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T cell subsets in bronchoalveolar lavage fluid in sarcoidosis and other diffuse parenchymal lung diseases

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
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T celler i bronkialskyllevæske ved sarkoidose og andre interstitielle lungesykdommer

Vi fant økt forekomst av T celler med naturlig drepecelle-markører (NKT celler) hos pasienter med hypersensitivitets pneumonitt sammenlignet med sarkoidose, og uttrykket av aktiveringsmarkøren HLA-DR på cytolytiske T celler var betydelig høyere ved hypersensitivitets pneumonitt. Tilsammen ser det ut til at disse to lymfocyttfenotypene kan gi en god diskriminering mellom pasienter med sarkoidose og hypersensitivitets pneumonitt. Videre undersøkte vi diagnostisk nøyaktighet av en test basert på immunceller i bronkoalveolær skyllevæske ved diagnostikk av sarkoidose. NKT celler og aktiveringsgrad på cytolytiske T celler ga en signifikant økning av testens diagnostiske nøyaktighet.
I den siste artikkelen viser vi at både fraksjoner av regulatoriske T celler og Th17 celler i bronkoalveolær skyllevæske var lavere hos pasienter med sarkoidose enn hos friske kontroller og fant at en stor andel av Th17 cellene hos pasienter med sarkoidose også produserte interferron-γ. Andelen interferron-γ+ Th17 celler var sterkt korrelert med andel Th1 celler, men også med alvorlighetsgrad av sykdommen. Dette kan være en effekt av en Th17-Th1 drift som følge av en intens Th1-respons.
Dette forskningsarbeidet viser at lymfocyttfenotyper kan øke diagnostisk nøyaktighet av immuncelleprofiler ved sarkoidose, og potensielt også ved hypersensitivitets pneumonitt.

Slimhinnen i luftveiene og lungene er kontinuerlig eksponert for partikler, støv og en stor mengde mikrober fra omgivelsene. T hjelpeceller orkestrerer forskjellige typer spesifikke immunresponser, passende for den aktuelle trusselen. Th1 celler og Th17 celler skiller ut de betennelsesfremmende signalstoffene interferron-γ og IL-17, mens regulatoriske T har en regulatorisk og dempende effekt på immunresponsen.
Immunceller fra lungene kan framskaffes ved fiberoptisk bronkoskopi med bronkoalveolar lavage, og undersøkes nærmere med flow cytometri.
Målet med dette forskningsprosjektet var å lete etter immuncelleprofiler og lymfocyttfenotyper som kan brukes i diagnostikk av sarkoidose eller hypersensitivitets pneumonitt, samt å undersøke balansen mellom de forskjellige undergruppene av T hjelperceller ved sarkoidose og friske kontroller.

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THE ROLE AND CONTRIBUTION OF THE PHD CANDIDATE

In papers 1 and 2 the PhD candidate has generated the study ideas and design, performed data collection, statistical analyses and writing of the articles, under advice and with contributions from all supervisors and co-authors. Study design and statistical analyses for study 1 has been supervised by co-author Arne Åsberg.

In paper 3 the candidate has generated the study idea and design, included all patients and healthy control subjects, performed flow cytometry of bronchoalveolar lavage fluid cells, collected data from patient records, performed statistical analyses and writing of the article, under advice and with contributions from all supervisors and co-authors. The laboratory work was performed under the supervision of Torolf Moen, and flow cytometry on unstimulated cells for detection of $FoxP3^+$ $CD4^+$ T (tube 3 in paper 3) was done by the Unit for cytometry, Department of Immunology and Transfusion Medicine, St.Olavs University Hospital.

The PhD candidate has performed bronchoscopy/bronchoalveolar lavages in some of the patients and in all the healthy control subjects.

The PhD candidate has planned, organized and led the project meetings. He has prepared and presented posters on international congresses for papers 1 and 3.

