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IMMUNOLOGICAL MARKERS AND CLINICAL CHARACTERISTICS OF PULMONARY INFLAMMATION

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IMMUNOLOGICAL MARKERS AND CLINICAL CHARACTERISTICS OF PULMONARY INFLAMMATION THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

Interstitial lung diseases (ILD) includes a broad spectrum of disorders affecting the lung parenchyma. Common to these conditions is the exacerbated pulmonary immunological response leading to inflammation, disordered tissue healing, and, in a proportion of patients, chronic pulmonary fibrosis. Current treatments consist mainly of immunosuppressive agents, however, they often fail to control the disease progression. Thus, there is a need for more effective treatment modalities. The development of such new therapies, however, requires an in depth understanding of the underlying immunopathological disease mechanisms.

The studies included in this thesis had two overall aims: first to describe the normal pulmonary cellular composition as reflected by bronchoalveolar lavage fluid (BALF), and second, to further characterize the immunological mechanisms and the pulmonary response to treatment in two disorders commonly associated with ILD, namely sarcoidosis and rheumatoid arthritis (RA).

Normal BALF cellular differential counts were established based on samples from 295 healthy individuals. Subgroup analysis demonstrated that BALF composition was independent of age, gender, season and sample collection site.

In sarcoidosis, the BALF cellular characteristics typical for the disease, namely the increased cluster of differentiation (CD) 4/CD8 T cell ratio, was not reflected in the lymph nodes (LNs). Furthermore, compared to LNs and blood, there was an accumulation of activated and differentiated T cells in BALF. This indicates that, in sarcoidosis, the lung is the primary target for the immune response. In treatment refractory sarcoidosis, the extracorporeal removal of leukocytes through granulocyte and monocyte apheresis (GMA) treatment, was associated with significant immunomodulation. Particularly, there was an increase in blood regulatory T cells. This has also been observed after GMA treatment in other inflammatory conditions, where it has been associated with a good clinical response.

In early RA, after six months of disease-modifying antirheumatic drug (DMARD) treatment, there was a radiological disease progression in one third of patients exhibiting ILD at baseline. Furthermore, there was an increase in airways obstruction, with all patients suffering a large decline in the forced expiratory volume in one second. This indicates that early RA may be associated with an underlying inflammation of both lung parenchyma and airways.

In conclusion, both sarcoidosis and RA are associated with an exacerbated pulmonary immune response. In sarcoidosis, the immune reaction seems to be focused to the lung, indicating an airborne disease trigger. In early RA, systemic inflammation appears to be accompanied by inflammation of both airways and lung parenchyma, with an inadequate response to DMARDs. In both diseases, there is a need for more effective therapies. GMA could be an interesting alternative in sarcoidosis. In RA, therapeutic efforts aiming to reduce the pulmonary inflammatory reaction, should be encouraged.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Interstitiella lungsjukdomar (ILD) inkluderar en rad olika åkommor som alla drabbar själva lungvävnaden. Sjukdomarna kan sitta primärt i lungan eller vara del av ett mera utbrett tillstånd, där flera organ är påverkade. Gemensamt för alla, är en överdriven immunologisk reaktion i lungvävnaden som leder till inflammation och ärrbildning. Detta i sin tur kan negativt påverka lungans syreupptagningsförmåga samt ge en nedsatt lungfunktion.

Sarcoidos och reumatoid artrit (RA) är två sjukdomar som båda kan engagera lungan på detta sätt. Sarcoidos drabbar primärt lymfkörtlar och lunga, men kan också påverka andra organ. RA, å andra sidan, är framför allt en ledsjukdom, men som också i vissa fall drabbar lungorna. Vad som orsakar dessa två sjukdomar är fortfarande okänd, dock vet man att både gener och miljöfaktorer är viktiga för sjukdomsuppkomsten.

Prognosen för ILD varierar mellan olika sjukdomar och från fall till fall. En andel patienter drabbas dock av kronisk progressiv sjukdom och behöver långvarig läkemedelsbehandling. I dagsläget ges oftast mediciner som trycker ned immunförsvaret. Tyvärr är dessa läkemedel ofta förknippade med biverkningar och behandlingseffekten är inte alltid tillfredställande. Därför behövs nya behandlingsmetoder med bättre effekt och en mera fördelaktig säkerhetsprofil. Utveckling av sådana nya behandlingar kräver en djupgående förståelse för de bakomliggande immunologiska sjukdomsmekanismerna. Vetenskapen har kommit en bit på vägen vad gäller att identifiera dessa mekanismer, dock är mycket fortfarande okänt.

Ett sätt att studera vad som händer i lungan hos sjuka patienter är att analysera celler från lungsköljvätska. Patienten undersöks då med hjälp av ett smalt fiberoptiskt instrument som förs ned i luftrören. Genom detta kan man skölja upp celler från de djupa luftvägarna, som sedan kan analyseras för celltyp och aktivitetsmarkörer. Proceduren kallas för bronkoskopi med bronkoalveolärt lavage (BAL) och används i stor utsträckning både för diagnostik och i forskningssammanhang. Trots detta, finns det begränsade data från tidigare studier som definierar normala BAL resultat.

Syftet med föreliggande avhandling är dels att beskriva vad som utgör normala cellfynd vid BAL samt att ytterligare karaktärisera immunologiska mekanismer, inkluderat dess svar på läkemedels behandling, vid de inflammatoriska lungsjukdomarna, sarcoidos och RA.

I delarbete I sammanställde vi BAL data från 295 friska, aldrig-rökande individer som hade undersökts med bronkoskopi under åren 1990-2009. Baserat på dessa data tog vi fram normalvärden för cellulär sammansättning av BAL vätska hos aldrig-rökare. Vi kunde också visa att dessa värden inte skiljde sig åt beroende på kön eller ålder, samt att cell kompositionen inte påverkades av vilken del av lungan som undersöktes eller vilken tid på året bronkoskopin gjordes.

I delarbete II evaluerade vi huruvida de celler som typiskt ses i bronksköljvätska vid sarcoidos också återfinnes i sjukdomsdrabbade lymfkörtlar. Prov från bronksköljvätska och från förstörade lymfkörtlar, lokaliserade vid lungbasen, togs från 15 patienter med sarcoidos. Lymfkörtelproven jämfördes också med prover från patienter som hade förstörade lymfkörtlar där biopsi visade på ospecifik inflammation. Resultaten visade att lymfkörtlarna inte uppvisar en ökning av inflammatoriska celler motsvarande den som ses i lungan hos sarcoidospatienter. Detta talar för att inflammationen vid sjukdomen främst är lokaliserad till lungan, vilket i sin tur kan tyda på att sjukdomen triggas i gång av en inhalerad miljöfaktor.

I delarbete III studerade vi effekten av granulocyt och monocytaferes (GMA) behandling hos sju patienter med sarcoidos, som inte hade svarat på sedvanlig immundämpande behandling. GMA innebär att man tar bort vissa immunceller (vita blodkroppar) från patientens blod, med hjälp av en apparat som bäst kan liknas vid en dialysmaskin. Dessa celler anses bidra till inflammationen och sjukdomsbilden vid flera olika inflammatoriska sjukdomar. Behandlingen har använts med god effekt vid inflammatorisk tarmsjukdom, men har inte tidigare studerats för sarcoidos. Efter behandling kunde vi se en ökning av en viss typ aktiverad immuncell i blodet. Detta skulle kunna tala för att dessa celler lämnat lungan, och vandrat tillbaka ut i blodbanan, vilket således borde leda till minskning av den inflammatoriska reaktionen i lungvävnaden. Utöver detta såg vi en ökning av regulatoriska immunceller i blod. Dessa celler har till uppgift att reglera immunförsvaret, och förhindra en överdriven och kronisk inflammation. En motsvarande stegring av regulatoriska celler i blod har observerats efter GMA behandling vid inflammatorisk tarmsjukdom, där det har förknippats med god behandlingseffekt. Således torde detta tala för en positiv effekt också vid sarcoidos.

I delarbete IV jämförde vi fynd från lungfunktionstester och datortomografi undersökning (röntgen) hos 143 patienter med nydiagnostiserad RA, före och efter sex månaders behandling. Vi observerade att en hög andel patienter (>60%) hade onormala fynd vid lungfunktion och/eller datortomografi undersökningarna redan vid diagnos. Efter sex månaders behandling sågs en ökning av ILD på datortomografi hos en tredjedel av de tolv patienter som hade denna sjukdom vid inkludering i studien. Vidare sågs en ökad luftvägsobstruktivitet hos alla patienter, efter sex månaders uppföljning. Detta talar för att patienter med tidig RA kan drabbas av inflammation både i lungvävnad och luftvägar, samt att denna inflammation inte svarar tillfredställande på den behandlingen som ges för ledsymptomen.

Sammanfattningsvis, är både sarcoidos och RA förknippade med en överdriven immun reaktion i lungan. I sarcoidos verkar denna inflammation vara lokaliserad primärt till lungorna, vilket kan tala för att sjukdomen triggas i gång av en inhalerad miljöfaktor. I RA, verkar ledinflammationen vara ackompanjerat av en inflammation både i lungvävnad och luftvägar, där effekt av den behandling som ges är otillräcklig. För båda sjukdomar finns ett behov av nya och mera effektiva behandlings metoder. GMA kan vara ett intressant alternativ för sarcoidos. För RA behövs nya studier där man fokuserar på att dämpa den inflammatoriska processen lokalt i lungan.

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following papers. They will be referred to in the text by their roman numerals:

- I. Olsen HH, Grunewald J, Tornling G, Sköld CM, Eklund A. Bronchoalveolar lavage results are independent of season, age, gender and collection site. PLoS One 2012;7(8):e43644. Epub 2012 Aug 31.
- 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. Olsen HH, Muratov V, Cederlund K, Lundahl J, Eklund A, Grunewald J. Therapeutic granulocyte and monocyte apheresis (GMA) for treatment refractory sarcoidosis: a pilot study of clinical effects and possible mechanisms of action. Clin Exp Immunol 2014;177:712-719.
- IV. Olsen HH, Reynisdottir G, Pawlowski J, Harju A, Karimi R, Klareskog L, Sköld CM, Nyrén S, Catrina AI, Eklund A, Grunewald J. Pulmonary manifestations in early rheumatoid arthritis: A six month follow up study. In manuscript.

