Lung T cells in inflammatory disorders
An Approach to Interstitial Lung Disease, Multiple Sclerosis and Smoking Induced Inflammation

Mahyar Ostadkarampour

Stockholm 2014
Cover photo by Mahyar Ostadkarampour. Fluorospot analysis of IFNγ and IL-17 producing cells from BAL of sarcoidosis patient. The green and red spots indicate IFNγ and IL-17 producing cells respectively after anti CD3 stimulation. The yellow circles show the cells that produce both cytokines simultaneously. The experiment was analyzed by an automated reader with filters for FITC and Cy3 in Mabtech company.

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Printed by Åtta.45 TRYCKERI AB
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Lung T cells in inflammatory disorders, An Approach to Interstitial Lung Disease, Multiple Sclerosis and Smoking Induced Inflammation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The lungs are constantly exposed to microorganisms and environmental irritants. Pulmonary inflammation is the result of an immune process to protect the body, and may sometimes eventually result in disease. T cells including various subsets are of major importance for orchestrating the protection of the lung as well as for inflammatory reactions. Activated pulmonary T cells not only have the potential to affect the lungs themselves, but they could contribute to immune responses in other organs as well. The overall aim of the study presented in this thesis was to investigate the potential effect of T cell immune responses for chronic lung inflammation from two different aspects. We thus first investigated antigen-specific T cell responses in patients with pulmonary sarcoidosis, and in the second part determined how smoking affected lung T-cell immunity, with regard to the influence of smoking in the development of autoimmunity.

Sarcoidosis is a granulomatous systemic inflammatory disorder which commonly affects the lungs. T cells and particularly activated CD4+ T cells are considered to be involved in the pathogenesis of the disease. A subgroup of sarcoidosis patients known as Löfgren’s syndrome differs strikingly from other patients by particular clinical symptoms. Spontaneous recovery within two years is particularly common in Löfgren’s syndrome patients who are HLA-DRB1*0301 positive, and these patients virtually always have accumulations of T cells expressing a particular T cell receptor (TCR) V gene segment, termed AV2S3, in the lungs. The aetiology of sarcoidosis is still not known. However, recently a specific mycobacterial protein, *M. tuberculosis* catalase-peroxidase (mKatG) was identified in sarcoidosis tissues.

BAL CD4+ T cells from HLA-DRB1*0301 positive Löfgren’s syndrome responded to mKatG with a more pronounced multifunctional cytokine profile, i.e. with simultaneous production of IFNγ and TNF compared to non-Löfgren’s syndrome patients. Non-Löfgren’s syndrome patients instead responded with a higher proportion of cells producing single cytokines, i.e. production of IFNγ alone. Moreover, AV2S3+ CD4+ T cells from both BAL and blood had a higher IFNγ production in response to mKatG compared to AV2S3- CD4+ T cells, while the opposite was found for BAL AV2S3+ CD4+ cells in response to PPD. Furthermore, BAL T cells from Löfgren’s syndrome patients had compared to T cells of non Löfgren’s syndrome higher frequencies of IL-17-producing cells in response to mKatG. Löfgren’s syndrome HLA-DRB1*03 positive patients clearly had higher levels of IL-17 in BAL fluid compared to healthy controls and to patients without Löfgren’s syndrome. Our results indicate that the quality of the T cell response in sarcoidosis patients may play a key role in disease presentation and clinical outcome. These findings imply that the presence of multifunctional BAL CD4+ T cells, higher activities of TCR AV2S3+ CD4+ T cells, and more pronounced IL-17 production in particular subgroups of sarcoidosis patients are involved in antigen elimination at the site of inflammation and may play a role in spontaneous recovery, typical for patients with Löfgren’s syndrome (in particular DRB1*03 positive).

Cigarette smoking is a well-known risk factor for several inflammatory and autoimmune disorders. The risk of developing multiple sclerosis (MS) is strongly increased by smoking in people with genetic susceptibility. Smoking is associated with both release and inhibition of pro-inflammatory and anti-inflammatory mediators that influence different T cell subsets. Our results indicate that cigarette smoke induces a decline in lung Th17 cells and alters the phenotype of T regulatory cells by decreasing the proportion of IL-10 producing Foxp3+ CD4+ cells and increasing the fraction of lung Foxp3+ Helios negative T cells. Thus, an imbalance between Th17/Tregs may be caused by cigarette smoking, which could result in an increased risk for infection and may also have consequences for autoimmune processes postulated to be initiated in the lung. Furthermore we studied the effect of smoking and conventional treatment in the lungs and blood of MS patients compared to healthy individuals. We found that the frequency of Foxp3+Helios- regulatory T cells, important in the context of autoimmunity, was reduced in BAL of MS patients. However, the frequencies of both this subset of Tregs and of total Foxp3+ CD4+ BAL Treg cells was increased after treatment particularly in IFNβ treated MS patients. If the lungs are involved in initiation and propagation of inflammatory processes in MS, the observed effects in IFNβ-treated patients may be involved in disease amelioration in MS patients following such treatment.
LIST OF SCIENTIFIC PAPERS


III. Mahyar Ostadkarampour, Malin Müller, Johan Öckinger, Susanna Kullberg, Anders Eklund, Johan Grunewald, Jan Wahlström; Cigarette smoke induces distinctive Tregs and a Treg/Th17 imbalance in the lungs (Submitted)

IV. Mahyar Ostadkarampour, Susanna Kullberg, Johan Öckinger, Anders Eklund, Fredrik Piehl, Tomas Olsson, Johan Grunewald, Jan Wahlström; Characteristics of lung T cell subsets in multiple sclerosis patients with respect to smoking and beta-interferon treatment (Manuscript)
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<table>
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>ACPA</td>
<td>Anti citrullinated protein antibody</td>
</tr>
<tr>
<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
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<tr>
<td>AM</td>
<td>Alveolar macrophage</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>AV2S3</td>
<td>Variable gene segment 2.3 of the T cell receptor α chain</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BHL</td>
<td>Bilateral hilar lymphadenopathy</td>
</tr>
<tr>
<td>CBD</td>
<td>Chronic beryllium disease</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte-associated antigen 4</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage Associated Molecular Pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DL&lt;sub&gt;co&lt;/sub&gt;</td>
<td>Diffusing capacity of the lung for carbon monoxide</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EAU</td>
<td>Experimental autoimmune uveitis</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FEV1</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box protein 3</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GITR</td>
<td>Glucocorticoid-induced TNF receptor family</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILD</td>
<td>Interstitial lung disease</td>
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<tr>
<td>iNKT</td>
<td>invariant natural killer T cell</td>
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<tr>
<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>iTreg</td>
<td>Induced regulatory T cell</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAIT</td>
<td>Mucosa-associated invariant T cells</td>
</tr>
<tr>
<td>MFI</td>
<td>Median (or mean) fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mKatG</td>
<td>Mycobacterial catalase-peroxidase</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD like receptor (nucleotide-binding oligomerization domain)</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
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<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>pTreg</td>
<td>Peripheral regulatory T cell</td>
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<tr>
<td>qIPCR</td>
<td>quantitative immuno-PCR</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SEA</td>
<td><em>Staphylococcus enterotoxin A</em></td>
</tr>
<tr>
<td>SEB</td>
<td><em>Staphylococcus enterotoxin B</em></td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tfh</td>
<td>Follicular helper T cell</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>tTreg</td>
<td>Thymus-derived regulatory T cell</td>
</tr>
<tr>
<td>Th cell</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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INTRODUCTION

1.1 General introduction

Inflammation is a biological process with accumulation of leukocytes and dissemination of fluid and proteins in the inflamed tissue in response to infection, irritation, injury or physical stress. Acute inflammation is an important immune response to protect against pathogens. The inflammation usually clears after pathogen elimination, while if e.g. autoimmunity is induced or a pathogen persists there may be chronic inflammation which may lead to tissue destruction or fibrosis. Inflammation is an essential component of many lung disorders. T helper lymphocytes have a pivotal role in orchestrating the inflammatory reaction following triggering by specific antigens. In this thesis my aim is to better understand the role of T-cell immunity in pulmonary inflammation by focusing on the inflammatory interstitial lung disease sarcoidosis and by studying the effect of smoking on lung T cells with regard to the influence of smoking in developing autoimmune disease.

1.2 The immune system

The immune system protects the host from a variety of pathogens including bacteria, viruses and parasites and is also activated by stressed cells like malignant cells or cells with damaged DNA. Innate immunity provides the first line and initial immune responses against pathogens, while adaptive immunity consists of highly specialized immune responses against pathogen specific antigens.

1.3 Innate immunity

Innate immunity is a complex of early response mechanisms that provide the first-line defense to control and eliminate pathogens. The innate cells eradicate pathogens by rapid phagocytosis or release of killing substances; moreover they induce delayed cellular responses by activating signaling pathways that promote recruitment of more effector cells.

Innate immunity components

The first line of defense by the innate immunity system consists of physical (epithelial) or chemical (specialized substances) barriers. There are several substances (mostly proteins) which can protect the body from pathogens. Lysozyme, surfactants and defensins are examples of substances which are found in mucosal epithelia and the respiratory tract and that protect the body from invading pathogens by different mechanisms. If pathogens evade these defenses, then they encounter the cellular responses of innate immunity.

The cellular components of innate immunity react against the invading pathogens via receptors named pattern recognition receptors (PRR). PRR can recognize special structures on pathogens or stressed cells, but not normal host cells. These special structures which are common to many different pathogens or damaged cells are named pathogen associated molecular patterns (PAMP) or damage associated molecular patterns (DAMP).

PRRs are receptors which are expressed on the cell surface or intracellularly by a variety of cells and they contribute in activation of innate immunity. Toll like receptors (TLR), NOD like receptors (NLR) and Retinoic acid-inducible gene-I-like receptors (RLR) are examples of PRRs that can recognize many types of pathogen molecules. Examples of such lignds are
lipopolysaccharide (LPS) or peptidoglycan (presented by gram negative and gram positive bacteria respectively), double stranded RNA and unmethylated CpG DNA (1).

Phagocytic cells including neutrophils and macrophages engulf pathogens which are subsequently destroyed inside the phagosome. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are two well-studied digestive mechanisms which have the ability to destroy phagosome contents. NADPH oxidase and inducible nitric oxide synthase (iNOS) are two important enzymes that generate bactericidal ROS and NO respectively. These enzymes are activated when pathogens bind to PRRs. Phagosome fusion with lysosomes containing proteolytic protease enzymes is another important mechanism which helps to destroy ingested pathogens (2).

Natural Killer cells (NK cells) are a class of lymphoid cells which react against pathogens and abnormal cells. Lack of MHC class I, often induced by viral infection of a cell, is a signal for activation of these efficient killer cells to secrete cytotoxic granules. Moreover, NK cells by secretion of cytokines help to activate other immune cells (3).

In addition, there are several unconventional lymphocyte subsets that are classified as parts of innate immunity. Cells like γδ T cells, invariant natural killer T (iNKT) cells, Mucosa-associated invariant T cells (MAIT) and B-1 cells are lymphocytes that are activated in response to pathogens immediately when they recognize molecular patterns and have the capacity to immediately express effector functions (4).

1.4 Adaptive immunity

The second form of immunity that protects the host from invading pathogens is adaptive immunity. In contrast to innate immunity which responds to shared structures of pathogens in the early phase of infection, the function of adaptive immunity is defined by highly antigen specific responses against pathogens. Adaptive immunity responses develop later in the secondary phase to recognize and eliminate the pathogens more specifically and efficiently. The receptors in innate immunity are encoded by the germline DNA pattern, while in order to make an antigen specific response in adaptive immunity, receptors are encoded by gene segments that undergo somatic recombination to generate an enormous diversity of receptors. Immunologic memory is one important feature of adaptive immunity that allows more effective and rapid responses to occur during the second exposure to the antigens that the immune cells encountered before. The adaptive consists of B and T lymphocytes.

1.4.1 B cells

B cells are lymphocytes that mature in the bone marrow. Antibody production is the most prominent feature of B cells. B cells express immunoglobulin on their cell surface as the B cell receptor (BCR). Once B cells with cell surface immunoglobulin bind to specific matched antigens, they can internalize the antigen via endocytosis. B cells start to process internalized proteins into peptides and consequently they present antigenic peptides on their surface by MHC class II molecules to CD4+ T cells. If the T cell via its TCR recognizes the presented antigen it can provide help for B cell maturation. This response of B cells to antigens is classified as a T dependent response. B cells can also recognize non protein antigens via immunoglobulin without T cell involvement, which is called a T independent response. Activated B cells can differentiate into plasma cells that secrete soluble BCRs in the form of different classes of antibody. Secreted antibodies can protect the host by different
mechanisms. Antibodies help to destroy or inactivate the pathogens by neutralization (binding to pathogens can inhibit them from infecting other cells), opsonization (binding to pathogens will facilitate the phagocytosis by phagocytes) and finally by complement activation (a complex of serum proteins that via a catalytic cascade form mediators that help to destroy pathogens or make them suitable for phagocytosis) (2).

Somatic recombination of the variable regions in the immunoglobulin heavy chain (V, D and J segment) and light chain (V and J segment) generates the diversity in BCR and antibodies (5). B cells initially produce IgM and IgD, while following interaction of B cells with T cells, class switching will happen and IgG, IgE and IgA are induced in a T cell mediated manner.

1.4.2 T cells

T cells are lymphocytes which are responsible for the so-called cell mediated immunity and they develop in the bone marrow followed by maturation in the thymus. T lymphocytes are divided into two main classes that are different in phenotype and function. T cells which carry the CD4 molecule on their cell surface are named helper T cells and they are involved in activating B cells and phagocytes to destroy the pathogens. The second class of T cells is named cytotoxic T cells; they carry the CD8 molecule on their surface and kill their target cells. Despite differences between CD4 and CD8 T cells, they share common features. Naïve T cells can only recognize pathogen-derived peptides which are presented by antigen presenting cells (APC). Both CD4 and CD8 T cells have T cell receptors on their surface which are highly specific and recognize a unique antigen that is presented by special molecules on the surface of APCs. When naive T cells recognize appropriate antigen presented by APCs in lymphoid organs they will differentiate into effector cells which have the ability to react against pathogens and migrate to peripheral sites to eliminate pathogens. Generation of memory T cells following activation of T cells is one of the most important features of T cells (quite similar to B cells) leading to a faster and more effective response when the host encounters a secondary challenge with the same pathogen (6).

1.4.2.1 CD4+ T cells

CD4 T cells constitute a major subset of T cells that perform a pivotal role in the immune system. When naive CD4 T cells encounter their cognate antigen presented on APCs, they differentiate into effector and/or memory cells. CD4 T cells are named T helper cells because they have the ability to e.g. help B cells produce antibody. CD4 T cells help B cells to mount antibody responses, provide feedback to DCs via costimulatory molecules, prime the immune system by cytokine and chemokine secretion and maintain CD8 T cells responses (7). Moreover, CD4 T cells have direct effector activity, including performing cytotoxic functions (8) and mediating macrophage activation. Dysregulation of CD4 T cells is associated with many chronic diseases. CD4 T cells according to their phenotype and function are categorized into different subsets. Th1, Th2, Th17 and Regulatory T cells are the most well-known T cell subsets (Figure 1). Polarizing cytokines, master gene regulators and cytokine signature are three major characteristics of each subset.

