined by flow cytometry. Lines indicate median values. * $p<0.05$.



Supplementary Figure S3. Correlation between the number of IL-$17A^+$ cells in the lamina propria and IL-22^+ cells in the subepithelium ($r=0.185$ Pearson test $p=0.05$).

CHAPTER 9

General discussion

Parts of this chapter were published in:

M.S. Paats, P.Th.W. van Hal, C.C. Baan, H.C. Hoogsteden, M.M. van der Eerden and R.W. Hendriks (2012). **Interleukin-17 and T Helper 17 Cells in Mucosal Immunity of the Lung**, Lung Diseases - Selected State of the Art Reviews, Dr. Elvisegran Malcolm Irusen (Ed.), ISBN: 978-953-51-0180-2

GENERAL DISCUSSION

The studies described in this thesis address the involvement of the Th17 lineage in the immunopathogenesis of human pulmonary diseases. By means of cytokine analysis, with a special focus on cytokines involved in Th17 lineage, we explored the local and systemic inflammatory response in patients with CAP (Chapters 2 and 3), CF (Chapters 5 and 6), COPD (Chapter 7) and sarcoidosis (Chapter 8).

The recent discovery of the Th17 cell subset and the critical biological functions of its main effector cytokines, IL-17A and IL-22, substantially advanced our understanding of the roles of $CD4^+$ T cells in adaptive mucosal immunity. With the potential of Th17 cells to induce a pronounced neutrophilic inflammation [1], which is a common feature of many pulmonary inflammatory diseases, Th17 cells are subject of great research interest. However, despite the recent progresses, including results from this thesis, many issues remain to be addressed (Figure 1). In many diseases previously considered typical Th1 or Th2 diseases, there is now evidence of Th17 involvement. However, most studies have been performed in experimental models and human data is still scarce. Moreover, the conditions promoting human Th17 differentiation are not universally established and the exact influence of the cytokine environment on Th17 cells in vivo under various inflammatory conditions is still largely unknown. Recent studies on $CD4^+$ T cell differentiation have revealed more plasticity in cytokine production than predicted by conventional models of $CD4^+$ T cell lineage commitment. Under changing stimulation conditions, activated memory T cells preserve plasticity to alter their cytokine profile and are also able to simul-

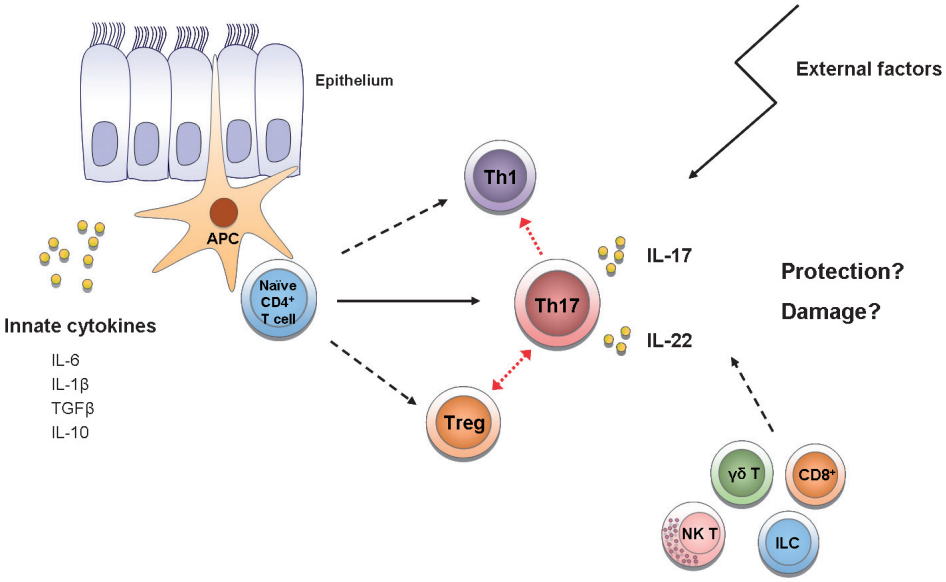


Figure 1. Th17 cells in the context of pulmonary inflammation (simplified overview)

taneously produce cytokines attributed to different T helper subsets. Furthermore, Th17 cells and their effector cytokines can have both pathological and protective roles during inflammation and infection. The balances of these functions during infectious diseases are not well understood. Lastly, Th17 cells are not the exclusive producers of IL-17 and IL-22. Other cell populations, including both adaptive and innate immune cells, are also capable of producing these cytokines and can be of influence in mucosal tissue inflammation in the lung.

Clarification of these issues is important for the development of future therapeutic strategies to treat various inflammatory and infectious pulmonary diseases.

Th17 cells: a novel T cell subset

T cells were shown to produce cytokines that could not be classified according to the classical Th1-Th2 scheme [2]. IL-17 was amongst these cytokines, and the T cells that preferentially produce IL-17, but not IFNγ or IL-4, were named Th17 cells [3].

Traditionally, many autoimmune diseases, such as rheumatoid arthritis and psoriasis, were considered Th1 diseases. However, the concept that organ-specific autoimmunity is a Th1 driven condition was challenged with the discovery that the IL-12 p40 subunit is shared by IL-23 and it became clear that IL-23, and not IL-12 was required for disease [4]. IL-23 is necessary for expansion and survival of lineage-committed Th17 cells[4] and the importance of the Th17 lineage in rheumatoid arthritis, psoriasis, and other autoimmune disease is recognized since [5]. Furthermore, also in asthma, typically regarded as a Th2 disease, evidence was found for a contribution of Th17 cells to disease pathology [6].

Pulmonary diseases such as COPD and sarcoidosis have previously been classified as Th1 diseases, but these and other lung conditions deserve special attention as Th17 cells might contribute to their pathogenesis. In our study in stable COPD patients (Chapter 7), we did not find evidence for a strong Th17 cytokine profile in circulating T cells. Because of ethical reasons we were not able to perform bronchoscopy in these patients and therefore our analysis was limited to the systemic component of the inflammatory response in COPD. The Th17 response in COPD seems to be compartmentalized to the lungs as others did report increased expression of Th17 cytokines in bronchial mucosal biopsies of stable COPD patients [7].

The accumulation in the lung of apparently oligoclonal IFNγ-producing T helper cells in sarcoidosis indicated an antigen-driven Th1 response [8-9]. Also because IL-17 had been implicated in the formation of mycobacterial infection-induced granuloma in the lung [10], we suspected a role for the Th17 lineage in this disease. By investigating Th17 cells by intracellular flow cytometry and immunohistochemistry in peripheral blood, bronchoalveolar lavage (BAL) and bronchial mucosal biopsies from a cohort of newly diagnosed sarcoidosis patients and healthy controls, we indeed found evidence for the involvement of the Th17 lineage in sarcoidosis (Chapter 8).

