MODULATORY ROLE OF IL-17 IN AIRWAY INFLAMMATION

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On the cover: Murine neutrophils from the bronchoalveolar space stained with the antineutrophil antibody NIMP-R14 and Liquid Permanent Red. Photo by Elin Silverpil.

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Att tänka rätt är stort, att tänka fritt är större och blir oftast rätt till slut

ABSTRACT

IL-17 orchestrates the accumulation of neutrophils to sites of infection and the release of microbicidal substances, and therefore plays a critical role in the innate immune response to infection. IL-17 is also involved in certain chronic inflammatory diseases in which dysfunctional control of neutrophil accumulation and turnover constitutes an important pathogenic factor. This pro-inflammatory potential of IL-17 in host defence and in inflammatory diseases has been studied extensively. However, there is now also published evidence that IL-17 has more complex actions, including inflammation-resolving potential under certain conditions. With this in mind, the aims of this thesis were to investigate endogenous and exogenous methods to regulate the production of IL-17 and to elucidate the role that IL-17 plays in resolving ongoing inflammation. More specifically, we looked at whether the cells in the lung produce IL-17 after exposure to lipopolysaccharide (LPS) from the Gram-negative *Escherichia coli* bacteria, and whether anti-inflammatory pharmacotherapies could be used to regulate the production of IL-17 in these cells. We also examined whether IL-17 contributes to neutrophil turnover through the regulation of macrophage phagocytosis of apoptotic neutrophils. Finally, we investigated whether IL-17 down-regulates the release of the upstream regulator IL-23.

We found that LPS induced sustained IL-17 production and release from T cells that reside in lung tissue and that are recruited to the bronchoalveolar space in a mouse model of acute inflammation *in vivo*. In addition, population of cells other than T cells contributed to IL-17 production in the lung tissues and in the bronchoalveolar space. LPS-induced IL-17 production from T cells in lung tissues and in the bronchoalveolar space was inhibited by the anti-inflammatory drug dexamethasone. Furthermore, we found that IL-17 stimulated macrophage phagocytosis of apoptotic neutrophils and particles, and induced neutrophil apoptosis in an *in vitro* study on isolated murine and human cells. Finally, we found that that IL-17 inhibited the release of the upstream regulator IL-23, both in the bronchoalveolar space in mice *in vivo* and in isolated human cells of the monocyte lineage.

A major finding is that the production of IL-17 can be regulated exogenously by anti-inflammatory drugs and endogenously by an IL-17-induced feedback loop, which, in turn, may protect against excessive, IL-23-induced IL-17 signalling. In addition, we demonstrate that IL-17 has both pro-inflammatory and inflammation-resolving actions; IL-17 accumulates neutrophils after stimulation with LPS, while it also induces the phagocytosis of apoptotic neutrophils, thereby controlling the total turnover of neutrophils. That IL-17 induces the apoptosis of neutrophils and increases the phagocytosis of these cells indicates a potentially valuable strategy to mitigate conditions in which necrotic neutrophils are an important contributor to severe and sometimes life-threatening conditions, such as chronic lung allograft rejection and acute respiratory distress syndrome.

Key words: IL-17, IL-23, phagocytosis, apoptosis, neutrophils, macrophages, airways

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LIST OF PUBLICATIONS

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-effects of anti-inflammatory pharmacotherapy
Pulm Pharmacol Ther. 2009:3;199-207.

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An inhibitory role for IL-17 in the release of IL-23 during airway inflammation
Manuscript in preparation

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ABBREVIATIONS

α-GalCer	α-galactosylceramide
ANOVA	One-way analysis of variance
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CBAD	C/EBPβ-activation domain
CCR6	CC chemokine receptor 6
C/EBPδ	CCAAT/enhancerbinding protein-δ
CFDA SE	Carboxyfluorescein diacetate succinimidyl ester
CFU	Colony forming units
CI	Calcium ionophore
C3	Complement factor
CR	Complement receptor
CXCL	CXC chemokine ligand
DAMPs	Damage associated molecular pattern molecules
DC	Dendrit cells
Dex	Dexamethasone
EAN	Experimental autoimmune neuritis
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FBS	Foetal bovine serum
FMLP	Formyl-methionyl-leucyl-phenylalanine
FOXP3	Forkhead box P3
FPR	N-formyl peptide receptor
GM-CSF	Granulocyte macrophage colony-stimulating factor
G-CSF	Granulocyte colony-stimulating factor
HMGB1	High mobility group box 1
i.n.	Intranasal
i.p	Intraperitoneally
i.v.	Intravenously
INF γ	Interferon γ
Lti	Lymphoid tissue inducer cells
LPS	Lipopolysaccharide
LTA	Lipoteichoic acids
MIP-2	Macrophage inflammatory protein-2
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular traps
NF-κB	Nuclear factor-κB
NK	Natural killer
ns	Not statistically significant
PBS	Phosphate buffered saline
PGN	Peptidoglycan
PMA	Phorbol 12-myristate 13-acetate
PAMPS	Pathogen-associated molecular pattern
PRR	Pattern-recognition receptors
Rac1	ras-related C3 botulinum toxin substrate 1
RAGE	Receptor for advanced glycation endproducts

ROR	Retinoic acid-related orphan receptor
ROS	Reactive oxygen species
SEFIR	SEF/IL-17R
SEM	Standard error of the mean
sLOX-1	Soluble lectin-like oxidized low-density lipoprotein receptor-1
TCR	T cell receptor
TIR	Toll/IL-1βR
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TRAF6	TNFR-associated factor 6

INTRODUCTION

Innate immunity

Host defence comprises innate and adaptive immune responses. Innate immunity relies on less-specific but more rapidly recruited cells that secrete antimicrobial peptides, complement factors, and reactive oxygen species (ROS) (1). Unlike the cells of the innate immune system, the cells of the adaptive immune system, which include lymphocytes, have specific receptors, e.g., T-cell and B-cell receptors, for the recognition of a specific antigens (2). Owing to their receptors, lymphocytes can act in a very specific and effective manner. The drawback is that the adaptive immune responses are usually delayed, as compared to innate immune responses. It takes 4–7 days for an adaptive response to occur, which is a relatively long time for the host in terms of defending against rapidly replicating microbial intruders (2).

Pathogen-associated molecular patterns and their receptors

The cells of the innate immune system also recognize antigens, such as bacterial peptides, although the strategy used by innate immune cells to recognize microbial 'danger signals' is based on the recognition of pathogen-associated molecular patterns (PAMPs). These are constitutive and conserved factors produced by microorganisms but not by the host. Usually, PAMPS are components that are essential for microbial survival. For example, lipopolysaccharide (LPS), peptidoglycan (PGN), and lipoteichoic acids (LTAs) are all components of bacteria, but are not made by eukaryotic cells. Therefore, these components are recognized as foreign materials by the innate immune system, which in turn signals the presence of infection (3-4).

To sense infection, the innate immune system has evolved pattern-recognition receptors (PRRs), which recognize and bind PAMPs (3). The innate immune system uses a variety of intracellular, cell-surface-localised, and secreted PRRs. The principal functions of PRRs include activation of proinflammatory signalling pathways, phagocytosis, opsonisation, and the complement and coagulation cascades (1, 3).

Neutrophils

The recruitment of neutrophils to the site of inflammation is one of the first lines of defence against invading pathogens. Neutrophils, which belong to the innate arm of the immune system, are professional phagocytes that take up and degrade microorganisms using combinations of reactive oxygen species (ROS), antimicrobial peptides, and proteases. Neutrophils also phagocytose apoptotic cells, although the extent of this phagocytosis is not entirely clear (5-6). Once inside the neutrophil, the phagocytosed microorganism or apoptotic cell is sequestered in a specialised compartment, called the phagosome, which subsequently fuses with a lysosome, which contains digestive enzymes, to form the phagolysosome. To ensure complete digestion of the ingested materials, the membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase system is activated. This system generates large quantities of ROS, which are subsequently released into the phagolysosome.

Neutrophilic granules

Neutrophils contain four types of granules that are designed to kill microbes during the different stages of neutrophil activation: 1) azurophilic granules, which contain myeloperoxidase (MPO), bactericidal permeability-increasing protein, defensins, and serine proteases; 2) specific granules, which contain lactoferrin, cathelicidin, lysozyme, and collagenase; 3) gelatinase granules, which contain gelatinase, leukolysin, and lysozyme; and 4) secretory granules, which contain complement receptors 1 and 3 (CR1 and CR3), N-formyl peptide receptors (FPRs), CD14, and CD16 (7). The different granules are produced at

specific stages of neutrophil maturation, and they differ in their propensities to be released upon neutrophil activation. Secretory granules are more readily released into the extracellular milieu than gelatinase granules, which in turn are more readily released than specific granules. Azurophilic granules are assumed to contribute mainly to degradation within the phagolysosome.

Neutrophil extracellular traps

Neutrophil extracellular traps (NETs) constitute a relatively newly discovered weapon in the lethal arsenal of neutrophils. NETs, which are web-like structures that contain chromatin and high concentrations of proteases and MPO, can be released from neutrophils (8). The formation of NETs occurs within minutes of the sensing of infection, and they trap and kill both Gram-positive and Gram-negative bacteria, as well as fungi (9-11).

Macrophages

Macrophages are also professional phagocytes, although whereas neutrophils have a focus in phagocytosing microbes, macrophages additionally removes cellular debris and apoptotic cells, and present antigens that have been phagocytosed. Monocytes that migrate from the blood to tissues can, depending on the environment and recruitment factors, differentiate into macrophages or dendritic cells (DCs). The prevailing milieu further decides the type of macrophage that is generated. Macrophages have been traditionally categorised into two subtypes: 1) the classically activated inflammatory type 1 macrophage; and 2) the alternatively activated macrophage or resident type 2 macrophage (12). Additional macrophage subtypes, with more or less distinct characteristics, have also been described (13).

Currently, the main macrophage subtypes are designated as classically activated macrophages, wound-healing macrophages, and regulatory macrophages (13). Classically activated macrophages are characterised by the production of cytokines, such as interferon-γ (INF-γ) and tumour-necrosis factor-α (TNF-α), enhanced microbe or tumour killing capabilities, and the secretion of high levels

of cytokines, such as IL-23, IL-1, and IL-6. These macrophages also support the development and proliferation of IL-17-producing cells (14-17), an aspect that will be discussed in detail later in this thesis. Wound-healing macrophages are distinguished by IL-4 production, and they promote wound healing by generating new extracellular matrix (18). These macrophages are poor presenters of antigens, do not express pro-inflammatory cytokines, have lower production levels of oxygen radicals and nitrogen radicals, and are weaker at intracellular killing of microbes (13). The third type of macrophage, the regulatory macrophage, differentiates in response to LPS in combination with immune complexes, prostaglandins, apoptotic cells, IL-10 or other cytokines (13, 19). These macrophages produce IL-10 and sphingosine kinase (13, 20).

Acute inflammation

Inflammation is initiated when a tissue suffers damage by microbial infection, injury from outside, or the loss of barrier function. PAMPs from the microbes or danger signals derived from host cell injury stimulate cells, such as macrophages and structural cells, to release chemoattractants and heat-shock proteins. Cells of the innate immune system, particularly neutrophils, and depending on the infectious triggers, other cells, such as eosinophils, are recruited by chemoattractants from the circulation to the site of the trauma (21). The endothelium of the vasculature opens to facilitate the passage of recruited cells, chemokines, and pro-inflammatory mediators (22). At the site of the trauma, neutrophils and macrophages secrete antibacterial peptides, complement factors, and ROS, so as to eradicate the potentially harmful stimuli, and the debris is scavenged by phagocytosis. Subsequently, if the resolution system is functioning properly, the inflammation is resolved and the tissue returns to normal homeostasis (23).

Necrosis

Necrosis is a form of cell death that results from toxic injury, hypoxia or stress. When there is excessive apoptosis and inefficient phagocytosis, i.e., during inflammation, the apoptotic neutrophils undergo necrosis, in what is termed 'secondary necrosis'. In both these types of necrosis, the membrane loses its integrity and toxic cellular contents and debris reach the surrounding tissues, thereby worsening the ongoing inflammation. The cellular remnants are cleared by macrophages, in a process that alters the macrophage phenotype to a more pro-inflammatory form (i.e., a classically activated macrophage). In many cases, the debris from cells that have undergone necrosis contains high levels of endogenous danger signals, such as heat-shock proteins, uric acid, histones, DNA, and high mobility group box 1 (HMGB1) protein (24). These damage-associated molecular pattern molecules (DAMPs), which are self antigens that normally are not accessible for immune system recognition but are produced or exposed during cell stress or necrosis, can be immunogenic and increase the risk for autoimmune reactions (25-26). In similarity to their microbial counterparts, the PAMPs, the DAMPs bind to various receptors, including the receptor for advanced glycation end-products (RAGE), TLR2, TLR4, and TLR9, and generate a danger signal, which in turn accelerates the inflammation cascade (26).