Th1 subset

Interleukin-12 (IL-12), IL-18 and Interferon γ (IFNγ) are key cytokines in Th1 polarization. IL-12 and IL-18 are cytokines that are produced by macrophages and DCs and which, together with IFNγ produced by T cells, induce Signal Transducers and Activators of
Transcription 4 (STAT4) (9) and up-regulate expression of T box expressed in T cells (T-bet) as master regulator of Th1 cells. T-bet expression promotes Th1 commitment and induces Th1 cells to produce IFNγ (10). IFNγ, TNF and lymphotxin α (LTα) are the most important Th1 cytokines (11). Th1 cells are involved in macrophage activation, antibody class switching (in B cells) and promotion of fully cytotoxic T cell activity. These functions by Th1 cells contribute to responses against viral and intracellular pathogens.

**Th2 subset**

IL-4 is the critical cytokine for polarization of Th2 cells and their differentiation is controlled by the master transcription factor GATA3. IL-4 acts by signaling via STAT6, which is the necessary signal transducer for Th2 differentiation (12, 13). IL-4, IL-5 and IL-13 are signature cytokines of Th2 cells (14). The main role of Th2 cells is clearance of extracellular parasitic infections. The secreted cytokines by Th2 cells induce eosinophil differentiation and promote production of IgE and IgG1 by B cells. Th2 cells also have important roles in mediating allergic reactions (15).

**Th17 subset**

The characterization of IL-17 producing T cells as a distinct T cell lineage was based on studies performed in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE) (16, 17). Differentiation of Th17 cells from naive CD4 T cells is controlled by RORc as master regulator (18). TGFβ (immunoregulatory cytokine) and IL-6 (proinflammatory cytokine) are two main cytokines which are required to induce IL-17 in naive T cells. Rorc expression is inhibited by a high concentration of TGFβ, while presence of TGFβ in combination with IL-6 causes induction of IL-17 expression (19, 20). IL-23 is another important cytokine in Th17 differentiation, and it has been considered as a cytokine needed in order to up-regulate IL-22 by Th17 cells (21) and necessary to attain full pathogenic potential through its maintenance and stabilization of the Th17 phenotype in autoimmunity (22). STAT3 is the main signal transducer in Th17 cells and not only causes increased expression of RORc molecule but it can directly bind to IL-17 and IL-21 promoters (23, 24). The main function of IL-17 in immunity against pathogens is due to its induction of pro-inflammatory cytokines and chemokines which orchestrate trafficking and activation of innate immune cells particularly neutrophils and macrophages (25). Many inflammatory or autoimmune diseases which were previously considered to be Th1-mediated disorders are now considered as Th17-mediated diseases.

IL-17 is actually a family of cytokines, which includes IL-17A (called IL-17), IL-17B, IL-17C, IL-17D, IL-17E (called IL-25), and IL-17F (19). Among the IL-17 family members, IL-17A and IL-17F have the highest degree of homology and both IL-17A and IL-17F are produced by a variety of cell types and have pro-inflammatory properties (26). IL-17 family receptors also consist of five members (IL-17RA, RB, RC, RD and RE), all of which, like their ligands, share sequence homology (27). IL-17A and IL-17F signal through the IL-17RA–IL-17RC heterodimeric receptor complex (28).

**Regulatory T cell (Treg) subset**

Regulatory T (Treg) cells is a distinct T cell subpopulation that is engaged in sustaining immunological self-tolerance and homeostasis (29, 30). The X-chromosome-encoded member of the Forkhead Transcription Factor box P3, named Foxp3 is a master regulator of
regulatory T cells (Treg) which controls differentiation and function of Tregs (31). Currently CD25 (IL-2 receptor subunit α) is the best surface marker to define Treg cells, although several markers have been described for Treg identification like CTLA-4 (cytotoxic T lymphocyte antigen-4), GITR (glucocorticoid-induced TNF receptor family), CD127 low and IL-1 receptor although they are not completely Treg specific (32-35).

The majority of Tregs develop in the thymus by high-avidity recognition of self-antigens and act to prevent damage caused by self-reactive T cells which evade the negative selection process (T cells with high affinity for self MHC/peptide); this group of T cells is named thymus-derived Tregs (tTreg) or natural Tregs (nTreg) (36). Another group of Tregs named peripheral Treg (pTreg) or induced Treg (iTreg) develop in the periphery in response to T cell receptor (TCR) stimulation in combination with several other signals, including IL-2 and TGF-β (31, 37). Foxp3+ Tregs, by migration to inflammatory sites are responsible for the suppression of various effector cells.

There is no well-accepted marker to distinguish tTregs from pTregs. Helios is a member of the Ikaros transcription factor family that has been considered as a marker of natural or thymic-derived Treg cells recently (38), although this notion has been challenged later on (39) and more recently additional roles for Helios has been suggested like a mediator of T-cell homeostasis or a T-cell activation and proliferation marker (40, 41).

Tregs exert their suppressive function by several modes of action that can be classified into five main mechanisms. Induction of suppressor cells or ‘infectious tolerance’ is mediated by TGFβ by induction of IL-10 expressing Tr1-like cells. IL-10, IL-35 and TGFβ are cytokines that are released by Tregs and have suppressive function. Tregs also can modulate APC cell function; for instance CTLA4 is a negative costimulatory factor that binds to CD80/CD86 (on APC) and mediates induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs. Tregs can also mediate cytolysis by granzyme A, granzymeB and perforin. Metabolic disruption is the last proposed mechanism in order to induce suppressive function. CD25 (part of the IL-2 receptor) is a surface marker on Tregs that can deprive effector T cells of IL-2 (42, 43). Dysregulation in the function of Tregs contributes to the pathogenesis of many chronic inflammatory diseases and autoimmune diseases (44, 45).

Tr1 and Th3 are other regulatory T cells with no Foxp3 expression. They have suppressive activity on effector cells by IL-10 and TGFβ production respectively (42).

**Other subsets of T cells**

In recent years the number of effector T helper subsets has grown further. Follicular helper T cells (Tfh), Th9 and Th22 are defined as distinctive T cell subsets but much still remains unknown about them.

Recent studies suggest follicular helper T cells (Tfh) are a distinct lineage of CD4 T cells which act in the germinal center of peripheral lymphoid tissue where they provide help to maintain and to regulate germinal center B cell differentiation into plasma cells and memory B cells (46-48). Differentiation of Tfh cells is under control of Bcl6 as a master regulator and dependent on IL-21, IL-6 and STAT3. Human Tfh cells have been implicated in various diseases like immunodeficiency and autoimmunity (49).
Th9 is another newly described subset of T cells with preferentially production of IL-9. They develop from naive T cells in the presence of TGFβ and IL-4 (50, 51). GATA3 and STAT6 are transcription factors and signal inducers that are required for Th9 differentiation. Involvement of Th9 cells in the pathophysiology of allergic asthma and autoimmunity has been demonstrated (52).

Th22 is a new subset of T cells that by a unique gene expression pattern and function make it distinct from Th17 cells. The aryl hydrocarbon receptor (AHR) is an important transcription factor in Th22 cells and IL-6 and TNF are cytokines that promote Th22 differentiation. Th22 cells, depending on the precise cytokine milieu and affected organ may modulate their functional profile showing both protective or pathogenic characteristics (53-55), and ongoing research aims to understand their complicated role in inflammatory and autoimmune diseases.

Figure 1: T cell subsets differentiation. Following recognition of cognate antigen that is presented by APCs, naive CD4⁺ T cells become polarized into distinct effector T helper cell subsets in lymphoid organs. The cytokine milieu plays an important role in orchestrating T cell polarization. (adapted from Nature Review Immunology 12:136-148) (56).
T cell plasticity

T cell plasticity is a phenomenon that has been recently described to occur in T cell subsets. Plasticity is defined as an ability of a T cell subset to display a flexible cytokine production pattern that includes cytokines normally considered to strictly belong to different T cell lineages. The most well-known example of a flexible T cell lineage was described in the case of Treg versus Th17 maturation, where TGFβ mediates differentiation of Foxp3+ Tregs from naive T cells in response to antigenic stimulation while the presence of TGFβ together with IL-6 leads to differentiation of Th17 cells from naive T cells (57). Among the T cell subsets, Th1 and Th2 cells cross-regulate each other by inhibiting the development of the other T cell subset, but following differentiation they have a stable phenotype and have achieved a lineage commitment state (58). In contrast, there is strong evidence indicating that a fraction of Foxp3+ Treg cells can acquire effector T cell function by losing Foxp3 expression under certain circumstances. The frequency of these ex-Foxp3+ Tregs increases particularly in the presence of inflammation (59). However, under inflammatory conditions a fraction of Foxp3+ Tregs can also acquire certain effector T cell functions such as IFNγ production with retained Foxp3 expression (60). These regulatory T cells have a high level of Foxp3, lack of IL-2 production and a maintained suppressive activity (61). Transition of Th17 cells that predominantly produce IL-17 to IFNγ producing cells has also been reported by different groups (62). The exact function of these flexible T cell subsets remains to elucidate but they may contribute to the protection against microbes, development of autoimmunity and anti-tumor activity (63). Regarding Th9 cells with IL-9 production it is not clear whether they should be considered a distinct T cell lineage or if they reflect an adaptation of Th2 cells under certain circumstances. Flexibility and similarity between Tfh and both Th1 and Th2 cells also makes it unclear whether Tfh is a separate T cell lineage or rather a 'chameleon' state of other subsets (64).

1.4.2.2 CD8+ T cells

CD8 T lymphocytes, named cytotoxic T lymphocytes (CTL), exert their main effect by killing target cells, such as virus-infected or tumour-transformed cells. CD8 T cells like CD4 T cells have T cell receptors on their surface and they recognize peptide antigens which are presented by MHC class I molecules on the surface of infected cells. Following recognition of antigen by their TCR they are activated and they kill the target cells by delivery of granule proteins. Granzymes and perforins are two major components that CTLs release to kill target cells. Granzymes are enzymes that induce apoptosis in target cells and perforin is a delivery protein that mediates transfer of granzymes. CD8 T cells also carry Fas ligand on their membrane that can induce cytotoxicity by binding to Fas (CD95, a TNF receptor family member) which delivers a death signal if binds to its ligand). Activated CTLs also have the ability to secrete many cytokines such IFNγ, TNF and IL-17 which are important in immune activation. CTL are critical T cells for eradication of intracellular pathogens like viruses (3).

1.5 Antigen presentation and human leukocyte antigen (HLA)

T cells can recognize cognate antigens by their surface T cell receptors, if antigens have been presented to them by major histocompatibility complex (MHC) molecules. Since the MHC molecules first were discovered on the surface of human leukocytes, they are also named human leukocyte antigen (HLA) complex. The MHC encoded genes in human are located on chromosome 6 as part of a locus which contains many genes which can classified in three
groups, named MHC class I, MHC class II and MHC class III. MHC class I and II are involved in presenting peptide antigens to T cells, while MHC class III contains genes for some cytokines and complement proteins and does not have any role in antigen presentation.

Despite high similarity in the structure and function of MHC class I and II they also differ in many characteristics, and some of the major differences are summarized in table 1 (65, 66).

<table>
<thead>
<tr>
<th>Source of antigen</th>
<th>MHC class I</th>
<th>MHC class II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responding cells</td>
<td>CD8(^+) T cells</td>
<td>CD4(^+) T cells</td>
</tr>
<tr>
<td>Composition of molecule</td>
<td>α1, α2, α3/ β2 macroglobulin</td>
<td>α1, α2/ β1, β2</td>
</tr>
<tr>
<td>Encoded genes</td>
<td>α chain encoded by MHC gene and β chain by non MHC genes</td>
<td>Both α and β chain encoded by MHC gene</td>
</tr>
<tr>
<td>Structure of molecule on cell surface</td>
<td>Only α chain span the cell membrane</td>
<td>Both α and β chain span the cell membrane</td>
</tr>
<tr>
<td>Presented cells</td>
<td>Expressed by most nucleated cells</td>
<td>Expressed by APCs (Macrophage, DC and B cell)</td>
</tr>
<tr>
<td>Peptide binding groove</td>
<td>Both ends close</td>
<td>Both ends open</td>
</tr>
<tr>
<td>Size of presented peptide</td>
<td>8-10 amino acids</td>
<td>13-18 amino acids</td>
</tr>
</tbody>
</table>

Table 1: Different characteristics of MHC class I and MHC class II molecules

MHC class I encodes in humans the three genes HLA-A, -B and -C, while MHC class II encodes HLA-DP, -DQ and -DR. MHC allelic genes inherited from both parents are expressed equally. The gene region of MHC molecules is highly polymorphic and contains multiple gene variants with the same function and slightly different structure, for instance HLA-DRB1 genes are encoded by 860 different allotypes (some are very common while others are very rare). This polymorphism leads to diversity in the expressed MHC repertoire and it is extremely rare to find two individuals with the same set of MHC molecules in the population (except for siblings) (1).

1.6 The T cell receptor

As described above, APCs present antigens via MHC molecules to the T cells. In order to induce appropriate immune responses against pathogens, the antigen should be recognized by specific cognate antigen receptors on the surface of T cells. T cell receptors like B cell receptors consist of a constant plus a variable domain, where the latter is antigen-specific and the enormous diversity of the total TCR repertoire enables T cells to recognize a wide variety of peptides.

The T cell receptor consists of an α polypeptide chain and a β polypeptide chain and each of them is composed of a variable and a constant region. The variable region of the TCR α chain
is encoded by V (variable) and J (junction) gene segments and the variable region of the β chain is encoded by V, D (diversity) and J gene segments. Each of these V, D and J segments can be selected from a number of alternative gene segments that after somatic gene rearrangement are combined to generate the complete variable α and β chains. This rearrangement creates vast diversity in variable regions of T cell receptors by the many different possible combinations of gene segments.

The three dimensional structure of a T cell receptor in the antigen binding sites reveals hypervariable loops, named complementarity determining regions (CDR). T cell receptors have three different CDR loops on each chain, among them CDR3 has the most variable loop and make the center of the antigen binding site of a T cell receptor. It is formed by contributions of D and J gene segments of the α and β chains. A major part of TCR diversity is generated by random inclusion/deletion of nucleotides at the junctions between V, D and J segments (1, 3).

A minority of T cells, termed γδ T cells, carry a different type of T cell receptor which instead of α and β chains is composed of γ and δ chains. These cells have completely different features compared to the αβ T cells and γδ T cells are part of the innate immunity system.

### 1.7 T cell activation

T cell maturation occurs in the thymus. During this process naive T cells expressing T cell receptors with capacity to recognize pathogenic antigens presented by MHC molecules develop following positive selection (to select T cells that recognize self MHC molecules by their T cell receptor) and negative selection (to eliminate T cells that bind to MHC with high affinity, a major mechanism to prevent autoimmunity). Naive T cells leave the thymus and circulate between lymphoid organs to meet the cognate antigen presented by APCs. In order for activation of T cells to occur, antigen recognition by the T cell receptor is essential but it is not sufficient. In addition to T cell receptors a complex of co-receptors and co-stimulatory factors are necessary for proper T cell activation (67).

