IMMUNE RESPONSES IN CLINICALLY AND GENETICALLY DEFINED SUBGROUPS OF PATIENTS WITH PULMONARY SARCOIDOSIS

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No matter how much you know, there is still much to know

To my family
ABSTRACT

This thesis focuses on inflammatory responses in the airways of sarcoidosis patients. Sarcoidosis is a T helper 1-mediated inflammatory disease with unknown aetiology. HLA-DRB1*0301<sup>pos</sup> sarcoidosis patients often present with an acute form of disease, i.e. Löfgren’s syndrome, usually with a very good prognosis. These patients also have expansions of CD4<sup>+</sup> T cells expressing the T cell receptor AV2S3 gene segment in their lungs. The overall aim of these studies was to investigate the inflammatory and immune regulatory responses in sarcoidosis and in specific subgroups of patients, compared to healthy controls.

The cytokine profile in bronchoalveolar lavage fluid (BALF) of patients revealed that the levels of mRNA and protein expression of pro-inflammatory and Th1 associated cytokines were in general increased in sarcoidosis patients compared to healthy individuals. In particular, HLA-DRB1*0301<sup>neg</sup> patients expressed significantly increased levels of pro-inflammatory and Th1 associated cytokines in their lungs as compared to HLA-DRB1*0301<sup>pos</sup> patients and controls. A tendency to a higher expression of TGF-β<sub>1</sub> was seen in DRB1*0301<sup>pos</sup> patients.

The study of BALF CD4<sup>+</sup> T cells in patients revealed decreased mRNA levels of the T regulatory cell-specific transcription factor, FOXP3, and of regulatory associated genes IL-10 and CCR2. Furthermore, at the protein level reduced frequencies of FOXP3-expressing BALF and blood CD4<sup>+</sup> T cells were observed in patients. The mean fluorescence intensity of FOXP3 expression in BALF FOXP3<sup>+</sup> CD4<sup>+</sup> cells of patients was also reduced. AV2S3<sup>+</sup> CD4<sup>+</sup> T cells expressed significantly reduced levels of FOXP3 and CCR2 compared to the other BALF CD4<sup>+</sup> T cells. We did not find any differences in the expression of CCR2, FOXP3, IL-10 and TGF-β1 between patient subgroups.

Sarcoidosis patients expressed decreased levels of T-cell immunoglobulin and mucin domain (TIM)-3 mRNA in their BALF CD4<sup>+</sup> T cells, as compared to healthy subjects, while IL-2 expression was increased in patients. TIM molecules have been suggested to be important regulators of immune functions. In addition, our data revealed an increased mRNA level of IFN-γ in non-Löfgren’s patients as compared to Löfgren’s patients, while the mRNA level of TIM-1 was decreased.

Analyzing alveolar macrophages, we detected a significantly lower expression of TLR2 in patients, in particular patients with Löfgren’s syndrome. We also observed that the gene expression of fibrosis-associated CCL18 was higher in patients compared to controls. There was a tendency to higher IL-23 levels in cultured BALF cells of patients, but upon LPS-stimulation it was markedly more upregulated in healthy controls.

In conclusion, the reduced immune regulatory response in the lungs of sarcoidosis patients may result in an uncontrolled inflammation particularly in non-Löfgren’s patients, contributing to the pathogenesis of this disease. AV2S3<sup>+</sup> T cells in the lungs of Löfgren’s patients seem to have an effector function and may contribute to the eradication of a postulated sarcoidosis antigen.

Key words: sarcoidosis, Löfgren’s syndrome, cytokine, regulatory T cell, TIM molecule, TLR
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AM</td>
<td>Alveolar macrophage</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<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>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BHL</td>
<td>Bilateral hilar lymphadenopathy</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen-4</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>EN</td>
<td>Erythema nodosum</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead box protein 3</td>
</tr>
<tr>
<td>Gal-9</td>
<td>Galectin-9</td>
</tr>
<tr>
<td>GITR</td>
<td>Glucocorticoid-induced tumor necrosis factor receptor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILD</td>
<td>Interstitial lung disease</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Monocyte inflammatory protein</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain receptor</td>
</tr>
<tr>
<td>nTreg</td>
<td>Natural regulatory T cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>TBL</td>
<td>Transbronchial lung biopsy</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th cell</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TIM</td>
<td>T cell immunoglobulin and mucin domain</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Tr1</td>
<td>Regulatory T cell type 1</td>
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1 INTRODUCTION

Inflammation is a defensive mechanism by the organism in order to remove invading pathogens as well as to make the first move of the healing process for the tissue. An acute inflammation presents a short-lived process with complete resolution, while a chronic inflammation is defined as a long-lasting phenomenon associated with tissue damage. The current thesis focuses on the difference in inflammatory response in the airways of clinically and genetically distinct subgroups of patients with pulmonary sarcoidosis.

1.1 THE IMMUNE SYSTEM

The immune system works throughout the body. It is, however, made of many cell types depending on each other that protect the body from bacterial, parasitic, fungal, viral infections and from growth of tumour cells. The job is done by two different parts of the immune system, innate and adaptive immunity. These two parts are not isolated and they are constantly interacting with each other. The less specific part, innate immunity provides the first line of defence against the pathogen. The specific part, adaptive immunity, takes over when antigen-specific lymphocytes have had time to proliferate.

1.1.1 Innate immunity

Innate immunity is activated very quickly after infection. Anatomic and physical barriers like skin and body temperature play an important role by inhibiting the entry and growth of microorganisms. Body secretions, such as stomach acid, bile salts, tears and urine, also contain proteins and peptides that inhibit or destroy invading microorganisms. When these nonspecific barriers are broken, the cells of the immune system begin with a series of responses, resulting in an inflammatory response. The characteristics of inflammation are elevated temperature, redness, swelling and pain, because of vasodilatation and increased permeability. Cells of the innate immune system are phagocytic cells (neutrophils, monocytes and macrophages), natural killer (NK) cells and cells that release inflammatory mediators (basophils, eosinophils and mast cells). In addition, dendritic cells (DCs) are also a part of innate immunity; they are specialized to take up antigen and present it for recognition by T lymphocytes.

The way that the innate immune system recognizes “foreign” is depending on recognition of common structures of microorganisms and in that respect the role of pattern recognition receptors (PRRs) is now well studied. The discrimination between “self” and “nonself” by PRRs through detection of pathogen molecules is a key mechanism of the immune system response.

1.1.1.1 Pattern recognition receptors

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs) and were first identified in the fruit fly, Drosophila, as type I transmembrane proteins. By now, about 15 mammalian TLRs have been described. For example, TLR4 rec-
ognizes lipopolysaccharide (LPS), a common constituent of the cell wall of gram-negative bacteria and TLR2 recognizes e.g. peptidoglycan, a common constituent of gram-positive cell walls, both of which are critical for the innate immune response against bacterial invasion. Other receptors like TLR3, TLR7 and TLR8 recognize nucleic acids produced during viral infection (5, 6). TLR9 recognizes unmethylated CPG-containing bacterial DNA (6). Unlike TLR2, 4, 5 and 6, which are expressed on the cell surface, TLR3, 7, 8 and 9 reside in endosomal compartments (7).

Nucleotide-binding oligomerization domain receptors (NODs) are other pattern recognition receptors that are expressed by antigen presenting cells. NODs are a family of intracellular receptors and recognize breakdown products of bacteria (3). Ligand recognition by TLRs and NODs activates DCs and other antigen presenting cells to secrete proinflammatory cytokines and, through stimulation of DCs, influence the differentiation of CD4+ T cells and in that way the adaptive immune system (3, 8). The adaptive immunity gives a well-organized immune response through the use of specificity and long-term protective immunity.

1.1.2 Adaptive immunity

When the pathogen breaks through the innate immune defence barriers, the adaptive immune system will take over. Adaptive immunity or antigen specific defence mechanisms are developed after several days. It has two key features; specificity and memory. The specificity describes that the adaptive immune system can differentiate between antigens and is capable of recognizing different structures on foreign antigen. When a naive lymphocyte has been activated, it goes through clonal expansion and differentiation into an effector cell. Once antigen is removed, most of the antigen specific cells undergo apoptosis. However, some persist even after elimination of antigen and guarantees a more rapid and effective response on the next encounter with the antigen. This characteristic is called memory (1). The cells which are responsible for these skills are B and T lymphocytes.

1.1.3 Antigen presentation

In adaptive immunity, antigens are recognized by two different sets of receptor molecules, either by BCRs and antibodies or by TCRs. Unlike antibodies and BCRs that recognize and bind to the intact antigen, TCRs recognize only antigenic peptides bound to the major histocompatibility complex (MHC) molecules. MHC is a set of glycoprotein molecules that present the processed antigens for T lymphocyte recognition. In humans, MHC is called human leukocyte antigen (HLA) and is located on chromosome 6, containing more than 200 genes. There are three main class I genes encoding for the α-chains of HLA-A, -B and -C. The HLA class II region includes three pairs of genes for α and β chains of HLA-DR, -DP and -DQ.

The MHC class I molecule is a heterodimer, consisting of two polypeptide chains; the α-chain and β2-microglobulin. These two chains are bound to each other noncovalently, but only the α-chain spans the membrane and forms with two of three domains the peptide-binding cleft. MHC class I molecules are expressed on all nucleated cells and
present small newly synthesized peptides, usually 8-10 amino acids long, from the endoplasmic reticulum (ER) or cytosol. The MHC-peptide binding happens in the ER and then the complex leaves ER and through the Golgi apparatus is transported to the cell surface. At the cell surface the complex of MHC-class I and peptide is presented to CD8\(^+\) cytotoxic T cells.

The MHC class II molecule consists of an \(\alpha\) chain and a \(\beta\) chain that are noncovalently bound to each other; both span the membrane and form the peptide-binding cleft. MHC class II molecules are expressed by antigen presenting cells (APCs) i.e. DCs, macrophages and B cells and thymic stromal cells and present peptides usually longer than 13 amino acids. These cells express constitutively MHC class II molecules but MHC class II molecules can also be induced on many cell types by cytokines, especially interferon (IFN)-\(\gamma\). MHC class II molecules are not able to bind peptides in the ER. The binding of invariant chain to the groove of newly synthesized MHC class II molecule blocks the binding of peptides before this molecule arrives to the acidified endocytotic vesicles. In endosomes, the invariant chain is released and allows the antigenic peptides to bind. The MHC class II molecule then travels to the cell surface and presents the peptide in the form of a MHC II: peptide complex to CD4\(^+\) T helper cells (1).

MHC molecules are highly polymorphic in humans and the sites of major polymorphism are located in the peptide-binding cleft, which determines antigen recognition by T cells. The interaction of T cells with MHC molecules and the antigenic peptides is the beginning of a series of immune responses.

### 1.2 CELLS OF THE IMMUNE SYSTEM

#### 1.2.1 Dendritic cells

Dendritic cells (DC) are central cells of the immune system, connecting the innate and adaptive immunity. The immature DCs carry receptors including Toll-like receptors (TLRs), which recognize common structures of many pathogens (9). They reside in the periphery where they are immature cells and take up antigens. Upon activation, they mature and travel to neighbouring lymphnodes, where they present the antigen to T cells. Activated DCs secrete cytokines that influence both innate and adaptive immune responses.