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACPA	Anti-citrullinated protein antibody
ACR	American college of rheumatology
AM	Alveolar macrophages
APCs	Antigen presenting cells
AV2S3	Variable gene segment 2.3 of the T cell receptor α chain
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BHL	Bilateral hilar lymphadenopathy
C	Constant
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
CTL	Cytotoxic T lymphocyte
CCP	Cyclic-citrullinated peptides
D	Diversity
DAS28	Disease activity score based on 28 joints
DLco	Diffusing capacity for carbon monoxide
DMARD	Disease-modifying antirheumatic drug
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
EULAR	European league against rheumatism
EUS-FNA	Endoscopic ultrasound guided - fine needle aspiration
FACS	Fluorescence-activated cell sorter
Fc receptor	Receptor for immunoglobulins
FEV1	Forced expiratory volume in one second
FOXP3	Forkhead box protein 3
GMA	Granulocyte monocyte apheresis
HLA	Human leukocyte antigen

HRCT	High resolution computed tomography
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILD	Interstitial lung disease
J	Joining
LN	Lymph node
MHC	Major histocompatibility complex
MTX	Methotrexate
NK	Natural killer
NSAID	Non-steroidal anti-inflammatory drug
PAD	Peptidylarginine deiminase
PFT	Pulmonary function test
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SE	Shared epitope
SVC	Slow vital capacity
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
V	Variable

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Interstitial lung diseases (ILD) refers to a broad spectra of disorders, involving the lung parenchyma. The diseases may be localised to the lung, or may be part of a systemic condition. Common to all disorders is an exacerbated pulmonary immune response, leading to inflammation, disordered tissue healing and, in a proportion of patients, the development of pulmonary fibrosis. This in turn results in impaired gas transfer, a restrictive lung function abnormality and in advanced disease, respiratory failure. Current therapies mainly aim to reduce the inflammatory response by general immunosuppression. However, these agents are associated with significant adverse events and are often unable to control the relentless disease progression. Thus, there is a pressing need for the development of new, effective treatment modalities with a more favourable benefit-risk ratio. In recent years, there has been an increased interest in specific immunomodulatory treatments such as biological agents and leukocyte apheresis. These therapies are designed to block or enhance specific immunological pathways, thereby reducing the pathological inflammatory response. The possibility to specifically tailor such treatments to the individual disease, holds great promise, however, it requires an in depth understanding of the immunopathological disease process. Although, in many diseases, science has come a long way to identify these specific disease mechanisms, for a majority of the pulmonary inflammatory conditions, much remains to be established.

The studies included in this thesis investigate the immunological mechanisms in two diseases commonly affecting the lungs, namely sarcoidosis and rheumatoid arthritis (RA). In both diseases, immunopathological findings have been linked to clinical parameters. Thus, hopefully, the studies in this thesis may contribute another few pieces to the complex puzzle of ILD.

1.2 THE IMMUNE SYSTEM

The main function of the immune system is to protect the host from pathogens and toxins, prevent tumour growth and participate in the clearance of dead cells. Two major defence mechanisms are in place to achieve these tasks, the innate and the adaptive immune systems, respectively (1).

1.2.1 Innate immunity

The innate immune system provides a first line of defence against pathogens and consists of five main components: the physical epithelial barriers, phagocytes (neutrophils and macrophages), dendritic cells, natural killer (NK) cells, and cytokines and plasma proteins. Together, these mechanisms serve to prevent pathogen intrusion and remove foreign substances from the host. The immune cells of the innate immune system react to invading pathogens in a non-specific manner. They are thereby capable of forming an immediate immune response against a variety of different invaders (1, 2). However, although not specific for any single pathogen, the phagocytic cells of innate immunity are able to recognize certain pathogenic features. The structures that they recognize are termed pathogen-associated molecular patterns, which binds to pattern recognition receptors, expressed on the cell wall of the phagocyte (3). Once activated, the innate immune system functions to eliminate invading pathogens, to recruit immune cells to sites of infection or inflammation, and to engage the second line of defence, the adaptive immune system.

1.2.2 Adaptive immunity

The adaptive immune system comes into play when a pathogen breaks through the barriers of the innate immune system. The primary functions of the adaptive immune system are to eliminate pathogens or pathogen-infected cells and to produce immunological memory. The latter ensures a more rapid and effective immune response upon reinfection with the same pathogen. Contrary to innate immunity, the adaptive immune system is highly specific and thus requires more time to develop. The cells of the adaptive immune system include T lymphocytes and B lymphocytes. T lymphocytes generally defend against intracellular pathogens and require activation by antigen presenting cells (APCs) in combination with co-stimulatory signals. B lymphocytes, on the other hand, secrete antibodies necessary for the protection against extracellular microbes. In addition to antigen binding, they usually depend on interaction with T cells for their activation (1).

1.2.3 Autoimmunity

Central to the immune system's function is the ability to distinguish self from non-self. Various mechanisms are in place to avoid an immune response against self-antigens. Together these mechanisms induce and maintain immunological tolerance. Tolerance can be classified as central or peripheral, depending on where the state occurs. Central tolerance refers to the elimination of self-reactive lymphocytes during their development in the thymus

or bone marrow. Peripheral tolerance ensures inactivation or destruction of self-reactive mature lymphocytes after they have left the primary lymphoid organs (4). Failure of self-tolerance constitutes the underlying pathology in autoimmune diseases. The exact mechanisms by which self-tolerance is lost are not clear. However, the failure may arise through a combination of susceptibility genes and exposure to environmental triggers (5).

1.2.4 Cells and mediators of innate and adaptive immunity

1.2.4.1 Inflammatory mediators

Inflammatory mediators are involved both in innate and adaptive immunity. They include a variety of molecules, which can act both locally and systemically, to modulate the inflammatory response. Cytokines are an example of an inflammatory mediator. These signal proteins are synthesised by a variety of different cell types. They regulate the activity and function of other cells, and they have both pro- and anti-inflammatory capacities (6).

1.2.4.2 Cells of the innate immune system

1.2.4.3 Neutrophils

Neutrophils are short lived cells that reside in the blood stream. They are among the first cells to be recruited to the site of inflammation, where their main role is to kill invading pathogens through phagocytosis and enzymatic digestion. They also produce a range of cytokines that modulate the immune response (7).

1.2.4.4 Macrophages

Macrophages originate from monocytes, and mature to macrophages as they are recruited to the tissues. Here they patrol for pathogens as well as clear dead tissue and debris through phagocytosis. Macrophages also participate in antigen presentation as well as immunomodulation through the production of various immune effector molecules (8).

1.2.4.5 Dendritic cells

Dendritic cells mainly function as APCs for naïve T lymphocytes. They are found in lymphoid or immune organs, in the surfaces of the airways and intestine and as Langerhans cells in the skin. Here they capture pathogens and convert them into antigenic peptides, which are subsequently displayed on their cell surface in the context of human leukocyte antigen (HLA) molecules. The dendritic cells then migrate to the lymph nodes (LNs) or the spleen, where the antigen is presented to T cells along with the appropriate co-stimulatory molecules. Dendritic cells are also involved in the maintenance of B cell function and the establishment of immunological memory (9).

1.2.4.6 Natural killer cells

NK cells constitute a small fraction of the circulating lymphocytes. They are, as the name implies, specialized at destroying target cells, and are particularly important for the defence against viral infections and various tumours (10). NK cells display a sophisticated repertoire

of both activating and inhibitory receptors, ensuring efficient elimination of pathogens, while preventing NK cell driven autoimmune reactions. Thus, NK cells are also regulatory cells that are able to both limit or exacerbate immune responses (11).

1.2.4.7 Natural killer T cells (NKT cells)

NKT cells are a subset of T cells that exhibit features of both T cells and NK cells. The cells thus possess both innate and adaptive immune functions (12). Several subsets of NKT cells have been identified. Generally, these cells are capable of promoting both inflammation and immune tolerance through the production of various cytokines (12, 13). Dysfunction or deficiency, but also inappropriate activation of NKT cells have all been shown to lead to the development of autoimmune diseases, highlighting their immunoregulatory functions (13).

1.2.4.8 Cells of the adaptive immune system

1.2.4.9 B lymphocytes

B lymphocytes are produced in the bone marrow and subsequently migrate to the spleen and other secondary lymphoid tissues. B cells are responsible for the generation of antibodies to specific antigens. Activation occurs following antigen recognition via the B cell receptor. A required co-stimulatory signal must also be provided. Most antigen responses require this signal to be delivered by T helper (Th) cells, however, antigens that are expressed in a highly repetitive form on the pathogen surface may activate B cells through the multivalent cross linking of antigen receptors. This results in B cell proliferation and differentiation into plasma cells or memory B cells. Plasma cells reside in the spleen and LNs where they secrete different classes of clonally unique antibodies. These antibodies protect the host from pathogens by neutralization of the pathogen and activation of other immune cells. Memory B cells survive for a longer period of time. They express surface immunoglobulins (Ig), which enable a rapid immune response upon re-challenge by the same antigen (2, 14).

1.2.4.10 T lymphocytes

T lymphocytes are produced in the bone marrow, and subsequently mature into functional T cells in the thymus. There are several subsets of T lymphocytes, including Th cells, regulatory T cells (Tregs) and cytotoxic T lymphocytes (CTLs) (1).

Th cells express the glycoprotein cluster of differentiation (CD) 4 on their cell surface and are therefore also called CD4⁺ T cells. They provide immunological support for other leukocytes through cell to cell interactions and through the production of cytokines (1). Based on their expression of certain cytokines, they can be further subdivided into Th1 cells, Th2 and Th17 cells. Th 1 cells typically express interferon (IFN)- γ , tumour necrosis factor (TNF) and interleukin (IL)-2 (15). They drive the activation of CTLs as well as enhance the phagocytic and bactericidal capacity of macrophages (16). Th2 cells, on the other hand, are important for the antibody mediated and allergic immune responses as well as the defence against parasites, and typically synthesize IL-4, IL-5, IL-10 and IL-13 (15, 16). Th17 cells mainly reside close to the epithelial barriers, where they defend against extracellular pathogens. They produce several cytokines, including IL-17, IL-21 and IL-22,

and promote neutrophil maturation and chemotaxis. They also have highly pro-inflammatory capacities and have been implicated in the development of several autoimmune diseases (17).

Tregs can be identified through their co-expression of CD4 and CD25^{bright} or through the expression of the transcription factor forkhead box protein 3 (FOXP3). The latter is more specific, as CD25 is also expressed by activated T cells (18). Tregs produce transforming growth factor (TGF)- β , IL-10 and IL-35. They are responsible for reducing the inflammatory response, through the suppression of activated T cells, and are important in maintaining peripheral tolerance (19).

