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Studies of Different Clinical Manifestations of Sarcoidosis and the Role of Genetic Factors

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To my family

ABSTRACT

Sarcoidosis is a systemic disease of unknown etiology characterized by the formation of non-necrotizing granulomas in the affected organs. Engagement of the lungs and/or thoracic lymph nodes (LN) are found in more than 90 % of all cases, but almost any organ such as the eyes, skin, heart and nervous system can be involved. Genetic factors influence the risk for disease as well as the clinical picture seen in sarcoidosis and especially the genes localized to the human leukocyte antigen (HLA) region on chromosome six are believed to be of importance. For example, the HLA-DRB1*0301 allele is found to be strongly associated with Löfgren's syndrome (LS). Characteristic for LS is an acute onset usually with fever, bilateral ankle arthritis and/or erythema nodosum and bilateral hilar lymphadenopathy with in some cases parenchymal infiltrates. The HLA-DRB1*0301 allele is also associated with an accumulation of T cells expressing the T cell receptor variable gene segment AV2S3 in bronchoalveolar lavage fluid (BALF) of sarcoidosis patients.

The aim of this thesis has been to identify risk factors for different clinical manifestations in sarcoidosis as well as markers of importance for the inflammatory cell response seen in sarcoidosis.

The results show that HLA-DRB1*04 positive sarcoidosis patients had an increased risk for the three organ engagements associated with Heerfordt´s syndrome. Heerfordt´s syndrome is a phenotype of sarcoidosis that in its complete form consists of uveitis, parotid and/or salivary gland enlargement and cranial nerve palsy.

In comparison to BALF where a high CD4/CD8-ratio is strongly associated with sarcoidosis, the CD4/CD8-ratio in the affected LNs of sarcoidosis patients had no diagnostic value. Further, in HLA-DRB1*03 positive patients the associated accumulation of AV2S3+ T cells was strictly compartmentalized in BALF. This finding indicates an airborne antigen as the triggering factor in sarcoidosis.

The risk for cardiac sarcoidosis (CS) was significantly higher in patients with an abnormal electrocardiography (ECG) compared to those with a normal ECG. The risk for CS was highest in patients who had a pathologic ECG in combination with cardiac related symptoms. Further, non-LS was associated with an increased risk for CS.

In LS patients was the absence of HLA-DRB1*03 a risk factor for extra-pulmonary manifestations (erythema nodosum and ankle arthritis excluded). Another risk marker in all patients was HLA-DRB1*04/*15 where half of the patients had extra-pulmonary manifestations.

In conclusion, the HLA-DRB1*04 allele is associated with an increased risk for involvement of the eyes, parotid and/or salivary glands and cranial nerves in sarcoidosis patients. Moreover, an increased CD4/CD8-ratio in enlarged LNs is not diagnostic for sarcoidosis in comparison to BALF where a high ratio is strongly associated with sarcoidosis. Further, a pathologic ECG is a risk marker for CS in sarcoidosis patients. Finally, not only the single HLA-DRB1 alleles are of importance for the risk of extra-pulmonary manifestations in sarcoidosis, but also the allele combinations and where especially the combination HLA-DRB1*04/*15 calls for an increased awareness and a more intensive follow-up.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Sarkoidos är en inflammatorisk sjukdom som vanligtvis drabbar unga vuxna i åldrarna 20-40 år. Orsaken till sjukdomen är okänd. I Sverige insjuknar årligen ungefär 1500-2000 personer i sarkoidos. Lungförändringar ses hos de allra flesta med sarkoidos, men inte bara lungorna drabbas utan även andra organ kan engageras. Detta kan yttra sig i form av ögoninflammationer, svullna lymfkörtlar, nytillkomna hudförändringar och/eller oregelbunden hjärtrytm. I de angripna organen ses de för sjukdomen karakteristiska granulomen. Granulomen är ett sätt för kroppen att avskärma icke-eliminerbara antigen. Sarkoidos läker hos en del ut spontant inom två års tid medan andra får ett sjukdomsförlopp av mer kronisk karaktär. Vid svårare former av sarkoidos brukar behandling med kortikosteroider prövas. Behandlingen har i regel en symtomlindrande effekt på kort sikt, men det saknas vetenskapliga bevis för att behandling på lång sikt påverkar sjukdomsutvecklingen.

Vid sarkoidos leder inflammationen i lungorna till att vita blodkroppar ansamlas, vilka främst utgörs av CD4 positiva T-celler men också till viss del av CD8 positiva T-celler. T-cellerna aktiveras i lungornas lymfknutor via de antigenpresenterande cellerna. De antigenpresenterande cellerna visar upp delar av potentiellt skadliga partiklar som de har tagit upp för T-celler i form a små proteinfragment, men även kroppsegna ämnen visas upp. Normalt ska dock endast de skadliga partiklarna leda till aktivering av immunförsvaret. Presentationen av olika proteinfragment sker via de antigenpresenterande cellernas s k human leukocyte antigen (HLA)-molekyler vilka är receptorer som sitter fästa i cellmembranet. T-cellerna har motsvarande receptorer, vilka kallas för T-cellsreceptorer och finns i miljontals olika varianter. Bara de T-celler som har den rätta passformen på sina receptorer kan binda ett specifikt komplex av HLA-molekyl med bundet antigen.

Generna som styr HLA-molekylernas utseende finns i olika varianter och kallas för HLA-alleler. Vid sarkoidos, men även vid många andra inflammatoriska sjukdomar finns det kopplingar mellan HLA-alleler och risk att utveckla sjukdom. Vid sarkoidos är exempelvis HLA-genen HLA-DRB1*03 klart överrepresenterad hos patienter med Löfgrens syndrom. Löfgrens syndrom är en form av sarkoidos som karakteriseras av ett akut insjuknande med feber, smärta och svullnad utav fotleder och/eller röda ömmande utslag på underbenen, så kallad knölros, och förstorade lymfkörtlar centralt kring luftrören samt ibland även på lungröntgen synliga infiltrat. HLA-DRB1*03 hos patienter med sarkoidos är även kopplat till en ansamling av av CD4 positiva T-celler i lungorna vilka uttrycker T-cellsreceptorer med ett segment som kallas AV2S3.

Syftet med studierna i denna avhandling har varit att undersöka samband mellan olika faktorer bl a HLA-alleler och risken att drabbas av olika organmanifestationer. Ett delmål har också varit att öka förståelsen kring de inflammatoriska cellerna som återfinns i lungorna och betydelsen av olika markörer för det inflammatoriska svaret.

Resultaten visar att HLA-DRB1*04 positiva sarkoidospatienter hade en ökad risk att drabbas av någon av de tre organmanifestationerna som ses vid Heerfordt´s syndrom.

Heerfordt´s syndrom är en typ av sarkoidos som i sin kompletta form består av ögoninflammation, svullnad av spottkörtlar och engagemang utav någon av kranialnerverna, vanligast är förlamning av ena ansiktsnerven.

Till skillnad från en förhöjd CD4/CD8-kvot (antalet CD4+ T-celler/CD8 + T-celler) i lungsköljvätska, vilken är starkt kopplad till sarkoidos, visar resultaten att en förhöjd CD4/CD8-kvot i de förstorade lymfkörtlarna i lungorna var ett ospecifikt fynd vid sarkoidos. De specifika lymfocyter (så kallade AV2S3+ T-celler) som ansamlas i lungorna hos HLA-DRB1*03 positiva patienter, återfanns inte i samma grad i de förstorade lymfkörtlarna hos dessa individer. T-celler från blod och lymfkörtlar var dessutom inte heller i lika hög grad differentierade jämfört med de i lungsköljvätska. Resultaten visar således att inflammationen vid sarkoidos främst är lokaliserad till de små luftvägarna.

Vidare var risken för hjärtengagemang hos patienter med sarkoidos starkt associerad med ett avvikande elektorkardiogram (EKG). Allra störst var risken hos de patienter som hade EKG-förändringar ihop med symtom i form av hjärtklappning eller svimningskänsla/svimning. En ytterligare riskfaktorer för hjärtsarkoidos var icke Löfgrens syndrom. Patienter med ett normalt EKG och utan symtom på hjärtbesvär vid insjuknandet hade således en mycket låg risk att senare utveckla hjärtsarkoidos.

Vi fann även att avsaknaden av HLA-DRB1*03 var en riskfaktor för engagemang av ytterligare organ utöver lungorna hos patienter med Löfgrens syndrom (symtom ifrån fotleder samt knölros borträknade). En annan riskfaktor för engagemang av andra organ än enbart lungorna var HLA-DRB1*04/*15 där detta sågs hos hälften av alla patienterna med sarkoidos

Sammanfattningsvis var förekomsten av den genvariant som kallas HLA-DRB1*04 hos patienter med sarkoidos kopplad till en ökad risk för engagemang utav ögon, spottkörtlar och hjärnnerver. Vidare så var en förhöjd CD4/CD8-kvot i de förstorade lymfknutorna i lungorna ett ospecifikt fynd i motsats till en förhöjd CD4/CD8-kvot i lungsköljvätska, vilken är starkt associerad med sarkoidos. Baserat på resultaten i denna avhandling bör alla sarkoidospatienter med ett avvikande EKG utredas för hjärtsarkoidos. Slutligen så visar fynden i denna avhandling att arvsmassan i form av HLA-DRB1 alleler har stor betydelse för risken att drabbas av olika organengagemang vid sarkoidos. Således kan HLA-typning utgöra ett bra komplement för att identifiera sarkoidospatienter med hög risk att drabbas av ytterligare organengagemang utöver lungorna.

LIST OF PUBLICATIONS

The present thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. Darlington P, Tallstedt L, Padyukov L, Kockum I, Cederlund K, Eklund A and Grunewald J. HLA-DRB1* alleles and symptoms associated with Heerfordt´s syndrome in sarcoidosis. *Eur Respir J 2011;38:1151-1157.*

II. Darlington P, Haugom-Olsen H, von Sivers K, Wahlström J, Runold M, Svjatoha V, Porwit A, Eklund A, Grunewald J. T cell phenotypes in bronchoalveolar lavage fluid, blood and lymph nodes in pulmonary sarcoidosis - indication for an airborne antigen as the triggering factor in sarcoidosis. *J Intern Med 2012;272:465-471.*

III. Darlington P, Gabrielsen A, Sörensson P, Cederlund K, Eklund A, Grunewald J. Cardiac involvement in Caucasian patients with pulmonary sarcoidosis. *Submitted.*

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
APC	Antigen presenting cell
AV2S3	Variable gene segment 2.3 of the T cell receptor α chain
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BHL	Bilateral hilar lymphadenophaty
C	Constant
CS	Cardiac sarcoidosis
CD	Cluster of differentiation
CDR	Complementary determining regions
CMR	Cardiovascular magnetic resonance
CTL	Cytotoxic T lymphocyte
D	Diversity
DC	Denditric cell
DOTA-TOC	68 Ga-DOTA-d-Phe(1)-Tyr(3)-octreotide
ECG	Electrocardiogram
EUS-FNA	Endoscopic ultrasound guided fine-needle aspiration
FACS	Fluorescence-activated cell sorter
FOXP3	Forkhead box protein 3
HLA	Human leukocyte antigen
HS	Heerfordt´s syndrome
IFN	Interferon
IL	Interleukin
ILD	Interstitial lung disease
J	Joining
LN	Lymph nodes
LS	Löfgren´s syndrome
MHC	Major histocompatibility complex
NK	Natural killer
Non-LS	Non-Löfgren´s syndrome
PAMP	Pathogen-associated molecular patterns
PET-CT	Position emission tomography-computer tomography
PRR	Pattern recognition receptors
Tc	T cytotoxic
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
TTE	Transthoracic echocardiogram
V	Variable

GENERAL INTRODUCTION

Sarcoidosis was first described in 1899 as a skin disease by the Norwegian dermatologist Caesar Boeck. He thought the typical foci of epitheloid cells and giant cells resembled sarcoma and called the condition “multiple benign sarcoid of the skin”. Later, in 1909 another manifestation of sarcoidosis was described by the Danish ophthalmologist Christian Heerfordt who had observed three patients with uveitis, parotid swelling and cranial nerve palsy. The triad of symptoms was named Heerfordt´s syndrome, but was first in 1937 classified as a distinct manifestation of sarcoidosis by the Swedish physician Jan Waldenström. Another form of sarcoidosis was described in 1952 by the Swedish physician Sven Löfgren, which he called “the bilateral hilar lymphoma syndrome”, later named Löfgren´s syndrome. Characteristic for this syndrome is illness, high fever, ankle arthritis and / or erythema nodosum and bilateral enlarged medistinal lymph nodes with or without bilateral pulmonary infiltrates.