The CD3 molecule (T cell specific marker) and ζ-chain are co-receptors that generate an activation signal upon peptide-MHC recognition by T cell receptors. T cell receptor, CD3 and ζ-chain all together make up the T cell receptor complex. CD4 and CD8 are two other co-receptors expressed on the surface of T helper and cytotoxic T cells, respectively. When T cell receptors recognize peptide and MHC complex, CD4 binds to MHC class II and CD8 binds to MHC class I, ensuring that, depending on the antigen source, the correct type of effector cell is engaged.

There are a couple of co-stimulatory receptors that are essential for T cell activation. CD28 is a co-stimulatory receptor that mediates initiating of the activation signal in naive T cells. CD28 binds to B7 (CD80 and CD86) molecules which are expressed on APCs surface (68, 69). A group of integrin molecules are expressed on T cells, whose ligands are found on APCs. Leukocyte function-associated antigen-1 (LFA-1) is one of the important integrins which is involved in T cell activation and its ligand on APCs is intercellular adhesion molecule-1 (ICAM-1) (70). CD40 ligand (CD40L) on T cells is another molecule which indirectly participates in T cell activation. CD40L binds CD40 on the APC surface, inducing APCs to secrete cytokines like IL-12 (a cytokine that is necessary for Th1 cell differentiation) and enhancing expression of B7 molecules (71). There is also a set of negative co-stimulatory
molecules like CTLA-4 and PD-1 which promotes negative signals and inhibit T cell receptor signaling (72). CTLA-4 is a receptor that is expressed on the T cell surface and can bind to a B7 molecule instead of CD28 (73) (Figure 2).

After antigen recognition by the T cell receptor and provided that necessary co-stimulatory signals are present, T cells initiate a process involving activation of biochemical signaling pathways and expression of a variety of proteins and transcription factors that leads to proliferation and clonal expansion, differentiation and obtaining full effector function. Activated effector T cells leave the peripheral lymph nodes and migrate to the site of infection or inflammation (74).

Figure 2: T cell activation initiated by three signals. Signal 1 is mediated by T cell receptors that recognize antigens presented by MHC molecule. Signal 2 is the co-stimulatory signal, mainly mediated by triggering of CD28 by CD80 and CD86. Signal 3 is the polarizing signal that mainly is provided by various soluble or membrane-bound factors, such as interleukin-12 (IL-12) that promote the development of Th1 cells (adapted from Nature Review Immunology 3: 984-993) (75).

1.8 The respiratory system

The lung is the main organ of the respiratory system whose responsibility is to provide the body with oxygen and remove carbon dioxide. The respiratory system is divided into two major parts: the upper and lower parts. The upper respiratory tract (upper airway) consists of the nose, nasal cavity, mouth, pharynx, larynx. The responsibility of upper respiratory system is to provide proper air conditioning (humidity and temperature), particle removal and air filtration and also to activate the immune defense against pathogens.
The lower respiratory tract consists of the trachea, bronchi, bronchioles and alveoli. The trachea enters the thoracic cavity and carries air from the throat into the lungs and vice versa. The trachea branches into bronchi and these are in each lobe of the lung further divided into smaller branches of airways called the bronchioles. Finally the bronchioles end into air sacs termed the alveoli. The alveoli have very thin walls surrounded by capillaries which permit exchange of gases with blood via passive diffusion; i.e. the main function of the respiratory system (Figure 3).

Figure 3: The major features of the lower respiratory system including the trachea, bronchi, the bronchioles, and the alveoli.

1.8.1 Immunology of the lungs

The lungs have the largest epithelial surface of the body and according to their function they are constantly exposed to microorganisms and environmental irritants. The lung epithelium is an important physical barrier, which is the first line of defense and acts as a shield to protect the body against irritants and pathogens including airborne toxins, cigarette smoke, pollutants, bacteria and viruses. However, the lung epithelium is not only a physical barrier but also it is activated in response to pathogen exposure or environment-induced damage to secretion of antimicrobial peptides and inflammatory mediators to recruit immune cells to the site of infection or damage (76).

The epithelium is composed of different cell types with a variety of functions which help the lung to protect itself against airborne insults. More than 50% of lung epithelial cells are named ciliated cells that by ciliary motility help mucous clearance (77). Goblet cells are another kind of epithelial cells that secrete mucous into the airway to trap pathogens and dust particles (78). The mucous layer in the airway is found from the trachea to the bronchioles.
and it consists of a mixture of highly glycosylated mucin proteins that help to maintain lung health (79). In the smaller airways the secretory cells are replaced by Clara cells which are non-ciliated bronchiolar secretory cells. Clara cells have been shown to produce bronchiolar surfactants and specific enzymes to protect and support lung function (80, 81). Epithelial cells in alveolar walls and the ciliated epithelium also have pattern recognition receptors (PRRs) which can sense pathogens (82).

In addition to these physiological barriers, the lung is recognized as a unique immunologic organ equipped to protect the body from inhaled invading pathogens. If the lung with specific physical and physiological clearance properties can’t control or remove inhaled particles, they can trigger the immune system in order to process foreign antigens. A variety of immune cells such as alveolar macrophages, lymphocytes and neutrophils are involved in lung protection. Alveolar macrophages account for approximately 95% of airspace leukocytes, lymphocytes with 1 to 4% and neutrophils only about 1% in the normal situation (82).

The alveolar macrophage (AM) is the resident mononuclear phagocyte in the alveolar air spaces. AMs can directly phagocyte inhaled harmful particles, moreover they are not only a potent orchestrator in the presentation of antigen to the T cells, but also they release different mediators to activate and recruit other immune cells (83). The AM can also produce reactive oxygen and nitrogen species and proteolytic enzymes to kill the ingested pathogens (84). Neutrophils also have an important role in lung immunity to pathogens. Neutrophil infiltration should be under control and in chronic airway diseases persistence of neutrophils in the lungs may lead to tissue damage due to their cytotoxic function (85, 86). Different types of lymphocytes are found in the lungs of healthy individuals such as T cells, B cells, NK cells, γδ T cells. Dendritic cells are professional APCs which are present just below the airway epithelium and by migration from the airway mucosa to the thoracic lymph nodes play an important role in the activation of T cells (87).

Insufficient or exaggerated immune responses to inhaled stimuli could lead to pathological conditions, such as pneumonia, asthma, chronic obstructive pulmonary disease (COPD) and interstitial lung disorders. Pulmonary inflammation affecting the airways or interstitium can be caused by many reasons and result in different manifestations. Both innate and adaptive immune systems have pivotal roles in orchestrating the inflammatory reaction and one of the most important cells in this process is T helper lymphocytes. Pulmonary inflammation is defined as increased inflammatory cells in the airspaces and lung tissue that are producing proinflammatory mediators such as hydrogen peroxide, interleukin-1 (IL-1), and interleukin-8 (IL-8) (88). The pulmonary inflammation can lead to obstructive symptoms like in asthma and COPD where inflammation causes narrowing of the airways or it can result in a restrictive pattern like in interstitial lung disease where inflammation leads to increased lung stiffness.

1.8.2 Interstitial lung diseases

The interstitial lung diseases (ILDs), also called diffuse parenchymal lung diseases, are a diverse group of pulmonary disorders classified together because of similar clinical, physiologic, or pathologic features (89, 90). They can be caused by a variety of factors and are clinically characterized by diffuse infiltrates on the chest radiograph and histologically by distortion of the gas exchanging portion of the lung. The physiologic correlates are restriction of lung volumes and impaired oxygenation (91, 92). Idiopathic pulmonary fibrosis (IPF) (a
progressive and lethal fibrotic lung disease), nonspecific interstitial pneumonia, chronic beryllium disease, silicosis and sarcoidosis are examples of ILDs.

1.8.2.1 Sarcoidosis

Sarcoidosis is a systemic inflammatory disorder. It is characterized by non-caseating granulomas that most commonly affect the lungs. The pathogenesis of sarcoidosis includes the accumulation of lymphocytes and macrophages in the alveoli; thus involving innate as well as adaptive immune mechanisms. The prevalence of sarcoidosis is about 4.7-64 in 100,000 and its incidence is about 1-35.5 in 100,000 per year. The northern European and African-American individuals have the highest rate of sarcoidosis (93, 94).

Clinical aspects

The main clinical symptoms in pulmonary sarcoidosis patients are non-productive cough and dyspnea, although other symptoms like fatigue (associated with impaired quality of life) and pain (especially arthralgia) are common in patients with pulmonary sarcoidosis (95, 96). Sarcoidosis can affect many different organs such as liver, spleen, lymph nodes, salivary glands, heart, nervous system; however the lung is the most commonly affected organ (97).

In general, a radiographic staging system is used in order to classify patients with pulmonary sarcoidosis. In this system a normal chest radiograph is classified as stage 0, stage I shows bilateral hilar lymphadenopathy (BHL) without pulmonary infiltrates, stage II BHL with pulmonary infiltrates, stage III pulmonary infiltrates without BHL and stage IV fibrosis with distortions. The extent of pulmonary engagement, reflected through the chest X-ray classification, is associated with the prognosis (this is the case in 90% of patients) (97).

Sarcoidosis patients generally have a rather good prognosis and nearly two-thirds of the patients recover spontaneously, however a chronic progressive disease course is seen in 10%–30% of patients (98, 99). In late course of chronic disease, pulmonary fibrosis may occur in 20 to 25% of patients and the rate of mortality is 1%-5% among sarcoidosis patients(97, 100).

Pathogenesis

The aetiology of sarcoidosis is still not known. However, associations with environmental factors, genetic susceptibility and bacterial triggers have been reported (101, 102). Due to involvement of lungs, eyes and skin, environmental causes have been considered for many years. An association of sarcoidosis with exposure to irritants such as tree pollen, inorganic particles, and moldy environment has been reported (103-105). Occupational studies have shown an increased risk for sarcoidosis in people who work in U.S. navy, firefighting and metalworking (100, 106, 107).

The presence of mycobacterial and propionic bacterial DNA in the lungs of sarcoidosis patients has been shown (108, 109). Accumulating data suggest that mycobacteria and in particularly M. Tuberculosis play a major etiologic role in sarcoidosis (110). It is not clear how infectious agents cause sarcoidosis, although one hypothesis is that sarcoidosis is caused by a viable, replicating mycobacterial or other infection with no microbiologic, pathologic and clinical evidence (111). However, most investigators favor a view of sarcoidosis as caused by remnants of an infectious agent in the form of poorly degradable antigens. The involvement of M. Tuberculosis in the pathogenesis of sarcoidosis has been validated by
immune assay studies (112). Ex vivo immune activation in response to several mycobacterial antigens have been studied and T cell responses to the mycobacterial virulence factor antigen 85A (Ag85A), early secreted antigenic target protein-6 (ESAT-6), culture filtrate protein-10 (CFP-10) and heat-shock proteins (hsp) have been reported in sarcoidosis patients (113-115).

**mKatG**

Homogenates of spleen and lymph node from sarcoidosis patients were previously used in the diagnosis of sarcoidosis. The test is named Kveim’s test and it is performed by intradermal injection of tissue homogenate that results, in sarcoidosis patients, in formation of granulomas at the site of injection (116). A proteomic approach was undertaken to identify antigens in sarcoidosis patients’ tissue samples, treated in the same way that Kveim’s reagent is prepared. *Mycobacterium tuberculosis* catalase-peroxidase (mKatG) was one of the non-self tissue antigens identified and antibodies to recombinant mKatG were detected in the sera of 12 of 25 (48%) sarcoidosis patients (117). This finding indicated that mycobacterial antigen is present, at least in a subset of sarcoidosis patients (112). T cell responses against mKatG have been observed in sarcoidosis lung and peripheral blood mononuclear cells (118, 119), and a study on lung mKatG-reactive, IFNγ-producing effector T cells indicated that T cell responses to mKatG in sarcoidosis are regulated in a manner expected for a pathogenic antigen (120).

**Immunopathogenesis**

Non-necrotizing granulomas are the histological hallmark of sarcoidosis. The granuloma formation occurs in the body whenever an antigen cannot be completely eliminated or degraded by macrophages. In this situation macrophages and epitheloid cells fuse together and make multinucleated giant cells; moreover accumulation of activated mononuclear cells (mostly T cells) and interaction between innate and adaptive immune cells by release of proinflammatory cytokines orchestrates the formation of granulomas and induce an immune stimulation in the affected organ (121-123) (Figure 4).

In bronchoalveolar fluid of sarcoidosis patients the proportion of T cells is increased (20–60% of the total cell count) with dominance of CD4+ T cells. An increased CD4/CD8 ratio in bronchoalveolar fluid of patients (>3.5) is one of that characteristics of sarcoidosis patients (124). The accumulated activated CD4+ T cells in alveoli are typically Th1 cells and they are considered to be of central importance for the inflammatory process, although CD4+ T cells in lung tissue and bronchoalveolar cells bearing a phenotype consistent with Th17 polarization and persistence of Th17 cells in sarcoidosis patients has been observed (125-127). By contrast, Th2 cell responses (IL-4 and IL-5 secretion) are downregulated at sites of inflammation in sarcoidosis patients (128, 129).

T cells in BAL fluid of sarcoidosis patients are highly activated and they express activation markers like CD69 and HLA-DR. A Th1 cytokine signature i.e. IFNγ is highly expressed, moreover cytokines that promote a Th1 response such as IL-12, IL-18 and IL-27 are upregulated in the lung of sarcoidosis patients (127, 130, 131). The expression of Th1-associated chemokine receptors such as CXCR3 and CCR5 has also been reported in lung of sarcoidosis patients (132).
Figure 4: Schematic speculation process of granuloma formation in sarcoidosis patients (Adapted from (133) with minor modification).

Treatment

There is no cure for sarcoidosis but conventional treatment improves the clinical consequences of disease by alteration of the granulomatous process (134). The decision of which treatment to use is dependent on clinical characteristics and severity of disease (135). Spontaneous resolution has been observed in many patients and treatment can usually be avoided, but oral corticosteroids are usually prescribed in patients with pulmonary sarcoidosis who suffer from lung dysfunction and persistent pulmonary infiltrates. The first line of treatment in severe sarcoidosis is corticosteroids (98, 136). There are other strategies in treatment of sarcoidosis patients such as cyclosporin to inhibit activated T cells or TNF blockade given the importance of TNF in the initiation and perpetuation of the granulomatous process, although there is no consensus regarding the efficiency of these treatments and their effect varies in different patients (137-139).

Löfgren’s syndrome

Sarcoidosis patients display phenotypic heterogeneity. A subgroup of sarcoidosis patients with specific clinical features, described the first time by the Swedish pulmonary physician Sven Löfgren (1910-1978), are said to have Löfgren’s syndrome. Löfgren’s syndrome (LS) is an acute form of sarcoidosis with bilateral ankle arthritis and/or erythema nodosum, usually fever, and bilateral hilar lymphadenopathy (140, 141). Löfgren’s syndrome has a good prognosis in general and spontaneous recovery within two years is particularly common in
Löfgren’s syndrome patients who also express the HLA allele DRB1*0301 (DR3) (142). HLA-DR3+ patients (two thirds of which have LS) virtually always have accumulations of TCR Vα2.3+ CD4+ T cells, i.e. T cells with a particular T cell receptor (TCR) alpha chain variable gene segment (AV2S3) (TCR-AV2S3) in the lungs (143). Such expansions were not found in DR3+ healthy individuals or in patients with other inflammatory pulmonary disorders (144, 145). More recently, these Vα2.3+ T cells in DR3+ patients were found to often use Vβ22 as part of their TCR β chain (146) (Kaiser et al, unpublished observations). The reasons for spontaneous recovery in some patients and disease progression in others remain to be elucidated. However, hypothetically in non-necrotising granuloma remnants of an assumptive antigen are not fully degraded and antigen persistence leads to progressive chronic disease (122, 147). In contrast, it can be hypothesized that the immune responses in patients with spontaneous resolution results in complete elimination of causative antigen (134).