CTLs express CD8 on their cell surface and are also called CD8⁺ T cells (1). They eliminate infected cells through the release perforin and granzyme onto the cell surface, or through the binding to death receptors on the target cells, thereby inducing apoptosis. CTLs are particularly effective in the defence against viral infections (20).

1.2.4.10.1 T cell activation

Naive T cells continuously circulate between the blood and LNs. In the LNs, T cells are activated when their T cell receptor (TCR) binds to its target antigen displayed by the HLA molecules on the surface of APCs (1).

1.2.4.10.2 The T cell receptor

T cells exhibit TCRs on their cell membrane. These receptors recognize peptide fragments of pathogenic proteins, with each T cell clone being specific for a certain antigen. Thus, upon encountering an antigen, only a small fraction of T cells will become activated.

In order for the immune system to provide an effective defence against a vast variety of pathogens, there must be a large repertoire of T cells with unique TCRs. This diversity is determined by the structure of the receptor antigen-binding site. The TCR consists of one α and one β chain, each with a constant (C) and a variable (V) region. The regions of the TCR in contact with the antigen is located in the V-regions of the α - and β chains and are called complementary determining regions. The V region of the TCR consist of variable (V), joining (J), and diversity ((D), β chain only) gene segments. During the development of T lymphocytes, these gene segments are rearranged in a random fashion, thus generating an immense receptor diversity (21).

The interaction between the T cells and the APCs is enhanced by co-receptors. These are the CD receptors (CD4 for CD4⁺ T cells and CD8 for CD8⁺ T cells) (22). Furthermore, in addition to binding antigen peptides, the full activation of T cells require co-stimulation. This is provided by co-stimulatory molecules, present on the surface of APCs. The main T cell co-receptor recognizing these co-signals is the CD28 receptor, expressed on the surface of virtually all T cells (23).

1.2.4.10.3 Human leukocyte antigen molecules

Antigenic peptides are presented to the T cells by APCs. These cells display antigenic fragments on their cell surface via their HLA molecule. Antigen presenting HLA molecules can be divided into two main classes, HLA class I and HLA class II. These are the human

equivalent of the major histocompatibility complex (MHC) found in most vertebrates. Allelic differences in HLA molecules are inherited in a Mendelian fashion.

HLA class I molecules are transmembrane glycoproteins present on the surface of all nucleated cells. They consist of an invariable β 2-microglobulin bound to a heavy chain (α). There are three variants of HLA-class I molecules, HLA-A, HLA-B and HLA-C. The α -chain genes are encoded on chromosome 6, whereas the β 2-microglobulin gene is encoded on chromosome 15 (24, 25). HLA class I molecules present peptides from intracellular pathogens to CTLs (1).

HLA class II molecules are expressed mainly on antigen-presenting cells (B cells, macrophages and dendritic cells). They consist of one α and one β chain, encoded by three different loci on chromosome 6, HLA-DP, HLA-DQ and HLA-DR respectively (24, 25). Variants of these genes code for different specific HLA molecules (26). HLA class II molecules display extracellular antigens to Th cells (1).

1.2.5 Autoimmune mechanisms

The exact mechanisms by which genetic and environmental factors contribute to the development of autoimmunity are still not clear. However, several autoimmune diseases have been strongly associated with specific HLA haplotypes, stressing their contribution to increased disease susceptibility (27, 28). The exact role of the HLA alleles in the development of autoimmunity remains elusive. In the thymus, T cells undergo positive selection if their TCRs has a certain degree of avidity for self-peptide–HLA complexes. However, T cells that interact strongly with such a complex, will be negatively selected and undergo apoptosis. Thus, one explanation for the genetic component may be that defective negative selection results from HLA molecules that are inefficient at presenting certain self-antigens in the thymus. Another hypothesis is that autoimmunity develops as certain HLA molecules are required to present autoantigenic peptides to autoreactive T cells in the periphery (27). Furthermore, it may be that self-peptides presented by these HLA alleles are unable to adequately stimulate Tregs (1).

In the circulation, peripheral tolerance mechanisms should lead to inactivation, suppression or elimination of these auto-reactive T cells. However, in case of an infection or an inflammatory challenge, cytokines secreted by APCs may lead to a more effective presentation of self-antigens to auto-reactive cells, thus allowing them to become activated (29). Another possible explanation for the breach of peripheral tolerance, is molecular mimicry. In this scenario, a pathogen may mimic a self-antigen. This in turn will lead to a cross reaction, where the immune response may be re-directed from a virus or a bacteria to self-tissues (30).

1.2.6 Cell surface markers

Leukocytes display numerous cell surface molecules, including adhesion molecules and receptors important for cell signalling. CD is the nomenclature used to define these molecules. The molecules allow immunological phenotyping of cells and can be identified by panels of monoclonal antibodies. They are useful for cell sorting, using methods such as flow cytometry. All T cells express CD3. Furthermore, as already mentioned, Th cells are CD4+ whereas CTLs are CD8+ (2). In addition, there are several markers of T cell activation. For instance, CD 69 is present on recently activated T cells, and is thus a marker of early T cell activation (31). CD27, on the other hand, is expressed by naïve T cells, upregulated during early T cell activation, and subsequently down regulated upon prolonged T cell activation (32, 33).

1.2.7 Lung immunity

The lungs primary function is to supply the body with oxygen. The lungs are thereby in immediate contact with the outside environment through the upper and lower airways. The epithelium lining the bronchial tree provides the first line of defence against inhaled pathogens or antigens. The epithelium consist of ciliated cells, mucus producing goblet cells and basal cells. In addition to providing a physical barrier, the cough reflex and the mucociliary clearance system are utilized to expel unwanted particles from the airways (34). Furthermore, epithelial cells produce antimicrobial peptides, which helps neutralize toxins and viruses and prevents bacterial entrance. Epithelial cells may also produce molecules such as chemokines, which leads to the recruitment of inflammatory cells (35, 36).

The basement membrane of the respiratory system contains a network of dendritic cells. These APCs engulf invading organisms and migrate to pulmonary LNs, where they activate T cells. Particles reaching the lower airways and the alveolar tissue of the lungs are generally caught by alveolar macrophages (AM), the most abundant cells in the airways of the healthy lung. In addition to their phagocytic activity, they secrete pro-inflammatory mediators, which helps recruit neutrophils and other inflammatory cells. AM also have bactericidal properties through the production of lysozymes, defensins and cationic proteins (36).

1.3 SPECIFIC INVESTIGATIONS AND TREATMENT

1.3.1 Bronchoalveolar lavage (BAL)

BAL involves the instillation and subsequent aspiration of saline solution from a specific lung lobe, usually the middle lobe or the lingula. The procedure is performed using a flexible bronchoscope and is safe and relatively non-invasive (37). In addition to providing diagnostic information in various pulmonary inflammatory diseases, it is an invaluable tool in research. Here it provides accessible samples of the epithelial lining fluid, thus facilitating the study of

pulmonary cells and non-cellular components. In inflammatory lung disease, BAL fluid (BALF) is analysed for cell differential count, as this may help discriminate between different diseases. In the healthy lung, the predominant cell population is macrophages, followed by lymphocytes and neutrophils (38). Upon infection or other insult, activation of the immune response may cause recruitment of large numbers of immune cells, thus changing the normal alveolar cell composition. Different pulmonary diseases may be associated with an overrepresentation of one or more cell type, something that is used for diagnostic purposes. For instance, in hypersensitivity pneumonitis and sarcoidosis, generally there is an increase in CD8+ and CD4+ T cells, respectively (39, 40), whereas in infectious disease, there is usually a pulmonary recruitment of neutrophils (41). An increase in BALF neutrophils may also be seen in idiopathic pulmonary fibrosis, where it is associated with a more severe prognosis (42).

1.3.2 Endoscopic ultrasound guided fine-needle aspiration (EUS-FNA)

EUS-FNA is performed via the oesophagus, and thereby represents a less invasive alternative to the traditionally used mediastinoscopy. It allows sampling from mediastinal LNs and is thereby useful for the diagnosis of sarcoidosis (43) as well as for differential diagnostic purposes (44).

1.3.3 Granulocyte and monocyte apheresis (GMA)

GMA is a non-pharmacological treatment associated with very few side effects (45). It involves the extracorporeal removal of activated granulocytes and monocytes from peripheral blood. Cell removal occurs through a system of cellulose acetate beads. The system is selective for granulocytes and monocytes/macrophages, eliminating only a small fraction of lymphocytes (46). Mainly, the GMA technology has been employed in the treatment of ulcerative colitis (UC) (45-47), however, there is emerging evidence of its efficacy also in other inflammatory conditions (48-50). The exact mechanism of action has still not been elucidated. However, it seems that the immune disruption caused by the cell removal leads to a modulation of the immune reaction. This in turn results in the reduction of pro-inflammatory cytokines and/or the increase in anti-inflammatory cytokines, as well as an increase in circulatory Tregs (51).

1.4 RHEUMATOID ARTHRITIS

1.4.1 Clinical features

RA is a chronic, autoimmune disorder, characterized by persistent synovitis, autoantibody formation and systemic inflammation (52). Typically, it presents with symmetric

polyarthritis of the small joints of the hands and feet, although any synovial joint may be involved. Affected joints exhibit swelling, pain, morning stiffness and a reduced range of movement. The clinical course is variable, ranging from a self-limiting condition, to rapidly progressive disease. In many patients, chronic synovitis will lead to progressive joint destruction, deformity and significant functional impairment, if left untreated (53). Extra-articular disease manifestations are common and include rheumatic nodules, vascular disease, ophthalmic disease, cardiopulmonary manifestation, nephritis and neurological manifestations (54, 55). These systemic complications are markers of disease severity and are associated with significant morbidity and an increased mortality (56, 57).

1.4.1.1 Pulmonary disease manifestations

Lung and/or bronchial abnormalities are evident on high resolution computed tomography (HRCT) in to thirds of patients or more (58-60). They may occur at any time during the course of the disease and can be present before there is clinical evidence of joint involvement (61). The spectrum of pleuropulmonary abnormalities is broad, spanning from pleural disease and airways obstruction to pulmonary nodules and ILD (see Table 1) (62, 63). In addition, patients may suffer from pulmonary infections and therapy-induced hypersensitivity pneumonitis, further complicating the picture (63, 64). ILD is the most severe pulmonary complication, contributing significantly to the excess mortality observed in RA (65-67). The reported prevalence of ILD depends on the mode of diagnosis and definition used, however, data indicate a lifetime risk of clinically evident RA-ILD of approximately 5-10% (65, 68).