#### 1.2.2 Mononuclear phagocytes (monocytes/macrophages)

Monocytes and macrophages are the basic cell types of the mononuclear phagocytes. Macrophages are the mature form of monocytes, which circulate in the blood and differentiate into macrophages upon migration from the blood to the tissues. Macrophages are distributed generally in the body tissues including macrophages in connective tissue (histiocytes), liver (Kupffer's cells) and lung (alveolar macrophages); therefore they can provide an immediate defence against foreign antigens before leukocyte immigration (1). Macrophages play an important role in inflammatory processes e.g. through the expression of chemokines which induces chemotaxis of distinct
cell types. In response to cytokines and microbial products, macrophages differ in receptor expression and effector function, as many refer to polarized macrophages, M1 and M2 cells. Classically activated M1 macrophages are induced by IFN-γ, TNF-α and microbial stimuli (e.g., LPS). In contrast, IL-4 and IL-13 induce an alternative M2 form of macrophage activation (10).

1.2.3 Lymphocytes

1.2.3.1 Natural killer (NK) cells

NK cells make up a major population of lymphocytes and are designed to kill certain abnormal cells like tumour cells and virus-infected cells without antigen presentation or MHC restriction (11). In humans, NK cells are identified by the absence of CD3 and surface expression of CD56 and CD16. NK cells differ from other lymphocytes, by lacking antigen specific receptors, but can kill their target by different mechanisms (12). They can use the perforin/granzyme-containing granule exocytosis pathway or the death receptor ligand pathway. Mature NK cells produce effector molecules like IFN-γ, which acts against tumors. NK cells also mediate antibody-dependent cell-mediated cytotoxicity (ADCC) by expressing Fc receptors for IgG. When the antibody binds to the antigen, the Fc portion of the antibody binds to Fcγ receptors on NK cells, leading to NK cell activation and destruction of target cells. Normally, NK cells are prevented from killing host cells through expression of inhibitory receptors, which are specific for MHC class I alleles. The expression of MHC class I on cells may be reduced upon viral infection or tumour transformation. Then, NK cells can recognize and kill cells that lack or express low levels of MHC class I (13).

1.2.3.2 B cells

The humoral immune response protects the extracellular spaces of our body, where antibodies produced by B cells cause the destruction of extracellular microorganisms and inhibit the spreading of intracellular infections. The surface antibody that serves as the B-cell antigen receptor (BCR) has two roles in B-cell activation. First, when BCR binds antigen, it transmits signals directly to the cell. Then the BCR delivers the antigen to intracellular sites, processes and presents it to the T cells as peptide bound to MHC class II molecules. The first expressed BCR is a membrane bound form of IgM antibody, but after activation, B cells go through immunoglobulin (Ig) class switching to a more effector specialized one, such as IgG (important neutralizing antibody), IgA (important for mucosal defence) or IgE (important in allergic response). B cells also differentiate into plasma cells, which will continue to secrete lots of Igs. The antibodies can destroy microorganisms through neutralization, opsonisation or activation of the complement system.

1.2.3.3 T cells

T cells like B cells originate in bone marrow, but their development is completed in the thymus. The first event in the thymus that T cells go through is the T cell receptor gene rearrangement. Each receptor is a heterodimer, which is consisting of two different polypeptide chains, α and β. Both TCR chains are made of one variable (V) and one
constant (C) region. The variable region forms the site for antigen recognition, which is created through rearrangement of V and J (joining) gene segments in TCRα, and V, J and D (diversity) segments in TCRβ. T cells are programmed to rearrange first the TCRβ and then the rearrangement process proceeds with TCRα. At this stage, T cells express both CD4 and CD8 molecules and are therefore called “double positive”. Upon expression of mature TCRs, the double positive T cells go through two selective steps. The first step is the positive selection of T cells that have a moderate affinity to MHC molecules. In the next step, i.e. the negative selection, those T cell clones that have high affinity for self antigens are deleted through apoptosis. Of all double positive T cells, only 2% survive the positive and negative selections. Each T cell carries around 3000 identical antigen receptor molecules on its surface (14). T cells with γδ TCRs are a minor population of peripheral T cells, which do not need antigen processing by APCs and presentation by MHC molecules to become activated. There are two major types of α:β T cells; CD4+ T helper (Th) cells CD8+ cytotoxic T cells (1).

1.2.3.3.1 CD4+ T helper cells

Our body can be protected from infectious disease if the correct set of immune responses is induced. CD4+ T cells play an important role in eliminating microbes by producing cytokines, which can regulate the immune response of other cells, like antibody production by B cells and phagocytosis by macrophages (15, 16).

Those CD4+ T cells that have passed selection in the thymus and travelled to the secondary lymphoid organs are called naïve T cells, which is before they meet the peptide-MHC II ligand. These cells enter the lymph node; interact with DCs, and search for the relevant antigen-MHC II ligand. DCs can either pick up an antigen in the lymph node
or have picked up the antigen at the site of antigen exposure. Those CD4⁺ T cells that encounter the antigen go through clonal expansion and leave the lymph node into the circulation, and travel to the original site of antigen exposure. In inflamed tissue, antigen specific T cells encounter the antigen in the context of MHCII molecules e.g. on macrophages, resulting in the persistence of T cells in the inflamed tissue (Fig. 1) (17).

The activated CD4⁺ T cells differentiate into two distinct populations defined by the cytokines they produce, Th1 or Th2 types (18). Th1 cells have an important role in the cellular immunity, mainly against intracellular pathogens, whereas Th2 cells induce humoral immunity, against extracellular pathogens such as parasites. IL-12 production by DCs promotes T cells to differentiate into the Th1 type. Th1 cells produce IFN-γ and thereby activate macrophages. In the absence of IL-12, naive T cells produce IL-4, promoting Th2 differentiation. Th2 cells produce IL-4, IL-5 and IL-13, activating mast cells and eosinophils and inducing B cells to produce IgE antibodies (1). Th2 cells have important roles in inducing immune response in allergic reactions.

### 1.2.3.3.1.1 TH-17 CELLS

Recently, another CD4⁺ T cell subpopulation was described, which is Th17 cells (19). These cells produce IL-17, a cytokine that induces the production of chemokines and antimicrobial peptides by tissue cells, leading to the recruitment of neutrophils and inflammation (20). In addition to defence mechanisms against certain extracellular bacteria, Th17 cells have been linked to the pathogenesis of several inflammatory and autoimmune diseases (19), such as systemic lupus erythematosus and collagen induced arthritis (21, 22). IL-23 is shown to be a requirement for IL-17 mediated effector function (23) but not for differentiation of Th17 cells (24). Studies in mice indicated that the differentiation of Th17 cells requires TGF-β and IL-6 (24-26), while in humans, Th17 polarization was induced by IL-1β and IL-6, but not TGF-β (27).

### 1.2.3.3.1.2 REGULATORY T CELLS

The majority of self-reactive T cells are destroyed in the thymus to prevent autoimmune reactions in the periphery. However, some of them may leave the thymus, but there are some mechanisms to regulate these cells in the periphery. Regulatory T cells mediate peripheral tolerance and control the adaptive immune responses that may cause damage to self tissues (28).

Natural regulatory T cells (nTreg) develop during the normal process of maturation in the thymus and express TCRs which are specific for self antigens (29, 30). These cells represent 5-10% of CD4⁺ T cells in healthy individuals (31), proliferate poorly to antigenic stimulation and do not express the key cytokines, such as IL-2 and interferon gamma (IFN-γ) in response to antigen. nTregs are identified through the surface expression of CD4, high level of interleukin (IL)-2 receptor (CD25), glucocorticoid-induced tumor necrosis factor receptor (GITR) (32) and cytotoxic T lymphocyte antigen-4 (CTLA-4) (33). A recent investigation has shown that co-expression of CD25 and CD27 could identify nTregs (34), while others suggested that the expression of CD103 (35) or the low expression of CD127 (IL-7R) on CD4⁺CD25⁺ cells may allow identification of nTreg cells (36, 37). However, the identification of transcription factor
forkhead boxp3 (FOXP3) as a Treg specific factor finally provided a useful marker for the study of Treg in mice and humans (38-40). FOXP3 is exclusively found in CD4+CD25+ cells and correlates with the suppressive activity of these cells. In humans, a mutation in FOXP3 results in the disease IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked) which is a rare and severe autoimmune disorder (41, 42).

Four basic mechanisms of Treg cell function have been suggested; suppression by the inhibitory cytokines IL-10, IL-35 and TGF-β (43-46), suppression by cytolysis through secretion of granzymes (47), suppression through the high expression of CD25 which allows Tregs to use IL-2 so that the dividing effector T cells starve (48, 49), and suppression through inducing DCs to produce immuno-regulatory molecules like indoleamine 2,3-dioxygenase (IDO) (Fig. 2) (50, 51).

Other T cells with regulatory functions are those called inducible regulatory T cells which are induced in response to infectious challenge in the periphery, and they may or may not express FOXP3 (53). These cells include T helper 3 (Th3) cells, which originate from conventional CD4+ T cells, and regulatory T cells type 1 (Tr1 cells), derived from CD4+ precursor cells (54-56). Th3 cells are induced by oral antigen and mediate their suppressive effect by secretion of transforming growth factor (TGF)-β. Tr1 cells can be produced by chronic activation of CD4+ cells in the presence of IL-10; they produce large amounts of IL-10 to prevent development of autoimmune diseases (Fig. 3).
1.2.3.3.2 **CD8+ cytotoxic T cells**

CD8+ cytotoxic T lymphocytes (CTLs) provide defence against virus infection and intracellular pathogens. They kill target cells that present peptide fragments of cytosolic pathogens, bound to MHC class I molecules at their surface. After clonal expansion of activated CTLs, they lyse the target cells by releasing cytotoxic molecules, forming pores in the cell membrane and thereby inducing cell death. Granzymes, perforin and cathepsins are the most commonly found proteins in CTL granules. CTLs also express Fas ligand which can bind Fas on target cells and induce apoptosis. CTLs can also through the expression of cytokines such as IFN-γ, tumor necrosis factor (TNF)-α and TNF-β contribute to host defence against viral infection (1).

1.2.3.4 **Cytokines**

Cytokines are a group of signalling proteins that are used in cellular communication. The cytokine family consists mainly of short-lived, water-soluble proteins and glycoproteins with a low-molecular weight between 8 and 30 kDa. Cytokines count as important mediators in both innate and adaptive immune responses. The secretion of cytokines by immune cells activates and recruits more immune cells and increase the immune response to the pathogen (57). The binding of each cytokine to its receptor leads to intracellular signalling and alteration in cell functions, including the upregulation and/or downregulation of several genes and their transcription factors. This may result in the production of other cytokines and surface receptors for other molecules. Cytokines may have different effects on target cells, or some of them may show similar functions. Over a hundred cytokines have been discovered, which may act locally or systemically.