1.8.3 Smoking induced-inflammation

Cigarette smoking by more than one billion consumers in the whole world is one of the most challenging health issues due to its effect on lung and other organ systems resulting in a high morbidity and mortality (148). Cigarette smoke consists of more than 6000 different chemicals and toxic components, such as nicotine, aromatic hydrocarbons, dioxin, tobacco glycoprotein, phenol, metals and ions which some of them are cytotoxic, antigenic and carcinogenic (149-151). Tobacco smoking is a well-known key factor in the pathogenesis of several diseases, such as infection, lung cancer, cardiovascular diseases, COPD and autoimmune disorders.

Cigarette smoke influences the airways by directly inducing activation of epithelial and immune cells. Cigarette smoke influences both innate and adaptive immunity at the local or systemic levels in different ways (151). The chronic exposure to components of cigarette smoke triggers a variety of pulmonary or systemic immune responses. Smoking can induce or inhibit release of both inflammatory and anti-inflammatory cytokines and mediators (151). Cigarette smoke through up-regulation of NF-κB (152) can induce secretion of inflammatory cytokines such as TNF, IL-1, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (153, 154). Induction of pro-inflammatory mediators leads to recruitment of immune cells and consequently inflammation. However, cigarette smoke can also down-regulate the release of IL-1β, IL-2, IFNγ and TNF via toll like receptors (155). Nicotine is one of the components of cigarette smoke that show direct anti-inflammatory effect by diminishing IL-6 production (156). Furthermore nicotine can activate α7 nicotinic acetylcholine receptor that leads to down-regulation of pro-inflammatory cytokines like TNF, IL-1b and IL-6 (157). Cigarette smoke by different mechanisms modulates epithelial and immune cell signaling to induce suppression of aspects of innate and adaptive immune cell activation which hinder immunity to infection (158, 159).

T helper lymphocytes at the center of adaptive immunity have a pivotal role in orchestrating the host defense and inflammatory reaction following triggering by specific antigens. Smoking has an influence on different T cell subsets with specific inflammatory or anti-inflammatory cytokine properties. Imbalance between pro-inflammatory and anti-inflammatory cytokines in the lungs can result in pathologic inflammatory conditions.
**Smoking-induced autoimmunity**

Cigarette smoking has been shown to be associations with autoimmunity. There is strong evidence of an increase in prevalence of many autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematos, Crohn's disease and systemic sclerosis in smokers (151, 160, 161).

Rheumatoid arthritis (RA) is an autoimmune disorder and a striking example of gene-environment interaction as the combination of smoking history and having two copies of HLA-DR “shared epitope” genes increased 21-fold the risk of the RA form characterized by antibodies to citrullinated protein antigen (ACPA) (162). Part of the mechanism appears to be that cigarette smoke induces the PAD enzymes responsible for citrullination (a post-translational modification) of proteins such as vimentin (163). Further examples of autoimmune disorders where cigarette smoking plays a role are systemic lupus erythematous (SLE), where a smoking history was associated with more active disease and increased levels of anti-dsDNA antibodies (164).

**Smoking-induced multiple sclerosis**

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), characterized by multifocal demyelination of nerves with relative preservation of axons and accompanied by oligodendrocyte loss (165-168). T-cells have a central role in the pathophysiology of MS and presence of T cells in CNS lesions and infiltrates of T-cells throughout the CNS is a hallmark characteristic in all stages of MS patients (169). Activated T-cells occur at a higher frequency in peripheral blood of MS patients (170).

The risk of developing multiple sclerosis (MS) is strongly influenced by smoking in individuals with genetic susceptibility (certain HLA alleles). An interaction between the presence of HLA-DRB1*15 and absence of HLA-A*02 among smokers leads to a 17-fold increased risk of MS compared with non-smokers without the genetic risk factors. Furthermore the risk increased further when the analysis was restricted to comparing current smokers with never-smokers (171). Smoking is associated with increased lesion volume and greater atrophy in MS patients (172).

Importantly, there is strong evidence in Lewis rat transfer experimental autoimmune encephalomyelitis (EAE) (a model of MS) that shows that the lung could contribute to the activation of potentially autoaggressive T cells and their transition to a migratory mode as a prerequisite to entering the central nervous system (CNS) (173).
2 AIMS

T cells of different subsets have a pivotal role in orchestrating the host immune protection and inflammatory reaction. Activated T cells in the lungs not only have the potential to affect that particular organ but due to lymphocyte trafficking they can also contribute to immune responses in other organs. The overall aim of the studies presented in this thesis was to investigate the characteristics of T cell immunity in chronic pulmonary inflammation in two different aspects:

A. Investigation of antigen-specific T cell responses in patients with pulmonary sarcoidosis. Project number 1 and 2 were done based of this objective.

- To elucidate the ability of T helper 1 cells in lung and blood of different sarcoidosis patient subgroups to respond with cytokine production to mycobacterial antigen (mKatG) stimulation,
- To study the single- or multifunctional cytokine profile of Th1 cells in response to mycobacterial antigen in the lung and blood of sarcoidosis patients.
- To evaluate the presence and levels of IL-17 and the frequency of IL-17 producing cells in response to mKatG and PPD in the lung and blood of patients with active sarcoidosis.
- To compare levels of IL-17 and the frequency of antigen specific IL-17 producing cells in lung and blood of different sarcoidosis patient subgroups.

B. Determining how smoking affects lung T-cell immunity, with regard to the influence of smoking in developing autoimmune disease

- To characterize of effector and regulatory T cell subsets in the lungs and blood of smoking and non-smoking healthy individuals.
- To elucidate the effector and regulatory T cell subsets in the lungs and blood of multiple sclerosis patients with regards to effect of smoking and treatment.
3 COMMENTS ON METHODOLOGY

The present thesis is based on studies of human samples from the airways and blood of sarcoidosis patients, MS patients and healthy individuals (smokers and non-smokers). The studies were performed after approval by the Regional Ethical Review Board (Stockholm, Sweden) and written consent was obtained from the all included subjects.

3.1 Study subjects

The sarcoidosis patients included in the present study were referred to the Lung and Allergy Clinic at the Karolinska University Hospital, Solna, Stockholm, Sweden for primary diagnostic investigation. The diagnosis was established using the criteria of the World Association of Sarcoidosis and other Granulomatous Disorders (WASOG) (97), based on chest radiographic picture, pulmonary function tests (vital capacity, forced vital capacity, forced expiratory volume in one second, and diffusing capacity of the lung for carbon monoxide), histology (positive biopsies), bronchoalveolar lavage (BAL) findings, and clinical symptoms compatible with sarcoidosis.

The clinical examinations of MS patients who participated in this study were performed by specialists in the Neurology clinic at the Karolinska University Hospital in Solna, Stockholm, Sweden and all patients diagnosed with MS fulfilled the McDonald criteria revised in 2010 (174). All MS patients were evaluated clinically at the time of sampling with the Expanded Disability Status Scale (EDSS). After diagnostic evaluation at the Neurology clinic, all patients were referred to the Lung and Allergy clinic for primary clinical examination with respect to the lungs and spirometry.

All healthy subjects in this study were examined at the Respiratory Medicine Unit (Karolinska University Hospital, Solna, Stockholm, Sweden). Included individuals had normal lung function and were in good health. They had normal chest X-rays and there were no signs of respiratory infection for at least one month prior to the bronchoscopy procedure.

DNA extraction was routinely done from whole blood of all subjects and PCR was run for analysis of HLA-DR by Olerup SSP™ PCR (Sequence Specific Primers for PCR).

3.2 Sampling

Bronchoalveolar lavage

Patients underwent bronchoscopy with bronchoalveolar lavage (BAL) in the morning. Morphine-scopolamine was injected intramuscularly, and topical anesthesia was sprayed into the nose and throat immediately before the BAL procedure (175). BAL was performed during flexible fiberoptic bronchoscopy after wedging the bronchoscope in a middle-lobe segmental bronchus. Sterile phosphate-buffered saline (PBS) solution was instilled in five aliquots of 50 mL. The solution was gently aspirated into a plastic bottle and kept on ice until use. In the laboratory, the BAL fluid was filtered to remove debris and mucus and the recovered volume was determined. BAL cells were washed two times with cold PBS and finally total cell number was determined by Bürker chamber counting.

The BAL cell differential count, including percentages of macrophages, lymphocytes, neutrophils, eosinophils, basophils and mast cells, was determined using May-Grünwald-Giemsa staining of cytospin slides.
Peripheral blood mononuclear cells

Whole blood was obtained in the morning of bronchoscopy and collected in heparinized tubes. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient separation (Pharmacia, Uppsala, Sweden). Equal volumes of blood and room-tempered (RT) phosphate buffered saline (PBS) solution (pH 7.4) were mixed and gently transferred to tubes containing Ficoll-Paque™ Plus. The tubes were centrifuged (400g for 25 min at RT) and thereafter PBMCs formed a whitish layer just beneath the plasma. The PBMCs were carefully transferred into a new tube and washed 2 times with cold PBS. After last washing PBMCs were counted in a Bürker chamber in order to obtain the total cell number.

3.3 Methodology

T cell stimulation

T cell stimulation was done in order to evaluate T cell cytokine production. We have used different agents for T cell stimulation depending on the specific aims. In paper I, T cell stimulation was done by mKatG, purified protein derivative (PPD) from Mycobacterium tuberculosis and superantigen stimulation. The recombinant mKatG protein was isolated and prepared at the John Hopkins University School of Medicine, using an Escherichia coli strain (176). PPD is an extract of Mycobacterium tuberculosis PPD and it is widely used as a diagnostic antigen for tuberculosis by skin test. A total of 171 different proteins have been identified in the PPD mixture. PPD is commonly used for in vitro stimulation to characterize B and T cell reactions (177). We have also used a mixture of Staphylococcus enterotoxin A (SEA) and Staphylococcus enterotoxin B (SEB) as a positive control in our experiments (superantigens). Superantigens are bacterial or viral proteins with the ability to bind simultaneously to the α chain of HLA class II and a Vβ chain from the T cell receptor. Each superantigen has specificity for several of the Vβ versions. Superantigens are potent mitogens and powerful tools for T cell stimulation (178). A mixture of SEA and SEB thus in a physiological way has the potential to stimulate a large fraction of T cells.

During antigen stimulation, whole blood cells were further co-stimulated with anti-CD28 and anti-CD49d. Unstimulated BAL cells in medium alone and unstimulated whole blood were used as negative controls.

Brefeldin A (BFA) (GolgiPlug derived from Penicillum) is a protein transport inhibitor and was added to stimulated cells in order to block the intracellular protein transport process. It was added to the BAL and blood samples during the last 4 hours of in vitro antigen stimulation. Following T cell stimulation cytokine production was evaluated by flow cytometry.

In paper II, T cell stimulation was done in order to study mKatG specific Th1 and Th17 responses by Elispot. 10 μg/ml mKatG was used for T cell stimulation and anti-CD3 as a polyclonal stimulation was used as a positive control for stimulation of Th17 cells and Phytohemagglutinin (PHA) for Th1 stimulation.

In paper III and IV, intracellular cytokine and transcription factor staining was analyzed by flow cytometry. We stimulated both BAL and PBMC by anti-CD3 together with anti-CD28...
in 37°C, humidified atmosphere of 5% CO₂ in air and unstimulated BAL and PBMC cells in medium alone were used as negative controls. Protein transport inhibitor (Brefeldin A) was added to the cells after 4 hours incubation followed by 12 hours incubation for a total of 16 h incubation. Stimulation in presence of Golgi Plug in cell culture for longer than 12 hours is lethal for the cells.

**Flow cytometry**

Flow cytometry was done on BAL cells, whole blood samples and PBMC for the study of phenotype and intracellular markers of T cells. A FACS Canto II (BD, Sweden) flow cytometer with filters to analyze eight colors was used.

The cell surface markers CD3, CD4 and CD8 were used to delineate T cell subsets in both BAL and blood. In paper I and II the study was done on samples from sarcoidosis patients, and staining of TCR AV2S3 was also performed in order to determine the frequency of T cells whose T cell receptors have this fragment in their variable region of the α chain. CD3, CD4, CD8 and TCR AV2S3 staining is part of the routine BAL evaluation which is performed on all sarcoidosis samples in Lung Research Lab. A CD4/CD8 ratio greater than 4 supports the diagnosis of sarcoidosis (179). Study of AV2S3 expression is one practical way to predict who are DRB1*03 positive before the results of HLA typing are obtained (180), since this is the case in virtually all patients who have an expansion of T cells with TCR AV2S3 in BAL (greater than 10.5% of CD4+ cells) (3 times the median frequency of TCR AV2S3+ cells in blood CD4+ T cells of normal healthy individuals). In paper II and IV all samples were stained with aqua fluorescent reactive dye (Live/Dead fixable dead cell stain kits, Invitrogen, USA) in order to discriminate between live and dead cells populations.

Intracellular cytokine staining was performed on both BAL and blood samples to evaluate cytokines and transcription factors. Following surface staining, fixation and permeabilization were performed on BAL and blood samples. Thereafter intracellular staining was done for the cytokines and transcription factors of interest. Percentages of T cell subsets expressing the various markers were determined. The median fluorescence intensity (MFI) for cytokines and transcription factors was calculated in order to indicate the quantitative amount of each marker per cell (181). Matched isotype controls were used for each cytokine and transcription factor to discriminate specific antibody staining from non-specific background staining.

**Elispot**

In paper II, Elispot assay was performed on PBMC and BAL cells from sarcoidosis patients and on PBMC from healthy controls for detection of IFNγ and IL-17 following mKatG stimulation. Elispot plates were treated with 70% ethanol. Thereafter plates were coated with anti-IFNγ coating antibody or anti-IL-17 coating antibody (Mabtech, Sweden) overnight. After washing, plates were blocked with complete tissue culture medium (CTCM) for 2 h at room temperature and freshly obtained PBMC (3×10⁵) or BAL cells (2×10⁵) were added to each well. Alveolar macrophages of BAL samples were partly depleted by plastic adherence during one hour incubation before adding the samples to the Elispot plate. Cells were stimulated with 10 μg/ml mKatG. Phytohemagglutinin (PHA) and anti-CD3 monoclonal antibody were used as positive controls for IFNγ and IL-17 respectively and unstimulated cells were used as negative control. Cells were incubated at 37 °C in a humidified atmosphere
of 5% CO2 for 16 h during which secreted cytokines were captured by the coating antibodies. Subsequently, biotinylated IFNγ monoclonal antibody or biotinylated anti-IL-17 monoclonal antibody were added and samples incubated for 2 h, thereafter plates were incubated with streptavidin–alkaline phosphatase for 1 h. Finally substrate (BCIP/NBT, Mabtech) was added and plates were developed for 10–20 min. We applied extensive washing between each step and after the last washing and overnight drying, plates were scanned and counted using the Elispot Reader system with AID Elispot Software 4.0. Results are presented as a mean value of triplicate wells and normalized to spot forming cell (sfc) in one million cells.