Table 1. Pleuropulmonary manifestations associated with rheumatoid arthritis.

Pleural	Airways	Interstitial	Vascular	Other
Pleuritis	Bronchiectasies	RA-ILD	Vasculitis	Chest wall disease
Effusions	Upper airways obstruction	BOOP	PAH	Drug reactions
Empyema	COPD	Rheumatic nodules		Infections
Pneumothorax	Bronchiolitis	Caplan's syndrome		

COPD = chronic obstructive pulmonary disease, RA-ILD = rheumatoid arthritis associated interstitial lung disease, BOOP = Bronchiolitis obliterans organizing pneumonia, PAH = pulmonary arterial hypertension.

Clinical features of ILD include cough and progressive exertional dyspnoea. Physical examination may reveal digital clubbing and “velcro” crackles on lung auscultation. Symptoms are due to the progressive development of pulmonary fibrosis, leading to a restrictive lung function pattern, impaired gas transfer, and in advanced disease arterial hypoxaemia (69). Risk factors for RA-ILD include male gender, longstanding disease, high autoantibody titres and smoking (61, 70, 71). Predictors of progression and therapeutic response remain largely unknown, however, high anti-citrullinated antibody (ACPA) titres and the specific histopathological subtype of “usual interstitial pneumonia” have been associated with a more severe outcome (70, 72, 73).

In recent years, obstructive airways disease as a manifestation of RA, has been gaining more focus. Studies have demonstrated an increased prevalence of chronic obstructive pulmonary disease (COPD) in RA patients, even after adjusting for smoking (74-76). Furthermore, it appears that COPD in combination with RA negatively influences patient

survival (74). Clinical manifestations of COPD are exertional dyspnoea and wheezing. Productive cough and recurrent respiratory tract infections are also common (77). Systemic manifestations include decreased fat-free mass, impaired muscle function, osteoporosis, anaemia, depression, pulmonary hypertension and heart failure (78).

1.4.2 Diagnosis

Diagnostic criteria for RA has be devised by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR, see Table 2) (79). These criteria replace the former 1987 ACR diagnostic criteria, and were developed to increase the likelihood of detecting early RA (80). However, for the purpose of clinical studies, the more rigid 1987 diagnostic criteria are still useful.

Table 2. ACR/EULAR Classification Criteria

<i>Symptom Duration (as reported by patient)</i>	Points
▪ < 6 weeks	0
▪ > 6 weeks	1
<i>Joint Distribution</i>	Points
▪ 1 large joint	0
▪ 2-10 large joints	1
▪ 1-3 small joints (with or without involvement of large joints)	2
▪ 4-10 small joints (with or without involvement of large joints)	4
▪ > 10 joints (at least 1 small joint)	5
<i>Serology</i>	Points
▪ RF- and ACPA-	0
▪ Low RF+ or ACPA+	2
▪ High RF+ or ACPA+	3
<i>Acute Phase Reactants</i>	Points
▪ Normal ESR or CRP	0
▪ Abnormal ESR or CRP	1

Adapted from Aletaha et al. (79). RF = rheumatoid factor, ACPA = anti-citrullinated protein antibodies, ESR = erythrocyte sedimentation rate, CRP = C-reactive protein. Low: < 3 x upper limit of normal (ULN). High: > 3 x ULN. Requirements: patients who have at least 1 swollen joint, not better explained by another disease. A score ≥ 6 points is required for classification as definite RA.

In patients fulfilling the diagnostic criteria, further clinical evaluation aims to assess disease activity and severity, including the presence of extra-articular involvement. Early clinical assessment usually entails x-rays of hands and feet as well as the disease activity score 28 (DAS28) (81). The radiological features in early disease are characterized by soft tissue swelling and juxta-articular osteoporosis, whereas bony erosions and joint deformities may be seen in more advanced disease (82). The DAS28 scoring system estimate disease activity using a combination of the number of swollen and tender joints (hands, arms and knees), patient's global assessment and the erythrocyte sedimentation rate (ESR) (83).

In patients with pulmonary symptoms, investigations with pulmonary function tests (PFTs) and HRCT are indicated (84). In manifest ILD bronchoscopy with BAL and lung biopsy may be conducted to establish a histopathological diagnosis and to exclude other underlying pathologies (38, 84).

1.4.3 Treatment

Current treatment strategies aim to alleviate symptoms and minimize disease activity. Analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) in combination with physiotherapy may mitigate pain and increase functional capacity (85). Disease-modifying antirheumatic drug (DMARD) treatment is initiated in all patients to induce remission and slow disease progression. DMARDs include a wide spectrum of different immunosuppressive agents with diverse mechanisms of action. Two main classes are synthetic chemical compounds (i.e. methotrexate (MTX), sulfasalazine and leflunomide) and biological agents (i.e. TNF inhibitors, the T cell co-stimulation inhibitor, the anti-B cell agent, the IL-6 receptor-blocking monoclonal antibody and the IL-1 inhibitor). In early RA, MTX is usually the first choice, administered in conjunction with low dose oral corticosteroids. In cases of MTX intolerance, sulfasalazine or leflunomide are suitable alternatives. In patients responding insufficiently to MTX or other DMARDs, treatment with a biological agent is indicated (86).

However, despite the high efficacy of these agents for treating joint manifestations, their effectiveness for RA-ILD appear limited, and controlled treatment studies are lacking. To date, therapeutic efforts in RA-ILD are therefore largely empirical.

1.4.4 Epidemiology

1.4.4.1 Frequency

RA affects 0.5-1% of the adult population with a 2:1-3:1 female preponderance. The disease may occur at any age, however, peak onset is seen in the fifth decade of life (87). Extra-articular organ involvement may occur at any time, but the prevalence increases with age (54).

1.4.4.2 Risk factors

Although the exact cause of RA is unknown, the disease is thought to be caused by a combination of environmental factors, genetic factors and chance. Based on twin studies, 50-60% of the risk of developing RA has been attributed to genetic factors (88).

The main genetic risk is conferred by HLA-DRB1. Although multiple HLA-DRB1 alleles have been associated with RA (DRB1*0401 and *0404 in particular), they all share a region of structural similarity termed the shared epitope (SE) (89, 90). The second most important gene appears to be the protein tyrosine phosphatase, non-receptor type 22, however, several other risk alleles for disease have been identified (90).

The most established environmental risk factor is cigarette smoking. Smoking increases the risk of RA in a dose dependent manner, and compared to never-smokers, smokers have approximately twice the risk of developing antibody positive RA (91). Furthermore, there appears to be a strong gene-environment interaction, with a combination of smoking history

and the presence of double copies of HLA–DR SE genes, increasing the risk for RA 21-fold (92). Other potential environmental risk factors include silica dust and periodontitis (93).

1.4.5 Pathogenesis

1.4.5.1 Autoantibodies

Two autoantibodies are commonly used as diagnostic tools in RA, rheumatoid factor (RF) and ACPA. Compared to RF, ACPAs have a better diagnostic value, with a higher specificity for RA (>90%) (94). Whereas RF is directed against epitopes on the Fc region of IgG, ACPAs recognize multiple proteins with citrulline residues. Citrulline is a non-standard amino acid generated by the posttranslational modification of arginine by peptidylarginine deiminase (PAD) enzymes. This process, termed “citrullination”, changes the structure and ionic charge of the peptide from positive to neutral, thus potentially making it more antigenic (95). There is emerging evidence suggesting that the autoantibodies, ACPA in particular, may also be pathogenic. In animal arthritis models, ACPAs are able to exacerbate the arthritic process (96). Furthermore, ACPAs and RF can be found in patient sera several years before clinical disease onset, and their presence are associated with a more severe disease course (97, 98). Thus, in addition to their diagnostic properties, autoantibodies are extremely valuable in that they allow classification of RA into two distinct phenotypes. Compared to seronegative patients, seropositivity is not only associated with an increased risk of comorbidities and a worse prognosis (98-100). Patients also differ with regard to genetic and environmental risk factors, with HLA-SE and smoking being primarily associated with ACPA positive disease (92).

1.4.5.2 Immunopathological mechanisms

The immunopathological mechanisms of RA are complex and incompletely understood. However, it seems a series of pro-inflammatory cascades are involved in the disease development. In the rheumatic joint, synovial inflammation is characterised by the presence and interaction of many different immune cells, including T cells, B cells, macrophages, dendritic cells, mast cells and plasma cells (101, 102).