1.2.3.5 **Th1/Th2 paradigm**

Since the 1980s, the Th1/Th2 dichotomy has been considered the basis of T cell immune responses (18). Initially it was thought that immune responses to infecting organisms could be grouped as either a Th1 or a Th2 response. Then it became clear that in
many cases a balance of these two types of T-cell responses was actually required to eliminate infection, as seen in the murine experimental Leishmaniasis model (58).

CD4+ T-cells have been divided into two subsets (Th1 and Th2) according to the cytokines they produce. Th1 cytokines include IL-2, IFN\(\gamma\) and lymphotoxin-\(\alpha\) (LT-\(\alpha\)), inducing cell-mediated immune responses, which are needed to resolve bacterial, viral and protozoal infections. Th1 cells are associated with inflammation and tissue injury (18). Th2 cytokines include IL-4, IL-5, IL-6, IL-10 and IL-13, induce humoral immunity and antibody responses (18). The Th1 pathway activates macrophages, NK cells, cytotoxic T cells and a prolonged inflammatory response, while the Th2 pathway with production of IL-4 and IL-6 supports B cell growth (59-61).

The factors that play a role in naïve (Th0) CD4\(^+\) cell differentiation include the nature of the antigen and the dose of the antigen. But the most important factor is the cytokine milieu in which antigen presentation occurs. The early presence of IL-12 and IFN-\(\gamma\) favour Th1 differentiation whereas IL-4 induce Th2 development (18, 60, 61).

### 1.2.3.6 Pro- and anti-inflammatory cytokines

Pro-inflammatory cytokines such as TNF-\(\alpha\), IL-1 and IL-6 promote inflammation and tissue destruction, while others termed anti-inflammatory cytokines suppress the activity of pro-inflammatory cytokines (62). TNF-\(\alpha\) is produced mostly by monocytes and macrophages in response to for example lipopolysacharide (LPS, bacterial endotoxin) and other cytokines (IL-1, IL-2, IFN-\(\gamma\)) (63). The expression of TNF-\(\alpha\) could be transcriptionally down-regulated by the anti-inflammatory cytokine IL-10 or corticosteroids (64). TNF-\(\alpha\) can activate vascular endothelium and stimulate the inflammatory cascade through induction of other pro-inflammatory cytokines, such as IL-1 and inflammatory mediators such as prostaglandins (57).

IL-1 is a pro-inflammatory cytokine produced by monocytes and macrophages and induced by bacterial products and other inflammatory stimuli such as complement factors and TNF-\(\alpha\). IL-1 activates vascular endothelium, lymphocytes and macrophages. The IL-1 cytokine family has three members; IL-1\(\alpha\), IL-1\(\beta\) and the specific receptor antagonist IL-1Ra. The other pro-inflammatory cytokine is IL-6, which is produced by different cell types and also promotes B cell growth (57). Recent data indicate that IL-1\(\beta\) an IL-6 could have a role in Th17 cell differentiation in humans (27).

Anti-inflammatory cytokines IL-10 and TGF-\(\beta\) are both secreted by T cells, having an important role in suppressing pro-inflammatory cytokines. IL-10 is also produced by monocytes/macrophages and down-regulates the expression of MHC class II and costimulatory molecules on macrophages. It is reported that TGF-\(\beta\) may execute its inhibitory effect through the induction of IL-1Ra, the natural antagonist of IL-1 (65).

### 1.2.4 T cell immunoglobulin and mucin domain (TIM) family

Molecules of the T cell immunoglobulin-mucin (TIM) family are thought to be involved in the regulation of immune responses (66-69). These molecules are transmembrane cell surface glycoproteins with common structural patterns, including a signal peptide, an IgV domain and a mucin like domain in their extracellular region, as well as
transmembrane and intracellular cytoplasmic domains (Fig. 4). The TIM gene family includes eight genes on mouse chromosome 11B1.1 and three genes on human chromosome 5q33.2. The three human genes TIM-1, TIM-3 and TIM-4 are similar to mouse corresponding genes (68). Unlike TIM-1, 2 and 3, TIM-4 lacks the signalling intracellular tyrosine phosphorylation motif.

Figure 4. The TIM family (adopted from Meyers et al. (69)).

TIM-3 and TIM-1 are differentially expressed by Th1 and Th2 cells, respectively (66, 69). TIM-1 was originally identified on human kidney and liver cells (70) and also described as the hepatitis A virus receptor (71). There is evidence that TIM-1 is expressed at low levels on naïve CD4^+ T cells, CD19^+ B cells and CD11c^+ DCs. Upon activation, the expression of TIM-1 is increased on T cells, but its expression is decreased on Th1 cells during the process of differentiation, while Th2 cells continue to express TIM-1 (72). Genetic polymorphisms in human TIM-1 have been associated with atopy, asthma and rheumatoid arthritis (RA) (73, 74). TIM-4, which is not expressed by T cells but by antigen presenting cells, serves as the natural ligand for TIM-1 (75). Data shows that TIM-1 functions as co-stimulatory molecule for T cell activation and proliferation of CD4^+ T cells (72).
Kuchroo’s laboratory was first to identify TIM-3 expression on differentiated Th1 cells (76). Subsequent functional studies indicated that blockage of the TIM-3 pathway accelerated Th1-mediated autoimmunity and prevented induction of immunological tolerance (76-78). Galectin-9 (gal-9) was identified as the natural ligand for TIM-3, and it is upregulated through IFN-γ induction (79). Its interaction with TIM-3 on Th1 cells causes calcium influx, cell aggregation and cell death (80). In addition, accumulating data indicate that the TIM-3-gal-9 pathway serves to dampen Th1 responses by eliminating effector Th1 cells. Andersson et al. suggested that in diseases such as multiple sclerosis (MS), the inability to up-regulate TIM-3 on Th1 cells (81), might allow Th1 cells to escape galectin-9 induced cell death (Fig. 5) (82).

A reduced expression of TIM-3 was suggested to contribute to inflammation in chronic disease states as it was reported that highly pathogenic Th17 T cells, express lower levels of TIM-3 compared to Th1 cells (83).

In addition to its expression in T cells, further investigations have shown that TIM-3 is constitutively expressed on mouse and human dendritic cells (DCs), and that it can synergize with Toll-like receptors (84). TIM-3 also has been found on cytotoxic CD8⁺T cells, Th17 cells, Tregs, monocytes and mast cells (76, 84-87). Thus, TIM-3 can both promote inflammation and terminate Th1 responses by its differential expression on cells of the innate and adaptive immune system. The importance of the TIM family in regulation of immune function is under intensive investigation.

Figure 5. TIM-3 mediated regulation of Th1 immunity, in healthy and in disease states, such as MS. Adopted from Anderson et al. (82) with minor modifications.
1.3 THE LUNGS

Main functions for the respiratory system are; air transfer, air filtration and gas exchange in the alveoli where oxygen is transferred to the blood and carbon dioxide is removed from the blood. Through the nose, pharynx and larynx the airways reach to the trachea. At the end of trachea, it divides into two stem bronchus that head into the respective lung. Each bronchus is then divided into bronchi, bronchioles and at the end into alveoli.

Figure 6. The human lungs

1.3.1 The lung immunity

The inspiration can supply the body with the oxygen, but at the same time with particles and microorganisms. Anatomical barriers, such as the cough reflex and mucus protect the lungs. Mucus is secreted from epithelial cells and glands and acts as a barrier for bacteria (88). Secretory immunoglobulin A (IgA) is also released by epithelial cells and it neutralises toxins and viruses and blocks the entry of bacteria across the epithelium (89). Upon bacterial stimuli, epithelial cells, through releasing chemokines like IL-8, recruit inflammatory cells to the airways (90). Epithelial cells can also up-regulate adhesion molecules, allowing the adhesion of neutrophils and mononuclear cells to the inflamed area. (91). In addition, DCs which are lying within the membrane in a resting state, matur upon inflammatory stimuli and migrate to the hilar lymph nodes, where they interact with naïve T cells and activate them (92).

1.3.2 Interstitial Lung Diseases

Interstitial lung diseases (ILDs), a heterogeneous group of parenchymal lung disorders (93), can be grouped into those with known and unknown causes. ILDs are differentiated by infiltration of cellular or noncellular material into the lung parenchyma (94).
The major effect of ILDs is disturbed gas exchange. Inhaled organic and inorganic substances, drugs and radiation are some of the known causes of ILDs. Idiopathic pulmonary fibrosis (IPF) and sarcoidosis count as ILDs where the causes are still unknown. Thus, when no cause can be identified for the ILD, a lung biopsy is often taken. The histological finding is then correlated with the clinical data and chest radiography (95).

1.3.3 Bronchoalveolar Lavage (BAL)

The development of flexible bronchoscopy and the usage of bronchoalveolar lavage (BAL) have improved the diagnostic accuracy of diffuse lung diseases. It is safe, minimally invasive, and associated with almost no morbidity. The use of bronchoscopy and BAL has made it possible to study cells from the site of inflammation. BAL is considered helpful in strengthening the diagnosis in patients with sarcoidosis especially in the absence of a positive biopsy (96). The results of BAL cell differentials show a mix of cellular patterns, some times dominated by lymphocytes, neutrophils or eosinophils, which can contribute to diagnosis (97). The BAL fluid also includes soluble components such as immunoglobulins, proteases and antiproteases, angiotensin-converting enzyme, antioxidants, oxidants, lipid mediators and cytokines (98).

1.3.4 Cells of BAL

1.3.4.1 Alveolar macrophages (AMs)

Alveolar macrophages (AMs) through their phagocytic function provide an early defence against pathogens or particles in the lower airways (99). These phagocytic cells can either neutralize pathogens and/or recruit neutrophils and other mononuclear cells to the airways. AMs interact with pathogens through PRRs such as TLRs. The activity of TLRs cause activation of several signalling pathways, leading to release of e.g. IL-12 and Th1 differentiation (6, 100). AMs initiate lung inflammation via secreting pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α, resulting in inflammatory cascades in the alveolar spaces, expression of adhesion molecules on endothelial cells and the release of chemokines. The secretion of cytokines such as IL-8 by macrophages contribute to the alveolitis in the lungs (101). In addition, these cells also have antibacterial activities and through production of lysozymes or defensins can attack microbial agents (102).

1.3.4.2 Lymphocytes

About 10% of BAL cells in healthy individuals are lymphocytes including CD4+ and CD8+ T cells, with a CD4 to CD8 T cell ratio of 1.5-2.0, which is similar to that in blood. Around 10-15% of alveolar lymphocytes are NK cells and 5% are B cells. There are some differences in the phenotype and function of lymphocytes in the alveolar spaces, compared to those in the peripheral blood. For example, most T cells have an increased expression of activation markers and a memory phenotype in the alveoli (103).
1.3.4.3 Neutrophils

In healthy non-smoking individuals, neutrophils represent less than 2% of the cells in the BAL fluid. Upon bacterial infection, when AMs are unable to control infectious agents, neutrophils are recruited to the lungs, which are especially important in the acute onset of infection (104). AMs produce mediators like leukotriene B4, and chemokines like IL-8, to recruit neutrophils. Once the neutrophils are activated, they eliminate bacterial agents by several different mechanisms, which involves phagocytosis, release of oxygen radicals as well as production of anti-bacterial peptides, such as defensins (1).

