Interleukin-17 in models of neutrophilic lung disease

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Abstract

Several acute or chronic lung disorders like adult respiratory distress syndrome, acute severe asthma, chronic obstructive pulmonary disease, chronic lung allograft rejection and cystic fibrosis, are associated with signs of an excess innate response in the bronchoalveolar space. Neutrophils may play a pathogenic role in these lung diseases by contributing to non-specific bronchial hyper-responsiveness and bronchial hypersecretion, as well as to epithelial damage and tissue remodelling. Previous studies employing stimuli of Gram-negative bacteria in mice have shown that the T-cell cytokine Interleukin (IL)-17 (also named IL-17A) can contribute to accumulation of neutrophils within the bronchoalveolar space. The general aims of this thesis were to determine a cellular source of IL-17 in the human bronchoalveolar space *in vivo* and to further characterize the role of IL-17 and the IL-17-inducing cytokine IL-23 in neutrophilic lung disease.

In study one, severe neutrophilic inflammation in the human bronchoalveolar space was caused by exposure of healthy, non-smoking volunteers to organic dust. The exposure increased IL-17 messenger (m) RNA in bronchoalveolar-lavage (BAL) cells and, because of an intracellular expression of IL-17 protein in lymphoid BAL cells, IL-17 may originate from lymphocytes residing within the bronchoalveolar space. The increased IL-17 mRNA expression was associated with an increased percentage of matrix metalloproteinase (MMP)-9-expressing BAL neutrophils. This is compatible with IL-17 controlling the local proteolytic burden of tissue-degrading enzymes like MMP-9, via its neutrophil-accumulating effect.

In study two, sensitized and allergen-challenged mice were pre-treated with a specific anti-IL-17 antibody in order to block endogenous IL-17. We showed that endogenous IL-17 may contribute not only to the accumulation of BAL neutrophils but also to the accumulation of BAL macrophages. IL-17 may serve as a direct chemotactic factor for macrophage precursor cells and as a survival factor for macrophages within the bronchoalveolar space. In addition, we present evidence that IL-17 might control the local proteolytic burden, since blocking endogenous IL-17 decreased the percentage of MMP-9-expressing BAL neutrophils and macrophages.

In study three, we determined the impact of the antigen-presenting cells cytokine IL-23 in the innate response of the lungs utilizing stimulation with bacterial structural components as well as with recombinant IL-23 protein. We showed that both Gram-negative and Gram-positive bacterial components promoted the release of IL-23 in lung tissue and BAL fluid. We also demonstrated that short-term stimulation with recombinant IL-23 protein caused accumulation of macrophages and neutrophils in the bronchoalveolar space via endogenous production of IL-17. This production of IL-17 occurred locally in IL-23-responsive CD4 cells. In addition, IL-23 did not seem to markedly affect the Th1 or Th2 polarization of these CD4 cells. Finally, stimulation with recombinant IL-23 protein increased the local MMP-9 activity, which was generated by neutrophils mainly.

In conclusion, there are lymphocytes residing within the human bronchoalveolar space, which are capable of producing IL-17. In mice, IL-17 production takes place in IL-23-responsive bronchoalveolar CD4 cells and IL-17 may contribute to the accumulation of both macrophages and neutrophils to the bronchoalveolar space. Thus, the antigen-presenting cell cytokine IL-23 and the T-cell cytokine IL-17 seem to form a functional immunological axis in mouse lungs *in vivo*. This IL-23-IL-17 axis is involved in the early innate immune response to Gram-negative and Gram-positive bacteria. If similar mechanisms exist in humans, IL-23 and IL-17 may constitute potential pharmacotherapeutical targets in severe lung diseases associated with an excess innate response.

Original Papers

This thesis is based on the following papers referred to in the text by Roman numerals (Paper I-III):

I. **Ivanov S.**, Palmberg L., Venge P., Larsson K., A. Lindén. Interleukin-17A mRNA and protein expression within cells from the human bronchoalveolar space after exposure to organic dust. *Respiratory Research* 2005 May 25; **6:** 44

II. Sergejeva S., **Ivanov S.**, Lötvall J., A. Lindén. IL-17 as a recruitment and survival factor for airway macrophages in allergic airway inflammation. *AJRCMB.* 2005; **33 (3):** 248-253.

III. **Ivanov S.**, Bozinovski S., Bossios A., Valadi H., Vlahos R., Malmhäll C., Sjöstrand M., Kolls J.K., Anderson G.P., A. Lindén. Functional relevance of the IL-23-IL-17 axis in lungs *in vivo*. Submitted to *AJRCMB*.

Abbreviations

ab	Antibody
BAL	Bronchoalveolar lavage
cDNA	complementary Deoxyribonucleic acid
CD	Cluster of differentiation
CI	Calcium Ionophore
COPD	Chronic obstructive pulmonary disease
CTLA	Cytotoxic T lymphocyte associated antigen 8
ELISA	Enzyme-linked immunosorbent assay
GCP-2	Granulocyte chemoattractant protein 2
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
Gro-α	Growth-related oncogene-alpha
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
ICAM	Intercellular adhesion molecule
IFN-γ	Interferone-gamma
IL	Interleukin
i.n.	Intranasal
LPS	Lipopolysaccharide
m	Messenger
MACS	Magnetic cell separation
MCP-1	Monocyte chemotactic protein 1
MMP	Matrix metalloproteinase
PBS	Phospate-buffered saline
PMA	Phorbol 12-myristate 13-acetate
R	Receptor
RNA	Ribonucleic acid
S.E.M.	Standard error of the mean
Th	T helper
TNF	Tumor necrosis factor

Contents

Introduction

Excessive accumulation of neutrophils in the bronchoalveolar space is linked with several acute and chronic lung disorders in which these cells may play an important pathogenic role.

Neutrophils

General

Neutrophils, also named polymorphonuclear leukocytes, are the most abundant type of white blood cells circulating in the blood (1). Their production and differentiation take place in the bone marrow from which they later enter the blood. Neutrophils remain in the circulation for only 6 to 10 hours before migrating to tissue sites of inflammation where they survive for one to two days. If not recruited, neutrophils undergo programmed cell death, i.e. apoptosis and are phagocytosed by the resident macrophages in the liver or spleen.

Neutrophils in host defence

Because of their function in first-line defence against bacterial pathogens, neutrophils are important cells of the innate immune system in mammals, including humans (2). Once the invading pathogen triggers an inflammatory response, neutrophil accumulation is one of the earliest and most consistent events. The reason for this is that neutrophils are capable of phagocytosing and killing microorganisms. In order to fulfil their role in host defence, neutrophils are equipped with a variety of protein-degrading or cytotoxic compounds such as neutrophil elastase, myeloperoxidase, matrix metalloproteinases (MMP) and oxygen free radicals.

Neutrophils in lung disease

The proteolytic capacity of neutrophils, in addition to their capability to move rapidly to sites of inflammation sometimes has harmful effects not only on the pathogens but also on the host. This is evident in severe airway diseases.

Elevated numbers of neutrophils have been found in patients with acute respiratory distress syndrome (3, 4), chronic bronchitis (5-7), cystic fibrosis (16, 17), bronchial bronchiectasis (20, 21) during acute and chronic lung allograft rejection (18, 19) or when healthy subjects are exposed to cotton, grain or swine dust (22-24). Two common obstructive lung diseases, chronic obstructive pulmonary disease (COPD) and bronchial asthma in its acute or severe forms are also characterized by neutrophilic inflammation. Thus, increased number of activated neutrophils is found in BAL fluid and sputum of patients with COPD (8-11). Cigarette smoking, which is the major environmental risk factor for COPD (12), has a direct stimulating effect on neutrophil production and release from bone marrow (13) and may also increase neutrophil retention in the lung (14). Even asymptomatic smokers display airway neutrophilia (15). Last but not least, the number of neutrophils is increased in certain forms of bronchial asthma (25). Neutrophils are the first cell type to enter the airways following allergen challenge (26). High neutrophil number is found in sputum and BAL fluid from patients undergoing acute exacerbations (27, 28) and in sputum from patients with sudden-onset fatal asthma (29). Neutrophilic inflammation is found in severe persistent asthma as well (30). Importantly, the most commonly used treatment in obstructive airway diseases, inhaled corticosteroids, have no effect on the increased neutrophil number. (31)

It is suggested that, via release of the protein-degrading and cytotoxic compounds mentioned above, neutrophils contribute to tissue damage and airway remodelling,

non-specific bronchial hyper-responsiveness and bronchial hypersecretion in these airway disorders (32). Therefore, determination of endogenous mechanisms controlling the accumulation and/or activity of neutrophils into the bronchoalveolar space might reveal new pharmaco-therapeutical targets in lung disease characterized by excessive neutrophil accumulation.

Neutrophil migration

Neutrophil recruitment to the lung occurring under physiological or pathological conditions is a multi-step process (33). It begins with transient attachment of neutrophils to endothelial cells using adhesion molecules followed by rolling of neutrophils along the vessel wall for firm adhesion. The latter is mediated by the transmembrane glycoproteins Integrins and by intercellular adhesion molecules (ICAMs). Thereafter, migration through the subendothelial matrix into the airway tissue follows (33, 34).