Fluorospot analysis of IFNγ and IL-17 producing cells was done on some samples. Following labeling and coating of the IPFL plate, cells were stimulated with mKatG. Secreted IFN-γ and IL-17 were captured and then stained with antibodies labeled with green and red fluorophore (FITC and Cy3). The plate was analyzed by an automated reader with filters for FITC and Cy3. The ability to detect cells that produce both cytokines simultaneously is the biggest advantage of Fluorospot compared to Elispot.

**Quantitative immune PCR**

The level (ie. concentration) of soluble IL-17 protein was measured in the cell-free BAL fluid of sarcoidosis patients with Löfgren’s syndrome (DR3+ and DR3−), sarcoidosis patients without Löfgren’s syndrome and healthy individuals. Due to the low absolute concentration of extracellular IL-17 protein in the diluted sample caused by the BAL procedure per se, we utilized a customized quantitative immuno-PCR (qIPCR) technology developed in A. Lindéns laboratory in collaboration with TATAA Biocenter™ (Göteborg, Sweden) (182). This qIPCR combines the high specificity of Enzyme-linked immunosorbent assay (ELISA) with the high sensitivity of quantitative PCR for detection of low concentrations of protein antigens (183). Plates were coated with anti-human IL-17 antibody and after blocking, 20 times concentrated BALF using ultrafiltration from each individual were added in triplicate. Thereafter each well was incubated with a detection IL-17 antibody/DNA conjugate (conjugated at TATAA Biocenter™; detection antibody from eBioscience™). A dilution series of recombinant human IL-17 was added to separate wells in triplicate and incubated in the same way as the samples for obtaining a standard curve. Real-time PCR was carried out by specific primers. After extensive washing, Ct values were determined for individual samples. The mean values of triplicate determinations were calculated after comparisons with a standard curve.

**Immunohistochemistry**

In order to visualize the expression of IL-17 in lung tissue from sarcoidosis patients we performed immunohistochemistry (paper III). Contiguous 4 μm thick transbronchial paraffin-embedded sections from sarcoidosis patients (patients with and without Löfgren’s syndrome) and healthy controls were deparaffinised in xylene and rehydrated by gradient ethanol. Antigen retrieval was done by boiling slides in citrate buffer (pH 6.0). After elimination of the endogenous peroxidase activity in the 0.3% H2O2, blocking was done by 5% goat serum. Rabbit polyclonal anti IL-17 as primary antibody was used for IL-17 staining. The staining was performed in parallel with rabbit IgG isotype control and tonsil tissue was processed as the positive tissue control. Thereafter slides were treated with biotinylated secondary antibodies (goat anti-rabbit). The staining continued with Vectastain Elite ABC Kit and the immune reaction was visualized using 3,3′-diaminobenzidine.
Sections were then counterstained with Mayer's hematoxylin, dehydrated, mounted and viewed under light microscope at a magnification of ×100, ×200 and/or ×400.

**Statistical analysis**

Statistical analysis of the data was performed by GraphPad PRISM 5.02 (GraphPad Software Inc., San Diego, CA, USA). Mann–Whitney U test or unpaired t-test was used for comparisons between two groups (non-parametric or parametric values, respectively). Analysis of data for more than two groups was performed by one-way ANOVA test and followed by Dunn’s or Tukey’s post-test for non-parametric or parametric values respectively. Wilcoxon signed rank test was used for comparisons of dependent samples. Correlation analysis was performed by Spearman’s rank correlation test. A p-value of less than 0.05 was considered to define statistical significance.
4 RESULTS AND DISCUSSION

4.1 Antigen-specific Th1 responses in sarcoidosis (Paper I)

Sarcoidosis is a multisystem granulomatous disease of unknown cause with commonly prominent lung involvement. Clinical manifestations of sarcoidosis vary from an asymptomatic state to a life-threatening condition (184). T cells are known to play a critical role in the pathogenesis of this disease and accumulation of activated CD4+ T cells in the airways of patients has been well documented. Given the increased levels of IFNγ and IL-2 in the lungs of sarcoidosis patients, it has been postulated that sarcoidosis is a Th1 mediated disease (185-187). However, accumulating data supports the importance also of other T cell subsets in the pathogenesis of sarcoidosis. The etiology of sarcoidosis is still unknown, although it has been proposed that mycobacterial species are involved in the pathogenesis of at least a subgroup of sarcoidosis patients (188). A specific mycobacterial antigen, mycobacterial catalase–peroxidase (mKatG) has been detected in sarcoidosis tissue. Furthermore, mKatG has been shown to trigger B and T cell responses in lung and blood cells of sarcoidosis patients (118-120). Here we wanted to investigate in detail Th1 and Th17 responses to mKatG in BAL and blood T cells from subgroups of sarcoidosis patients with distinct clinical manifestations.

Sarcoidosis patients enrolled in this study were classified as patients with Löfgren’s syndrome (LS) and patients without Löfgren’s syndrome. In addition, all patients with LS were selected to be HLA-DR3+ (DRB1*0301) and with an accumulation of CD4+ TCR AV2S3+ BAL T cells (n=13). In a sharp contrast all non Löfgren’s syndrome patients should in addition fulfil the criterium to be HLA-DR3- (n=10). Since Löfgren’s syndrome in combination with HLA-DRB1*14 and –DRB1*15 alleles are strongly associated with a chronic disease course, at high risk of developing pulmonary fibrosis (141, 142).

IFNγ and TNF are important cytokines in mycobacterial clearance (189-191) and IL-2 is involved in T cell clonal proliferation (192). All three cytokines can be produced by CD4+ and CD8+ T cells. We selected these three cytokines to evaluate the production of each in response to mKatG in sarcoidosis patients.

Stimulation with mKatG led to activation of BAL and blood CD4+ T cell cytokine responses in both Löfgren’s and non Löfgren’s syndrome patients when compared to spontaneous cytokine production i.e. un-stimulated sample.

An important role of Th1 cells and related cytokines in protection against *M. tuberculosis* has been reported by different groups. Splenocyte T cells from (Bacillus Calmette–Guérin) BCG immunized mice could adoptively induce protection and control growth of *M. tuberculosis* in recipient mice (193). Although IFN-γ alone is insufficient to control *M. tuberculosis* infection, it is required for protection against *M. tuberculosis* and an early response of IFNγ producing T cells is critical to induce resistance to infection with *M. tuberculosis* (194, 195).

The requirement for TNF in control of *M. tuberculosis* infection is complex, e.g. it has a role as a mediator of macrophage activation. Moreover TNF in synergy with IFNγ induces nitric
oxide synthase-2 expression (Absence of NOS2 causes increased susceptibility to *M. tuberculosis* infection (196). TNF in *M. tuberculosis* infection is also involved in cell migration and cell localization within lungs. It also affects formation of functional granulomas in infected tissues by influencing the expression of adhesion molecules, chemokines and chemokine receptors (197).

CD8+ T cells in sarcoidosis patients exhibited a cytokine profile similar to CD4+ T cells following mKatG stimulation. Our data regarding responses of CD4+ and CD8+ T cells to mKatG is in accordance with a previous study showing positive IFNγ response after mKatG stimulation in U.S. patients (120).

Since MHC class I presentation is most efficient with cytoplasmic antigens, a possible role for CD8+ T cells in the immune response to *M. tuberculosis* received little attention for many years, since mycobacterial antigens are normally restricted to phagosomes and presented on MHC class II to CD4+ T cells (197). However, mice with a genetic deficiency in β2 microglobulin, and thus deficient in MHC class I molecules were quite susceptible to *M. tuberculosis* infection (198). There is strong evidence that indicated migration of CD8+ T cells to the lungs in *M. tuberculosis* infection occurs with the same kinetics as that of CD4+ T cells (199) and these CD8+ T cells are capable to produce IFNγ and to lyse infected macrophages (200). Rapid accumulation of CD8+ T cells in the lung of mice infected with *M. tuberculosis* has been reported before (201).

Both CD4+ and CD8+ T cells are capable to release cytokines and mediators to activate macrophages. Our data on the cytokine profile of CD8+ T cells indicated that CD8+ T cells not only contribute in cytotoxic activity but also that they are activated to cytokine production and to protect against *M. tuberculosis*. CD8+ T cells are found within granulomas and can help to prevent spreading of mycobacteria (202). Although the relative frequency of CD8+ T cells in BAL of sarcoidosis patients is reduced at onset, their relative proportion increases at later stages of disease. Our data suggests that both CD4+ and CD8+ T cells play an important role in mycobacterial clearance, which is also supported by other studies (203).

Comparing T cell activity in response to mKatG between BAL and blood demonstrated that BAL T cells responded to mKatG with higher IFNγ production than blood T cells in both Löfgren’s and non Löfgren’s patients. This indicated that mKatG specific T cells are accumulated in the affected organ. Moreover, it has been shown that alveolar T cells in sarcoidosis patients are more activated than peripheral cells (204).

We didn’t compare BAL T cell reactivity to mKatG with irrelevant antigens in sarcoidosis patients. However, reactivity of BAL cells from sarcoidosis patients to mycobacterial antigen but not to Keyhole Limpet Hemocyanin has been shown by others (118, 205). Furthermore, T cell responses in blood of most sarcoidosis patients to mycobacterial antigen but not lysate from *Trypanosoma brucei* have been demonstrated by other groups (206).

In contrast to mKatG responses, IFNγ responses to PPD was observed only in CD4+ T cells, and not in CD8+ T cells, which indicates that there might be a selective recognition of the mycobacterial epitopes among T cells. Some mKatG-derived peptides may be constituents of PPD, but if so they would clearly form a small fraction of the whole protein mixture.

There were no major differences between patient subgroups regarding the production of individual cytokines in response to mKatG and PPD.
Among the T cells some can exhibit a multifunctional capacity, i.e. secrete two or more cytokines. As we mentioned before IFN$_\gamma$ and TNF are two important cytokines for combating mycobacterial infections. (189, 190, 194, 207). When combined, IFN$_\gamma$ and TNF synergize in their capacity to mediate effective killing (208, 209). There is some evidence that shows T cells secreting IFN$_\gamma$, TNF and IL-2 simultaneously were found to possess the best protective capacity towards Mycobacterium tuberculosis (210).

We therefore investigated multifunctional T cells, i.e. CD4+ and CD8+ T cells that simultaneously produce two cytokines in response to mKatG and PPD. Among CD4+ and CD8+ T cell subsets, we calculated the percentage of total mKatG-reactive cells that produced single IFN$_\gamma$, single TNF, or IFN$_\gamma$ and TNF in combination (the same calculation was made for IFN$_\gamma$ in combination with IL-2). We found that mKatG stimulated the BAL CD4+ T cells to less single IFN$_\gamma$ production, but more simultaneous production of IFN$_\gamma$ and TNF, in patients with Löfgren’s syndrome as compared to non-Löfgren’s syndrome patients. In contrast, PPD stimulation gave rise to similar cytokine pattern in both patient subgroups. This finding suggests that DR3+ sarcoidosis patients with Löfgren’s syndrome i.e. patients with favorable prognosis have a more potent immune response towards a minor number of antigens, in which mKatG could be one of them, which consequently could lead to antigen elimination followed by recovery. We may speculate that patients with non-Löfgren’s syndrome have a less potent response to mKatG and other antigens, allowing antigen persistence. Additionally, they may exhibit an epitope spreading within the lungs, leading to involvement of more antigens and more IFN$_\gamma$ production with solid inflammation and prolonged disease, since analysis of total CD4+ T cell IFN$_\gamma$ mRNA expression showed higher levels in DR3- patients (211).

The median fluorescence intensity (MFI) of cytokine staining was measured in patients with Löfgren’s syndrome. The MFI value is an indication of the quantity of cytokine content on a per-cell basis (212). Our findings demonstrated that the multifunctional CD4+ T cells i.e. IFN$_\gamma$/TNF double producing cells, following mKatG stimulation, had the highest MFI values of each cytokine, suggesting that they produce more of the two respective cytokines from each cell, compared to single cytokine-producing CD4+ T cells and it indicates these multifunctional T cells have a more potent effector capacity with regard to each respective cytokine. Seder et al. have reported the importance of a potent and durable T cell response with a high frequency of T cells that are antigen specific in vaccine development (213). The frequency of IFN$_\gamma$ producing cells is the most common parameter used to evaluate vaccine responses due to the important role of IFN$_\gamma$ in pathogen clearance (190, 214). However, IFN$_\gamma$-producing T cells are not sufficient for protection (215, 216) and presence of TNF is also important to induce protection. The role of TNF in immunopathology of tuberculosis is complex. Rheumatoid arthritis patients who were treated with anti TNF antibody were susceptible to developed fatal tuberculosis(217) which indicated the importance of TNF in protection against infection. The contribution of both IFN$_\gamma$ and TNF lead to enhanced killing of M. tuberculosis compared to either cytokine alone (208, 209, 218).

The importance of multifunctional T cells in immune reactions against tuberculosis (219), in BCG-vaccinated infants (220) and people who live in high infected areas (221) has been studied before. Multifunctional, high-level cytokine-producing Th1 cells in the lungs of mice have been associated with enhanced protection against M. tuberculosis (208, 210, 222). There is some evidence that protection against pathogens such as Leishmania major is associated with multifunctional T cells making IL-2, IFN$_\gamma$ and TNF (181). The induction of
multifunctional T-cells is a new approach to the process of vaccine development (223, 224). We speculate that a high frequency of mKatG specific multifunctional T cells with a high per cell cytokine content may help clearance of the assumptive pathogenic antigen in sarcoidosis patients with Löfgren’s syndrome. Probably the potency of multifunctional T cells is due both to their double cytokine production, and the higher per cell cytokine content of each cytokine.

As we described before sarcoidosis patients who are HLA-DR3+ most commonly have Löfgren’s syndrome and a good prognosis. The accumulation of AV2S3+ T cells in the lungs (>10.5\% of total CD4+ T cells) is an interesting characteristic of the patients in this group. TCR AV2S3+ T cell proliferation of PBMC from healthy DR3+ BCG vaccinated individuals following in vitro stimulation with *Mycobacterium tuberculosis* extract has been shown by our group before (225). Our group in collaboration with John Hopkins University has reported a correlation between the frequency of BAL TCR AV2S3+ T cells and BAL IFNγ producing cells in response to mKatG by Elispot (120). In the current study we show, for the first time, the capability of BAL TCR AV2S3+ T cells to react against mKatG.

Our data demonstrated that TCR AV2S3+ T cells responded with IFNγ production to a significantly higher extent than the TCR AV2S3- T cells (median 0.65\% vs. 0.48\%, \(p=0.016\)). This finding indicates that mKatG could be a specific disease-related antigen that is capable to trigger different subsets of T cells. It is important to remember that mKatG is a large protein, approximately 700 amino acids, and likely contains several T cell epitopes that can be recognized by various TCRs. It is therefore not surprising that both TCR AV2S3+ and AV2S3- T cells can respond to the same protein.

