The Interleukin-23 axis and innate immunity in the airways

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Cover illustration: Macrophages containing IL-22, by Marit Stockfelt

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To Leo

Abstract

The Interleukin-23 (IL-23) axis is a communication system that integrates innate and adaptive immunity. When triggered by microbial stimuli, antigen presenting cells can secrete the cytokine IL-23, leading to the production of IL-17 and IL-22. These cytokines facilitate the recruitment of neutrophils that can eliminate microbes, but may also cause epithelial damage through extensive inflammation. At the same time, the IL-23 axis protects the epithelium through the production of antimicrobial peptides.

The protective role of the IL-23 axis for local epithelial defence led us to ask whether inflammatory cells of the airway epithelium can produce IL-22, a cytokine associated with the IL-23 axis. We showed that airway macrophages responded to IL-23 and a bacterial stimulus with the secretion of IL-22. This constitutes a local and accessible source of IL-22 during activation of the innate arm of pulmonary host defence.

The IL-23 axis leads to neutrophil recruitment which risks damaging epithelial tissue. Therefore, a strict regulation of the production of these cytokines is necessary. We showed that IL-17 exerts a negative feedback effect on IL-23, thus decreasing its own production. Further, the IL-17 receptor was present on macrophages demonstrating a prerequisite to this response.

The airway epithelium is protected by antimicrobial peptides functioning as innate antibiotics, several of which are regulated by the IL-23 axis. We demonstrated the expression of two antimicrobial peptides, calprotectin and LL-37, in healthy human airways. Of these, only LL-37 was induced by the gram-negative bacterial stimulus endotoxin in this setting. This demonstrates the involvement of LL-37 in the innate immune response against gram-negative bacteria.

Finally, we quantified cytokines associated with the IL-23 axis in smokers with and without chronic obstructive pulmonary disease. Airway IL-17 did not differ significantly between the groups, but plasma IL-22 was increased in smokers, demonstrating a smoking induced systemic effect on the IL-23 axis. Neutrophils in the airways displayed signs of activation and could be further activated by TNFα, indicating that the local microenvironment can affect neutrophil activation.

Populärvetenskaplig sammanfattning

Inflammation är grunden i vårt försvar mot bakterier och virus, men för mycket inflammation leder till barriärskada och sjukdom. Signalmolekylerna i IL-23-axeln knyter samman de medfödda och de adaptiva delarna av immunförsvaret. Vid till exempel en bakteriell lunginflammation kan IL-23 bildas av antigenpresenterande celler. Detta leder till utsöndring av signalmolekylen IL-17 som kallar till sig neutrofiler. Dessa är immunceller som kan döda bakterier, men samtidigt riskerar att orsaka kraftig inflammation som kan skada kroppens egna barriärer. Samtidigt bildas IL-22, som skyddar barriärcellerna, och får dem att bilda kroppsegna antibiotika. Vi visade att två sådana kroppsegna antibiotika, calprotectin och LL-37, förekom i lungsköljvätska hos friska individer, och att det bakteriella ämnet endotoxin ökade förekomsten av LL-37.

Den skyddande rollen av IL-23-axeln för barriärfunktionen gjorde att vi ville undersöka om lokala immunceller kan delta i produktionen av dessa signalmolekyler. Vi visade att lungans makrofager reagerade på IL-23 och ett bakteriellt ämne med att bilda IL-22. Vi förstår därför att celler på lungans yta lokalt kan bilda denna barriärskyddande cytokin.

Balansen mellan produktiv inflammation som hindrar infektion, och destruktiv inflammation som skadar vävnaden upprätthålls av olika regulatoriska system. Vi visade att det i IL-23-axeln finns en negativ feedback-mekanism, där IL-23 uppreglerar produktion av IL-17, som i sin tur nedreglerar produktion av IL-23. Detta kan tänkas vara ett skydd mot en alltför kraftig neutrofil inflammation i kroppen.

Slutligen studerade vi IL-23-axeln hos rökare med och utan kronisk obstruktiv lungsjukdom, KOL. Eftersom denna sjukdom kan innebära kraftig neutrofil inflammation ter det sig sannolikt att IL-23-axeln är inblandad. Vi såg en tendens till minskat IL-17 hos rökare med och utan KOL, och IL-22 var ökat i blodet hos rökare, vilket visar att rökning kan påverka IL-23-axeln. Vidare såg vi att luftvägs-neutrofiler var aktiverade jämfört med neutrofiler i blod, och kunde aktiveras ytterligare av cytokinen TNFα. Detta tyder på att neutrofilernas aktivering kan påverkas av den lokala mikromiljön i luftvägarna.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Hansson M, Silverpil E, Lindén A, Glader P. Interleukin-22 produced by alveolar macrophages during activation of the innate immune response. Inflammation Research 2013. Jun;62(6):561-9.

II. Silverpil E, Wright AK, Hansson M, Jirholt P, Henningsson L, Smith ME, Gordon SB, Iwakura Y, Gjertsson I, Glader P, Lindén A. Negative feedback on IL-23 exerted by IL-17A during pulmonary inflammation. Innate Immunity 2013 Oct; 19(5):479-92.

III. Smith ME*, Stockfelt M*, Tengvall S, Bergman P, Lindén A, Qvarfordt I. Endotoxin Exposure Increases LL-37 – but Not Calprotectin – in Healthy Human Airways. Journal of Innate Immunity. 2017. *Joint first authorship.

IV. Stockfelt M, Christenson K, Andersson A, Björkman L, Padra M, Sun J, Levänen B, Ganguly K, Asgeirsdottir H, Qvarfordt I, Bylund J and Lindén A. Neutrophil activation and associated cytokines before and after extravasation into the airways of smokers with and without COPD. Manuscript in preparation.

CONTENT

ABBREVIATIONS

AMP	Antimicrobial peptide
BAL	Bronchoalveolar lavage
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
IL	Interleukin
GM-CSF	Granulocyte macrophage colony-stimulating factor
FEV_1	Forced expiratory volume in 1 s
FVC	Forced vital capacity
LPS	Lipopolysaccharide, endotoxin
MHC	Major histocompatibility complex
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
PBS	Phosphate buffered saline
Th	T helper
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha

INTRODUCTION

The airway epithelium – setting the stage

The airways function as a filter for our environment. The inhaled air travels through the conductive airways to its destination, the alveoli [1] where gas exchange occurs (Figure 1). Most airborne microbes are prevented from causing infection by the mucus covered epithelium, the barrier cell layer that coats the airways. The epithelium makes up around 75 square meters of surface area, providing a large area for gas exchange [2].

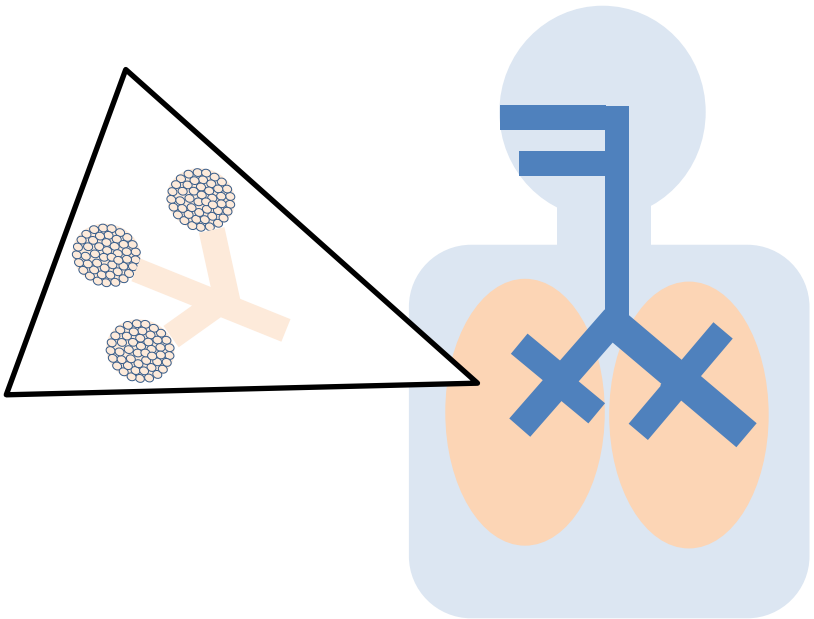

Figure 1. Schematic picture of the human airways. Air enters the nose and mouth and travels through the conducting airways (in blue) branching into the bronchioles and finally reaching the alveoli (magnified) where gas exchange occurs.

The conductive airways are lined with a pseudostratified columnar epithelium, dominated by ciliated cells (Figure 2). Secretory cells produce mucus. The mucus layer prevents adherence of bacteria to the epithelium, and clears the bacteria upwards, driven by ciliated epithelial cells. Immune cells such as dendritic cells are interspersed, sampling antigens from microbes in the

airways. The columnar epithelium is gradually transitioned, and in the terminal bronchioles and alveoli, the epithelium is cuboidal, and ciliated cells are absent [3]. The alveoli have thin epithelial walls that enable gas exchange, but this also makes them vulnerable to microbe assaults.

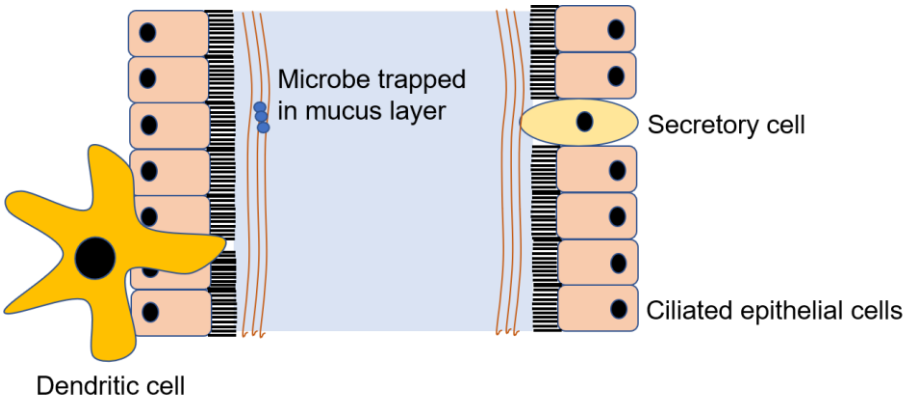

Figure 2. The airway epithelium in a bronchiole. Ciliated epithelial cells coat the airways, with secretory cells producing mucus. Microbes can be trapped in the mucus layer, and dendritic cells are interspersed, sampling their antigens.

Airway immunity and the IL-23 axis

The airways are naturally exposed to large numbers of microbes, and the airway epithelium and mucosal layer hinders most microbes from entering the tissue. A productive immune response is based upon the recognition of danger signals on microbes by immune cells, the communication of these signals to other immune cells and the elimination of the microbes. A balance between productive and destructive inflammation is necessary, hindering infection while not unnecessarily harming the tissue.

If a microbe crosses the epithelial cell barrier, it meets antigen presenting cells of the innate immunity, such as dendritic cells and alveolar macrophages. The action of these cells is dependent on the recognition of microbes through pattern recognition receptors. These recognize a relatively small number of conserved microbial patterns.

If this is insufficient, signals are communicated to the adaptive immune system. For adaptive immunity, specificity is key, and the T and B cell

receptors have the potential to recognize almost all possible antigenic structures that are not tolerated or belong to the self [4]. An airway dendritic cell that has met a microbe can travel to the lymph node and present its antigens to naïve T cells. This polarises the T cell in different directions depending upon the antigen, co-stimulation and cytokine production. This interaction decides the nature of the subsequent immune response to the microbe [5]. The adaptive system then uses and directs the effectors of the innate system to eliminate the microbe. Traditionally, innate and adaptive immunity are described as separate systems, but in reality, they are closely inter-connected.

The IL-23 axis is a signalling system that integrates innate and adaptive immunity. The production of IL-23 originates in the innate immune system. Antigen presenting cells that sense danger signals from microbes can produce IL-23. This induce the production of IL-17 and IL-22 from adaptive T helper cells and tissue macrophages. The cytokines IL-17 and IL-22 signals to the epithelium. This leads to the production of neutrophil mobilising cytokines such as GM-CSF and IL-8, as well as the production of antimicrobial peptides and wound healing. The result is neutrophil recruitment that leads to the killing of bacteria, and upregulation of the epithelial defence.

Recognizing danger

Pattern recognition receptors – sensing danger

Microbes express conserved molecular patterns, absent from human cells. These function as danger signals that our immune system has evolved to recognize through pattern recognition receptors [6]. Recognition of a microbe through a pattern recognition receptor can activate the antigen presenting cells, so that they can express the cytokines and co-stimulatory molecules necessary to activate the adaptive immunity [7]. One important group of pattern recognition receptors is the Toll-like receptors (TLRs). These include TLR4 that recognizes endotoxin expressed on the gram-negative bacterial wall, and TLR2 that recognizes peptidoglycan, which makes up most of the gram positive bacterial cell wall.

When antigen presenting cells are stimulated through their pattern recognition receptors they become activated and can secrete IL-23 [8]. Signals through both TLR2 [8] and TLR4 [9] stimulate the secretion of IL-23, and after only 1,5 hours of challenge with endotoxin or peptidoglycan, the IL-23 concentration is increased in bronchoalveolar lavage (BAL) fluid and lung tissue [10]. The secretion of IL-23 is increased by the signalling through several other pattern recognition receptors as well, such as TLR5, 7 and 8 [11], NOD2 [12] and the dectin-1 receptor [13]. Thus, the expression of IL-23 is triggered by the binding of microbes to pattern recognition receptors on antigen presenting cells.

Macrophages – guarding the gate

Alveolar macrophages constitute the most numerous type of antigen presenting cell during steady state of the airways [14]. The recognition of microbes through pattern recognition receptors leads to the production of proinflammatory chemokines and cytokines such as IL-1, IL-6, IL-8 and TNFα, which lead to the recruitment of other immune cells to the tissue.