1.4 SARCOIDOSIS

Caesar Boeck, the Norwegian dermatologist, described in the late 19th century a case with cutaneous lesions as “multiple benign sarcoid of the skin”, which consisted of accumulated epithelioid and giant cells. He used the term sarcoid because he thought the lesions were similar to sarcoma. He also described sarcoidosis as “a bacillary infectious disease”. Subsequently, similar changes were described in the lungs and other organs. In the 1940s the Norwegian Ansgar Kveim discovered the skin test, known as Kveim-Siltzbach test, after further development by Louis Siltzbach. This test was based on intradermal injection of tissue obtained from spleen or lymph nodes affected by sarcoidosis. A granulomatous reaction was elicited. The Swedish pulmonary physician, Sven Löfgren (1910-1978) in early 50th described an acute form of sarcoidosis with a combination of fever, bilateral hilar lymphadenopathy (BHL), erythema nodosum (EN) in women and sometimes ankle arthritis (mostly in men). This form of the disease is known as Löfgren’s syndrome.

Sarcoidosis is now considered to be a multiorgan inflammatory disease, primarily affecting the lungs, but other organs including the skin, lymph nodes, eyes, heart, bone and central nervous system may also be involved (105).

1.4.1 Epidemiology

Sarcoidosis affects people throughout the world, though the incidence differs between populations. The disease occurs at all ages, although it usually develops in young adults, with the incidence peaking at 20 to 39 years (106), but is rare in childhood. One of the highest incidences has been observed in Scandinavia, with a range from 5 to 40 cases per 100,000 people (107). In Sweden, the estimated yearly incidence of sarcoidosis was reported to be 19 cases per 100,000 people (108). The reported incidence of sarcoidosis varies in different parts of the world, possibly because of differences in environmental and genetic factors (109). Sarcoidosis is more common in African Americans than Caucasian Americans and there is also a high frequency of disease in Japan (109). Sarcoidosis can affect both males and females. Seasonal clustering has also been described with a peak incidence of acute sarcoidosis in early spring (110, 111).
1.4.2 Aetiology
Although there is some evidence to suggest that sarcoidosis might be caused by bacterial infections such as *Mycobacterium* or *Propionibacterium* (112, 113), the aetiology is still unknown. A number of non-infectious agents, such as pine tree pollen (114) and aluminum (115) have also been suggested as possible causes of sarcoidosis, but enough evidence is missing (105). Differences in incidence between ethnic groups and familial clustering suggest that genetic factor influence the susceptibility for sarcoidosis (106, 116). Among candidate genes in sarcoidosis are HLA molecules and cytokines (109). In addition, occupational studies have also indicated a positive association of metal-working, fire fighting, and the handling of building supplies, with the incidence of sarcoidosis (117).

1.4.3 Clinical features
The majority of sarcoidosis patients have a good prognosis and more than half of the patients have a spontaneous recovery. The other patients could develop a prolonged disease course and in some cases chronic progressive disease (108, 118), with symptoms such as dry cough, low grade fever, fatigue, shortness of breath, weight loss and more pronounced chest radiographic changes. The morbidity rate is between 1-5%, caused by severe pulmonary complications, myocardial or central nervous system involvement (105). Chronic disease course occurs more often at age onset over 40 years (119), black race (120) and extra pulmonary disease (121). On the other hand, the acute course of the disease L"ofgren’s syndrome, has a good prognosis especially in patients that are HLA-DRB1*0301 positive (122).

1.4.4 Diagnosis
The diagnosis of sarcoidosis is based on clinical, radiologic, histological and lung function findings (105). The histological finding of non-caseating epithelioid cell granulomas and exclusion of known infectious causes supports the diagnosis. A diagnosis of sarcoidosis in patients with L"ofgren’s syndrome may be made without biopsy. In the absence of biopsy, a ratio between CD4+ and CD8+ T cells in BALF higher than 3.5-4.0 and an increased serum angiotensin-converting enzyme (sACE) levels strongly support the diagnosis of sarcoidosis (123).

The extent of pulmonary involvement is reflected through radiographic changes. There are four radiologic stages of intrathoratic changes, which are shown in table 1.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal chest radiograph</td>
</tr>
<tr>
<td>I</td>
<td>Bilateral hilar lymphadenopathy (BHL)</td>
</tr>
<tr>
<td>II</td>
<td>BHL plus parenchymal infiltrations</td>
</tr>
<tr>
<td>III</td>
<td>Parenchymal infiltrations (without BHL)</td>
</tr>
<tr>
<td>IV</td>
<td>Pulmonary fibrosis</td>
</tr>
</tbody>
</table>

Recently, the integrin CD103 has been suggested to be of value in the diagnostic evaluation of sarcoidosis (124), but if this marker in combination with the CD4/CD8 ratio can provide a specific tool for discriminating sarcoidosis is still under investiga-
tion. More recently, our group showed that increased levels of nerve growth factor (NGF) in BALF of sarcoidosis patients could also contribute to the diagnosis of disease (125).

Pulmonary function tests are important to measure initial lung injury and a sensitive tool that provide a baseline for future follow-up of lung function. In case of sarcoidosis, pulmonary function tests often show disturbed gas exchange and reduced lung volumes (126).

**1.4.5 Treatment**

Since many patients with sarcoidosis recover spontaneously, treatment should usually be avoided. Sometimes non-steroidal anti-inflammatory drugs may be administered e.g. for ankle arthritis and fever. Patients with involvement of the eye, heart and central nervous system are however often treated already at an early stage. The treatment is based on suppression of the inflammatory reaction and usually long-term oral corticosteroids is prescribed (109). Asymptomatic patients with pulmonary involvement and mild disease are usually not treated, while patients with severe pulmonary dysfunction will be offered a trial with corticosteroids, eventually in combination with other immunosuppressants, e.g. methotrexate. Treatment with corticosteroids for a longer period may be associated with serious side-effects. Another less harmful treatment option is inhaled corticosteroids (127). The use of TNF-α inhibitors (128) is still under evaluation.

**1.4.6 The granuloma formation**

One characteristic feature of sarcoidosis is the formation of noncaseating granulomas. The granuloma formation starts with an accumulation of activated CD4+ T cells and macrophages. Macrophages fuse to each other to form multinucleated giant cells and epithelioid cells. The release of IL-12 by macrophages and DCs direct naïve T cells to differentiate into Th1 phenotype.

![Figure 7. Hypothetical model of granuloma formation in sarcoidosis. Adopted from Grunewal et al. (129), with minor modification.](image-url)
The activated T cells accumulate in the lungs of patients and through production of IL-2, IFN-γ and TNF-α contribute to granuloma formation (Fig. 7). The immune reactions subsequently result in the creation of the noncaseating epithelioid cell granuloma. Granuloma formation is a defence mechanism by immune cells to protect against infectious and other harmful antigens that cannot be completely eliminated (105, 130).

1.4.6.1 T cells in sarcoidosis

CD4+ T lymphocytes are believed to play a central role in directing the immune response in sarcoidosis (131). The accumulation of activated CD4+ T cells in the alveolar space of the lungs creates a T-cell alveolitis, resulting in an increase in CD4/CD8 ratio (123). These T cells may either be derived from the periphery and redistributed to the lungs, or have proliferated in the lungs. The oligoclonal expansions of T cells expressing specific T cell receptor (TCR) gene segments in the lung, support the idea that sarcoidosis is an antigen driven process (132-136). The finding by Moller et al, showing a restricted usage for the TCR Vβ8 gene segment of T cells obtained from BAL and blood in sarcoidosis (137), was confirmed by other studies showing a restricted usage of TCR-Vα or Vβ segments of BAL CD4+ T cells in sarcoidosis (133-136). A study on Scandinavian patients demonstrated that HLA-DRB1*0301-positive patients have remarkable expansions of CD4+ T cells using the TCR AV2S3 gene segment in their lungs (135). The number of lung T cells expressing AV2S3 TCR correlates with disease activity (138) and with a better prognosis (139).

BALF T cells from sarcoidosis patients express activation markers to a high degree, such as CD69, CD25 (IL-2R) and HLA-DR, which indicate recent or continuous activation (140-142).

Increased levels of IL-12 and IL-18 expression by alveolar macrophages seem to act synergistically to direct Th1 responses in sarcoidosis (143, 144) and TNF-α has been associated with a chronic form of disease (145).

Chemokines of importance for the typical T cell accumulation in the lungs of sarcoidosis patients include monocyte inflammatory protein (MIP) 1α and 3β, monocyte chemoattractant protein 1 (MCP-1) and RANTES. These chemokines have the capacity to attract and immobilize T cells at the site of inflammation, and are expressed at high levels in patients with sarcoidosis (146, 147). As a result, T cells expressing the corresponding chemokine receptors, e.g. CXCR3, CCR5 and CCR7, migrate and accumulate in the lungs (148).

1.4.6.2 NKT cells in sarcoidosis

Natural killer T (NKT) cells are considered to have immunoregulatory effects. These cells are a subset of CD3+ T cells, express markers common to NK cells, namely CD56 and CD16, and express T cell receptors (149). They recognize glycopeptides presented by CD1d molecules (150) and are capable of producing large amounts of both Th1 (IFN-γ) and Th2 (IL-4) cytokines. NKT cells have been found at reduced levels in blood and BAL of sarcoidosis patients (151, 152). Interestingly, the level of NKT cells in the blood of Löfgren’s patients who have a good prognosis, was found to be at nor-
mal levels (152). There are conflicting data regarding NKT cells in granulomas of pa-
tients (151, 152).

1.4.6.3 Regulatory T cells in sarcoidosis
A couple of studies on regulatory T cells in sarcoidosis suggested increased numbers of
CD4⁺CD25^{bright} Treg cells in blood and BALF of sarcoidosis patients (153, 154). How-
ever, these Treg cells were found to have an anti-proliferative activity, while they were
unable to completely down-regulate the production of inflammatory cytokines TNF-α
and IFN-γ (153), therefore allowing granuloma formation.

1.4.6.4 Apoptosis in sarcoidosis
Apoptosis is an essential mechanism for normal development and tissue homeostasis in
multicellular organisms. There are conflicting data concerning apoptotic events in sar-
coidosis. Some data indicate high expression of pro-apoptotic molecules on BAL
macrophages and T lymphocytes (155, 156). On the other hand, BALF cells and the
immune cells involved in the granuloma formation in sarcoidosis have shown a non-
apoptotic phenotype and seemed to be resistant to apoptosis (157, 158). Survival of
immune cells involved in the granuloma formation goes well with a decreased apop-
totic ability that could contribute to the increased amount of cells in the lungs.