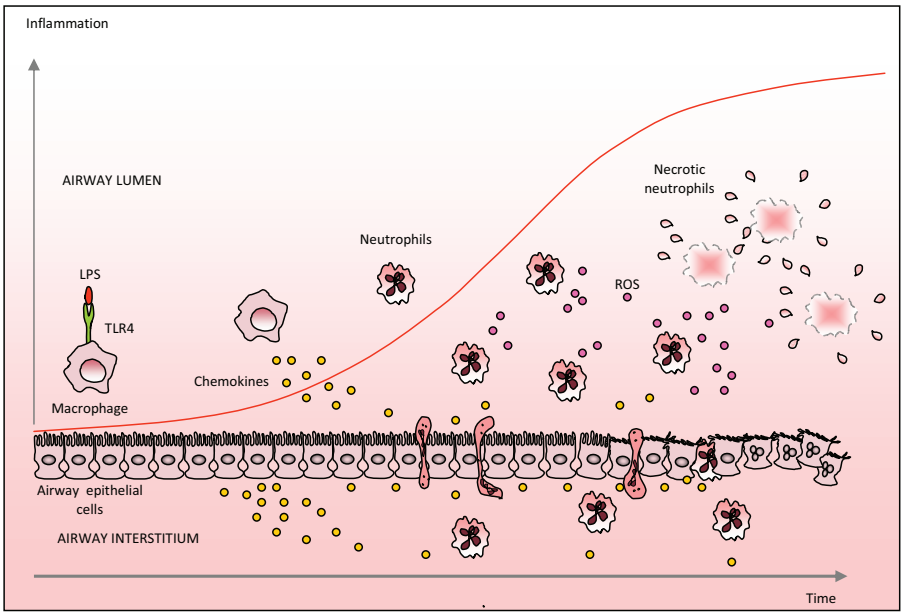

Figure 1. Acute inflammation in the airways. Inflammation is initiated by LPS binding to TLR4 on resident macrophages, which in turn release chemoattractants that recruits neutrophils. The endothelium becomes leaky to facilitate the passage of recruited neutrophils, and the epithelium is gradually destroyed. Macrophages and neutrophils secrete antibacterial mediators to eradicate the potentially harmful stimuli. The neutrophils become necrotic and the toxic cellular contents and debris exacerbate the inflammation. The macrophages and some of the epithelial cells are adapted from *Nature Reviews Immunology*.

IL-17

Cellular sources of IL-17

T helper-17 cells

Differentiation of T helper-17 cells

The differentiation of naïve T cells to T helper-17 (Th17) cells is promoted by the cytokines transforming growth factor β (TGFβ) and IL-6 (15, 27-28). TGFβ and IL-6 induce the Th17 lineage-specific transcription factors retinoic acid-related orphan receptor (ROR)γt and RORα (29-31). TGFβ and IL-6 are required for TH17-cell differentiation, partly owing to their inhibition of T helper-1 (Th1) and T-regulatory (Treg) cell differentiation. Thus, TGFβ blocks

Th1 cell commitment by inhibiting the transcription factor T-bet, and IL-6 reduces the expression of the transcription factor forkhead box P3 (FOXP3), which is critical for Treg differentiation (27). The Th17 lineage-specific transcription factors RORγt and RORα are nuclear hormone receptors that belong to a family of retinoic acid receptor-related orphan nuclear hormone receptors. Although the ligands for these nuclear hormone receptors have not been completely mapped out, studies have shown that provitamin D3 (cholesterol and 7-dehydrocholesterol) is a natural ligand for RORα (32). RORγt and RORα have been shown to have redundant and synergistic functions; in RORγt-deficient mice, Th17 cells persist, albeit in lower numbers than in wild-type mice (30). Th17 cells have also been shown to be promoted by stimulation of the aryl hydrocarbon receptor (AhR), which is a ligand-dependent transcription factor that binds many types of exogenous and endogenous ligands (33-34). These ligands include environmental toxins (e.g., dioxins), resveratrol, and lipoxin A4 (35-37).

Expansion and stabilisation of Th17 cells

Three cytokines expand and stabilise the phenotype of Th17 cells: IL-21, IL-23, and IL-1 (30, 38-44). IL-21 is an IL-6-induced cytokine that appears to act in an autocrine loop, since it is both produced by Th17 cells and promotes the expansion of Th17 cells (29-30, 39-40). IL-23 is a member of the IL-12 cytokine family, which includes IL-12, IL-23, IL-27, and IL-35, all of which are involved in the regulation of T-cell responses. IL-23 is produced by cells of the monocytic lineage, i.e., monocytes, dendritic cells, and macrophages, in response to infectious stimuli (42). The IL-23 molecule is a heterodimer of the p19 and p40 subunits. The p40 subunit is shared with IL-12, while the p19 subunit appears to be unique to IL-23 (42). IL-23 was the first cytokine implicated in the regulation of IL-17 expression, and IL-23 was initially thought to be critical for the differentiation of Th17 cells (43-44). However, it has been shown that naïve T cells do not express the IL-23 receptor (IL-23R) and do not differentiate into Th17 cells in response to IL-23 *in vitro*, suggesting that IL-23 is not required for the initial differentiation of Th17 cells (14, 45). In contrast, IL-23 inhibits the

Th1 and Treg differentiation transcription factors T-bet and FoxP3 in $CD4^+$ T cells from naïve mice, and expands Th17 cells from *in vivo*-primed memory $CD4^+$ T cells (14, 46-47). Therefore, IL-23 is important for the stabilisation and expansion of the Th17-cell phenotype. The role of IL-1 in the promotion of Th17 cells is suggested to be primarily linked to synergy with IL-23 (38).

Th17-specific cell surface markers

To date, no single Th17-specific marker unique for only Th17 cells has been identified, although marker combinations that distinguish Th17 cells have been proposed.

The receptor for IL-23 (IL-23R), which is expressed on many IL-17-producing cells, as well as on cells that apparently do not produce IL-17, is an important marker ofTh17 cells (48-49). The CC-chemokine receptor 6 (CCR6) is another receptor that is expressed on Th17 cells, although it is also found in abundance on B cells, DCs, and other subsets of T cells (50-51). CCR6 binds to CC-chemokine ligand 20 (CCL20), which is produced in various tissues, such as the joints, intestines, and lungs (50, 52-53). CCL20 recruits haematopoietic cells, and it has been associated with the generation of lymphoid tissue (50, 52-53). Th17 cells are able to produce their own CCL20, which suggests that Th17 cells use a paracrine mechanism to steer their chemotaxis to inflamed tissues (48, 50). The C-type, lectin-like receptor CD161 is expressed on subsets of NK, CD4, and CD8 T cells, and has been suggested to be a surface marker for Th17 cells (54-55). CD161 binds to the proliferation-induced lymphocyte-associated receptor (PILAR), and it has been demonstrated to function as a co-activating receptor that promotes antigen-dependent T-cell proliferation (56).

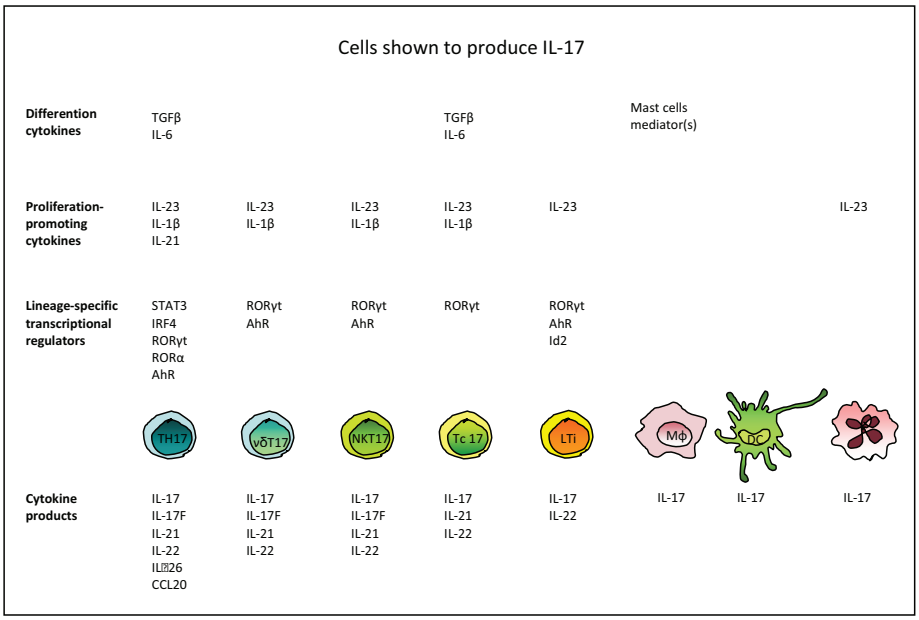

Table 1. Cells shown to produce IL-17. The macrophage and dendritic cell are adapted from *Nature Reviews Immunology*.

Other mediators produced by Th17 cells

In addition to IL-17, Th17 cells produce IL-17F, IL-21, and IL-22. It is not yet clear whether all these TH17-cell-associated cytokines can be secreted from a single cell. IL-17F will be described in more detail below. As mentioned earlier, IL-21 induces Th17 cell proliferation in an autocrine manner (29, 40, 57). IL-21 is a member of the common γ-chain family of cytokines, which includes IL-2, IL-4, IL-7, IL-9, and IL-15. The receptor for IL-21 is expressed by NK, T and B cells, DCs, macrophages, and keratinocytes, and IL-21 appears to increase the proliferation and survival of most of these cell types (58-60).

IL-22, which is a member of the IL-10 family, is important for the maintenance of tissue integrity during inflammation. The receptors for IL-22 have only been found on structural cells, and they mediate tissue production of pro-inflammatory mediators, mucins, and antimicrobial peptides (61-63). Interestingly, IL-22 production by Th17 cells appears to be dependent exclusively upon the transcription factor AHR, rather than RORγt (33).

Other IL-17-producing T cells

γδT cells

γδT cells are positioned at the border between the innate and adaptive immune systems. Although these cells are present in low numbers in the bloodstream, they comprise up to 50% of all the cells in the skin, gut, and reproductive tract (64). To date, only a few antigens have been shown to bind to the γδ TCR. The γδ TCR is usually not required for antigen recognition by γδT cells (65-68). Owing to the absence of TCR restriction, γδT cells recognize a wide array of antigens in a direct manner. These antigens include protein and non-protein antigens that are expressed endogenously by host cells or microbes. Most γδT cells have the $CD4^{-}CD8^{-}$ phenotype, with the exception of the intestinal intraepithelial γδT lymphocytes, which have the $CD8\alpha\alpha^{+}$ phenotype.

γδT-17 cells

The IL-17-producing γδT cells, termed γδT-17 cells, are the main producers of IL-17 during infections with *Listeria monocytogenes*, *Mycobacterium bovis* Bacille Calmette-Guérin, and *Salmonella enterica* (69-71). γδT-17 cells have been shown to produce IL-17 in mice stimulated with IL-23 and IL-1 and in mice in which there is direct microbial triggering of the TLR2 and dectin-1 receptors (16, 67-68, 72). The same factors needed for the differentiation of Th17 cells have been shown to be important for the development of γδT-17 cells, i.e., RORγt, AhR, IL-23 receptor, and CCR6 (67). γδT-17 cells have also been shown to produce many of the cytokines that are produced by Th17 cells, including IL-17, IL-17F, IL-21, and IL-22 (67-68).

NKT cells

Similar to the γδ T cells, NKT cells express a restricted array of TCRs. The TCRs of NKT cells are αβ TCRs that have specificities for the different glycolipid antigens presented by CD1d (73-76). The antigen-presenting molecule

CD1d is related to major histocompatibility complex (MHC) I and MHC II, and is expressed on antigen-presenting cells. The first discovered antigen presented by CD1d was the glycosphingolipid α-galactosylceramide (α-GalCer), which was originally isolated from the marine sponge *Agelas mauritianus* (76). Subsequently, other glycolipid antigens, including bacterial glycolipids, have been found to be recognized by the TCRs of NKT cells (73-74). NKT cells are categorised into subsets based on the $CD4^+$ or $CD4^-CD8^-$ phenotype and the expression of CD161. TGF-β has been implicated as a differentiation factor for NKT cells (77-78).

NKT-17 cells

The population of NKT cells that produce IL-17, NKT-17 cells, have the $CD4^-CD161^-$ phenotype (79-80). NKT-17 cells have been shown to express the integrin α-chain α_E (CD103) and IL-1R type I (CD121a) in the skin and peripheral lymph nodes of mice (80). In similarity to Th17 cells and γδ T-17 cells, NKT-17 cells express RORγt, CCR6, and the IL-23 receptor (80-83). In similarity to γδ T-17 cells, NKT-17 cells can be stimulated directly by LPS to produce IL-17 within a few hours (80). In addition to IL-17, NKT-17 cells produce IL-21 and IL-22 (84-86).

CD8 T cells

Culturing of $CD8^+$ T cells under Th17-polarising conditions results in IL-17 production and the release of IL-17 from these cells. IL-17-secreting $CD8^+$ T cells (Tc17) are observed in mice that are deficient for the TH1-polarising transcription factor T-bet (87-88). It appears that these cells lose their cytotoxic capabilities upon acquisition of IL-17 production capacity. Tc17 cells have also been detected in a population of $CD8^+$ T cells in the blood of healthy human subjects, and these cells express CCR6 (89). The IL-17 produced by Tc17 cells has been demonstrated to be important for mouse survival after primary challenge with influenza A virus into the lungs (90).

IL-17-producing cells other than T cells

Neutrophils, macrophages, and Paneth cells have been reported as being able to produce IL-17 (91-97). Although one of the first studies to demonstrate that cells other than T cells could produce IL-17 was published in 2003 (91), relatively little information on this topic has been reported since then. This area clearly warrants further investigation.

Lymphoid tissue inducer cells

Several different subtypes of lymphoid tissue inducer (LTi) cells have been identified. As these cells were discovered only relatively recently, novel subtypes are emerging continuously. As a result, the nomenclature and phenotypes that define the different subtypes of LTi cells are currently rather confusing. Human and murine LTi cells share the characteristics that they both have the $CD3^-$ phenotype and use RORγt together with AHR or Id2 (98). To date, LTi cells have been detected in the thymus, developing lymph nodes, Peyer's patches, and nasopharynx-associated lymphoid tissue (NALT) (98-99). The development of these lymphoid organs is dependent upon interactions between LTi cells and stroma lymphoid tissue organiser cells (100-101).