Influence of cytokine milieu on T cell differentiation

Differentiation of naïve $CD4^+$ T cells into distinct T helper subsets is a process that is largely controlled by various environmental factors, including the cytokine milieu in particular. The current consensus is that IL-6 induces human Th17 differentiation together with TGFβ. At the same time, IL-6 inhibits TGFβ-induced regulatory T cell (Treg) differentiation. Hence, IL-6 acts as a potent pro-inflammatory cytokine in T cells through promotion of Th17 differentiation and concomitant inhibition of Treg differentiation, indicating that IL-6 is a decisive factor in determining Th17/Treg balance. Either an excess in Th17 function, increased numbers of Th17 cells, defects in Treg function or reduced numbers of Tregs, may trigger inflammatory disorders. In contrast, when an inflammatory response already has been initiated such as in an infection, Tregs play an essential role by restraining excessive effector T cell responses and restoring a homeostatic environment. Most available studies in human and experimental models support the idea that a limitation in Treg cell function or number is usually associated with enhanced immune responses and subsequent better control of the infection [11]. Moreover, an imbalanced ratio between Th17 cells and Treg cell subsets has been reported in various diseases, including systemic sclerosis [12] and systemic lupus erythematosus [13].

In this context, it is worth mentioning that in our cohort of CAP patients we found significantly increased proportions of FoxP3 positive Tregs in the peripheral blood of severe patients when compared to non-severe patients and healthy individuals (Paats *et al.* unpublished data; Figure 2). Even one month after admission, proportions of Tregs were still elevated in severe CAP patients, irrespective of the causative pathogen. In Chapter 2 we have demonstrated a strong positive correlation between the PSI and serum IL-6 levels, meaning that CAP patients with more severe disease had higher circulating IL-6 levels. This could explain the higher proportions of circulating IL-17/IL-22 double produc-

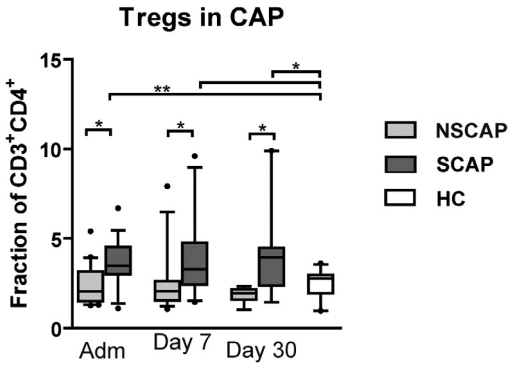


Figure 2. Fractions of $CD25^{high}CD127^{low}FoxP3^+$ regulatory T cells in peripheral blood of patients with community-acquired pneumonia (CAP) upon admission and at day 7 and day 30 after admission respectively. Data are presented as box and whisker plots with medians and 10^{th} and 90^{th} percentiles.
CAP: community-acquired pneumonia. Adm: admission. NSCAP: non-severe CAP. SCAP: severe CAP. HC: healthy control.

ing Th17 cells in blood of severe CAP (Chapter 3), but cannot directly explain the higher Treg proportions. Other factors must therefore influence the proportions of circulating Tregs and it would be very interesting to study this finding in more detail by investigating activation status and cytokine production of these cells, by identifying different Treg subpopulations [14] and also by studying Tregs locally at the site of infection, i.e. the lungs.

Th17 effector cytokines

Although IL-17 and IL-22 are both effector cytokines of Th17 cells and can be expressed simultaneously, recent reports demonstrate that there are some differences in the induction [15] and function [16] of these two cytokines. Whereas IL-17 is thought to promote chemokine and proinflammatory cytokine production and consequent recruitment and activation of neutrophils[1], IL-22 controls tissue responses during inflammation for instance by increasing lung epithelial proliferation and transepithelial resistance to injury [17-18].

The importance of IL-22 for the improvement of the lung mucosal barrier has been illustrated in CF patients. Although adult patients with CF have large numbers of *Ps. aeruginosa* in the lung, bacteriaemia with *Ps. aeruginosa* is rare. We showed that adult CF patients, have significantly elevated basal IL-22 responses in lung tissue (Chapter 6) and it has also been reported by others that CF patients have enhanced IL-22 responses in their hilar lymph nodes[17]. This indicates that IL-22 might well be required for the mucosal immunity that prevents bacteraemia in CF [11]. Despite rare bacteraemia in CF, we and others have shown that there is systemic inflammation present in CF patients when in pulmonary exacerbation as well as when clinically stable (Chapter 5). Systemically however, this does not seem to be a Th17 response. Interestingly, in our COPD patient cohort we found that smokers had higher proportions of circulating IL-22$^+$ T helper cells of a memory phenotype (Chapter 7). This again could reflect the local activation of the mucosal barrier which is translated to the periphery, but it is also conceivable that IL-22 production is promoted by direct effects on T cells of AHR agonists present in cigarette smoke.

Co-expression of IL-17 and IL-22 by Th17 cells is common. IL-17 and IL-22 can synergistically or additively increase antimicrobial proteins in skin keratinocytes [19]. Co-expression of IL-17 and IL-22 is particularly of interest, as we observed high proportions of IL-17/IL-22 double-producing T helper cells in BAL and peripheral blood of CAP patients (Chapter 3). This supports the hypothesis that IL-17/IL-22 double producing T helper cells contribute to a vital beneficial host response in human acute pulmonary infection. In Chapter 3 we have shown that IL-17 and IL-22 single positive CD4$^+$ T cells were only increased in BAL but not in peripheral blood of CAP patients. Furthermore, CAP patients had higher proportions of IL-17 and, to a lesser extend, IL-22 CD4$^+$ T cells in BAL than in peripheral blood, indicating that these cells preferentially home to or are generated locally at the site of infection. Also in sarcoidosis patients (Chapter 8) we found increased proportions of IL-17/IL-22 double producing CD4$^+$ T cells in peripheral blood when compared with

proportions in the blood of healthy individuals. However, in contrast to CAP patients, proportions of IL-17/IL-22 positive CD4+ T cells in BAL of sarcoidosis patients were low compared with systemic levels. This is most likely due to the low IL-22+ cells in the BAL of sarcoidosis patients. Together with the different anatomical localization of IL-17 and IL-22 in bronchial biopsies of sarcoidosis patients, this data points to differential roles of IL-17 and IL-22 in this disease.

Our data obtained in sarcoidosis patients, however, point to a possible role for IL-17/IFNγ and IL-17/IL-4 double positive T cells, which are normally very rare. Recent studies on T helper differentiation have revealed more plasticity in cytokine production than predicted by conventional models of T helper cell lineage commitment. Activated memory T cells preserve plasticity to alter their cytokine program according to stimuli they receive [20]. A cytokine restricted to one T helper subset can therefore be secreted by another subset under changing stimulating conditions. In this way, Th17 cells can acquire characteristics of Th1 and Th2 cells by co-production of IFNγ and IL-4, respectively.