Almost any organ can be affected in sarcoidosis, although the lungs are involved in more than 90% of all cases. What causes the disease is, however, still unknown. Sarcoidosis is found throughout the world, but the clinical presentation and frequency differ. The disease outcome in sarcoidosis varies from spontaneous resolution to progressive disease with development of pulmonary fibrosis.

The overall aim of this thesis has been to improve our understanding on why the clinical picture in sarcoidosis is so highly variable and to identify risk factors which can be used in the clinic for early identification of high risk patients with regard to extra-pulmonary manifestations.

Hopefully, the results from this thesis will be applicable to the everyday clinical practice.

1 THE IMMUNE SYSTEM

The immune system is divided into the innate and adaptive part. The role of the innate immune system is to provide a first line of defense against pathogens. If a pathogen breaks through the barriers of the innate immune system, the adaptive immune system will take over. However, the defense mechanisms of the adaptive immune system take days to develop and during this period the innate immune system plays a critical role (1).

1.1 INNATE IMMUNITY

The principal defense components of the innate immune system are several and where the epithelial cells serve as a structural barrier, cytokines as directing signal proteins, macrophages and dendritic cells (DC) are important for activation of the adaptive immune system, neutrophils together with the macrophages are major phagocytes and recruit other cells to the site of inflammation. There are also natural killer (NK) cells capable to directly kill infected cells. Finally, the proteins of the complement system can cause damage to the membrane of pathogens or coat them so that they are more easily phagocytized (2).

1.1.1 Inflammatory mediators

Cytokines are signal proteins involved in the communication between cells in both the innate and adaptive immune system. Some cytokines promote inflammation (pro-inflammatory cytokines) and others suppress inflammation (anti-inflammatory cytokines). Cytokines also influence the differentiation of cells, for example T cells, into either T helper (Th)1 or Th2 cells (2, 3). Chemokines are another group of signal proteins which have chemotactic properties. They are important for recruitment of inflammatory cells from the circulation into infected tissues (4).

1.1.2 Macrophages

Macrophages originate from monocytes and differentiate into macrophages when they migrate from the blood to the lungs (5). The macrophages are phagocytes responsible for clearance of pathogens and have housekeeping functions. They can either be activated through the classic pathway or be alternatively activated macrophages (AAMs) (6). The classic pathway is induced by the Th1 cytokine interferon (IFN)-γ (7). Macrophages activated via the classic pathway produce interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, all important cytokines for the clearance of intracellular and bacterial pathogens. The differentiation of macrophages into AAMs is stimulated by the Th2 cytokines IL-4 and IL-13. The role of AAMs is not fully clear, but they are thought to be involved in the eradication of extracellular parasites and in several chronic airway diseases (8, 9).

1.1.3 Dendritic cells

DCs link the innate immune system with the adaptive immune system. For the recognition of pathogens the DCs have receptors termed pattern recognition receptors (PRR). After activation, the DC travels to a neighboring lymph node (LN) and there present the antigen it has encountered to T cells (10, 11).

1.1.4 Neutrophils

Neutrophils are normally found in the blood stream and are one of the first cells that migrate to the site of inflammation. They are phagocytes and recognize antigens that for example are opsonized (covered for recognition) with antibodies (12). Neutrophils will eliminate microbes by first phagocytize and then enzymatically digest them (13). They also produce cytokines that are important for the inflammatory answer (14).

1.1.5 Natural killer cells

NK cells are lymphocytes that are able to directly kill target cells and are important for the defense against viral infections. NK cells are activated by infected cells that express ligands for the NK cell´s activating receptors. There are also other receptors with inhibitory functions on NK cells, identifying the major histocompatibility complex (MHC) class I molecules found on all healthy cells. A loss of MHC class I receptors due to infection or transformation into cancer cells will lead to activation of NK cells (15-17). There are also cytokines that enhance the activity of NK cell such as the virus-induced type 1 interferons, IFN-α and β (18, 19). NK cells produce cytokines themselves such as IFN-γ and TNF-α (20).

1.1.6 Innate immune recognition

PRRs of the cells of the innate immune system recognize pathogens through molecules in the bacterial cell membrane. The structures that the PRRs bind to are termed pathogen-associated molecular patterns (PAMP) (13, 21, 22). Among the PRRs, the most well-known is the toll-like receptors (TLR) (23, 24). The TLR was first discovered in fruit-flies and later on in humans (25-27). Mutations in the TLR genes have been associated with severe bacterial infections (28, 29). For the discovery of the critical role of the TLRs, Jules Hoffman and Bruce Beutler were rewarded with the Nobel Prize in 2011.

1.2 ADAPTIVE IMMUNITY

Characteristics for the adaptive immune system are specificity, memory and diversity. The main cells of the adaptive immune system are the lymphocytes which are divided into T cells and B cells. Example of T cells are the Th1 cells which enhance macrophages ability to phagocyte and kill pathogens and the Th2 cells important for the activation of B cells, cytotoxic T lymphocytes (CTL) which kill cells infected with viruses or intracellular bacteria, T regulatory cells (Treg) that suppress other T cells from reacting against self-antigens and Th17 cells which are believed to be involved in

the defense against bacterial infections and may be of importance for developing autoimmune diseases. The B cells are able to transform into antibody producing plasma cells which neutralize bacterial toxins, activate the complement system and opsonize bacteria and thereby facilitate the phagocytosis of them (2).

1.2.1 Antigen presentation

For the activation of T cells the antigen presentation is important. Involved in this process are macrophages, DCs and B cells, termed antigen presenting cells (APC). T cells not yet activated by APC are named naïve T cells. Naïve T cells circulate continuously between the blood and lymph nodes (LN). When a naïve T cell meet within a LN an APC which presents the right antigen, the naïve T cell will become activated (30, 31). APCs have specific receptors on the cell surface used for antigen presentation termed major histocompatibility complex (MHC), which in humans is named human leukocyte antigen (HLA) receptor (Figure 1) (32). T cells have receptors named T cell receptors (TCR) by which they bind to the MHC receptor. TCRs are only able to bind to a complex of an antigen bound to an APCs´ MHC molecule if it has the right pass form (33-37). For this discovery of MHC restricted antigen recognition, Rolf Zinkernagel and Peter Doherty were rewarded with the Nobel prize in 1996 (38).

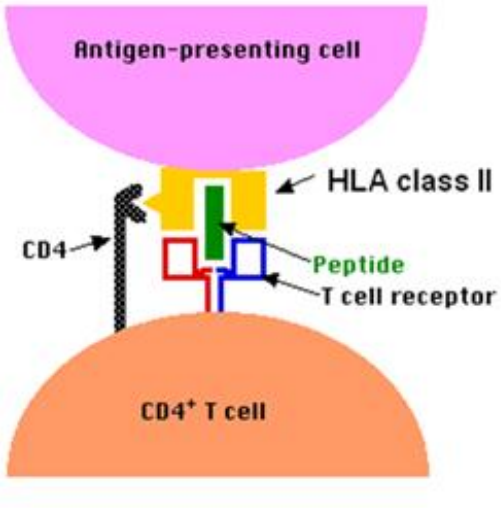

Figure 1. Antigen presentation.

The interaction between T cells and APCs are enhanced by specific co-receptors named cluster of differentiation (CD) 4 (found on CD4+ T cells) and CD8 (found on CD8+ T cells) (39). The CD4 co-receptor only binds to MHC class II molecules and CD8 to MHC class I molecules. After activation the naïve T cell will proliferate and all T cells produced will have the same specificity of their TCR as the initially stimulated T cell had. These new T cells are named effector T cells since they produce molecules able to eliminate antigens. The effector T cells will eventually leave the LNs and accumulate at site of inflammation, stimulated by the antigen that the naïve T cell initially was presented to (40). Still, after several years there are specific T cells able to recall antigens they once have been stimulated by. These T cells are named memory T cells and there are two different types: effector memory T cells and central memory T

cells. Effector memory T cells react immediately upon antigenic stimulation, but lack LN homing receptors. The central memory T cells on the other hand have LN homing receptors and do therefore confer a more superior protection compared to the effector memory T cells (41).

1.2.2 Human leukocyte antigen class I and II

The HLA-molecules are coded by genes that belongs to either HLA class I or II (42). The third class, HLA class III, encodes for proteins involved in the complement system and cytokines such as TNF. The genes of the HLA region are localized on chromosome six (43). HLA class I genes encodes for HLA class I molecules found on the cell-surface of all cells. HLA class I molecules are used for the presentation of intracellular peptides to CD8+ T cells. HLA class II is found on the cell-surface of APCs which use the receptor to present peptides they have taken up and degraded to T cells and B cells (44).

The HLA class I and II molecules have one α- and β-chain (45). There are three classical variants of HLA class I: HLA-A, -B and –C. The genes of HLA class II are named HLA-DR, -DP and –DQ (Figure 2) (46, 47). There are separate genes encoding the α- and β-chains. Variants of these genes ("alleles") code for distinct HLA molecules and the HLA-DRB1 gene includes variants termed e.g. HLA-DRB1*01,*03,*04,*07, *08, *09, *10, *11, *12,*13,*14,*15,*16. Since there is one allele inherited from each parent, a combination may for example be HLA-DRB1*01/*03 (48). Since the HLA class I and II alleles encode for receptors involved in the antigen presentation, the high diversity minimizes the risk for a pathogen to escape detection (49). The variation of alleles have developed during the evolution and as a result there are differences between ethnic groups (50).

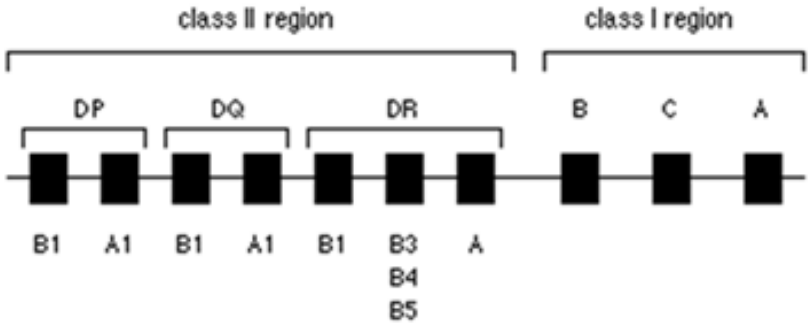

Figure 2. HLA alleles. Adopted from Hurley et al (51).

Several autoimmune diseases are associated with the HLA alleles (52, 53). One of the first described is ankylosing spondylitis strongly linked to HLA-B27 (54). Another disease is diabetes type I where there is an increased risk in individuals who carry HLA-DRB1*03 or *04, and highest is the risk in those who carry both the alleles (55). HLA-DRB1*04 is also in the Northern Europe associated with rheumatoid arthritis (56). One theory proposed about how the HLA-alleles contribute to the autoimmunity

is that individuals with an autoimmune disease-associated MHC phenotype will allow potentially self-reactive T cell clones during the process of positive and negative selection in the thymus. The varying penetrance of autoimmune diseases in individuals with a MHC-associated phenotype would then depend on the likelihood that T cells with the correct set of V(variable) - J (joining) α and V- D (diversity) - J β heterodimers will be formed (see next chapter) (57).

1.2.3 The T cell receptor

The TCR consists of one α- and β-chain and each chain has a constant (C) and a V region. The V region is responsible for the antigen recognition (58). The V region of the α-chain consists of randomly combined V- and J- segments and the same region of the β-chain of V-, D and J segments (59). In the V region of each α-chain and β-chain there are three highly diverse loops termed complementary determining regions (CDR). Out of these three, CDR3 is the most variable and play a major role in the interaction with antigen peptides presented on MHC molecules (60). The TCR´s structure resembles the antibody's and there are important structural sites on antibodies also found on TCRs (61). Similar to antibodies the antigen binding site of the TCR is created by gene rearrangements, which generates an immense diversity. TCRs are therefore able to interact with virtually any antigen (62, 63). During this recombination process, there is a random addition or removal of nucleotides at the junctions between the segments, in order to increase diversity of the TCR. The exon is then spliced together with the C region (59).