The macrophage can directly phagocyte microbes and present their antigens to other immune cells. It is an effective killing machine and contain several dangerous components that can harm the tissues. When activated by T helper type 1 cells (Th1), the macrophage becomes effective at killing intracellular

bacteria [15]. Macrophages can produce IL-23, and we show in this thesis that they can also respond to IL-23 and bacterial stimuli with the secretion of the IL-23 axis cytokine IL-22 (paper I). Several studies have shown that macrophages from different tissues can also produce IL-17 [16-19].

Dendritic cells – presenting danger

Antigen presenting cells such as dendritic cells are constantly sampling antigens in the airway lumen and presenting them on major histocompatibility complex (MHC)-II molecules. When the dendritic cell is activated through pattern recognition receptors, it travels to a lymph node to present the antigen to, and cross-talk with, naïve T cells. To become activated a T cell needs several signals from the antigen presenting cell (Figure 3).

First, the T cell must have a T cell receptor specific for the same antigen that is expressed on the MHC-II molecules on the dendritic cell surface. Second, co-stimulatory molecules must be expressed on the dendritic cell. CD80 or CD86 on the dendritic cell interacts with CD28 on the T cell. For optimal proliferation, differentiation and expansion of the T cell, cytokine signals are needed from the dendritic cell [20]. These cytokine signals polarise subsequent immune responses, and when the dendritic cell secretes IL-23, this contributes to the T helper 17 (Th17) cell population being formed [21].

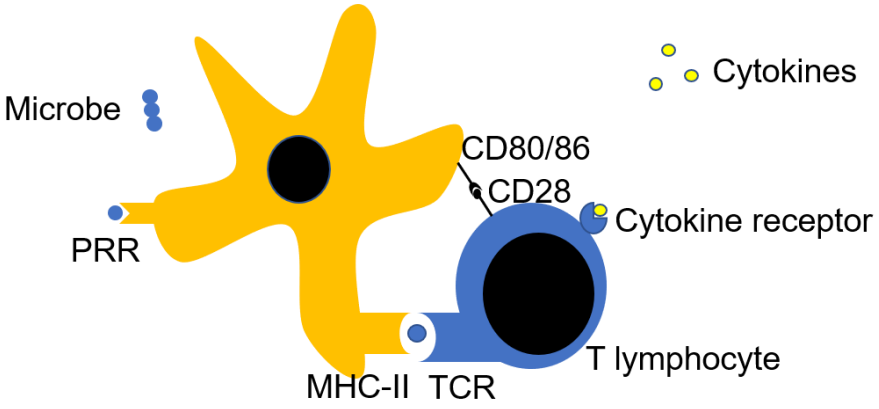

Figure 3. Activation of a naïve T helper cell by a dendritic cell. Microbial patterns are presented on the MHC-II molecule of the dendritic cell and recognized by the T cell receptor. Then, co-stimulation with CD80/86 and CD28 is needed. Then cytokine signals from the dendritic cell are needed for optimal proliferation, differentiation and expansion. PRR, pattern recognition receptor. TCR, T cell receptor.

Once the T cell is activated, it starts to secrete IL-2 that binds to receptors on its own surface. This drives clonal expansion and during this time, the T cell surface adhesion molecules are changed. The T cell then circulates through the blood and into the tissue, guided by chemokines and chemokine receptors expressed on the endothelium. It is now ready to secrete cytokines that polarise subsequent immune responses [5].

T helper cells – polarising responses

Fundamental to understanding adaptive immunity is the polarisation of immune responses. When the dendritic cell presents its antigen to the naïve T cell, different classes of T helper cells are generated. At least four T helper (Th) subtypes are defined, Th1, Th2, Th17 and regulatory T cells. While Th1 and Th2 cells have been defined for over 20 years [22, 23], the Th17 cell population was described in 2005 and shown to possess many of the autoimmune properties previously attributed to Th1-cells [24-26].

The different types of T helper cell can lead the immune response in four different broad directions. Simplified, Th1 cells take part in the cell mediate defence and activate macrophages and cytotoxic T cells to kill intracellular microbes through the secretion of IFNγ. Th2 cells are part of the humoral defence and activate B cells and eosinophils to produce neutralising antibodies to kill extracellular parasites through the secretion of IL-4. Th17 cells recruit neutrophils to tissue to kill extracellular bacteria and protects the epithelium through the production of IL-17 and IL-22. Finally, regulatory T cells regulate immune responses through the production of TGFβ and IL-10 [27] (Figure 4).

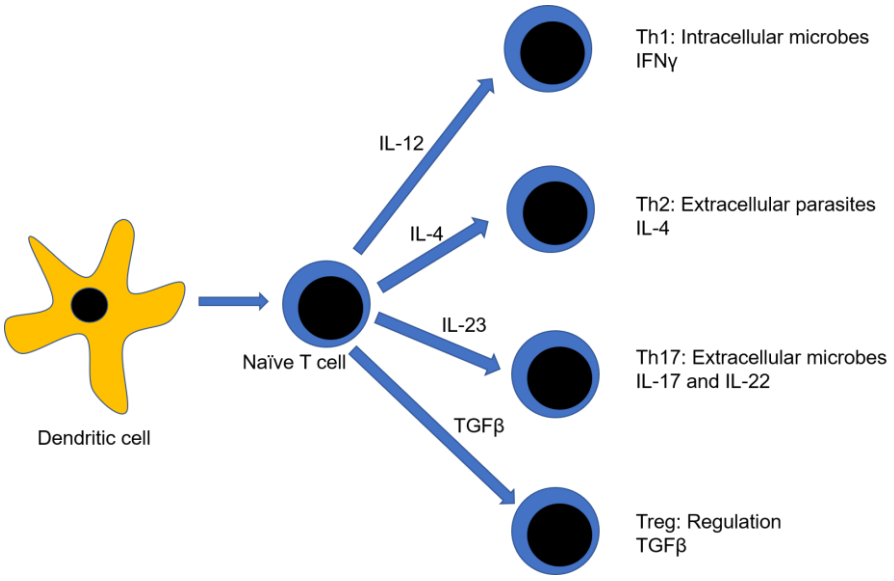

Figure 4. Differentiation and stabilisation of naïve T cells into T helper cells through the cytokine production from dendritic cells.

Once the T helper cell has differentiated, it will migrate back to inflamed tissue following chemokine gradients and direct the local immune response through its own cytokine production.

The Th17 cell – changing paradigms

As described, the Th17 cell polarises subsequent immune responses towards the defence against extracellular bacteria through the production of IL-17 and IL-22. Several studies have been performed to elucidate the cytokines necessary for Th17 differentiation and the role of IL-23. In mice, IL-6 and TGFβ seem to be necessary for Th17 cell differentiation [28, 29]. In humans IL-6, TGFβ, IL-21 and IL-1β can be involved in the process [30]. The role of IL-23 seems to be to control the expansion and stability of Th17 cells once they have differentiated [21, 31, 32]. Stimulating memory T cells with IL-23 induces IL-17 production [33] arguing that IL-23 plays yet another important role once the Th17 phenotype is set.

The Th17 phenotype (Figure 5) includes the chemokine ligands CCR4 and CCR6, that enables their recruitment to tissue. Further, the IL-23 receptor [34] and the surface molecule CD161 which is the human orthologue of NK1.1 [35]. They also express the transcription factor RORC and secrete the cytokines IL-17, IL-17F, IL-22 and IL-21 [30] as well as IL-26 [36].

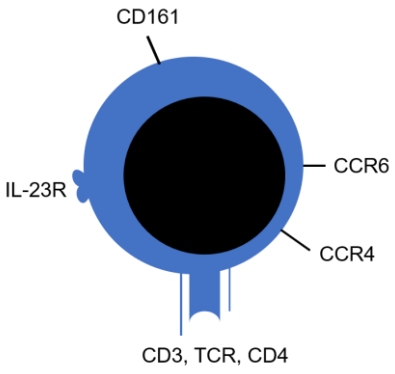

Figure 5. A Th17 cell, expressing the T cell specific molecules CD3, CD4 and the T cell receptor (TCR), as well as the receptor for IL-23 (IL-23R), CD161 and the chemokine ligands CCR6 and CCR4.

The discovery of the Th17 cell led to a major revision of the Th1-Th2 paradigm. In an elegant study, IL-23 and not IL-12 was shown to be responsible for the mouse model disease experimental autoimmune encephalomyelitis (EAE) [37], and the same was demonstrated for collagen induced arthritis (CIA) [38]. After this, it was discovered that IL-23 could promote IL-17 production from CD4+ T cells, that Th17 cells could promote autoimmunity and that the Th17 cells are a cell subset distinct from the Th1 and Th2 cells [39]. These discoveries changed the Th1-Th2 paradigm and since then, efforts have been made to elucidate the roles of these T helper cell subsets and their signature cytokines in different diseases.

Signalling danger

Cytokines – communication, communication

No cell is an island. They function in the chemical microenvironment of the extracellular fluid that surrounds them. The chemicals originate from nearby or even the same cell, or are transported in the blood stream as endocrine hormones. The receptors on the cellular surface and inside the cell will determine to which degree the cell will respond to the chemicals in its surroundings; greatly, somewhat, or not at all, and cells can change their phenotype and functional characteristics depending on the composition of the cytokine microenvironment [40].

Cytokines are an important communication tool used by immune cells. These are loosely defined as small soluble proteins produced by one cell and acting on another. Monocytes, macrophages, and dendritic cells are prominent cytokine producers, but almost every cell in the body can produce and respond to cytokines. They can work in synergy, or inhibit each other in complex manners, depending on the composition of the chemical microenvironment and the receptor flora of the cell. When a cytokine binds its receptor, intracellular signalling pathways are activated affecting gene transcription and thereby protein synthesis [41].

The IL-23 axis – a finger in every pie

The production of IL-23 originates in the innate immune system triggered by the recognition by a microbe by pattern recognition receptors. Thus, antigen presenting cells that encounter microbes can produce IL-23, that induce the production of IL-17 and IL-22 [42].

IL-17 and IL-22 are produced by adaptive Th17 cells, but they can also be produced by other lymphocyte subtypes from both adaptive and innate immune defences: CD4+ and CD8+ T cells NK-cells, NKT-cells and innate lymphoid cells [43]. The IL-23 receptor is expressed on numerous cell types such as T cells, NK-cells, NKT-cells, eosinophils, dendritic cells and macrophages, which makes it possible for these cells to respond to this cytokine [44].

IL-17 and IL-22 affect structural cells such as epithelial cells and fibroblasts inducing further cytokine and chemokine production. A main effect of IL-17 is the induction of neutrophil mobilising cytokines [45, 46] while IL-22 induces the production of antimicrobial peptides [47-49] (Figure 6). However, the two cytokines have both overlapping and additive effects.

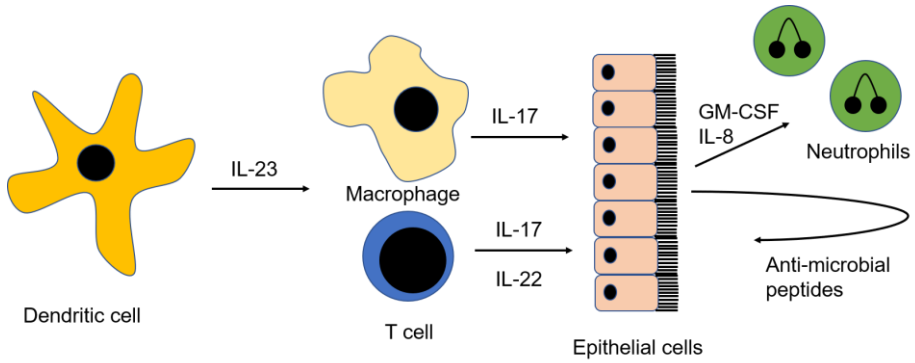

Figure 6. The IL-23 axis. The expression of IL-23 induces production of IL-17 and IL-22 which in turn stimulates epithelial cells to produce cytokines such as GM-CSF and IL-8, as well as antimicrobial peptides. The net effect includes the recruitment of neutrophils to infected tissue and increased epithelial defence.

We define the IL-23 axis as IL-23 and the cytokines that are produced and released as a direct response to IL-23. Here, we focus on IL-17 and IL-22 but other cytokines triggered by IL-23 include IL-17F [50] and IL-26 [51]. Through the action of these cytokines and their downstream molecules, the IL-23 axis exerts effect on both the innate and the adaptive immune system.

IL-17 and family

The IL-17 family consists of several cytokines of which IL-17 (synonymous with IL-17A) is the most well-defined. IL-17 is secreted by immune cells but acts primarily on structural cells such as airway epithelial cells [45, 52]. It signals through the dimer receptor formed by the IL-17 receptor A and C [53]. IL-17 induces molecules that can be roughly divided into two different groups: neutrophil mobilising cytokines and antimicrobial peptides. There are also other members of the IL-17 family, namely IL-17 B, C, D, E and F. Of these, IL-17F is most similar to IL-17, and share several functions. IL-17 and IL-17F can form both homodimers and heterodimers. The highest activity comes from

IL-17 A/A, followed by A/F and F/F as measured when inducing cytokines and chemokines such as IL-6, CXCL-1, GROα and IL-8 [54].

IL-22 – barrier protection

IL-22 is a cytokine with both pro- and anti-inflammatory effects. It affects epithelial cells and fibroblasts in organs such as the skin, airway and the intestines [47, 48, 55]. The IL-22 receptor is expressed on human bronchial epithelial cells, and when these cells are stimulated with IL-22, it leads to the upregulation of antimicrobial peptides and the strengthening of the epithelial barrier [56]. A role in wound healing has been demonstrated for IL-22 as well. In keratinocyte [49] and intestinal epithelial [57] cell lines, the addition of IL-22 increase migration to sterile wounds, and wounds in diabetic mice healed faster with IL-22 treatment [58].