Abstracts presented on international congresses:

1. 46th Nordic Lung Congress, Reykjavík, Iceland, 13–15 June, 2013: Poster discussion. Activated $CD8^+$T and NKT cells in BAL fluid improve diagnostic accuracy in sarcoidosis. Tøndell A, Rø A, Åsberg A, Børset M, Moen T, Sue-Chu M.

2. 6th International WASOG Conference, Paris, France, 6-7 June, 2013: Poster discussion. Th17 cells in bronchoalveolar lavage fluid produce IFN-γ in sarcoidosis. Tøndell A, Moen T, Børset M, Sue-Chu M.

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LIST OF ABBREVIATIONS

AM	Alveolar macrophage
APC (-Cy7)	Allophycocyanin (-with cyanine dye)
ARDS	Adult respiratory distress syndrome
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DPLD	Diffuse parenchymal lung disease
FC	Flow cytometry
FEV_1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
FSc	Forward Scatter
FVC	Forced vital capacity
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HC	Healthy control
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HP	Hypersensitivity pneumonitis
HRCT	High-resolution computed tomography
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
i or nTreg	inducible or natural regulatory T cell

IPF	Idiopathic pulmonary fibrosis
IQR	Inter quartile range
LR	Likelihood Ratio
M2	M2 polarization
MALT	Mucosa-associated lymphoid tissue
MHC	Major Histocompatibility complex
MyD88	Myeloid differentiation primary response gene (88)
NETs	Neutrophil extracellular traps
NK	Natural killer cell
NKT	Natural killer T cell
NSIP	Non-specific interstitial pneumonia
PAMP	Pathogen-associated molecular patterns
PE (-Cy7)	Phycoerythrin (-with cyanine dye)
PerCP-Cy5.5	Peridinin chlorophyll protein with cyanine dye
PRR	Pattern recognition receptors
RA	Rheumatoid arthritis
RAG	Recombination-activating gene
ROC curve	Receiver operating characteristic curve
SSc	Side Scatter
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th1, 2, 17	T helper cell types 1, 2 and 17
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor alpha

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LIST OF PAPERS

I. Tøndell A, Rø AD, Børset M, Moen T, Sue-Chu M.
Activated CD8+ T cells and natural killer T cells in bronchoalveolar lavage fluid in hypersensitivity pneumonitis and sarcoidosis.
Sarcoidosis Vasculitis and Diffuse Lung Disease 2014; 31; 316-24.

II. Tøndell A, Rø AD, Åsberg A, Børset M, Moen T, Sue-Chu M.
Activated CD8+ T Cells and NKT Cells in BAL Fluid Improve Diagnostic Accuracy in Sarcoidosis.
Lung: 2014, 192 (1), 133-140.

III. Tøndell A, Moen T, Børset M, Salvesen Ø, Rø AD, Sue-Chu M.
Bronchoalveolar lavage fluid Inf-γ+ Th17 cells and regulatory T cells in pulmonary sarcoidosis.
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NORSK SAMMENDRAG

Slimhinnen i luftveiene og lungene er kontinuerlig eksponert for partikler, støv og en stor mengde mikrober fra omgivelsene. Ufarlige mikrober og partikler fjernes med minst mulig betennelsesreaksjon i vevet, slik at lungenes gassutveksling kan opprettholdes. I normalsituasjonen sørger alveolære makrofager for dette, mens de skiller ut immundempende signalstoffer. Lymfocyttene i lungene er hovedsakelig T celler. T hjelpeceller utrykker co-reseptoren CD4 på celleoverflaten, og orkestrere forskjellige typer spesifikke immunresponser, passende for den aktuelle trusselen, og deles inn etter hvilke signalstoffer de produserer og skiller ut. Th1 celler skiller ut det betennelsesfremmende signalstoffet interferron-γ, Th2 celler skiller ut IL-4 og Th17 celler skiller ut IL-17. Regulatoriske T celler er en populasjon med $CD4^+$ T celler som uttrykker transkripsjonsfaktoren FoxP3, og har en regulatorisk og dempende effekt på immunresponsen.