In contact with an antigen, only a small fraction of T cells will proliferate and differentiate into effector cells (64). Expansion of T cells with a certain TCR able to identify specific antigens have been described in both infectious (65, 66) and inflammatory diseases (67-70). Since the V region is important for the antigenic recognition, a mutation in this region may lead to an altered antigen specificity of the TCR (71). In addition to the αβ TCR there is also a few percentages of TCRs built up by γ and δ chains. The function of the γδ TCR is not yet known (72, 73).

During the T cell development all T cells go through positive and negative selection processes (74-76). During this procedure it is also decided if a T cell will be CD4+ or CD8+. Initially all T cells are double positive. Later, the T cells that bind to MHC class I will lose expression of co-receptor CD4 and be only CD8+, and those who bind to MHC class II will lose expression of CD8 and be only CD4+ (77). Since some self-reactive T cells will escape the system of negative selection in the thymus, there are several back-up systems in the periphery to protect the body against autoimmune reactions. These defense mechanisms include deletions of self-reactive T cells and unresponsiveness if there is no co-stimulation (anergy) (78, 79) and suppression via Tregs (80).

There are several mechanisms proposed to explain how self-reactive T cells can be activated despite the protective systems in the periphery. One of them is that cytokines released by APCs during an infection may lead to a more efficient antigen presentation of self-antigens to self-reactive T cells (81). Another is molecular mimicry, i.e. that a

pathogen mimics a self-antigen and which leads to a cross-reaction where the immune response against a viral or bacterial infection is re-directed to self-cells and tissues (82, 83). Furthermore, superantigens produced by viruses or bacteria (for example staphylococcal enterotoxins) cause non-specific T cell activations. Superantigens are able to bind to MHC class II molecules and the complexes that are formed can interact with certain T cells by binding to their TCRs´ Vβ element and thereby stimulate these T cells, irrespective of their fine-specificity. T cell populations stimulated by superantigens are therefore polyclonal, but have certain TCR Vβ gene expressions in common (84-87). Moreover, a viral infection may lead to exposure of cellular proteins normally shielded from the immune system by making them aberrantly expressed (88-91).

1.2.4 Cell surface molecules

The nomenclature CD is used for identification of cell surface molecules found on leukocytes such as adhesions molecules or receptors important for cell signaling. They are identified by panels of monoclonal antibodies used to investigate a particular cell type or stage of cell differentiation, for example do Th cells express the co-receptor CD4 and cytotoxic T cells CD8. T helper cells are therefore said to be CD4+ and cytotoxic T cells to be CD8+. Both CD4+ and CD8+ T cells are said to be CD3+ since they express another marker, CD3, which consists of a complex of three proteins closely attached to the TCR (2). CD69 and CD27 are examples of markers of activation and differentiation. CD69 is expressed on very early activated T cells, already 1-2 hours after stimulation, and persists for at least three days (92), while CD27 is expressed by naïve T cells and is up-regulated during T cell activation, but gradually down-regulated as the T-cells differentiate (93).

1.2.5 T helper cell 1 and 2

Th cells are divided into several subgroups. This chapter will focus on the two first discovered types, Th1 and Th2. The Th1 cells are mainly responsible for the defense against intracellular pathogens by activation of macrophages, enhancing their ability to phagocyte and kill pathogens. The Th2 cells are important for the extra-cellular immunity and humoral response, i.e. clearance of extracellular pathogens, parasites and toxins and for the proliferation and differentiation of B cells (94, 95). The differentiation of T cells into Th1 or Th2 cells is influenced by cytokines and the two subsets themselves do also produce cytokines (Figure 3) (96). For example IL-12 produced by APCs promotes naïve T cells to differentiate into Th1 cells (97-99) and IL-4 induces Th2 differentiation (100). Typical cytokines produced by Th1 cells are IL-2, TNF and INF-γ and by Th2 cells IL-4, IL-5, IL- 10 and IL-13. Th1 and Th2 cells also regulate each other so that the Th1 cytokines inhibit the proliferation of Th2 cells and vice versa (101).

1.2.6 T helper 17 cells

Previously it was believed that the Th cells consisted only of Th1 or Th2 cells. In allergy research it was much discussed that an imbalance of these two could cause allergic diseases. The discovery of a third subgroup named Th17 has therefore filled an

essential gap in the understanding of inflammatory processes. The hallmark cytokine of Th17 is IL-17 (IL-17A) which has pro-inflammatory properties and stimulates production of anti-microbial peptides, cytokines, chemokines and growth factors leading to recruitment of neutrophils and inflammation (102). IL-17 binds to IL-17 receptors found on fibroblasts, epithelial cells and keratinocytes (2, 103). Th17 cells also produce IL-17F, IL-6, IL-21, IL22 and TNF-α (104). Th17 cells are mainly found close to epithelial cells and protect against extracellular bacteria and fungi (105, 106). One of the markers for Th17 cells is the transcription factor retinoid orphan receptor gamma T (RORγT) (107). Th17 cells have been associated with several autoimmune and inflammatory disorders, e.g. rheumatic arthritis, asthma and chronic obstructive pulmonary disease (COPD), where an accumulation of Th17 has been reported (108-111).

1.2.7 CD8+ T cells

CD8+ T cells, i.e. CTLs, kill viruses and intracellular pathogens. They recognize antigens presented by HLA class I molecules (112). Upon stimulation, the CD8+ T cells differentiate into effector CD8+ T cells able to induce cell death; either through release of cytotoxic molecules such as perforin (113) or through binding to death receptors (Fas) on target cells and thereby stimulate apoptosis (114). Like CD4+ T cells, CD8+ T cells can upon stimulation by cytokines differentiate into T cytotoxic (Tc) 1 or Tc 2 cells with a cytokine profile similar to Th1 and Th2 cells (115-117).

1.2.8 T regulatory cells

Tregs is a subset of T cells which balance inflammatory and antigen specific responses. Abnormalities in the function of Tregs have been described in many autoimmune and chronic inflammatory disorders (118-121). In the protection against potential hazardous self-reactive T cells and to maintain immunologic self-tolerance there a two systems involved, commonly referred to as central and peripheral tolerance. The system of central tolerance is localized to the thymus. Since some self-reactive T cells will escape there are additional defense mechanisms in the periphery. For the peripheral tolerance the Tregs play an important role (122). Tregs develop in the thymus and characteristic for them is their lack of proliferative response towards antigenic stimulation. Instead, the Tregs suppress the proliferation of naïve T cells that respond to self-peptides (123).

The first research about Tregs began with studies of cell induced multiorgan autoimmunity in recipient animals by adoptive transfer of T cells depleted of CD4+CD25+ (124). Tregs were therefore initially described as CD4+CD25+ T cells, which constitute 5-10% of all CD4+ T cells. Later, it was shown that they specifically express the transcription factor forkhead box protein 3 (FOXP3), today used as a marker for Tregs (119, 125-128). The transcription factor FOXP3 is critical for the development and function of Tregs (129). There is a rare disease in humans caused by a mutation in the FOXP3 gene named IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked) associated with severe autoimmunity (130, 131).

Normally functioning Tregs are proposed to use four suppressive mechanisms; by expression of the inhibitory cytokines IL-10, IL-35 and transforming growth factor-β (TGF-β) (132-135), via cytolysis (136), through consuming IL-2 produced by effector T cells and thereby starve the self-reactive T cells, leading to a decrease in T cell activity and proliferation (137, 138) and by inhibition of dendritic cell maturation and function (Figure 3) (139, 140).

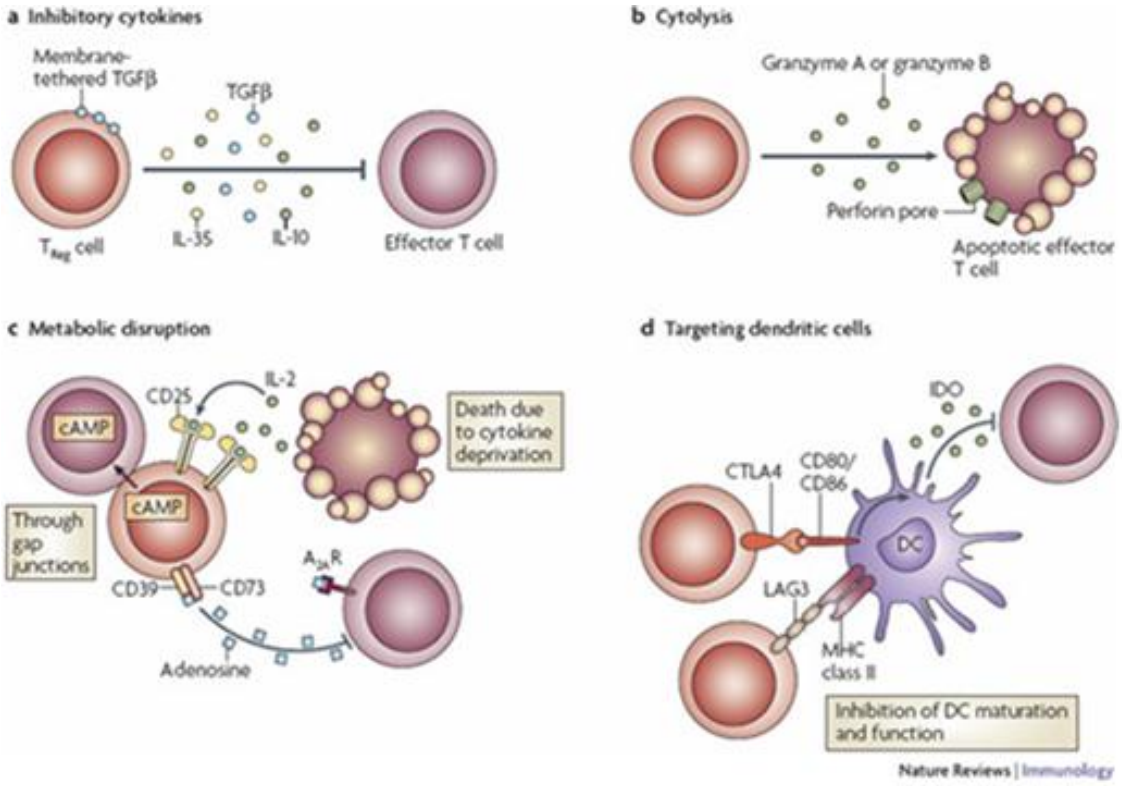

Figure 3. Basic mechanisms used by Tregs: a) by expression of the inhibitory cytokines IL-10, IL-35 and TGF-β, b) via cytolysis, c) through consumption of IL-2, d) by inhibition of dendritic cells maturation and function. Adopted from Vignali et al (141).

The Tregs developed in the thymus are said to be natural occurring Tregs but there are also other subsets of Tregs, generated from mature T cell populations under certain conditions of antigenic stimulation such as infection. These adaptive Tregs produce anti-inflammatory cytokines which inhibit inflammation (142, 143) and they include IL-10 secreting T regulatory (Tr)1 cells, inducible FOXP3+ Tregs, Th3 cells (producing TGF-β) and double negative Tregs (144). They are believed to be induced by DCs with an activation status distinct from the DCs that promote differentiation of T cells into Th1 or Th2 cells (145).

1.2.9 B cells

B cells produce antibodies, known as immunoglobulins, and also function as APCs. The activation of B cells is either performed in a T cell dependent or a T cell

independent way. Upon the T cell-dependent activation the B cell will first internalize an antigen bound to the B cell receptor (BCR) and thereafter process it and then present parts of it as peptides bound to the B cell´s HLA class II molecule. The T cell that binds to this complex consisting of an antigen and HLA class II molecule will activate the B cell. B cells are sometimes also activated in a T cell independent way by binding directly to an antigen (146, 147). Activated B cells will transform into plasma cells able to produce antibodies and some undergo immunoglobulin (Ig) class switch to become more effector specialized (148). There are also B cells which will become long-lived memory cells (149).

1.3 THE RESPIRATORY SYSTEM

The main function of the respiratory system is to supply the body with oxygen and exhale carbon dioxide. The respiratory system is divided into the upper and lower part. The upper part of the respiratory system consists of the nose, nasal cavity, pharynx and larynx. The lower parts include trachea, bronchi, bronchioles and where the alveoli are the most distal part (Figure 4). Gas exchange takes part in the small alveoli by passive diffusion (150).