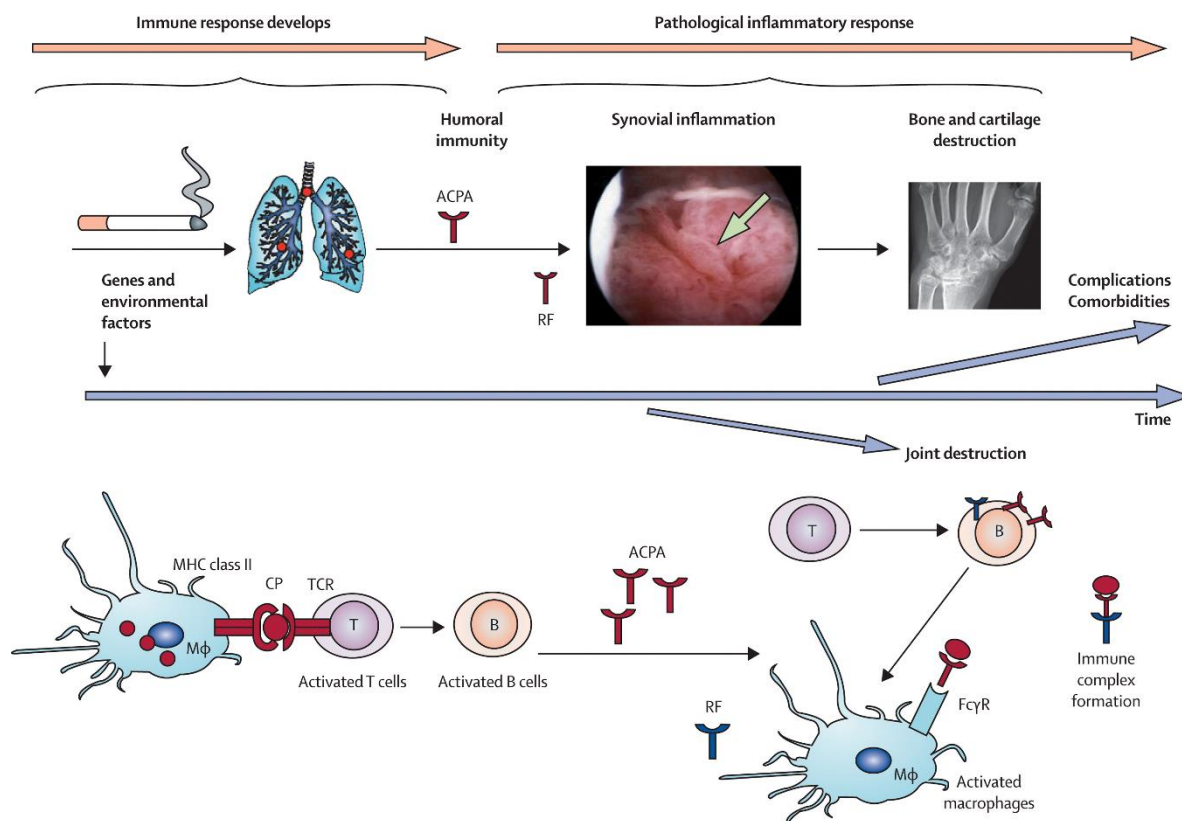
T cells infiltrate the synovial membrane subsequent to activation by APCs. This leads to the downstream activation of other immune cells through the production of cytokines and via cell to cell interactions. Both Th1 and Th 17 cells have been implicated in the disease process, and are capable of exacerbating and maintaining the inflammatory response (101-103). B cells produce autoantibodies, which form immune-complexes, and trigger immune responses via Fc and complement receptors. In addition, they may act as APCs as well as produce pro-inflammatory mediators (101-102). Macrophages are activated by T cell signalling and immune complexes and are major sources of pro-inflammatory cytokines (including TNF, IL-1 and IL-6) (102-103). Tregs are present but appear to have an impaired regulatory function (103). The net result of all these activities, is angiogenesis, enzymatic degradation of cartilage and synovial expansion into underlying bone.

1.4.5.3 The lung in the immunopathogenesis of RA

It has been well known for years that smoking is a risk factor for ACPA positive RA. Furthermore, studies suggest that this risk increases in a dose-dependent manner (91). Combined with the fact that autoantibody production predates the clinical disease by several years (97), these findings suggest that early immunological events in the lungs may be a critical initiating factor in the development of RA. In recent years, evidence has emerged to further support this hypothesis. Our group has previously shown an up-regulation of citrullinated proteins and enhanced PAD enzyme expression in BALF of healthy smokers compared to non-smokers (104). Furthermore, in patients with early RA we have identified increased citrullinated protein expression in the lungs of ACPA positive patients compared to ACPA negative patients. In addition we have demonstrated that ACPA positive patients exhibit significantly higher ACPA titres in BALF compared to blood (105), indicating local production of ACPA.

This suggests that smoking triggers pulmonary generation of citrullinated proteins. These citrullinated proteins may in turn bind with high affinity to HLA class II molecules in individuals possessing the SE alleles. Antigen presentation to CD4⁺ T cells causes T cell activation, with subsequent activation of B cells and the production of ACPAs. Binding of these autoantibodies to Fc receptors on the surface of synovial macrophages may eventually contribute to, and perpetuate the synovial inflammation (57).

Figure 1. Hypothetical model for the pathogenesis of ACPA-positive rheumatoid arthritis



Adapted from Klareskog et al (57). ACPA = anti-citrullinated protein antibody, CP = citrullinated proteins and peptides. RF = rheumatoid factor, MHC = major histocompatibility complex, TCR = T cell receptor.

1.5 SARCOIDOSIS

1.5.1 Clinical features

Sarcoidosis is a systemic, inflammatory disorder of unknown aetiology. It involves the lungs and/or mediastinal LNs (LN) in more than 90 % of cases, but almost any organ may be affected (106). The clinical features vary widely, depending on the disease activity and site of organ involvement. A large proportion of patients may be asymptomatic, with the disease identified on routine chest x-ray (107, 108). Symptomatic patients are commonly divided into two subgroups, depending on the clinical phenotype. Approximately 1/3 of patients present with Löfgren's syndrome, characterized by an acute disease onset, fever, bilateral ankle arthritis and/or erythema nodosum and bilateral hilar lymphadenopathy (BHL) with or without parenchymal infiltrates (107, 109). The majority of patients, however, have an insidious onset of disease, the first symptoms being a dry cough, fatigue and low grade fever. Other systemic symptoms include weight loss, weakness and night sweats (106). Extrapulmonary organ involvement is common, and may give rise to significant symptoms. Particularly, the disease may affect the liver, spleen, peripheral LNs, eyes, skin, nervous system, heart, kidneys and bone marrow (110). Whereas generally the prognosis is

favourable, 10-30% of patients experience a chronic progressive disease. Overall mortality is 1-5 % and is mainly due to respiratory failure or neurological or cardiac involvement (111).

1.5.2 Diagnosis

The hallmark feature of sarcoidosis is the non-caseating, epithelioid granulomas found in affected organs. They consist of centrally organized collections of macrophages and epithelioid cells, surrounded by lymphocytes (112). Granulomas may be identified on transbronchial biopsies or biopsies from airway mucosa or mediastinal LNs, retrieved via bronchoscopy or EUS-FNA. Other easily accessible locations, if involved, are the skin and peripheral LNs (43, 106). In patients where histopathological evidence of disease cannot be obtained, the diagnosis is based on a combination of typical clinical, laboratory and radiological features. BALF classically exhibits lymphocytosis, with an accumulation of CD4+ T lymphocytes. A raised CD4/CD8 ratio (>3.5) is highly specific for the disease (93-96%), with an acceptable sensitivity (53-59%). In patients with Löfgren's syndrome, an increased CD4/CD8 ratio is considered to have the same diagnostic impact as a positive biopsy (40). Blood tests may show elevated acute phase proteins, as well as an elevated calcium and serum angiotensin-converting enzyme (ACE) level. ACE is produced by the granuloma epithelioid cells, and is considered to reflect the granuloma burden, whereas calcium may be increased in blood and urine due to enhanced intestinal calcium absorption induced by high serum calcitriol concentrations (113, 114). Liver function tests and creatinine are monitored, as they may reflect hepatic or renal involvement (106).

PFTs are conducted routinely in all patients. They may be normal, or demonstrate an obstructive or restrictive pattern. Either way, they provide a baseline status for later assessment of pulmonary disease progression or improvement (115).

Chest x-ray typically shows BHL and/or pulmonary infiltrates, but x-ray findings may range from normal to severe pulmonary fibrosis. In addition to its diagnostic value, chest x-ray is used for classification of the disease into five stages (see Table 3) (111), where the higher stages correlate with a worse disease prognosis (116).

Table 3. Chest radiological staging in sarcoidosis.

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

HRCT may be indicated for differential diagnostic purposes or for a more detailed assessment of the pulmonary involvement (116). Suspicion of extra-pulmonary organ involvement may prompt further investigations. Magnetic resonance imaging in particular has proven valuable in the diagnosis and evaluation of cardiac and neurological disease manifestations (117, 118).

1.5.3 Treatment

The therapeutic options depend on the disease course, and range from purely symptomatic treatment to immunosuppressive agents, aiming to control the exaggerated inflammatory response. In patients with Löfgren's syndrome, the prognosis is good, particularly in HLA-DRB1*03 positive patients. Typically, in these patients, the disease will resolve spontaneously within 2 years of onset, and NSAIDs are often sufficient to control symptoms in the acute phase (119). In patients with extensive lung involvement, progressive loss of pulmonary function or extra-pulmonary organ disease, immunosuppressive treatment is usually indicated. Generally, oral corticosteroids is administered first line. Other immunosuppressive agents, such as MTX, may be initiated in patients that do not respond satisfactorily to corticosteroids (106). Biological treatments, such as TNF- α inhibitors, are still under evaluation, and to date there is no convincing evidence for their efficacy in sarcoidosis (120). In a subgroup of patients, available therapeutic regimens are unable to control the disease, and some of these patients will require referral for a lung transplantation (121).

1.5.4 Epidemiology

1.5.4.1 Frequency

Sarcoidosis is a disease of all ethnicities, but the prevalence, disease presentation and severity varies widely between different ethnic groups (122, 123). In Sweden there are approximately 1,500 - 2,000 new cases diagnosed annually (124). Sarcoidosis is more common in young adults, with a peak incidence between 20-40 years, and a second peak occurring in females >50 (122, 125). There is a slight female preponderance (125), and the disease is more common in non-smokers compared to smokers (126).

1.5.4.2 Risk factors

The exact aetiology of sarcoidosis remains to be established. However, as is true for RA, sarcoidosis likely results from an interplay between genetic and environmental factors. The magnitude of the genetic influence is difficult to estimate, however, the disease has been reported to occur five times as often in close relatives of sarcoidosis patients, compared to controls (127). The main genetic risk stems from genes localized in the HLA region on chromosome 6 (24, 25). Both HLA class I and class II molecules have been implicated,