Immunceller fra lungene kan framskaffes ved fiberoptisk bronkoskopi med bronkoalveolar lavage, der man skyller et lungesegment med saltvann eller buffer via arbeidskanalen i bronkoskopet. Cellene i skyllevannsprøven kan undersøkes nærmere med flow cytometri, som muliggjør analyser av blant annet uttrykk av antigener på celleoverflaten, produksjon av signalstoffer, funksjonell status og aktiveringsgrad på hver enkelt celle. Samtidig kan man måle relativ forekomst av de forskjellige immuncelletypene. Enkelte inflammatoriske sykdommer kan ha et mer eller mindre spesifikt mønster av immunceller, og kunnskap om dette kan i noen sammenhenger brukes til å sannsynliggjøre hvilken sykdom en pasient lider av. Dessuten kan man øke kunnskapen om de forskjellige sykdommene ved å studere mønstre i immunresponsen. Forholdet mellom forekomst av forskjellige undergrupper av T celler har blitt knyttet til enkelte autoimmune sykdommer.

Sarkoidose er en inflammatorisk sykdom som kan ramme nærmest alle vev i kroppen, og kjennetegnes av akkumulering av Th1 celler og betennelsesknuter kalt granulomer i lunger og lymfevev. En lignende granulomatøs lungesykdom med uttalt Th1 respons er hypersensitivitets pneumonitt, som kan oppstå ved eksponering for en rekke forskjellige stoffer, hovedsakelig antigener fra inhalert organisk støv, som sopp, bakterier eller proteiner fra fugler. Årsaken til sarkoidose er ennå ukjent, men man mistenker at inhalasjon av et eller flere ukjente antigen utløser sykdommen..

Målet med dette forskningsprosjektet var å lete etter immuncelleprofiler og lymfocyttfenotyper som kan brukes i diagnostikk av sarkoidose eller hypersensitivitets pneumonitt, samt å undersøke forekomsten av og balansen mellom de forskjellige undergruppene av $CD4^+$ T celler ved sarkoidose og friske kontroller.

Vi fant økt forekomst av T celler med naturlig drepecelle-markører (NKT celler) hos pasienter med hypersensitivitets pneumonitt sammenlignet med sarkoidose (median 5.5% versus 0.7% av leukocyttene, $p<0.0001$). Videre var uttrykket av aktiveringsmarkøren HLA-DR på cytolytiske T celler ($CD8^+$) betydelig høyere hos pasienter med hypersensitivitets pneumonitt sammenlignet med sarkoidose. (median 79% versus 43%, $p<0.0001$). Tilsammen ser det ut til at de to lymfocyttfenotypene NKT celler og HLA-DR^+ $CD8^+$ T celler kan gi en god diskriminering mellom pasienter med sarkoidose og hypersensitivitets pneumonitt. Videre undersøkte vi diagnostisk nøyaktighet av en test basert på data fra bronkoalveolær skyllevæske hos pasienter som ble utredet med mistanke om sarkoidose eller andre lignende lungesykdommer. Ved hjelp av ROC (receiver operating characteristic)-kurve analyser kunne vi anslå testens diagnostiske nøyaktighet med eller uten tillegg av NKT cellefraksjon og HLA-DR^+ $CD8^+$ T celler. Arealet under ROC kurven var henholdsvis 0.937 og 0.898 ($p=0.008$). Tillegg av disse to lymfocyttfenotypene i vår prediksjonsmodell ga en signifikant økning av testens diagnostiske nøyaktighet.

I den siste artikkelen viser vi at både fraksjoner av regulatoriske T celler og Th17 celler i bronkoalveolær skyllevæske var lavere hos pasienter med sarkoidose enn hos friske kontroller (median: 3.4% versus 5.3%, $p=0.017$ og 2.5% versus 4.5%, $p=0.01$). Videre fant vi at en stor andel av Th17 cellene hos pasienter med sarkoidose også produserte interferron-γ (median: 72.4% versus 31% hos friske kontroller, $p=0.0005$). Andelen interferron-γ^+ Th17 celler var sterkt korrelert med andel Th1 celler, men også med alvorlighetsgrad av sykdommen uttrykt ved radiologisk stadium ($n=23$, $rho=0.45$, $p=0.03$).