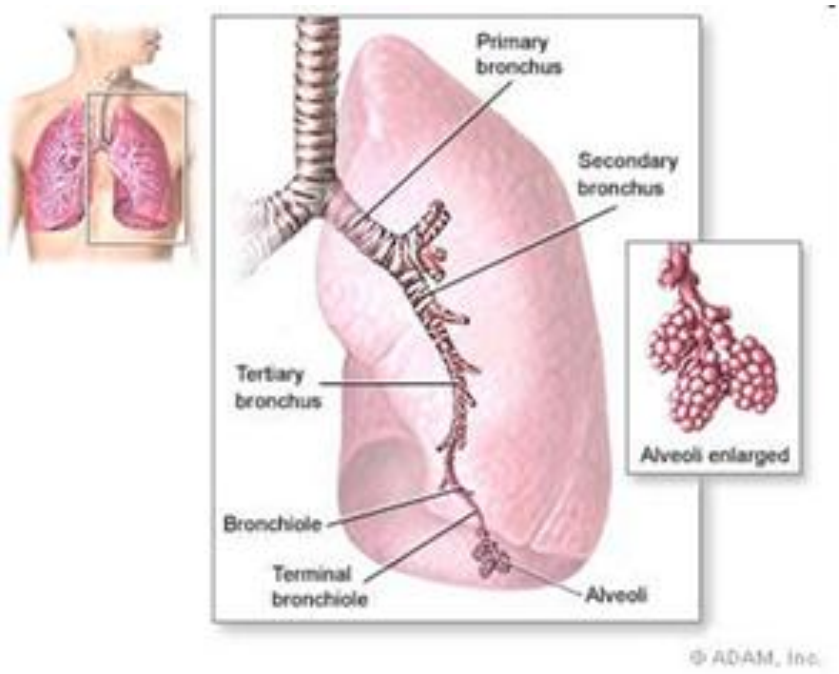

Figure 4. Illustration of the respiratory system.

Inflammatory diseases of the respiratory system can be either obstructive where inflammation leads to narrowing of the airways (e.g asthma and chronic obstructive pulmonary disease) (151) or restrictive i.e. interstitial lung diseases where the inflammation causes increased lung stiffness (e.g sarcoidosis and idiopatic pulmonary fibrosis) (152).

1.3.1 Lung immunity

The epithelium that lines the bronchial tree represents the first defense against harmful agents in the lung and acts as a physical barrier. The epithelium in the upper airways is mainly built up by ciliated cells that transport particles away from the lungs. Between the ciliated cells there are mucus producing goblet cells which together with the cough reflex helps to protect the airways (Figure 5) (153). The Epithelial cells produce IgA which neutralizes toxins and viruses and blocks bacteria to entry across the epithelium (154). Epithelial cells also send out signals, i.e. chemokines, which lead to recruitment of inflammatory cells into the airways (155). Important APCs in the lung are the DCs that upon inflammatory stimulation will migrate to the hilar LNs and there activate T cells (156). Even for the initial activation of DCs are the multifunctional epithelial cells important (155). For identification of pathogens, cells of the innate immune system in the lung have PPRs by which they recognize microbial products such as lipopolysaccharides (LPS), which are components in the membrane of gram-negative bacteria (157). The key mediators of the adaptive immune system in the lung are the lymphocytes which directly influence important events in pulmonary inflammation and repair (158).

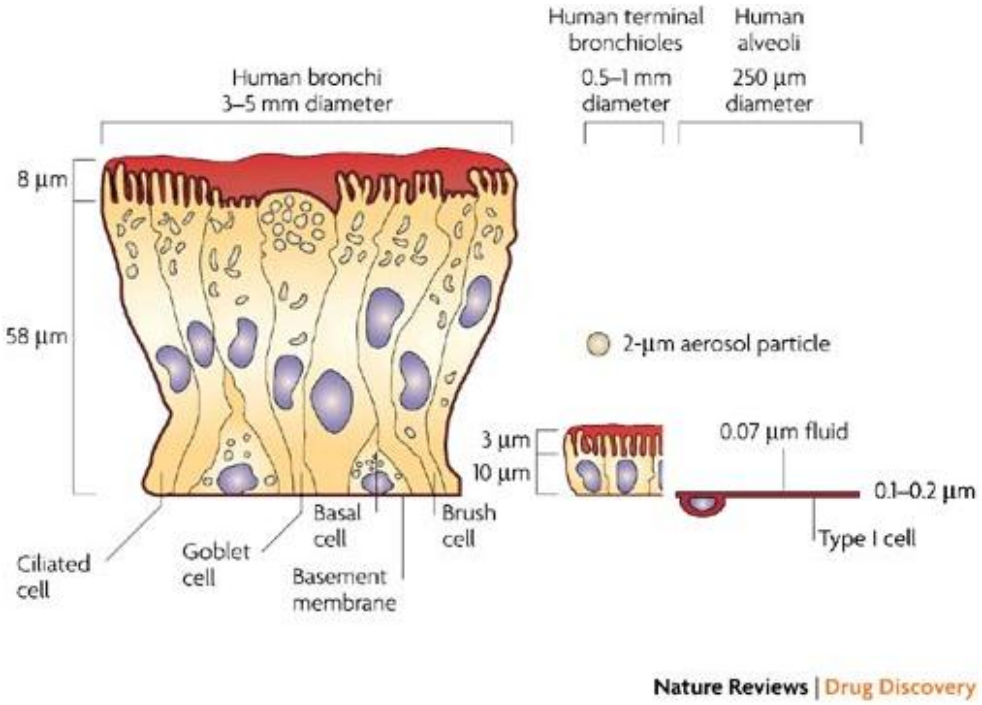

Figure 5. Illustration of airway epithelia. Adopted from Patton et al (159).

Macrophages are the predominant cells in the airways of the healthy lung. They are except from being phagocytes able to engulf harmful foreign particles such as bacteria and dead or dying cells, also able to secrete pro-inflammatory mediators i.e. cytokines and chemokines. These signal proteins are important for the recruitment of other immune cells (158). Macrophages arise originally from monocytes and are a very heterogeneous population. Based on the anatomical location in the lung, there are alveolar, interstitial and intravascular macrophages, all with different functions (160).

1.3.2 Interstitial lung diseases

Interstitial lung diseases (ILD) consist of a heterogeneous group of parenchymal lung disorders. Some ILDs are caused by inhaled organic or inorganic substances, e.g. hypersensitivity pneumonitis and asbestosis. Others are parts of a rheumatic systemic disease and interstitial pulmonary infiltrates may occur in patients with for example rheumatic arthritis and scleroderma. Furthermore, drugs used in the treatment of rheumatic systemic diseases sometimes cause ILD themselves (161). It is often not possible to differentiate between the types of ILDs with just a histological sample. Therefore, a good patient history and a careful examination of the patient are important. Questions that could give valuable information are for example about exposure to organic or inorganic substances and what medicines the patient takes. Investigation methods that can be essential in the diagnostic procedure are high resolution computed tomography (HRCT), bronchoalveolar lavage (BAL) and sometimes an open lung biopsy is required (162). Since the accumulated cells in the lung differ between ILDs, the findings in BAL may give guidance (163-165).

1.3.3 Bronchoalveolar lavage

The development of the technique with flexible bronchoscope and BAL has made it possible to study inflammatory cells in the lung. The method is safe and non-invasive and has been used since the late 1960s (166). To perform a BAL, saline solution is first installed via a bronchoscope in a selected location in the lung and thereafter as much as possible of the fluid is aspirated. The BAL is later analyzed with regard on how many percent there are of macrophages, neutrophils, eosinophils or lymphocytes (167). The results can be indicative or diagnostic for certain diseases together with findings from for example chest X-ray. In the healthy lung, the macrophages are the predominant cell population found in bronchoalveolar lavage fluid (BALF) (168). In diseases such as sarcoidosis and hypersensitivity pneumonitis there is instead an increased percentage of lymphocytes, consisting mainly of T cells (169). In infectious diseases of the lower respiratory tract the number of neutrophils are usually increased (170), which is also the major lavage finding in patients with idiopathic pulmonary fibrosis (171). Except from counting the cells can analysis of cell-surface markers via a fluorescence-activated cell sorting (FACS) machine of BALF be of diagnostic help. Lymphocytes can for example be marked for CD4 and CD8 and where a CD4/CD8-ratio >3,5 strongly supports the sarcoidosis diagnosis (165).

1.3.4 Endoscopic ultrasound guided fine-needle aspiration

As a complement to bronchoscopy it is often of differential diagnostic interest to examine enlarged mediastinal LNs in patients with pulmonary infiltrates. Until recently, sampling of mediastinal LNs has required mediastinoscopy (172). Therefore, the availability of the less invasive method with endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) via esophagus has become a valuable tool for the diagnostic procedure. The specificity and sensitivity in sarcoidosis patients have been shown to be 94% and 100%, respectively (173). Another way to take samples from the mediastinal LN is via endobronchial ultrasound guided transbronchial needle aspiration

(EBUS-TBNA). For the choice of method it is important to determine the position of the mediastinal LNs since some are possible to reach only via EUS-FNA, while others only can be accessed via EBUS-TBNA (174).

1.4 SARCOIDOSIS

1.4.1 Epidemiology

Sarcoidosis affects mainly young adults (25-35 years) with a second peak in women aged 45-65 (175). The risk of contracting sarcoidosis is higher for non-smokers than smokers (176). In Sweden there are approximately 1 500 - 2000 people diagnosed annually with sarcoidosis (175). Sarcoidosis is found throughout the world, but the frequency varies between different countries and ethnic groups (177). Afro-Americans are for example diagnosed three times more often than Caucasians and often get a more aggressive form of disease (178-182). Another example of differences between ethnic groups is the ocular manifestation uveitis, where the anterior form is reported to be more common in Afro-Americans (70-75%) while posterior uveitis is more often seen in Caucasians (65-83%) (183). Moreover, eye symptoms are much more frequently seen in Orientals in comparison to Scandinavians where instead Löfgren´s syndrome (LS) with bilateral hilar lymphadenopathy (BHL), fever and erythema nodosum and/or ankle arthritis is common (184-186). There is also a seasonal variation in the onset of LS with two peaks, a first in January and a second in April and May (187, 188). Moreover, cardiac sarcoidosis is several times more frequent in Japan where it is the leading cause of death related to sarcoidosis (189). Elsewhere, respiratory failure is the most common cause. The overall mortality rate due to sarcoidosis is believed to be between 1 and 5 % (190).

1.4.2 Etiology

The etiology of sarcoidosis is still unknown, but there are several antigens proposed such as organic and inorganic particles, for example insecticides (191-194). Furthermore, there are data supporting that mycobacteria may be a triggering factor of sarcoidosis (195, 196). Studies have shown that lung and blood CD4+ and CD8+ T cells from sarcoidosis patients respond to multiple mycobacterial peptides (197, 198) and one identified candidate antigen is the Mycobacterium tuberculosis catalase-peroxidase (mKatG) protein (199, 200). There are also reports about other bacteria associated with sarcoidosis such as propionibacterium acne (201, 202). Furthermore, differences between ethnic groups indicate that genetic factors play an important role for the risk of developing disease (203, 204)

1.4.3 Genetic factors

Genetic factors influence the clinical picture seen in sarcoidosis and especially the genes localized in the HLA region on chromosome six are believed to be of importance (205). In sarcoidosis, there are associations reported to both HLA class I and II genes (204, 206, 207) as well as to the genes involved in the activation of T cells and macrophages (208-210). One example of a HLA class II allele linked to sarcoidosis is

HLA-DRB1*03 associated with LS (188). LS is commonly seen in Scandinavia but very rare in patients of Japanese origin in whom the HLA-DRB1*03 allele also is virtually missing (211, 212). In LS patients the HLA-DRB1*03 allele strongly influences the disease course with complete recovery within two years in almost all DRB1*03 positive patients, but only in half of the DRB1*03 negative patients (188). In contrast, the allele HLA-DRB1*15 is associated with a non-resolving disease in sarcoidosis (213). Furthermore, there is a strong association between HLA-DRB1*03 and an oligoclonal expansion in BALF of CD4+ T cells expressing a TCR V gene segment termed AV2S3 (214). A similar accumulation of TCR AV2S3+ T cells in BALF is also seen in HLA-DRB1*13 positive patients (215, 216). This correlation likely occurs because HLA-DRB1 *13 positive patients sometimes carry the HLA-DRB3*0101 allele, which is known to be structurally similar to HLA-DRB1*0301 and therefore able to present comparable antigenic peptides (217).