Control of neutrophil accumulation

Two major groups of compounds can cause accumulation of neutrophils. First, nonchemotactic cytokines like TNF-α and IL-1β facilitate the migration process by stimulating the expression of adhesion molecules on neutrophils and endothelial cells, without being chemotactic factors for neutrophils themselves (33). They also induce the release of chemotactic cytokines (33). The latter, named chemokines represent the second group of compounds involved in the accumulation of neutrophils. Chemokines stimulate the migration of cells expressing the cognate chemokines receptors. The best-studied neutrophil-mobilising chemokine are the C-X-C chemokines such as Interleukin (IL)-8, Gro-α, GCP-2 (35).

Macrophages and T-lymphocytes in lung disease

Macrophages and T-lymphocytes are other cells increased in the lung disorders referred to above.

Macrophages

Like neutrophils, macrophages are phagocytic for bacteria (1) and, thus, play a role in innate immunity. Macrophages are the predominant cells in the bronchoalveolar space in healthy individuals (36) but these cells may also be involved in the pathogenesis of COPD and emphysema, where the number of macrophages has been associated with disease severity (37, 38). The macrophage number is increased in asthmatics as well (39). Macrophages may participate in airway inflammation through multiple mechanisms, including cell recruitment, altered vascular permeability and removal of cells that are undergoing apoptosis. Macrophages also release protein-degrading compounds including MMPs. The predominant proteolytic enzyme secreted by alveolar macrophages in COPD patients is MMP-9 (37).

T-lymphocytes

Given their regulatory capacity, T-lymphocytes may be important for the control of virtually any immune response. One aspect of their effects is recruiting effector cells to the site of inflammation. It is well established that T-lymphocytes can accumulate bronchoalveolar eosinophils through Th2 cytokines (40-42). Although not so extensively studied, it has been suggested that T-lymphocytes can contribute to accumulation of bronchoalveolar neutrophils as well. Thus, in murine *in vivo* models, an antibody against the T-lymphocyte activation marker IL-2 as well as an antibody against CD4 attenuates allergen-induced neutrophil accumulation (43-45). Studies in humans have shown that an increased neutrophil number is associated with increased

numbers of T-lymphocytes in lung disorders such as bronchial asthma and COPD (46-48), bronchiectasis (20), chronic lung transplant rejection (49) and after exposure to organic dust (24). There is a growing body of evidence that Interleukin (IL)-17 constitutes an important link between T-lymphocytes and the accumulation of neutrophils within the bronchoalveolar space.

Interleukin-17

IL-17 (also termed IL-17A) is a homodimeric cytokine with a molecular weight between 30 and 35 kDa containing 155 amino acids (50, 51). IL-17 was first identified as a rodent cDNA transcript, named cytotoxic T lymphocyte associated antigen 8 (CTLA8), isolated from an activated T-lymphocyte hybridoma (50).

IL-17 Family

IL-17 is the prototypic member of the IL-17 family of cytokines. It includes five more members named IL-17B, -C, -D, -E (now re-named IL-25) and –F (52-57). Among these five additional members, IL-17F appears to have the greatest degree of structural and functional similarity with IL-17 (57-58).

Structure

The archetype molecular structure of the IL-17 family includes canonical cystein knot fold of β-strands, disulphide linkage and relatively similar C-terminal amino acid sequence (57). Mouse and rat IL-17 display structural homology with human IL-17 and this is particularly true for the glycolisation site of the cytokine. This fact is compatible with IL-17 playing an important role in the mammalian immune system (50, 51, 59).

Source

IL-17 was initially described on a messenger level as a product of activated CD4 memory cells from peripheral blood (59, 60). Later studies in mice and humans have demonstrated that CD8 cells can also produce IL-17 (61-63). Interestingly, IL-17 production is not restricted to CD4 with a ''typical'' Th1 or Th2 profile. In a subset of T-lymphocytes stimulated with bacterial lipopeptides *in vitro*, IL-17 is co-expressed with TNF-α and GM-CSF but not with Th1 or Th2 cytokines (64). Therefore, IL-17 may define a new subset of T lymphocytes; thus not fitting within the classical Th1/Th2 paradigm.

It has been claimed that eosinophils and neutrophils might also be sources for IL-17 since IL-17 mRNA or intracellular IL-17 protein has been detected in these cells (62, 65). However, the actual release of free, soluble IL-17 protein has not been demonstrated by either of these cells.

Receptor

IL-17, and possibly IL-17 F (58), signal through the IL-17 receptor (R), a transmembrane protein, which has a unique structure and does not display homology with previously identified cytokine receptor families (66). Unlike the relatively restricted expression of IL-17, its receptor is ubiquitously expressed and has been found in a variety of cell types in humans and mice, including fibroblasts, epithelial cells and lymphocytes (66). *In vitro* studies show that IL-17 has the ability to signal at its receptor at low concentrations but has a relatively low affinity to it (66). As is the case with IL-17, human and mouse IL-17R display great degree of structural homology (58).

Four additional IL-17 receptors have been described, IL17RB-E, and the IL-17B receptor was subsequently renamed IL-17 receptor homologue 1 (67-69). The latter serves as a receptor for IL-17 B and E, whereas the function of IL-17RC-E is still unknown (58).

Function

There is now substantial evidence from the literature that IL-17 can contribute to accumulation of neutrophils into the bronchoalveolar space (70-77). *In vitro*, IL-17 does not have any direct chemotactic effect on neutrophils (70). It contributes to neutrophil accumulation indirectly, by stimulating the production and secretion of secondary mediators like IL-8, Gro-α, GCP-2, IL-6, G- and GM-CSF from the local stromal cells (bronchial epithelial cells, fibroblasts, smooth muscle cells, venous endothelial cells) (60, 78-84). Furthermore, pre-treatment with specific anti-IL-17 antibody attenuates IL-17-induced effect on bronchoalveolar neutrophilia in rats and mice (70, 76), arguing that the neutrophil-accumulating effect of IL-17 is specific.

It has also been suggested that IL-17 controls the local burden of protein-degrading enzymes (32). Because no direct effect of IL-17 on neutrophil activation *in vitro* has been shown (73, 75) it is likely that IL-17 exerts this control via its neutrophil-accumulating effect. Local stimulation with recombinant mouse IL-17 protein increases the concentration and the corresponding gelatinase activity of matrix MMP-9 in the mouse bronchoalveolar space *in vivo*, although IL-17 does not increase the production or gelatinase activity of MMP-9 per neutrophil (75).

IL-17 and lungs

In vivo studies in mice show that IL-17 plays an important role in the host defence of the lungs. Local administration of endotoxin from *E coli* increases the level of IL-17

mRNA as well as IL-17 protein concentration in the bronchoalveolar space of mice *in vivo* (76, 85). In this model, IL-17 mediates the late phase of neutrophil accumulation (76). In another Gram-negative infectious model, using inoculation with *Klebsiella pneumoniae*, IL-17 is induced in dose- and time-dependent manner (86). In the same model, IL-17's role is further supported by the fact that IL-17-receptor deficient mice have impaired neutrophil accumulation, bacterial clearance and increased death rate compared to their wild-type controls (87).

The knowledge of the role for IL-17 in human lung disease is still very limited. Studies in mouse models of allergic airway inflammation have suggested that IL-17 is involved in allergen-induced accumulation of neutrophils and eosinophils to the bronchoalveolar space (74) and that it might also mediate the allergen induced non-specific bronchial hyper-responsiveness (88). An increase in the concentration of IL-17 protein within the bronchoalveolar space of certain asthmatic patients has been reported (65). Another study claims that the concentration of IL-17 in the sputum correlates with the bronchial hyper-responsiveness to methacholine (89). Elevated levels of IL-17 have been demonstrated in the sputum during pulmonary exacerbation in cystic fibrosis patients colonised with *Pseudomonas aeroginosa* (90). Another demonstration of an increased concentration of IL-17 protein in the human bronchoalveolar space is after exposure of healthy individuals to organic dust (91).

Up-stream regulation of IL-17 production

IL-23, an IL-12-related cytokine secreted by certain activated antigen-presenting cells, has been forwarded as a proximal regulator of IL-17 production (92). IL-23 is a heterodimeric cytokine consisting of two subunits: p40, which is shared with IL-12, and p19 that is unique for IL-23 (93). Although they share structural homology, the biological activities of IL-12 and IL-23 are distinct. Whereas IL-12 is an important

factor for the differentiation of naïve T-cells into INF-γ-producing Th1 cells (94), IL-23 acts mainly on CD4 cells of the memory subtype (93, 95). Unlike IL-12, IL-23 stimulates CD4 cells to produce IL-17 *in vitro* (95). Studies in mouse models of autoimmune disease in the central nervous system and in joints indicate that IL-23 drives the proliferation of a unique subset of T-cells, named the T_H17-subset (96-100). Besides IL-17, IL-23 induces the secretion of IL-17F, IL-6, TNF in this subset *in vitro*. Recent studies demonstrate that IL-23 can stimulate CD8 cells to produce IL-17 as well (77, 101).

It has also been suggested that IL-2, IL-15, IL-18 as well as IL-21 might regulate IL-17 production by T-cells *in vitro*, but their role *in vivo* is still uncertain (62, 102).