1.4.7 Genetic basis of Löfgren’s syndrome
Löfgren’s syndrome, often with a favourable clinical outcome, presents acute with fe-
ver, erythema nodosum (EN), bilateral hilar lymphadenopathy (BHL) and/or ankle ar-
thritis, and has been associated with distinct HLA-II genes (159, 160). The association
of good prognosis of sarcoidosis and HLA-DR3 alleles is reported in several studies
(159-161). British and Dutch studies have shown that DQB1*0201 (which is in close
linkage disequilibrium with HLA-DR3) is also associated with milder disease e.g.
Löfgren’s syndrome and strongly protective against severe sarcoidosis (162). In con-
trast, HLA-DRB1*14, HLA-DRB1*1501 and HLA-DQB1*0602 were associated with
more severe disease (159, 163).
There is a strong association between HLA-DRB1*0301 and expansions of CD4⁺ T
cells expressing TCR AV2S3 gene segment in BALF of Scandinavian sarcoidosis pa-
tients (136, 164). These patients usually have Löfgren’s syndrome. HLA-DRB3*0101-
positive patients is another group of sarcoidosis patients with lung accumulated
AV2S3⁺ T cells (165). Interestingly, the similarities between HLA-DRB3*0101 and
HLA-DRB1*0301 suggest that these HLA-alleles can present identical antigen pep-
tides (165, 166). Sarcoidosis has also been associated with other genes than HLA-II,
including TNF and lymphotoxin (LT). A German study indicated a higher frequency of
HLA-DR3 and the TNF-A2 allele in German patients with Löfgren’s syndrome (167).
Together, the good prognosis of Löfgren’s patients and the association with the expan-
sion of AV2S3⁺ T cells in the lungs of patients with HLA-DR3 alleles could be based
on the peptide-binding properties of the HLA molecules of these patients.
2 AIMS OF THIS THESIS

The aim of this thesis project was to study the pulmonary inflammation in relation to disease phenotypes in sarcoidosis, with focus on the inflammatory response in the airways of patients with Löfgren’s syndrome and those patients with more severe disease.

The specific aims were:

I. To investigate the pattern of pro- and anti-inflammatory cytokine expression in bronchoalveolar lavage cells and fluid from sarcoidosis patients and healthy individuals. Furthermore, to investigate possible differences in cytokine expression in the lungs of patient subgroups.

II. To study whether the airway inflammation was associated with alterations of regulatory T cells in the lungs of patients.

III. To further investigate other aspects of immune functions, and their role in the disease course in patient subgroups, e.g. TLR expression, macrophage polarization and T-cell expression of TIM molecules.
3 METHODS

3.1 SUBJECTS AND CELLS
All the experimental work was performed on human immune cells. The patients, who participated in the studies, were diagnosed with active pulmonary sarcoidosis. Disease activity was assessed on the basis of symptoms, chest radiography and pulmonary function tests, using the criteria by the World Association of Sarcoidosis and Other Granulomatous disorders (WASOG) (105). The total BALF cells were obtained by bronchoalveolar lavage (BAL) (paper I-IV), and BALF CD4+ T cells (paper II and III) as well as BALF macrophages (paper IV) were sorted by means of flow cytometry. In case of Löfgren’s patients, BAL samples were usually obtained within 3 months after disease onset. All patients were non-smokers and none of them was treated with corticosteroids at the time of the BAL. Healthy individuals included in these studies were all non-smokers, had normal chest radiographs and no signs of any disease. All subjects had given their informed consent and the local ethics committee approved the studies.

3.2 BRONCHOALVEOLAR LAVAGE (BAL)
Fibreoptic bronchoscopy was performed under local anaesthesia on patients and healthy subjects. A flexible fiberoptic bronchoscope was passed transorally and wedged into the middle-lobe bronchus and sterile phosphate-buffered saline (PBS) solution at 37°C was instilled in five aliquots of 50 ml. After each instillation the BALF was gently aspirated and collected in a siliconized plastic bottle that was kept on ice (168). The BAL fluid was strained through a Dacron net (Millipore, Cork, Ireland) and centrifuged at 400 g for 10 min at 4°C, to separate BAL cells from the supernatant. The supernatant was stored in a -70°C freezer until use. The cell pellet was resuspended in RPMI-1640 medium and the viability was determined by trypan blue exclusion. Cell differential counts were determined by May-Grünwald- Giemsa staining of cytopin slides.

3.3 SEPARATION OF CELLS
To sort CD4+ and CD4+ AV2S3+ T cells (papers II and III), BALF cells were stained with anti-CD4-Phycoerythrin (PE) (DAKO) and anti-AV2S3 TCR-Flourescein isothiocyanate (FITC) (Pierce Biotechnology, Rockford, USA). The stained cells were sorted by FACSVantage (BD Biosciences, Montain View, CA, USA). BALF cells were gated on lymphocytes, identified by forward- and side-scatter characteristics, and sorted into different populations; CD4+ T cells from patients and controls, and CD4+ AV2S3+ and CD4+ AV2S3− T cells from patients with lung accumulated T cells expressing the AV2S3 TCR gene segment.
In paper IV, using FACSVantage, alveolar macrophages were sorted by their forward- and side-scatter characteristics.
The purity of the sorted populations, which was determined by FACS, was 98% on average.
3.4 RNA EXTRACTION AND CDNA SYNTHESIS

Total RNA was extracted from the cells through the guanidium thiocyanate phenol-chloroform technique (169), using RNA Bee (Nordic Biosite, Stockholm, Sweden), according to the manufacture’s protocol.

RNA was transcribed to cDNA, using random hexamers primers (Pharmacia Biotech, Uppsala, Sweden), Superscript™II RNase H- Reverse transcriptase (Invitrogen, Lidingö, Sweden), dNTP mix, RNasin (Pharmacia Biotech) and with buffers supplied by the manufacturer, according to the protocol. The procedure was performed in papers I-IV.

3.5 REAL-TIME QUANTITATIVE PCR

Cytokine and other gene expression were quantified by real-time PCR using ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Equal amounts of total RNA were used from all samples to synthesize cDNA and equal volumes of cDNA were used in all assays. β-actin was used as a house-keeping gene to normalize the values of other genes. In paper I, MgCl2 concentration was optimized for each primer to obtain maximum efficiency. To avoid the amplification of contaminating genomic DNA, primers and probes were designed to span either related exon junctions or multiple exon junctions separated with long introns.

The assay-on-demand products and universal master mix were purchased commercially (Applied Biosystems) (papers II-IV). The PCR condition was described in paper I. All samples were run in duplicates and the mean values calculated.

The relative quantitative expression of different genes in various cell populations were determined using the arithmetic formula 2^{-ΔΔCT} according to Perkin Elmer instruction manual (170), where the amount of target gene was normalized to β-actin (ΔCT) and the relative increase of a gene in BALF cells was calculated in relation to the mean value of gene expression in a healthy control group (ΔΔCT).

3.6 CYTOMETRIC BEAD ARRAY (CBA)

The BD™ CBA human inflammation and Th1/Th2 cytokine kits were used for detection of secreted cytokines in BAL supernatant, according to manufacturer’s instruction and was applied in paper I. BAL supernatant was concentrated 650x using Amicon Ultra-15 Filters (Millipore Corporation, Bedford, MA, USA), the day before cytokine testing was to be performed. Standards and samples were processed by FACScan (BectonDickenson, Montain View, CA, USA) and data analyzed by the CBA software program (BD). The concentration of cytokines was calculated using standard curves. The detection range of BD™ CBA kit is from 20 to 5000 pg/ml, but according to BD, the values below 20 pg/ml could be acceptable, if further diluting of the standards (down to 5 pg/ml) was performed.
3.7 HUMAN LEUKOCYTE ANTIGEN TYPING

HLA class II (HLA-DR) typing was carried out on DNA through use of PCR and amplification with sequence specific primers (171). This procedure was done by the laboratory personnel of Olerup SSP AB.

3.8 CELL PHENOTYPING BY FLOW CYTOMETRY

BALF CD4/CD8 T-lymphocyte ratio and TCR AV2S3 expression in BALF cells were determined by flow cytometric analysis (FACScan and FACSCanto II) using monoclonal antibodies (Mabs) against CD3⁺, CD4⁺ and CD8⁺ (Dako Cytomation Norden AB, Solna, Sweden) and anti-human TCR AV2S3-specific Mab clone F1 (Serotec, Oxford, UK; in papers I and II, and Pierce Biotechnology, Rockford, USA; in paper III).

3.9 INTRACELLULAR STAINING OF FOXP3

FoxP3 protein expression was analyzed in blood and BALF CD4⁺ T cells as well as in BALF CD4⁺ AV2S3⁺ and CD4⁺AV2S3⁻ T cells, using flow cytometry. Anti-FoxP3-PE (clone PCH101-PE) and isotype control (rat IgG2a-PE) and staining kit were purchased from eBioscience (Biosciences, San Diego, CA, USA). Expression of cell-surface markers: CD3, CD4 and AV2S3 TCR and intra-cellular FOXP3 were determined by flow cytometry after gating on CD4⁺ lymphocytes. The data were analysed using FACS Diva software (BD Biosciences).

3.10 STAINING OF TIM MOLECULES

The analysis of TIM-1 and TIM-3 cell surface expression in blood and BALF CD4⁺ cells was performed on BD FACSCanto II flow cytometer with FACSDiva software (BD Biosciences). 1x10⁶ BALF cells or 100 μl heparinized blood were surface stained with monoclonal antibodies against human TIM-1 (R&D system, Minneapolis, MN, USA; followed by APC-conjugated goat anti-mouse antibody), CD3-pacific blue (BD Biosciences), CD4- APC H7 (BD Biosciences), AV2S3-FITC (Pierce Biotechnology) and TIM-3-PE (R&D system). Mouse IgG1-FITC (BD Biosciences), mouse IgG2b (Nordic Biosite AB, Stockholm, Sweden) and rat IgG2a (BD Biosciences) were used as isotype controls. The red blood cells were lysed after the end of incubations. Expression of cell surface markers was determined by flow cytometry after gating on CD3⁺CD4⁺ lymphocytes. This procedure was used in paper III.

3.11 IN VITRO STIMULATION OF TOTAL BALF CELLS

Total BALF cells (1x10⁶/ml) were stimulated for 24 hours with LPS (1.6 μg/ml) or with medium alone (RPMI-1640 medium, supplemented with 1% penicillin streptomycin, 1% L-glutamine and 2% heat-inactivated human AB serum) for four and 24 hours in humidified air with 5% CO₂ at 37°C. After incubation, the cells were harvested, RNA Bee added, and cells were stored at -70°C until use. The supernatant of cell cultures were collected for future analysis.
3.12 STATISTICAL METHODS

The significance levels were calculated according to non-parametric tests, using the Kruskal–Wallis test followed by Dunn’s post-test for comparisons between groups or Mann–Whitney $U$-test for comparison between two groups. The non-parametric Wilcoxon matched pairs statistical test was used for calculation of statistical significances of differences in TIM-3 expression between BAL and blood in patients and controls. The correlations between different parameters were determined with Spearman’s rank correlation test. Values of $P < 0.05$ were regarded as significant. No correction was done for multiple comparisons.
4 RESULTS AND DISCUSSION

4.1 INTRODUCTION

Although the cause of sarcoidosis is still unknown, the granulomatous inflammation in sarcoidosis is suggested to involve an exaggerated immune response against an undefined antigen (112, 113), which persists at different sites of disease involvement. In human disease models, when the invading antigen is removed, the inflammatory response is generally resolved, while the persistence of the antigen may lead to a prolonged inflammatory response (146). Sarcoidosis patients with an acute form of disease, Löfgren’s syndrome, especially those patients that are HLA-DRB1*0301-positive have a very good prognosis, with a short disease course (122), while the other group of patients has an insidious onset of disease and is associated with a longer disease course (159, 172). Thus, there are two patient groups with quite distinct forms of disease, and in which inflammatory and immune regulatory responses are investigated here.