Previously, it has been shown that IL-23 stimulation of cells isolated from the spleens of Rag 2-/- mice, which lack lymphocytes, resulted in about 30% of the amount of IL-17 produced by the wild-type mice. The cells that produced this IL-17 were not macrophages, DCs or neutrophils, but turned out to be LTi cells (102). Seemingly, all LTi cells produce IL-22 in the absence of stimulation, and produce IL-17 after stimulation with IL-23 (103-104).

IL-17 family members

The IL-17 family comprises IL-17A, IL-17B, IL-17C, IL-17D, IL-25 (formerly IL-17E), and IL-17F. Many studies have focused on IL-17A and IL-17F, and these two family members are the most closely related (28). As described in the

previous section, IL-17 is produced by many different types of haematopoietic cells. Whereas IL-17F can be produced by most of these cells, it has also been shown to be produced by non-haematopoietic cells, such as lung epithelial cells (105).

Downstream actions of IL-17

IL-17R

The mRNA for IL-17 receptor A (IL-17RA) is expressed in most cell types (106). The corresponding IL-17RA protein is produced by structural cells and haematopoietic cells, such as neutrophils, macrophages, dendritic cells, and T cells (107-110). IL-17RA is a type I transmembrane protein that lacks sequence similarity to any other known cytokine receptor (108). However, certain similarities with the IL-1β receptor (IL-1βR) and TLR regarding the signalling cascade have been noted. One of these similarities is that IL-17RA contains a conserved motif in the cytoplasmic domain, SEF/IL-17R (SEFIR), which is homologous to the Toll/IL-1βR (TIR) domain (111). It has recently been demonstrated that IL-17 binds and signals through a heterodimeric receptor complex that consists of IL-17RA and IL-17RC (112). Both homodimers (composed of IL-17 or IL-17F) and heterodimers (composed of IL-17 and IL-17F) can bind to this receptor complex, albeit with somewhat different affinities (107, 113-114).

IL-17A and IL-17F receptor affinities differ between mice and humans

In humans, IL-17 binds to IL-17RA with high affinity, whereas IL-17F binds to IL-17RA with an almost 1000-fold lower affinity, which implies that IL-17RA cannot bind IL-17F under physiological conditions (115). It has also been shown that an anti-IL-17RA antibody blocks the responses to IL-17 and IL-17F, suggesting that both of these cytokines are dependent upon a functional IL-17RA for signalling (116). In contrast, in mice, while only IL-17F can bind IL-17C, both IL-17 and IL-17F can bind IL-17RA (115).

IL-17RA and IL-17RC have different distribution patterns

The distribution patterns of IL-17RA and IL-17RC are divergent in different tissues. The expression of IL-17RA is higher in haematopoietic tissues, while IL-17RC is expressed at higher levels in structural cells of the prostate, liver, kidney, thyroid, and joints (115, 117). In addition, $CD4^+$ cells express IL-17RA, but not IL-17RC (107). IL-17 alone has been shown to induce signals in these cells, suggesting that a heterodimeric receptor complex consisting of IL-17RA together with IL-17RC is not essential for signalling (107).

IL-17R family receptor-ligand interactions

It has been demonstrated that the IL-17-related cytokine IL-25 (formerly IL-17E) binds to IL-17RA when it is present in a heterodimeric receptor complex with IL-17RB (114, 118-119). IL-17RD is also able to form a heteromeric receptor complex with IL-17RA, although the ligand for this receptor complex is currently unknown (120). Since IL-17RA is used by several IL-17-related cytokines and can form different heterodimeric receptor complexes, it appears to be analogous to gp130, which is a shared cytokine receptor subunit and a common signal transducer for molecules of the IL-6 family (121-122).

Signalling downstream of IL-17R

As described earlier, IL-17RA engages the SEFIR domain-containing adaptor ACT1 to mediate various downstream events (123). ACT1 recruits TNFR-associated factor 6 (TRAF6), which is a key adaptor protein and an upstream activator of the canonical nuclear factor-κB (NF-κB) pathway. The transcription factor CCAAT/enhancer-binding protein-δ (C/EBPδ) seems to be downstream of NF-κB (124-125). A distinct arm of the signalling network is linked to another functional domain on IL-17RA, termed the C/EBPβ-activation domain (CBAD), which instead of activating the NF-κB pathway induces expression of the transcription factor C/EBPβ (126-127).

Negative regulation of IL-17RA signalling

Two inhibitory pathways are initiated by binding to IL-17RA, both of which lead to the phosphorylation of the C/EBPβ domain, resulting in down-regulation of the transcriptional capacity of C/EBPβ. The first inhibitory pathway includes extracellular signal-regulated kinase (ERK)-mediated phosphorylation of C/EBPβ at threonine 188 (125), and in the second inhibitory pathway, CBAD induces the phosphorylation of C/EBPβ at threonine 179 (125).

Effects of IL-17

IL-17 acts as an important player in the host defence against both extracellular and intracellular bacteria, fungi, and viruses by promoting the mobilisation of neutrophils to the site of inflammation and inducing the release of microbicidal substances (49, 128-132). IL-17 induces neutrophil accumulation by increasing the proliferation of neutrophils through the stimulation of structural cells to produce the neutrophilic growth factors granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) (133-136). IL-17 also contributes to the recruitment of neutrophils by stimulating structural cells to release CXC-chemokines, such as KC (also called CXCL1), macrophage inflammatory protein 2α (MIP2α; also called CXCL2), as well as the human-specific CXC-chemokine IL-8 (91, 137-140). IL-17 induces microbial killing by enhancing the production and secretion of microbicidal peptides, such as cathelicidin (also called LL-37), β-defensin, and S100A8, by structural cells and neutrophils (61, 141-143). In addition, IL-17 up-regulates the constitutive release of other angiogenic factors from synovial fibroblasts, all of which are involved in the proliferation of endothelial cells (144). IL-17 mediates the induction of IL-6, and also stimulates the production of the pro-enzymatic inactive forms of MMP-2, MMP-3, MMP-9, and MMP-13 (145-148). IL-17 has also been shown to enhance osteoclast differentiation and activity through the

promotion of receptor activator of NF-κB ligand (RANKL) and other osteoclastogenic factors, such as prostaglandin E2 (149-152).

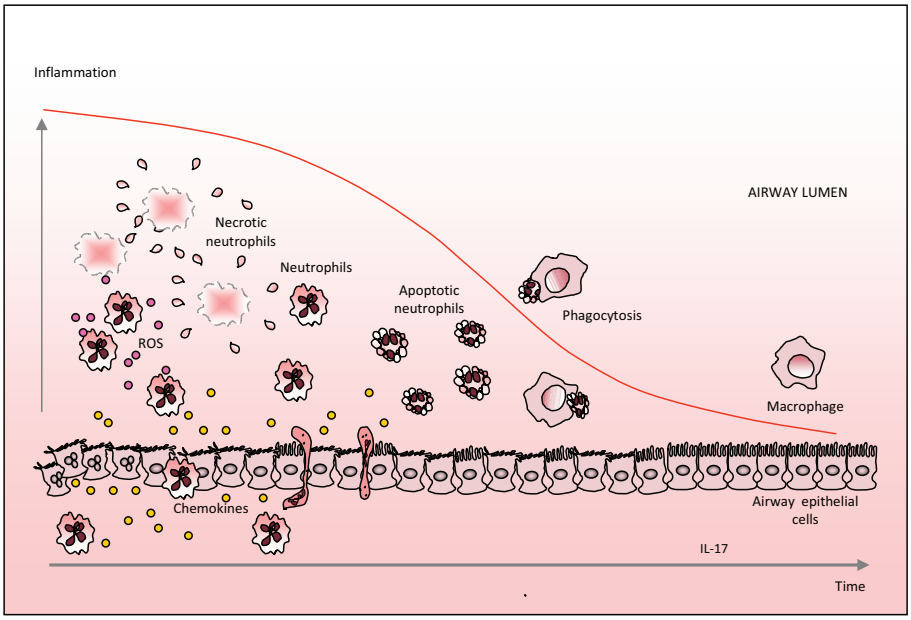

Figure 2. Resolution of inflammation. When the infectious stimuli are removed, the inflammatory triggers are diminished, which reduces the recruitment of inflammatory cells. Pro-resolution mediators induce the phagocytosis of apoptotic neutrophils and block neutrophil recruitment from the blood to the tissues. Phagocytosis of bacterial remnants and apoptotic cells further promotes the resolution of inflammation through the generation of anti-inflammatory cytokines. The macrophages and some of the epithelial cells are adapted from *Nature Reviews Immunology*.

Resolution of inflammation

When microbes are eradicated from the body, the inflammatory triggers are diminished, and the levels of residual effectors, which include both PAMPs, such as LPS, zymosan, and peptidoglycan (PAMPs), as well as DAMPS, are gradually reduced. With fewer microbial stimuli, the recruitment of cells to the site of inflammation declines. With fewer inflammatory cells, there are fewer apoptotic and necrotic cells. Thus, the production of pro-inflammatory

mediators is reduced, and those mediators that are already produced are eventually degraded (153-154).

Clearance of inflammatory cells

Apoptosis of neutrophils

Neutrophils are recruited to the site of infection or injury so as to kill microbes and to phagocytose cells and debris. Neutrophils are short-lived, and after they exert their activities at the inflammatory site, they undergo apoptosis. Neutrophils retain membrane integrity during the different stages of apoptosis, which include shrinking, chromatin condensation, cell-membrane blebbing, and DNA fragmentation. Thereafter, the neutrophil disintegrates into numerous membrane-bound apoptotic bodies, without releasing their intracellular contents to the surrounding tissues (155). The apoptotic bodies are subsequently phagocytosed, mainly by macrophages, but also by other cells in the vicinity. Apoptosis followed by clearance by macrophages is important for proper resolution of acute inflammation (155-156). Macrophages that phagocytose apoptotic neutrophils release anti-inflammatory mediators, such as TGFβ and IL-10, which further dampen the inflammation (157-158).

Phagocytosis of apoptotic neutrophils

The phagocytosis of apoptotic cells involves the following steps: 1) sensing of the presence of apoptotic cells that display 'find-me' signals, such as lipid lysophosphatidylcholine (LPC); 2) recognition of apoptotic cells that have 'eat-me' signals, such as phosphatidylserine (PtdSer). The phagocytic receptors that recognize 'eat-me' signals as well as other apoptotic cell characteristics include scavenger receptors, phosphatidylserine receptors, the thrombospondin receptor, integrins, and complement receptors (159); and 3) reorganisation of the cytoskeleton to form the phagocytic cup, which gradually surrounds the

apoptotic cell and internalises it, thereby forming an endosome. The ingested apoptotic cell is processed and degraded following fusion of the phagocytic endosome with the lysosome. Various anti-inflammatory signals participate in the different stages of apoptotic cell phagocytosis (160)

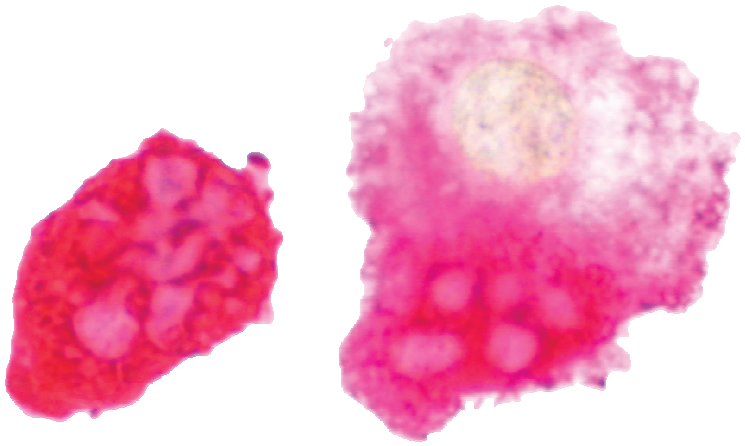

Figure 3. Photographs of an apoptotic murine neutrophil (left) and a bronchoalveolar macrophage phagocytosing an apoptotic neutrophil (right) from the bronchoalveolar space. The cells are stained with the anti-neutrophil antibody NIMP-R14 and Liquid Permanent Red.

Chronic inflammatory diseases

When an acute inflammation that has been initiated by infection with a microbe or an injury does not resolve, the result may be a chronic inflammation. The reason why an inflammation sometimes turns into a chronic inflammation is not clear. It is known that chronic inflammation often results from a combination of several factors that act concomitantly. These factors include genes, microbes, and environmental parameters, such as stress, pollutants, and lifestyle. On the cellular level, the mechanisms that contribute to chronic inflammation include necrosis and immunogenic responses to self antigens. Whichever factors are responsible, the outcome is more or less the same, i.e., tissue infiltration of cells

and inflammatory mediators, granuloma formation, and fibrosis (161). Chronic inflammatory diseases of the respiratory tract are characterised by infiltration of the tissues and airways by lymphocytes, neutrophils, and monocytes, which release proteases, such as matrix metalloproteinases, leading to elastin degradation and emphysema. Neutrophil-produced elastase causes mucous hypersecretion by goblet cells. Epithelial cells and macrophages release TGFβ, which induce the proliferation of fibroblasts, resulting in fibrosis of the small airways (162-163).

Anti-inflammatory pharmacology

Anti-inflammatory drugs constitute a large group of drugs with multiple mechanisms of action. Many of these drugs, such as dexamethasone, originate from endogenous mediators that are proven to exert anti-inflammatory actions, whereas others originate from microbes, e.g., cyclosporine and rapamycin. Currently, many new anti-inflammatory drugs in the form of antibodies directed against endogenous pro-inflammatory mediators, such as TNFα and the p40 subunit shared by IL-12 and IL-23, are employed.