Circulating IL-17A+ CD4+ T cells co-expressing IFNγ were also found in a cohort of patients who received lung transplantation because of end-stage COPD (Paats *et al.* unpublished data; Figure 3). In previous studies in lung transplantation patients, IL-17 has been implicated in ischemia reperfusion injury, acute rejection, infection and chronic rejection (bronchiolitis obliterans syndrome; BOS) [21-23]. Recent observations in our own group indicate that IL-17 and other Th17 cytokines might also be important in stable lung transplantation patients. We found enhanced Th17 differentiation of peripheral blood mononuclear cells (PBMC) in a group of stable lung transplantation patients, compared with both healthy individuals and patients on the waiting list for lung transplantation. The increase in the proportions of circulating Th17 cells was not linked to donor-specific haploreactivity as was verified in a donor-specific ELISPOT assay. Interestingly, increased

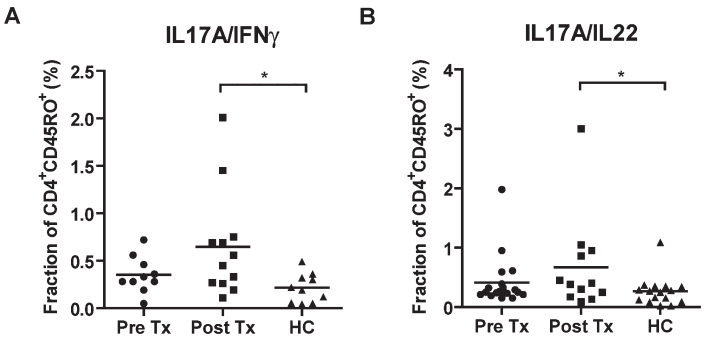


Figure 3. Enhanced systemic Th17 differentiation in COPD patients after lung transplantation. Proportions of IL-17+ CD4+ T cells co-expressing IFNγ **(A)** and IL-22 **(B)**.
Pre Tx: End stage COPD, on the waiting list to receive lung transplantation. Post Tx: Patients 1 year after lung transplantation without signs of transplant rejection. HC: healthy control. Dots represent values of individual patients with median. *: p<0.05

proportions of IL-17/IFNγ and IL-17/IL-22 double positive $CD4^+$ T cells were found in the peripheral blood, indicating that these specific Th17 subpopulations may have a functional role in stable lung transplantation patients (Paats *et al*., unpublished data; Figure 3). However, it is important to keep in mind that transplantation procedures themselves may have a direct effect on the cytokine profile within the graft. Following an organ harvest the ischemia-reperfusion injury results in the release of a number of inflammatory mediators. Additionally, patient heterogeneity may also cause conflicting results, for instance by including both unilateral and bilateral transplant patients.

Non T cell sources of IL-17 and IL-22

Th17 cells are not the exclusive producers of IL-17 and IL-22. Within the adaptive arm of the immune system, a subset of $CD8^+$ T cells is also capable of producing these cytokines as we have demonstrated in our cohorts of CAP patients (Chapter 3), COPD patients (Chapter 7), and sarcoidosis patients (Chapter 8). However, proportions of $IL\text{-}17^+$ and $IL\text{-}22^+$ $CD8^+$ T cells are much lower compared with proportions of $CD4^+$ T helper cells and might therefore play a less significant role in the pathogenesis of these diseases than $CD4^+$ T cells.

Recent studies have shown that also γδ T cells are important innate-like IL-17 and IL-22 producing cells especially during infectious diseases [24-25]. They have a critical role in the mucosal barrier as they are able to provide a rapid cytokine source [26]. The results from this thesis confirm the reported production of IL-17 and IL-22 by γδ T cells in both acute infection (CAP and CF exacerbations) and inflammation (sarcoidosis and clinically stable CF). Nevertheless, similar to results in $CD8^+$ T cells, proportions of IL-17 and IL-22 producing γδ T cells in the circulation of these patients were almost negligible compared with proportions in $CD4^+$ T cells.

Recently it was recognized that innate lymphoid cells (ILCs) can be considered a family of non-T/non-B lymphocytes that also includes cells that produce Th2 or Th17 associated cytokines. ILCs react rapidly to a wide array of signals [27-29] and serve important roles in lymphoid tissue formation, repair of damaged tissue, and tissue homeostasis, as well as in immunity against infectious microorganisms [30]. Several distinct subsets of ILCs have been described which mediate different functions [30]. $Ror\gamma t^+$ ILCs are particularly of interest because of their importance in innate immunity against bacteria and their potential to produce IL-17 and IL-22 [28,31]. In this context, it is very interesting that we have found high proportions of circulating $IL\text{-}17^+$ non-$CD3^+$ cells in our CAP patient cohort as well as in our CF patients when in pulmonary exacerbation (Paats *et al.* unpublished data; Figure 4A,B). In CAP patients, proportions of the circulating $IL\text{-}17^+CD3^-$ cells remain elevated up until one week after admission when compared with healthy individuals (Figure 4A). More extensive phenotyping of this non-T cell fraction of PBMC was only possible in a small subset of CAP patients that showed considerable numbers of circulating IL-17 expressing

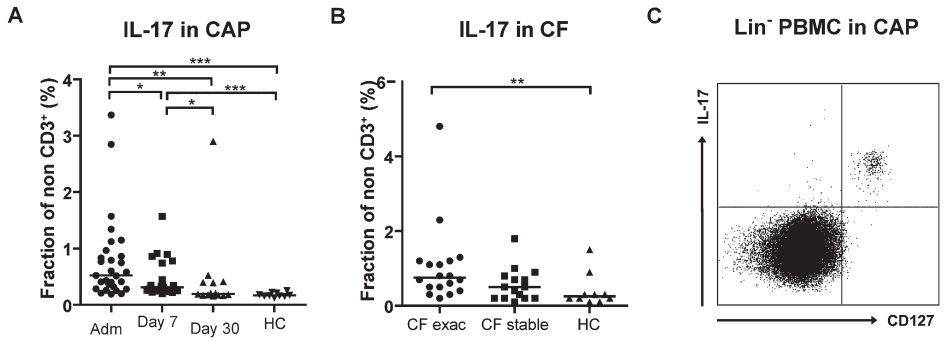


Figure 4. IL-17 production by non T cells in CAP and CF patients. **A.** Proportions of IL-17$^+$ cells other than CD3$^+$ T lymphocytes in peripheral blood of CAP patients upon admission and 7 and 30 days after admission respectively, compared with healthy controls. CAP: community-acquired pneumonia. Adm: Admission. **B.** Proportions of IL-17$^+$ cells within the fraction of CD3$^-$ cells in peripheral blood of CF patients when in pulmonary exacerbation (exac) and when clinically stable, compared with healthy individuals. *: p<0.05; **: p<0.01; ***: p<0.001. **C.** Flow cytometric identification of IL-17$^+$ CD3$^-$ cells from peripheral blood of a CAP patient. Plot shows expression of IL-17 and CD127 (IL7Ra) on cells negative for lineage (lin) markers (CD3$^-$CD11c$^-$CD14$^-$CD19$^-$CD94$^-$). PBMC: peripheral blood mononuclear cells.