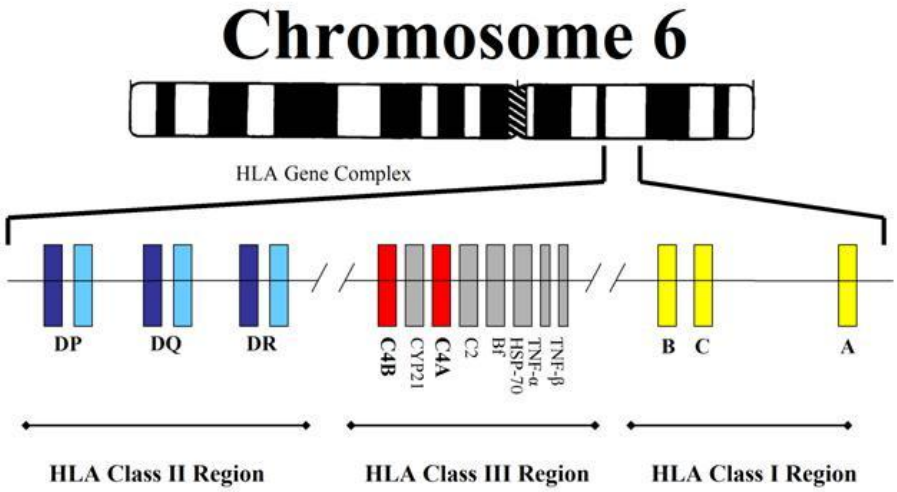

Figure 6. A simplified map over the HLA-region.

There are also other genes closely linked to the HLA class II alleles that have been associated with sarcoidosis (Figure 6). Examples are the associations between uveitis caused by sarcoidosis and the heat shock protein (HSP)70/Hom rs2075800 G allele in patients from the United Kingdom and Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) gene polymorphism in patients from Japan (218, 219). Furthermore, mutation in the Butyrophilin-like 2 gene (BTNL2) located close to the HLA genes has also been associated with an increased risk for sarcoidosis. The BTNL2 gene is thought to influence T cell activation and regulation (220). There are also reports about a higher frequency of the TNF allele TNF-A2 in patients with LS (221, 222). Moreover, it has been reported about associations between complement C4 and sarcoidosis (223). The role of linkage disequilibrium (LD), defined as a tendency for genetic variants located close to each other on the same chromosome to be associated within a population more often than if they were unlinked, have been discussed in sarcoidosis. Because of the strong LD within the HLA-region there are difficulties to determine which gene/s

represents the primary association and whether nearby located non-HLA genes have any role (224).

1.4.4 Pathogenesis

Sarcoidosis is a multisystem inflammatory disease of undetermined etiology. In the lungs of sarcoidosis patients there is commonly seen an increased percentage of lymphocytes, causing alveolitis. Among the lymphocytes found in BALF there are usually more CD4+ T cells then CD8+ T cells. The CD4+ T cells consist mainly of Th1 lymphocytes, reflected by a cytokine profile with high levels of IL-2 and IFN-γ (225-231). Further, cell surface markers of activation/differentiation on the CD4+ T cells such as CD69 and CD27 in bronchoalveolar lavage fluid (BALF) of patients with sarcoidosis show that these T cells are highly activated (232).

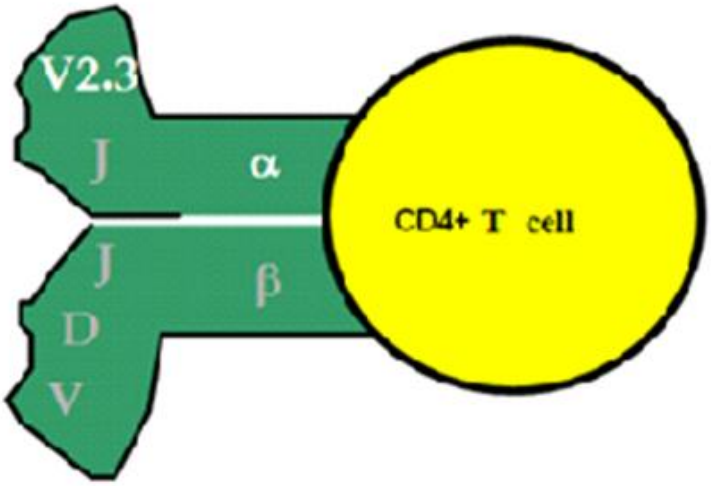

Figure 7. T cell receptor with the V-segment AV2S3.

Another marker of active disease in BALF of sarcoidosis patients is the expansion (>10%) of T cells expressing the T cell receptor V gene segment AV2S3 which are seen in patients who are HLA-DRB1*03 positive (Figure 7). The accumulation of AV2S3+ T cells in BALF is believed to be due to a response against a specific antigen (232). However, the expansion is oligoclonal since the α-chain could be paired with different β-chains (233). Analysis of cell-surface markers on AV2S3+ T cells together with the intracellular marker FOXP3 show that these AV2S3+ T cells are highly activated effector cells and not Tregs (234). Moreover, an increased percentage of AV2S3+ T cells in sarcoidosis is associated with a better prognosis (235). The ratio of AV2S3+ T cell is later normalized when the patient has recovered (185).

The CD8+ T cells found in BALF of sarcoidosis patients are also highly activated with a high capacity to produce IFN-γ after *in vitro* stimulation, which is even more pronounced for the CD8+ T cells compared to the CD4+ T cells (230). In sarcoidosis, there are also activated B-cells, probable as a result of an increased cytokine secretion

by the T cells. This leads to production of autoantibodies which form the immune complexes associated with erythema nodosum seen in patients with LS (236).

In sarcoidosis, not only the lymphocytes are important for the disease course but also the macrophages. The production of the pro-inflammatory cytokine TNF-α by macrophages is central for the formation of granulomas seen in sarcoidosis. The level of TNF-α is found to be increased in sarcoidosis patients with active disease (237-239). It has been suggested that Tregs in patients with sarcoidosis have reduced inhibitory capacities (240, 241) and are unable to inhibit the production of TNF-α (242). The activated macrophages in the lungs of sarcoidosis patients also overproduce 1,25-dihydroxy-vitamin D_3 (calcitriol) which leads to an increased absorption of calcium in the gut, sometimes leading to hypercalcemia (243).

Characteristic for sarcoidosis is the typically found non-necrotizing granulomas. In infectious diseases, the formation of granulomas for example in Tuberculosis is to prevent spreading of harmful antigens that the body cannot eliminate. In sarcoidosis, the function of the granulomas is not believed to be protective, but instead contribute to the tissue pathology. There are other systemic non-infectious diseases that also go with granuloma formation. However, typically for sarcoidosis is the presence of multinucleated giant cells in the granulomas. In sarcoidosis, the granulomas are formed by an aggregation of lymphocytes, macrophages, epitheloid cell, mast cells and fibroblasts (Figure 8). The epitheloid cells of the granulomas overproduce angiotensin-converting enzyme (ACE), and an elevated ACE level is therefore common in sarcoidosis patients (244). The ACE level is believed to reflect the total burden of granulomas (245).

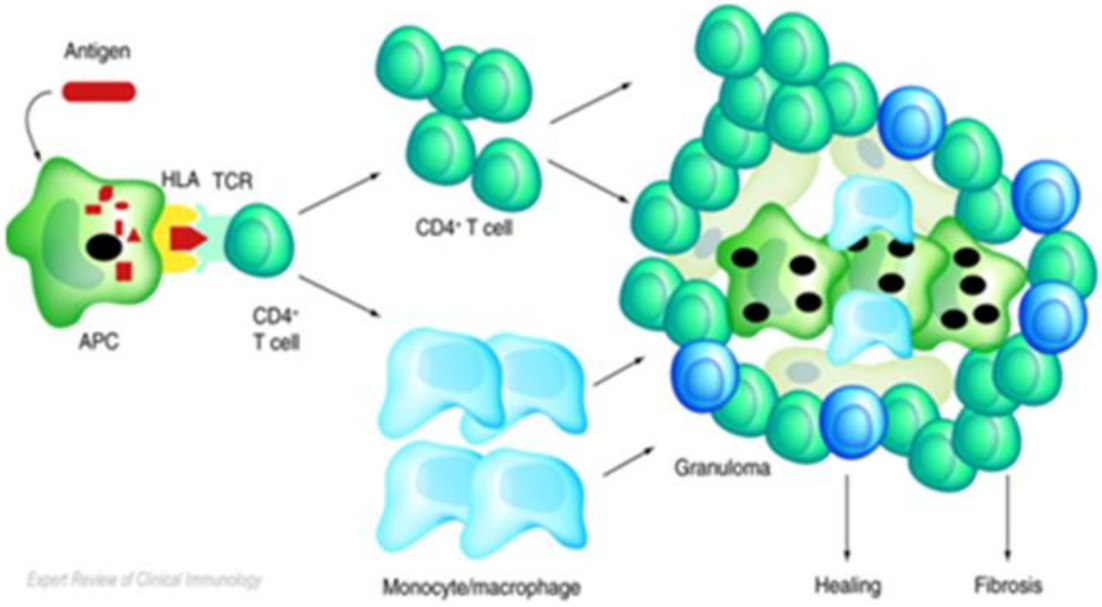

Figure 8. Granuloma formation. Adopted from Grunewald et al (246).

The granuloma formation is initiated by an accumulation of CD4+ T cells and macrophages (204, 247). The CD4+ T cells are found mainly within the central portion of the granuloma and in the periphery there are CD8+ T cells. In a sarcoid granuloma

that undergoes fibrotic changes the process starts in the border and travels centrally. CD8+ T cells, fibroblasts and regulatory cells will become more prominent as the activity of the granuloma diminishes and fibrosis ensues (190, 248-250). In patients who develop fibrosis in their granulomas it has been suggested that there is a switch from a Th1 phenotype to Th2 phenotype, and where the Th2 cytokines IL-4 and IL-13 functions as chemo-attractants for the fibroblasts (251). In summary, pulmonary sarcoidosis begins as an alveolitis with development towards a multinucleated giant cell granulomatous disease, followed by either resolution with no or little sequele, or progressive fibrosis leading to an end-stage lung disease.

1.4.5 Clinical features

Sarcoidosis is a granulomatous disease that affects the lungs and/or intrathoracic LNs in more than 90% of all cases, but almost any organ can be involved such as the skin, peripheral LNs, eyes, nervous system, kidneys, heart, liver, spleen and bone marrow (252, 253). A specific phenotype of sarcoidosis is LS, characterized by acute disease onset with erythema nodosum and/or ankle arthritis, fever and bilateral hilar lymphadenopathy and occasionally with pulmonary infiltrates (254, 255). LS was first described by the Swedish physician Sven Löfgren in 1952 who had studied patients with erythema nodosum and its various causes. He described a condition that he termed "the bilateral hilar lymphoma syndrome", later known as LS (256). LS usually has a very favorable outcome with a spontaneous and complete resolution (188). However, the most common variant of sarcoidosis is non-LS with a more insidious onset with dry cough and fatigue (252).

Extra-pulmonary manifestations are commonly seen in patients with sarcoidosis and one example is cardiac involvement which can manifest itself in the form of benign arrhythmias, but in the worst case as sudden death (257-259). Findings from autopsy studies indicate that the incidence of cardiac sarcoidosis (CS) likely is higher than what is diagnosed in the clinic (189, 260). Another extra-pulmonary manifestation is ocular sarcoidosis and where uveitis is the most common manifestation (261). Examples of other ocular engagements are lacrimal gland inflammation and conjunctival granulomas (262). The ocular manifestation uveitis is associated with a distinct phenotype of sarcoidosis named Heerfordt´s syndrome (HS). The complete form of HS consists of uveitis, parotid and/or salivary gland enlargement and cranial nerve palsy, especially the facial, and also fever (263). In addition, engagement of the cranial nerves is also the most common form of neurosarcoidosis (264-266). Furthermore, the presence of skin involvement in sarcoidosis can be very unsightly if localized to the face and can sometimes appear in old scars. However, skin reactions seen in patients with LS, i.e. erythema nodosum, usually disappears within three weeks (253). Findings of increased levels of creatinine may indicate kidney involvement (267, 268).

1.4.6 Diagnosis

The diagnosis of sarcoidosis is based on evaluation of clinical symptoms, chest X-ray, laboratory findings and if possible positive biopsies showing the characteristic

granulomas. Chest-X-ray in sarcoidosis patients are divided into five stages (Table 1) (246).