Dexamethasone and cyclosporine A

Dexamethasone and cyclosporine A are two of the most commonly used anti-inflammatory drugs for acute and chronic inflammation (164-165). Dexamethasone is a glucocorticoid derived from the endogenous anti-inflammatory mediator cortisol, which is synthesised in the cortex of the adrenal gland (166). Glucocorticoids are pleiotropic and mediate their multiple anti-inflammatory effects through many effector molecules. In addition to their anti-inflammatory properties, glucocorticoids promote the resolution of inflammation by stimulating the production of annexin I and promoting macrophage phagocytosis of apoptotic neutrophils (167). Glucocorticoid receptors are ubiquitously expressed on cells. Binding to its receptor leads to either activation or repression of gene transcription. There are also

glucocorticoid receptors that are independent of gene transcription, and they mediate a faster response (166).

Cyclosporine A is primarily used for the prevention of transplant rejection and to treat severe forms of autoimmune disease (168). Cyclosporine A exerts its immunosuppressive activities by forming a complex with the cytosolic protein cyclophilin. This complex inhibits calcineurin, which is required for dephosphorylation of the cytosol-phosphorylated transcription factor NF-AT. (169) This failure to dephosphorylate NF-AT results in decreased transcription of the genes for cytokines, such as IL-2, IL-3, IL-4, and IFN-γ (169). This blocks T-cell activation, and also has other effects, such as the prevention of mitochondrial permeability and cytochrome c-induced apoptosis (170-171).

Paradigms of IL-17 over the years

IL-17 was first discovered about 15 years ago, at a time when IL-17 was mainly associated with host defence and some pro-inflammatory effects. Ten years later, the IL-17-producing memory CD4+ T cell was found to be a specific T-helper cell that was distinct from Th1 and Th2 cells. This Th17 cell has been strongly associated with autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, and psoriasis. More recently, it was shown that Th17 could induce the production of cytokines other than IL-17, and that cells other than Th17 cells could produce IL-17. The biology of IL-17 became more complex when it emerged that IL-17 and IL-17F in many cases have redundant roles, and that their binding affinities to the corresponding heterodimeric receptor complex differ, which also appear to differ between humans and mice.

Thus, over the years, research on IL-17 has undergone major reviews, and undoubtedly additional changes to the paradigm are yet to come. One example of this type of development is the new concept that IL-17 might act as a mediator of inflammation resolution. Clearly, the various roles of IL-17 in different disease settings need to be studied further.

AIMS

General aims

The overall aims of this thesis are to investigate endogenous and exogenous methods to regulate the production of IL-17 and to elucidate the role that IL-17 plays in resolving ongoing inflammation.

Specific aims

The specific aims of the thesis are:

I. To determine whether cells in the lung produce IL-17 after exposure to LPS isolated from the Gram-negative *E. coli* bacteria and whether anti-inflammatory pharmacotherapy can be used to regulate the production of IL-17 in these cells;

II. To ascertain the contributions of IL-17 to neutrophil apoptosis, as well as to neutrophil turnover through the regulation of macrophage phagocytosis of apoptotic neutrophils;

III. To determine whether IL-17 down-regulates the release of the upstream regulator IL-23 both *in vivo* and *in vitro*.

METHODS

In vivo experiments

Mice

Male BALB/c, C57BL/6, and C57BL/6 IL-17-knockout mice were maintained under standard conditions with access to food and water *ad libitum* (172). Permission for all experiments was obtained from the Ethics Committee for Animal Studies, Gothenburg, Sweden, in accordance with national animal welfare legislation.

Intranasal instillation

To perform intranasal (i.n.) instillation, mice were anaesthetised transiently using isofluorane, and instilled i.n. with lipopolysaccharide (LPS) or recombinant IL-17 in 50 μl of phosphate-buffered saline (PBS). This volume was chosen because a study on the distribution of i.n. instillations in mice showed that i.n. administration of a 50-μl volume achieved maximal efficacy, with approximately 60% of the test substance being deposited in the bronchoalveolar space (173). Increasing the volume to 75 μl did not increase the yield (173). An LPS dosage of 10 μg was chosen in our experiments because this has been described as the sub-maximal effective dosage for i.n. instillation (174-175). Three μg of the recombinant mouse IL-17 protein were used, as earlier studies have shown that dosages in the range of 1.5 to 10.0 μg of recombinant mouse IL-17 protein are relevant i.n. dosages (176-177).

Intravenous injections

Some mice were inoculated intravenously (i.v.) in the tail vein with 0.22×10^8 *Staphylococcus aureus*. The *S. aureus* bacteria used were derived from the toxic shock syndrome toxin 1–producing LS-1 strain (178-179).

Intraperitoneal injections

Some mice were injected intraperitoneally (i.p.) with a neutralising monoclonal anti-mouse IL-17 antibody (aIL-17 Ab) or the isotype control, IgG2a Ab. Other mice were injected i.p. with the glucocorticoid receptor agonist dexamethasone (180) or the inhibitor of the endogenous calcium/calmodulin-dependent phosphatase calcineurin, cyclosporine A, or vehicle (PBS) (181).

Euthanasia and blood sampling

Mice were anesthetised i.p. using ketamine (Ketalar) and xylazine (Rompun). The mice were euthanised by puncture of the left heart ventricle, and blood was drawn. The red blood cells were lysed in lysis buffer, and the remaining white blood cells were washed three times.

Bronchoalveolar lavage

After tracheotomy, mouse airways were washed with PBS to obtain bronchoalveolar lavage (BAL) samples. Although the technique used for BAL was always performed in the same way, the volume of lavage fluid obtained varied between different experiments. Low volumes (0.2-0.25 mL) and 4 × 0.25 mL sampling were utilised to sample cells and proteins from the upper airways, while larger volumes (4 × 0.5-1.0 mL) were collected when cells from the whole bronchoalveolar space were needed or when it was desirable to collect as many cells and proteins from the airways as possible. All the BAL samples recovered from a single mouse were pooled, the recovery volume was recorded, and the BAL samples were kept on ice until centrifugation. After centrifugation, the cell-free BAL fluid was frozen for subsequent IL-17 protein assays. The BAL cells were resuspended in PBS that contained bovine serum albumin (BSA), and the total cell numbers were determined using a Bürker chamber. When BAL samples are taken, the recovered volume of lavage differs somewhat for different mice due to unintentional methodological variations. Since the cells are centrifuged and pelleted, these variations in recovery volume affect the number of cells present in each BAL sample. To compensate for these variations, the

quantity of cells is presented as the number of cells per recovery volume. As the concentration of proteins in the cell-free BAL fluid used for ELISA is considered to be independent of the recovery volume, the ELISA data are not corrected for BAL recovery.

Lung tissues and spleens

Lungs used for mRNA analysis were perfused with PBS *via* the right ventricle of the heart. The lungs were removed, snap frozen in liquid nitrogen, and stored at -80 °C until further processing. The lungs used for flow cytometry were perfused with PBS, removed, and placed in a buffered solution (Hanks balanced salt solution with Golgi Stop protein secretion blockage). The lungs from *S. aureus*-infected mice were aseptically dissected, placed on ice, homogenised, serially diluted in PBS, and spread on blood agar plates. The colony forming units (CFU) per lung were assessed after 24 h of incubation at 37°C. The spleens were surgically removed from mice, and splenocytes were isolated as described below.

In vitro experiments

Bronchoalveolar lavage in humans

The study protocol was approved by the Ethics Committee in Gothenburg, and the full protocol has recently been published by Glader et al. (182). Briefly, during a first bronchoscopy, a balloon-tipped catheter was inserted through the bronchoscope, placed in a segmental bronchus, and inflated with air to occlude the segments chosen for challenge. Ten millilitres of PBS followed by 10 mL of air were then instilled into the bronchial segment. The bronchoscope was then retracted and the head end of the operating table was elevated with the subject in place for 1 hour, to minimise spread of the instilled PBS. During a second bronchoscopy, the same protocol was followed but with the inclusion of a BAL procedure (3 × 50 mL of PBS) instead of PBS instillation. Endobronchial

photographs were taken bilaterally on both occasions, to ensure that the BAL sampling was performed in the PBS-exposed segment.

Isolation of cells

The total cell numbers in the BAL samples and blood samples were determined using a Bürker chamber, and cytospin slides were prepared. Differential cell counts was carried out by counting 400 cells per cytospin slide stained with May-Grünewald-Giemsa reagent, as described elsewhere (138).

Isolation, labelling, and ageing of neutrophils

Neutrophils from the blood of naïve BALB/c mice were isolated using the Anti-Ly-6G MicroBead Kit, as described in the product manual. Positively selected mouse neutrophils were labelled with a fluorescent marker, carboxyfluorescein diacetate succinimidyl ester (CFDA SE), and cultured (1×10^6 neutrophils/mL) for 48 hours.

Isolation of human monocytes and differentiation of monocyte-derived macrophages

Monocytes were harvested from the blood samples of healthy human volunteers. Peripheral blood mononuclear cells were collected by density centrifugation over a Ficoll gradient. Monocytes were then isolated from the fraction of mononuclear cells by negative selection using Monocyte Isolation Kit II as described in the product manual. To derive monocyte-derived macrophages, monocytes were cultured at 37°C in 5% CO_2 for 5 days in supplemented medium (RPMI 1640 with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin, 1 mM sodium pyruvate, and 2 mM L-glutamine, together with 10 ng/mL of recombinant GM-CSF.

Isolation of BAL macrophages

Human BAL macrophages and BAL macrophages from naïve mice were isolated from the total cell population in the BAL fluid by adherence for 2 hours

or 3 hours in non-supplemented RPMI 1640 medium at 37°C in 5% CO_2. After the incubation period, the cells were washed two or three times to remove non-adherent, non-macrophage cells.

Isolation of murine CD3+ splenocytes

For the enrichment of $CD3^+$ cells, mouse spleens were minced, the red blood cells were lysed using a hypotonic solution, and the remaining cells were filtered through a 40-μm pore size cell strainer, to obtain a single-cell solution. The cells were washed in PBS with 0.5% BSA and the $CD3^-$ cells were depleted using a magnetically labelled biotin-conjugated antibody cocktail (Pan T-cell isolation kit, as described in the product manual.

Stimulation of cells

Neutrophils and cells of the monocytic lineage

Murine bronchoalveolar and human monocyte-derived macrophages for phagocytosis experiments were cultured in 16-well chamber slides and primed in supplemented medium that contained LPS (1 ng/mL) for 48 hours. All human cells of the monocytic lineage and the murine BAL macrophages were subsequently stimulated with 100 ng/mL LPS, together with 0.1, 1, 10 or 100 ng/mL recombinant IL-17 or its vehicle (supplemented medium alone) for 24 hours. There are few data on the local physiological levels of IL-17 in humans and mice. However, one published study on patients with rheumatoid arthritis gives the mean concentration of IL-17 in the synovial fluids as approximately 1 ng/mL (170).

Some of the human monocyte-derived macrophages were also stimulated with the Ras-related C3 botulinum toxin substrate 1 (Rac1) inhibitor NSC23766 at concentrations of 0 (vehicle for the Rac1 inhibitor), 50, 100, 200 or 300 μM.
After 24 hours of stimulation, some of the human cells of the monocytic lineage and the murine BAL macrophages were harvested. The conditioned medium

was aspirated and centrifuged. The cell-free centrifugation supernatant was then collected and frozen at -80°C for later analysis. This supernatant is hereinafter referred to as 'cell-free medium'.

Co-culturing of macrophages with aged neutrophils or latex beads

After 24 hours of stimulation, the conditioned media from the murine bronchoalveolar and human monocyte-derived macrophages were aspirated. The cell-free supernatants were frozen at -80°C for later analyses of soluble lectin-like oxidised low-density lipoprotein receptor-1 (sLOX-1) and macrophage inflammatory protein-2 (MIP-2). Fresh supplemented medium that contained recombinant IL-17, LPS or vehicle alone was then added to the macrophages, thereby maintaining the previous stimuli (see above). The macrophages were subsequently exposed to either the fluorescently-labelled aged neutrophils or to fluorescent yellow-green carboxylate-modified latex beads (diameter of 1 μm). After 2 hours of exposure (see above), the culture medium was aspirated, and neutrophils or beads that had not been phagocytosed by the macrophages were removed by washing three times with with PBS. The remaining cells were then fixed in 4% formaldehyde. The walls of the chamber slide were removed, and a cover glass was mounted on the slide. The nuclei of the macrophages were fluorescently labelled by adding 7AAD to the mounting medium.

CD3+ cells and adherent BAL cells

Negatively selected CD3-positive cells from spleens and adherent mononuclear BAL cells were isolated as described above. CD3-positive cells were seeded insupplemented medium together with the adherent mononuclear BAL cells. This mixed culture was pre-treated with the calcineurin phosphatase inhibitor cyclosporine A (10^{-6} M) (181), the glucocorticoid receptor agonist hydrocortisone (10^{-6} M) (180, 183) or vehicle alone. Ethanol was used as the solvent for these chemicals; the final concentration of ethanol did not exceed 0.1% and it was added at the same concentration to the negative and positive

controls. Hydrocortisone was chosen as the glucocorticoid receptor agonist because it is more water soluble than dexamethasone. Thirty minutes after the pre-treatment, the cells were stimulated with 100 ng/mL LPS, with a positive control (calcium ionophore A 23487 [CI] at 1 μg/mL plus phorbol 12-myristate 13-acetate [PMA] at 2 ng/mL) and a negative control (RPMI 1640 only) and incubated for 20 hours, after which the conditioned cell media were harvested. The culture media were then centrifuged to remove cells, and subsequently frozen at -80°C for subsequent assays of IL-17.