4.2 BALF AND LUNG PARAMETERS (PAPERS I-IV)

BAL cell analyses in these studies showed higher BAL cell concentrations (papers I-IV) and higher frequencies of lymphocytes in BALF from each patient subgroup compared to controls (papers I, III, IV). In paper II, the increased frequency of lymphocytes was detected only in non-Löfgren’s patients. The comparisons of BAL cell concentration and frequency of BALF lymphocytes and macrophages between patient subgroups (papers II) showed significant differences. A higher BAL cell concentration and higher frequency of BALF lymphocytes in the lungs of patients without Löfgren’s syndrome may reflect a more pronounced inflammatory activity in their lungs.

Pulmonary function tests reflected a more marked pulmonary disease in non-Löfgren’s patients (papers II-IV (in paper I; HLA-DRB1*0301 neg patient group)), as defined by vital capacity (VC), forced expiratory volume in 1 second (FEV1) and diffusing capacity of the lung for carbon monoxide (DLco).

4.3 CYTOKINE PATTERN IN TOTAL BAL CELLS AND FLUID (PAPER I)

Reduced Th1 response in the lungs of HLA-DRB1*0301 patients with pulmonary sarcoidosis (Eur Respir J 2006; 27: 451-459)

The cytokine pattern at the site of inflammation has been suggested to be of importance for the outcome of the inflammatory response in sarcoidosis (131, 146, 173). This issue is of interest with regard to our patient subgroups. In this study, the patients were stratified according to their HLA-type. We studied cytokine gene expression in the following two patient subgroups: A) HLA-DRB1*0301pos patients (n=12) with an acute onset of the disease, in all but 4 cases having Löfgren’s syndrome, and all with lung accumulations of CD4+ T cells expressing the TCR AV2S3 gene segment and B) HLA-DRB1*0301neg patients (n=13), all except one (who had Löfgren’s syndrome) with an insidious disease onset. None of these patients had an AV2S3 lung T-cell expansion.

We had the same subgroup characteristics when we studied the levels of released cyto-
kines in BALF; HLA-DRB1*0301^pos patients (n=14, 12 having Löfgren’s syndrome) and HLA-DRB1*0301^neg patients (n=20, one having Löfgren’s syndrome). Eleven healthy adults were included as controls.

4.3.1 Th1-mediated immune response in patients

The concept of sarcoidosis as a Th1-mediated disease (174-176) was confirmed here. We detected a higher mRNA level of IFN-γ as well as higher levels of released cytokines IL-2 and IL-12p70 in patients versus controls. In addition, pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in BALF were also increased in patients, while the IL-4 level was too low for detection, in line with previous studies reporting a preferred Th1 cytokine expression in the lungs of patients (132, 143, 177). We found no difference in RNA transcript levels of TNF-α and IL-12p40, when comparing patients and controls. These results contrasted with some previous studies (143, 178) but was in line with others (144, 174, 179, 180). There may be various explanations for such discrepancies. One could be the choice of patient groups, treatment history of patients, or the methodology. However, the increased secreted levels of TNF-α and IL-12p70 in BALF of patients was noted, which could be explained by the fact that other pulmonary cells, like pulmonary epithelial cells (181-183) may contribute to the secretion of for example TNF-α in BALF. In addition, mature macrophages may develop a post-transcriptional mechanism to potentiate TNF-α protein secretion, while transcription of the message is much slower (184, 185).

Finally, our results also demonstrate an increased mRNA level of the anti-inflammatory mediator, IL-10, in freshly isolated BAL cells of patients, which strongly correlated with the secreted level in the BALF. Since IL-10 is a potent downregulator of IL-12 and TNF-α (186, 187) increased levels of this cytokine may have a suppressive effect on the transcription of cytokines like IL-12 and TNF-α.

4.3.2 Immune response in patient subgroups

4.3.2.1 HLA-DRB1*0301^pos versus HLA-DRB1*0301^neg patients

The HLA-DRB1*0301/ HLA-DQB1*0201 allele has been strongly associated with good prognosis. The HLA-DRB1*0301 allele is found in Scandinavian patients with sarcoidosis, who have remarkable expansions of AV2S3^+ T cells in their lungs (188). In this study, we found that the mRNA levels of IFN-γ and TNF-α were significantly increased in DRB1*0301^neg patients as compared to DRB1*0301^pos patients (Fig. 8a-b). Our data also indicate significantly elevated protein levels of TNF-α, IL-2 and IL-12p70 in DRB1*0301^neg patients but not DRB1*0301^pos patients, compared to controls.

With regard to anti-inflammatory cytokines, Zissel et al. showed that elevated protein levels of TGF-β1 in cell cultures of sarcoidosis patients associated with good prognosis (189), which can suggest TGF-β as a regulatory cytokine in the lungs of sarcoidosis patients. In this study, we found a tendency towards higher TGF-β1 transcript levels in freshly isolated BALF cells from HLA-DRB1*0301^pos patients versus DRB1*0301^neg patients (Fig. 8c). In summary, down-regulation of the Th1 immune response and a
tendency to higher levels of TGF-\(\beta\)1 were associated with HLA-DRB1*0301\(^{pos}\) patients, which may explain the spontaneous disease resolution and good prognosis in these patients. This issue needs further investigation.

4.3.2.2 Löfgren’s versus non-Löfgren’s patients

To be able to compare the expression of cytokines in the same subgroups as in the other studies in this thesis, we redefined the patient subgroups from study I into patients with Löfgren’s syndrome and those without. Nine out of 25 patients were defined as Löfgren’s syndrome when we analysed cytokine gene expression and 13 out of 34 when the protein level of cytokines were analysed. By investigating the statistical differences in cytokine mRNA expression, we found the same significant differences between groups as when they were defined as DRB1*0301\(^{pos}\) or DRB1*0301\(^{neg}\), i.e. higher IFN-\(\gamma\) and TNF-\(\alpha\) mRNA levels in non-Löfgren’s patients and a tendency to higher TGF-\(\beta\)1 mRNA levels in Löfgren’s patients, except that non-Löfgren’s patients expressed significantly higher mRNA level of IL-10 as compared to controls (Fig. 9).

New significant differences were also detected when we analysed the protein levels of cytokines in BALF. Significantly elevated protein levels of TNF-\(\alpha\), IL-2 and IL-6 were found in BALF of non-Löfgren’s patients versus Löfgren’s patients (Fig. 10), which further supports our demonstration that patients with a less favourable prognosis have a more pronounced Th1 response in their lungs.
4.4 REGULATORY T CELLS IN SARCOIDOSIS (PAPER II)

*Analysis of regulatory T cell associated forkhead box P3 expression in the lungs of patients with sarcoidosis (Clin Exp Immunol 2008; 152:127-137)*

Regulatory T cells play an important role in controlling immune responses and provide protection from autoimmune diseases (190). On the other hand, high numbers of Tregs make it possible for cancer cells to evade the host immune response (191). Previous studies of Tregs in pulmonary sarcoidosis have demonstrated that the number of Tregs, then defined as CD4+CD25+ T cells, present in the lungs of patients are higher compared to control subjects (153, 154). Since in humans CD25+ cells contain both regulatory and activated effector T cells (192, 193), we chose to analyse the forkhead transcription factor FOXP3 expression in BALF cells, since expression of FOXP3 rather than CD25 is suggested to be specific for the regulatory phenotype of T cells (38).

FACS-sorted BALF CD4+ T cells from sarcoidosis patients (n=24, 11 with Löfgren’s syndrome) and healthy controls (n=7) were used to measure the mRNA expression of chemokine receptor 2 (CCR2), FOXP3, IL-10 and TGF-β1. In addition, to analyse FOXP3 protein expression we performed FACS analysis on BALF T cells of 14 patients (7 with Löfgren’s syndrome) and nine controls.

4.4.1 Reduced FOXP3 expression in sarcoidosis patients

In this study, we demonstrated a down-regulation of FOXP3 at both mRNA and protein levels in BALF CD4+ T cells in sarcoidosis patients. The significant reduction in FOXP3 mRNA expression (Fig. 11a) was accompanied by the reduction in mRNA levels of CCR2 and IL-10 in isolated BALF CD4+ T cells from patients. Previous studies have shown that CCR2 is expressed on Treg cells (194, 195) and directly associated CCR2 with the amounts of FOXP3 mRNA in atherosclerosis lesions (196). Recently, however, it has also been suggested that CCR2 may be used as a marker for Th17 cells (197). Our results confirm that CCR2 can be a suitable candidate molecule to be analysed in relation to Tregs. The decreased FOXP3 mRNA expression in BALF cells of patients was confirmed by further investigating the expression of this molecule by flow
cytometry, which showed a reduced number of FOXP3+ CD4+ T cells present in the lungs of sarcoidosis patients, compared to healthy subjects (Fig. 11b).

In addition, there was also a reduced FOXP3 intensity in CD4+ FOXP3+ BALF T cells of patients, which may reflect a lower suppressive activity in Treg cells from patients. This would confirm the results from Miyara et al., showing a limited functional capacity of CD4+CD25bright cells from sarcoidosis patients to suppress TNF-α and IFN-γ secretion (153). Moreover, the fraction of blood CD4+ T cells expressing FOXP3 was also decreased in patients (Fig. 11b).

It was also of interest to compare the frequency of FOXP3-expressing T cells in paired BALF and blood cells. Our data indicate that in healthy controls, the frequency of FOXP3+ T cells (Fig. 11b) and even their intensity of FOXP3 expression were much higher in the lungs compared to blood. These results were in line with data from Hartl et al. showing higher Treg activity in the lungs compared to blood of healthy individuals (198). One could speculate that such Treg activity could be of importance for the maintenance of immune homeostasis in the lungs. In patients, we found only a slightly increased number of FOXP3-expressing T cells in the lungs (Fig. 11b), while no difference in the intensity of FOXP3 between BALF and blood T cells was observed.