The IL-23 axis – working together

The cytokines in the IL-23 axis complement each other: while the main net effect of IL-17 seem to be to recruit neutrophils to the infected tissue, IL-22 protects the epithelium through promotion of wound healing and production of antimicrobial peptides. The effects of can be additive as well. IL-17 and IL-22 can together, or together with vitamin D3, increase mRNA and protein expression of the precursor to LL-37 in epithelial cells [59, 60]. In primary human keratinocytes IL-22 exerted synergistic effects together with IL-17 or IL-17F in inducing antimicrobial peptides such as human β-defensin 2 and calprotectin [61]. In the airway of mice, IL-22 and IL-17 have synergic effects in the production of G-CSF, IL-6 and mRNA for β-defensin 2 [47] and the combination of IL-22 and IL-17 induce matrix metalloproteinases [62]. Whether IL-22 exerts proinflammatory effects or not may depend upon the presence of IL-17 [63] and in airway inflammation caused by bleomycin in mice, the presence of IL-17 decides whether IL-22 exerts a pro- or anti-inflammatory effect [64].

Responding to danger

Neutrophils – the rescue patrol

The cytokines associated with the IL-23 axis lead to two different ways to defend against extracellular bacteria. While the main effect of IL-22 seems to be to induce antimicrobial peptides and epithelial protection, IL-17 is instrumental in promoting neutrophil infiltration. Neutrophils are short lived phagocytes circulating in the blood waiting for a signal to enter the tissue [65]. During inflammatory conditions their life span is prolonged [66] and in inflamed tissue, they soon become the most common phagocyte. In tissue, they function as effector cells that rapidly eliminate microbes and clear debris.

IL-17 and IL-22 regulate several cytokines and chemokines responsible for the mobilisation of neutrophils, and IL-17 is shown to stimulate neutrophilic infiltration into the airways [67]. These cytokines lead to the production of GM-CSF which stimulate neutrophil production in the bone marrow increasing the numbers circulating in blood [45, 68]. They induce the production of the chemokines IL-8 and CXCL-2, which attract neutrophils to infected tissue [46, 64]. IL-17 and IL-22 also stimulate other neutrophil mobilising cytokines such as IL-1 and IL-6 [68].

For neutrophils to exert their effects in infected tissue, they must migrate from blood to tissue. Neutrophil migration is a multistep process. The migration can be induced by tissue resident macrophages that secrete cytokines to activate endothelial cells, inducing surface molecules such as selectins and integrins. Neutrophil tertiary granules as well as secretory vesicles contain membrane bound molecules needed for migration such as CD11b and CD16. The granules fuse with the plasma membrane, leaving the molecules exposed on the surface [69].

The first step in neutrophil migration is tethering and rolling. Endothelial cells express pre-stored P-selectin, E-selectin and L-selectin ligands which bind weakly to ligands such as CD62L (L-selectin), PSGL-1, GlyCAM-1 and MAdCAM-1 on the neutrophil surface. The neutrophil rolls, binds weakly, falls off and binds again, now moving slower along the endothelium. CD62L is cleaved off in this process by surface bound proteolytic enzymes such as ADAM-17 [70]. In the next step, CD11b (CR3) is upregulated on the neutrophil surface through degranulation [71]. A firmer adhesion is

established as ICAM-1 on the endothelial cells binds CD11a or CD11b on the neutrophil. Then the neutrophil moves into the tissue through diapedesis and migrates through the tissue following chemokine gradients such as IL-8 [69] (Figure 7).

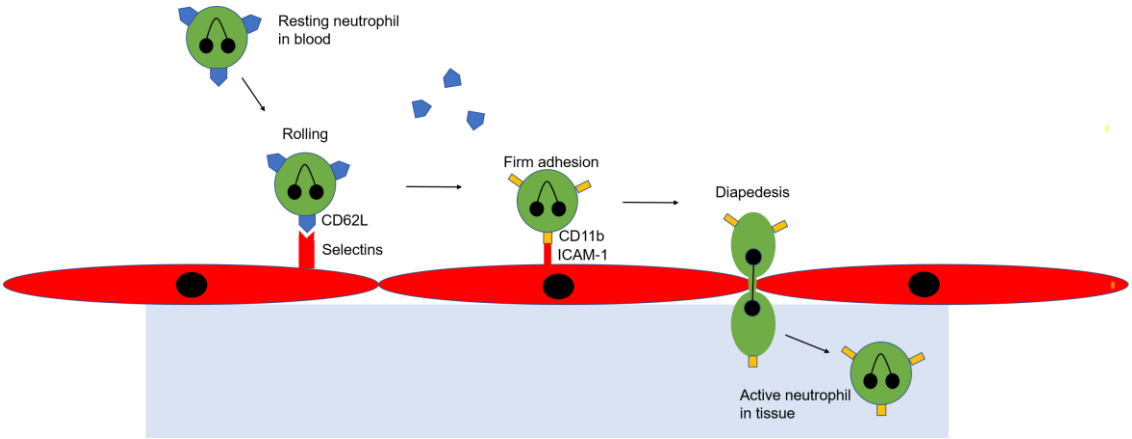

Figure 7. Neutrophil migration is a multistep process, and involves the shedding of CD62L and the upregulation of CD11b.

During those steps, the neutrophil is activated from a relatively dormant phenotype in the blood stream, and migrate through the tissue where it is ready to use its arsenal to kill microbes [72, 73]. *In vitro*, the activation of neutrophils can be mimicked using endotoxin [74] or TNFα [72]. TNFα is a rapid and potent activator of neutrophils. It stimulates degranulation of vesicles through which CD11b can be exposed on the neutrophil surface [71]. It also stimulates surface bound proteolytic enzymes such as ADAM-17, that cleaves CD62L [70].

Antimicrobial peptides – epithelial defenders

The airway epithelial cells and several types of immune cells, produce small antimicrobial peptides [75]. These function as “endogenous antibiotics” that can clear microbes through diverse mechanisms, such as interaction with bacterial membranes or deprivation of nutrients. The antimicrobial peptides can also function as signalling molecules. A number of antimicrobial peptides are secreted in the conducting airways, such as β-defensins, lysozyme [6] lactoferrin, LL-37 and surfactant proteins A and D [3].

LL-37 is the only human antimicrobial peptide of the cathelicidin family, found primarily in neutrophil granules but also in epithelial cells and macrophages. It is stored as the precursor protein hCAP-18 [76], but activated through cleavage by serine proteases to LL-37 [77]. It can damage bacterial lipid layers, have a chemotactic effect [78, 79] and induce chemokine release from epithelial cells [80]. It is induced by inflammatory stimuli such as IL-17 and IL-22, pathogen-associated molecular patterns (PAMPs) or tissue injury [59, 81].

Calprotectin (also named S100A8/A9 or calgranulin A/B) is expressed in various tissues and was first isolated from granulocytes [82]. It can exert both bacteriostatic and cytokine-like effects [83]. In inflammatory bowel disease, it is used as a clinical biomarker for inflammation.

In human bronchial epithelial cells, IL-22 upregulates antimicrobial peptides and strengthens the epithelial barrier [56]. Mouse lung epithelial cells respond to IL-22 by secreting antimicrobial peptides and primary human bronchial epithelial cells accelerate their wound repair [47]. The effect of IL-22 has been more extensively studied in the skin epithelium, and human keratinocytes react to IL-22 by increasing antimicrobial peptides such as β-defensin 2 [48], psoriasin and calprotectin [49]. Molecules involved in differentiation are decreased and molecules associated with mobility are increased [49, 84], Antimicrobial molecules upregulated by IL-17 include LL-37, calprotectin and lipocalin-2 as well as CCL20, human β-defensin 4 and MUC5B/AC [85].

Chronic obstructive pulmonary disease

In chronic obstructive pulmonary disease (COPD), inhaled particles lead to inflammation in the airways. They cause bronchiolitis, inflammation in the small airways, presumably because the particles are deposited here. They may also cause emphysema, destruction of peripheral airway tissue, leaving less surface area for gas exchange. The inflammation is progressive if smoking is continued and largely irreversible [86]. Tobacco smoke is an important risk factor for chronic obstructive pulmonary disease (COPD), but biomass fuel exposure and air pollution may contribute, as well as some genetic conditions [87]. Only a subgroup of smokers develops COPD and it is still not clear why these individuals are susceptible to this disease.

Patients with COPD experience airflow limitation and respiratory symptoms such as dyspnoea, cough and sputum production. For diagnostics, spirometry is required and a forced expiratory volume in 1 s divided by forced vital

capacity (FEV_1/FVC) < 0.70 after use of a bronchodilator confirms the COPD diagnosis. In these patients, the predicted FEV_1 is then used to classify the severity of disease according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria, and a patient with FEV_1 % predicted of ≥ 80 is classified with mild COPD, 50-80 with moderate COPD, 30-50 with severe disease and <30 with very severe disease [86].

Patients with COPD may experience periods of more or less acute deterioration. These are called exacerbations, and are often caused by viral and bacterial infections, and associated with increased airway inflammation. Systemic inflammation is common, especially during severe disease and exacerbations, and is associated with increased morbidity [88] and mortality [89]. In severe COPD, both innate and adaptive immunity are involved. Neutrophils are increased in the airways of patients with COPD [90] and further increased in acute exacerbations [91]. A similar pattern of airway inflammation and increase in mediators can be seen in smokers, but is amplified in COPD patients [87].

The IL-23 axis in the airways

The neutrophil recruitment caused by the cytokines associated with the IL-23 axis has led to studies of whether these cytokines are involved in COPD, a disease with increased neutrophil infiltration. The number of IL-23 positive cells are increased in the lung epithelium of patients with stable COPD [92]. Several studies have shown increased immunoreactivity for IL-17 in bronchial submucosa [92-95] and increased numbers of IL-22+ and IL-23+ cells in the bronchial epithelium [92] in COPD compared to healthy controls.

As for secreted protein, increased systemic concentrations of IL-23, IL-17 and IL-22 has been shown to be increased in moderate and severe COPD compared to healthy individuals [96, 97]. However, in one study, smokers with COPD and chronic bronchitis had lower systemic concentration of IL-17 protein compared to never-smokers [98]. In the airways, patients with COPD did not express significantly higher local IL-17 protein in BAL fluid compared to healthy controls [99]. The importance of the IL-23 axis in COPD has not yet been fully understood.

In this thesis, we show different aspects on the IL-23 axis in the airways. We demonstrate that airway macrophages can produce IL-22, contributing with a local and accessible source of this cytokine to the airway epithelial defence.

The cytokine IL-17 exerts a negative feedback effect on IL-23 in the airways, changing the dynamics of the IL-23 axis signalling. The antimicrobial peptides LL-37 and calprotectin are expressed in healthy human airways and LL-37 is induced by bacterial gram-negative stimuli, showing its involvement in local innate immune response against gram-negative bacteria. Airway neutrophils are activated compared to blood neutrophils, and can be further activated by TNFα indicating that the local microenvironment may influence neutrophil activation. Finally, IL-22 is increased in plasma from smokers, showing a smoking induced systemic effect on a cytokine of the IL-23 axis. This thesis contributes information about the local expression of the IL-23 axis in the airways.

AIM

The overall aim of this project was to characterise the role of the IL-23 axis in the airways. More specifically, we wanted to investigate if:

I. Macrophages are sources of IL-22 in mouse and human airways.
II. IL-17 exerts a negative feedback effect on IL-23.
III. Endotoxin induces the antimicrobial peptides LL-37 and calprotectin in healthy human airways.
IV. Airway neutrophils are activated compared to blood neutrophils, and if the cytokines associated with the IL-23 axis are increased in plasma and BAL fluid of smokers with and without COPD.

METHODS

Mouse experiments

Balb/c mice, IL-17 knock-out mice and C57BL/6 mice were kept at the animal facilities of the University of Gothenburg under conditions approved by the Animal Ethics Committee in Gothenburg.

Inflammation was caused by different exposures. In experiments with intranasal exposure, the mice were transiently anaesthetised with isoflurane, and given endotoxin (LPS), peptidoglycan, recombinant IL-17 or phosphate buffered saline (PBS) intranasally once daily during 3 consecutive days. In experiments with systemic exposure, the mice were inoculated with Staphylococcus Aureus in the tail vein. In experiments with intraperitoneal exposure, the mice were injected with a neutralising monoclonal anti-mouse IL-17 antibody or its isotype control. When the experiments ended, the mice were injected intraperitoneally with a mixture of ketamine and xylazine. After reaching deep anaesthesia, they were euthanised by bleeding of the right ventricle of the heart.

To obtain lung samples, the lungs were perfused with PBS through the right ventricle of the heart to remove blood cells and the lungs were then excised and homogenised. Lung homogenates were depleted of CD3 positive cells using antibody coupled magnetic beads and cultured for 24 h in the presence of medium alone, endotoxin, peptidoglycan, or recombinant mouse IL-23. Cytospins were prepared with mouse lung or BAL cells for immunocytochemical analyses.

To obtain BAL fluid samples, mouse airways were washed with PBS or Hank's balanced salt solution. After centrifugation, the cell-free BAL fluid was frozen for further analyses.

Bronchoscopy and BAL

The study protocols were approved by the Regional Committee for ethical review in Gothenburg. For paper I-III, the subjects were non-smoking and non-atopic individuals without regular medication. In paper IV, the subjects were recruited into three groups. Healthy never-smokers had normal ventilatory

lung function, with a forced expiratory volume in 1 s (FEV_1) of >80% of the predicted value and a FEV_1/FVC ratio of >70%. Smokers were defined as having smoked at least 10 cigarettes per day for at least 10 years. Smokers were included in the COPD group if FEV_1/FVC was <70 after bronchodilation, and in the asymptomatic group if FEV_1/FVC was ≥70 and FEV_1 >80%.

For paper I, II and IV, bronchoscopy with BAL was performed on each subject using 3 x 50 ml of PBS. In the endotoxin model used in paper III, a balloon-tipped catheter was inserted through the bronchoscope, placed in the chosen segment, and inflated with air to close off the segment. The segment was then challenged with vehicle followed by air. The bronchoscope was moved to the contralateral segment and the sequence repeated followed by instillation of endotoxin. During a second bronchoscopy, the bronchial segments were re-identified and bronchoscopy with BAL was performed with the instillation of 3 x 50 ml of PBS in the vehicle and endotoxin segments respectively.