Neutrophil apoptosis assay

Positively selected neutrophils (see above) were cultured at 37°C in 5% CO_2 in supplemented medium and stimulated with 1, 10, or 100 ng/mL recombinant mouse IL-17 protein or medium alone (vehicle; a negative control). After 48 hours of incubation, the cell-free supernatants were frozen at -80°C for later analysis of myeloperoxidase (MPO), and neutrophil survival was assessed using the Annexin V–PE apoptosis detection kit. Cells were sorted using a FACScan flow cytometer, and the data were analysed using the CellQuest software. The results are presented as percentages of viable (Annexin V^-, $7AAD^-$), apoptotic (Annexin V^+, $7AAD^-$), and necrotic (Annexin V^+, $7AAD^+$) neutrophils.

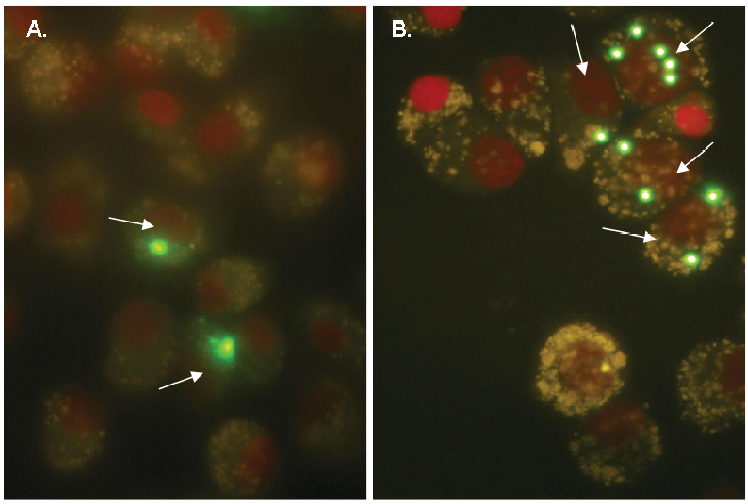

Figure 4. Fluorescence images of IL-17-stimulated murine bronchoalveolar macrophages that have phagocytosed. Macrophages were primed with LPS for 48 hours and incubated with rmIL-17 for 24 hours in vitro before 2 hours of co-incubation with aged neutrophils or latex beads. (a) Phagocytosis of CFDA SE-labeled neutrophils (green). (b) Phagocytosis of latex beads (green; indicated by arrows) (original magnification ×100).

Assessment of phagocytosis

The chamber slides with adhered macrophages were photographed using a fluorescence microscope, and at least 500 randomly selected macrophages from each of the wells of the chamber slides were counted (Figure 4). The percentage phagocytosis was calculated by dividing the number of macrophages (red fluorescent nuclei) that had phagocytosed aged neutrophils (green fluorescence) or latex beads (green fluorescence) by the number of counted macrophages. A phagocytic index was calculated as the total number of phagocytosed latex beads found in the macrophages divided by the total number of macrophages that were counted.

The phagocytic index, which provides more information than the percentage phagocytosis, could not be used when quantifying the phagocytosis of apoptotic neutrophils, since the phagocytosed neutrophils occasionally were fragmented into smaller pieces (Figure 4a), which meant that the exact number of

neutrophils phagocytosed by each macrophage could not be determined. Therefore, the percentage of neutrophil-containing macrophages was used when quantifying the phagocytosis of apoptotic neutrophils.

Enzyme-linked immunosorbent assay

The cell-free BAL fluids from mice and the cell-free media from *in vitro*-cultured cells were assayed for IL-23 (p19/p40), IL-12 (p70), IL-17, MPO, sLOX-1, and MIP-2 using enzyme-linked immunosorbent assays (ELISAs). The instructions in the product manuals for the respective ELISA kits were followed

mRNA measurements

Total RNA was isolated from 15 mg of frozen lung tissue, which was ground to a fine powder under liquid nitrogen, using the RNeasy kit according to the manufacturer's instructions. The purified total RNA preparation was used as a template to generate first-strand cDNA, as described previously (184). Quantitative Real-Time PCR was performed as described previously using the ABI PRISM 7900 HT Sequence Detection System (185) and validated Assays-on-Demand TaqMan primers for IL-17. Briefly, IL-17 gene expression was quantified using multiplexing single reactions, whereby the gene of interest was standardised to the control (18S rRNA). An individual sample from the control group was then arbitrarily assigned as a calibrator, against which all the other values are expressed as fold-differences.

Flow cytometry

To study in greater detail the IL-17-containing T lymphocytes, flow cytometric analysis of the intracellular expression of IL-17 in CD3-positive cells from lung tissues and BAL suspensions was conducted. Flow cytometry was performed as described previously (186). Briefly, BAL was performed using PBS with Golgi

Stop, and the lungs were mechanically disrupted in a 100-μm nylon mesh cell strainer, followed by filtration (40-μm pore size) to remove tissue fragments. Then, both the BAL and lung cells were incubated with a blocking solution, incubated with a PerCP-conjugated anti-CD3 antibody, and fixed in paraformaldehyde (4%) at room temperature. After re-suspension in saponin buffer, the cells were incubated with a PE-conjugated rat anti-mouse IL-17 monoclonal antibody, followed by two washes with saponin buffer. Finally, the cells were washed, re-suspended, and analysed using the FACScan flow cytometer. Data were analysed using the CellQuest software.

Statistical analysis

Data are expressed as mean with standard error of the mean (SEM) [mean (SEM)]. Differences were considered to be statistically significant for p-values < 0.05 and not statistically significant (ns) for p-values > 0.05. Unless otherwise stated, all n values refer to the numbers of independent experiments for each treatment group.

Paper I

Correlation analyses were conducted utilising the Spearman rank correlation test. The Mann-Whitney U-test (preceded by the Kruskal-Wallis test for multiple comparisons) was utilised for comparison of groups.

Paper II

The results of the neutrophil survival assay and ELISA assays for MIP-2 and sLOX-1 ($n \geq 6$), were analysed using a non-parametric paired test, i.e., the Wilcoxon signed rank test. The results of the phagocytosis and MPO ELISA assay were analysed as maximum induced increase (i.e., change). The maximum induced change was calculated by subtracting the maximum response caused by the most effective concentration of recombinant IL-17 with the response

caused by the vehicle in that particular experiment. The Mann-Whitney *U*-test was used for statistical analysis of the difference between the maximum induced change response and the vehicle response.

Paper III

One- or two-tailed Student's *t*-tests were used for statistical analysis of the data for mouse BAL fluids and human monocytes *in vitro*, as appropriate. One-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test, was used for statistical analysis of the data for the BAL samples when more than two groups were compared. The concentration-response data from human monocyte-derived macrophages *in vitro* were analysed using repeated measures ANOVA. The term *n* refers to the number of mice or to the number of different blood donors used in each treatment group.

RESULTS

Paper I

LPS treatment increases IL-17 release and neutrophil numbers in the murine bronchoalveolar space

Intranasal stimulation with LPS increased the concentration of free, soluble IL-17 protein in BAL fluid from the bronchoalveolar space (Figure 5) and it also increased the number of neutrophils and other inflammatory cells in the BAL sample. The IL-17 concentration was highest on Day 1 post-treatment, and gradually decreased in a time-dependent manner. Two days after LPS stimulation, there was a correlation between the concentration of IL-17 and the number of neutrophils in the BAL samples.

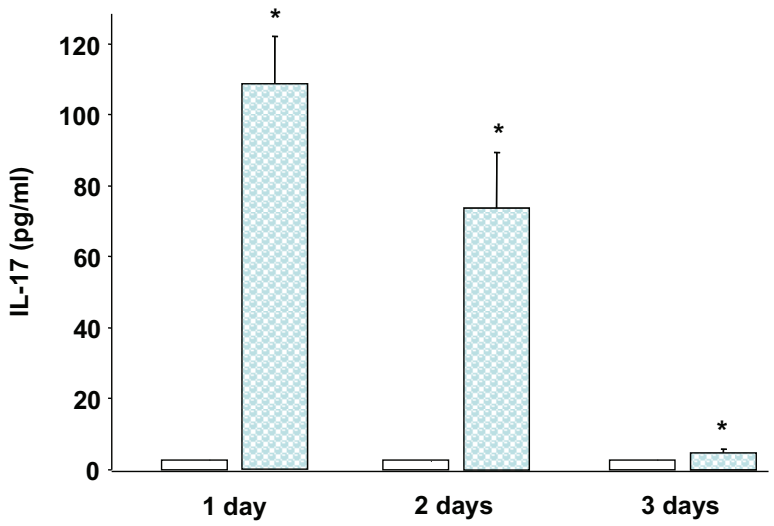

Figure 5. Concentrations of IL-17 in BAL fluid. The IL-17 levels on Days 1, 2 and 3 after exposure to LPS (blue columns) are compared to those of the negative control (PBS, white columns) (*$p<0.05$, n=3-9). Data are presented as mean ± SEM.

Pharmacotherapy decreases IL-17 release and BAL cell numbers in the bronchoalveolar space

Pre-treatment with a high dose of dexamethasone attenuated the LPS-induced increase in free, soluble IL-17 protein in BAL fluid. Pre-treatment with cyclosporine A and low dosage of dexamethasone reduced the LPS-induced increase in IL-17 in the BAL samples, albeit to a lesser extent. Considering the LPS-induced increase in the number of BAL cells, the high dosage of dexamethasone exerted a general inhibitory effect on the numbers of all cell types, whereas cyclosporine A exclusively decreased on the numbers of BAL neutrophils.

Pharmacotherapy decreases numbers of CD3+ IL-17+ cells in lung tissues and in the bronchoalveolar space

Intranasal LPS exposure increased the total number of cells in the lung tissues of the mice, as compared with the negative control. This increase was fully attenuated by the high, but not by the low dose of dexamethasone or cyclosporine A. However, for the $CD3^+$ cells in the lung tissue samples, there was no substantial effect of either treatment (Figure 6A). Interestingly, LPS treatment also increased the number of IL-17-containing $CD3^+$ cells in this compartment, from a reproducibly low level to a substantially higher level, and only the high dose of dexamethasone attenuated this increase (Figure 6B).

Similar to the results observed for the lung tissues, intranasal LPS exposure increased the total number of BAL cells compared to control treatment. The high dosage of dexamethasone and the dosage of cyclosporine A partially inhibited this increase in BAL cell numbers. It is noteworthy that in contrast to the situation in the lung tissues, intranasal LPS exposure increased both the total number of $CD3^+$ cells and the number of IL-17-expressing $CD3^+$ cells in the BAL samples (Figure 6C and D). Only the high dosage of dexamethasone totally attenuated these responses to LPS (Figure 6C and D).

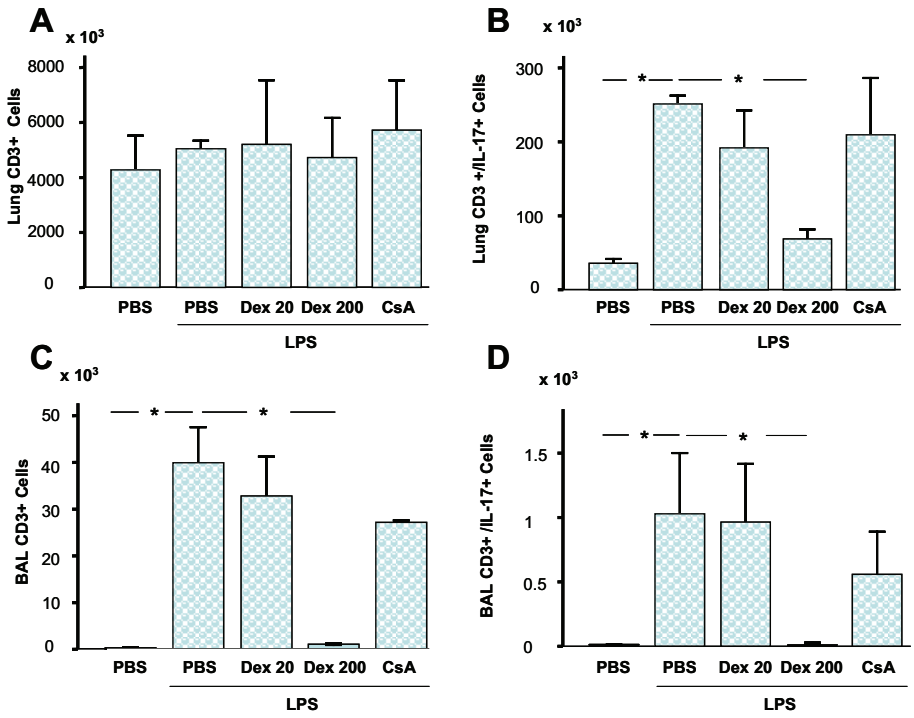

Figure 6. Cells in lung tissues and in the bronchoalveolar space after treatment with LPS and pharmacotherapy. Effects of pre-treatment (i.p.) with dexamethasone (Dex, 20 or 200 mg) and cyclosporine A (CsA, 500 mg) on the total number of CD3+ cells and IL-17-containing CD3+ cells in mouse lung tissue (A, B) and BAL samples (C, D) harvested two days after exposure to LPS compared to negative and positive control. Data presented as mean ± SEM (*= $p \leq 0.05$; n=3).

High proportion of CD3-IL-17+ cells in lung tissues and in the bronchoalveolar space

In the lung tissues of mice, the percentage of $CD3^+$ cells was similar to that of $CD3^-$ cells among all the IL-17-containing cells after LPS exposure (Figure 7). However, in BAL samples, the percentage of $CD3^+$ cells was lower than that of $CD3^-$ cells among all the IL-17-containing cells after LPS exposure (Figure 7).