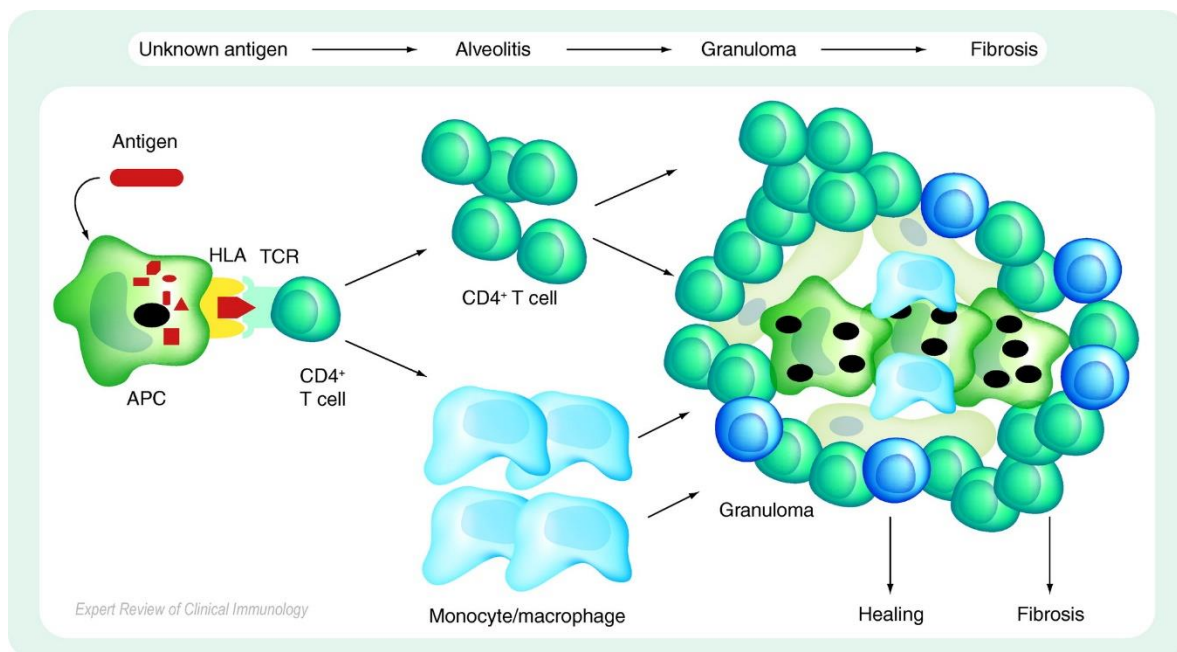
including HLA-B8 as well as antigens encoded by HLA-DR1 and HLA-DQ1 (128, 129). Furthermore, HLA genes also influence the clinical phenotype and disease outcome. For instance, HLA-DRB1*03, common in Scandinavian patients, is associated with a good prognosis (119), whereas HLA-DRB1* 14 and HLA-DRB1* 15 generally predispose for a chronic disease course. In addition, several candidate non-HLA susceptibility genes have been identified, such as the immune regulatory genes coding for butyrophilin-like 2 and annexin 11 (130).

In sarcoidosis, there is a familial, spatial, work-associated and seasonal clustering of disease (111). This indicates a common environmental trigger. Although, several environmental agents have been implicated, no single exposure has yet convincingly been associated with a majority of cases. Examples of suspected environmental triggers include inorganic and organic particles, such as insecticides, and mouldy environments (131). Furthermore, several infectious agents have been associated with the disease, with most studies focused on the role of mycobacteria and/or propionibacteria (132, 133). Particularly for mycobacteria, there are data showing that lung and blood T cells from sarcoidosis patients respond to multiple mycobacterial proteins (134, 135). Furthermore, using molecular techniques, the odds of finding mycobacteria in samples of patients with sarcoidosis is almost 10 times that of controls (133).

1.5.5 Pathogenesis

The immunopathology in sarcoidosis is consistent with an antigen-triggered cell-mediated immune response, leading to the formation and accumulation of granulomas in affected organs. Granulomas are generally formed to confine poorly degradable pathogens, thus restricting inflammation, and protecting surrounding tissue. However, in sarcoidosis, the granuloma formation contributes to the disease process. The granuloma formation is initiated by CD4+ T cells after interaction and activation by APCs. This leads to an accumulation of CD4+ T cells and macrophages. The macrophages make up the centre of the granuloma. Here they differentiate into epithelioid cells, which subsequently fuse and form multinucleated giant cells. Furthermore, they contribute to the persistent inflammation, through the production of pro-inflammatory cytokines (such as TNF- α), essential for granuloma formation. The central core is surrounded by CD4+ T cells interspersed with CD8+ T cells, Tregs and B cells (112, 136, 137). In more mature granulomas, fibroblasts also encircle the cluster of cells (137).

Figure 2. Granuloma formation.



Adapted from Grunewald et al (136). APC = antigen presenting cell, HLA = human leukocyte antigen, TCR = T cell receptor, CD = cluster of differentiation.

In line with these observations, BALF of sarcoidosis patients exhibit an increased percentage of lymphocytes. The majority of these lymphocytes are CD4⁺ T cells, showing a clear Th 1 cytokine profile, secreting high levels of IL-2 and IFN- γ (138-140). Cell surface markers of activation (such as CD69) are usually present, suggesting that these cells are highly activated (141). This is true also for the CD8⁺ T cells, which demonstrate a high capability of producing IFN- γ after *in vitro* stimulation (142). Another marker of active disease is the oligoclonal expansion of T cells expressing the variable gene segment 2.3 of the TCR α chain (AV2S3). This is observed in HLA-DRB1*03 positive patients, where it is associated with a good prognosis. The expansion suggest selective activation of the immune system, and are thus indicative of a specific antigen trigger (141, 143, 144).

Disease remission occurs when the offending antigen has been cleared, or with the suppression of macrophage and Th cell activity. The immune mechanisms leading to a fibrotic outcome are not clear, but could be due differences in the initial inflammatory response. In patients with new onset sarcoidosis, differences in BALF granulocyte counts and TGF- β release have been observed between patients who undergo clinical remission and those who develop chronic disease (139, 145, 146). Failure of Tregs to downregulate inflammation may be another factor contributing to chronic disease. It is, however, controversial whether the Tregs are increased, with impaired regulatory function, or whether they are present in decreased numbers in sarcoidosis (147-151). In addition, Th17 cells have been observed in tissue and BALF of sarcoidosis patients. However, their exact role in the pathogenesis of the disease remains to be defined (151-153).

2 AIMS OF THIS THESIS

The overall aim of the studies presented in this thesis, was to further characterize the immunological mechanisms involved in pulmonary inflammation and the pulmonary response to immunomodulatory treatment.

Specific aims were:

- To establish normal values for BALF cell differential counts, cell concentration and return volume in healthy non-smoking adults, and to investigate the influence of age, gender, season and site of BALF collection on these parameters.
- To compare the immunological response in BALF, blood and LNs of sarcoidosis patients, and to establish whether the pulmonary accumulation of CD4+ T cells classically seen in these patients are also present in mediastinal LNs.
- To investigate the immunological response to granulocyte and monocyte apheresis treatment in BALF and blood of sarcoidosis patients.
- To describe pulmonary manifestations in early RA, and to characterise the clinical and immunological response to antirheumatic drug treatment.

3 METHODS

The studies presented in this thesis were conducted using human samples. Written informed consent was obtained from all the participants and all studies were approved by the local ethics committee.

Below follows a short presentation of the methodology employed in the four studies included in this thesis. The individual papers are referred to by their roman numerals (I-IV).

3.1 SUBJECTS

The four papers included healthy volunteers (I), sarcoidosis patients (II-III) and patients with early RA (IV).

Paper I was a retrospective study of BALF samples from healthy volunteers who had participated as controls in various studies conducted at the pulmonary clinic, Karolinska University Hospital, from 1990-2009. Paper II-III prospectively included sarcoidosis patients attending the pulmonary clinic at the Karolinska University Hospital and at Södersjukhuset (paper III only). Sarcoidosis was diagnosed in accordance with the criteria outlined by the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) (111). Paper II included patients with radiological stage I and II only, whereas subjects in paper III were confined to patients with treatment refractory disease. The latter was defined as the presence of markers of disease activity despite recent or ongoing immunosuppressive therapy. Paper II also included a control group of patients with enlarged superficial LNs where histopathology demonstrated unspecific inflammation. Paper IV consecutively included patients with early RA attending the Early Arthritis Clinic at the Karolinska University Hospital in Solna and Huddinge. Diagnosis of RA was according to the 1987 ACR classification criteria (80). All patients were naïve to glucocorticoids and DMARD treatment. The main baseline patient characteristics have been summarised below:

Table 4. Main baseline characteristics of subjects included in paper I-IV

	Number of patients	Age, median (range)	Female gender %
Paper I	295	27 (18-65)	55
Paper II (patients)	15	38 (29-66)	13
Paper II (controls)	6	42 (25-63)	50
Paper III	7	47 (35-59)	14
Paper IV	143	58 (20-84)	66

3.2 INVESTIGATIONS

Investigations were performed at baseline (papers I-IV) and after treatment (paper III and IV).

3.2.1 Bronchoalveolar lavage (paper I-IV)

The bronchoscopy procedure and handling of the BAL fluid was conducted as previously described (154). For the BALF sample, two hundred and fifty ml of sterile, phosphate buffered saline solution was installed (5 x 50 ml), and immediately aspirated. The fluid was kept on ice and, filtered through a Dacron net (Millipore, Cork, Ireland) and centrifuged. May-Grünwald Giemsa staining of cytopsin slides was used to determine the differential cell counts.

3.2.2 Endoscopic ultrasound guided fine-needle aspiration (paper II)

EUS-FNA was conducted via the oesophagus. Aspirates were obtained using a 25-gauge needle. Smears were air-dried and immediately evaluated using a modified May-Grünwald Giemsa quick stain.

3.2.3 Flow cytometry (paper II-IV)

The expression of cell surface molecules was analysed by flow cytometry (fluorescence-activated cell sorter (FACS) CANTO II) and data were processed using the FACSDiva 6.1.2 (BD Bioscience) and Infinicyt (Cytognos, Salamanca, Spain) software.

Fresh BAL cells or whole blood were stained for a variety of surface markers: AV2S3 (paper II), CD3, CD4, CD8, CD27 and CD69 (paper II-IV), CD19, CD25, CD45RO and CD56/16 (paper III and IV), and CD28, CD45RA, CD57, CD103, HLA-DR, CXCR3, and CCR5 (paper IV). The cell suspensions were incubated in the dark for 20 minutes (4 °C for BAL cells and room temperature for blood cells). Erythrocytes were removed from whole blood, through incubation with FACS lysing solution. The blood and BAL cells were thereafter washed twice with cell wash (BD Bioscience). Cells from LNs were surface stained using the same procedure as for BALF cells (paper II).

FOXP3 was analysed using the FOXP3 intracellular staining kit (eBioscience) after the cells had been fixed and permeabilised (paper II-IV). The cells were then washed twice with permeabilisation buffer and incubated with 2% rat serum for 15 minutes. Subsequently, the cells were stained with anti-FOXP3 for 30 minutes. Finally, the cells were washed twice with permeabilisation buffer.

3.2.4 Enzyme-Linked ImmunoSorbent Assay (ELISA) (paper III and IV)

Soluble CD27, TNF α (paper III) and serum IgG ACPA levels (paper IV) were measured using commercial ELISA kits, following the manufacturers instructions (Human sCD27 instant ELISA and Human TNF- α total platinum ELISA (eBioscience) and Immunoscan RA Mark 2 (Euro-Diagnostica)). ACPA positivity was defined as having serum anti-IgG cyclic-citrullinated peptides (CCP)-2 antibody titres over 25 units/ml.