Similar to our findings in BAL, the blood TCR AV2S3+ T cells also responded to mKatG to a higher extent than blood TCR AV2S3- T cells. We speculate that the TCR AV2S3+ T cells are re-circulating throughout the body. The alveolar T lymphocytes can reach regional lymph nodes by leaving the alveoli through the alveolar epithelium. Via the regional lymph nodes they can be distributed all over the body to rejoin the systemic immune system (226). Alternatively, the sarcoidosis antigen that stimulates the TCR AV2S3+ T cells may be distributed systemically.

Our group has previously shown that the frequency of CD4+ AV2S3+ T cells in BAL of patients after clinical recovery is normalized (180). This indicates that the number of BAL TCR AV2S3+ T cells correlates with disease activity. Furthermore we know that there is an association between the number of CD4+ AV2S3+ T cells and good prognosis (227). Previously, our group also reported that expression of activation markers such as CD26, CD28, CD69, and HLA-DR were enhanced in AV2S3+ BAL CD4 T cells compared to AV2S3- subsets. These data suggested that AV2S3+ CD4+ T cells in the lung are significantly more activated and differentiated compared to AV2S3- CD4+ lung T cells (228, 229). Moreover, in another study we showed a sharply reduced expression of the regulatory T cell transcription factor Foxp3 in BAL AV2S3+ CD4+ T cells of DR3+ sarcoidosis patients compared to AV2S3- cells (230) This indicates that BAL AV2S3+ CD4+ T cells are effector cells rather than regulatory T cells. These findings together with our observation of Th1 cytokine production by these cells and a higher response of TCR AV2S3+ T cells to mKatG compared to the other CD4+ T cells suggests that TCR AV2S3+ T cells are associated with good prognosis and spontaneously resolving disease because of their ability to secrete effector cytokines upon mycobacterial antigen stimulation, possibly leading to antigen elimination.
The TCR AV2S3+ T cell subset is not a T cell clone but it constitutes an oligoclonal T cell subset. The variable (V) α chain can associate with different Vβ chains. Most recently our group identified 16 different Vβ CD4+ T cell expansions in BAL of sarcoidosis patients and a preference of TCR AV2S3+ T cells to pair with Vβ22, Vβ7 and Vβ18 has been shown before using PCR (146, 231). We also have data based on flow cytometric analysis that indicates a preference for Va2.3 to pair with Vβ22, although not in a majority of Va2.3+ cells (Kaiser et al, unpublished observations). T cell clones can exhibit different avidity towards a given antigen; one study showed that some clones only proliferated, whereas others both proliferated and produced cytokines, in response to a given concentration of the same antigen (232).

BAL T cells preferentially exhibited a CD27- phenotype while in blood the majority of CD4+ T cells were CD27+ T cells. Our findings thus revealed that differentiated T cells localized in inflamed organ. BAL CD27- T cells produced more IFNγ in response to mKatG, while in blood the CD27+ T cells were the major cytokine producing cells. Similarly in chronic beryllium disease (CBD), i.e. a granulomatous disease like sarcoidosis, characterized by infiltration of beryllium-specific CD4+ T cells in the lungs, the majority of cytokine producing T cells are CD27- (233). CD27, a co-stimulatory molecule (member of the TNF/NGF-R family), is expressed on the naïve and memory T cells. Activation of T cells via TCR/CD3 induces high CD27 surface expression. However, following prolonged activation, CD27 becomes gradually switched off (234-236). Our findings are therefore compatible with long-term antigen stimulation in the lungs.

4.2 IL-17 and antigen-specific IL-17-responses in sarcoidosis (Paper II)

T helper 17 is a subset of CD4+ T helper cells that less than a decade ago was identified as a distinct T cell lineage (237) and production of interleukin IL-17 (synonymous to IL-17A), a pleiotropic cytokine with widespread effects, is the hallmark of this subset of T cells (26). IL-17 is an essential player in host defense in several mammalian organs including the lungs of humans (182, 238). Th17 cells were recognized as a key factor in protection against extracellular bacterial and fungal pathogens like Klebsiella pneumoniae (239), Citrobacter rodentium and Candida albicans (240), while other studies showed the role of Th17 cells in protection against intracellular bacteria like Mycobacterium tuberculosis and Salmonella enterica (241). An impaired function of IL-17 results in increased bacterial burden and reduced overall host survival (242). IL-17 and IL-17-producing cells are thought to play a key role in chronic inflammation like mycobacterial infection, rheumatoid arthritis and other autoimmune disorders and also granuloma formation (243-245). Involvement of Th17 and IL-17 producing cells in inflammatory lung disorders such as asthma, COPD and ILDs have also been reported in several studies. Up-regulation of IL-17 has been reported in patients with asthma (246), although the exact role of IL-17 in asthmatic responses is difficult to define. It can be speculated that IL-17 in asthma doesn’t have a prominent role and the influence of IL-17 in pulmonary inflammation is mostly restricted to neutrophil dependent disorders or Th2 independent chronic inflammation (247).

The involvement of IL-17 and IL-17-producing T cells in sarcoidosis has been reported by different groups, although there is some discrepancy regarding the role of IL-17 in pathogenicity of sarcoidosis. Facco et al. detected persistence of Th17 cells in both BAL and blood of sarcoidosis patients. They could also detect IL-17 producing cells surrounding the
central core of the granuloma from sarcoidosis patients. They also reported trafficking of Th17 cells to the lungs of sarcoidosis patients mediated by CCL29 (CCR6 ligand) (125). An increased population of Th17 cells in blood of sarcoidosis patients has been reported by Ten Berge et al. (126). They have also shown an increased proportion of IL-17/IFNγ double producing cells in BAL and blood of patients and increased numbers of IL-17 producing cells in and around granulomas. A significant correlation between IL-17 and IL-6 levels was detected by Urbankowski et al. in BAL of sarcoidosis patients (248). In contrast to Ten Berge, Furusawa et al. reported a decline in IL-17 mRNA level in PBMCs from patients with sarcoidosis compared to controls and they didn’t observed any response to ESAT-6 stimulation (mycobacterial antigen) (249). Wonder Drake’s group showed a greater number of Th17 cells in BAL and blood of sarcoidosis patients versus controls. In a sharp contrast with Furasawa’s findings, they demonstrated an increase in ESAT-6 specific Th17 cell responses in BAL and blood of sarcoidosis patients compared to controls. Moreover they reported a reduction in expression of IFNγ by Th17 cells in sarcoidosis patients (250). Tondell et al. finally reported a lower fraction of Th17 cells in sarcoidosis patients compared to controls. However, they found a higher proportion of IFNγ producing Th17 cells in sarcoidosis patients and this was correlated with radiologic stage (251).

Our data reveals the presence of T cells making IFNγ as well as IL-17 in response to the mycobacterial antigen mKatG in sarcoidosis patients. mKatG-reactive IL-17 producing cells were found in the affected organ, the lung, as well as in peripheral blood. We and others previously showed the presence of mKatG-reactive IFNγ producing effector T cells in sarcoidosis patients (120). We have also reported a more pronounced IFNγ production by CD4+ TCR AV2S3+ T cells compared to CD4+ TCR AV2S3- T cells in response to mKatG (252). Altogether these findings indicated mKatG behaves in a manner expected for a pathogenic antigen.

Here for the first time, we have shown mKatG-specific IL-17 responses in BAL of sarcoidosis patients with higher frequency of mKatG-reactive IL-17 producing cells in BAL of sarcoidosis patients with Löfgren’s syndrome compared to patients without Löfgren’s syndrome (Figure 5a). Furthermore sarcoidosis patients with Löfgren’s syndrome and more precisely sarcoidosis patients with Löfgren’s syndrome who are DR3+ (patients with accumulation of CD4+ TCR AV2S3+ T cells in the lungs) had higher levels of IL-17 in BALF (Figure 5b). Importantly this pioneer finding could be a validation for the pathogenic role of mKatG in a subset of sarcoidosis patients. The specific capacity of the HLA-DR3 molecule in presenting sarcoidosis-specific antigen(s) such as mKatG for T cells followed by induction of Th17 cells in the lungs of DR3+ sarcoidosis patients with Löfgren’s syndrome can be a possible explanation for higher levels of soluble IL-17 in this subset of patients.

Our findings demonstrated that mKatG-reactive IFNγ producing cells are dominant compared to mKatG-reactive IL-17 producing cells in BAL of sarcoidosis patients. However, given the importance of Th17 and IL-17 producing cells in the immunopathogenesis of several inflammatory and autoimmune diseases (253), we propose that the observed Th17 responses against the mycobacterial protein mKatG could contribute to the inflammation in sarcoidosis. The accumulation of IL-17 producing cells reactive to mKatG in the lungs of sarcoidosis patients may be a result of trafficking or local proliferation of antigen-specific IL-17 producing cells at the site of granulomatous inflammation.
The finding of more IL-17-producing cells responding to mKatG in sarcoidosis patients with Löfgren’s syndrome locally at the site of inflammation suggests further qualitative differences in the mKatG-specific responses between patients with and without Löfgren’s syndrome. Due to the very good prognosis in DR3+ sarcoidosis patients with Löfgren’s syndrome we postulate that IL-17-producing cells play a role in the presumed elimination of antigen and spontaneous recovery that is characteristic for DR3+ patients with Löfgren’s syndrome.

Figure 5: mKatG-specific IL-17 responses and IL-17 levels in patients with pulmonary sarcoidosis. (a) Elispot assay for evaluation of mKatG specific IL-17 producing cells in BAL of patients with and without Löfgren’s syndrome. (b) IL-17 levels in BAL fluid determined by Immuno-PCR and compared in healthy controls, sarcoidosis patients without Löfgren’s syndrome and with Löfgren’s syndrome. Sarcoïdosis patients with Löfgren’s syndrome were divided into two groups (HLA-DR3+ and HLA-DR3-).

Both Th1 and Th17 cells are required and interact in different steps for host defence against mycobacterial infection. In the lungs of vaccinated animals after M. tuberculosis challenge, expression of the CXCR3 ligands i.e. CXCL9, CXCL10 and CXCL11 have been induced by IL-17, thereby IL-17 can regulate trafficking of Th1 cells to the site of infection (241). IFNγ and IL-17 T cell responses to M. tuberculosis antigens are time dependent and IL-17 producing T cells accumulate in the lungs more rapidly than IFNγ-producing T cells; furthermore IFNγ and IL-17 may act in synergy to initiate inflammation (254). IL-17 stimulates the activity of the transcription factor NF-κB (255); activation of NF-κB in antigen presenting cells in turn affects Th1 responses and leads to development of IFNγ producing T-cells (256). IL-17 is not only involved in neutrophil-mediated inflammatory responses, but also it is an important cytokine in the induction of an optimal Th1 response and protective immunity against mycobacterial infection. We speculated that the higher expression of IL-17 and mKatG specific IL-17-producing cells in sarcoidosis patients with Löfgren’s syndrome help to induce Th1 responses, and contribute to a more rapid and diverse effector T cell response, which then contributes to the protection against unknown antigen(s).

The inflammatory properties of IL-17 and IL-17 producing cells are irrefutable and involvement of IL-17 in the pathogenesis of several inflammatory disorders, in particular autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), psoriasis and inflammatory bowel disease (IBD) has been reported before (19, 257). IL-17 has been
considered as a potent pro-inflammatory cytokine that induces expression of different inflammatory mediators such as IL-6, TNF-α, IL-1β, GM-CSF, CXCL1, CXCL8, CCL2, CCL7 and CCL20 by different cell types like epithelial cells, endothelial cells and macrophages (258). Th17 and possibly other IL-17 producing cells in sarcoidosis patients can be considered as potent mediators of inflammatory responses because of their ability to induce trafficking and activation of inflammatory innate immune cells at the site of inflammation (259, 260).

More recently there is some evidence for an immune-modulatory or non-pathogenic role for IL-17. The role of IL-17 and antigen specific IL-17 producing cells in the pathogenesis of experimental autoimmune uveitis (EAU) has been demonstrated before (261). Yan Ke et al., however, identified an anti-inflammatory role for IL-17 in the EAU model. They found that in EAU-susceptible rats, treatment with small doses of IL-17, rather than exacerbating the clinical score of the disease, lead to suppression of EAU development and unexpectedly ameliorated the clinical score of EAU. They thus concluded that IL-17 has both pro- and anti-inflammatory effects on the development of EAU (262). O’Connor et al have demonstrated a protective function for IL-17 in a mouse IBD model and suggested that this function is exerted partly by suppressing Th1 differentiation. They also showed that absence of IL-17 led to an accelerated and severe disease accompanied by higher expression of genes encoding Th1 type cytokines (263). Thus, we cannot exclude this type of beneficial effect of IL-17, particularly in the DR3+ Löfgren’s patients.

Another recent and intriguing finding is that Th17 cells can be polarized to become pathogenic or non-pathogenic depending on the environmental cytokines or mediators (22). Kuchroo’s group has shown a critical role of TGF-β in development of Th17 cells. They reported that although the presence of IL-23 has been considered to be essential for developing the pathogenic Th17 phenotype, TGF-β3 can differentiate naïve T cells into pathogenic Th17 cells without any need for further exposure to IL-23. In fact both TGF-β1 and TGF-β3 are able to induce Th17 cells, but Th17 cells induced by TGF-β1 are not pathogenic whereas TGF-β3 has an important role in induction of pathogenic and pro-inflammatory Th17 cells. We know from previous studies that the levels of some cytokines like TGF-β1 that may influence differentiation of Th17 cells, differ between sarcoidosis patients with or without Löfgren’s syndrome (211).

Our findings indicate that in sarcoidosis patients, there is a subset of T cells which has the ability to produce both IFNγ and IL-17 simultaneously. In fact, we found that a majority of T helper 17 cells can also produce IFNγ. Existence of these hybrid T cells has been reported before in other inflammatory diseases and also sarcoidosis (126). Monteleone et al. demonstrated that as a consequence of cytokines and microenvironmental molecules in gut mucosa of IBD patients, conditions are generated that result in that some IFNγ producing cells originate from previously IL-17 producing T cells (264). Conversion or switching of T helper cells from IL-17 producing to IFNγ producing cells is a phenomenon that is dependent on the cytokine environment. IL-1, IL-6 and TGF-β have important roles in the cytokine signature of Th17 and IL-17 producing cells. TGF-β is essential for sustained expression of IL-17A by Th17 cells. In the absence of TGF-β, both IL-23 and IL-12 cause suppression of IL-17 expression and instead enhance IFNγ production (265). Importantly, there is evidence that hybrid Th1/Th17 T cells are more pathogenic than conventional Th17 cells (266). CD4+ AV2S3+ T-cells and CD4+ AV2S3- T-cells displayed differences with regard to the capacity
to express IFNγ and IL-17. However, further studies are needed to investigate the frequencies of such hybrid T cells in patient subgroups versus healthy control subjects.