non-CD3$^+$ cells (5/39). This detailed analyses revealed that most of these IL-17$^+$ non-CD3$^+$ cells lack typical lineage specific markers such as CD19 (B lymphocytes), CD11c (dendritic cells), CD11b and CD14 (monocytes), FcεRIα (mast cells), and CD94 and CD56 (NK cells) (Paats *et al.* unpublished data; data not shown). They did express IL-7Ra (CD127) which is typically expressed on ILCs [32], and therefore indicates that these cells might be so-called ILC17 cells that express the transcription factor RORγt (Figure 4C). However, flow cytometric detection of RORγt is methodologically challenging. In our limited analysis of BAL fluid cells of CAP patients, however, we did not find evidence for a significant contribution of non-CD3$^+$ cells in the production of IL-17. Further research should show whether circulating IL-17$^+$ non-CD3$^+$ cells also express RORγt, RORalpha or AHR, all of which have been implicated in the induction of IL-17 production by T helper cells [33-34].

Clinical implications: Th17 based biomarkers

The studies in this thesis contribute to our understanding of the involvement of Th17 lineage cytokines in different pulmonary diseases. Ultimately this knowledge will translate to the clinical practice and it will become clear if these cytokines can for instance be useful as biomarkers. Ideal biomarkers of pulmonary infection and inflammation are minimally invasive and allow for a rapid diagnosis, are useful in monitoring disease progression, facilitate therapeutic decision making, or evaluate response to therapy.

CAP

The analyses of patients with CAP presented in this thesis show that soluble levels of IL-6, IL-10 and IFNγ in serum could distinguish non-severe from severe CAP patients (Chapter 2). As expected, cytokine levels in BAL of CAP patients were higher than those in healthy

individuals. However, surprisingly, in contrast to systemic concentrations, local levels of cytokines did not correlate with disease severity. As systemic and local IL-6 levels in CAP patients showed a strong correlation with each other, systemic IL-6 measurements might be valuable to improve prognosis predictions in CAP patients. T cell cytokine profiles in BAL and peripheral blood of CAP patients, however, did not discriminate between non-severe and severe CAP patients (Chapter 3).

CF

Airway inflammation plays a central role in CF and biomarkers of inflammation may be useful for monitoring disease progression and evaluating response to therapy. In Chapter 5 we measured a wide range of cytokines in sputa, nasal lavages and plasma of CF patients when in exacerbation as well as when clinically stable. Interestingly, levels of IL-6 and IL-10 in nasal lavages are higher in CF patients when in exacerbation than when clinically stable, indicating that these measurements may provide an excellent minimally invasive tool in the assessment of an exacerbation in CF. Furthermore, IL-6 concentrations in plasma of CF patients correlate with lung function and CRP levels in these patients, independent of exacerbation status. Because of this, systemic IL-6 might also in CF be a valuable marker to improve prognosis predictions or to monitor disease. In addition, systemic IL-6 might also be useful to define pro-inflammatory status and potentially identify a subpopulation of CF patients who would benefit from anti-inflammatory therapies. Notably, the results of this thesis did not provide evidence that IL-17 or IL-22 are useful as biomarkers in CAP or CF.

COPD and sarcoidosis

In Chapter 7 we showed that more advanced COPD patients with severely reduced diffusing capacity had lower systemic proportions of IL-17$^+$CD4$^+$ memory T cells as well as lower proportions of IFNγ or TNFα expressing CD8$^+$ T cells. These findings might imply that progression of emphysema results in lower circulating proportions of these cells or conversely that patients with relatively low numbers of these cells have an increased susceptibility to progress to more severe lung disease. In sarcoidosis, measuring proportions of IL-17/IFNγ and IL-17/IL-4 CD4$^+$ memory T cells might be useful in the diagnosis of this disease since proportions of these cells were increased in peripheral blood and BAL of sarcoidosis patients (Chapter 8).

Clinical implications: Th17 targeted therapy

As accumulating evidence suggests that IL-17 and other cytokines associated with the Th17 lineage play an important role in the pathogenesis of various lung diseases, Th17-directed immunotherapy is likely to be an effective treatment modality. Interference with the activity of Th17 cells or the inflammatory mediators that either influence their

differentiation (IL-6, TGFβ, IL-23 and IL-1β), are produced by them (IL-17, IL-22, TNFα), or work downstream of IL-17 could provide potential therapeutic benefits. One of these novel treatment modalities is cell blockade by monoclonal antibodies [35]. Most antibody therapies have not yet been tested in lung pathology, which is remarkable because of the lung's continuous exposure to external triggers and pathogens. The risks of monoclonal antibody therapies must, however, always be kept in mind. Adverse effects including infections, cancer and autoimmune disease are all issues that need consideration before antibody treatment can be introduced [36].

The most direct way to limit the biological effects of Th17 cells would be to target production of their effector cytokines. Monoclonal antibodies against IL-17 or its receptor (IL-17R) and a soluble IL-17R have been developed for clinical application. Intravenous administration of LY2439821, an anti-IL-17 monoclonal antibody, has recently been used in a phase 1 clinical trial in rheumatoid arthritis patients and improved signs and symptoms of the disease, without significant side effects [37]. In addition, very recently two phase 2 clinical trials in which patients with psoriasis were treated with antibodies directed against IL-17 or its receptor were published [38-39]. In these studies, the antibodies were subcutaneously administered to patients with moderate-to-severe psoriasis. After 12 weeks of study, the results of both trials showed that patients receiving the antibody had marked improvements in clinical scores. Importantly, few adverse effects were observed, and few patients withdrew from the trials. Nevertheless, a 12-week follow-up period is too short to assess the safety of treatments targeting IL-17 [40]. Inhibitors of other products of Th17 cells such as IL-22 have not reached the clinical setting yet [41-42].

CAP and CF

Reviewing the data presented in this thesis, IL-17 or IL-22 mediated immunotherapy might prove beneficial especially in the treatment of pulmonary infections. Our data combined with those from experimental studies suggests that in (Gram negative) pulmonary infection, augmentation of Th17 responses through the administration of IL-17 or IL-22 may be able to decrease morbidity or mortality in these acute infections [17,43]. In the case of chronic *Ps. aeruginosa* infection in CF, neutralization of IL-17 might significantly diminish unnecessary neutrophil recruitment and the related airway damage, while the effects of IL-22 may be very important in containing the pathogen to the airway in CF. Thus, neutralization of the Th17 response in this scenario can work both ways and illustrates that we need to be well aware of the differential roles that individual Th17 cytokines can play in infections when developing immunotherapies.