3.2.5 Pulmonary function tests (paper III-IV)

Patients in study III underwent dynamic spirometry only, whereas in study IV patients performed both dynamic spirometry and body plethysmography. The diffusing capacity for carbon monoxide (DLco) was measured using the single-breath method and corrected for haemoglobin.

3.2.6 HRCT (paper IV)

Images were independently reviewed by two blinded investigators. Discrepant findings were re-evaluated by both investigators until consensus was reached. Pathological findings were categorized as parenchymal abnormalities and airway abnormalities in accordance with the

International Classification of HRCT for Occupational and Environmental Respiratory Diseases criteria (155).

3.3 STATISTICAL ANALYSIS (PAPER I-IV)

Analyses were performed and graphs generated using Graph Pad Prism (GraphPad Software Inc., San Diego, CA, USA). In paper I, descriptive statistics were used to define reference values (5th -95th percentile). Furthermore, comparison between groups were performed by analyses of variance, using the Satterthwaite approximation in case of unequal variance between groups. In paper II, the non-parametric Mann Whitney test, Friedman's test followed by Dunn's post hoc test, was used as appropriate. In paper III and IV, the non-parametric Wilcoxon signed rank test was used for paired observations, with the exception of lung function parameters, where the parametric paired T-test was used (paper IV). Non-paired observations were analysed using the non-parametric Mann Whitney U test (paper IV). Correlations were analysed using Pearson correlation coefficient (paper I), and Spearman's rank correlation (paper II-IV), as appropriate. In all studies a $p < 0.05$ was considered significant.

4 RESULTS AND DISCUSSION

4.1 INTRODUCTION

Therapeutic advances in inflammatory pulmonary disease depends to a large extent on the identification of key components of the disease mechanisms. Furthermore, a prerequisite for studying these pathological mechanisms, is the recognition of what constitutes normality. The results of the papers included in this thesis contribute further to the understanding of the immunological and immunopathological pulmonary mechanisms. Thus, we hope that the information derived from these studies may eventually lead to better patient care.

4.2 NORMAL COMPOSITION OF BALF - PAPER I

BAL provides accessible samples from the lower respiratory tract. It is used extensively in clinical practice to aid the diagnosis of different pulmonary inflammatory disorders (38). Despite this, previous reference values for normal BALF composition have been based on small sample sizes and restricted patient populations, thus limiting their applicability (156-169). Attempting to overcome these limitations, reference material based on data collected from several previous studies have been produced (38, 170). However, the validity of such studies may be questioned, due to large methodological differences between the included trials. Furthermore, whereas the same reference values have been used for all patients, independent of age, gender and BALF collection site, there is little data to support their validity in these distinct subgroups. Aiming to compensate the deficiencies of these previous publications, we conducted a retrospective analysis of BALF data from 295 healthy never-smoking volunteers. Repeat lavages were performed in 47 individuals.

Table 5 shows the cut off points for BALF parameters from our study compared to the two main previous reviews, each including data from multiple different studies. The publication by Meyer et al is the Official American Thoracic Society Clinical Practice Guideline.

Table 5. Comparison of BALF cellular differential counts.

	Olsen et al [†]	Balbi et al [‡] (170)	Meyer et al (ATS)* (38)
No of subjects	295	478	502
Smoking status	Never-smokers	Never-smokers	Non-smokers
Macrophages %	72-96	NR	>85
Lymphocytes %	2-26	≤16.7	10-15
Neutrophils %	0-4	≤2.3	≤3
Eosinophils %	0-1	≤1.9	≤1

NR = not reported. [†]Values are based on 5th-95th percentile. [‡]Values are mean+2SD. *The definition used for cut-off is not reported.

Although generally similar, our values allow a larger span of normality, particularly for macrophages and lymphocytes. There are several possible explanations for this. First, our study is the single largest study to date. The inclusion of such a large population may also increase the chances of capturing individuals at the far ends of the normal spectra. We did indeed observe a large inter-individual variability. Particularly this was evident for macrophages and lymphocytes, where 10% of individuals had values under 76% and over 20%, respectively. Furthermore, whereas previous investigators generally have used the mean \pm 2SD as cut-off points, we elected to use the 5th-95th percentile. The reason for this is that the SD is an approximation, which assumes the data are normally distributed. This is not the case for several of the BALF parameters. Thus, using percentiles should more accurately capture the normal variability.

In some of the individuals undergoing more than one bronchoscopy, we observed a transient increase in one or other parameter. This is important information, as it suggests that isolated high values may be observed without clinical evidence of disease.

With regards to the different subgroup analyses, we demonstrated an age dependent decrease of lavage fluid return. This has also been observed by other investigators (162, 163), and can probably be explained by the loss of elastic recoil seen in older individuals (171, 172). There was no age dependent correlation with any of the other BALF parameters. Furthermore, the BALF cell parameters were independent of gender, season and collection site (lingula vs. middle lobe). These findings are significant, as they justify the use of the same reference values for all patients. Furthermore, they suggest that in older individuals a lower fluid recovery is acceptable, and does not influence the BALF composition.

4.3 T CELL PHENOTYPES FROM THREE COMPARTMENTS IN SARCOIDOSIS – PAPER II

Sarcoidosis is one of the inflammatory pulmonary diseases where bronchoscopy and BALF findings may be diagnostic as well as provide prognostic information (113, 139, 141, 144, 145). The diagnosis can be considered established if bronchial biopsies show non-caseating epithelioid granulomas (111). However, characteristic BALF findings may also be diagnostic, particularly in patients with Löfgren's syndrome. These include lymphocytosis, with an accumulation of CD4+ T lymphocytes and a raised CD4/CD8 ratio (>3.5 or 4) (113). In HLA-DRB1*03 patients there is also an oligoclonal expansion of CD4+ T cells expressing the AV2S3 gene segment. These AV2S3+ T cells are associated with a favourable prognosis, and they are thus thought to be effector cells (144). Although, these findings are often sufficient for diagnosis, there are situations where an alternative diagnostic approach is required. The availability of EUS-FNA has made the sampling from mediastinal LNs less invasive and more easily available. However, to date it is not known whether the lymphocytes found in BALF, reflect those in enlarged LNs. This is interesting both from a clinical and immunopathological perspective. Clinically, it may provide additional diagnostic information in patients where granulomas are not identified, whereas immunopathologically it may further increase our understanding of the underlying disease mechanisms.

In order to characterize and compare the cellular characteristics in blood, BALF and enlarged LNs, 15 sarcoidosis patients underwent bronchoscopy, blood tests and EUS-FNA. Cells were analysed by flow cytometry, focusing on markers of T cell activation. LN findings were also compared to six controls who had enlarged LNs due to unspecific inflammation.

Compared to blood, there was an accumulation of CD4⁺ T cells in LNs of sarcoidosis patients. The accumulation was similar to that observed in inflammatory LNs and less pronounced than in BALF. Thereby, it can be concluded that the CD4/CD8 ratio in LNs is of limited diagnostic value in sarcoidosis.

With regards to T cell activation markers, in accordance with previous studies, we found that both CD4⁺ and CD8⁺ T cells in BALF of sarcoidosis patients were highly activated and differentiated (141,142). Activated T cells were also present in the LNs, but the proportion of cells was significantly lower than in BALF.

Regulatory FOXP3⁺ T cells were found in higher proportions in the LNs compared to BAL and blood, however, there was a positive correlation between the percentage of Tregs in LNs and BALF. Furthermore, there were no differences in the percentage of Tregs in the LNs of patients and controls. In six patients there was an expansion of BALF AV2S3⁺ T cells (>10% of CD4⁺ T cells expressing AV2S3). A similar accumulation did not occur in the LNs. In these patients, there was an inverse relation between the frequency of AV2S3⁺ T cells and the percentage of BALF Tregs. This strengthens previous observations, suggesting that the AV2S3 T cells are mainly effector cells (144). Thereby, patients with an expansion of these cells may be able to more efficiently eliminate the offending antigen, and thus are not dependent on Tregs for disease resolution.

Altogether, the findings indicate that the lungs are the main focus of the exaggerated immune response observed in sarcoidosis. Perhaps as a result of repeated exposure to an inhaled antigen, T cells activated in the LNs circulate through the blood to the alveoli. Here they accumulate and exercise their effector functions, thereby leading to clinically manifest disease.

4.4 GMA FOR TREATMENT REFRACTORY SARCOIDOSIS – PAPER III

Chronic progressive sarcoidosis occurs in 10-30% of patients (111). In these patients, inflammation may persist for years, leading to pulmonary fibrosis and loss of lung function. Immunosuppressive agents are frequently used to dampen the exaggerated inflammatory response (106), however, they are associated with significant toxicities and often fail to control the relentless disease progression. Thus, there is a pressing need for new treatment modalities with a more favourable benefit-risk ratio.

GMA is a non-pharmacological treatment almost devoid of side effects (45). There is evidence of its efficacy reported for several inflammatory diseases (45-50), particularly in ulcerative colitis the experience with GMA is more extensive (45-47). The treatment causes immune modulation through the removal of circulating granulocytes and monocytes, however its mechanism of action is not fully established, and is thought to extend beyond the simple removal of these cells (51, 173). Particularly, GMA seems to cause an increase in

circulatory regulatory T cells, accompanied by a decrease in tissue Tregs. Furthermore, this seems to correlate with a favourable clinical response (174-176). Tregs are important also in the immunopathology of sarcoidosis, where they may be present in reduced numbers and/or have an impaired regulatory function (147-151).

To determine if GMA could potentially be a therapeutic option in sarcoidosis, we conducted a pilot study evaluating the clinical and immunological response to GMA in seven patients with chronic treatment refractory sarcoidosis. Particularly, we analysed cells from BALF and peripheral blood for markers of activity, differentiation and T-regulatory function. Investigations were performed at baseline, and at 2-4 weeks and again at three months (except bronchoscopy) after ten GMA treatment sessions.

In accordance with what has been reported in ulcerative colitis, we found a significant increase in circulating Tregs at 2-4 weeks after treatment. The increase was accompanied by a decrease in BALF Tregs, although this did not reach statistical significance. Furthermore, there was a correlation between the pulmonary decrease in Tregs and clinical response, as reflected by an improvement in dyspnoea score. These findings are encouraging, as they indicate that the suggested immunomodulatory effect of GMA on Tregs also occurs in sarcoidosis.