In most cases the first option for reducing the inflammation in sarcoidosis patients is to prescribe corticosteroids. In recent years, the blocking and controlling of IL-17 and IL-17 producing cells is one of the strategies that have been used for treatment of inflammatory disorders, for instance administration of antibody against IL-17 described in RA and psoriasis (267, 268). On the other hand, a protective effect of IL-17A in inflammatory bowel diseases (IBD) and uveitis has been reported before (24, 262). Given our findings that indicated higher level of IL-17 and increased IL-17 responses among a subgroup of sarcoidosis patients with good prognosis, it remains to be elucidated whether inhibiting this signaling will offer clinical benefits for patients. Therefore, a more detailed understanding about the pathogenic or protective role of Th17 cells in various subsets of sarcoidosis patients is needed.

4.3 Characteristics of effector and regulatory T cell subsets in the lungs of smoking and non-smoking healthy individuals (Paper III)

Cigarette smoke intrinsically induces local inflammation; however, it is associated with both release and inhibition of pro-inflammatory and anti-inflammatory mediators that influence different T cell subsets. T helper lymphocytes have a pivotal role in orchestrating the host defense and inflammatory reaction following triggering by specific antigens. Our group has demonstrated an impact of smoking on the distribution of BAL T cell subsets in the context of COPD studies (269). In the present study, in order to better understand the influence of cigarette smoking on T cells in relation to the pathogenesis of smoking-induced diseases, we investigated frequencies and characteristics of lung and blood CD4+ T cell subsets such as T helper 1 (Th1), Th17 and Treg cells (and corresponding subsets in CD8+ T cells) in 18 healthy young and moderate smokers and 15 never-smoking individuals.

Our findings indicated a lower CD4/CD8 ratio of BAL T cells in healthy smokers. A higher rate of apoptosis in alveolar cells and alterations in glycoproteins in smokers can be possible explanations for changing this ratio in BAL and blood. Cigarette smoke components and even the gaseous phase can directly induce both apoptosis and necrosis of lymphocytes at the site of exposure (270, 271).

Our data demonstrated that healthy smokers had a lower frequency of BAL CD4+ IL-17 producing cells compared to healthy never smokers. Inflammatory responses can be induced by smoking and involvement of IL-17 producing cells in this has been reported before (158, 245). A higher frequency of IL-17 producing cells in the bronchial biopsies of COPD patients compared to control non-smokers has been documented. Although COPD patients are different from healthy smokers with normal lung function, studies on the role of IL-17 in COPD patients can be informative.

COPD is an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases in particularly cigarette smoke and is characterized by persistent and progressive airflow limitation (272). Increased production of IL-17A in the bronchial submucosa and infiltrating inflammatory cells in small airways has been reported (273, 274). Chang et al. reported expression of IL-17A by both CD4 and CD8 T cells in the lung submucosa from COPD patients and suggested that IL-17 producing cells play an important role in the pathogenesis of COPD by inducing an accumulation of neutrophils in the lungs of
COPD patients (275). Airway epithelial cells were induced by IL-17 to produce mucus and matrix metalloproteinase-9 (MMP-9) and dysregulation of MMPs contribute to the destruction of lung tissue in COPD (276). Neutrophils can contribute in the pathogenesis of COPD by secretion of proteolytic enzymes such as neutrophil elastase (277). Elastin is a major constituent of the extracellular matrix in the lungs and it can be degraded by neutrophil elastase, leading to mucus hyper-secretion (278). Moreover it has been suggested that elastin can act as an auto-antigen in the lung of COPD patients by inducing of Th1 and Th17 cells against elastin (276). It has been suggested that in COPD patients with frequent exacerbations and excessive mucus production, these phenomena can be attributed to IL-17 mediated neutrophilia (245). Shen et al. has demonstrated not only that the concentration of IL-17 and neutrophil percentage decline in the lungs of tobacco-smoke-exposed mice which were treated with anti-IL-17 antibodies, but also the pathological score of small airway inflammation was improved (279). This indicated the importance of IL-17 in the pathogenesis of COPD. Doe et al. demonstrated a similarity in IL-17 expression between COPD patients and healthy smokers but no association with increased neutrophilic inflammation (280).

There is no consensus on the effect of smoking on IL-17 producing cells in healthy smokers with normal lung function and this is partly related to variations in methodology and differences regarding the characteristics of studied populations. Tobacco smoking can induce an antigen specific Th17 response in certain individuals (281) and higher relative expression of IL-17 mRNA in lung tissue of smokers has been reported before (282). Our finding regarding the frequency of BAL CD4+ Th17 cells are in contrast with these studies. We found a reduced IL-17 expression by BAL CD4+ T cells of light smokers but an increase in the number of cigarettes per day caused an increased IL-17 expression. We should emphasize that most studies related to the effect of smoking on T cells focused on differences between never-smokers and smokers who were matched with COPD patients with regard to smoking history, while the present study was performed on samples from mild smokers.

The frequency of IFNγ-producing CD8 T-cells increased in BAL of healthy smokers after anti-CD3/CD28 stimulation and this increase was more intensive in heavy smokers. Freeman et al. demonstrated increased production of inflammatory cytokines by CD8+ T cells in COPD patients. The lung CD8+ T cells respond to danger signals by up-regulating inflammatory mediators and cytotoxic molecules (283, 284). IFN-γ is a key cytokine in the development of the airway obstruction and pulmonary destruction in COPD by induction of different mediators (285).

Bacterial burden and susceptibility to develop some infections increase among smokers (286). Cigarette smoke exposure can inhibit T cell responses to M. tuberculosis and influenza virus in a mouse model (287). Lugade et al. demonstrated a negative effect of cigarette smoke exposure on the generation of adaptive immune responses to nontypeable Haemophilus influenzae (NTHI) (288). Although in contrast to our findings they reported increased IL-17 response and decreased IFNγ response to NTHI chronic infection in mice with chronic cigarette smoke exposure. There may however be several reasons for this discrepancy, such as species differences, the level of smoke exposure, infection in mice versus in vitro stimulation of human T cells with anti-CD3 antibodies. Due to the important role of Th17 cells in host defense against bacterial or viral infections, we propose that the decline in expression of IL-17 in smokers can increase infection susceptibility.
The multifunctional T cells (cells that produce two or more cytokines simultaneously) as well as hybrid cells (T-cells which are capable to produce cytokines from two different T cell lineages) have been studied in BAL and blood of healthy smokers and non-smokers. The multifunctional IFNγ/TNF producing cells are associated with either a protective role or are indicative of active disease in tuberculosis (289-291). IFNγ/IL-17 producing T cells is an example of hybrid T cells and indicates plasticity between Th1 and Th17 cells. Less is known about the function of these hybrid T cells but there is some evidence to suggest that Th17/Th1 T cells are more pathogenic than conventional Th1 or Th17 cells in the context of autoimmunity (266, 292). We believe these multifunctional or hybrid cells are potent functional T cells which play an important role in host defense. Our findings demonstrated a decline in BAL CD4+ IFNγ/IL-17 and IL-17/TNF producing cells in healthy smokers compared to never smokers. The reduction of these potent T cells could negatively impact on the capacity of the immune system in smokers to control pulmonary infections.

Our findings on regulatory T cells (Treg) showed no difference in the proportion of Foxp3+ CD4+ T cells in BAL of smokers and never-smokers. Similarly to the situation for IL-17, there is no consensus on the impact of smoking on Treg cells and an augmented or abated frequency of Foxp3 expression (293, 294) and even variation between smokers and non-smokers in different compartments of lung tissue has been reported. Isajevs et al. have demonstrated increased Foxp3 expression in large airways of smokers and COPD patients but a decreased percentage of Foxp3+ cells in small airways of COPD patients (295). Furthermore, we found that Foxp3+ CD4+ regulatory T cells in BAL of smokers did not show any IL-10 production (Figure 6a). IL-10 is a suppressive cytokine that hinders transcription of many genes which are normally induced via pattern recognition receptors. Many immune cell types including effector or regulatory T-cells can produce IL-10 (296).

IL-10 can be produced by Foxp3+ Treg cells in tissues such as colon or lungs and it is needed for restraining immunological hyperreactivity at these interfaces with the environment, but is not needed to control systemic autoimmunity (297). We speculate that the lack of IL-10 production by Foxp3+ CD4+ regulatory T cells in BAL of smokers may reflect a defective function of regulatory T-cells which is caused by cigarette smoke components. Roos-Engstrand et al. have shown that despite similar frequencies of T cells expressing Foxp3 in smokers and non-smokers, the numbers of non-functional Tregs with a CD4+ CD25+ phenotype that do not express Foxp3 is higher in healthy smokers compared to non-smokers (298).

Tregs constitute a heterogeneous population with diverse sub-populations, e.g. thymus-derived Tregs (tTregs), and peripherally derived Tregs (pTregs). These Treg variants have different functions and it has been proposed that tTregs modulate T-effector cell trafficking, while pTregs inhibit T-effector priming (37, 44). Helios is a member of the Ikaros transcription factor family and recently has been considered as a marker of natural or thymic-derived Treg cells (38), although this has later been challenged (39) and more recently, Helios has been suggested to be a marker of anergic effector cells or a T cell activation and proliferation marker (40, 41). Regardless of Helios qualification as a tTreg or activation marker our findings indicate a remarkable increase in the proportion of Helios+ Foxp3+ Tregs in healthy smokers (Figure 6b). Raffin et al. found that Helios− Tregs, but not Helios+ Tregs has capacity to secrete IL-17 and IL-10 (299). In agreement with their findings, our data indicated that expression of IL-10 and IFNγ in Foxp3+ Tregs was almost exclusively confined to the Helios− subset. Moreover we found that the fraction of IL-10 positive cells in
the Helios- Tregs was significantly reduced in smokers (Figure 6c) and no such difference was observed for IFNγ production. The lower expression of IL-10, but not IFNγ, in Foxp3+Helios- BAL Tregs of smokers may indicate that smoking induces a defective function in Foxp3+Helios- Tregs. Alternatively, if Helios- Treg cells remain potent suppressors, the observed decrease in Th17 cells and other inflammatory cytokine producing cells in smokers may be partly ascribed to a high frequency of these Helios- Tregs in smokers. Such a scenario would be in accordance with Raffin’s finding that demonstrated Helios- Tregs have higher suppressive capacity versus to Helios+ Tregs (299). Our finding of a reduced ratio of IL-17- to IL-10-producing CD4+ BAL T cells in healthy smokers is in contrast to the findings in smoke-exposed mice by Wang, although their model was based on heavy smoke exposure that induced a COPD-like disease (300).

Figure 6: Analysis of regulatory T cells, (a) comparison of the frequency of BAL CD4+ Foxp3+ T cells that produce IL-10 between smokers and non-smokers. (b) Comparison of fractions of BAL CD4+ Foxp3+ T cells that are Helios- in smokers and non-smokers. (c) Comparison of IL-10 production by Foxp3+Helios- BAL CD4+ T cells between smokers and non-smokers.

There are discrepancies between different studies as to the effect of smoking on T-cell responses and several explanations are plausible. Daily quantity of exposure, smoking history, intensity of smoking inhalation varies between different smokers and those are just some of the factors that have an influence on T-cell function studies. Genetic background and socioeconomic status are other factors that contribute to the results. Whether studies were performed on human or mouse samples, and design of study ex vivo or in vitro as well as the choice of experimental techniques are other factors that affect the T-cell responses observed.

In conclusion, our findings implied that a change in subset composition of Tregs and reduced IL-10 production by Tregs may be of importance for induction of smoking-induced diseases such as COPD and autoimmune disorders.
4.4 Characteristics of lung T cell subsets in multiple sclerosis patients (Paper IV)

Cigarette smoke is recognized as a major risk factor for developing several diseases. In addition to the lung related disorders smoking has also been associated with risk of disease or to disease phenotype in a number of important autoimmune disorders (151, 301).

Rheumatoid arthritis (RA) is one of the immune mediated diseases with a striking example of gene-environment interaction. Certain variants of HLA-DRB1*01 and HLA-DRB1*04 are called the shared epitope genotype. A study on RA patients demonstrated a gene-environment interaction as the combination of smoking history and having two copies of HLA-DR “shared epitope” genes increased 21-fold the risk of the RA in the subgroup of patients who are positive for antibodies to citrullinated protein antigen (ACPA) (162, 302). Part of the mechanism appears to be that cigarette smoke induces the PAD enzymes responsible for citrullination (a post-translational modification) of proteins such as vimentin (163).

MS is another example of autoimmune disease where cigarette smoking strongly increases the risk of developing MS in people with genetic susceptibility, in this case carriage of HLA-DRB1*15 and absence of HLA-A*02 (171). Furthermore Odoardi et al. by study on Lewis rat transfer experimental autoimmune encephalomyelitis (EAE, a model of MS) have recently demonstrated that the lungs can contribute to the activation of potentially autoaggressive T cells and their transition to a migratory mode as a prerequisite to entering their target tissues and inducing autoimmune disease (303) and they concluded that T cells become educated in the lung to enter to the central nervous system.

T cells play an important role in orchestrating inflammation. However, very little is known about how smoking may lead to autoimmunity. To better understand the mechanisms behind the T cell activation in the lungs we investigated frequencies and characteristics of lung and blood CD4+ and CD8+ T cell subsets in seventeen MS patients (8 smokers and 9 non-smokers) (10 treated with IFNβ, 3 with natalizumab and 4 untreated patients) and twenty three healthy individuals (14 smokers and 9 non-smokers). IFNβ and natalizumab are two common, and the most effective, treatments in MS patients.

Pulmonary complications are reported in some MS patients, caused by pulmonary muscle dysfunction and destruction of cranial nerves (304). We didn’t find any pulmonary complications in our patients and all enrolled patients had normal lung function.

Increased level of inflammatory cytokines such as IFNγ and IL-17 in CNS and blood from MS patients has been well documented (305-307). A dominant response of either Th17 or Th1 type has been reported to be associated with differences in preferential involvement of brain or spinal cord respectively and disease phenotype (166, 308). Our findings regarding BAL T cell cytokine production demonstrated that IL-17 production in BAL CD4+ T cells declines in MS patients regardless of smoking status and this effect is probably related to the treatment. The suppression of IL-17 production by IFNβ has been reported before and it has been suggested that this effect is exerted via induction of IL-27 secretion by dendritic cells (309, 310).

Sweeney et al. reported a correlation between reduced frequency of Tregs and severity of clinical symptoms of MS patients (310). Moreover, there are some studies that show defective suppressive function of Tregs in MS patients (43). Treatment with IFNβ leads to up-regulation of GITRL on dendritic cells and down-regulation of CTLA-4 on Tregs. CTLA-
4 induces negative signal into T cells to inhibit T cell proliferation, while GITR delivers costimulatory signals to induce T cell proliferation. Thus up-regulation of GITRL on dendritic cells and down-regulation of CTLA-4 on Foxp3+ T cells could provide a signal to motivate proliferation of regulatory T cells. It has been shown that there is an increased frequency of Foxp3+ Tregs and a partial improvement of Treg suppressive function in MS patients treated with IFNβ (311, 312). In accordance with other studies, we detected no significant difference in the frequency of Foxp3+ CD4+ T cells in BAL and blood of MS patients compared to controls (313). However, IFNβ treated patients have the highest frequency of Foxp3+ cells among our patients. We believe that the similarity in frequency of Foxp3+ T cells between patients and controls is likely related to the effect of IFNβ treatment. We propose that an increased frequency of BAL Foxp3+ T cells in both natalizumab and IFNβ treated patients and decrease in IFNγ and IL-17 producing cells in BAL of treated patients might indicate that treatment with IFNβ or natalizumab restored the frequency and function of Foxp3 regulatory T cells in BAL of patients, but the reason for this effect to occur in the lung and not in peripheral blood remains to be addressed.