Specific aims

The specific aims of this thesis were to determine:

I. Whether severe neutrophilic inflammation in the human bronchoalveolar space is associated with:

- Local up-regulation of IL-17 transcripts

- Increased local expression of IL-17 protein

II. Whether allergic airway inflammation involves endogenous IL-17 in:

- The accumulation of local inflammatory cells

- The control of local proteolytic burden

III. Whether IL-23:

- Is involved in the innate response of lungs to structural components of Gram-positive and Gram-negative bacteria

- Causes local IL-17 production and subsequent accumulation of inflammatory cells

- Affects the local proteolytic burden

Methods

In Vivo studies

Study in humans

Healthy, non-smoking volunteers gave their written consent to participate in the study after receiving both written and oral information. All the participants were non-atopic, non-asthmatic as determined by history and questionnaire. They were exposed to organic dust while weighing pigs in a swine confinement, during three hours. The procedure incorporates exposure to aeroxidised organic dust, which causes severe neutrophil inflammation. Normal lung function and normal airway responsiveness were ascertained in all subjects prior to exposure (see Methods in Paper I).

Bronchoalveolar lavage

The subjects underwent bronchoscopy with bronchoalveolar lavage 2 weeks before and 24 hours after the exposure.

After pre-medication and under local anaesthesia, a flexible fibreoptic bronchoscope was inserted through the nose. The bronchoscope was wedged in a middle lobe bronchus and sterile saline was instilled. After each instillation, the BAL suspension was gently aspirated. After sterile filtration, the BAL suspension was centrifuged. The cell-free supernatant was kept frozen until analysis. The cell pellet was re-suspended, cell viability was assessed using trypan blue exclusion and the total cell number (ie. concentration) was determined for each sample in a Bürker chamber. BAL cytospin slides were prepared. For differential cell counts, slides were stained utilizing the May-Grünwald-Giemsa method. The latter allows distinguishing the different cell types by their shape and colour using light microscopy.

Messenger RNA for IL-17 in BAL cells

Total RNA was isolated from BAL cells and, after measuring its quantity and purity by spectrophotometry, used to generate first-strand cDNA by reverse transcription. PCR technique was then utilized for amplification of the cDNA using commercially available primers for IL-17. During the amplification phase, DIG Labelling mix was added to the samples. The DIG-labelled PCR products were incubated with an internal probe specifically designed to hybridize with each PCR-gene product. The detection was performed using PCR ELISA DIG-detection kit. The expression of transcripts for IL-17 mRNA were normalized to the expression of transcripts for the house-keeping gene HPRT and expressed as percentage of it.

Immunocytochemical (ICC) detection of intracellular proteins in BAL cells

ICC was used in other to characterize the cellular origin of the protein of interest. BAL cytospin slides were fixed with formalaldehyde followed by blocking of unspecific binding and of endogenous biotin.

ICC detection of IL-17

For ICC detection of IL-17, a polyclonal goat anti-human IL-17 antibody was utilized. As secondary antibody, a biotinylated donkey anti-goat IgG was used, and bound antibodies were visualized with Vector Red staining.

ICC detection of MMP-9

For ICC detection of MMP-9, a polyclonal goat anti-human MMP-9 was used. The secondary antibody and the visualization of the bound antibodies were the same as for IL-17.

Data presentation and statistics

Data are presented as median (range) values. The analysis of differences between measurements before and after the exposure, were conducted utilizing the sign test,

assuming binominal distribution. Compensation was made taking into the account the limited number (*n)* of the participants in the study.

Studies in mice

BALB/c mice, 6–8 wk old, were maintained under conventional animal housing conditions and provided with food and water *ad libitum*.

Allergen sensitization and challenge

In paper II, the mice were sensitized by intraperitoneal injections of aluminum-precipitated antigen containing ovalbumin bound to aluminum hydroxide in phosphate-buffered saline (PBS) twice, 5 days apart. Eight days after the second sensitization, the animals were briefly anaesthetized and challenged intranasally (see also Intranasal stimulation below) with OVA or PBS.

Intranasal stimulation

Animals were transiently anaesthetized using isofluorane and were subsequently intranasally (i.n.) stimulated.

To determine the conditions for IL-23 release, mice were i.n. stimulated with either PBS, endotoxin (LPS from E.Coli) or peptidoglycan (PepG from Staphylococcus aureus).

To determine the impact of IL-23, mice were i.n. stimulated with recombinant IL-23 protein or its negative control Vehicle (Phosphate-buffered saline [PBS] + bovine serum albumin [BSA] + lipopolysaccharide [LPS]) in corresponding concentration and volume for 3 days (i.e. three daily doses). LPS was added to the vehicle in order to compensate for the maximal possible endotoxin content of the recombinant IL-23 protein (to avoid a false positive outcome of the study). The animals recovered fully after each anaesthesia and did not display any clinical signs of long-term side effects.

Collection and processing of samples

Animals were anaesthetized with a mixture of xylazin and ketamine. They were euthanized by bleeding of the right ventricle of the heart and blood samples were collected. Unless stated otherwise, the sampling took place 24 hours after the last intranasal stimulation.

Harvest of BAL fluid (Paper II and III)

Tracheotomy was performed and the airways were washed twice with PBS through a tracheal cannula, followed by gentle aspiration. The recovered BAL suspension was kept on ice. After centrifugation of the suspension, the cell-free BAL supernatant was frozen until further analyses. The cell pellet was re-suspended in PBS containing BSA and the total cell number was determined. BAL cytospin slides were prepared. Cell differential counts were performed after May-Grünwald-Giemsa staining (see also Bronchoalveolar lavage above).

Harvest of lungs (Paper III)

Lungs were washed with PBS through the tracheal cannula, perfused with warmed PBS through the right ventricle of the heart and then were carefully excised. For determination of IL-17 mRNA and IL-23 protein, the lungs were immediately placed in Eppendorf tubes, snap-frozen in liquid nitrogen and stored until further use.

For flow cytometry analisys of lung cells after stimulation with IL-23, the lungs were placed in a mixture of Hank's Balanced Salt Solution (HBSS) and Goldgi stop in order to block protein secretion until cell staining. Afterwards, the lungs were grinded and tissue fragments removed via filtration through cell strainers. The cell suspension was centrifuged; the cells were re-suspended in PBS containing BSA and kept on ice until further use.

Systemic blockade of endogenous IL-17

In Paper II, a neutralizing anti-mouse IL-17 monoclonal antibody or its isotype control antibody - rat IgG2α, were administrated intravenously into the lateral tail vein of the mouse three hours before OVA challenge.

In Paper III, the mice were pre-treated with the same anti-mouse IL-17 antibody or its isotype control intraperitoneally, 12 hours before each stimulation i.n.

Immunostaining

Immunocytochemical detection

As in Paper I, ICC was used to characterize the cellular origin of the protein of interest. BAL cytospin slides were fixed with formalaldehyde followed by blocking of unspecific binding and of endogenous biotin.

ICC detection of FAS antigen (Paper II)

For FAS antigen detection, an anti-mouse FAS monoclonal antibody was utilized. As secondary antibody, a biotinylated donkey anti-goat IgG was utsed. Bound antibodies were visualized by Vector Red staining.

ICC detection of MMP-9 (Paper II and III)

For MMP-9 detection, a polyclonal goat anti-mouse MMP-9 antibody was used. The secondary antibody and the visualization of the bound antibodies were the same as for FAS antigen with the modification that, in Paper III, we used Liquid Permanent Red instead of Vector Red. Although provided by different manufacturers, these two staining techniques are similar.

Flow cytometry analysis of lung cells after intranasal stimulation with IL-23 (Paper III)

After blocking the unspecific binding, the cells were incubated with a PerCP-conjugated anti-CD4 antibody or its isotype-matched control. Surface-immunostained cells were fixed in paraformaldehyde and cell membranes were permeabilized by saponin buffer. Thereafter, the cells were incubated with a Phycoerytrin (PE)-conjugated rat anti-mouse IL-17 monoclonal antibody, Fuorescein-conjugated rat anti-mouse IFN-γ antibody or Alexa-Fluor labeled anti-mouse IL-4 or their respective isotype controls. The analysis was performed using a FACScan flow cytometer. Ten thousand cells were computed in a list mode and analyzed using the CellQuest Software.

Determination of IL-23 protein levels by one-dimensional western blot analysis

In Paper III, mice were i.n. stimulated with PBS, LPS or peptidoglycan (see "Intranasal stimulation" above) and lung tissue and BAL fluid were harvested 1.5 hours later (see "Harvest of BAL fluid" and "Harvest of Lungs" above).

The lung tissue was finely homogenized and the protein concentration was determined using commercial protein assay kits. The cell lysate was loaded onto a SDS-polyacrylamide gel and, after electrophoresis, the gel was electro-blotted onto a Hybond ECL nitrocellulose membrane. After blocking non-specific binding, a rat anti-mouse IL-23 p19 monoclonal antibody was added followed by horseradish Peroxidise-conjugated anti-rat IgG. Bound antibodies were visualized with ECL Plus. The level of IL-23 protein was determined by exposure in a charge-coupled device camera.

In addition to lung tissue, we processed cell-free BAL fluid in a similar manner.

An individual sample from the PepG-stimulated group was then arbitrarily assigned as a calibrator against which all other samples are expressed as a fold change.

Messenger RNA for IL-17

In Paper III, total RNA was isolated from 10 mg of lung tissue and used to generate first-strand complementary DNA reverse transcription. Quantitative real-time PCR was performed using commercially available primers. Gene expression was quantified by multiplexing single reactions, where the gene of interest (IL-17) was standardized to control (18s ribosomal RNA). An individual sample from the control group was then arbitrarily assigned as a calibrator against which all other samples are expressed as a fold change.

Measurement of cytokines

In Paper III, cell-free BAL fluid was analyzed for free, soluble IL-17 and total pro-MMP-9 using commercially available enzyme linked immunosorbent assay (ELISA) kits.