Stimulation of human cells

BAL fluid samples were filtered for removal of mucus and debris. Cell-free BAL fluid was separated from the cells through centrifugation and frozen. The cell pellet was resuspended, cell viability assessed and a total cell count carried out. Differential counts were performed on cytospin preparation after May Grünwald Giemsa staining.

In paper I and II, human BAL macrophages were isolated through adherence for 2 h, after which the cells were washed to remove non-adherent cells. The alveolar macrophages were cultured during 24 h and stimulated with peptidoglycan, recombinant IL-23 or medium alone.

In paper II, monocytes were isolated from human blood samples of healthy human volunteers through centrifugation over a Ficoll gradient. The monocytes were further isolated from the mononuclear cell fraction using negative selection in a Monocyte Isolation kit. The monocytes were cultured in 37ºC in 5% CO_2 during 5 days in supplemented medium and GM-CSF to produce monocyte-derived macrophages.

In paper IV, polymorphonuclear leukocytes were isolated from the BAL cells through centrifugation over a Ficoll gradient. The BAL cells and the whole blood cells were incubated at room temperature or at 37°C with addition of TNFα for 20 minutes. Red blood cells were lysed, once for BAL cells and twice

for whole blood cells. The cells were resuspended in PBS before flow cytometry analysis.

Antibody-based methods

The samples in this thesis were analysed with different antibody based methods, especially flow cytometry, ELISA and immunocytochemistry. Enzyme labelled antibodies were introduced over 40 years ago to identify antigens in tissue. Several methods exist, each with their advantages and disadvantages. The basis of the method is the reaction between the antibody (Figure 8) and its antigen, and the specificity of the antibody is key. Monoclonal antibodies are preferentially used. These are generated toward a specific epitope of an antigen, while a polyclonal antibody is generated toward several different epitopes of the same antigen. All antibodies need to be titrated to the right concentration as to give a specific visual staining but avoid unspecific staining.

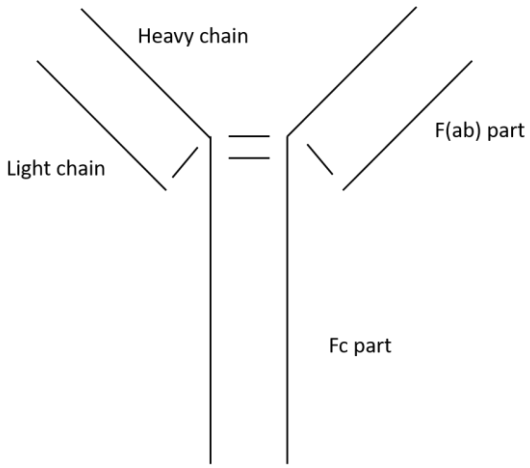

Figure 8. The antibody, or immunoglobulin, is made up by two heavy chains and two light chains. The F(ab) part (fragment antigen binding) or variable region recognizes microbial peptides and gives the antibody its specificity. The Fc part (fragment constant) determines the mechanism of microbial clearance, and divides the antibodies into the groups IgM, IgD, IgG, IgA or IgE.

Immunocytochemistry

In paper I, we used immunocytochemistry (ICC) to study the expression of IL-22 in mouse and human macrophages. With this method, the presence and cellular distribution of an antigen can be visualised. However, there is no generally accepted scoring system and no consensus as to what constitutes a positive signal. The method in brief is shown in Figure 9.

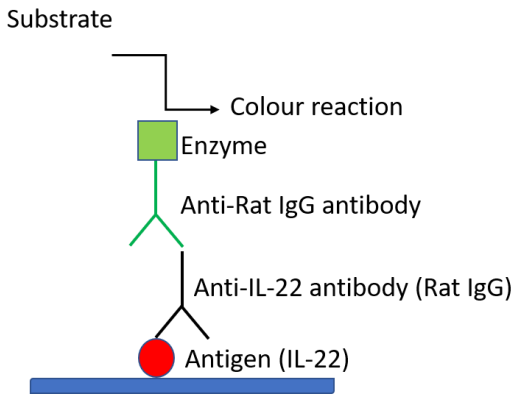

Figure 9. Immunocytochemistry. Soluble cells are put on a cytospin slide and fixated. A primary antibody is added, specific to the antigen of interest, in our case IL-22. A secondary antibody (anti-Rat IgG) is applied, specific to the primary antibody, with an enzyme attached. A substrate is added that reacts with the enzyme. This leads to the formation of a color precipitate, which is visible under the light microscope.

In Paper I, mouse lung and BAL cells were put on cytospin slides. The slides were washed with saponin to allow for intracellular staining. The intracellular expression of IL-22 was detected using a monoclonal rat anti-mouse IL-22 antibody and a matched isotype was used as control. As secondary antibody, a Rabbit F(ab')2 anti-rat IgG HRP was added together with mouse serum. Bound antibodies were visualised with DAB colour substrate.

The same cells were then immunostained for F4/80. The extracellular expression of F4/80 was detected using a monoclonal biotinylated rat anti-mouse F4/80 antibody or its isotype matched control. Streptavidin-AP was added as secondary reagent and bound antibodies were visualised with liquid permanent red. Mayers Hematoxylin was used for counter staining. The number of cells with positive immunostaining for F4/80 and IL-22 were evaluated using light microscopy.

For immunostaining of human alveolar macrophages in paper I, in four independent experiments, total BAL cells were collected for cytospin preparation. The slides were washed with Saponin to allow for intracellular staining. The intracellular expression of IL-22 was detected using a polyclonal goat anti-human IL-22 antibody. Total goat Ig was used as negative control. The slides were incubated with a biotinylated swine anti goat IgG together with an in house human serum, and then with streptavidin-AKP. Bound antibodies were visualised with LPR. The slides and numbers of macrophages with positive immunostaining for IL-22 were evaluated using light microscopy.

ELISA

In all papers of this thesis, commercially available sandwich ELISAs were used for the detection of secreted proteins in cell-free conditioned medium or cell-free BAL fluid according to the manufacturer's instructions. Measurements of mouse and human IL-22 (paper I), mouse IL-12, IL-17, IL-22 and IL-23 (paper II), human LL-37 and calprotectin (paper III) and human IL-17 (paper IV) were performed. This method gives a quantitative answer, and uses two different antibodies, the capture and detection antibodies, increasing the specificity of the method. However, the cellular source of the protein remains unknown. A direct sandwich ELISA is shown in Figure 10.

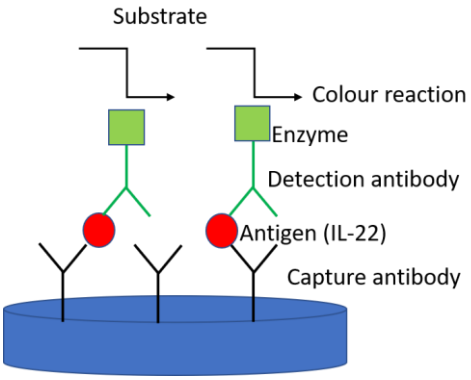

Figure 10. Sandwich ELISA. The bottom of the well is coated with a capture antibody, in our case an antibody specific towards IL-22. We add cell-free BAL fluid to determine if there is any IL-22 in this solution. A detection antibody is added, coupled to biotin. Streptavidin is added, which binds to biotin, and is coupled with an enzyme. Finally, TMG substrate is added, and reacts with the enzyme to form a color reaction. The amount of color can be measured with a spectrophotometer and is proportional to the amount of IL-22 in the solution. A standard curve with a known concentration of protein is used to determine the concentrations in the samples.

U-plex®

The U-plex® method is an antibody based method that allows the analysis of several proteins at the same time. In Paper IV, IL-22 among other cytokines was quantified in concentrated cell-free BAL fluid and plasma using the U-Plex assay®, MesoScale DiscoveryTM platform according to the manufacturer's instructions.

Flow cytometry

In paper I, II and IV, flow cytometry was used to study the expression of cellular markers at the surface of or within single cells. Flow cytometry is a powerful tool which can be used to measure relative size, internal complexity and cellular proteins as well as distinguish between and sort cell populations. The flow cytometry method gives a quantitative answer, can measure several cellular proteins at once and can generate large amounts of information from small sample volumes. However, setting up a large-scale flow cytometry experiment is complicated and there can be a high risk of false positive results.

In flow cytometry, cells are stained with antibodies coupled to fluorescent probes. Single cells are delivered to the detector, where light scatter and fluorescence is measured. The light is converted to a voltage pulse and the signal is amplified and measured.

In paper I, the purity of mouse CD3 depleted cells was determined using antibodies directed against CD4-positive and CD8-positive cells. The cell suspension was incubated 30 min at 4°C with a PE-conjugated anti-CD4 antibody and a PerCP-conjugated anti-CD8 antibody or their isotype-matched controls. The cells were washed, re-suspended and analysed using a FACScan flow cytometer.

In paper II, the expression of IL-17 receptor A and C were evaluated. Human BAL cells were collected for flow cytometry analysis of IL-17RA and IL-17RC. The cells were incubated for 15 min on ice with AF647-conjugated mouse anti-human IL-17RA antibody or APC-conjugated mouse anti-human IL-17RC antibody or their isotype matched controls. The cells were washed, resuspended and analysed using a BD LSR 2 flow cytometer.

In paper IV, whole blood and BAL cells were incubated with a PE conjugated anti-CD11b antibody or a PE conjugated anti-CD62L antibody. Neutrophils from blood were gated based on FSC/SSC characteristics. For gating of BAL neutrophils, the neutrophil gate from blood cells was used in combination with exclusion of autofluorescent cells, since alveolar macrophages, but not

neutrophils, are highly autofluorescent. The cells were washed, resuspended and analysed using a BD Accuri flow cytometer.

Western blot

In the Western blot, gel electrophoresis can be used to separate proteins. In Paper III, the pro- and active forms of LL-37 were analysed using Western blot. In short, proteins were enriched on reversed phase OASIS columns, after which eluation was done with Acetonitrile. Next, 20 µg peptide-extract was applied to each lane on gradient NuPAGE-gels. The pro-form and the active form of LL-37 were detected by monoclonal anti-LL-37 antibody. A rabbit anti-human IgG-antibody coupled to horse radish peroxidase was used as a secondary reagent. The signal was developed by the ECL-prime system. For densitometry, all films were scanned and analysed in ImageJ. Data was normalised to the internal control for each gel. Nondetectable levels were arbitrarily defined as 0.01.

Statistical methods

In all papers, each donor contributed to one independent experiment and n represents the number of independent observations. Differences were considered to be statistically significant with two-sided p-values of ≤ 0.05.

In paper 1, logarithmic transformation was performed to get more equal variances and symmetric distributions. In the mouse experiments, two-way variance analysis was performed on log-transformed data, with stimuli as a fixed factor and animal as a random factor. This was followed by multiple comparisons with the Dunnett method. On human data, differences between groups were tested using paired samples t-test.

In paper II, the IL-23 concentration in the cell culture medium of the human monocyte-derived macrophages and the alveolar macrophages was logarithmically transformed to stabilise variances. Two-tailed student's t-test was used for statistical analysis of data from mouse BAL samples and human monocyte-derived macrophages and alveolar macrophages in vitro.

In paper III, differences between endotoxin- and vehicle-exposed segments of the lungs within the same volunteer were analysed using the non-parametric Wilcoxon Signed rank test. For differences between time points in samples

from different volunteers, comparisons were made by calculating the difference between results from the endotoxin- and vehicle-exposed segment within each volunteer, at each time point, followed by a comparison of these differences between 12 and 24 h, using the Mann Whitney test. Data are presented as median and individual values with range. For correlations between individual data in different groups, Spearman´s two-tail rank correlation test was used.

In paper IV, logarithmic transformation was performed to get more equal variances and symmetric distributions. For differential counts and analysis of IL-22 and IL-17 unpaired T test was used. For the other analyses, two-way variance analysis was used, where the two factors were group and stimulated (yes/no). An interaction term between the factors were added to the model. The SAS procedure mixed was used for the calculations.

RESULTS

Paper I

Previous studies have suggested an important role of IL-22 for local epithelial defence. This led us to ask whether inflammatory cells residing in the airway epithelium are involved in the production of this cytokine. In Paper I we demonstrate that macrophages can contain and release IL-22 in the airways.

First, we determined whether there are cells other than T cells in the airways that can release IL-22. Mice were intranasally primed with the bacterial stimuli endotoxin or peptidoglycan. Whole lungs were collected and we removed T cells with magnetic beads binding to CD3 on the T cells. The resulting lung cell suspension without T cells was cultured and stimulated *in vitro* for 24 h with IL-23 or additional endotoxin or peptidoglycan. These stimuli significantly increased IL-22 secretion in conditioned medium compared to vehicle, showing that cells other than T cells can secrete IL-22 in the airways.

To characterise the cell type involved in this IL-22 signalling, we immunostained mouse lung cells with an antibody directed towards IL-22. We observed that the IL-22 immunoreactive cells displayed a morphology resembling macrophages. Therefore, we counterstained with an antibody directed towards the macrophage marker F4/80, showing in three separate experiments that the IL-22 immunoreactive cells were positive for F4/80 as well (Figure 11). This shows that mouse airway macrophages contain IL-22.