Non-selective blockade of the adaptive immune system by steroids or cyclosporine does not seem effective enough in patients with severe CF or CAP. This is possibly due to the reported steroid resistance of the Th17 cell-neutrophil axis [44]. Other medication capable of dampening the innate immune system might be an alternative way to interfere

with the Th17 pathway. The best documented therapy for reducing IL-17-T cell-mediated neutrophilia is macrolide therapy, which is effectively being used in clinical practice in patients with CF [45-46]. Macrolides can inhibit neutrophil accumulation by affecting IL-17-induced CXCL-8 production [47].

Sarcoidosis and COPD

Modulating the Th17 lineage might also be a possible treatment target in sarcoidosis. Ustekinumab, a human monoclonal antibody directed against the p40 subunits of IL-12 (Th1) and IL-23 (Th17) is currently tested in patients with chronic sarcoidosis [48]. Antibodies, antagonists or receptor antagonists to IL-1β, which induces Th17 cells, and TNFα, a product of Th17 cells, are already in use for a range of autoimmune and chronic inflammatory conditions, including sarcoidosis [26,49]. Unfortunately, recurrence of immunoinflammatory disease when treatment with TNFα inhibitors is discontinued is common [50]. A combination of IL-17 and TNFα inhibitors administered either simultaneously or sequentially, might be a good alternative to better control inflammation [51-52].

Like in infectious pulmonary diseases, non-selective blockade of the adaptive immune system by steroids does not seem to be effective enough in COPD and sarcoidosis. Macrolide therapy, however, is successfully being used in patients with COPD and BOS [45-46]. In addition, although the exact mechanism is still unknown, *in vitro* studies have shown that vitamin D inhibits Th17 cells [46,53-54]. Hence, vitamin D therapy might have potential in controlling Th-17-mediated lung diseases. Clinical trials that could prove the importance of vitamin D in chronic lung diseases are currently in progress [55].

Concluding remarks

With their potential to induce a pronounced neutrophilic inflammation, which is a common feature of many pulmonary inflammatory conditions, Th17 cells are subject of great research interest in the field of pulmonary medicine.

With further exploring the role of the Th17 lineage in pulmonary diseases, one must bare in mind several aspects. First of all, it is important to realize that besides Th17 cells there are also other sources of IL-17 and IL-22, including $CD8^+$ T cells, γδ T cells, NKT cells and ILCs. Furthermore, for analysis of the Th17 lineage in pulmonary diseases, it is important to consider anatomical localization. Many studies focus on the analysis of peripheral blood or sputum. However, it is not always clear to what extend these compartments reflect what is happening within the lung. The studies in this thesis revealed great differences between cytokines and T cell cytokine profiles derived from peripheral blood or BAL, sputum or nasal lavages, or bronchial mucosal biopsies. Therefore, to make real progress in understanding lung pathology, in-depth analyses of players and processes in the affected organs are required. Moreover, location-specific differences within the organ also have to be considered, which is exemplified by the very different location of $IL\text{-}17^+$

and IL-22$^+$ cells in granuloma-containing biopsies from sarcoidosis patients (Chapter 8). Finally, and maybe most importantly, depending on stage of disease, the tissue, and the local microenvironment, IL-17 and IL-22 secreting cells appear to be able to play both beneficial and detrimental roles in lung immunity and disease. The exact balance of these opposing roles during the processes of many autoimmune, inflammatory and infectious diseases is one of the major future challenges in respiratory medicine. Therefore, it is crucial to further define this delicate balance and to uncover strategies to maximize the protective effects of the Th17 lineage cytokines while simultaneously preventing these key inflammatory mediators from causing immune-mediated host damage.

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Summary

SUMMARY

The lungs are the central organ of the respiratory tract and its enormous surface area ensures efficient gas exchange. Constant exposure to the outside world requires this organ to have a unique defense mechanism against potential harmful particles and pathogens. T lymphocytes play a central role in the immune system because of their unique antigen recognizing capacity. In reaction to an antigen, T cells may react in different ways: $CD4^+$ T helper cells produce various cytokines that direct the immune response, whereas $CD8^+$ cytotoxic T cells produce toxic granules that induce death of infected cells.

Dependent on the cytokine milieu during their activation, naïve $CD4^+$ T cells can differentiate into one of the several subsets of T helper (Th) cells. Originally, two types of $CD4^+$ T lymphocytes were described: type 1 helper T cells (Th1 cells) and type 2 helper T cells (Th2 cells). Th1 cells produce large quantities of interferon (IFN)γ and are essential for the defense against intracellular pathogens such as in tuberculosis, but may also mediate autoimmunity. Th2 cell produce mainly interleukin (IL)-4, are important in clearing parasitic infections, but also play a central role in asthma pathogenesis. Recently, the novel Th17 subset, characterized by the production of IL-17 and IL-22, was identified. The discovery of these Th17 cells, which are induced by IL-6 and TGFβ, has since provided crucial new insights into immunoregulation, host defense and the pathogenesis of many conditions, including autoimmune diseases, allergy and infection. However, most of the evidence for an essential role for Th17 cells has been generated in mouse models and data on the contribution of Th17 cells in human diseases, including pulmonary disorders, remains limited.

Inflammatory processes are central to many pulmonary diseases, including acute and chronic infections, smoking related disorders, and interstitial lung diseases. In this thesis we explored the involvement of the Th17 lineage in the context of innate and adaptive immune responses in different pulmonary diseases. **Chapter 1** provides an overview of the current knowledge on IL-17 and Th17 cells and focuses on the mucosal immunity of the lung. Because IL-17 and Th17 cells play a role in regulating neutrophilic inflammation in the lung, a potential role of the Th17 lineage seems legitimate in many different lung diseases, including community-acquired pneumonia (CAP) and cystic fibrosis (CF), but also in chronic obstructive pulmonary disease (COPD) and sarcoidosis.

Community-acquired pneumonia

CAP is an acute infection of the lung and a common and serious illness despite the use of antimicrobial therapy. The immunopathology in CAP is characterized by a strong local and systemic acute phase response in which components of the innate and adaptive immune response have been identified as important players. To determine the presence

and possible participation of the Th17 lineage in acute pulmonary infection, we studied patients with a CAP and followed these patients over time while the pneumonia resolved. Uniquely, we determined the immune response not only systemically but also locally in both non-severe and severe CAP patients.

In **Chapter 2** we quantified levels of soluble innate and adaptive cytokines in peripheral blood and bronchoalveolar lavages (BAL) of CAP patients and healthy individuals. The results of these analyses showed the importance of the systemic inflammatory response in CAP and emphasized the importance of IL-6, but also of IFNγ, in the local and systemic inflammatory response in patients with CAP.