Assessment of the local proteolytic burden

To assess the local proteolytic burden (Paper III), we ascertained the identity [pro-MMP-9 ELISA (see Measurement of cytokines) and zymography], activity (gelatinase substrate assay) and cellular source (See ICC for MMP-9) of gelatinases in BAL fluid.

For zymography cell-free BAL fluid was run in SDS-PAGE mini-gels at constant voltage under non-reducing conditions. The gels were then washed, incubated over night in zymography buffer and stained with Coomassie blue .

Cell-free BAL fluid was also tested for net gelatinase activity using fluorescence-conjugated gelatin. After incubation with BAL fluid from IL-23 or vehicle-stimulated mice, the fluorescence intensity of a gelatin substrate was measured to detect quantitative differences in protease activity in BAL fluid.

In Vitro studies

Cell enrichment

For the *in vitro* experiments, cells were enriched by positive selection using magnetic cell sorting system (MACS) according to manufacturer's instructions. MACS allows to magnetically sorts cells that are labeled with biotinylated antibodies or molecules.

Enrichment of BAL macrophages and blood monocytes (Paper II)

Mice were sensitized and challenged with OVA or PBS and BAL fluid and blood were harvested as described previously (see Allergen sensitization and challenge as well as Collection and processing of samples). BAL macrophages or blood monocytes were enriched using a biotinylated *Griffonia simplicifolia* lectin 1 antibody. The latter recognizes galactose-containing carbohydrate residues present on macrophages and monocytes.

Enrichment of BAL CD3 cells (Paper III)

To yield high cell number, mice were primed *in vivo* with endotoxin (See also Intranasal stimulation). BAL was harvested as described above (see Collection and processing of samples). CD3 BAL cells were enriched by positive selection using biotinylated monoclonal anti-CD3ε antibody.

Cell cultures

Culture of BAL macrophages (Paper II)

The enriched macrophages were re-suspended in complete medium (RPMI culture supplemented with fetal calf serum, penicillin-streptomycin, L-glutamine and sodium pyruvate), seeded into 96-well plate and incubated for 20 hours. BAL-fluid macrophages were stimulated with recombinant mouse IL-17 protein or vehicle

(complete medium). Cell viability was assessed by Trypan Blue test at baseline and at the end of the 20-hour incubation of BAL-fluid macrophages.

Culture of BAL CD3 cells (Paper III)

BAL CD3 cells were incubated for 12 hours as described for BAL macrophages above (see Culture of BAL macrophages). The cells were incubated in complete medium only (negative control) or stimulated with recombinant mouse IL-23 protein, phorbol myristate acetate (PMA) and calcium ionophore (CI). During the last 6 hours of the incubation, Brefeldin A solution was added to the culture in order to stop protein secretion.

Chemotaxis assay

The chemotaxis assay for blood monocytes was performed in a 48-well microchemotaxis chamber. The bottom wells were filled with either RPMI containing BSA (negative control), recombinant MCP-1 (positive control), or recombinant mouse IL-17 protein. A polycarbonate filter was placed over the bottom wells. The silicon gasket and upper piece of the chamber were applied and monocyte suspension was pipetted into upper wells. After incubation, the filter was removed, stained and mounted on a glass slide. In each well, monocytes that completely migrated through the filter were counted using a light microscope. The chemotactic response was expressed as a migration index. For each chemotactic stimulus (recombinant MCP-1 or IL-17 protein), a migration index was calculated by dividing the number of migrated cells in response to the cytokine by the number of cells that migrated randomly, that is, in response only to RPMI containing BSA. Thus, a reference index exceeding 1 indicates chemotaxis.

Flow cytometry analysis of intracellular IL-17 protein in bronchoalveolar CD3 cells

BAL CD3 cells cultured and stimulated as described above (see Culture of BAL CD3 cells), were incubated with a PerCP-conjugated anti-CD4 antibody or anti-CD8 or their isotype-matched controls. After fixation, cell membranes were permeabilized by saponin buffer. Thereafter, the cells were incubated with a Phycoerytrin (PE)-conjugated rat anti-mouse IL-17 monoclonal antibody or its isotype control – a PE-conjugated rat IgG1. The flow cytometry analysis was performed using a FACScan flow cytometer. Ten thousand cells were computed in a list mode and analyzed using the CellQuest Software. (See also Flow cytometry analysis of lung cells after stimulation with IL-23)

Data presentation and statistics

Data are presented as mean with SEM. p less than 0.05 was considered statistically significant. Non-parametric (Paper II) or parametric tests (Paper III) were used as appropriate (see Methods: statistical analysis in Paper II and III for details).

Results and comments

IL-17 in the human bronchoalveolar space after exposure to organic dust

A previous study utilizing organic dust exposure of healthy, non-smoking volunteers, as a model of severe neutrophilc airway inflammation, showed that this exposure increases the concentration of free, soluble IL-17 protein in BAL fluid (91). In Paper I, we found that the exposure to organic dust significantly increases the levels of IL-17 mRNA in BAL cells as well (Fig 1).
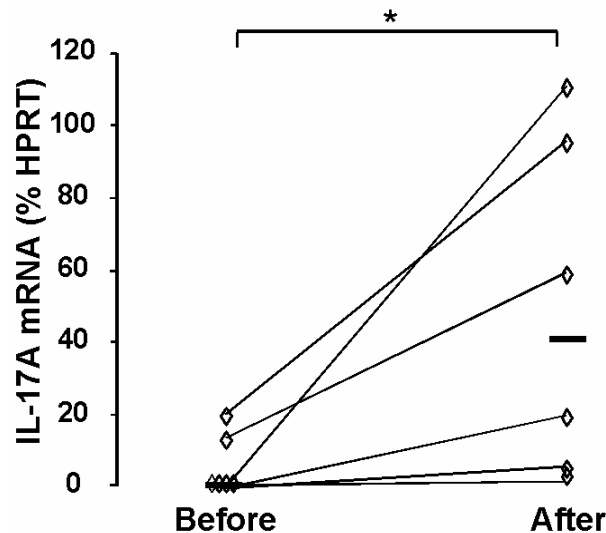

Fig.1 *Messenger RNA for IL-17A in BAL cells (percentage of the house-keeping gene HPRT), measured 2 weeks before and 24 hours after exposure to organic dust in a swine confinement. Data are shown as individual (rhombs) plus median (bold horizontal lines) values. *: p<0.0167; n=6*

To determine a cellular source of IL-17 in the human bronchoalveolar space *in vivo*, we stained BAL cells for intracellular IL-17 protein utilizing ICC. We demonstrated that it was ''lymphoid'' BAL cells that stain positively for IL-17 and the fraction of IL-17-positive lymphoid cells increases 8-fold after the exposure (Fig 2 A & B). Exposure to organic dust increased the percentage of neutrophils stained positively for MMP-9 protein as well (Paper I, Fig 4 and 5).

Taken together, the results from the previous study on IL-17 protein release and our study suggest that exposure to organic dust induces *de novo* synthesis of IL-17, which most probably takes place in ''lymphoid'' BAL cells. The fact that the increase of IL-

17 mRNA is associated with an increase in MMP-9-positive neutrophils is compatible with IL-17 controlling the local proteolytic burden through its neutrophil
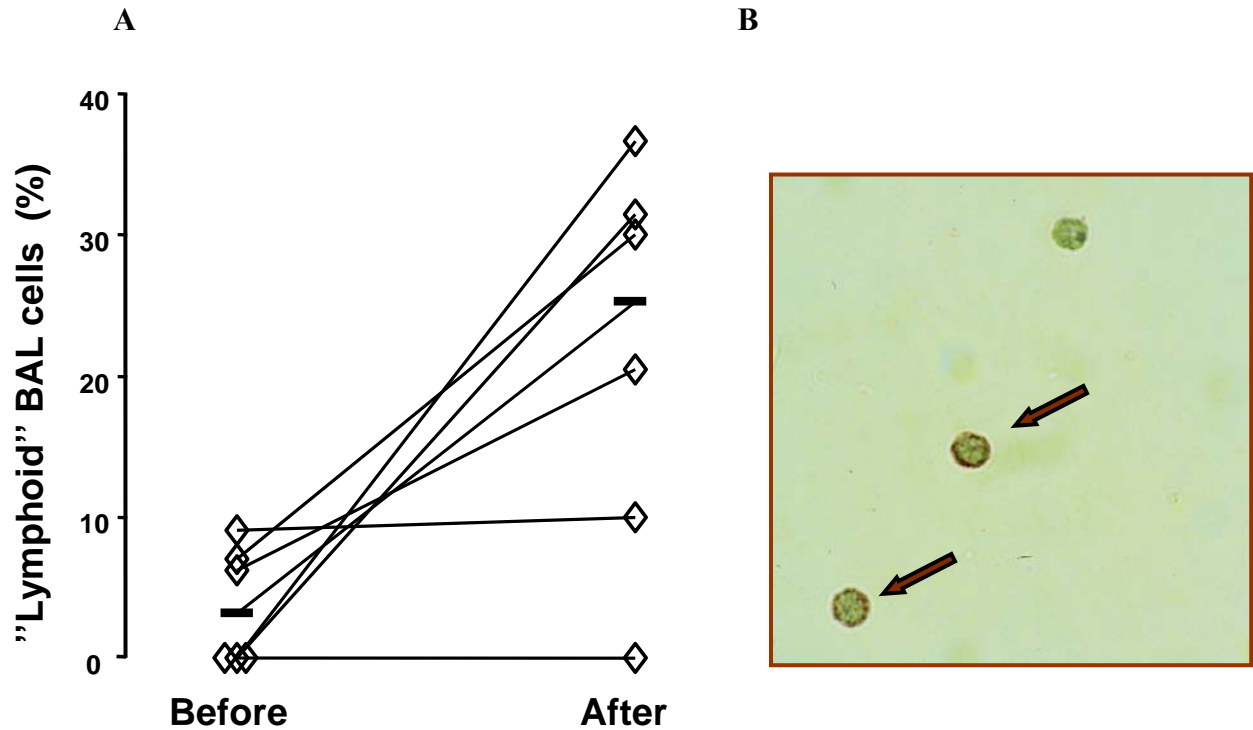
Fig. 2 *IL-17 protein in the human bronchoalveolar space*
A. *Percentage of ''lymphoid'' BAL cells expressing IL-17 protein before and after the exposure to organic dust in a swine confinement. Data are shown as individual (rhombs) plus median (bold horizontal lines) values; p>0.0167, i.e. not statistically significant; n=6.*

B. *Immunocytochemical detection of IL-17 protein (arrows) in ''lymphoid'' BAL cells after exposure to organic dust*

accumulating effect.