Foxp3 instability and Treg plasticity is another reason for impaired function of Tregs. Komatsu et al. demonstrated conversion of Foxp3+ Tregs into Th17 cells leading to increased numbers of pathogenic Th17 cells in arthritis and IL-17 producing exFoxp3 cells have pathogenic function with higher affinity to self-antigens (314). Dominguez et al. reported higher frequency of IFNγ producing Foxp3+ T cells, i.e. Th1-like Tregs, in untreated MS patients. These Th1-like Tregs are regulatory T cells affected by functional plasticity with reduced suppressive function (315) and after treatment with β-interferon, their frequency were normalized in MS patients. In accordance with findings of Dominguez, we did not detect any differences in BAL Th1-like Tregs between MS patients and healthy controls since the most of enrolled patients were treated. However, we found that these plastic Tregs in BAL were affected by environmental signals since the frequency of Th1-like Tregs declined in both healthy smokers and MS smokers compared to non-smoking individuals.

IL-10 is a regulatory cytokine expressed by numerous cell types. It is a potent anti-inflammatory cytokine, which through its suppressive effects on many cells inhibits various inflammatory pathologies (316). IL-10-producing CD4+CD25+ T cells are present mainly within the intestinal lamina propria and resolution of murine colitis is dependent on the presence and enrichment of IL-10-producing CD4+CD25+ T cells in the intestine (317). Ranatunga et al. described that colitis resistance is associated with accumulation of IL-10 producing Foxp3+ T cells within the lamina propria. They also reported that IL-10 producing cells control expression of inflammatory cytokines and reduce the accumulation of pathogenic Th17 cells in the gastrointestinal tract (318). IL-10 has also been found to be of importance for immune regulation at the epithelial surfaces in the lungs. It has been reported that lung CD4+CD25+Foxp3+ T cells from naive mice can reduce airway hyper-responsiveness by a mechanism dependent on the induction of both IL-10 and TGF-β production (319, 320). Our findings demonstrated a down-regulation of IL-10 production by Foxp3+ Tregs in BAL of both healthy smokers and MS patients (regardless of smoking status). Thus, smoking-induced impairment of Treg function may be one factor that promotes autoimmunity, but several other mechanisms likely can contribute to such immune dysregulation. It remains to elucidate the exact role of IL-10 producing CD4+ Foxp3+ regulatory T cells in MS patients.
Regulatory T cells play a critical role in immune homeostasis and Foxp3 is considered as a master transcription factor in the development of Tregs. Tregs can be categorized into two main subgroups: natural regulatory T cells (tTreg) and adaptive regulatory T cells (pTreg) (321). tTregs that develop in the thymus and their main responsibility is to prevent autoimmunity in response to self-antigens, whereas pTregs are generated in the periphery in response to antigen stimulation and their function is to control and suppress T cell activity against foreign or neo-antigens (322). Helios is another transcription factor that plays an important role in the function of regulatory T cells. Raffin et al demonstrated that Helios-Tregs are potent regulatory T cells with higher suppressive capacity (299). They suggested Helios can be a marker of tTregs, although the validity of this is controversial, and alternatively Helios has been considered as an activation marker. They have shown that Foxp3+/Helios- Tregs have capacity to secrete IL-17 and IL-10. Moreover, these cells lose suppressive function under specific circumstances and switch to a pro-inflammatory phenotype. Our study demonstrated an increase in the frequency of Foxp3+Helios- Tregs and a corresponding decrease in Foxp3+Helios+ Tregs in BAL of MS patients. Thus we could detect in the BAL of MS patients a down-regulation of a Treg subset which is associated with control on self-reactive T cells and with a stable suppressive phenotype. Moreover we found an increase in the frequency of Foxp3+Helios+ Tregs in IFNβ treated patients that might be one of the main benefits of IFNβ treatment.

Finally, the balance between Treg and Th17 cell subsets plays an important role in the development of autoimmune and inflammatory diseases (323). A high frequency of IL-17 producing CD4+ T cells (307) and low frequency of Foxp3+ T cells have been reported in MS patients with active disease (324). In contrast, we found an increase in the ratio of Foxp3/IL-17 cells in both BAL and blood of non-smoking MS patients compared to healthy controls and this ratio was elevated in patients who treated with β-interferon. The Foxp3/Th17 ratio was increased in patients treated with β-interferon, mainly because the treatment caused an increased frequency of Foxp3+ T cells. However, we believe that the notion of a Treg/Th17 imbalance may be too simplistic to explain important features of the immunopathogenesis of MS, and the characteristics of Th17 and Treg cells need to be considered separately.
5 CONCLUSIONS

- There was a higher frequency of mKatG reactive IFNg producing CD4+ T cells within the AV2S3+ subset compared to AV2S3- cells of both BAL and blood from HLA-DR3+ sarcoidosis patients with Löfgren’s syndrome and a lung accumulation of TCR AV2S3+ CD4+ T cells.

- Sarcoidosis patients with Löfgren’s syndrome had higher proportions of multifunctional cytokine producing T cells (IFNg /TNF) in response to mKatG compared to patients without Löfgren’s syndrome.

- Patients with Löfgren’s syndrome had a significantly higher frequency of IL-17-producing cells in BAL following mKatG stimulation, compared to patients without Löfgren’s syndrome.

- HLA-DR3+ sarcoidosis patients with Löfgren’s syndrome had a significantly higher concentration of IL-17 in BAL fluid compared to healthy controls and patients without Löfgren’s syndrome.

- BAL CD4+ T cells in healthy smokers with normal lung function have significantly lower frequencies of IL-17 producing cells compared to healthy never smokers.

- IFNg/TNF double-producing BAL CD4+ T cells had a significantly higher per cell content (MFI value) of both IFNg and TNF compared to cells that produced either of the cytokines alone. This was observed in both sarcoidosis patients and healthy individuals.

- IL-10 expression was reduced in BAL CD4+ Foxp3+ T regulatory cells from smokers with normal lung function compared to healthy never smokers.

- The fraction of Foxp3+/Helios- Tregs was higher in healthy smokers compared to never smokers and IFNg and IL-10 cytokine production in both smokers and non-smokers was mainly confined to Foxp3+/Helios- Tregs subset.

- There was a significantly lower ratio of IL-17+ to IL-10+ cells in BAL CD4+ T cells in healthy smokers compared to never smokers.

- The cytokine pattern of MS patients indicated a sharp down-regulation of IL-17 expression by BAL CD4+ T cells in MS patients who received β-interferon or natalizumab compared to patients who didn’t received any treatment.

- MS patients who were treated with β-interferon or natalizumab had a tendency toward higher percentages of Foxp3+ CD4+ T cells in BAL compared to untreated patients. MS non-smokers and MS smokers had significantly fewer IL-10 expressing Foxp3+ cells compared to healthy non-smokers.

- The proportion of Foxp3+ BAL CD4+ cells that are Helios+ declined in MS patients, while patients who received β-interferon had a tendency towards higher relative frequency of Foxp3+ Helios+ cells in the BAL CD4+ T cell compartment.
6 CONCLUDING REMARKS AND PERSPECTIVES

The observed heterogeneous responses to mKatG in sarcoidosis patients strengthen the hypothesis that mKatG (and other mycobacterial antigens) are pathogenic antigens in a large group of patients, and that the quality of the T cell responses against such antigens may determine disease outcome. An important question for future studies to address will be to find out if there is a way to enhance multifunctional T cell or IL-17 responses, and if so is it beneficial at least in a subgroup of sarcoidosis patients? Finding dominant peptide epitopes of mKatG in the context of different HLA types is another important task that should shed light on the precise pathogenic mechanisms. In the future, antigen-specific therapy with T cell epitopes may be possible for patients with non-resolving disease.

If the lungs are involved in initiation and also propagation of the inflammatory process in MS, alterations of BAL Treg phenotype may be of relevance for the pathogenesis. The observed effects of treatment on frequency and function of lung Treg cells, and the Treg/Th17 ratio in IFNβ-treated patients, may be parts of the mode of action whereby this treatment leads to disease amelioration in MS patients. Important questions that remain to answer are: what are the functional consequences of the elevated frequency of Foxp3+/Helios+ Tregs in IFNβ-treated MS patients? What are the relative roles of different Treg subsets in the suppression of auto-reactive T cells? How much similarity is there between phenotype and function of the lung and CNS T cells and effect of treatment on Treg phenotype in different organs considering that the CNS is the site of auto-aggressive attack? What is the role of the lung in the induction versus the regulation of autoimmunity, not only in MS but also in other autoimmune disorders such as RA? Further studies of lung T cells in MS should help to better understand the immunopathogenesis of this disease, and may suggest new approaches to control the inflammatory process.
I would like to express my sincere gratitude to all people who have supported and helped me to make this study possible. In particular I would like to thank:

My main supervisor Jan Wahlström. Thank you for your trust and provide me this opportunity to work under your supervision. All your encouragement, continuous support, memorable discussions, constructive criticisms, optimism attitude and particularly excellent knowledge in immunology are admirable.

My co-supervisor Johan Grunewald. Thank you to give me this opportunity to work in pulmonary division lab and provide outstanding research environment. Your brilliant knowledge in lung immunology, excellent leadership, realistic attitude and supervision are extra ordinary.

My unofficial co-supervisor Anders Eklund, Thank you for all your support, valuable knowledge in clinical perspectives and helpful comments.

My former supervisor Mahmood Jeddi-Tehrani. Thank you to trust me, taught me the basic of scientific perspectives, leading me to research and all your elegant advices. Your excellent knowledge in molecular immunology, great attitude and pleasant personality are all exemplary.

Hodjattallah Rabbani, You gave me a lot of impression, scientific ambition and taught me how to solve the scientific problems. Thank you for your support during the first days that I came to Sweden. Mohammad Mehdi Akhondi, Thank you to provide such a unique scientific environment during my past studies which encourage me to follow the research. Farah Idali for introducing me to the sarcoidosis world for the first time.

Anders Linden and David Muller, for your wonderful scientific discussions and all the encouragement in IL-17 and mKatG respectively.

Pejman Soroosh, Omid Akbari, Amir Hasan Zarnani and Mohammad Abolhassani for all your inspirational discussions which made me interested in science. You are wonderful advisors.

My great co-authors Caroline Olgart Höglund for being so gentle and all helpful consult to run IHC; Maria Wikén for nice team work and ICS set up, Malin Müller for being so nice and helpful. Your advices to select the right fluorophore and idea to add Helios to my panel were fantastic; Johan Öckinger for your great scientific consults; Susanna Kullberg for all your wonderful clinical advices; Fredrik Piehl for providing patient information with a short notice; Matthew Willett, Edward Chen, Pernilla Glader and Tomas Olsson for all comments and discussions.

All the present and past members of the Lung Research Lab: Benita D you are the best lab manager and trip companion; Benita E for your accurate lab works and great tips for living in Sweden. Presence both of you made our work in the lab much easier. All my office-mates, Tove for being so positive and svenska translation, Natalia you are completely new in the lab but I feel I know you since a long time ago, Tina for always being supportive and Ernesto for our great conversations. Muntasir for being so helpful, kind and generous, all our conversations were pleasant; Helena for all your support, friendship and kindness; Kerstin your positive personality was wonderful [all your shoes look great :-)]; Ylva you are the most scientific travel guide in the world [my majesty ;-]); Michael for good team
work and collaboration; **Pernilla** you are the first person taught me BAL preparation; **Marcus** for your positive attitude; **Mantas** and **Maria A** both of you have given me lots of energy, working with you are so pleasant; **Magnus S** for pleasant scientific personality; **Åsa** for your support, **Ming-xing, Heta, Chuan-xing, Tobias, Daniel, Louis, Yvonne** and all the former members of the Lung Research Lab **Abraham, Bettina, Charlotte, Micke, Kie, Karin** and **Maxie** thank you all for making the Lung Research Lab a nice place to work in.

Wonderful research nurses **Helen Blomqvist, Gunnel de Forest** and **Magitha Dahl** for your kindness and all your support to provide the main material of research.

**Eva-Marie Karlsson** and **Lillemor Melander** for your perfect administrative support; **Magnus Mossfeldt** for your wonderful support in the virtual world.

**Heidi Wähämaa, Eva Gelius** (in Mabtech) for all your help and support for setting up and reading the Elispot plates. **Annika van Vollenhoven, Birgitta Wester** and **Eva Lindroos** for helping out with cell sorting and fluorescence microscopy. **Konrad, Maria Jose, Ludvig** and **Emma** from Translational Immunolology for being nice every time I see you.

**Ali Zirakzadeh**, for all your support and priceless friendship. Our intriguing scientific and particularly non-scientific discussions are unforgettable to me. Thank you for all your help and being such an amazing friend.

**Behnaz Babaei, Amin Nasri, Tohid Kazemi, Mostafa Ghaffari, Elham Tabee, Mohammad Hojat Farsangi** and **Maryam Nikmaram** for friendship, support and having a wonderful time together in Sweden.

My dear friends in ARI, **Saeed Talebi, Roya Ghods, Pouneh Dokouhaki, Ahmad Reza Mahmoudi, Ebrahim Mirzadegan, Jamile Ghasemi, Jafar Mahmoudian** and **Reza Hadavi** and my close friends **Bahman, Farhad and Shahin**, your valuable friendship was always like a box of treasure for me. We have had a lot of fun together. Thank you for being such great friends.

My close relatives (**aunts and uncles**), particularly my cousins **Babak, Soheil, Behzad, Omid** and **Behnam**, you are amazing. All the fun, enjoyable time and wonderful chats we have had are memorable.

My father and mother in-law **Fariborz and Mehrafarid** for believing in me and for all your support and encouragements. Your persistence and your phone calls are always encouraging for us. Borzou and Behnoush thank you for nice company and good memories.

**Mitra** you are a fantastic sister with amazing source of support and kindness. **Mehrdad** you are an amazing brother, always having a lot of ambition to make me progress in my life. I am always proud to have both of your support. **Farzaneh** for all the good times and your well wishes. My lovely niece and nephew **Jasmin and Sam** (you are always in my mind).

**My mother** and my **father** (RIP) for your unconditional love, support, encouragement and for your prayers and well wishes. I am so proud to be your son. You have given me everything that has brought me to where I am today.

Finally, last but not least my wife, **Behnaz** and my little princess, **Baran**. Thank you for everything. Especially your endless love, being such a wonderful life companions with such great patience. You have brought joy into my life. Your encouragements, understandings and support made this step of my life much easier. I love you both.
This work was financially supported by the Swedish Heart Lung Foundation, the Swedish Research Council, through the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet, Novartis collaborative grant, Torsten and Ragnar Söderberg’s Foundation, The King Oscar II Jubilee Foundation, Karolinska Institutet and the Mats Kleberg Foundation.
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