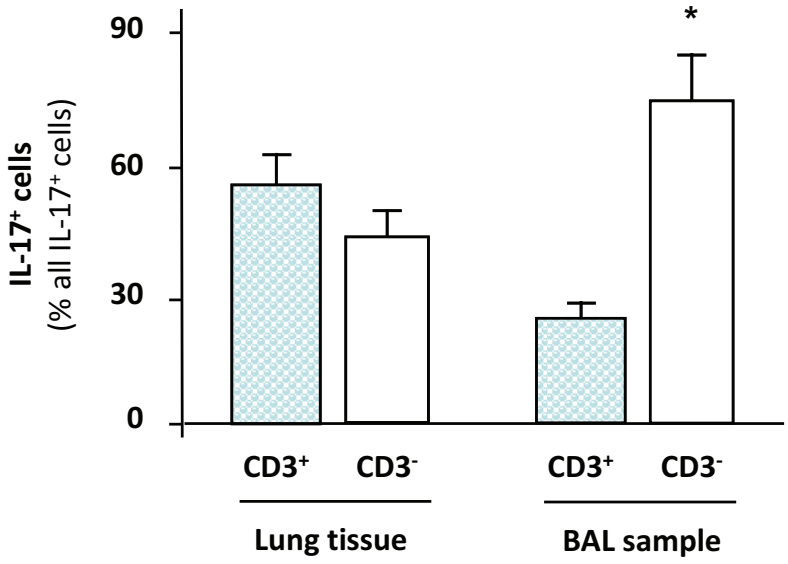

Figure 7. Percentages of CD3+ and CD3- cells in the population of IL-17-containing cells in lung tissues and in the bronchoalveolar space. A. In the lung tissues, the percentage of CD3+ cells is similar to that of CD3- cells among all the IL-17-containing cells after LPS exposure ($p=0.66$, $n=3$). B. In the BAL samples, the percentage of CD3+ cells is lower than that of CD3- cells among all the IL-17-containing cells after LPS exposure (*$p<0.05$, $n=3$). Data presented as mean ± SEM.

Pharmacotherapy decreases IL-17 mRNA expression in lung tissues and reduces IL-17 release in vitro

Both doses of dexamethasone attenuated the LPS-induced increase in IL-17 gene transcripts in lung tissue samples. LPS stimulation caused an increase in the concentration of IL-17 protein released into the conditioned medium of co-cultures of $CD3^+$ spleen cells and adherent mononuclear BAL cells. Both hydrocortisone and cyclosporine A attenuated the LPS-induced increase of released IL-17.

Paper II

IL-17 enhances neutrophil apoptosis and MPO release

Stimulation of murine blood-derived neutrophils with 100 ng / mL of IL-17 increased the percentage of apoptotic neutrophils in the *in vitro* cultures. In contrast, the percentage of viable murine neutrophils was decreased compared with the negative control (Figure 8A). However, stimulation with IL-17 caused no clear change in the percentage of necrotic neutrophils, as compared with the negative control. IL-17 increased the release of MPO from the murine neutrophils, corresponding to a relative increase (% of control) in MPO concentration of 75% over the level in the negative control.

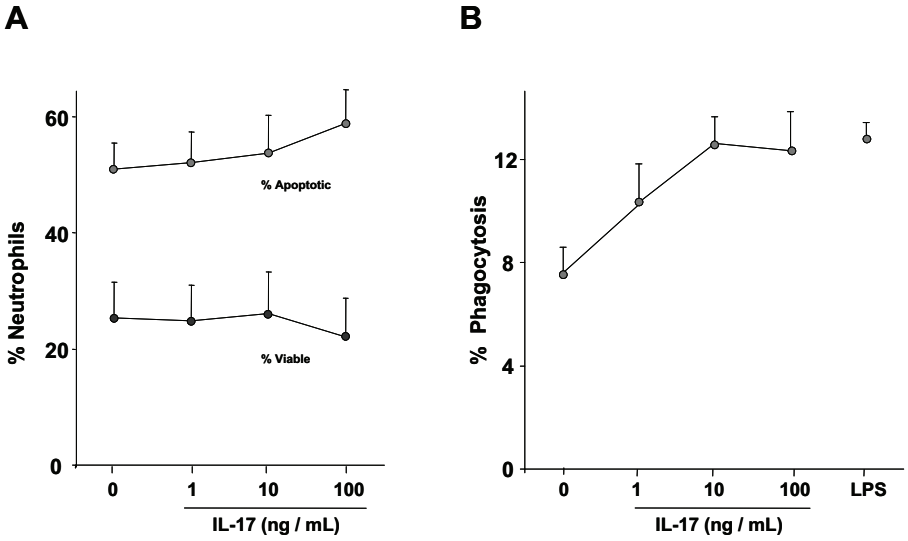

Figure 8. Impact of IL-17 on neutrophil apoptosis and viability as well as macrophage phagocytosis of aged neutrophils in vitro. A. Apoptotic neutrophils (Annexin V+, 7AAD- cells) and viable neutrophils (Annexin V-, 7AAD- cells) were quantified using flow cytometry after 48 hours of ageing in vitro with rmIL-17 (n=6; p=0.03 [for both apoptotic and viable neutrophils] 100 ng/mL vs. negative control). Macrophages were primed incubated 24 hours in vitro together with rmIL-17 or LPS before 2 hours of co-incubation with aged neutrophils. B. Phagocytosis of aged neutrophils by mouse bronchoalveolar macrophages (n=4; p=0.01 for maximum IL-17-induced change in the concentration range up to 100 ng/mL vs. negative control).

IL-17 increases the phagocytosis of murine neutrophils and latex beads

Stimulation with IL-17 increased the phagocytosis of aged murine neutrophils by murine bronchoalveolar macrophages by 95% above the negative control (Figure 8B). IL-17 increased the phagocytic index for murine bronchoalveolar macrophage phagocytosis of fluorescent latex beads by 103% above the negative control. In experiments using human monocyte-derived macrophages, IL-17 increased the phagocytic index by 52% above the negative control.

IL-17 stimulates the release of sLOX-1 but not MIP-2

Stimulation with IL-17 resulted in a clear increase in the concentration of sLOX-1 in the conditioned medium from cultures of murine bronchoalveolar macrophages. However, the release of MIP-2 from murine bronchoalveolar macrophages was not changed by IL-17 treatment, as compared with the conditioned medium from cultures of non-IL-17-treated murine bronchoalveolar macrophages.

Paper III

Following S. aureus infection, IL-17-knockout mice release more IL-23 than wild-type mice

To test the hypothesis that IL-17 inhibits the release of IL-23 in airways during inflammation, we administered *S. aureus* i.v. to wild-type and IL-17-knockout C57BL/6 mice and subsequently measured the local concentrations of IL-23. We found that the concentrations of IL-23 and the concentration of alveolar macrophages in the cell-free BAL fluids were significantly lower in the wild-type

mice than in the IL-17-knockout mice 24 h after, but not before, i.v. inoculation of *S. aureus*.

Following LPS stimulation, anti-IL-17 antibody-treated mice release more IL-23 than isotype control-treated mice

To test further our hypothesis without the influences of bacterial growth and systemic inflammation, we stimulated mice i.n. with LPS for 24 h, to create a model of local airway inflammation (184, 187). As an intervention, some mice were pre-treated with an anti-IL-17 antibody (aIL-17 Ab). Using this model, we observed a significant decrease in the concentration of IL-23 protein in the cell-free BAL fluids from mice stimulated locally with LPS, as compared with vehicle-treated control mice. The IL-23 concentrations in the corresponding samples from LPS-stimulated mice pre-treated with the aIL-17 Ab were significantly increased compared with mice stimulated with LPS only. The numbers of macrophages were also decreased in the BAL samples from mice stimulated locally with LPS, as compared with vehicle-treated control mice, although there was no difference in the concentrations of macrophages when comparison was made with LPS-stimulated mice that were pre-treated with the aIL-17 Ab. There were measurable levels of IL-17 protein in the cell-free BAL fluids from all mice stimulated locally with LPS but not in the vehicle-treated control mice.

IL-17 treatment decreases IL-23 in mice

We also performed local stimulation with IL-17 using i.n. instillation in the absence of LPS. Two hours after instillation, the mice stimulated with IL-17 displayed a significantly decreased concentration of IL-23 protein in the cell-free BAL fluid, as compared with the vehicle-treated control mice. There was no detectable difference in the concentrations of macrophages between the BAL samples from mice stimulated with IL-17 and the BAL samples from the vehicle-treated control mice.

IL-17 treatment decreases IL-23 release from human cells of the monocytic lineage in vitro

To investigate the underlying cellular mechanisms and to test whether IL-17 affects the release of IL-23 from a fixed number of human cells of the monocyte-lineage, we isolated and cultured human monocytes, monocyte-derived macrophages, and BAL macrophages *in vitro*. These cells were stimulated with LPS together with increasing concentrations of IL-17. For the human monocytes, only the lowest concentration of IL-17 (0.1 ng/mL) clearly decreased the concentration of IL-23 protein in the cell-free medium, while for the human monocyte-derived macrophages, there was a concentration-dependent decreasing effect of IL-17 on the IL-23 concentration. For both cell types, when IL-17 was administered without LPS, no IL-23 was detected after stimulation with any of IL-17 concentrations.

We also tested whether IL-17 could influence the release of the IL-23-related cytokine IL-12 from the *in vitro*-cultured human monocytes and monocyte-derived macrophages. Negligible amounts of IL-12 were detected; with all levels were below the lowest value in the ELISA standard curve. In addition, in experiments on monocyte-derived macrophages, we utilized NSC23766, a specific inhibitor of the GTPase Rac1, with or without concomitant stimulation with IL-17. We found that the concentration of IL-23 in the conditioned medium was decreased by the Rac1 inhibitor in a concentration-dependent manner. This decrease reached statistical significance in the IL-17-stimulated cell cultures. Notably, in one confirmatory experiment using human BAL macrophages, we observed a lower concentration of IL-23 in the cell-free medium from a culture of BAL macrophages stimulated with IL-17, as compared to the cell-free medium from a culture of control BAL macrophages treated with vehicle only.

DISCUSSION

This discussion complements the respective discussions in the three papers upon which this thesis is based. This section therefore focuses on placing our results in a larger and updated context and expanding upon plausible explanations for the mechanisms underlying the results.

Paper I

IL-17-containing cells

The aim of this study was to characterise the level of induced IL-17 production in T cells in a mouse model of lung infection caused by Gram negative bacteria. At the time that we started this study, it was believed that only $CD4^+$ memory T cells could produce IL-17. Therefore, the study was not designed to characterise further the different types of IL-17-containing T cells and non-T cells. As studies with new data of different types of IL-17-containing cells have emerged after this study was conducted, our results will be discussed in the light of recent discoveries regarding IL-17-producing cells.

T cells

In this study, we show that stimulation with LPS increases the number of IL-17-containing T cells in both lung tissues and the bronchoalveolar space. According to the literature, the T cells ($CD3^+$ cells) that produce IL-17 are Th17 cells, γδ T-17 cells, NKT-17 cells, and Tc17 cells. Since γδ T-17 and NKT-17 cells have TCRs that allow direct stimulation by LPS, it is very likely that these cells contributed to the release of IL-17 in our experiments (16, 67-68, 80). Th17 and Tc17 cells have TCRs that exclusively recognise peptide antigens. Therefore, it is unlikely that these cells were directly stimulated by LPS. However, one study

has shown that IL-23 can augment the production of IL-17 by memory T cells in the absence of TCR engagement (47). Th17 cells are prone to self-antigen specificity and one possibility for the observed LPS activation of memory Th17 cells is that these cells recognise phagocytosed endogenous peptide antigens from necrotic cells (188-189). When these endogenous peptide antigens are presented on the MHC II molecule together with LPS-induced co-stimulatory molecules on macrophages and DCs, Th17 cells are activated and start to produce IL-17 (188). Likewise, we cannot exclude that memory Tc17 cells are activated in a similar manner to Th17 cells, although in this case, by cross-presentation through the MHC II (190-191).

Thus, all the IL-17-containing T cells described in the literature are potential contributors to the IL-17 levels observed in the present study.

Non-T cells

We found that a large proportion of the IL-17-containing cells in the lung tissues and bronchoalveolar spaces were not T cells, i.e., they had the $CD3^-$ phenotype. From the literature, non-T cells that can produce IL-17 and that may be present in the lungs include: lymphoid tissue inducer cells (LTi), macrophages, and neutrophils (91-96, 102-103). From our data, we it is no possible to conclude how many LTi cells were present, and as even though the IL-17 levels were increased by LPS stimulation, there was no associated increase in the number of macrophages. Given that the number of neutrophils correlated positively with the IL-17 levels after LPS stimulation, the focus hereinafter will be on the neutrophils. Since IL-17 is a neutrophil-recruiting cytokine, the correlation between neutrophil numbers and IL-17 levels probably reflects IL-17-induced accumulation of neutrophils, although we cannot exclude the possibility that IL-17-containing neutrophils contributed to IL-17 production (133-136, 138-140, 192).

Even though it does not seem very likely, there is a possibility that the non-T cells that were intracellularly stained positive for IL-17, and which constitute a

substantial proportion of the total number of IL-17-containing cells, are in fact cells that have phagocytosed IL-17-containing T cells.