Macrophage-accumulating effect of endogenous IL-17 in allergic airway inflammation

In Paper II, we utilized specific anti-IL-17 antibody in a mouse model of allergic airway inflammation. Blocking endogenous IL-17 decreased the number of bronchoalveolar neutrophils (Fig. 3). However, to our surprise, blocking endogenous IL-17 decreased the number of bronchoalveolar macrophages as well without any statistically significant effect on the other types of inflammatory cells (Fig. 3).

IL-17 as a chemotactic and survival factor for bronchoalveolar macrophages

In Paper II, we showed that recombinant IL-17 protein induces migration of blood
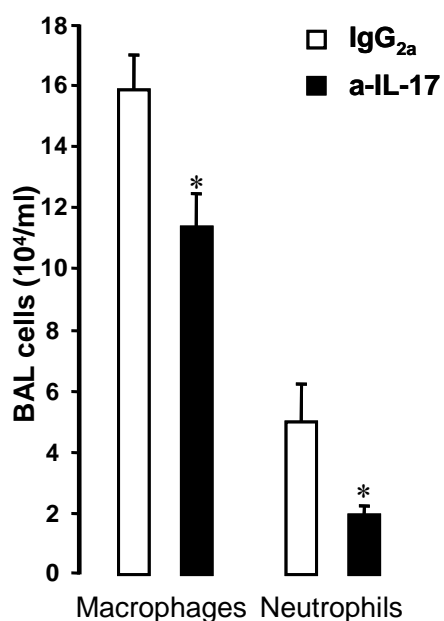
Fig. 3 *Effect of systemic pre-treatment with anti-IL-17 ab or its isotype control ab (IgG2α) on the number of BAL macrophages and neutrophils in OVA-sensitised and challenged mice. Data are presented as mean ± SEM. *: p<0.05; n=12-13*

monocytes from OVA-challenged mice. This migration was of similar magnitude as the migration induced by MCP-1, a known chemotactic factor for monocytes (Fig.4).

In vivo, blocking endogenous IL-17 increased the percentage of FAS-antigen-positive macrophages only (Fig. 5), (see also Fig. 3 in Paper II); without any substantial effect on the other BAL cells (data not shown). Stimulation with recombinant IL-17 protein *in vitro* increased the survival of bronchoalveolar macrophages in a concentration dependent manner (Fig. 6). The IL-17–induced (1 ng/ml) increase in survival was significantly higher for macrophages from OVA-challenged mice compared with those from PBS-exposed animals.

Blockade of endogenous IL-17 decreases MMP-9-positive neutrophils and macrophages

In Paper II, we also demonstrated that systemic blockade of IL-17 substantially decreases the proportion of MMP-9-expressing bronchoalveolar neutrophils and macrophages (Paper II, Fig. 4, 5).
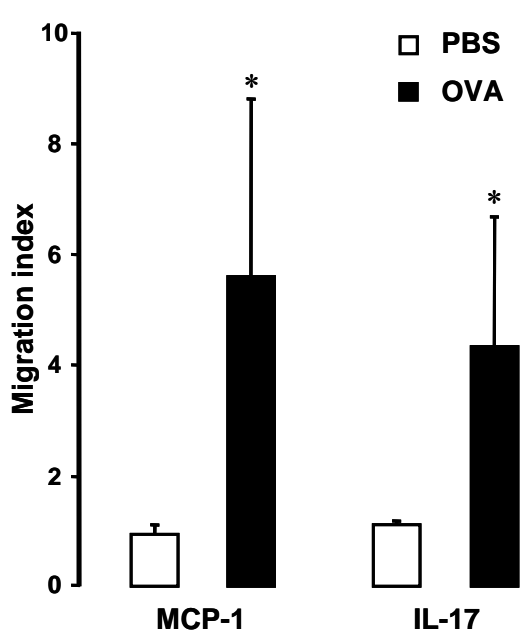
Fig. 4 *Effect of 1-hour stimulation with MCP-1 or IL-17 on the migration of blood monocytes harvested from PBS-exposed or OVA-challenged mice. Data from three experiments is shown as mean ± SEM. *: p<0.05*
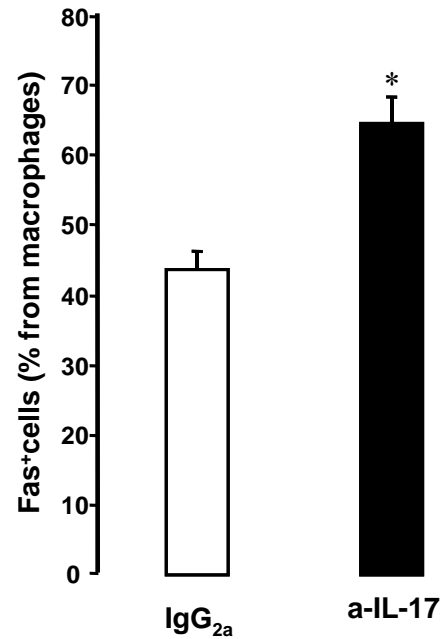
Fig. 5 *Effect of systemic pre-treatment with anti-IL-17 ab or its isotype control ab (IgG2α) on the percentage of Fas⁺ BAL macrophages from OVA-sensitised and challenged mice. Data are presented as mean ± SEM . *: p<0.05; n=12-13*
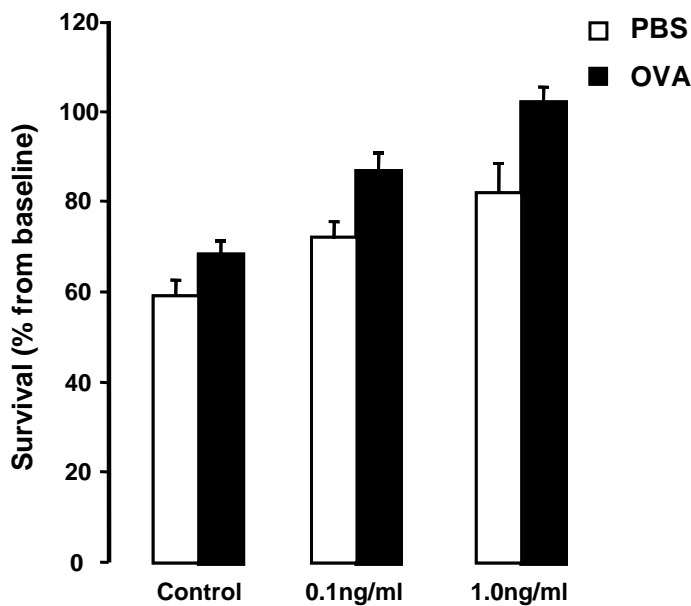
Fig. 6 *Effect 20-hour stimulation with IL-17 on the survival of BAL macrophages harvested from OVA-sensitised and challenged mice. Data from three experiments are presented as mean ± SEM .*

IL-23 protein is increased and released in response to Gram-positive and Gram-negative bacterial components *in vivo*