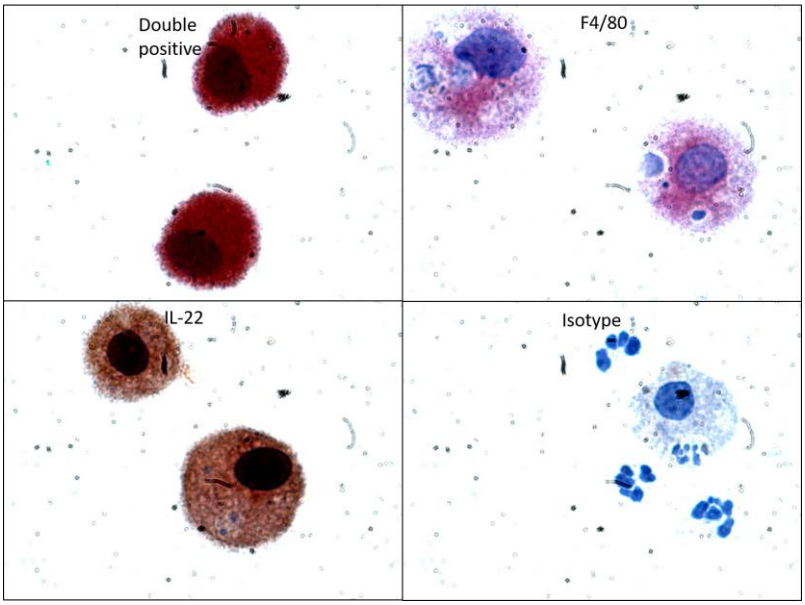

Figure 11. A majority of the cells with positive immunostaining for the macrophage marker F4/80 (red) displayed positive immunostaining for IL-22 (brown) as well, seen as double staining (dark red). Magnification x100.

To evaluate if there is a human correlate to the mouse macrophage production of IL-22, we collected BAL from healthy human volunteers, and enriched macrophages through adherence. Stimulation with IL-23 and peptidoglycan led to the release of IL-22 from human BAL macrophages (Figure 12) showing that human airway macrophages can release IL-22 protein to the extracellular space.

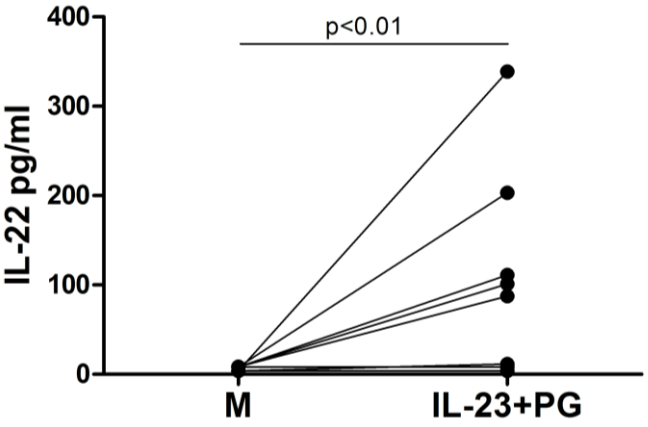

Figure 12. Human BAL fluid macrophages were stimulated with IL-23 and peptidoglycan (PG) or medium as control (M) for 24 h. With stimuli, IL-22 was secreted.

To visualise the IL-22 positive cells from humans, BAL cells were immunostained with an antibody directed towards IL-22. We demonstrate in four separate experiments that, similar to the mouse setting, the cells that contained IL-22 displayed a macrophage morphology.

Thus, we show that mouse and human alveolar macrophages can produce IL-22 during activation of the innate arm of pulmonary host defence.

Paper II

Since the cytokines associated with the IL-23 axis may cause neutrophil recruitment which risks damaging the epithelium through inflammation, we examined whether the IL-23 axis can be downregulated. In paper II, we show that IL-17 exerts a negative feedback effect on IL-23. To study this negative feedback effect in mice, we used three different approaches.

In a model of sepsis-induced pneumonia, the bacteria Staphylococcus Aureus (S. Aureus) was administered intravenously to wild-type and IL-17 knock-out mice. IL-23 protein was measured in cell-free BAL fluid, and was significantly higher in IL-17 knock-out mice compared to wild-type mice compared 24 h after inoculation with S. Aureus (Figure 13). This shows that mice lacking IL-17 can produce larger amounts of IL-23.

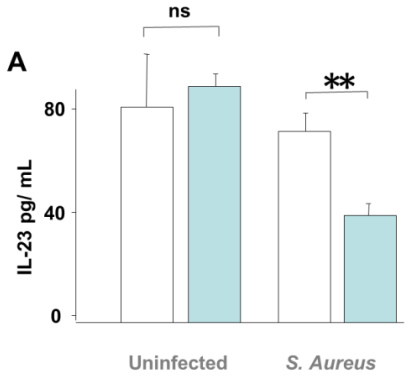

Figure 13. IL-17 knock-out mice (white bars) had higher levels of IL-23 in cell-free BAL fluid after systemic S. Aureus treatment compared to wild type mice (blue bars).

Second, in a model of gram-negative airway infection, BALB/c mice were pre-treated intra-peritoneally with anti-IL-17 antibody or its isotype control, then exposed to endotoxin intranasally. Endotoxin decreased the release of IL-23, but pre-treatment with anti-IL-17 antibody increased IL-23 concentration in cell free BAL fluid after 24 h compared to isotype control. At the same time, concentrations of IL-17 were increased with a similar tendency for IL-22. This suggests that when IL-17 is blocked, greater amounts of IL-23 can be secreted in BAL fluid, which may lead to secretion of IL-17 and IL-22 (Figure 14).

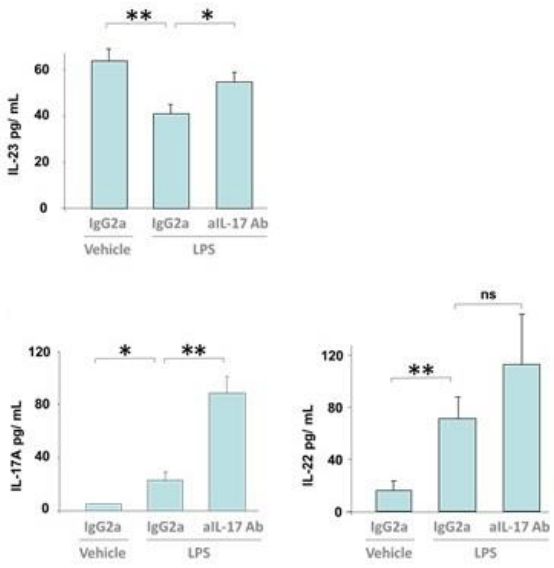

Figure 14. Stimulation with endotoxin intranasally decreased IL-23 release in BAL fluid from mice, but intraperitoneal pre-treatment with anti-IL-17 antibody increased IL-23 concentration after 24 h compared to isotype control. Concentrations of IL-17 were increased with a similar tendency for IL-22.

Third, recombinant IL-17 protein or vehicle was administered intranasally, and cell free BAL fluid was collected after only 2 h. Local treatment with recombinant IL-17 decreased the concentration of IL-23 in cell-free BAL fluid in comparison to the corresponding treatment with vehicle (Figure 15).

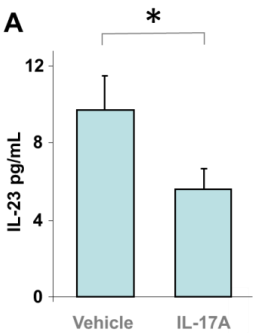

Figure 15. Intranasally administered recombinant IL-17 decreased the concentration of IL-23 in cell-free BAL fluid 2 h later.

To study the prerequisites for this negative feedback effect, the expression of the IL-17 receptors RA and RC was studied on alveolar macrophages, known sources of IL-23. Fresh isolated human alveolar macrophages were immunostained and analysed by flow cytometry. Macrophages in BAL samples from eight human healthy volunteers expressed both these receptors showing that it is possible for those cells to respond to IL-17.

Human monocyte derived macrophages were activated by exposure to endotoxin and stimulated with recombinant IL-17. This stimulation decreased IL-23 protein concentration in conditioned medium compared to vehicle. Similar results were obtained for human alveolar macrophages but not for primary human monocytes.

Thus, in mouse and human models, we demonstrate that IL-17 exerts a negative feedback effect on IL-23, and on human alveolar macrophages we demonstrate the presence of IL-17 receptors, a prerequisite to this response.

Paper III

The airway epithelium is protected by antimicrobial peptides functioning as endogenous antibiotics, several of which are regulated by the cytokines associated with the IL-23 axis. In paper III, we investigated whether the antimicrobial peptides LL-37 and calprotectin are expressed in normal human BAL fluid and are induced by endotoxin. Healthy volunteers were exposed to endotoxin in one bronchial segment and phosphate-buffered saline (PBS) in the contra-lateral segment. After 12 or 24 h, BAL fluid was collected from both segments, and comparisons between endotoxin and saline exposure were made within each subject.

The concentrations of LL-37 were increased by endotoxin stimulation after 12 and 24 h. Calprotectin was detected in cell-free BAL fluid in ELISA in both vehicle and endotoxin exposed segments but was not markedly affected by endotoxin (Figure 16). There was a positive correlation between the expression of LL-37 and the concentration of neutrophils, supporting previous reports that the neutrophil may be an important producer of LL-37 [77].

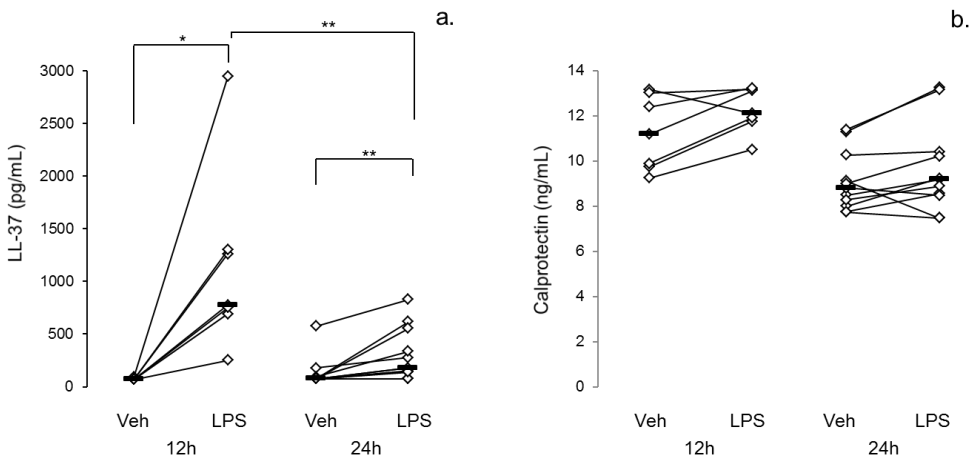

Figure 16. The concentrations of LL-37 in cell-free BAL fluid were increased by endotoxin stimulation after 12 and 24 h. Calprotectin was detectible, but not increased by endotoxin stimulation in this setting.

LL-37 is produced as the precursor hCAP18, but needs to be activated to exert effects. To ensure that not only the precursor but also the active form of LL-37 was present, both these forms were analysed with Western Blot. The precursor

form was significantly increased in endotoxin exposed segments after both 12 and 24 h, and the active form was increased after 24 h (Figure 17).

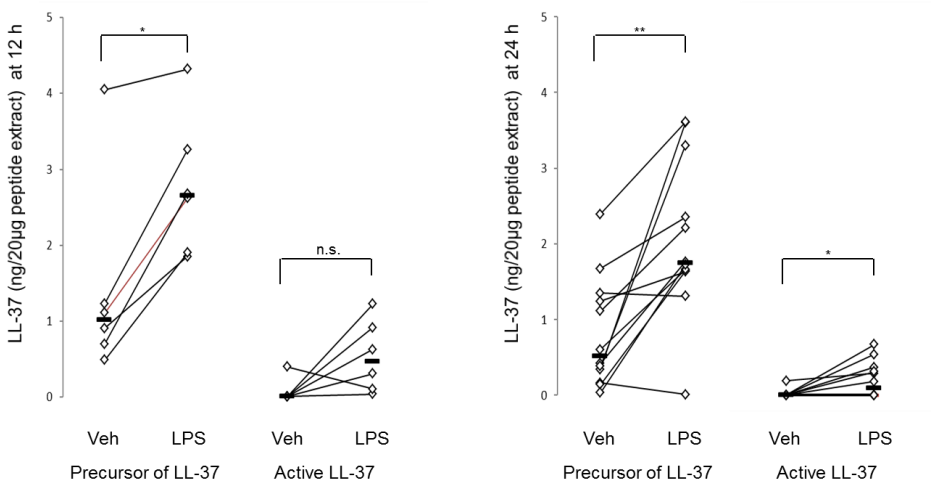

Figure 17. The concentration of the precursor form of LL-37 was significantly increased after 12 and 24 h, while the active form of LL-37 was increased after 24 h.

Thus, we show that calprotectin and LL-37 are expressed in human BAL fluid but only LL-37 is induced by endotoxin in this setting.

Paper IV

Neutrophils carry out their most important functions after leaving the blood stream and migrating to a site of inflammation in extravascular tissues. Despite this, the vast majority of human studies use cells isolated from peripheral blood, and our understanding about the degree of activation of human neutrophils is limited. In paper IV, we studied the activation of airway and blood neutrophils and their expression of neutrophil mobilising and IL-23 axis cytokines.

Un-stimulated blood neutrophils displayed low expression of CD11b and high expression of CD62L, both characteristics of a resting cell. The un-stimulated blood neutrophils in smokers with and without COPD displayed significantly lower expression of CD62L as compared to healthy never-smokers, demonstrating a smoking induced activation of neutrophils in the circulation of these subjects (Figure 18). Short-term ex vivo stimulation with TNFα induced neutrophil activation with up-regulated CD11b expression, and down-regulated CD62L expression.

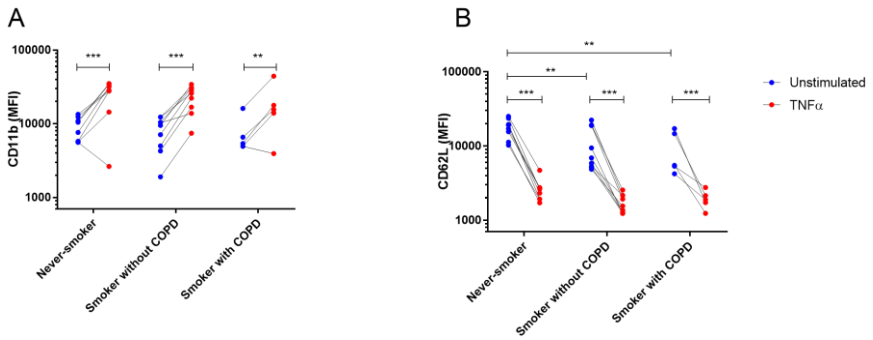

Figure 18. Unstimulated blood neutrophils displayed low level of CD11b and high level of CD62L, showing a resting state. Stimulation with TNFα led to signs of activation with increased CD11b and decreased CD62L.