In addition to soluble cytokine analyses, we also studied local and systemic populations of $CD4^+$ T cells in CAP patients and healthy controls, to determine the proportions of these cells that produced IL-17, IL-22 or both of these cytokines (**Chapter 3**). We evidently demonstrated that $CD4^+$ T cells with a Th17 profile are engaged in local and systemic immune responses in human pneumonia and that especially IL-17/IL-22 double positive Th17 cells are involved in the immunopathogenesis of CAP.

Cystic fibrosis

CF is a disease characterized by the excessive production of aberrantly hydrated mucus in the airways, resulting from mutations in the ion channel cystic fibrosis transmembrane conductance regulator (CFTR). This increased mucus production, blocks normal ciliary function and thereby enhances recurrent pulmonary infections with bacteria such as *Pseudomonas aeruginosa*. The chronic respiratory infections in patients with CF result in progressive tissue damage and ongoing deterioration in lung function which is the major cause of morbidity and mortality in these patients.

In **Chapter 4** we report the incidence of viral respiratory pathogens at the start of a pulmonary exacerbation in a cohort of adult CF patients. By using conventional and molecular techniques for viral isolation, we show that a respiratory virus is detected in 29% of pulmonary exacerbations in adult CF patients.

Subsequently, we studied chronic infection and inflammation in *Ps. aeruginosa*-infected adult cystic fibrosis patients. In **Chapter 5**, cytokines associated with the Th17 lineage were quantified in sputa, nasal lavages and plasma of CF patients when in pulmonary exacerbation as well as when clinically stable. Our results suggest that determination of IL-6 and IL-10 in nasal lavages of CF patients may provide a minimally invasive tool in the assessment of a pulmonary exacerbation in adult CF patients. Furthermore, systemic IL-6 levels might be used as a marker to improve prognosis predictions in adult CF patients, or be useful to define a subpopulation of patients that might benefit from anti-inflammatory therapy.

We also studied IL-17 and IL-22-positive T cells in peripheral blood and mucosal bronchial biopsies of *Ps. aeruginosa*-infected adult CF patients (**Chapter 6**). Although

proportions of IL-17 and IL-22 positive cells in the peripheral blood were not elevated, we found increased amounts of IL-17 and IL-22 positive cells in bronchial biopsies of CF patients demonstrating the importance of both cytokines in the local mucosal infection and inflammation in CF.

COPD and sarcoidosis

COPD is a chronic inflammatory disease that is marked by a progressive and irreversible airway obstruction. It is believed that the innate and adaptive immune responses that cooperate in the inflammatory process are not only localized to the lungs but also have a systemic component. In **Chapter 7** we evaluated the systemic inflammatory status in COPD patients and provided a comprehensive cytokine analysis of circulating $CD4^+$ and $CD8^+$ T cells in COPD patient groups with different disease severity. Proportions of IFNγ and TNFα producing $CD8^+$ T cells were increased in the circulation of COPD patients compared with healthy individuals and this was most evident in patients with less severe disease. We also identified a novel and interesting correlation between smoking and the proportions of IL-22 expressing $CD4^+$ memory T cells in the peripheral blood of COPD patients.

In **Chapter 8** we provide evidence for the involvement of the Th17 lineage in sarcoidosis. Sarcoidosis is a systemic inflammatory disease characterized by non-caseating granulomas in various organs with pulmonary involvement in over 90% of patients. Enhanced IL-17 expression was found in granulomas in the lungs and increased proportions of Th17 cells were present in the peripheral blood of these patients.

As discussed in **Chapter 9**, the results presented in this thesis point to an important contribution of the novel Th17 subset in various inflammatory processes that are crucially involved in different pulmonary diseases. Although our findings contribute to our understanding of Th17 cell involvement, we still have much to learn about the phenotype, function and regulation of human Th17 cells in pulmonary disease. Depending on stage of disease, localization, and the local cytokine environment, the Th17 effector cytokines appear to be able to play both beneficial and detrimental roles in lung homeostasis and disease. Therefore, the challenge lies in further defining this delicate balance and to uncover strategies and therapies to maximize the protective effects of the Th17 lineage cytokines while simultaneously preventing these key inflammatory mediators from causing immune-mediated host damage. At present, blocking antibodies to IL-17 or its receptor have been developed for clinical application and provide promising opportunities for future treatment of various pulmonary disorders.

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

De longen zijn het belangrijkste onderdeel van het ademhalingsstelsel en hebben een zeer groot inwendig oppervlak. Hierdoor is een efficiënte uitwisseling van zuurstof en koolzuur tussen de ingeademde lucht en het bloed mogelijk. Het longweefsel staat in voortdurend contact met de buitenwereld en heeft daarom verschillende verdedigingsmechanismen om iedere in principe schadelijke factor die in de inademingslucht aanwezig is, onschadelijk te maken en te verwijderen. In alle zoogdieren, inclusief de mens, speelt het afweersysteem een belangrijke rol in de verdediging tegen schadelijke organismen (pathogenen), zoals bacteriën en virussen.

Het afweersysteem bestaat uit twee delen: het aangeboren en het verworven (adaptieve) immuunsysteem. Het aangeboren immuunsysteem zorgt voor een snelle maar niet specifieke reactie op een pathogeen, het verworven immuunsysteem heeft daarentegen tijd nodig om zich te ontwikkelen maar zorgt voor een zeer specifieke afweer. T cellen zijn witte bloedcellen die van groot belang zijn in de verworven afweerreactie. De afweerreactie wordt in gang gezet door eiwitten die door T cellen en andere cellen van het immuunsysteem herkend worden, zogenaamde antigenen. Na het herkennen van een antigeen, kan een T cel op verschillende manieren reageren. De zogenaamde $CD8^+$ cytotoxische T cellen zullen geïnfecteerde cellen aanvallen en doden. Wanneer $CD4^+$ T helper cellen worden geactiveerd, maken zij mediatoren genaamd cytokinen, die helpen bij het klaren van de ontsteking. Afhankelijk van de aanwezige mediatoren in de omgeving van $CD4^+$ T helper cellen wanneer zij worden geactiveerd, differentiëren deze cellen in verschillende soorten T helper cellen. Deze verschillende soorten worden onderverdeeld aan de hand van het cytokineprofiel dat zij hebben na activering. Van oudsher worden 2 verschillende soorten $CD4^+$ T helper cellen onderscheiden: T helper 1 (Th1) en T helper 2 (Th2) cellen. Th1 cellen zijn in staat grote hoeveelheden van het cytokine interferon (IFN) γ te produceren en zijn essentieel voor de verdediging tegen intracellulaire pathogenen zoals in tuberculose, maar spelen ook een rol in auto-immuunziekten. Th2 cellen produceren voornamelijk het cytokine interleukine (IL)-4 en zijn van belang in parasitaire infecties en spelen een rol in astma. De afgelopen jaren is het gebleken dat er ook $CD4^+$ T helper cellen bestaan die cytokinen produceren die niet passen bij Th1 of Th2 cellen. Een daarvan is IL-17 en T helper cellen die wel IL-17 maar geen IFNγ en IL-4 produceren worden sindsdien geclassificeerd als Th17 cellen. Naast IL-17 produceren deze Th17 cellen ook o.a. IL-22, en zijn zij belangrijk voor de verdediging tegen infecties met extracellulaire pathogenen en wordt er gedacht dat zij een belangrijke rol spelen in de ontwikkeling van verschillende chronische ontstekingen en auto-immuunziekten. Informatie over Th17 cellen is echter voornamelijk gegenereerd met behulp van onderzoek in diermodellen en tot op heden is er slechts beperkte informatie over de bijdrage van Th17 cellen in menselijke aandoeningen.