Anti-inflammatory drugs

Dexamethasone

We found that the high dose of the glucocorticoid dexamethasone attenuated the LPS-induced increases in; 1) the numbers of macrophages, neutrophils, T cells, and IL-17-containing T cells, as well as the levels of IL-17 protein in the bronchoalveolar space; and 2) the numbers of IL-17-containing T cells and levels of IL-17 mRNA in the lung tissues.

The finding that the number of IL-17-containing T cells but not the total number of T cells in the lung tissues were decreased by dexamethasone treatment suggests that dexamethasone has a specific effect on IL-17-producing cell numbers. In the bronchoalveolar space, the high dose of dexamethasone reduced both the LPS-induced increase in total number of T cells and the number of IL-17-containing T cells. Since there were no T cells in the bronchoalveolar space in the negative control group, we do not know whether dexamethasone reduces the IL-17 level simply by decreasing the recruitment of T cells to the bronchoalveolar space or decreases the number of IL-17-containing T cells in a specific manner. Interestingly, the low dosage of dexamethasone, which did not show much efficacy in cell number in our study, was able to reduce the level of IL-17 released in the bronchoalveolar space, whereas it did not reduce the number of IL-17-containing T cells. These findings suggest that while low-dose dexamethasone does not affect the number of IL-17-containing T cells, it negatively influences the amount of IL-17 that is released by each cell. These results also suggest that the dexamethasone-induced reduction in IL-17 release is due to an effect on the IL-17-containing non-T cells. If this population of IL-17-containing non-T cells consists mainly of neutrophils, this observation seems somewhat contradictory, since neutrophils

are known to be resistant to glucocorticoid treatment, and glucocorticoid treatment may even increase the survival of neutrophils (193). A possible explanation for this is that dexamethasone can decrease the production of IL-17 by neutrophils, while at the same time increasing their survival. This hypothesis is supported by the results of our second study (Paper II), in which we show that IL-17 decreases the viability and increases the apoptosis rate of neutrophils. Therefore, we speculate that in this study, dexamethasone increases neutrophil survival indirectly through a reduction in the level of IL-17.

Cyclosporine A

We found that cyclosporine A attenuated the LPS-induced increases in the number of neutrophils and IL-17 protein levels in the bronchoalveolar space and the increase in IL-17 protein levels *in vitro*, whereas it did not attenuate the LPS-induced increases in the numbers of T cells or IL-17-containing T cells in the bronchoalveolar space or lung tissues.

Cyclosporine A is a calcineurin inhibitor that acts by inhibiting the production of IL-2, IL-4, IFN-γ, and TNF-α (194-195). IL-2 and IL-4 are members of the common γ-chain family of cytokines, which are important for the activation, proliferation, and apoptosis of Th1, Th2, Treg, and $CD8^+$ cells (196). However, both IL-2 and IL-4 have been shown to be negative regulators of Th17 cells, which mean that cyclosporine A could theoretically increase the number of Th17 cells (105, 197). The fact that Th17 cells are not as dependent upon IL-2 and IL-4 as other T cells may partially explain the observed limited effect of cyclosporine A on IL-17-producing T cells. Whether IL-2 and IL-4 affect γδ T-17 cells and NKT-17 cells remains unknown. The only cell type that was reduced in number by cyclosporine A was the neutrophil. This could be due to the fact that cyclosporine A reduces the level of IFN-γ, which is an important factor for neutrophil activation and survival (194-195, 198-201).

In the present study, we show that both the IL-17 level and the number of neutrophils in the bronchoalveolar space are decreased by dexamethasone or

cyclosporine A treatment. The relevance of our results was confirmed in a clinical study that was published shortly after the publication of our study, which showed that three months of cyclosporine A and dexamethasone treatment of patients with uveitis lowered the concentration of IL-17 in the bloodstream and improved the uveitis (202).

Paper II

Apoptosis

In this study, we demonstrate that IL-17 stimulates neutrophil apoptosis. A hypothetical mechanism for IL-17 induction of neutrophil apoptosis relies on the fact that IL-17 increases the production and release of the microbicidal peptides S1008A and S1009A (61). These peptides are released from neutrophils, and in addition to their microbicidal actions, they form a complex, called calprotectin, which induces apoptosis (203-206). The identity of the receptor for this peptide complex is not yet known, although RAGE has been excluded as a candidate. Nevertheless, the apoptosis-inducing properties of this complex are manifested as rapid decreases in the mitochondrial membrane potential ($\Delta\Psi_m$) and the anti-apoptotic proteins Bcl2, bak, and Bcl-X_L, as well as increases in the activities of the pro-apoptotic mediators caspase-3 and caspase-9 (205-206).

As mentioned in Paper II, studies other than ours have verified that IL-17 increases neutrophil apoptosis (207-208). One of these studies (208) has shown that IL-17, in similarity to the microbicidal peptides S1008A and S1009A, decreases the mitochondrial membrane potential and increases caspase-3 activity. Therefore, it can be speculate that IL-17 augmentation of neutrophil apoptosis involves the microbicidal peptides S1008A and S1009A.

Myeloperoxidase

In parallel with the stimulation of apoptosis of mouse neutrophils, IL-17 induces the release of the microbicidal compound MPO. Hypothetically, the increased MPO could, *via* its inherent capacity to induce the production of ROS, allow the neutrophils to remain tolerogenic while undergoing apoptosis (209-210). Both apoptotic and necrotic cells release the potential danger signal high mobility group protein B1 (HMGB1). In apoptotic cells, ROS oxidise cysteine 106 of HMGB1, which neutralises HMGB1 and thereby promotes tolerance when recognised by antigen-presenting cells (210-212). In necrotic cells, HMGB1 is not oxidised, and the lack of oxidation contributes to the immunogenic reaction upon recognition by antigen-presenting cells. From our experiments, we could not deduce whether the MPO was released from viable, apoptotic or necrotic neutrophils. As mentioned in Paper II, MPO has been shown to be released from viable neutrophils, and we did not see any correlation between the concentration of MPO and the percentage of necrotic neutrophils (213-215). In addition, to be sure that we had not missed a population of neutrophils that might have been necrotic in the first place but then had exploded and turned to cell debris, we re-checked our flow cytometry data to see if there was an increase in cell debris in the samples that had higher levels of MPO; we found that this was not the case. Based on the above, it seems plausible that IL-17 exerts tolerogenic properties through the oxidation of HMGB1 in apoptotic neutrophils, which is attributed to the MPO-induced production of ROS.

MIP-2 and soluble LOX-1

Bronchoalveolar macrophages stimulated with IL-17 showed increased release of the soluble portion of the scavenger receptor LOX-1, sLOX-1, but did not show increased release of the neutrophil recruitment chemokine MIP-2. IL-17 has been shown to increase the production and release of MIP-2 in mesangial cells, fibroblastoid L929 cells, astrocytes, microglia, as well as in the

bronchoalveolar space (175, 216-218). However, IL-17 did not increase the release of MIP-2 in a co-culture of lymphocytes and airway macrophages (175). This confirms our result and suggests that IL-17 increases the release of MIP-2 from structural and supporting cells but not from macrophages.

LOX-1 is a scavenger receptor that aids phagocytosis by binding to apoptotic cells, as well as to bacteria and oxidised low-density lipoproteins. It has been proposed that the amount of sLOX-1 represents the amount of LOX-1 expressed on the membrane of the phagocytic cell, and thereby reflects the level of phagocytosis (219-221). Soluble LOX-1 has been shown to correlate with the severity of atherosclerosis, a disease in which one of the hallmarks is the phagocytosis of oxidised low-density lipoproteins (219-221). Other studies have proposed a role for soluble scavenger receptors in chemotaxis (222-223). Applying this hypothesis to our study, for macrophages to recruit their prey by shedding sLOX-1 receptors, the apoptotic cells that bind sLOX-1 must have the capability to migrate up a chemotactic gradient. Even though one study has shown that apoptotic neutrophils have an impaired propensity to migrate towards formyl-methionyl-leucyl-phenylalanine (FMLP), cells in the first stages of apoptosis may still be able to migrate towards phagocytic cells (215, 224-225).

We have shown that IL-17 increases macrophage phagocytosis of apoptotic neutrophils and increases macrophage release of sLOX-1. It is theoretically possible that these macrophages, when stimulated by IL-17, release more sLOX-1, which then functions as a chemotactic factor for apoptotic neutrophils (226).

Phagocytosis

We found that incubation of macrophages with IL-17 increased the phagocytosis of both aged neutrophils and latex beads. The literature contains two potential explanations for how IL-17 increases phagocytosis. First, as mentioned in Paper II, the small GTPase Rac1 constitutes a theoretical link for the intracellular signalling underlying IL-17-induced phagocytosis. Rac1 is

induced by IL-17 and facilitates phagocytosis by rearrangement of the actin cytoskeleton (227-229).

Second, IL-17 has been shown to increase complement factor 3 (C3) gene and protein expression in fibroblasts, as well as in mucosal samples from patients with inflammatory bowel disease (230-231). Since C3 can be enzymatically cleaved to the opsonin C3b, it can enhance phagocytosis by binding to CR3 (CD11b/CD18) and CR4 (CD11c/CD18) on apoptotic neutrophils (232).

Paper III

Negative regulation downstream of IL-17RA

We found that the release of IL-23 from cells of the monocyte lineage was decreased after stimulation with IL-17 and LPS. Our results and those of others imply that the level of IL-23 is decreased by IL-17RA signalling. As described in Paper III, the cytokine IL-25 (formerly IL-17E) decreases macrophage production of IL-23 (233-235). Both IL-17 and IL-25 bind to the receptor subunit IL-17RA, although the complementing receptor subunit in the respective heterodimeric receptor complexes differs (114, 118-119). Since studies of IL-25 (233-235), in similarity to our study, have demonstrated decreased levels of IL-23, it appears that the shared receptor subunit IL-17RA is responsible for the reduction in IL-23 expression.

It has also been shown that IL-17 downstream signalling *via* IL-17RA phosphorylates the CCAAT/enhancer-binding protein β (C/EBPβ), which in turn leads to downregulation of the transcriptional capacity of C/EBPβ (127). Synthesis of the p40 protein, which is shared by IL-12 and IL-23, is dependent upon the activation of C/EBP, which may explain how the IL-17-induced decrease of IL-23 is executed (236-239). However, regulation of C/EBPβ is complex, and the role of C/EBPβ phosphorylation is suggested to be dependent on both the upstream signals as well as the specific downstream promoter system (127).

IL-17 dose dependency

The IL-17-induced decrease in the level of IL-23 was most prominent when lower concentrations of IL-17 were used. A similar phenomenon was described in another study, in which a low dosage (0.1 μg/rat/day) or a high dosage (0.9 μg/rat/day) of IL-17 was administered intranasally to rats with experimental autoimmune neuritis (EAN). During the initial acute phase, both dosages of IL-17 enhanced the severity of EAN, as compared with EAN rats that received PBS only (240). The rats that received the low dosage of IL-17 recovered completely after Day 80 post-infection, the rats that received the high dosage of IL-17 recovered completely after Day 98 post-infection; and the control rats that received PBS only still had neuritis on Day 120 post-infection. These results suggest that IL-17 can have a pro-resolving effect, and that this effect is most pronounced at low concentrations of IL-17.

A similar observation regarding IL-17 and dose-dependency was made in the *in vitro* studies (referred to in Paper III) of human synovial fibroblasts and mouse lung fibroblasts, revealing an inhibitory role for IL-17 at low concentrations and a stimulatory role for IL-17 at higher concentrations (241).

What happens when IL-17 decreases IL-23?

By decreasing IL-23, IL-17 limits its own production. Concomitantly, the production levels of other IL-23-dependent cytokines, such as the IL-17-related cytokines IL-17F, IL-21, and IL-22, are reduced. These cytokines have been shown to mediate disease in the absence of IL-17 (113, 242). Cytokines produced by subsets of macrophages and dendritic cells that express IL-23R exhibit decreased production of mediators, such as IL-6, TNF-α, and IL-1 (44, 243-244). Thus, when IL-17 decreases IL-23 it has an impact that is larger than the negative feedback of IL-17 alone.

GENERAL DISCUSSION

Effect of IL-17 on macrophage phenotype

As described in the *Introduction*, macrophages can be divided into subtypes depending on the stimuli that activate them. Regulatory macrophages, which suppress immune responses, are suggested to be induced by LPS, immune complexes, prostaglandins, apoptotic cells, and IL-10 (13, 19, 245). Although many subtypes of regulatory macrophages exist, most of these regulatory cells require two different types of stimuli to induce their anti-inflammatory activities. The first stimulus has no or moderate effect by itself but when it is combined with the second stimulus, usually a TLR ligand, the macrophage becomes a regulatory-type cell. The fundamental characteristics of regulatory macrophages are that they produce IL-10 and are down-regulated for IL-12 production. Although we did not assess the IL-10 levels in our experiments, we measured the levels of IL-12. We found no measurable release of IL-12 from the human monocyte-derived macrophages that were stimulated with IL-17 together with LPS. The macrophages that were stimulated with IL-17 together with LPS in Studies II and III displayed a regulatory and anti-inflammatory phenotype. We found that these cells did not produce measurable levels of IL-12, and the addition of IL-17 decreased IL-23 production, without increasing MIP-2 production (216-217). Last but not least, these macrophages showed an increased capacity to phagocytose both apoptotic cells and particles. Based on these findings, we propose a new regulatory macrophage subtype: the Mreg-17.