Compared to their blood counterparts, un-stimulated BAL neutrophils were significantly more activated with up-regulated CD11b and down-regulated CD62L. Ex vivo stimulation with TNFα further decreased CD62L expression, indicating that these neutrophils are not yet maximally primed (Figure 19).

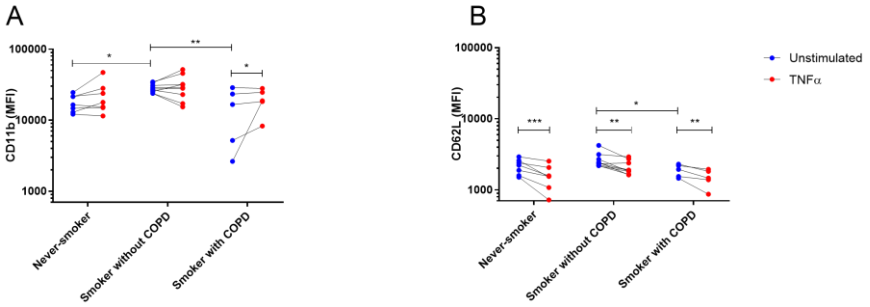

Figure 19. BAL fluid neutrophils displayed signs of activation with high level of CD11b and low level of CD62L. Stimulation with TNFα further decreased CD62L expression, showing that BAL neutrophils have the potential to be further activated.

The concentration of IL-22 in plasma was increased in smokers without COPD compared to healthy never-smokers and smokers with COPD. In BAL fluid there was a tendency towards a decrease in IL-17 in smokers with and without COPD compared to never-smokers (Figure 20).

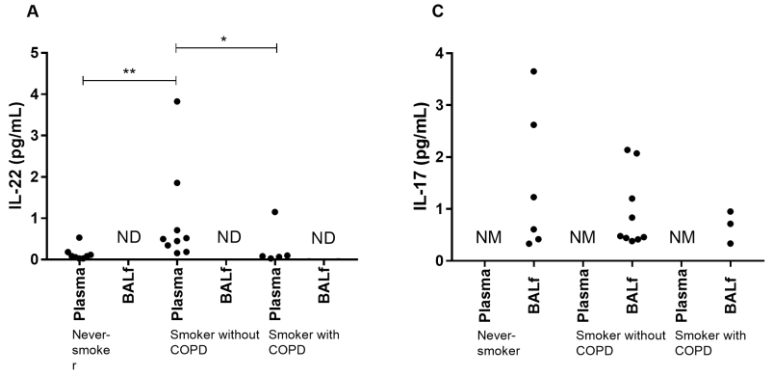

Figure 20. The plasma concentration of IL-22 was higher in smokers without COPD. The BAL fluid concentration of IL-17 tended to be lower in smokers with and without COPD.

Thus, we show that human airway neutrophils are activated and that this mechanism is maintained in smokers with and without COPD. However, they can be further activated by TNFα stimulation indicating that local inflammatory mediators can affect neutrophil activation. Smoking per se induced systemic neutrophil activation as well as the expression of IL-22.

DISCUSSION

Cellular sources

Our immune cells have different functions, are active at different time points during an immune response and respond to different stimuli. Thus, knowing the cellular source of a cytokine contribute information about its function as well. The IL-23 receptor is expressed on several different cell types, providing the prerequisite to respond to IL-23, and several cell types of the innate and adaptive immunity produce IL-22 and IL-17 as a response to IL-23. The most well-characterised IL-23-responsive cell is the Th17 cell, which directs adaptive immune responses. However, several other T cell subsets are involved in the expression of IL-17 and IL-22 as well. In fact, these cytokines can be produced by a broad range of airway lymphocytes; CD4 and CD8 positive T cells, γδ T cells, innate lymphoid cells and related subsets NKT cells and NK cells [43]. These cells play roles in both innate and adaptive immunity.

In Paper I we investigated cellular sources of IL-22 in the airways. We demonstrated the presence of cells other than T cells in mouse airways, which secreted IL-22 in response to IL-23 as well as gram-positive and gram-negative bacterial stimuli. We immunostained mouse lung and BAL cells as well as human BAL cells, and showed that the IL-22 immunoreactive cells displayed a macrophage morphology. Further, we enriched human BAL macrophages and showed that these cells secreted IL-22 in response to IL-23 and a gram-positive stimulus. It is known that macrophages can express the receptor for IL-23 [100] and it can also produce IL-23 [101]. This illustrates a potential autoregulatory mechanism by which macrophages could respond to a bacterial stimulus with the production of IL-23, and then respond to IL-23 with the release of IL-22.

The time course of the responses from these various cell types can differ. The response of the Th17 cell is antigen dependent, necessitating antigen presentation and differentiation before a response, and the Th17 cells need stimulation with IL-6 to sufficiently express the IL-23 receptor [102]. In contrast, the more evolutionary conserved γδ-T cells constitutively express the IL-23 receptor [103] with a potentially quicker response to IL-23. We observed that unstimulated airway macrophages from mice and humans contained IL-22, although stimulation was needed for release of this cytokine. Potentially, these cells can quickly release their cargo of prestored IL-22 when stimulated with microbial stimuli and IL-23.

The localisation of the various cell types can also differ. The abundant alveolar macrophages are strategically positioned close to the alveolar epithelium, recognizing and killing microbes, recruiting inflammatory cells and presenting antigens to T cells. While γδ T cells preferentially locate to the lamina propria and smooth muscle of the airways, αβ T cells preferentially locate to the alveolar epithelium [104]. The abundance of IL-17 and IL-22 producing cells may also indicate redundancy, with several potential sources of the cytokines associated with the IL-23 axis in response to different or the same microbe.

Although several sources of the cytokines associated with the IL-23 axis have been identified, their relative contributions at different time points, locations and diseases are unclear. As knowledge is gathered, more fine-tuned models of pulmonary host defence will be put together. This may provide more well-adjusted ways to modulate the immune response for optimal anti-inflammatory therapies while keeping infectious defences intact.

Regulation

A properly functioning pulmonary host defence is dependent on the initiation of inflammation when challenged by microbes, as well as the resolution of inflammation when the challenge is cleared. This is partly accomplished by the elimination of the microbe, which removes the stimulation of the immune defence. However, a chronic inflammation can be maintained by the remaining inflammatory cells and the cytokine milieu, the mechanisms for which are insufficiently known.

In paper II, we showed that IL-17 exerted a negative feedback effect on IL-23, which also tended to decrease the secretion of IL-22. This may explain previous findings that the absence of IL-17 led to increased IL-22 protein in a mouse model of pulmonary inflammation [105] as well as increased IL-22 mRNA in a mouse model of colitis [106]. It may also explain why IL-17 suppressed IL-22 secretion [105] and decreased IL-22 mRNA from Th17 cells [107]. A similar negative feedback effect has previously been observed by IL-25 (also called IL-17E) on IL-23 [108, 109]. It is possible that the receptor subunit IL-17RA, which is shared between IL-25 and IL-17, is responsible for this downregulation.

We showed in three different mouse models that IL-17 decreased the extracellular concentration of its archetype regulator IL-23. We confirmed this in humans, where IL-17 receptors as well as a negative feedback effect of IL-

17 on IL-23 protein release were demonstrated in human alveolar macrophages. This negative feedback effect may constitute a mechanism protecting from excessive IL-17 stimulation during pulmonary infection and inflammation.

Antimicrobial peptides

For the studies in Paper III, we used an endotoxin model, a well-characterized and medically safe model of the innate pulmonary host defence in the airways, where healthy human volunteers were given the gram-negative bacterial stimuli endotoxin. Gram-negative bacteria are common causes of pneumonia and exacerbations of COPD [110], and this model enabled us to examine the normal immunological response to a single dose of this stimuli. The endotoxin was given in one lung segment, while the opposite lung segment functioned as internal control. The dose of endotoxin (4 ng/kg) has been tested before and gave a prompt influx of inflammatory cells to the airways within 12 h. It caused a slight systemic reaction, as demonstrated by an increase in CRP and white cell count, but only mild to moderate symptoms [111]. Previously other members of our group have demonstrated the presence of IL-22, IL-17 and IL-23 in this setting after 12 h, with IL-17 still lingering after 24 h [112].

We found in the studies of Paper III that two antimicrobial peptides, LL-37 and calprotectin were both expressed in the human airways. LL-37 was induced by endotoxin stimulation, while calprotectin seemed to be constitutively expressed in this setting. We showed an increase in LL-37 already at 12 h, and it had started to recede at 24 h. To become active, LL-37 must be cleaved by from its precursor hCAP-18 molecule [3]. To ensure that not only the precursor but also the active form of LL-37 was present, both these forms were analysed with Western blot. The concentration of the precursor was significantly increased in endotoxin exposed segments after both 12 and 24 h, and the concentrations of the active form was significantly increased after 24 h with a similar tendency after 12 h. This shows that the activate form of LL-37 is secreted as well, and may affect the airways. LL-37 exerts direct microbe killing effects, as well as immune modulating effects [113]. Its induction by endotoxin challenge demonstrates the involvement of LL-37 in the innate arm of pulmonary host defence against gram-negative bacteria.

Neutrophil activation

The mobilisation of neutrophils is a multistep process that can be measured with the expression of activation markers. In Paper IV, we used the downregulation of CD62L and the upregulation of CD11b to measure the activation profile of systemic and extravasated neutrophils. In previously published papers, neutrophils that have extravasated to tissues such as human skin lesions and pus decrease their expression of CD62L [70]. We showed in concordance that airway neutrophils became activated during extravasation into the airways, and that this mechanism was maintained in smokers with and without COPD. We found that airway neutrophils were further activated upon stimulation with TNFα ex vivo, indicating that these cells can be affected by the local microenvironment in the airways.

Neutrophils from smokers with and without COPD displayed increased activation in their blood neutrophils, with decreased CD62L compared to those from healthy never-smokers. Earlier studies have shown similar systemic activation in an autoinflammatory disease [114] and increased CD11b was demonstrated in smokers with COPD compared to healthy individuals [115]. It seems probable that the neutrophils are pre-activated rather than activated, since activated neutrophils in circulation would be expected to quickly extravasate into tissue. If so, the actual activation might be caused by the act of removing them from circulation and performing the experiments. In any case, the neutrophils from smokers with COPD have an increased tendency to become activated, which seems to be related to smoking.

The IL-23 axis in COPD

In Paper IV, we studied the secretion of the cytokines associated with the IL-23 axis systemically and in the airways of smokers with and without COPD. IL-23 was not detectable in BAL fluid despite the sensitive detection method (U-plex®) and the concentration of the sample. However, cytokines are potent mediators even at low concentrations, and the lack of detection does not exclude the presence or alteration of IL-23 in the airways.

Previous studies have demonstrated increased immunoreactivity for IL-17 in bronchial submucosa in COPD compared to healthy controls [92-95]. These methods show the cellular expression but do not assess the extracellular concentrations of the secreted protein. When analysing cytokines secreted to the extracellular space, one previous study revealed no differences in terms of concentrations for IL-17 protein in smokers with COPD [99]. Other previous studies suggested a reduction of IL-17 in severe COPD [94, 116]. Although

our material is too limited for definite conclusions, we did observe a tendency towards lower IL-17 in BAL fluid from smokers with and without COPD compared to never-smokers. A potential explanation is the bronchial epithelial damage in severe COPD with emphysema, that could also disrupt epithelial cell production of IL-17. Another explanation might be that the effects of IL-17 could be protective in COPD, and the lack of IL-17 would thus promote the development of disease.

The IL-23 axis cytokine IL-22 was increased in plasma in smokers without COPD, demonstrating a smoking-induced systemic effect on the IL-23 axis. Previously, published studies have indicated that smoking per se increases cytokine concentrations at the systemic level [117], and that smoking causes local inflammation that is distinct from that in COPD [118]. In large quantities, cytokines can also leak from the tissue out into the blood stream, but since IL-22 was not measurable in BAL fluid, a smoking-induced systemic effect on IL-22 seems more likely.

Timing is everything

In the secretion of cytokines, there is commonly a lag period, followed by an increased secretion, and then a reduction caused by the elimination of the protein. It is important to choose a time point to measure when the protein concentration is at its peak. Different antibody based methods contribute with different ways to do this. In Flow cytometry and Immunocytochemistry, protein secretion is often blocked during the last hours of incubation. This prevents the cells from secreting their intracellular proteins which may otherwise occur during the experimental process. Counting on the relative stability of the protein, cumulative secretion can be measured in an ELISA such as in Paper I, where we measured the secretion of IL-22 after 24 h in cell culture supernatant with an ELISA.

Several time points can also be included in the study, as was the case in paper III, where we measured LL-37 and Calprotectin in BAL fluid after 12 and 24 h. We observed no endotoxin induced increase in Calprotectin in this model, but if Calprotectin were increased quickly and had a short half-life we might miss it. The half-life of Calprotectin in serum has been reported to be 5h [119], but knowledge about Calprotectin in the airways is lacking. However, Calprotectin is a stable marker for inflammation in the intestines where the milieu is oriented towards the degradation of nutrients, indicating that it would be fairly stable in the airways as well.

Correlation does not equal causation, and the presence of a cytokine is easier to study than the importance of the same cytokine. A cytokine may be present because it is protective in host defence, it can be a by-product or it could contribute to the disease. When the presence of a molecule has been demonstrated in a particular setting, its role in this setting needs further study experimentally.

Location, location, location

The airway tissue can be studied with several different methods, each with their advantages and disadvantages. In the papers of this thesis, we used BAL and whole lung preparations as well as peripheral blood to gain understanding of what happens in the airways. Biopsy is another method that show a specific part of the airways, and the histology of this part. However, if the inflammation is intermittent or sparse, it may be missed. We use whole lung preparations from mice, which is a cruder but also more encompassing method to gain access to the cells of the airways. Compared to BAL fluid, whole lung preparations add epithelial cells and other structural cells, as well as inflammatory cells that have not yet migrated into the bronchoalveolar space.