Een ontsteking, ofwel inflammatie, is een reactie van het lichaam op beschadiging van weefsel of op prikkels van buiten. Dit kan door een microbiologische prikkel (bacteriën, virussen), door een chemische of fysische prikkel (bijv. irriterende stoffen of straling), maar dit kan ook het gevolg zijn van een auto-immuun reactie van het lichaam. In bijna alle longaandoeningen is er in meer of mindere mate sprake van lokale (in de long) of systemische (in de bloedbaan) inflammatie. Th17 cellen en gerelateerde cytokinen spelen mogelijkerwijs een rol in de aanwezige inflammatie in verschillende longaandoeningen. In **Hoofdstuk 1** wordt een overzicht gegeven van de huidige kennis over de rol van Th17 cellen en IL-17 in verschillende longziekten, zoals longontstekingen, taaislijmziekte, chronische obstructieve longziekte (COPD) en sarcoïdose.

Een longontsteking of pneumonie is een ontsteking van de longblaasjes en het omringende weefsel, vaak veroorzaakt door een infectie. Mits tijdig herkend kan een bacteriële longontsteking worden behandeld met een antibioticum. Waarom het ene individu een longontsteking ontwikkeld na contact met een pathogeen en een ander niet, is tot op heden nog onduidelijk maar de ontstekingsreactie die in het lichaam tot stand komt, heeft daar hoogstwaarschijnlijk mee te maken. In **Hoofdstuk 2 en 3** onderzoeken wij of Th17 cellen en Th17-gerelateerde cytokinen een rol spelen bij de ontstekingsreactie die plaatsvindt in longen en in het bloed van patiënten met een longontsteking. Onze resultaten laten zien dat er een rol is weggelegd voor Th17 cellen aangezien $CD4^+$ T helper cellen die IL-17, IL-22 of beide cytokinen produceren, in verhoogde mate aanwezig zijn in de longen en bloedbaan van deze patiënten. Ook tonen wij aan dat de systemische reactie van het lichaam in patiënten met een longontsteking van zeer groot belang is en dat naast Th17 cellen, IL-6 en IFNγ belangrijke cytokinen zijn in deze reactie.

Taaislijmziekte (cystische fibrose; CF) is een ernstige aangeboren aandoening, waarbij slijm dat op diverse plaatsen in het lichaam wordt afgescheiden, abnormaal taai is. Als gevolg daarvan ontstaan slijmophopingen in diverse organen welke in de longen leiden tot infecties. Deze infecties resulteren in littekenvorming en een voortschrijdend functieverlies van de longen. In **Hoofdstuk 4** rapporteren wij de incidentie van virale verwekkers in acute luchtweginfecties (exacerbaties) in volwassen CF patiënten. In **Hoofdstuk 5** analyseren wij verschillende Th17-gerelateerde cytokinen in sputum, neusspoelingen en bloedplasma van CF patiënten wanneer zij in een exacerbatie verkeren evenals wanneer zij klinisch stabiel zijn. De resultaten beschreven in dit hoofdstuk suggereren dat het bepalen van IL-6 en IL-10 in neusspoelingen van CF patiënten kan bijdragen aan het vaststellen van de aanwezigheid van een exacerbatie bij deze patiënten. Bovendien zouden systemische IL-6 waarden in de toekomst wellicht kunnen worden gebruikt om betere voorspellingen te doen over de prognose van CF patiënten. Ook zouden er met behulp van deze relatief eenvoudige bepaling groepen patiënten kunnen worden

geïdentificeerd die baat zouden kunnen hebben bij het gebruik van anti-inflammatoïre medicijnen.

In **Hoofdstuk 6** bestuderen we IL-17 en IL-22 positieve T cellen in het bloed en in longbiopten van CF patiënten en tonen aan dat deze cytokinen belangrijk zijn in de lokale infectie en inflammatie die aanwezig is in volwassen CF patiënten.

COPD is een verzamelnaam voor onder meer de longziekten chronische bronchitis en longemfyseem. Bij deze aandoening zitten de longen vol met kleine ontstekingen die blijvende schade veroorzaken. Het is aangetoond dat zowel het aangeboren als het verworven immuunsysteem betrokken is bij deze aandoening, en dat de inflammatie zich niet beperkt tot de longen maar ook een systemische component omvat. In **Hoofdstuk 7** beschrijven we de cytokineprofielen van circulerende $CD4^+$ en $CD8^+$ T cellen in het bloed van COPD patiënten. De proporties van IFNγ en tumor necrosis factor (TNF)α producerende $CD8^+$ T cellen zijn verhoogd in het bloed van COPD patiënten wanneer we dit vergelijken met gezonde individuen. Opvallend was dat dit verschil het best zichtbaar is in de groep COPD patiënten met de minst ernstige vorm van deze ziekte. Ook tonen we in dit hoofdstuk aan dat er een correlatie is tussen roken en de hoeveelheid IL-22 producerende $CD4^+$ T helper cellen in het bloed van COPD patiënten.

In **Hoofdstuk 8** onderzoeken we de rol van Th17 cellen in de inflammatie bij sarcoïdose patiënten. Sarcoïdose is een relatief zeldzame aandoening die verschillende organen van het lichaam kan aantasten en waarbij bepaalde ontstekingen, zogenaamde granulomen, gevonden kunnen worden in o.a. de huid, spieren en ogen. In meer dan 90% van de gevallen zijn de longen bij het ziekteproces betrokken. De resultaten in dit hoofdstuk laten de aanwezigheid en lokalisatie van Th17 cellen in het bloed en longen van sarcoïdose patiënten zien. In vergelijking met gezonde individuen hebben sarcoïdose patiënten meer Th17 cellen in hun bloed, en ook in longspoelsel en rondom granulomen in de longen zijn meer IL-17 producerende T cellen aanwezig.