In Paper III, we showed that IL-23 protein is rapidly increased in lung tissue (Fig. 7 A) and released in BAL fluid (Fig. 7 B) after stimulation with endotoxin (LPS), a component of Gram-negative bacteria and peptidoglycan (PepG), a Gram-positive bacterial component.
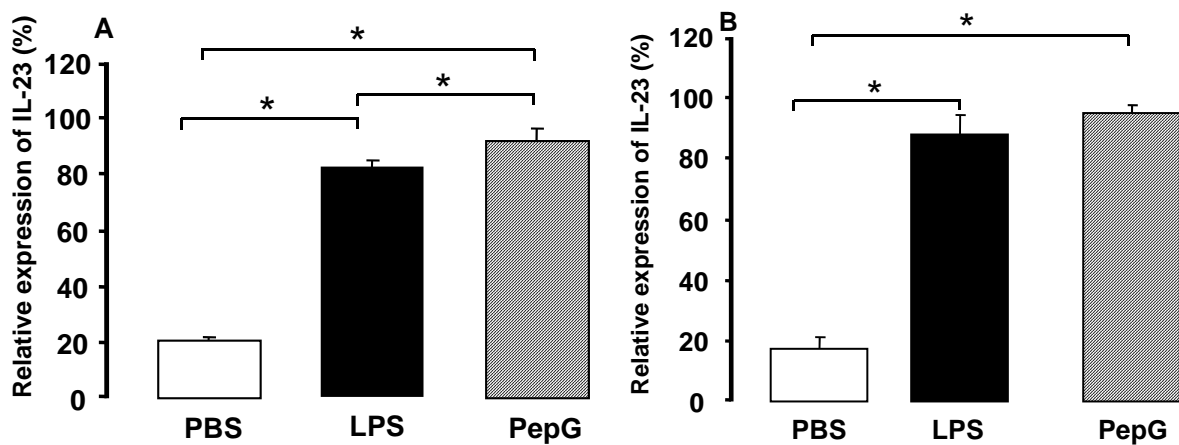
Fig. 7 *IL-23 protein levels determined 1.5 hours after intranasal (i.n.) stimulation in vivo with PBS, LPS (10μg) or Peptidoglycan (PepG) (50μg). Data are shown as mean ± SEM; n=3-5 per group *: p<0.05 **A.** Lung tissue; **B.** BAL fluid.*
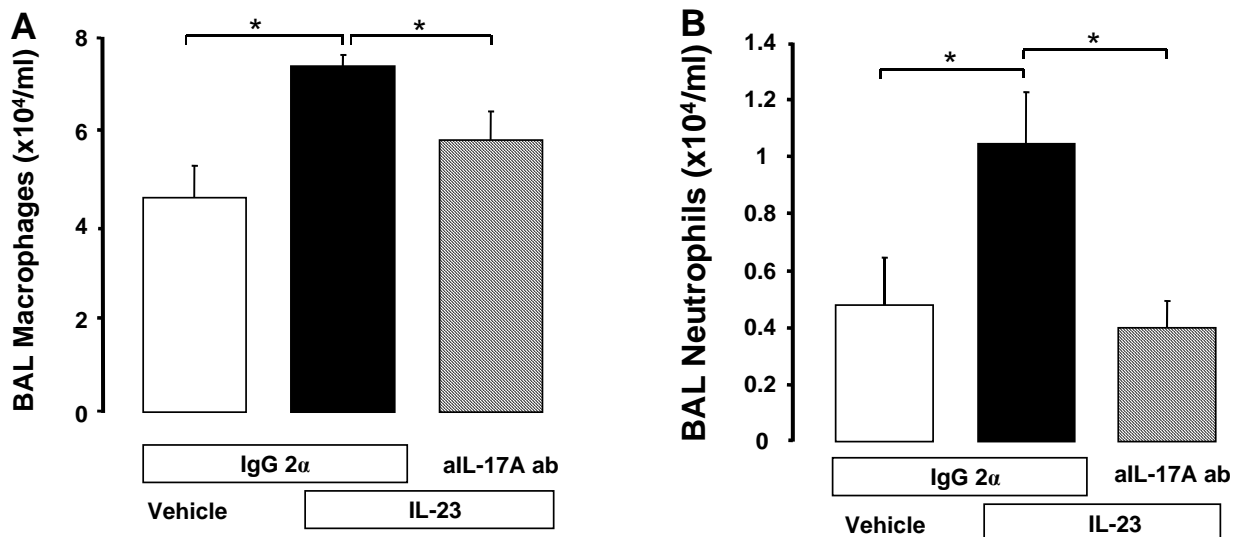

Fig. 8 *Effect of neutralizing endogenous IL-17 on IL-23-induced increase in BAL macrophages (**A**) and neutrophils (**B**). Animals were pre-treated with a-IL-17 ab or its isotype control (IgG 2α) 12 hours before each i.n. stimulation with IL-23 (1μg) or vehicle. Data are shown as mean±SEM; *: p<0.05; n=5-8 per group*

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IL-23 accumulates bronchoalveolar macrophages and neutrophils via endogenous IL-17 *in vivo*

Short-term stimulation with recombinant IL-23 protein (1 µg) increased the number of bronchoalveolar macrophages and neutrophils whereas blocking endogenous IL-17 with a specific antibody attenuated this IL-23-induced increase (Fig. 8 A & B).

IL-23 induces production of IL-17 protein in CD4 cells

Stimulation with IL-23 (1 µg) increased the level of IL-17 mRNA in lung tissue (Fig. 9 A) and the concentration of IL-17 protein in BAL fluid (Fig 9 B).
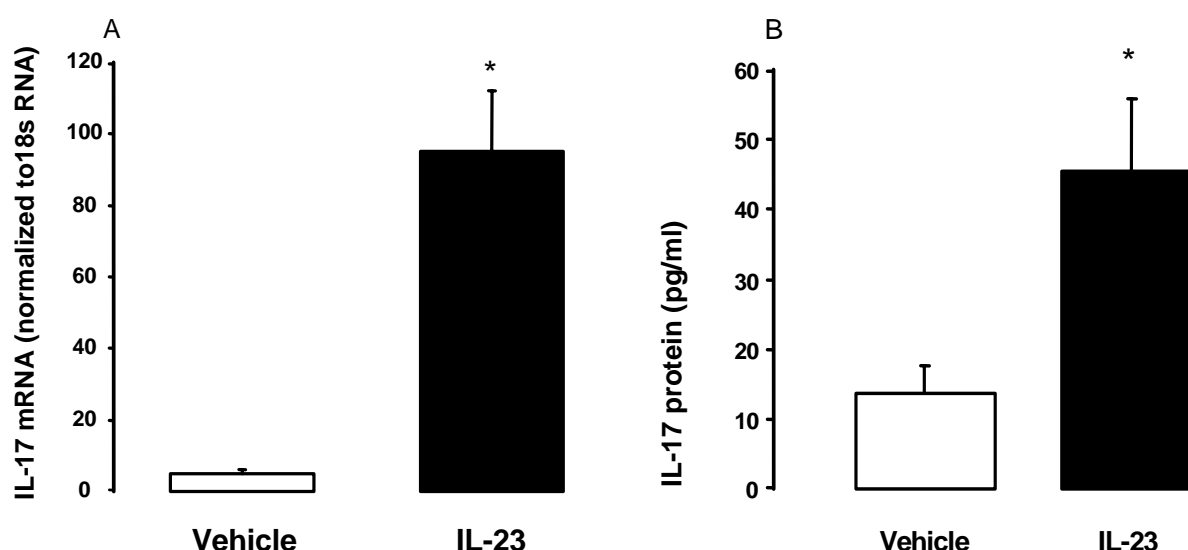
Fig. 9 *Assessment of the impact of IL-23 on IL-17 production in vivo. **A.** Relative expression of IL-17 mRNA in lung tissue cells after i.n.stimulation with IL-23 (1µg) or vehicle. Data are shown as mean±SEM; *: p<0.05; n=7 per group. **B.** Concentration of soluble IL-17 protein in cell-free BAL fluid after i.n.stimulation with IL-23 (1µg) or vehicle. Data are shown as mean±SEM; *: p<0.05; n=13 per group.*

To determine a cellular source of IL-17 in the bronchoalveolar space, BAL CD3 cells from LPS-primed mice were analysed for intracellular IL-17 protein content, after *in vitro* culture in three different conditions: in plain medium or stimulated with IL-23 (100ng/ml) or PMA+CI. The flow cytometry analysis showed that *in vivo*-primed CD4 and CD8 cells express intracellular IL-17 protein without additional stimulation (ie cultured in plain medium only) *in vitro* (see Intracellular protein expression after

stimulation with IL-23 in Paper III). Stimulation with IL-23 increased the fraction of CD4 cells that stained positively for IL-17 without affecting the CD8 fraction (Fig 10 A). Furthermore, IL-23 increased the intensity of IL-expression in each of these CD4 cells (Fig 10 B).