BAL is a method through which a sterile and buffered saline solution is instilled into the airways and collected [120]. This allows sampling of cells and molecules of the airway lining. An unknown dilution factor is introduced by this sampling technique, which may hinder measurements of molecules in low concentrations and efforts have been made to standardise the method [121]. In Paper IV, we studied the expression of neutrophil mobilising cytokines in the BAL fluid, and concentrate the BAL fluid around ten times to be able to detect the cytokines.

Peripheral blood concentrations of inflammatory proteins and cells can give information of the systemic aspects of the airway inflammation. To find biomarkers that can be measured in blood but mirror the activity of tissue, simultaneously sampling airway tissue and peripheral blood may be helpful. Our knowledge of airway tissue is limited by research to a large degree being performed on the easily accessible blood cells, while knowledge about the less accessible tissue cells is projected from the peripheral results. Many differences exist between immune cells in tissue and in the blood. In the blood, cells must be stable and strictly controlled so as not to cause a widespread immune reaction. In contrast, transmigrated cells are activated and ready to meet and eliminate microbes.

The differences between mice and men are necessary to keep in mind when analysing data from animal studies. The cytokine IL-26 is a relatively newly discovered cytokine which, like IL-17 and IL-22, has effects on epithelial cells [122]. However, a mice orthologue has not yet been described, even though there is an IL-26 receptor complex in mice. Thus, mouse studies on the IL-23 axis cannot directly be translated to all mammals, but need to be verified in humans as well.

Limitations of the model

The immune system is complex with multiple parts that interact with each other. We use models to aid us in understanding the processes, and our models will necessarily be reductive. Rather than processes being strictly pathogenic or protective, diseases may be regarded as balances that are being tipped too far in one or the other direction.

In our models, hierarchical structures are often employed in which one cell or molecule dominate or decide. This is correct in that there are certain gateways in which the fate of an immune response is decided, such as the interaction of the dendritic cell with a naïve T cell in the lymph node. However, the result of this interaction is dependent on several factors, including the nature of the microbe, the cytokine signalling from the dendritic cell and its costimulatory molecules. Further, whether the T cell will be able to return from the lymph node to the infected tissue depends on the adhesion molecules expressed by the endothelium. In our models, the T helper cell is often casted as the hero, steering the immune response. However, other immune cells such as the neutrophil, that is often seen as the brawn of immune responses, have regulatory functions as well [123]. Rather than one cell shouting orders, there is a constant babble of communication between cells locally and in the blood.

It is tempting to describe the IL-23 axis as a step by step system, as in Figure 6, in which IL-23 leads to IL-17 and IL-22 which further down the line leads to neutrophil mobilisation and epithelial defence. The use of the model of the cytokine axis is a way to pin down a multidimensional system into a two-dimensional picture. It helps us to understand the concepts, but we must be aware that there are several unknowns. In this thesis the IL-23 axis is defined as 23 and the cytokines that are produced and released as a direct response to IL-23. We focus on IL-17 and IL-22, but at least IL-17F and IL-26 would also fit in this definition and the secretion of several other cytokines is to some degree affected by IL-23. The effect of IL-23 is probably different on a naïve

T cell compared to a memory T cell. The same concentration of IL-23 may give a different signal depending on whether a high or low concentration of IL-23 receptor is expressed on the responding cell. The combination of cytokines may give rise to drastically different effects compared to a single cytokine. Concentrations of IL-22 may be protective alone, but pathogenic together with IL-17. It is also important to note that apart from the IL-23 axis, several other systems affect neutrophil mobilisation and antimicrobial peptides. These may play different roles in different microbial challenges, diseases and locations in the body. The more we understand, the more functional our models will become.

CONCLUSION

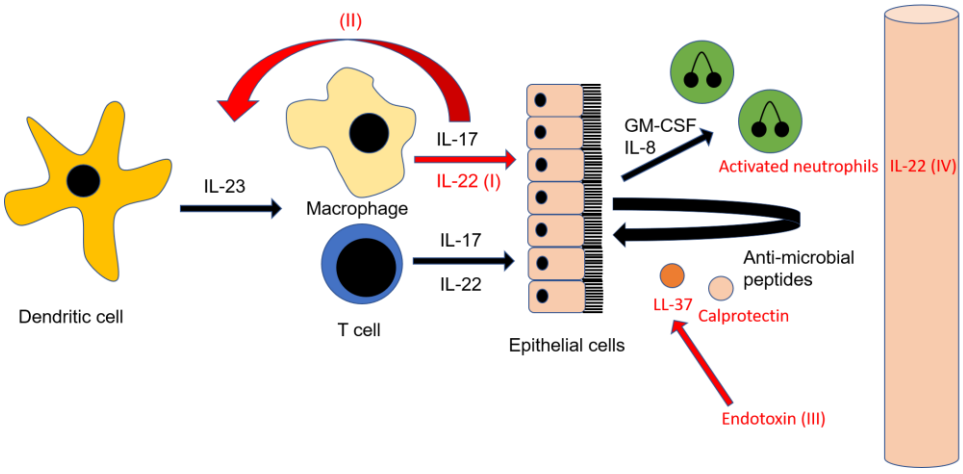

Figure 21. A schematic picture of the findings of the papers included in the thesis (in red). The numbers indicate the Papers I-IV.

We add to the complex picture of the role of the cytokines associated with the IL-23 axis in innate immunity. Our contributions to the field are marked in red above (Figure 21). In this picture, dendritic cells secrete IL-23 after being stimulated by microbes through their pattern recognition receptors. This IL-23 acts on T cells and on macrophages in the airways. This induces the production of IL-17, and we show that airway macrophages produce IL-22 as well. The macrophages constitute a local and accessible source of IL-22 (I).

The IL-17 produced exerts a negative feedback effect, reducing the concentration of IL-23 and thus decreasing its own production. The IL-17 receptor is present on human alveolar macrophages as a prerequisite to this response. This may constitute a self-protecting mechanism that restricts IL-17 activity during pulmonary inflammation and limit the tissue damaging effect of neutrophil infiltration (II).

Antimicrobial peptides can be induced by IL-17 and IL-22. We show that two antimicrobial peptides, LL-37 and calprotectin, are present in normal human airways. LL-37 is increased after stimulation with bacterial endotoxin, indicating its involvement in the innate immune defence in healthy human airways (III).

Finally, we examine neutrophil activation and the expression of the cytokines associated with the IL-23 axis in COPD, a disease characterised by neutrophil infiltration and frequent infections. We show that airway neutrophils are activated in this disease, however, the neutrophils can be further activated, indicating that the local microenvironment can affect neutrophil activation after extravasation. There are measurable systemic concentrations of IL-22, and this cytokine is increased in smokers showing a smoking-induced effect on the IL-23 axis (IV).

FUTURE PERSPECTIVES

The IL-23 axis is a communication system integrating innate and adaptive immunity. Whether its effects are protective or pathogenic may depend upon the surrounding cytokines and the tissue microenvironment. The airway immune defence is a complex system and we need more knowledge to be able to predict the local expression and effects of the cytokines associated with the IL-23 axis. These effects may differ in vitro and in vivo, because of their interaction with each other and with the chemical microenvironment of the airways.

Several cellular sources of the cytokines in the IL-23 axis have been described, both of the innate and adaptive immunity, and we show that airway macrophages can produce IL-22. Further studies are needed to address the relative contributions of the different cellular sources in steady state and their importance in different airway diseases.

We show that IL-17 exerts a negative feedback on IL-23 in the airways of mice and humans. This could have implications for the use of monoclonal antibodies against the cytokines associated with the IL-23 axis, and may lead to different dynamics when targeting IL-23 compared to IL-17 or its receptor. However, the in vivo effects of this negative feedback need to be studied further.

The cytokines associated with the IL-23 axis as well as antimicrobial peptides are potential areas for the development of new medications. We demonstrate a smoking induced effect on systemic IL-22 and on neutrophil activation, but observed no increase in IL-17 in the airways of stable COPD patients. Further studies on patients with COPD in different stages and with and without exacerbations would be helpful. The perceived importance of neutrophil infiltration in COPD makes this disease an attractive target for the use of monoclonal antibodies against the cytokines associated with the IL-23 axis.

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REFERENCES

1. Weibel, E., Morphometry of the human lung. Berlin and New York: Springer Verlag, 1963.
2. Ross, M.H., Histology. A text and atlas. 2003, Philadelphia, United States: Lippincott Williams and Wilkins.
3. Whitsett, J.A. and T. Alenghat, Respiratory epithelial cells orchestrate pulmonary innate immunity. Nat Immunol, 2015. **16**(1): p. 27-35.
4. Gonzalez, S., et al., Conceptual aspects of self and nonself discrimination. Self Nonself, 2011. **2**(1): p. 19-25.
5. Delves, P.J., S.J. Martin, and D.R. Burton, Roitt's Essential Immunology. 2011, Hoboken: John Wiley & Sons, Incorporated.
6. Bals, R. and P.S. Hiemstra, Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. Eur Respir J, 2004. **23**(2): p. 327-33.
7. Medzhitov, R. and C.A. Janeway, Jr., Decoding the patterns of self and nonself by the innate immune system. Science, 2002. **296**(5566): p. 298-300.
8. Re, F. and J.L. Strominger, Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. J Biol Chem, 2001. **276**(40): p. 37692-9.
9. Happel, K.I., et al., Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to Klebsiella pneumoniae infection. J Immunol, 2003. **170**(9): p. 4432-6.
10. Ivanov, S., et al., Functional relevance of the IL-23-IL-17 axis in lungs in vivo. Am J Respir Cell Mol Biol, 2007. **36**(4): p. 442-51.
11. Roses, R.E., et al., Differential production of IL-23 and IL-12 by myeloid-derived dendritic cells in response to TLR agonists. J Immunol, 2008. **181**(7): p. 5120-7.
12. van Beelen, A.J., et al., Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. Immunity, 2007. **27**(4): p. 660-9.
13. Lyakh, L., et al., Regulation of interleukin-12/interleukin-23 production and the T-helper 17 response in humans. Immunol Rev, 2008. **226**: p. 112-31.
14. Subhra K. Biswas, A.M., Macrophages: Biology and Role in the Pathology of Diseases. 2014, New York, Heidelberg, Dordrecht, London: Springer.
15. Hussell, T., T.J. Bell, and T. Fujimori, Airway Macrophages: Sentinels of Health. Macrophages: Biology and Role in the Pathology of Diseases, ed. S.K. Biswas and A. Mantovani. 2014, New York: Springer. 171-182.

16. Gu, Y., et al., Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. Eur J Immunol, 2008. **38**(7): p. 1807-13.
17. Kasahara, D.I., et al., Pulmonary inflammation induced by subacute ozone is augmented in adiponectin-deficient mice: role of IL-17A. J Immunol, 2012. **188**(9): p. 4558-67.
18. Vazquez, N., et al., Modulation of innate host factors by Mycobacterium avium complex in human macrophages includes interleukin 17. J Infect Dis, 2012. **206**(8): p. 1206-17.
19. Jiang, X.D., et al., The characterization of IL-17A expression in patients with chronic rhinosinusitis with nasal polyps. Am J Rhinol Allergy, 2011. **25**(5): p. e171-5.
20. Janeway, C.A., et al., Immunobiology. 2001, New York: Garland Publishing. 304-317.
21. Volpe, E., et al., A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. Nat Immunol, 2008. **9**(6): p. 650-7.
22. Mosmann, T.R., et al., Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol, 1986. **136**(7): p. 2348-57.
23. Coffman, R.L. and J. Carty, A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-gamma. J Immunol, 1986. **136**(3): p. 949-54.
24. Park, H., et al., A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nature immunology, 2005. **6**(11): p. 1133-1141.
25. Harrington, L.E., et al., Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol, 2005. **6**(11): p. 1123-32.
26. Langrish, C.L., et al., IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med, 2005. **201**(2): p. 233-40.
27. Reiner, S.L., Development in motion: helper T cells at work. Cell, 2007. **129**(1): p. 33-6.
28. Bettelli, E., et al., Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature, 2006. **441**(7090): p. 235-8.
29. Mangan, P.R., et al., Transforming growth factor-beta induces development of the T(H)17 lineage. Nature, 2006. **441**(7090): p. 231-4.
30. Korn, T., et al., IL-17 and Th17 Cells. Annu Rev Immunol, 2009. **27**: p. 485-517.
31. Chen, Z., et al., Distinct regulation of interleukin-17 in human T helper lymphocytes. Arthritis Rheum, 2007. **56**(9): p. 2936-46.

32. Veldhoen, M., et al., TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity, 2006. **24**(2): p. 179-89.
33. Aggarwal, S., et al., Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem, 2003. **278**(3): p. 1910-4.
34. Crome, S.Q., A.Y. Wang, and M.K. Levings, Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease. Clin Exp Immunol, 2010. **159**(2): p. 109-19.
35. Cosmi, L., et al., Human interleukin 17–producing cells originate from a CD161+ CD4+ T cell precursor. The Journal of Experimental Medicine, 2008. **205**(8): p. 1903-1916.
36. Manel, N., D. Unutmaz, and D.R. Littman, The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. Nat Immunol, 2008. **9**(6): p. 641-9.
37. Cua, D.J., et al., Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature, 2003. **421**(6924): p. 744-748.
38. Murphy, C.A., et al., Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med, 2003. **198**(12): p. 1951-7.
39. Gaffen, S.L., et al., The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. Nat Rev Immunol, 2014. **14**(9): p. 585-600.
40. Stout, R.D., et al., Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. J Immunol, 2005. **175**(1): p. 342-9.
41. Turner, M.D., et al., Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2014. **1843**(11): p. 2563-2582.
42. Oppmann, B., et al., Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity, 2000. **13**(5): p. 715-25.
43. Cua, D.J. and C.M. Tato, Innate IL-17-producing cells: the sentinels of the immune system. Nat Rev Immunol, 2010. **10**(7): p. 479-89.
44. Abraham, C. and J.H. Cho, IL-23 and autoimmunity: new insights into the pathogenesis of inflammatory bowel disease. Annu Rev Med, 2009. **60**: p. 97-110.
45. McAllister, F., et al., Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene-alpha and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis. J Immunol, 2005. **175**(1): p. 404-12.