Concluderend, Th17 cellen en Th17 gerelateerde cytokinen zijn betrokken bij de longinflammatie die aanwezig is in verschillende longaandoeningen. Ondanks dat de resultaten gepresenteerd in dit proefschrift bijdragen aan onze kennis over de rol van de Th17 familie in verschillende longaandoeningen, is meer onderzoek noodzakelijk om de exacte functie en regulatie van deze cellen te identificeren. Afhankelijk van het stadium van de ziekte, de lokalisatie en het aanwezige cytokinemilieu, lijken Th17 cellen zowel een beschermende als een schadelijke rol te kunnen spelen. Het doel van vervolgonderzoek is dan ook om deze delicate balans nader te definiëren en om therapieën te ontwikkelen die de beschermende effecten van deze cellen verstreken en tegelijkertijd de schadelijke effecten remmen. Momenteel worden onderzoeken verricht met blokkerende antilichamen tegen

IL-17 waarbij de eerste resultaten veelbelovend zijn en wellicht in de toekomst kunnen worden ingezet in de behandeling van verschillende longaandoeningen.

About the author

ABOUT THE AUTHOR

Marthe Paats was born on September 6th 1982 in Ede. Six weeks after her birthday she made her first great journey and returned with us to Mongu in Zambia where she spent the first few years of her life. After returning from Zambia we settled in Enschede where she completed high school (Gymnasium, bilingual stream) at the Stedelijk Lyceum in 2000. The same year she started medical school at the Rijksuniversiteit Groningen. During her study she did a research internship in paediatric pulmonology at the University of Oslo under supervision of Prof. Dr. E.J. Duiverman and Prof. Dr. K.H. Carlsen. After finishing her regular clinical internships in 2006, she did an additional rotation at Same District Hospital in Tanzania. We are very pleased that she did not only study medicine, but also found the time to represent the interests of the medical students at the RUG as president of the Medical Student Association and student representative in the Alumni Association.

In 2007 she transferred to the Erasmus MC Rotterdam, where she started as a resident (ANIOS) at the department of pulmonary medicine. Supervised by Prof. Dr. R.W. Hendriks and Prof. Dr. H.C. Hoogsteden she started her PhD research in 2008 at the research laboratory of the department of pulmonary medicine at the Erasmus MC, resulting in the present thesis. In July 2012 she started her pulmonary medicine training with two years of internal medicine in the Sint Franciscus Gasthuis in Rotterdam (supervisor Dr. A.P. Rietveld). The final part of the training will be undertaken at the department of pulmonary medicine in the Erasmus MC in Rotterdam (Head of department: Prof. Dr. H.C. Hoogsteden).

We are very proud of the result of the research that lies in front of you.

Katinka Jansen and Fred Paats
Parents of Marthe

PhD portfolio

PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: M.S. (Marthe) Paats
Erasmus MC Department: Pulmonary Medicine
Research School: Molecular Medicine

PhD period: 2008-2012
Promotors: Prof.dr. R.W. Hendriks
Prof.dr. H.C. Hoogsteden
Copromotor: Dr. M.M. van der Eerden

1. PhD training	Year
General Courses	
▪ Photoshop and Illustrator CS5	2011
▪ PubMed and EndNote	2010
▪ Statistics and survival analysis	2010
▪ Biomedical English Writing and Communication	2010
▪ SPSS	2009
In-depth seminars, workshops and courses	
▪ Masterclass David L. Woodland, AMC Amsterdam	2011
▪ Research Seminar and workshops on Molecular and Cellular Aspects of Chronic Lung Disease, Erasmus MC Rotterdam	2009
▪ Good Clinical Practice (GCP) / Basiscursus regelgeving en organisatie voor klinisch onderzoekers (BROK), Erasmus MC Rotterdam	2009
▪ Molecular Immunology course, Molecular Medicine Postgraduate School, Erasmus MC Rotterdam	2009
▪ Biomedical Research Techniques VII, Molecular Medicine Postgraduate School, Erasmus MC Rotterdam	2008
▪ 3rd Symposium & Masterclasses on Mucosal Immunology, Erasmus MC Rotterdam	2008
▪ 2nd Symposium & Workshops on Molecular Microbiology of Infectious Diseases, Erasmus MC Rotterdam	2008
▪ Periodic presentations at the Department of Pulmonary Medicine and collaborating departments	2008-2012
(Inter)national scientific presentations	
▪ American Thoracic Society (ATS) International Conference, San Francisco, USA (poster)	2012
▪ 16th Molecular Medicine Day, Rotterdam, the Netherlands (poster)	2012
▪ European Respiratory Society (ERS) Annual Conference, Amsterdam, the Netherlands (poster discussion)	2011
▪ Masterclass on Respiratory Infections, Barcelona, Spain (oral)	2011
▪ Netherlands Respiratory Society (NRS) 3rd Spring Meeting, Utrecht, the Netherlands (poster)	2011
▪ ERS Lung Science Conference, Estoril, Portugal (poster)	2011
▪ 15th Molecular Medicine Day, Rotterdam, the Netherlands (poster)	2011
▪ ERS Annual Conference, Barcelona, Spain (oral)	2010

▪ ATS International Conference, New Orleans, USA (poster discussion)	2010
▪ NRS 2nd Spring Meeting, Driebergen, the Netherlands (poster)	2010
▪ 14th Molecular Medicine Day, Rotterdam, the Netherlands (poster)	2010
▪ Annual Meeting Dutch Society for Immunology (NVVI), Noordwijkerhout, the Netherlands (poster)	2009
(Inter)national conferences	
▪ 4th Mucosal Immunology Symposium, Rotterdam, the Netherlands	2011
▪ 2nd NRS Young Investigators Symposium, Amersfoort, the Netherlands	2010
▪ NRS 1st Spring Meeting, Arnhem, the Netherlands	2009
▪ ERS Annual Conference, Vienna, Austria	2009
▪ Keystone Meeting "Th17 cells in Health and Disease", Vancouver, Canada	2009
▪ Symposium "New Perspectives on Severe Asthma", Erasmus MC, Rotterdam, the Netherlands	2008
▪ Annual Meeting NVVI, Noordwijkerhout, the Netherlands	2008
2. Student coaching and teaching	
▪ Study courses for fifth- and sixth-grade VWO students. Erasmus University, Faculty of Medicine	2009-2012
▪ Journal Club courses for first-year medical students. Erasmus University, Faculty of Medicine	2009-2012
▪ Supervising master of science-student. Molecular Medicine Postgraduate School, Erasmus MC Rotterdam	2011
3. Awards	
▪ Best Poster Presentation Award, 16th Molecular Medicine Day, Rotterdam, the Netherlands	2012
▪ Best Abstract Award, ERS Lung Science Conference, Estoril, Portugal	2011
▪ ATS Travel Award, New Orleans, USA	2010

Dankwoord

DANKWOORD

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