A

B

C
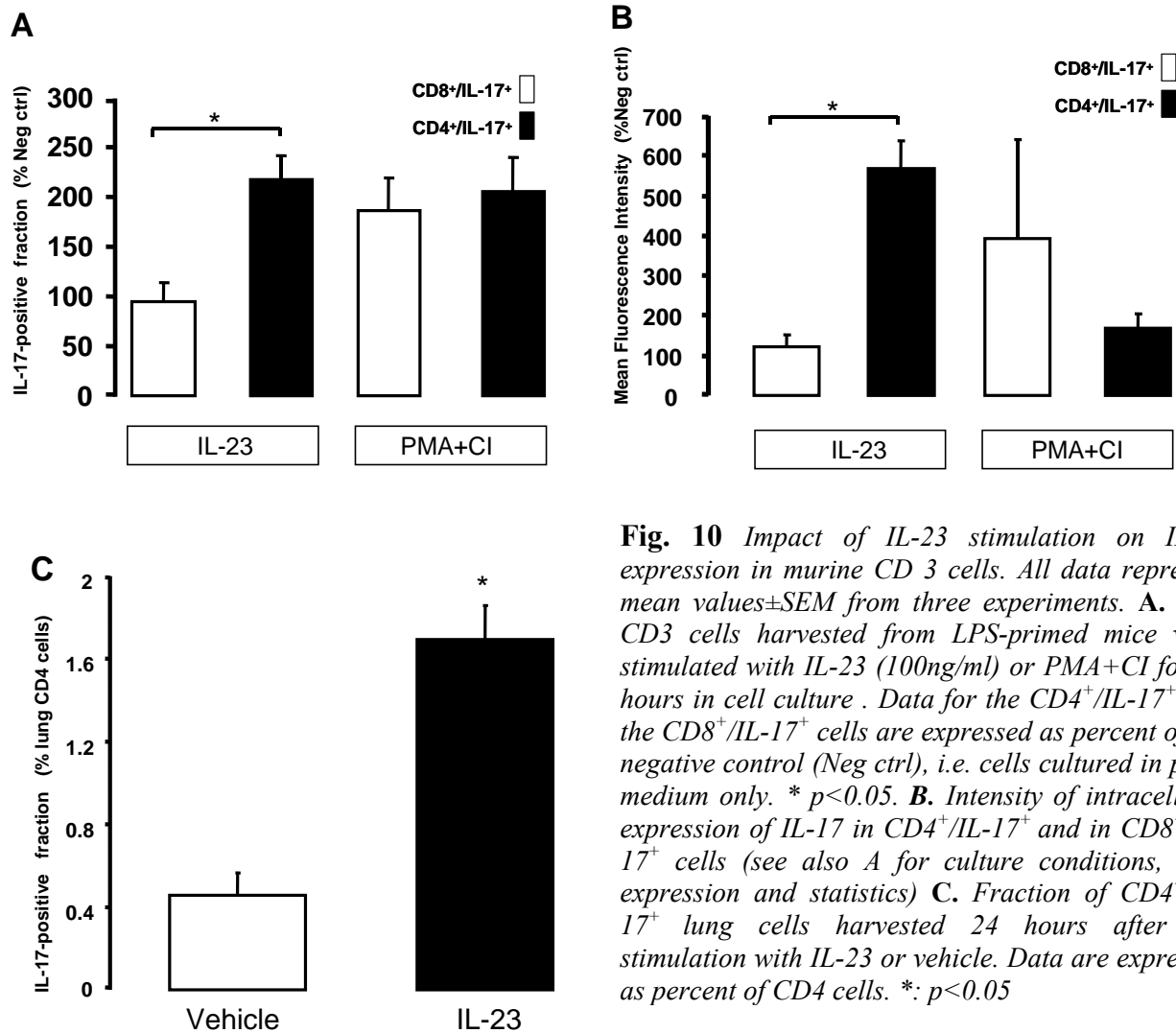
Fig. 10 *Impact of IL-23 stimulation on IL-17 expression in murine CD 3 cells. All data represent mean values±SEM from three experiments. **A.** BAL CD3 cells harvested from LPS-primed mice were stimulated with IL-23 (100ng/ml) or PMA+CI for 12 hours in cell culture . Data for the CD4^{+}/IL-17^{+} and the CD8^{+}/IL-17^{+} cells are expressed as percent of the negative control (Neg ctrl), i.e. cells cultured in plain medium only. * p<0.05. **B.** Intensity of intracellular expression of IL-17 in CD4^{+}/IL-17^{+} and in CD8^{+}/IL-17^{+} cells (see also A for culture conditions, data expression and statistics) **C.** Fraction of CD4^{+}/IL-17^{+} lung cells harvested 24 hours after i.n. stimulation with IL-23 or vehicle. Data are expressed as percent of CD4 cells. *: p<0.05*

To further characterise, whether IL-23 (1 μg) polarises the CD4 positive IL-17-positive cells towards Th1 or Th2 phenotype, lung CD4 cells from IL-23- and vehicle-stimulated mice were analysed for intracellular IL-17 protein as well as for INF-γ or IL-4 protein by flow cytometry. In accordance with our *in vitro* data, IL-23 significantly increased the percentage of IL-17-producing CD4 cells (Fig 10 C). However, IL-23 did not have any pronounced effect on the fraction of cells expressing

either INF-γ or IL-4. (see Intracellular protein expression after stimulation with IL-23 in Paper III).

IL-23 increases the proteolytic burden within the bronchoalveolar space *in vivo*

Stimulation with a higher dose (3 μg) IL-23 significantly increased the concentration of total, pro-MMP-9 protein in BAL fluid (Fig. 11 A). Zymography analysis of BAL fluid from mice stimulated with IL-23 (1 μg) identified a 92 kDa band accounting for most gelatinase activity (Paper III, Fig 7 B). This molecular weight corresponds to the molecular weight of MMP-9. Furthermore, net gelatinase activity of BAL-fluid from IL-23-stimulated mice (1 μg) was increased as well (Fig 11 B). Immunocytochemistry analysis revealed that it was BAL neutrophils, which stained strongest and in a reproducible manner, for MMP-9 after stimulation with IL-23 (1 μg) (Paper III, Fig 7 D).
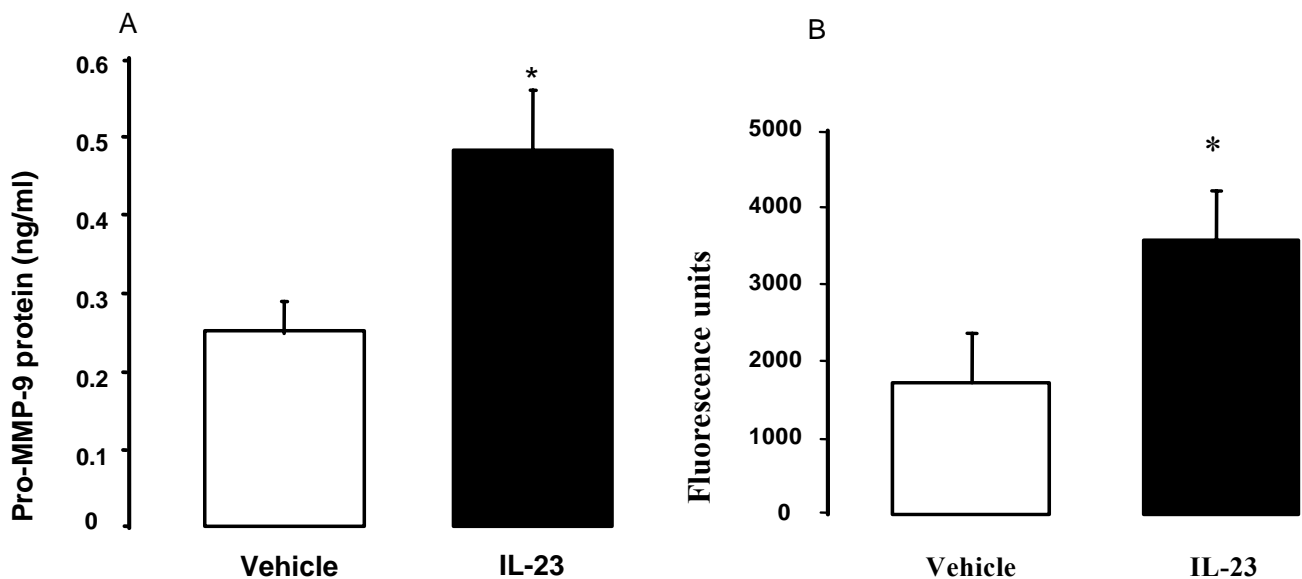
Fig. 11 *Assessment of impact of IL-23 on the proteolytic burden in cell-free BAL fluid. Data are presented as mean values±SEM. *: p<0.05*
A. *Concentration of total pro-MMP-9 protein after i.n. stimulation with IL-23 (3μg) or vehicle. n=7-8 per group*
B. *Net gelatinase activity after i.n. stimulation with IL-23 (1μg) or vehicle. The gelatinase activity was determined using a gelatin conjugated fluorescence substrate assay. n=11-13 per group*

General Discussion

Source of IL-17 in lungs *in vivo*

Current knowledge indicates that IL-17 is released from certain activated T lymphocytes but there is no corresponding information on lymphocytes in human lungs. With respect to the source of IL-17 in lungs, a recent study shows that CD3 cells isolated from mouse lung tissue and co-cultured with airway macrophages, release free soluble IL-17 protein upon PMA+CI stimulation *in vitro* (76). In Paper I, we now demonstrate that a fraction of ''lymphoid '' BAL cells from humans stains positively for intracellular IL-17 protein, following exposure of healthy volunteers to organic dust. In addition, we present evidence for increased IL-17 mRNA in the collective population of BAL cells after such an exposure. Therefore, our results suggest that there are cells, most probably lymphocytes, residing within the human bronchoalveolar space *in vivo*, which are capable of producing IL-17. These results, as well as the results on IL-17-protein release from our previous study utilising the same model of organic dust exposure (91), forward IL-17 as a cytokine having a potential role in human airway disorders characterized by an excessive innate response.

In our experimental mouse model in Paper III, we further characterise this bronchoalveolar cell source for IL-17 in terms of CD4 and CD8 origin. In line with data from previous studies (59-63), we show that both CD4 and CD8 BAL cells, primed and harvested *in vivo*, can produce IL-17 protein *in vitro* without additional stimulation. Furthermore, upon stimulation with IL-23, the fraction of IL-17-producing CD4 cells as well as the individual intensity of each CD4 cell does increase. Importantly, we confirm these results *in vivo* as well, by demonstrating that short-term IL-23 stimulation increases the IL-17-positive CD4 cell fraction in mouse

lungs. The latter impact of IL-23 is exerted without any pronounced effect on Th1/Th2 polarization in the responsive CD4 cells, as assessed by co-expression of IL-4 and IFN-γ. This observation is compatible with the existing evidence that the IL-23-driven immune response is not associated with IFN-γ or IL-4; instead this response is fulfilled by the T_H17-subset (96-100). Whether the IL-17-producing cells observed in Paper III are really unique T_H17's, i.e. distinct from the ''classical'' Th1 and Th2 cells, remains to be proven but, importantly, we demonstrate that, upon IL-23 stimulation, there are IL-17-producing-CD4 cells in the lungs *in vivo*. It can be hypothesized that, according to their intracellular protein expression assessed in Paper III, these IL-23-responsive CD4 lung cells could be divided into three groups: cells expressing intracellular IL-17 alone (CD4$^+$/IL-17$^+$); cells co-expressing IL-17 and IFN-γ (CD4$^+$/IL-17$^+$/IFN-γ^+), and cells co-expressing IL-17 and IL-4 (CD4$^+$/IL-17$^+$/IL-4$^+$). Taking into consideration that, *in vitro*, the T_H17's do not express either IFN-γ or IL-4 (96-100), it would be intriguing to determine whether only cells belonging to the first group, i.e. the CD4$^+$/IL-17$^+$cells, represent the T_H17 subset in lungs *in vivo* and whether the cells belonging to the other two groups have the potential to differentiate into either of the T-helper subtypes – Th1, Th2 or T_H17, depending on the surrounding cytokine milieu.