Dose dependency

One of the conclusions from Paper III is that when the concentration of IL-17 is low IL-23 production is held back, which in turn means that the production of IL-17 is decreased. This is exemplified in Figure 10, which shows that in the

presence of a weak infectious stimulus, the antigen-presenting cells weakly stimulate IL-17-producing cells, which results in negligible release of IL-17. This low concentration of IL-17 reduces the production of IL-23 and promotes the resolution of inflammation. However, if the infectious stimulus is strong, the antigen-presenting cells are highly activated and produce high levels of IL-17-inducing factors, such as IL-1β, IL-6, and TGFß, which increase the IL-17 concentration. This in turn releases the IL-17-induced blockade so that IL-23 production is increased once again, and the major inflammation is initiated. During inflammation, when the IL-17 concentration is high, IL-17 induces both neutrophil apoptosis and the phagocytosis of apoptotic neutrophils and other particles. At the end of the inflammatory reactions, when the infectious stimuli have been cleared, the reduced infectious stimuli lead to decrease in the IL-17 concentration. This process of resolution is supported by the phagocytosis of apoptotic neutrophils by macrophages, thereby decreasing the release of IL-23, which also lead to decrease in the IL-17 concentration (246). When the IL-17 concentration is low, again IL-17 can start the restriction of IL-23 expression and thereby down-regulates not only itself, but also other cytokines that are dependent upon IL-23 signalling. Furthermore, the use of an anti-inflammatory drug, such as dexamethasone or cyclosporine A, helps to decrease the concentration of IL-17, so that IL-17 limits its own production and that of the other cytokines that are dependent upon IL-23 signalling.

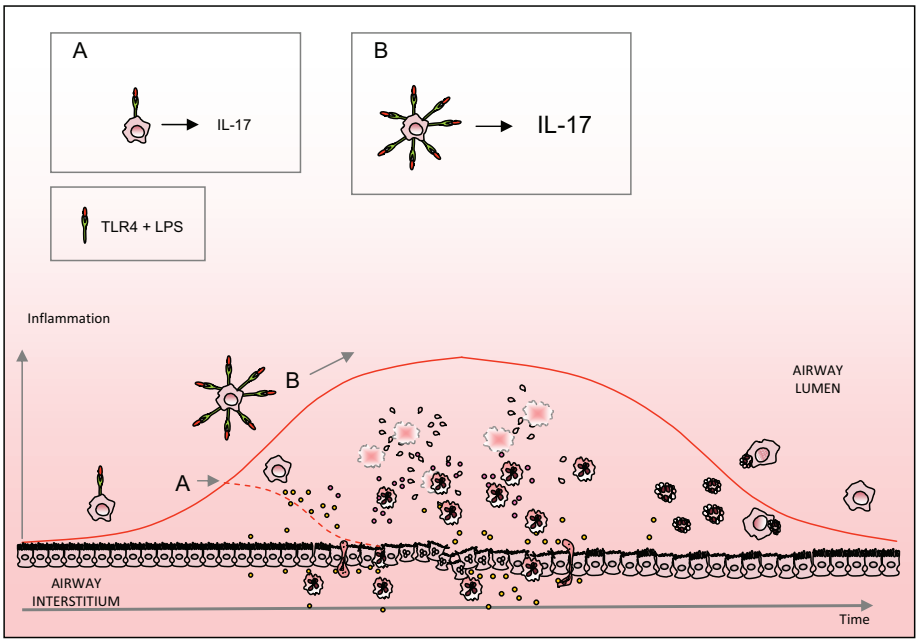

Figure 9. Dose–dependency of IL-17 activity. A weak infectious stimulus (A) produces weak activation of the antigen-presenting cell, which in turn generates low levels of IL-17 released from IL-17-producing cells. The low concentration of IL-17 reduces the production of IL-23 and promotes resolution of the inflammation. When the infectious stimulus is strong (B), the antigen-presenting cell is highly activated and produces high levels of IL-17-inducing factors, in addition to IL-23, thereby increasing the IL-17 concentration. This in turn releases the IL-17-induced blockade, so that IL-23 production is once again increased and the inflammation is initiated. The macrophages and some of the epithelial cells are adapted from *Nature Reviews Immunology*.

Why IL-17 is considered to be a pro-inflammatory cytokine

In two of our studies, we show that IL-17 exerts actions that can be viewed as pro-resolving. This observation is supported by other studies, based on animal models, which show that the inhibition of endogenous IL-17 actually worsens airway allergy, inflammatory bowel disease, and atherosclerosis (247-252). In line with these findings, treatment with recombinant IL-17 protein exerts anti-inflammatory effects in gastritis, experimental autoimmune neuritis and in chronic relapsing uveitis in animal models (240, 253-254).

An important question then arises: if IL-17 can exert all these anti-inflammatory activities, why then is IL-17 considered to be a pro-inflammatory cytokine? In my view, IL-17 exerts both pro-inflammatory and pro-resolving activities, depending on the situation. The predominant perception of IL-17 as a pro-inflammatory cytokine may be in part a reflection of the methods that have been used to assay this cytokine.

The usual suspect

In the literature, the actions of Th17 and "the IL-23/IL-17 axis" are often alluded to as being equivalent to those of IL-17. The effects of IL-17 have been studied using various methods, ranging from direct blocking using anti-IL-17 antibodies, to very indirect methods, such as blocking the IL-12 and IL-23 shared p40 subunit. Many of the biological functions attributed to IL-17 have been based on studies of Th17 cells or the IL-17 receptor.

Do not mistake IL-17 for Th17

The activities attributed to Th17 cells differ from those of IL-17, in that Th17 cells also produce cytokines, such as IL-17F, IL-21, and IL-22, all of which have been shown to mediate disease independently of IL-17 (113, 242). In addition, Th17 cells have an inherently high specificity for self-antigens, and therefore have increased potential to cause autoimmune disease (188). Cells other than Th17 cells can produce IL-17. Thus, even if Th17 cells are associated with autoimmune diseases, this does not necessarily mean that other IL-17-producing cells acts in the same way or that IL-17 produced by, for example, LTi cells mediates responses similar to those induced by the IL-17 produced from Th17 cells.

Much information on the IL-23/IL-17-axis and Th17 cells has been derived using experimental animal models, even though the results have varied depending on the particular model chosen. For example, the numerous experimental models of multiple sclerosis, inflammatory bowel disease, and

rheumatoid arthritis have produced divergent outcomes regarding the actions and properties of the IL-23/IL-17-axis and TH17 cells (255-259). Obviously, there is also a risk that some experimental models become more widely used because they generate unambiguous results for IL-23/IL-17-axis or Th17 cells but may not necessarily reflect the real disease.

SUMMARY

IL-17 has the capacity to decrease IL-23 release in several ways. IL-17 induces the accumulation of neutrophils at the site of inflammation by increasing the proliferation and recruitment of neutrophils through stimulation of G-CSF and GM-CSF, and CXC chemokines, respectively. After the neutrophils have killed the invading bacteria, IL-17 drives them to apoptosis and accelerates their phagocytosis by macrophages. Phagocytosis decreases the release of IL-23, so once the infectious stimuli are cleared there will be no more IL-17. Furthermore, when the IL-17 levels are low (at the beginning and end of the cycle), the pro-inflammatory cascade is retarded by IL-17 through its inhibition of IL-23 release.

CONCLUSIONS

General conclusion

The results of the work described in this thesis reveal that the production of IL-17 is regulated by both anti-inflammatory drugs and an IL-17-induced feedback loop, which in turn may protect against excessive, IL-23-induced IL-17 signalling. Moreover, we demonstrate that IL-17 has both pro-inflammatory and inflammation-resolving actions; IL-17 induces the accumulation of neutrophils after stimulation with LPS, while it also induces the phagocytosis of apoptotic neutrophils, thereby controlling the overall turnover of neutrophils. The studies presented in this thesis demonstrate that IL-17 exerts different actions depending on the inducing stimulus, the cell that produces it, the concentration of the cytokine itself, the cell that expresses its receptors, and of course, the prevailing milieu. Therefore, IL-17 can no longer be regarded simply as a pro-inflammatory or anti-inflammatory cytokine.

That IL-17 induces the apoptosis of neutrophils and increases their phagocytosis may represent a valuable strategy for modulating conditions in which necrotic neutrophils are an important contributor to severe and sometimes life-threatening conditions, such as chronic lung allograft rejection and acute respiratory distress syndrome.

Specific conclusions

Paper I

The results of this study using a mouse model of acute inflammation indicate that LPS induces sustained IL-17 production and release from T cells that reside in lung tissues and that are recruited to the bronchoalveolar space *in vivo*. In

addition, we identify a population of cells other than T cells that contributes to IL-17 production in lung tissues and in the bronchoalveolar space. Finally, we show that LPS-induced IL-17 production by T cells in lung tissues and in the bronchoalveolar space can be inhibited by treatment with the anti-inflammatory drug dexamethasone.

Paper II

This *in vitro* study on isolated cells from mice and humans demonstrates that IL-17 not only induces neutrophil apoptosis, but also stimulates macrophage phagocytosis of apoptotic neutrophils and particles. These findings suggest that IL-17 is involved in controlling the overall turnover of neutrophils, in addition to its previously described activities in the recruitment and accumulation of neutrophils.

Paper III

In this study, we provide *in vivo* and *in vitro* evidence that IL-17 inhibits the release of the upstream regulator IL-23. These findings indicate that IL-17 *per se* participates in a negative feed-back loop that protects against excessive, IL-23-induced IL-17 signalling.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Modulerande roll för IL-17 vid inflammation i luftvägarna

IL-17 är en signalmolekyl som produceras av celler som tillhör immunförsvaret. När IL-17 frisätts från en cell kan den binda till en IL-17-specifik receptor (IL-17-receptor) på en mottagarcell. Denna receptorbindning ger en signal till mottagarcellen att börja tillverka olika ämnen. IL-17 produceras oftast under olika inflammatoriska tillstånd som innefattar akut inflammation så som bakteriell infektion och kroniska inflammatoriska sjukdomar så som astma, reumatoid artrit och multipel skleros.

Tidigare har man trott att IL-17s främsta uppgift är att bidra till ökad inflammation, men under den senaste tiden har flera nya studier visat att IL-17 också kan ha en anti-inflammatorisk effekt. Syftet med denna avhandling var att studera metoder för att reglera produktionen av IL-17 samt att undersöka en eventuell roll för IL-17 som begränsande faktor för inflammationer.

Avhandlingen består av tre separata studier där vi främst har fokuserat på effekter av IL-17 vid den typ av akut inflammation som uppstår vid en bakteriell infektion. Vi har genomfört försök i lungan hos möss samt odlat celler *in vitro*. För att efterlikna en bakteriell infektion har vi använt oss av komponenter från *E. coli* bakterier (LPS).

Delarbete I

I den första av avhandlingens studier kom vi fram till att IL-17 kan öka inflammation. I denna studie använde vi möss och genom att låta dem andas in LPS kunde vi studera akut inflammation i lungan. Vi såg att celler i immunförsvaret producerade och frisatte mer IL-17 i lungorna, och att IL-17

bidrog till att rekrytera inflammatoriska celler, neutrofiler, från blodbanan till lungorna. I samma studie behandlades några av mössen med de anti-inflammatoriska läkemedlen dexametason och cyklosporin A, och hos de mössen minskade både produktionen av IL-17 och rekryteringen av neutrofiler till lungan.

Slutsats

LPS stimulerar produktion av IL-17 som i sin tur bidrar till rekrytering av neutrofiler till lunga. Detta går att minska med hjälp av antiinflammatoriska läkemedel.

Delarbete II

I avhandlingens andra studie kunde vi visa att IL-17 kan hjälpa till att avsluta en inflammation. I denna studie odlade vi celler från immunförsvaret, mamrofager och neutrofiler *in vitro*. Dessa celler stimulerades med IL-17 och LPS. Överlevnaden hos inflammatoriska neutrofiler minskade av IL-17, och makrofagers förmåga att äta upp (fagocytera) dessa döende neutrofiler ökade av IL-17.

Slutsats

Genom att IL-17 både minskar överlevnaden av inflammatoriska neutrofiler samt ökar makrofagers fagocytos av dessa döende neutrofiler, kan IL-17 bidra till att begränsa en pågående inflammation.

Delarbete III

I avhandlingens tredje studie kom vi fram till att IL-17 kan verka anti-inflammatoriskt genom en negativ feedback mekanism. IL-23 är en signalmolekyl som är nödvändig för att celler ska kunna producera IL-17. I denna studie undersökte vi om IL-17 kan påverka cellers produktion av IL-23.

Efter att ha undersökt detta, både i lungor hos möss och hos makrofager odlade in vitro, kunde vi visa att IL-17 kan minska produktionen och frisättningen av IL-23.

Slutsats

IL-17 kan minska produktionen av IL-23 i lunga och eftersom IL-23 är nödvändig för att celler ska kunna producera IL-17, så minskar IL-17 indirekt sin egen produktion.

Sammanfattningsvis har vi visat att det går att stoppa IL-17s inflammatoriska effekter med hjälp av antiinflammatoriska läkemedel, samt att IL-17 kan begränsa inflammation och dessutom under vissa omständigheter även dämpa sin egen produktion genom en negativ feedback mekanism.
Genom att visa att IL-17 både kan medföra ökad inflammation, men även kan begränsa den, bidrar denna avhandling till att nyansera bilden av IL-17 i inflammation. Förmodligen är effekten av IL-17 beroende av det förhållande som föreligger när IL-17 ska utföra sin verkan. Detta förhållande kan bero på vad som startat inflammationen, mängden IL-17, samt vilka celler som finns i närheten. Att IL-17 kan minska neutrofilers överlevnad samt öka fagocytosen av dessa, skulle kunna innebära en värdefull strategi för att modulera tillstånd där neutrofilerna i sig, kombinerat med en otillräcklig fagocytos av dessa neutrofiler, utgör en väsentlig del i allvarliga inflammatoriska tillstånd så som kronisk avstötning av transplanterade lungor och akut respiratorisk distress-syndrom (ARDS).

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