Another finding supporting a beneficial effect of GMA in the treatment of sarcoidosis, was the observed increase in the percentage of circulating CD4+ and CD8+/CD27- T cells. These fully differentiated effector T cells have been observed in high numbers in BALF of sarcoidosis patients, where they are thought to augment the inflammatory process (177). Their increase in peripheral blood could indicate migration of these cells from the pulmonary compartment to the circulation. This should in turn translate into a dampening of the localized pulmonary immune response, and eventually prevent further disease progression. We were unable to demonstrate an accompanying decrease in BALF CD27- T cells, however, this could be due simply to the relatively short follow-up period.

Recent studies suggest that GMA may be more effective in early UC as well as in mild disease, compared to moderate-severe disease (178, 179). Similarly, it is possible that the immunomodulatory effect of GMA would be more pronounced in sarcoidosis patients at the early stages of the disease or in patients with a lower radiological stage. Particularly it would be interesting to evaluate the treatment effect in patients presenting with stage II and stage III disease. In these patients, spontaneous remission occurs in 20% to 70% (stage II) and 10% to 30% (stage III), respectively (180). However, often it is not possible to predict the clinical outcome at the early stages of the disease. Due to the significant adverse events associated with standardised immunosuppressive regimens, treatment decisions in these patients are difficult. The low rate of side effects associated with GMA, however, would make this an attractive treatment option.

Furthermore, more recently, the opportunity to tailor the leukapheresis treatment to target specific immune cells, has become available. This could lead to an even more effective modulation of the immune response, and would be an interesting basis for future studies.

Either way, the findings from this study are interesting, as they indicate a GMA induced modulation of the pulmonary inflammatory response in sarcoidosis. Although larger studies are needed to confirm these findings, the treatment seems to cause modulation of Tregs similar to that being reported in ulcerative colitis. Furthermore, it may reduce the proportion of pulmonary effector T cells, something that should translate into a favourable clinical response.

4.5 THE PULMONARY RESPONSE TO DMARD TREATMENT IN PATIENTS WITH EARLY RA – PAPER IV

Pulmonary manifestations in RA are common, and may contribute significantly to the increased morbidity and mortality associated with the disease (58-60, 65-67, 74). Particularly disabling are RA-ILD and COPD, which may lead to respiratory failure and significant loss of functional capacity. Despite this, little information is available concerning the natural history of RA associated lung disease and the response to standard DMARD treatment. This is particularly true for early, treatment naïve arthritis, with most previous studies evaluating patients with a longer disease duration. This is of great concern, as an understanding of the disease process is essential for making valid treatment decisions. Furthermore, the instigation of appropriate treatment early in the disease course, may have a better chance at improving the long-term outcome.

To characterize the clinical and immunological response to standard DMARD treatment in patients with early, treatment naïve RA, we prospectively included 143 patients, investigated with HRCT and PFTs before and after six months of treatment. Bronchoscopy with BAL was conducted in a subgroup of 23 patients. BAL cell differential counts were recorded, and cells from BAL and peripheral blood were analysed by flow cytometry, focusing on markers of T cell activation. In accordance with clinical treatment guidelines (86), MTX was the DMARD administered to the majority of patients (92%), in conjunction with corticosteroids in ~80%. The baseline findings from these patients have been published previously (105).

4.5.1 Clinical response

There were three main findings in this study. First, as previously reported (105), a large proportion of patients had radiological evidence of pulmonary pathology at baseline (see Table 6). This was true also for the PFTs, where 62% of patients had an abnormal baseline spirometry. The most common abnormality was a reduced DLco (<80% of predicted, (52%)), with or without an obstructive pattern (forced expiratory volume in one second (FEV1)/slow vital capacity (SVC) ratio <0.7, (32%)). Although pulmonary manifestations are common in patients with RA (3, 4), it is interesting to note the presence of such changes also at the early stages of the disease.

Table 6. HRCT findings at baseline stratified by ACPA status

	ACPA – negative RA	ACPA – positive RA
<i>All subjects</i>		
No. of subjects	35	70
Parenchymal findings	13 (37)	44 (63)
Emphysema	5 (14)	13 (19)
Fibrosis	4 (11)	8 (11)
Ground-glass opacities	2 (6)	5 (7)
Opacities	5 (14)	14 (20)
Nodules (all sizes)	22 (63)	42 (60)
Nodules > 3 mm	9 (25)	24 (34)
Airway findings	23 (66)	46 (66)
Bronchiectasies	4 (11)	14 (20)
Wall thickening	11 (31)	22 (31)
Air trapping	15 (43)	30 (43)
<i>Never smokers</i>		
No. of subjects	8	20
Parenchymal findings	0	9 (45)
Airway findings	6 (75)	14 (70)

Adapted from Reynisdottir et al. (105) Values are presented as number (%) of subjects.

Second, after six months of treatment there was a radiological progression of RA-ILD in every third (four of 12) patients exhibiting interstitial changes on the baseline HRCT. In addition, three patients with normal baseline HRCTs developed changes consistent with early fibrosis after six months follow up. This is in accordance with previous reports of RA-ILD (181-183). However, in these previous studies, patients generally had a longer disease duration or were on DMARD treatment prior to inclusion, while our baseline data reflect the occurrence of RA-ILD in early, treatment naïve disease. Furthermore, the progression of interstitial changes occurred after initiation of treatment and despite a good clinical response with regards to articular disease. This suggests that standard DMARDs, although successful at controlling the joint manifestations, are not effective at alleviating the pulmonary inflammation.

The third, and perhaps most unexpected, finding was the large decline in FEV1 observed in all patients after six months follow up. The reduction was most pronounced in smokers (mean decline of 150 ml), however, even the group of non-smokers demonstrated a significant loss of function (30 ml). Although, a decline in FEV1 typically occurs with aging, the annual reduction observed in smoking and non-smoking individuals normally lies around 50-60 ml and 20 ml, respectively (184). Thus, in both ever-smoking and never-smoking patients with early RA, the loss of FEV1 is greater than what would be normally expected. This indicates that airways inflammation is in fact a disease manifestation of RA, rather than being an isolated smoking effect. As was true for the interstitial lung changes, the decline in FEV1 occurred despite treatment, further substantiating a lack of pulmonary anti-inflammatory DMARD effect.

4.5.2 Immunological response

There was a significant increase in BALF NKT cells after treatment. Furthermore, in ever-smokers, there was a negative correlation between the absolute number of BALF macrophages at baseline and the change from baseline in FEV1. In addition, in all patients, there was a positive correlation between the absolute number of NK-cells in blood at baseline and change from baseline in FEV1.

These findings may offer a mechanistic explanation for the exacerbated loss of FEV1. Macrophages have been implicated in the pathogenesis of both RA and COPD. They are found in large numbers at the site of disease, where they seem to significantly contribute to the persistent and destructive inflammatory response (185, 186). Similarly, whereas the role of NKT cells in RA is still elusive, in asthma and COPD, NKT cells have been found in high numbers in BALF, suggesting that these may contribute to the airways inflammation (187, 188). NK cells on the other hand may serve a protective function in RA, with low blood levels being related to increased disease severity, and treatment induced restoration of circulating NK-cells being associated with a good clinical response. Thus, the observations fit well with our findings, where high numbers of BALF macrophages seem to exacerbate the loss of pulmonary function, whereas a higher proportion of PBL NK-cells seems to serve a protective function (189-191).

In PBL, there was a reduction of CD4+ T cells expressing the activation markers HLA-DR, and the chemokine receptors, CCR5 and CXCR3. This is an expected finding, as DMARD treatment should induce disease remission and a reduced systemic inflammatory response. However, to further substantiate this finding, it would have been interesting to also analyse synovial fluid T cells of the same individuals.

Altogether the findings indicate that RA patients may have significant pulmonary manifestations already at the early stages of the disease. Particularly, there seems to be an increased inflammatory reaction affecting both the lung parenchyma and the airways. The inflammation responds poorly or slowly to DMARD treatment. This is alarming, particularly with regards to airways inflammation in smokers, where many patients will progress to develop COPD. Although further studies are needed to confirm our findings, they indicate a need for active screening for pulmonary disease in early RA. Furthermore, they highlight the importance of early smoking cessation and should prompt the instigation of interventional studies aiming to suppress the local pulmonary immune process.

5 CONCLUDING REMARKS

- BALF cell differential counts are independent of age, gender, season and collection site. There is an age dependent decline in BALF return volume, however, generally this does not appear to affect the BALF cellular composition.
- In sarcoidosis, the CD4/CD8 ratio does not have the same diagnostic value in LNs compared to BALF, where a ratio >3.5 has a high diagnostic yield.
- In sarcoidosis patients demonstrating a lung expansion of AV2S3+ T cells, there is an inverse correlation between the BALF levels of these cells and the fraction of Tregs. This suggests that the AV2S3+ T cells contribute to effective elimination of a presumed disease-triggering antigen, reducing the need for Tregs.
- GMA in treatment refractory sarcoidosis is associated with immunomodulatory changes in Tregs similar to that observed in ulcerative colitis, where it has been associated with clinical benefit. Thus, GMA could potentially be an alternative treatment option in sarcoidosis.
- In early RA, a large proportion of patients demonstrate abnormalities on HRCT and/or PFTs at diagnosis. Furthermore, during the first six months of DMARD treatment, there is a significant reduction in FEV1 in all patients, suggesting that airways inflammation is a disease manifestation of RA. These findings highlight the importance of smoking cessation. In addition, it should prompt the instigation of interventional studies to treat the local pulmonary immune process.

6 FUTURE PERSPECTIVES

- In sarcoidosis patients impaired T regulatory function may play a part in the immunopathology of the disease. In paper II, regulatory FOXP3⁺ T cells were found in higher proportions in the LNs compared to BAL and blood. In a future study, it would be interesting to compare the functional capacities of FOXP3⁺ T cells derived from LNs and BALF of sarcoidosis patients, to determine if these are similar.
- Leukaphereses treatment may be an attractive alternative in sarcoidosis. Therefore, it would be interesting to evaluate this treatment modality in a larger clinical study, including a relevant control group. Furthermore, it would be of value to investigate if apheresis treatment specifically tailored to sarcoidosis patients, would lead to a more effective immune modulation and a better clinical response.
- In the investigation of early RA, a follow up study within five years may contribute more information concerning the natural development of airways obstruction in smoking and non-smoking patients. Furthermore, it may help elucidate any potential protective or toxic effect of MTX treatment. In addition, a larger study of BALF parameters in RA could help to further characterise the immunopathological mechanisms of the disease, and the pulmonary response to DMARD treatment.

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