Table 1. Chest radiographic staging

Stage	Finding
0	Normal chest radiograph
I	Bilateral hilar lymphadenopathy (BHL)
II	Parenchymal infiltrations with BHL
III	Parenchymal infiltrations without BHL
IV	Volume reduction and signs of fibrosis

The investigation of sarcoidosis patients normally includes measurements of the lung function, and where static and dynamic spirometry tests usually reveal signs of a restrictive and/or obstructive disease (269). Often is also a bronchoscopy with BAL performed and where the cell analysis from the BAL fluid typically show an accumulation of CD4+ T lymphocytes resulting in an increased CD4/CD8-ratio >3.5. In patients with LS who have a characteristic clinical and radiological picture, an increased CD4/CD8 -ratio is considered to be diagnostic and equivalent with a positive biopsy (165).

A laboratory parameter that strengthens the diagnosis, even if not specific for sarcoidosis, is an increased level of ACE (270). ACE is produced by epitheloid cells in the sarcoid granulomas (271) . The ACE level is believed to reflect the granuloma burden (272). It has therefore been suggested that the ACE level can be used to predict relapse or improvement of sarcoidosis (270, 273). Further, liver enzymes are usually controlled at onset of disease to see if signs of hepatic involvement, but not all patients with engagement of the liver have an abnormal liver function (274, 275). Enlargement of the liver on an abdominal CT scan can also indicate liver involvement (276). Further, creatinine is controlled to see if the kidneys are affected which could result in kidney failure (268). The levels of calcium is controlled in both blood and urine, since hypercalciuria is more commonly seen than hypercalcemia (277). In addition, it should be kept in mind that hypercalcemia can be seen in also in several other diseases.

In patients investigated for sarcoidosis, efforts are made to obtain histological evidence in the form of a positive biopsy showing the typical granulomas, especially in patients with non-LS. If possible, the biopsies are taken from easily accessible locals such as peripheral enlarged LNs or skin lesions secondary to sarcoidosis. An alternative are to take samples from mediastinal LNs which until recently have required mediastinoscopy (172). Therefore, the availability of the less invasive technique with EUS-FNA via esophagus has become a valuable tool in the diagnostic procedure (173). A positive biopsy can be taken not only to ensure the diagnosis but also to confirm extra-pulmonary engagement. However, there are extra-pulmonary manifestations difficult to confirm with a biopsy such as cardiac involvement and often are several different

investigation methods used instead. For example can 24-hour ambulatory electrocardiogram (ECG) show ventricular tachycardia, atrioventricular blocks or ventricular extra systoles (278, 279). Cardiovascular magnetic resonance (CMR) can detect edema as a sign of inflammation or show fibrotic changes (280-284). Furthermore, investigation with position emission tomography-computer tomography (PET / CT) can show defects in the uptake of contrast (285-289). A cardiac biopsy is an alternative in patients with a high suspicion of CS, but the chance to get representative samples with granulomas is low and not without risk (290). Thus, proposal for criteria of cardiac sarcoidosis have been developed as an alternative to the histological diagnosis (291). Other examples of organ manifestations where alternative criteria have been developed for the diagnosis are engagement of the eyes and nervous system (292, 293). For the diagnosis of neurosarcoidosis can investigation with CMR be useful in the same way as for CS (294, 295).

Important to remember is that there are other diseases that may be mistaken for sarcoidosis, for example lymphoma, tuberculosis, histoplasmosis, Wegners granulomatosis and chronic beryllium disease. Tests that can be used to exclude these diseases are needle aspiration or biopsies from enlarged LNs if there is a suspicion of lymphoma (296), culture for mycobacteria can be taken to rule out tuberculosis, serology for histoplasmosis, blood test for detection of anti-neutrophil cytoplasmic antibodies (ANCA) in Wegners granulomatosis and beryllium lymphocyte proliferation test on blood or BALF for chronic beryllium disease (252).

1.4.7 Treatment

The disease course in sarcoidosis varies significantly, from complete resolution to pulmonary fibrosis with respiratory failure. Patients with LS usually have a very good prognosis and non-steroidal anti-inflammatory drugs are often sufficient as symptomatic treatment in the initial acute phase (188). There are patients with sarcoidosis who recover spontaneously, but also those who develop chronic disease with pulmonary fibrosis leading to respiratory failure. The first line of treatment for these patients is oral corticosteroids. Treatment is usually initiated if there is a progressive loss of lung function or extra-pulmonary manifestations such as hypercalcemia, involvement of the heart or nervous system (252). Treatment with corticosteroids is in general considered to have a suppressive effect in the acute phase but long term benefits are less certain (297-299). In addition, many patients with pulmonary sarcoidosis do also improve spontaneously (300). For individuals that require high doses of corticosteroids, methotrexat may be added in order to reduce the dose of cortisone (301). Treatment with TNF-α inhibitors as an alternative are still under evaluation (253, 302). However, no study has clearly demonstrated that medication prevents the progression of pulmonary inflammation towards fibrotic changes (303). In patients with cardiac involvement is the aim with corticosteroid therapy to prevent development of heart failure and to reduce disturbances in the cardiac conduction system which in the worst case can lead to life-threatening arrhythmias. Medication is usually continued for a minimum of one year but may be discontinued if there is a lack of efficacy or if the pathologic changes completely disappear. (284). Furthermore, inhaled corticosteroids may ease the symptoms in

patients with cough (304). For patients with ocular engagement, topical corticosteroids may sometimes be sufficient for maintenance (186).

2 AIMS OF THIS THESIS

The general aim of the work presented in this thesis was to study risk factors for different organ involvements and markers of importance for the inflammatory response seen in sarcoidosis patients.

Specific aims were:

- To analyze links between HLA-DRB1* alleles and symptoms associated with Heerfordt´s syndrome such as uveitis, engagement of parotid and/or salivary glands or cranial nerve palsy, with particular focus on uveitis.

- To concomitantly compare the adaptive immune response seen in BALF, blood and lymph nodes of sarcoidosis patients. Furthermore to investigate if the increased CD4/CD8-ratio found in BALF is reflected by a similar type of accumulation of CD4+ T cells in the thoracic lymph nodes.

- To examine the role of pathologic ECG changes for the risk of developing cardiac sarcoidosis and as well phenotypic and genotypic factors.

- To investigate the role of both single and pairs of HLA-DRB1* alleles for the risk of developing extra-pulmonary manifestations in sarcoidosis patients.

3 METHODS

The present thesis is based on studies of human samples, including analyses of BALF, blood and lymph nodes (LN). The studies were performed after approval by the local ethics committee and all subjects included in the studies gave their written informed consent to participate.

3.1 SUBJECTS (PAPER I-V)

The included sarcoidosis patients were all, except for in **paper II**, consecutively recruited after referral to the Chest Clinics at either Karolinska University Hospital in Solna or Södersjukhuset, Sweden, for diagnostic investigation. In **paper II**, only patients with a chest X-ray showing radiological stage I (bilateral hilar lymphadenopathy) or II (bilateral lymphadenopathy with parenchymal infiltrates) were included. In the other three papers were also patients included with stage III (solely parenchymal infiltrates) and IV (volume reduction). All patients in **paper I-IV** were HLA-typed except from three patients in **paper III**. Patients were diagnosed with sarcoidosis in **paper I-IV** through typical clinical and radiographic manifestations, findings at bronchoscopy with BAL including an elevated CD4/CD8-ratio and positive biopsies, using the criteria outlined by the World Association of Sarcoidosis and other Granulomatous disorders (WASOG) (190). Löfgrens´ syndrome (LS) was defined as bilateral hilar lymphadenopathy with or without parenchymal infiltration, fever, erythema nodosum and/or ankle arthritis. Clinically resolved disease was defined as disappearance of symptoms (dry cough, fever, fatigue and extra pulmonary manifestations) and a normalized chest X-ray. For non-resolving disease the criteria outlined by Costabel et al. were used (305). Ever smokers were defined as patients who previously had smoked or were current smokers. Healthy subjects were included as controls in **paper I** and **IV** for comparisons regarding the frequency of HLA-DRB1* alleles. In **paper II** a control group was included with six patients who had enlarged superficial LNs, punctured for diagnostic purposes with findings of unspecific inflammation.

3.2 BRONCHOALVEOLAR LAVAGE (PAPER II)

The BAL was performed according to procedures earlier described (306). The BALF was afterwards kept on ice, and later strained through a Dacron net (Millipore, Cork, Ireland) and centrifuged. Thereafter the supernatants were removed. The cell pellet was resuspended in PBS for further analyzes. Cell differential counts were determined by May-Grünwald Giemsa staining of cytospin slides.

3.3 ENDOSCOPIC ULTRASOUND GUIDED FINE-NEEDLE ASPIRATON (PAPER II)

EUS-FNA was carried out via an esofagus ultrasound endoscope and aspirates were obtained with a 25-gauge needle. Smears of aspirates obtained by EUS-FNA were

initially air-dried and evaluated on-site by using a modified May-Grünwald Giemsa quick staining method.

3.4 FLOW CYTOMETRY (PAPER II)

The cell pellet from the centrifuged BALF was resuspended in PBS and antibodies were added, and thereafter incubated at 4^{o} C for 20 minutes. Monoclonal antibodies used for surface staining were CD4, CD8, CD69, CD27 (BD Bioscience, Mountain View, CA, U.S.A.) and AV2S3 (Thermo scientific, Waltham, MA, U.S.A.). After incubation, the cells were washed twice. Surface markers expressed on T cells were analyzed with flow cytometry using a FACS CANTO II flow cytometer (BD Bioscience). Data were processed with FACS Diva 6.1.2 software (BD Bioscience).

The blood was stained with the same mixture of antibodies as for BALF and incubated at room temperature for 20 minutes. Thereafter, erythrocytes were removed through incubation with lysing solution (BD Bioscience) for 8 minutes. The cells were afterwards washed twice and analyzed by flow cytometry. Data were processed with FACS Diva 6.1.2 software (BD Bioscience).

Cells from LN were stained with surface markers using the same procedure as for BALF cells. Data were processed using FACS Diva 6.1.2 software (BD Bioscience) and Infinicyt software (Cytognos, Salamanca, Spain).

A FOXP3 staining kit (eBioscience, San Diego, CA, USA) was used for intracellular staining. First, the cells were fixed and permeabilized for 45 minutes at 4^{o} C in dark. Then washed twice with permeabilization buffer and incubated with 2% rat serum for 15 minutes. Next, the cells were stained intracellularly with anti-FOXP3 and incubated for 30 minutes at 4^{o} C. Finally, the cells were washed twice with permeabilization buffer and analyzed by flow cytometry.

3.5 HUMAN LEUKOCYTE ANTIGEN TYPING (PAPER I-V)

HLA-class II (HLA-DRB1) typing of patients **paper I-IV** was done on DNA from blood samples through the use of polymerase chain reaction (PCR) and amplification with sequence-specific primers (SSP) (307). For the controls in **paper I and IV** the majority was HLA-typed using PCR-SSP but some of the HLA-DRB1* alleles were determined with restriction length polymorphism (308, 309).

3.6 DEFINITION OF ORGAN INVOLVEMENT (PAPER I-V)

A positive biopsy was in general required for patients with engagement of the skin, LNs, parotid and/or salivary glands, liver, spleen and bone marrow. Patients diagnosed with sarcoidosis were defined to have hypercalcemia and/or kidney involvement if they had repeated blood samples with p-calcium >2.60 mmol/L and/or p-creatinine > 90 µmol/L for women and > 100 for men. The criteria outlined by Herbort et al (293) were used for ocular sarcoidosis in **paper I and IV** and the Japanese guidelines by Hiraga et

al (291) was used for cardiac sarcoidosis in **paper III and IV**. Patients were defined as having neurosarcoidosis according to Zajicek criteria in **paper I and IV** (292). Erythema nodosum and ankle arthritis in patients with LS were excluded from the extra-pulmonary manifestations in **paper IV**.