Figure 11. The expression of FOXP3 in CD4+ T cells. (a) Relative mRNA expression of FOXP3 in BALF cells was decreased in patients (n=24) versus controls (n=7) and (b) flow cytometric analysis of FOXP3 protein expression in paired BALF and blood cells showed a down regulation of FOXP3 in both compartments in patients (n=7) versus controls (n=9). Horizontal bars indicate median values and the lines indicate T cell sub populations from the same individual. (Figures are reproduced from paper II)

When comparing the levels of FOXP3 and other genes between patient subgroups, we did not detect any significant differences. However, the decreased levels of CCR2 and IL-10 were most pronounced in non-Löfgren’s patients. In addition, a tendency to increased expression of TGF-β1 in BALF CD4+ T cells of Löfgren’s patients versus non-Löfgren’s patients was observed.

The outcome of this study may reflect that the suppressive function of regulatory T cells is lacking in the lungs of sarcoidosis patients, resulting in an augmented Th1 immune response.
4.4.2 The AV2S3\(^+\) T cells in the lungs of patients

One possible explanation for the association of CD4\(^+\) TCR AV2S3\(^+\) T cells with resolving disease in Scandinavian patients is that these cells may have the ability to produce high levels of TGF-\(\beta\) or IL-10 and be of a regulatory phenotype. To investigate this, we sorted CD4\(^+\) T cells expressing TCR AV2S3 from BALF cells obtained from patients with such T cell expansions in their lungs (n=12). Contrary to the above hypothesis, there were lower mRNA levels of FOXP3 (Fig. 12a), CCR2 and IL-10 (although not statistically significant) in AV2S3\(^+\) T cells compared with AV2S3\(^-\) T cells, which was confirmed by flow cytometric analysis of FOXP3 (Fig. 12b). This is consistent with the results from a previous study, which demonstrated a reduced expression of regulatory T cell associated molecules, CD25 and CD27, and increased expression of the effector T cell associated molecule CD28 on the surface of AV2S3\(^+\) T cells (142). In addition, no difference was detected in mRNA expression for TGF-\(\beta\)1. It is therefore likely that these AV2S3\(^+\) lung T cells act as effector T cells rather than Treg cells and could be important in eradicating an offending antigen.

![Figure 12](image.png)

Figure 12. The expression of FOXP3 in BALF AV2S3\(^+/-\) CD4\(^+\) T cells. (a) The mRNA level of FOXP3 was decreased in AV2S3\(^+\) CD4\(^+\) T cells versus AV2S3\(^-\) CD4\(^+\) T cells. The lines indicate T cell subpopulations from the same individual. (b) One representative dot plot out of three, showing the decreased frequency of FOXP3-expressing cells in AV2S3\(^+\) T cells. (Figures are reproduced from paper II)

4.5 TIM MOLECULES IN SARCOIDOSIS (PAPER III)

*Altered expression of T cell Immunoglobulin-Mucin (TIM) molecules in bronchoalveolar lavage CD4\(^+\) T cells in sarcoidosis. (Submitted)*

TIM-3 has been described as a Th1-specific molecule, contributing to the regulation and control of immune responses and the pathogenesis of Th1-driven diseases (76-78). Until now, there is still no data concerning the expression of TIM-1 and TIM-3 in patients with sarcoidosis. Using FACS-sorted CD4\(^+\) T cells from BALF of patients (n=28; 13 with Lofgren’s syndrome) and healthy controls (n=8), we measured the mRNA expression of TIM-1, TIM-3 and Th1/Th2 cytokines by means of real-time PCR. TIM-1 and TIM-3 were also analysed at the protein level in matched BAL and blood samples in a few individuals.
4.5.1 Decreased TIM-3 expression in sarcoidosis patients

In sarcoidosis patients, we found a significant reduction in TIM-3 mRNA expression (Fig. 13a). We also analysed the protein expression of TIM-3 on BALF and blood cells of 4 patients and 4 controls. The results showed a strong tendency to lower frequencies of TIM-3-expressing CD4+ T cells in patients versus controls. TIM-3 has been suggested to be downregulated in autoimmune and inflammatory diseases and therefore allowing exaggerated immune responses in such disease conditions (81, 199). Another observation in this study was an increased mRNA level of IL-2 in patients (Fig. 13b), indicating a proliferative and Th1-associated response in patients, in line with previous studies (175, 176). The decreased level of TIM-3 was associated with an increased CD4+/CD8 ratio in BALF of patients, indicating that a reduced TIM-3 level on BALF CD4+ T cells may result in a more intensive T cell alveolitis. Recent data suggest that the interaction of TIM-3 with its ligand, galectin-9, may inhibit Th1 immune responses and therefore be crucial for the induction of peripheral tolerance (77, 78, 80). However, when we analysed the levels of gal-9 we found that the mRNA level of gal-9 in BALF T cells of patients was similar to that in healthy subjects, leaving TIM-3 as a possible factor involved in the uncontrolled Th1 response in the lungs of patients. Further analyses on TIM-1, IFN-γ and IL-13 expression showed no difference between patients and controls, while the Th2 cytokines IL-4 and IL-5 were detected only in BALF T cells of two patients and one control.

![Figure 13](image)

Figure 13. The relative mRNA expression of (a) TIM-3 and (b) IL-2 in BALF CD4+ T cells of patients and healthy controls. The horizontal bars indicate median values. * p< 0.05, ** p< 0.01. (Figures are reproduced from paper III)

4.5.2 BALF CD4+ T cells of Non-Löfgren’s patients express reduced TIM-1 mRNA levels

Since in paper I we observed that patients with prolonged disease had an increased Th1 immune response, it was of interest to investigate the expression of TIMs in patient subgroups to explore their possible involvement in the regulation of disease. We did not find any differences in the transcript levels of TIM-3 and IL-2 between patient subgroups, but the mRNA levels of TIM-1 and IFN-γ were significantly different in Löfgren’s and non-Löfgren’s patients. The levels of TIM-1 were decreased in non-Löfgren’s patients versus Löfgren’s patients, while a higher level of IFN-γ in CD4+ lung T cells of non-Löfgren’s patients was observed (Fig. 14a,b). The data regarding IFN-γ confirms the results from paper I, which indicated that IFN-γ is a cytokine affect-
ing the progression of disease. A previous study from another group revealed that a higher expression of TIM-1 could be associated with clinical remission and low expression of IFN-γ in MS (200). Their findings are in line with our present data, suggesting a regulatory role for TIM-1 in modulating the inflammation.

Figure 14. The relative TIM-1 and IFN-γ mRNA expression in BALF CD4+ T cells of patient subgroups. Non-Löfgren’s patients showed (a) a reduced TIM-1 mRNA level and (b) an increased IFN-γ mRNA level versus patients with Löfgren’s syndrome. (Figures are reproduced from paper III)

Our findings in paper II suggested an effector function for lung accumulated AV2S3+ T cells. In study III, we analysed these cells for the expression of TIM molecules and Th1/Th2 cytokines. Using real-time PCR, we did not find any significant differences in expression of the above mentioned genes between AV2S3+ T cells. Further investigations of effector molecule expression by AV2S3+ T cells are needed to elucidate their functional role.

4.6 TLR EXPRESSION AND ALVEOLAR MACROPHAGE POLARIZATION IN SARCOIDOSIS (PAPER IV)

Altered expression of TLR2 and IL-23 in bronchoalveolar lavage cells in sarcoidosis. Submitted

The finding of bacterial antigens in sarcoidosis tissues (112, 113) suggest a role for pattern recognition receptors such as TLRs in the pathogenesis. Therefore, the specific objective of this study was to study whether the TLR expression pattern of AM from sarcoidosis patients was different compared to healthy controls. In addition, we studied the degree of macrophage polarization, M1 versus M2 types, in the lungs of patients to find out any possible relation to the course of sarcoidosis. Activated M1 macrophages are induced by IFN-γ and TNF-α and are characterized by a high capacity to present antigens and to produce toxic intermediates such as nitric oxide, while M2 macrophages are induced by IL-4 and IL-13 and are associated with fibrosis development (10).
AMs were sorted by flow cytometry from patients (n=22; 11 with Löfgren’s syndrome) and healthy controls (n=11). In addition, total BALF cells from patients (n=11, two with Löfgren’s syndrome) and controls (n=5) were cultured for 4 or 24h in medium or stimulated with LPS for 24 hours. The mRNA expression of selected TLRs and genes associated with macrophage polarization were analysed in AM and in vitro stimulated BALF cells.

4.6.1 Decreased TLR2 mRNA levels in AMs of Löfgren’s patients

We detected a decreased mRNA level of TLR2 in AMs from patients compared to controls, and this down-regulation was mainly due to the low levels of TLR2 in Löfgren’s patients (Fig. 15a, b). TLRs are believed to play an important role in the early detection of microbial infections (201). Studies on TLR2-deficient mice indicated that these mice were more susceptible to both Gram-positive and Gram-negative bacteria (202, 203), suggesting that TLR2 expression may influence the possibility of being infected.

The decreased TLR2 expression of AM in Löfgren’s patients goes well with a reduced IFN-γ mRNA level in BALF CD4+ T cells of these patients (paper III), since a down-regulated TLR2 may lead to a reduced ability for AM and T cells to interact, which might result in reduced IFN-γ levels. Although the reduced level of TLR2 in the lungs could be caused by genetic factors, which contributed to the disease development, environmental factors like tobacco smoking may also change TLR2 expression (204, 205). Despite most findings on TLR2 suggesting an important role for TLR2 in driving Th1 responses (206, 207), there is also data indicating that TLR2-mediated signals preferentially induce a Th2 profile in APCs through expression of IL-10 (208-210). These differences may be explained by the use of different ligands in these studies, since the choice of the ligand is of importance for the outcome of TLR2-mediated immune responses.

The results regarding TLR2 expression in the lungs of patients contrast with findings in blood, since our group’s previous data indicated a higher expression of TLR2 on blood monocytes of sarcoidosis patients (211). The reason for differences between these two compartments remains to be investigated.

We did not detect any significant differences in transcript levels of either TLR4 or TLR9 between patients and controls.

We also investigated the mRNA levels of M1- and M2-associated molecules in AM. In general, patients did not differ from controls with regard to M1/M2 polarization. Unlike other studies on AM in sarcoidosis which showed increased expression of M1 markers CXCL10 (212), CXCL11 (213) and CXCL16 (214) in sarcoidosis patients, we could only detect a tendency to an increased CXCL10 expression in Löfgren’s patients compared to controls. In our study, freshly isolated AMs were used, while in the previous studies either AM or total BALF cells were cultured before the analysis of gene expression. These discrepancies may suggest a need of in vitro stimulation of AM before analysing these genes.

Further analyses of M2 markers showed a significant up-regulation of CCL18 in patients compared to controls (Fig. 15c), which could indicate an increased risk of developing fibrosis in patients. This is because CCL18 has the ability to increase the collagen
production of lung fibroblasts (215). The up-regulated CCL18 correlated positively with the frequency of BAL lymphocytes. We could not find any correlations between the level of CCL18 and X-ray stage, or lung function parameters.

The expression of several genes was undetectable in freshly isolated AMs. Therefore, in the next stage, we analysed the expression of selected genes in BALF cells, cultured for 4 and 24h in medium or 24h with LPS. Our results indicated that BALF cells from healthy subjects had the highest capacity to be stimulated with LPS after 24h in culture, as analysed by expression of various inflammatory-related genes. This might depend on the fact that BALF cells from patients were already activated in vivo and that the kinetics could differ from that in controls, or alternatively that there might be a defect in BALF cells of patients to respond to microbial agents.