46. Kuestner, R.E., et al., Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F. J Immunol, 2007. **179**(8): p. 5462-73.
47. Aujla, S. and J. Kolls, IL-22: A critical mediator in mucosal host defense. Journal of Molecular Medicine, 2009. **87**(5): p. 451-454.
48. Wolk, K., et al., IL-22 Increases the Innate Immunity of Tissues. Immunity, 2004. **21**(2): p. 241-254.
49. Boniface, K., et al., IL-22 Inhibits Epidermal Differentiation and Induces Proinflammatory Gene Expression and Migration of Human Keratinocytes. The Journal of Immunology, 2005. **174**(6): p. 3695.
50. Happel, K.I., et al., Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. The Journal of Experimental Medicine, 2005. **202**(6): p. 761.
51. Donnelly, R.P., et al., Interleukin-26: An IL-10-related cytokine produced by Th17 cells. Cytokine & growth factor reviews, 2010. **21**(5): p. 393-401.
52. Laan, M., et al., Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. J Immunol, 1999. **162**(4): p. 2347-52.
53. Toy, D., et al., Cutting Edge: Interleukin 17 Signals through a Heteromeric Receptor Complex. The Journal of Immunology, 2006. **177**(1): p. 36.
54. Chang, S.H. and C. Dong, A novel heterodimeric cytokine consisting of IL-17 and IL-17F regulates inflammatory responses. Cell Res, 2007. **17**(5): p. 435-40.
55. Wolk, K., et al., IL-22 Induces Lipopolysaccharide-Binding Protein in Hepatocytes: A Potential Systemic Role of IL-22 in Crohn's Disease. J Immunol, 2007. **178**(9): p. 5973-5981.
56. Aujla, S.J., et al., IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. Nat Med, 2008. **14**(3): p. 275-81.
57. Brand, S., et al., IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. American Journal of Physiology - Gastrointestinal and Liver Physiology, 2006. **290**(4): p. G827.
58. Avitabile, S., et al., Interleukin-22 Promotes Wound Repair in Diabetes by Improving Keratinocyte Pro-Healing Functions. Journal of Investigative Dermatology, 2015. **135**(11): p. 2862-2870.
59. Sakabe, J., et al., Calcipotriol increases hCAP18 mRNA expression but inhibits extracellular LL37 peptide production in IL-17/IL-22-stimulated normal human epidermal keratinocytes. Acta Derm Venereol, 2014. **94**(5): p. 512-6.
60. Peric, M., et al., IL-17A Enhances Vitamin D(3)-Induced Expression of Cathelicidin Antimicrobial Peptide in Human Keratinocytes. Journal of immunology, 2008. **181**(12): p. 8504-8512.

61. Liang, S.C., et al., Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. The Journal of Experimental Medicine, 2006. **203**(10): p. 2271-2279.
62. Chen, K., et al., IL-17RA is required for CCL2 expression, macrophage recruitment, and emphysema in response to cigarette smoke. PLoS One, 2011. **6**(5): p. e20333.
63. Dudakov, J.A., A.M. Hanash, and M.R. van den Brink, Interleukin-22: immunobiology and pathology. Annu Rev Immunol, 2015. **33**: p. 747-85.
64. Sonnenberg, G.F., et al., Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. The Journal of Experimental Medicine, 2010. **207**(6): p. 1293-1305.
65. Thieblemont, N., et al., Human neutrophils in auto-immunity. Semin Immunol, 2016. **28**(2): p. 159-73.
66. Summers, C., et al., Neutrophil kinetics in health and disease. Trends Immunol, 2010. **31**(8): p. 318-24.
67. Ivanov, S. and A. Lindén, Interleukin-17 as a drug target in human disease. Trends in Pharmacological Sciences, 2009. **30**(2): p. 95-103.
68. Andoh, A., et al., Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts. Gastroenterology, 2005. **129**(3): p. 969-84.
69. Seely, A., J. Pascual, and N. V Christou, Science review: Cell membrane expression (connectivity) regulates neutrophil delivery, function and clearance. Vol. 7. 2003. 291-307.
70. Kuhns, D.B., D.A. Long Priel, and J.I. Gallin, Loss of L-selectin (CD62L) on human neutrophils following exudation in vivo. Cell Immunol, 1995. **164**(2): p. 306-10.
71. De Clerck, L.S., et al., Expression of neutrophil activation markers and neutrophil adhesion to chondrocytes in rheumatoid arthritis patients: relationship with disease activity. Research in Immunology, 1995. **146**(2): p. 81-87.
72. Sengelov, H., et al., Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J Immunol, 1995. **154**(8): p. 4157-65.
73. Follin, P., G. Briheim, and C. Dahlgren, Mechanisms in neutrophil priming: characterization of the oxidative response induced by formylmethionyl-leucyl-phenylalanine in human exudated cells. Scand J Immunol, 1991. **34**(3): p. 317-22.
74. Almkvist, J., et al., Lipopolysaccharide-induced gelatinase granule mobilization primes neutrophils for activation by galectin-3 and formylmethionyl-Leu-Phe. Infect Immun, 2001. **69**(2): p. 832-7.
75. Hiemstra, P.S., et al., Antimicrobial Peptides and Innate Lung Defenses: Role in Infectious and Noninfectious Lung Diseases and Therapeutic Applications. Chest, 2016. **149**(2): p. 545-51.

76. Golec, M., et al., Cathelicidin LL-37 in bronchoalveolar lavage and epithelial lining fluids from COPD patients and healthy individuals. J Biol Regul Homeost Agents, 2012. **26**(4): p. 617-25.
77. Sorensen, O.E., et al., Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood, 2001. **97**(12): p. 3951-9.
78. Tjabringa, G.S., et al., Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. Int Arch Allergy Immunol, 2006. **140**(2): p. 103-12.
79. De, Y., et al., LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J Exp Med, 2000. **192**(7): p. 1069-74.
80. Bowdish, D.M., et al., Impact of LL-37 on anti-infective immunity. J Leukoc Biol, 2005. **77**(4): p. 451-9.
81. Yang, D., et al., Alarmins link neutrophils and dendritic cells. Trends Immunol, 2009. **30**(11): p. 531-7.
82. Johne, B., et al., Functional and clinical aspects of the myelomonocyte protein calprotectin. Molecular Pathology, 1997. **50**(3): p. 113-123.
83. Striz, I. and I. Trebichavsky, Calprotectin - a pleiotropic molecule in acute and chronic inflammation. Physiol Res, 2004. **53**(3): p. 245-53.
84. Wolk, K., et al., IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. European Journal of Immunology, 2006. **36**(5): p. 1309-1323.
85. Tsai, H.C., et al., IL-17A and Th17 cells in lung inflammation: an update on the role of Th17 cell differentiation and IL-17R signaling in host defense against infection. Clin Dev Immunol, 2013. **2013**: p. 267971.
86. GOLD, From the Global Strategy for the Diagnosis, Management and Prevention of COPD. Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017, 2017.
87. Barnes, P.J., Inflammatory mechanisms in patients with chronic obstructive pulmonary disease. Journal of Allergy and Clinical Immunology, 2016. **138**(1): p. 16-27.
88. Thomsen, M., et al., Inflammatory biomarkers and comorbidities in chronic obstructive pulmonary disease. Am J Respir Crit Care Med, 2012. **186**(10): p. 982-8.
89. Agustí, A., et al., Persistent Systemic Inflammation is Associated with Poor Clinical Outcomes in COPD: A Novel Phenotype. PLOS ONE, 2012. **7**(5): p. e37483.
90. Pesci, A., et al., Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. European Respiratory Journal, 1998. **12**(2): p. 380.

91. Drost, E.M., et al., Oxidative stress and airway inflammation in severe exacerbations of COPD. Thorax, 2005. **60**(4): p. 293.
92. Di Stefano, A., et al., T helper type 17-related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients. Clin Exp Immunol, 2009. **157**(2): p. 316-24.
93. Eustace, A., et al., Identification of cells expressing IL-17A and IL-17F in the lungs of patients with COPD. Chest, 2011. **139**(5): p. 1089-100.
94. Doe, C., et al., Expression of the T helper 17-associated cytokines IL-17A and IL-17F in asthma and COPD. Chest, 2010. **138**(5): p. 1140-7.
95. Chang, Y., et al., CD8 positive T cells express IL-17 in patients with chronic obstructive pulmonary disease. Respir Res, 2011. **12**: p. 43.
96. Wang, H., et al., Imbalance of peripheral blood Th17 and Treg responses in patients with chronic obstructive pulmonary disease. Clin Respir J, 2015. **9**(3): p. 330-41.
97. Zhang, L., et al., Expression of Interleukin (IL)-10, IL-17A and IL-22 in Serum and Sputum of Stable Chronic Obstructive Pulmonary Disease Patients. COPD: Journal of Chronic Obstructive Pulmonary Disease, 2013. **10**(4): p. 459-465.
98. Andelid, K., et al., Systemic cytokine signaling via IL-17 in smokers with obstructive pulmonary disease: a link to bacterial colonization? Int J Chron Obstruct Pulmon Dis, 2015. **10**: p. 689-702.
99. Levanen, B., et al., Impact of tobacco smoking on cytokine signaling via interleukin-17A in the peripheral airways. Int J Chron Obstruct Pulmon Dis, 2016. **11**: p. 2109-2116.
100. Liu, Z., et al., The increased expression of IL-23 in inflammatory bowel disease promotes intraepithelial and lamina propria lymphocyte inflammatory responses and cytotoxicity. Journal of Leukocyte Biology, 2011. **89**(4): p. 597-606.
101. Pirhonen, J., S. Matikainen, and I. Julkunen, Regulation of virus-induced IL-12 and IL-23 expression in human macrophages. The Journal of Immunology, 2002. **169**(10): p. 5673-5678.
102. Yang, X.O., et al., STAT3 Regulates Cytokine-mediated Generation of Inflammatory Helper T Cells. Journal of Biological Chemistry, 2007. **282**(13): p. 9358-9363.
103. Croxford, A.L., F. Mair, and B. Becher, IL-23: one cytokine in control of autoimmunity. Eur J Immunol, 2012. **42**(9): p. 2263-73.
104. Wands, J.M., et al., Distribution and leukocyte contacts of gammadelta T cells in the lung. J Leukoc Biol, 2005. **78**(5): p. 1086-96.
105. Sonnenberg, G.F., et al., Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. J Exp Med, 2010. **207**(6): p. 1293-305.

106. O'Connor, W., Jr., et al., A protective function for interleukin 17A in T cell-mediated intestinal inflammation. Nat Immunol, 2009. **10**(6): p. 603-9.
107. von Vietinghoff, S. and K. Ley, IL-17A controls IL-17F production and maintains blood neutrophil counts in mice. J Immunol, 2009. **183**(2): p. 865-73.
108. Caruso, R., et al., Interleukin-25 Inhibits Interleukin-12 Production and Th1 Cell-Driven Inflammation in the Gut. Gastroenterology, 2009. **136**(7): p. 2270-2279.
109. Kleinschek, M.A., et al., IL-25 regulates Th17 function in autoimmune inflammation. Journal of Experimental Medicine, 2007. **204**(1): p. 161-170.
110. Prina, E., O.T. Ranzani, and A. Torres, Community-acquired pneumonia. The Lancet, 2015. **386**(9998): p. 1097-1108.
111. O'Grady, N.P., et al., Local inflammatory responses following bronchial endotoxin instillation in humans. Am J Respir Crit Care Med, 2001. **163**(7): p. 1591-8.
112. Glader, P., et al., Interleukin-17-producing T-helper cells and related cytokines in human airways exposed to endotoxin. Eur Respir J, 2010. **36**(5): p. 1155-64.
113. Turner, J., et al., Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. Antimicrob Agents Chemother, 1998. **42**(9): p. 2206-14.
114. Sundqvist, M., et al., Increased Intracellular Oxygen Radical Production in Neutrophils During Febrile Episodes of Periodic Fever, Aphthous Stomatitis, Pharyngitis, and Cervical Adenitis Syndrome. Arthritis & Rheumatism, 2013. **65**(11): p. 2971-2983.
115. Blidberg, K., et al., Adhesion molecules in subjects with COPD and healthy non-smokers: a cross sectional parallel group study. Respir Res, 2013. **14**: p. 47.
116. Zhang, X., et al., Increased interleukin (IL)-8 and decreased IL-17 production in chronic obstructive pulmonary disease (COPD) provoked by cigarette smoke. Cytokine, 2011. **56**(3): p. 717-725.
117. Selvarajah, S., et al., Multiple Circulating Cytokines Are Coelevated in Chronic Obstructive Pulmonary Disease. Mediators of Inflammation, 2016. **2016**: p. 9.
118. Chung, K.F. and I.M. Adcock, Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. European Respiratory Journal, 2008. **31**(6): p. 1334-1356.
119. Meuwis, M.A., et al., Serum calprotectin as a biomarker for Crohn's disease. Journal of Crohn's and Colitis, 2013. **7**(12): p. e678-e683.
120. Meyer, K.C., Bronchoalveolar lavage as a diagnostic tool. Semin Respir Crit Care Med, 2007. **28**(5): p. 546-60.

121. Haslam, P.L. and R.P. Baughman, Report of ERS Task Force: guidelines for measurement of acellular components and standardization of BAL. Eur Respir J, 1999. **14**(2): p. 245-8.
122. Dambacher, J., et al., The role of the novel Th17 cytokine IL-26 in intestinal inflammation. Gut, 2009. **58**(9): p. 1207-1217.
123. Mantovani, A., et al., Neutrophils in the activation and regulation of innate and adaptive immunity. Nat Rev Immunol, 2011. **11**(8): p. 519-531.