3.7 STATISTICAL ANALYSIS (PAPER I-V)

In **paper I and II** statistical analyses and graphs were performed with Graph Pad Prism 4.03 (GraphPad Software Inc., San Diego, CA, USA) and in **paper III and IV** with Graph Pad Prism 6. For comparison of different allele frequencies, $p<0.003$ was regarded as significant [after Bonferroni correction number of alleles (n=13) i.e. dividing 0.05 with 13] and for combinations of HLA-alleles $p<0.0005$ (n=91). Otherwise $p<0.05$ was regarded as significant. The non-parametric Mann Whitney test, Friedman´s test followed by Dunn´s post-test, was used when appropriate. Correlations were analyzed using Spearman´s rank correlation test. HLA-data were analyzed by Chi-square test or in the case of small numbers by Fisher´s Exact Test. In **paper IV** relative risk (RR) was calculated from the cross product ratio of the data of interest. In **paper IV** sensitivity, specificity, positive and negative predictive value of ECG-screening for identifying the presence or absence of CS were calculated considering a patient with a pathologic ECG diagnosed with CS as true positive and false positive if the ECG was pathologic but CS was not diagnosed during the follow-up.

4 RESULTS AND DISCUSSION

4.1 INTRODUCTION

Sarcoidosis is a systemic granulomatous disease that in more than 90% affects the lungs and/or intrathoracic LNs, and almost any organ such as skin, peripheral LNs, eyes and heart can be involved (252, 253). Genetic factors have an influence on the clinical picture seen in sarcoidosis (310-313). For example is the HLA-DRB1*0301 allele overrepresented in patients with LS (314, 315). Therefore, it could be other specific forms of sarcoidosis where the HLA-DRB1 alleles are of importance. Characteristic for sarcoidosis is an increased percentage of CD4+ T cells in BALF (165). In HLA-DRB1*03 positive patients there is an accumulation among the CD4+ T cells of AV2S3+ T cells (>10%) (214). However, it has not been known whether cells found in BALF reflect those found in the enlarged regional LNs. Engagement of the heart is a potentially life-threatening condition. Previously, there has been no consensus with regard to the optimal clinical evaluation and diagnostic procedures of patients with suspected cardiac involvement in sarcoidosis, and improvement in patient evaluation strategies might help defining the patients at high risk (281). In type 1 diabetes is the HLA-DRB1 alleles *03 and *04 separately associated with an increased risk, but were the risk is highest for those who carry both the alleles (316). The role of HLA-DRB1 combinations in sarcoidosis has not previously been thoroughly investigated.

The aim of this thesis was therefore to further evaluate risk factors for distinct clinical manifestations of sarcoidosis and the inflammatory cell response seen in sarcoidosis.

4.2 SYMPTOMS ASSOCIATED WITH HEERFORDT´S SYNDROME (PAPER I)

HS originally termed “Febris uvea-parotidea subchronica”, is a specific phenotype of sarcoidosis that was characterized by Christian Heerfordt in 1909 (317). He described three patients with uveitis, parotid swelling, cranial nerve palsy and fever. However, HS was first in 1937 classified as a distinct manifestation of sarcoidosis by Jan Waldenström (318). Since HS like LS is a specific phenotype of sarcoidosis, we wanted to investigate whether there also here were links to the HLA-DRB1* alleles.

One thousand patients with sarcoidosis, out of which 83 had one or more symptoms associated with HS, were included in the study together with a group of 2000 healthy individuals from the same population, matched for gender and age. HLA-DRB1* alleles were determined for all individuals and comparisons were made between patients with HS associated symptoms and healthy controls.

4.2.1 Risk factors for Heerfordt´s syndrome

The complete form of HS is rare and we choose therefore in accordance with Scadding´s modification of HS, to divide patients as having incomplete or complete forms of HS (263). We thus added patients with only uveitis and patients without

uveitis but with one or both of the other two manifestations, i.e. engagement of parotid and/or salivary glands and cranial nerve palsy, and described them as having symptoms associated with HS. We found in patients with HS associated manifestations an overrepresentation of HLA-DRB1*04, and the risk seemed to be even higher in homozygote patients. HLA-DRB1*04 has otherwise, in this as well as in previous studies, shown to be protective against overall sarcoidosis (319, 320). In patients with HS associated symptoms, ocular engagement was the most common manifestation. Half of the patients with parotid and/or salivary gland enlargement did not have ocular engagement and more than half of them were HLA-DRB1*04 positive. This indicate that the connection to HLA-DRB1*04 is not only driven by uveitis. The fact that almost all patients with cranial nerve palsy also had uveitis strengthen that it is a part of a syndrome. Further, about half of the patients with other ocular inflammatory manifestations than uveitis were also HLA-DRB1*04 positive. Therefore, the correlation seems not to be limited only to uveitis, but instead include all ocular manifestations.

Our results of a genetic association to the different organ manifestations seen in HS are supported by other studies. For example have associations between HLA-DRB1*0401 and ocular sarcoidosis been suggested, but in significantly smaller patient cohorts and where patients of different ethnic origins were included (310, 321). Further, correlations between HLA-DRB1* 0401 and involvement of parotid and salivary glands were noted in a study by Rossman et al (310), however the correlation was significant only for Afro-Americans and not for Caucasians. In a more recent study by Sato et al, links between DRB1*0803 and neurosarcoidosis was reported in Japanese patients and between DRB1*0401/DQB1*0301 and uveitis in UK (321).

One possible explanation to the genetic linkage to HLA-alleles is cross reactivity, i.e. that there are proteins for example in the eye that resembles the antigen which the immune system initially reacted against. HLA-DRB1*04 allows according to this theory an adequate antigen presentation of eye derived proteins. Since patients homozygous for HLA-DRB1*04 had an even higher risk for HS associated symptoms compared to heterozygous, those who are homozygous may have a more efficient antigen presentation, leading to an inflammatory reaction.

In conclusion, with regard to our findings it seems reasonable to suggest that HLA-DRB1*04 positive patients should be closely monitored for particularly uveitis, but also parotid and salivary gland enlargement as well as cranial nerve palsy.

4.2.2 The protective role of human leukocyte antigens

We also investigated if there were HLA alleles with a reduced risk for ocular engagement. We found that the presence of the HLA-DRB1*03 allele, which is associated with a good prognosis in Scandinavian patients (206, 315), was significantly reduced in patients with ocular sarcoidosis. However, in all patients the frequency of the allele was higher compared to healthy controls and seemed to be associated with an increased risk of contracting sarcoidosis. Also HLA-DRB1*01, previously found to be

strongly protective against non-LS was found to protect also against eye involvement (320).

4.2.3 Ocular involvement and non-resolving disease

Since the majority of the patients had ocular engagement we focused extra on this group. The prevalence of ocular sarcoidosis in our study was relatively low compared to what has been reported by others. One explanation may be that we had relatively strict criteria for the diagnosis of ocular sarcoidosis, and where only patients with obvious symptoms were examined for eye involvement. For example patients with conjunctivitis sicca were excluded in our study but included in others (322, 323). We also wanted to investigate if the HLA-DRB1*04 allele had any influence on the disease course. The majority of patients had been followed for at least two years, and there were no significant differences in outcome between the HLA-DRB1*04 positive or negative patients. Compared with patients without ocular sarcoidosis, patients with eye involvement had a significantly poorer prognosis with a higher frequency of non-resolving disease. We concluded therefore that ocular engagement seems to be of greater prognostic importance than the HLA-DRB1* type. This in contrast to patients with LS, where the HLA-type (HLA-DRB1*03) is a prognostic marker (315).

Ethnicity is also likely to have an impact on the prevalence of ocular sarcoidosis, and it is known that for example in Japan the frequency of ocular engagement is considerably higher than in Scandinavia (324). Most of the patients with ocular sarcoidosis in our study had non-resolving disease, which sharply differs from the Japanese sarcoidosis patients where ocular engagement often is associated with good prognosis (325). In a study by Pietinalho et al where Finnish and Japanese patients with sarcoidosis were compared, ocular sarcoidosis was found in more than half of the Japanese patients compared with 5 % in the Finnish cohort. The majority of the Japanese patients had a normalized chest radiograph after two years (324). In conclusion, involvement of ocular engagement in Scandinavian patients calls for increased awareness of possible non-resolving disease and a more intensive follow-up.

4.3 T CELL PHENOTYPES FROM THREE COMPARTMENTS (PAPER II)

Sarcoidosis is characterized by the formation of non-necrotizing granulomas in the affected organs and with an increased CD4/CD8-ratio in BALF (165). The increased ratio is caused by an accumulation of CD4+ T cells. In HLA-DRB1*0301 positive patients there is also an expansion of CD4+ T cells that express the TCR V-gene segment AV2S3 (68). Since accumulation of AV2S3+ T cells are associated with good prognosis, AV2S3+ T cells are proposed to be effector cells (234). However, it is not known whether the lymphocytes found in BALF simultaneously reflect those in the enlarged regional LNs. Therefore, we wanted to investigate and compare T cell phenotypes in BALF, blood and mediastinal LNs in patients with sarcoidosis.

The fifteen included patients underwent clinical investigation including bronchoscopy with BAL. Blood samples were drawn simultaneously, and EUS-FNA of enlarged mediastinal LNs via esophagus was also performed. The cells from the three different

compartments were then stained with markers/antibodies and analyzed by flow cytometry. Analysis were performed regarding T cells (CD4+ and CD8+), markers of activity (CD69), differentiation (CD27), T regulatory cells (FOXP3) and AV2S3+ cells. A control group was included with six patients who had enlarged superficial LNs, punctured for diagnostic purposes with findings of unspecific inflammation.

4.3.1 CD4/CD8-ratio in lymph nodes of less diagnostic value

In BALF of sarcoidosis patients a CD4/CD8-ratio >3.5 is used to strengthen the diagnosis (165). We wanted therefore to analyze if an elevated CD4/CD8-ratio in BALF correlates with a similar elevated CD4/CD8-ratio in the regional LNs. We found that the CD4+ T cells did accumulate in the LNs compared to peripheral blood, resulting in an elevated CD4/CD8 ratio in LN. However, the accumulation of CD4+ T cells in the LNs was not as pronounced as in BALF. An accumulation of CD4+ T cells was also seen in the controls´ LNs, in line with what has previously been reported (326). In summary, the CD4/CD8-ratio in LNs seems to be of less value as a diagnostic tool in comparison to findings in BALF in patients with a suspicion of sarcoidosis.

4.3.2 Differentiated T cells in bronchoalveolar lavage fluid

The cell surface marker CD27 is known to be expressed by naïve T cells and to be initially up-regulated during T cell activation but gradually down-regulated as the T cells differentiate and become capable to produce more cytokines (93). CD27 and other markers of activation such as CD69 are known to be expressed differently in sarcoidosis patients in comparison to healthy controls, indicating that CD4+ T cells in BALF of sarcoidosis patients are more activated and differentiated (232). Therefore, we wanted to investigate the enlarged LNs and compare the findings with what is seen in BALF of sarcoidosis patients. Our results show that T cells are more differentiated in the peripheral airways than in the LNs and thus contributing to the exaggerated inflammation seen in the small airways. In sarcoidosis patients, a possible explanation to the excessive levels of CD4+ T cells in BALF in comparison to LNs and blood, may be that the CD4+ T cells activated in the LNs circulate through the bloodstream to the alveoli and there accumulate in response to an unknown antigen. In addition, our findings also show that not only CD4+ T cells, but also the CD8+ T cells are highly activated in the lungs of sarcoidosis patients. This is supported by a previous study that showed a high capacity of BALF CD8+ T cells to produce cytokine IFN-γ in sarcoidosis patients after *in vitro* stimulation, which was even more pronounced for CD8+ than CD4+ T cells (230).

Mutations in the gene coding for FOXP3 has previously been associated with severe autoimmune diseases, illustrating the importance of T regulatory cells for which FOXP3 is a marker (131). Our results of an increased level of FOXP3+ T cells in LNs of sarcoidosis patients is also supported by findings from previous studies, though the authors then investigated LN biopsies while we analyzed aspirates from EUS-FNA (327). The similarity of the results, illustrates the clinical value of EUS-FNA, which is less invasive compared to taking biopsies from LNs through mediastinoscopy. For EUS-FNA we used a thin 25 gauge needle to avoid blood contamination of the

samples, but which at the same time was thick enough to provide sufficient cell material to obtain a definite diagnosis (328, 329).