As mentioned earlier, IL-17 can contribute to accumulation of neutrophils to the bronchoalveolar space under various inflammatory conditions (70-77). Our current studies confirm this effect of IL-17 in two different *in vivo* models. In Paper II, we show that endogenous IL-17 promotes allergen-induced neutrophil accumulation in mouse airways. This finding is consistent with a similar observation in a previously published study (74) and supports the idea of IL-17 having a potential role in allergic airway disease. In Paper III, we show that endogenous IL-17 mediates neutrophil

accumulation upon IL-23 stimulation. This finding is compatible with the critical role of IL-23 for IL-17 production *in vitro* (77, 98, 103) and the known neutrophil-accumulating effect of IL-17. Importantly, we demonstrate that IL-23 *per se* induces this IL-17-mediated neutrophil accumulation within the bronchoalveolar space *in vivo*.

Macrophage accumulation mediated by IL-17

In Paper II, we also expand the role of IL-17 in allergic airway inflammation by demonstrating that endogenous IL-17 can increase the allergen-induced accumulation of bronchoalveolar macrophages as well.

Unlike neutrophil accumulation, to which IL-17 contributes in an indirect manner by promoting the release of secondary mediators (78-84), it seems that IL-17 contributes to macrophage accumulation directly. First, pre-treatment with anti-IL-17 did not have any significant effect on the concentration of BAL cytokines like TNF, M-CSF or MCP-1, which in turn can act as potential monocyte-mobilising mediators. Second, the IL-17-induced migration of blood monocytes from sensitised and allergen-challenged mice, serves as evidence that IL-17 acts as a direct chemotactic factor for macrophage precursor cells. Finally, another mechanism explaining the reduction of the number of bronchoalveolar macrophages caused by anti-IL-17 pre-treatment is that IL-17 increases the survival of these cells. This is supported by our *in vivo* finding that blocking endogenous IL-17 increased the expression of FAS antigen selectively in bronchoalveolar macrophages. The FAS antigen-FAS ligand system is recognized as the major extrinsic pathway of apoptosis (105). When Fas antigen, which belongs to the TNF receptor family, binds to FAS ligand apoptosis is induced in FAS-bearing cells (104, 105). In other words, IL-17 seems to promote macrophage survival by down-regulating the programmed cell death. This *in vivo* observation was

supported by the fact that *in vitro* stimulation with IL-17 increased the survival of bronchoalveolar macrophages in a concentration dependent manner.

The functional IL-23-IL-17 axis *in vivo*

Paper III indicates that IL-23 forms a functional axis with IL-17 in lungs *in vivo.* This axis seems to be involved in the early immune response to bacterial stimuli because IL-23 protein is rapidly increased in lung tissue and released in BAL fluid after stimulation with LPS and peptidoglycan. Indeed, there are data in the literature supporting the idea of a functional link between IL-23 and IL-17 playing a role in host defence of lungs when Gram-negative bacteria are invading microbes. In a mouse model of *Klebsiella pneumoniae* infection, it has been shown that mice deficient in IL-23 are more susceptible to this infection and demonstrate increased mortality (106). Similar observations in IL-17 receptor–deficient mice infected with the same pathogen (87) lend further support for the existence of an IL-23-IL-17 axis. Also, IL-23 seems to play a critical role in mediating pulmonary responses to *Pseudomonas aerugenosa* - another Gram negative bacterium (107). Noteworthy, by showing that Gram-positive bacterial components such as peptidoglycan induce IL-23 protein release *in vivo*, we demonstrate that the IL-23-IL-17 axis might be engaged in the early pulmonary innate response to Gram-positive bacteria as well.

A reason for the involvement of the IL-23-IL-17 axis in the early innate response of the lungs is that the interaction between these two cytokines leads to accumulation of inflammatory cells into the bronchoalveolar space. We show that short-term stimulation with IL-23 mobilises bronchoalveolar macrophages and neutrophils via endogenous IL-17 protein. By demonstrating that IL-23 *per se* increases the release of IL-17 protein in lungs *in vivo*, we further consolidate the recent findings that lung homogenates (106) and BAL fluid (107) from IL-23-deficient mice have less IL-17

protein than the respective wild-type controls in the mouse models of Gram negative bacterial infection referred to above. As discussed above, our demonstration that endogenous IL-17 mediates the IL-23-induced mobilisation of bronchoalveolar neutrophils is fully compatible with the role of IL-23 on the production of IL-17 and the known neutrophil-accumulating effect of the latter. Interestingly, the fact that blocking endogenous IL-17 attenuated the IL-23-induced macrophage accumulation as well is in line with our findings on the role of IL-17 in allergen-induced accumulation of these cells in Paper II. It is also supported by a recent observation that this type of IL-17 blockade caused reduction in the IL-23-induced macrophage accumulation in a mouse model of autoimmune disease in the central nervous system (100).

One important aspect of the biological effects of IL-17 and IL-23 is their ability to control the local proteolytic burden with respect to MMP-9. This has potential pathogenic importance for lung disease since MMP-9 is believed to play an important role in the processes of tissue remodelling, due to its capacity to cleave structural proteins like collagen and elastin (108). Thus, MMP-9 concentration is increased in patients with COPD, bronchial asthma, pulmonary fibrosis and smokers with subclinical emphysema (109-112). It is also claimed that MMP-9 has a functional impact in lung disease as well because it increases the allergen-induced non-specific bronchial hyper-responsive in the mouse bronchoalveolar space in vivo (113).

Two of our current studies lend further support to the previously published evidence that IL-17 increases the local proteolytic burden. In Paper I, we show that the increase of IL-17 mRNA during severe neutrophilic bronchoalveolar inflammation in humans is associated with increased percentage of MMP-9-positive BAL neutrophils *in vivo*. In Paper II, we demonstrate that blocking endogenous IL-17 decreases MMP-9-

positive BAL neutrophils in a mouse model of allergic airway inflammation. The observation that IL-23 also increases the concentration and the corresponding activity of MMP-9 in the bronchoalveolar space *in vivo* is a novel previously unrecognised effect of IL-23 (Paper III). This effect of IL-23 is likely to be at least partially mediated by IL-17 even though we have not specifically proven it. The reason for forwarding such a claim is that it has previously been shown that IL-17 *per se* has an increasing effect on MMP-9 concentration and activity (75). Among bronchoalveolar cells, it is neutrophils mainly, which express MMP-9 protein after stimulation with IL-23 (Paper III) as well as after stimulation with IL-17 (75).

Our experimental results suggest that by forming a functional axis, the antigen presenting cell cytokine IL-23 and the T-cell cytokine IL-17 contribute to linking the innate and adaptive immune responses in the lungs *in vivo*. Besides the involvement in host defence, there is mounting evidence that the IL-23-IL-17 axis, if disregulated, might play an important pathogenic role in autoimmune diseases of the central nervous system, joints and the gut (97-100, 114, 115). Increased expression of IL-23 and IL-17 has been found in patients with psoriasis (116) and in patients with active Crohn's disease (117). Administration of human IL-12 p40 antibody in these patients, i.e. an antibody recognizing a molecular subunit shared by IL-12 and IL-23, attenuates the increased expression of IL-23 and IL-17 and has clinically beneficial effects (116, 117). Future studies are needed to further determine the pathogenic relevance of the IL-23-IL-17 axis in human lung disorders. If down-regulated, this axis could be enhanced, for example by administration of IL-23, in lung disorders associated with immunodeficiency and leukopenia, in order to facilitate inflammatory cell accumulation and improve the immune response. If up-regulated, the IL-23-IL-17 axis could also be pharmacologically blocked in lung disorders associated with

exaggerated local accumulation of macrophages and neutrophils as part of an excess innate response.

Conclusions

I. Severe neutrophilic inflammation in the human bronchoalveolar space is associated with:

- Local up-regulation of IL-17 transcripts
- Increased expression of IL-17 protein in ''lymphoid'' BAL cells.

II. During allergic airway inflammation endogenous IL-17:

- Contributes to allergen-induced accumulation of neutrophils and macrophages within the bronchoalveolar space.
- Controls local proteolytic burden by increasing the number MMP-9-expressing neutrophils and macrophages.

III. IL-23:

- Is involved in the innate response of lungs to structural components of Gram-positive and Gram-negative bacteria
- Causes production of IL-17 in bronchoalveolar CD4 cells *in vivo*
- Accumulates bronchoalveolar macrophages and neutrophils via endogenous IL-17
- Increases the local proteolytic burden, which is generated mainly by MMP-9-expressing neutrophils.

Acknowledgements

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