However, after 4 and 24h culture in medium alone, we found strong tendencies to an up-regulated IL-23p19 mRNA expression in patients, suggesting that BALF cells of patients are already activated in vivo. Upon LPS stimulation, healthy subjects showed much stronger up-regulation of IL-23p19 gene expression compared to culture in medium alone than patients, which might indicate a defect in BALF cells of patients to respond properly to a postulated sarcoidosis pathogen. IL-23 regulates the maintenance of Th17 cells and has been linked to several autoimmune diseases (85, 216). Its role in sarcoidosis is unclear and needs further investigation.

### 4.7 PRELIMINARY DATA ON TGF-BETA1

To find out if the lung cells from sarcoidosis patients have the ability to up-regulate the release of TGF-β1, we cultured total BALF cells of sarcoidosis patients and healthy subjects in complete medium (10^6 cells /0.3ml) alone, or with PHA stimulation for 24 hours. Using ELISA, we measured the released TGF-β1 in cell supernatant in patient subgroups; HLA-DRB1*0301^{pos} patients (n=11) with an acute onset of disease and HLA-DRB1*0301^{neg} patients (n=15) with an insidious disease onset as well as in healthy adults (n=16) (Fig. 16).
Our data showed significantly elevated protein levels of TGF-β1 in cell cultures (medium alone and with PHA stimulation) of both patient subgroups as compared to controls. In particular, DRBI*0301pos patients showed markedly higher levels of TGF-β1 in medium without PHA stimulation. These are preliminary data and further investigations are needed.
4.8 INTERPRETATION OF PAPERS I-IV

In this project, we found several distinct immune response patterns in specific patient sub-groups, i.e. in DRB1*0301<sup>pos</sup> versus DRB1*0301<sup>neg</sup> patients or alternatively in Löfgren’s patients versus non-Löfgren’s patients. In general, an increased Th1 immune response, localized to the lungs, was found in patients associated with a more severe disease course.

4.8.1 Patients versus controls

In paper I, we demonstrated that pro-inflammatory and Th1-associated cytokines were up-regulated at both mRNA and protein levels in the lungs of patients. Also, an increased expression of IL-10 was observed in the lungs of patients, which may indicate a regulatory action by BALF cells to inhibit the exaggerated Th1 response in the lungs. The increased IL-2 expression in the lungs of patients was noted in papers I and III. In contrast, the observed increased level of IFN-γ in total BALF cells in the lungs of sarcoidosis patients in paper I was not confirmed by our analysis in paper III. In the latter paper, we could not show any significant increase of the IFN-γ mRNA level in BALF CD4<sup>+</sup> T cells of patients as compared to healthy controls. The discrepancy in results between paper I and III may indicate that other BALF cells besides CD4<sup>+</sup> T cells contribute to the increased levels of IFN-γ in total BALF cells of patients. For example, BALF NK-cells have previously been shown to produce increased levels of IFN-γ in sarcoidosis (217).

In papers II and III, we investigated aspects of immune regulatory mechanisms in BALF T cells through the analysis of the regulatory T cell associated transcription factor FOXP3 and the recently identified TIM molecules. We demonstrated a reduction of FOXP3, the specific marker of Tregs, and of TIM-3 in BALF CD4<sup>+</sup> T of sarcoidosis patients, which could indicate a defect of BALF T cells in regulating Th1 responses in the lungs of these patients.

In paper II and III, the observed reduction of FOXP3, CCR2 and TIM-3 in sarcoidosis was further supported by the findings of a negative association of the mRNA levels of these genes with the frequency of BALF lymphocytes in the case of FOXP3 and CCR2, and with the CD4/CD8 ratio of BALF lymphocytes in the case of TIM-3. These data suggest that a reduced regulatory activity in the lungs may result in a more intensive accumulation of CD4<sup>+</sup> T cells and a more pronounced alveolitis in patients.

In paper IV, we found that upon LPS-stimulation there was a markedly lower ability to up-regulate IL-23, of importance for Th17 responses, in patients. This may however in part be explained by the patients’ cells being activated <i>in vivo</i>, since there were strong tendencies to higher IL-23 mRNA levels in total BAL cells from patients after culture in medium alone.

4.8.2 Patient subgroups

In paper I, when analysing the cytokine mRNA expression in total BALF cells as well as the released cytokines in BALF of patient subgroups, DRB1*0301<sup>pos</sup> patients were found to express increased levels of Th1 cytokines in their lungs, while a tendency to a higher TGF-β1 mRNA level was seen in DRB1*0301<sup>pos</sup> patients.
In paper III, further analysis of the mRNA level of IFN-γ in BALF CD4⁺ T cells of patient subgroups showed an increased IFN-γ level in non-Löfgren’s patients versus both Löfgren’s patients and controls. In paper II, we found no significant differences between patient subgroups in the expression of regulatory T cell associated genes. However, we observed that non-Löfgren’s patients had more pronounced decreased mRNA levels of CCR2 and IL-10, while Löfgren’s patients showed a tendency to a higher ability to express TGF-β₁ mRNA in BALF CD4⁺ T cells. The observed tendencies to higher TGF-β₁ mRNA levels in Löfgren’s patients in paper I and II, may explain the clinical characteristic of this group, namely spontaneous disease resolution. Interestingly, paper III showed a decreased mRNA level of TIM-1 in non-Löfgren’s patients versus patients with Löfgren’s syndrome. Previous studies on TIM-1 have indicated that higher mRNA expression of TIM-1 associated with clinical remission and lower levels of IFN-γ (200). Here, we found that Löfgren’s patient expressed TIM-1 at the same levels as healthy controls. The levels of TIM-1 correlated with the levels of IFN-γ in BALF CD4⁺ T cells of Löfgren’s patients and controls, but not of non-Löfgren’s patients, suggesting a regulatory role for TIM-1 in the inflammatory response.

In paper IV, a decreased mRNA level of TLR2 was demonstrated in AM of Löfgren’s patients. The positive correlation between TLR2 mRNA levels in AM and IFN-γ mRNA expression in CD4⁺ T cells of the same patients (paper III), suggest that the level of TLR2 could influence the capacity of AM to interact effectively with BALF T cells, thereby inducing IFN-γ production.

### 4.8.3 BALF TCR AV2S3⁺ CD4⁺ T cells

Expansions of TCR AV2S3⁺ T cells in BALF are strongly associated with DRB1*0301 positive patients and with a better prognosis (139), which may suggest a protective role for these cells. However, we found that these T cells expressed significantly lower levels of FOXP3 and CCR2 in addition to a strong tendency of reduced IL-10 levels as compared to AV2S3neg CD4⁺ BALF T cells. The expression of TGF-β1 did not differ between T cell subsets. Further investigation of AV2S3⁺ T cells showed no difference in the expression of TIM molecules or of IL-2, IL-13 and IFN-γ versus AV2S3neg T cells. Together, the data in paper II and III suggest that this particular T cell subset has more of an effector function than other BALF CD4⁺ T cells and may contribute to eradicating a postulated sarcoidosis antigen. This is in line with previous findings of higher expression of activation markers on AV2S3⁺ T cells (142). However, further investigations are needed to clarify the exact function of AV2S3⁺ T cells in sarcoidosis.
4.9 CONCLUDING REMARKS

Altogether, these studies indicate an increased Th1 response in the lungs of patients with sarcoidosis compared to healthy subjects. The decreased levels of FOXP3 and TIM-3 in the lung CD4+ T cells of sarcoidosis patients likely reflect a defect in the T cell ability to control inflammation and granuloma formation. In addition, the study revealed that an increased production of pro-inflammatory and Th1 associated cytokines, i.e. IL-2, IL-6, IL-12p70, TNF-α and IFN-γ by BALF cells is associated with non-Löfgren’s patients. The higher mRNA level of IL-10 in BALF cells of non-Löfgren’s patients could suggest an ability of alveolar cells that counteracts the elevated levels of IFN-γ in BALF. The ability to produce higher levels of TGF-β1 by BALF cells is associated with a favourable prognosis, i.e. Löfgren’s syndrome. The reduced level of TIM-1 in non-Löfgren’s patients goes well with the exaggerated Th1 response in the lungs of these patients, suggesting a regulatory role for this molecule in inflammation. Reduced levels of regulatory associated genes FOXP3 and CCR2 in BALF AV2S3+ CD4+ T cells suggests an effector function rather than a regulatory role for this T cell subset, which is thus implicated in the eradication of a sarcoidosis antigen. Furthermore, the decreased level of TLR2 in AM of Löfgren’s patients might be of relevance for macrophage interaction with a postulated sarcoidosis pathogen, and thereby subsequent reduced macrophage activation and stimulation of T cells.

Figure 17. Hypothetical model of inflammatory reaction in sarcoidosis, comparing the immune response in the lungs of patients with Löfgren’s syndrome and those without. Illustration inspired by D.R. Moller (218).
5 CONCLUSION

This thesis has focused on inflammatory responses including regulatory functions in BAL fluid and cells of sarcoidosis patients. In particular, we compared immune responses in the lungs of patients with and without Löfgren’s syndrome and the critical factors that could contribute to their respective disease course. Based on our findings we conclude that:

- A predominant Th1 cytokine profile in BALF cells of sarcoidosis patients was observed. We demonstrated higher levels of pro-inflammatory and Th1 associated cytokines in the lungs of HLA-DRB1*0301\(^{\text{neg}}\) patients versus HLA-DRB1*0301\(^{\text{pos}}\) patients.

- A decreased level of expression of regulatory associated genes in BALF CD4\(^{+}\) T cells of patients was detected. Also a decreased FOXP3 intensity in the CD4\(^{+}\)FOXP3\(^{+}\) BALF T cells of patients was observed at the protein level. No difference between patient subgroups was found.

- A reduced level of regulatory associated genes in BALF TCR AV2S3\(^{+}\) CD4\(^{+}\) T cells versus BALF AV2S3\(^{\text{neg}}\) CD4\(^{+}\) T cells suggested an effector function rather than a regulatory one for these T cells.

- A higher ability to produce TGF-β1 in BALF cells was observed in patients with Löfgren’s syndrome.

- A reduced level of TIM-3 in BALF CD4\(^{+}\) T cells of sarcoidosis patients was noted. A down-regulated TIM-1 expression was associated with a higher expression of IFN-γ in the lung CD4\(^{+}\) T cells of non-Löfgren’s patients, and thus a dysregulation of TIM-1 may be of importance for the exaggerated Th1 response in these patients.

- A lower level of TLR2 in AM of patients with Löfgren’s syndrome was observed, which associated with the reduced levels of IFN-γ in CD4\(^{+}\) T cells of these patients. This may suggest a reduced capacity to be stimulated by a sarcoidosis pathogen.

- A lower ability of BALF cells to release IL-23 upon stimulation with LPS was detected in sarcoidosis patients. This may result in a reduced capacity to mount a Th17 response.
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