4.3.3 Specific T cells in bronchoalveolar lavage fluid

Our group has previously shown that HLA-DRB1*03 positive patients of Scandinavian origin with sarcoidosis more or less always have an accumulation in the lungs of CD4+ T cells expressing the TCR V gene segment AV2S3 (defined as >10% of CD4+ T cells express AV2S3 in BALF) (215). In this study we found that in comparison to BALF, there was an absence of LN-accumulated AV2S3+ T cells, suggesting that the focus of the immune response is localized to the alveoli. This perhaps as a result of repeated inhalations of triggering antigens alternatively by protein deposits following a bacterial or viral infection. Further, our results show in patients who harbored an expansion of AV2S3+ T cells, an inverse relation between the frequency of BALF AV2S3+ T cells and the fraction of FOXP3 expressing BALF T cells. It has in previous studies been discussed that the AV2S3+ T cells appear to be effector cells, rather than regulatory cells (234, 241). In line with this hypothesis, patients with an expansion of AV2S3+ T cells in BALF may consequently be able to more efficiently eliminate a presumed sarcoidosis-specific antigen, and there is therefore less need of FOXP3 expressing T cells in such patients.

4.4 RISK FACTORS FOR CARDIAC SARCOIDOSIS (PAPER III)

Clinical manifestations in CS occurs in about 5%, but biopsy samples from deceased patients with sarcoidosis show that the incidence of cardiac involvement is likely to be higher (190, 260). The septum is commonly engaged in patients with CS, leading to damage of the conduction system (258). Engagement of the heart may therefore manifest itself as benign arrhythmias or give rise to severe conduction blocks and in worst case life threatening arrhythmias (259). The overall objective of this study of a large cohort of well-characterized Scandinavian patients with a primary diagnosis of sarcoidosis was to investigate the value of resting ECG and clinical symptoms for early identification of CS and to estimate their relative importance. In addition, different phenotypic and genotypic factors were also included in the analysis.

A cohort of 1017 Caucasian patients with sarcoidosis were screened with ECG at disease onset and investigated for CS according to clinical routine.

4.4.1 An abnormal electrocardiogram and cardiac sarcoidosis

We found that CS was very rare in Caucasian sarcoidosis patients with a normal ECG at disease onset. The risk for CS was significantly higher in sarcoidosis patients presenting with a pathologic ECG, in particular if they simultaneously had cardiac related symptoms such as palpitations, pre-syncope or syncope. Furthermore, symptoms indicating CS may develop later on in the disease course and therefore repeated investigations with ECG may be needed (287). In our study the average patient had had sarcoidosis for a couple of years before the diagnosis of CS. In addition, even small ECG changes are important to take seriously since they later can progress.

In our material, CS with pathologic changes developed later on in three patients who initially had a normal ECG. Moreover, the frequency of CS in the whole study population may have been even higher than the one we noticed if we had investigated all patients with a pathologic ECG in a more extended way. However, it was not possible because of this study´s retrospective nature. Based on the results, it seems reasonable to suggest that all sarcoidosis patients who have a pathologic ECG should be further investigated for CS. Patients who have a normal ECG at first presentation and lack the above symptoms of suspected cardiac involvement appear to be at a low risk for CS.

4.4.2 Additional risk factor for cardiac involvement

We also found phenotypic and genotypic factors associated with a pathological ECG as well as with CS. For example there was a similar male dominance among patients with a pathologic ECG, as also seen in patients finally diagnosed with CS. This finding differs slightly from what was reported in a previous study of autopsy material from diseased sarcoidosis patients, where in Caucasians there were almost an equal number males and females with CS (189). In our study, an increased risk for a pathological ECG, and a similar tendency of an increased risk for CS, was seen in patients with parenchymal lung infiltrates, i.e. more advanced disease, on chest radiography. Moreover, LS patients had a significantly reduced risk for CS compared to non-LS patients. However, HLA-DRB1*03 negative LS patients need to be more closely controlled with respect to CS. This finding is consistent with the observation that Scandinavian LS patients, especially HLA-DRB1*03 positives, have a very favorable prognosis (188). A genotypic factor that seemed to be associated with CS was HLA-DRB1*15 as half (50.0%) of the HLA-typed patients with CS had this allele compared to a third of the patients without CS (non-significant, probably due to the small number of patients with CS). In contrast, HLA-DRB1*01 seemed to protect against CS. However, these findings did not reach statistical significance probably due to too few observations. The finding of a genetic link is supported by results from Japanese patients where HLA-DQB1*0601 was found to be significantly increased in patients with CS (330). In conclusion, male gender, advaned radiographic stages, non-LS and HLA-DRB1*15 call for an increased awareness of CS.

4.5 EXTRA-PULMONARY MANIFESTATIONS AND HLA ALLELES (PAPER IV)

The HLA-DRB1*03 allele is known to be overrepresented in patients with LS compared to healthy controls (314, 315). A genetic association is also found in patients with ocular sarcoidos, where there is an increased frequency of HLA-DRB1*04 (213). We therefore aimed to further investigate the role of specific HLA alleles as well as combinations of HLA alleles for the risk of extra-pulmonary manifestation in sarcoidosis.

Thousand patients with sarcoidosis, out of which 288 had extra-pulmonary manifestations, were included in the study. There were 383 patients with LS and 617 patients with non-LS. Extra-pulmonary manifestation was defined as an organ engagement secondary to the sarcoidosis disease, involving other organs than the lungs and/or thoracic lymph nodes but excluding erythema nodosum and ankle arthritis. The different organ manifestations studied were engagement of the skin, superficial LNs, eyes, nervous system, kidneys, hypercalcemia, parotid and salivary glands, heart, liver, spleen and bone marrow. HLA-DRB1 alleles were determined for all individuals, and comparisons were made between different subgroups.

4.5.1 The role of single human leukocyte antigen alleles

We found that the risk for extra-thoracic engagement such as skin, ocular and peripheral lymph nodes was very low in LS patients and that the frequency was several times higher in non-LS patients. Moreover, there were significant differences between HLA-DRB1 alleles with respect to the risk for extra-pulmonary manifestations. Lowest was the risk for patients who carried the HLA-DRB1*03 allele. In contrast, the HLA-DRB1*04 allele was a risk factor for extra-pulmonary manifestations. Compared to DRB1*03, the alleles DRB1*04, *07, *08 and *15 were associated with a significantly increased risk for extra-pulmonary manifestations. Moreover, HLA-DRB1*03 negative compared to DRB1*03 positive LS patients had a significantly increased risk for extra-pulmonary manifestations. Correlation between disease progression and different HLA alleles has been seen also in other diseases than sarcoidosis; leprosy caused by *Mycobacterium leprae* is one example. In leprosy HLA alleles are believed to not only influence the susceptibility or resistance against disease, but also if the patient will develop a tuberculoid or lepromatous form of leprosy. The tuberculoid form of leprosy is associated with T helper 1 (cellular) immune response while the lepromatous form is related instead to a T helper 2 (humoral) response. How HLA molecules present peptides derived from *M. leprae* to T cells of the host is believed to be of importance for the immunologic reaction (331). Since there in sarcoidos are links between HLA-type and disease expression, the differences in how the HLA-alleles present the sarcoidosis related antigen may influence the subsequent immune reaction. It has previously been shown that HLA-DRB1*03 in Scandinavian sarcoidosis patients is associated with LS and an expansion (>10%) of CD4+ T cells expressing a T cell receptor with a V gene segment, AV2S3, in bronchoalveolar lavage fluid (214). In conclusion, extra-pulmonary manifestations are common in non-LS sarcoidosis. Further, HLA-typing could be of clinical value for identification of patients with an increased risk for extra-pulmonary manifestations.

4.5.2 Combinations of human leukocyte antigens

We found that the risk for extra-pulmonary manifestation not only depended on the single HLA-DRB1 alleles, but also on how the alleles were combined. Patients with the combination HLA-DRB1*01/*03 had a relatively low risk of about 7% for extra-pulmonary manifestations compared to the general risk for extra-pulmonary manifestations in patients who were either HLA-DRB1*01 or HLA-DRB1*03 positive where the risk was close to 20% in both cases. The risk was even lower in HLA-DRB1*01/*03 positive patients who also had LS. A similar, but inverse phenomena is

seen in patients with type 1 diabetes where the HLA-DRB1 alleles *03 and *04 separately are associated with an increased risk, but were the risk is highest for those who carry both the alleles. The presented theory in type 1 diabetes is not that there is a primarily link to the HLA-DRB1 alleles, but instead to the combinations of the strongly associated HLA-DQB1 and HLA-DQA1 alleles (316). The role of linkage disequilibrium, defined as the tendency for genetic variants located close to each other on the same chromosome to be associated within a population more often than if they were unlinked, have been discussed also in sarcoidosis. Since there is a strong linkage disequilibrium within the HLA-region there are difficulties to determine which gene/s represent the primary association, and whether non-HLA genes located in the region have any role for the risk of developing disease as well as for disease progression (224). As a summary, not only single HLA-DRB1 alleles influence the risk for extra-thoracic manifestations, but also combinations of certain alleles are of importance.

4.5.3 Human leukocyte antigen DRB1*04/*15 a risk factor

We found that HLA-DRB1*04 positive patients in general had a high risk for extra-thoracic engagements, and among the five most common HLA-DRB1 allele pairs the risk was highest for those patients who had the combination HLA-DRB1*04 /*15, where 50% had some form of extra-thoracic engagement. This shows that HLA-typing of sarcoidosis patients could be of clinical value for the identification of high risk patients and where especially the combination HLA-DRB1*04/*15 calls for an increased awareness and a more intensive follow-up.

5 CONCLUDING REMARKS

- There was a correlation between HLA-DRB1*04 and symptoms associated with HS, which consists in its complete form of uveitis, parotid and/or salivary gland enlargement and cranial nerve palsy.

- There was a prolonged disease course in sarcoidosis patients with ocular engagement.

- The CD4/CD8-ratio in LNs of sarcoidosis patients seem to be of less diagnostic value, compared to BALF where a level >3.5 is used to strengthen the diagnosis.

- In patients who harbored an expansion of AV2S3+ T cells, there was an inverse relation between the frequency of BALF AV2S3+ T cells and the fraction of BALF T cells expressing FOXP3. This indicates that patients with an expansion of AV2S3+ T cells more efficiently eliminate a presumed sarcoidosis-specific antigen and there is therefore a less need of FOXP3 expressing T cells (Tregs).

- A pathologic ECG was found to be a risk marker for CS. With respect to our findings we therefore recommend that all sarcoidosis patients with an abnormal ECG should be further investigated for CS.

- Male gender, advanced radiographic stages, non-LS and HLA-DRB1*15 were additional risk factors for CS.

- The absence of HLA-DRB1*03 in LS patients was a risk factor for extra-pulmonary manifestations (erythema nodosum and ankle arthritis excluded). Furthermore, HLA-DRB1*04 was in all sarcoidosis patients a risk factor for extra-pulmonary manifestations.

- Not only single HLA-DRB1 alleles influenced the clinical picture seen in sarcoidosis patients, but also the allele combinations. Among the five most common HLA-DRB1 allele pairs in sarcoidosis patients, the highest risk for extra-pulmonary manifestations was seen in those with the combination HLA-DRB1*04/*15.

6 FUTURE PERSPECTIVES

- It would be of interest to investigate if HLA-DRB1*04 positive patients with uveitis express autoantibodies that are not found in HLA-DRB1*04 positive patients without ocular engagement.

- Functional analyses could give us information about the regulatory capacity of FOXP3+ cells in BALF versus LN.

- In sarcoidosis and as well as in other diseases such as rheumatoid arthritis, which also sometimes goes with pulmonary infiltrates, it has been discussed if there is an air-borne antigen that act as a triggering factor. One way to further investigate this theory could be to compare BALF from patients with sarcoidosis with findings from BALF of patients with other systemic diseases that also goes with pulmonary involvement.

- Since a pathologic ECG seems to be a risk factor for cardiac involvement, it would be of interest to do a prospective study of ECG findings and the risk for cardiac sarcoidosis.

- Not all patients with cardiac engagement develop arrhythmias and by comparing findings on CMR with findings from for example 24-hour ambulatory ECG, it may in the future be possible from CMR images to predict patients with a high risk for life-threatening arrhythmias.

- AV2S3+ T cells are known to accumulate in BALF of HLA-DRB1*03 positive patients with active disease. Since patients with the HLA-DRB1*01/*03 combination have a low risk for extra-pulmonary manifestations in comparison to patients with HLA-DRB1*03/*04, there might be differences also in the expression of BALF AV2S3 + T cells.

- Sarcoidosis patients are often non-smokers and it may therefore be of interest to compare smokers with non-smokers who have sarcoidosis, to see if the disease progression differs between the two groups.

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