Dette forskningsarbeidet viser at lymfocyttfenotyper kan øke diagnostisk nøyaktighet av immuncelleprofiler fra bronkoalveolær skyllevæske ved sarkoidose, og potensielt også ved hypersensitivitets pneumonitt. Videre fant vi at hovedparten av Th17 celler i bronkoalveolær skyllevæske hos pasienter med sarkoidose produserte interferron-γ. Dette kan være en effekt av en Th17-Th1 drift som følge av en intens Th1-respons.

SUMMARY IN ENGLISH

The lungs and airways are constantly exposed to inhaled particles and microbes from the environment. To maintain the integrity of the lung epithelial surfaces, non-virulent microbes and particulate matter must be removed without unnecessary inflammation, whereas an effective immune response must be mounted towards potentially harmful pathogens. In health, this multifaceted task is handled by lung epithelial cells and the dominating population of scavenger cells, alveolar macrophages, which exert an array of immunosuppressive signals. The mucosal immune cells also include a population of lung homing lymphocytes of the memory phenotype, mostly T cells, capable of mounting a specific immune response towards formerly encountered pathogens or virus infected cells. T helper cells express CD4, and can release proinflammatory cytokines when activated via the T cell receptor, orchestrating an appropriate response to the offending threat. $CD4^+$ T cells are principally subdivided into subsets by their cytokine profile, including IFN-γ (Th1), IL-4 (Th2) and IL-17 (Th17). Another subset of $CD4^+$ T cells expresses the transcription factor FoxP3 (regulatory T cells), and exerts regulatory effects on the immune response.

During bronchoscopy, immune cells from the mucosa of the lungs and airways can be obtained by bronchoalveolar lavage, in which fluid is instilled and retrieved from a segmental bronchus. Flow cytometry is an essential tool in immunology, and enables us to characterize the phenotype, cytokine profile and quantify the frequencies of T cell subsets in bronchoalveolar lavage fluid. The immune cell profile in affected tissues of an inflammatory disorder may in some settings be of diagnostic value, as different disorders tend to have particular patterns of immune cell phenotypes or activation states. Additionally, altered balance of T helper cell subsets may be harmful to the patient. As an example, the failure to produce functional regulatory T cells causes devastating autoimmunity.

Sarcoidosis is a multisystem inflammatory disorder characterized by an accumulation of Th1 cells in the lungs and lymphatics. Granulomas, nodular collections of giant cells, epithelioid histiocytes, and chronic inflammatory cells, are frequently seen. Another granulomatous lung disease with accumulation of Th1 cells in the lungs and alveoli is hypersensitivity pneumonitis. The granulomatous reaction in hypersensitivity pneumonitis can be caused by a vast number of antigens, mostly proteins from inhaled organic dusts, whereas the offending agent in sarcoidosis is unknown.

The aim of the present studies was to identify bronchoalveolar lavage fluid profiles and lymphocyte phenotypes that can be used in the diagnosis of sarcoidosis and hypersensitivity pneumonitis, and to explore the frequency and balance of regulatory T cells and Th17 cells in the Th1-polarized disorder sarcoidosis and in healthy control subjects.

In patients with hypersensitivity pneumonitis, the frequency of T cells expressing the natural killer cell markers (NKT cells) was prominently increased compared to sarcoidosis (median 5.5% versus 0.7% of leucocytes, $p<0.0001$). The fraction of cytolytic ($CD8^+$) T cells expressing the activation marker HLA-DR was also considerably higher in hypersensitivity pneumonitis (median 79% versus 43%, $p<0.0001$). The expression of HLA-DR in $CD4^+$ T cells was higher in hypersensitivity pneumonitis, although not as markedly (89% versus 69%, $p=0.0002$). When viewed together, fraction of NKT cells and HLA-DR^+ $CD8^+$ T cells seem to discriminate patients with hypersensitivity pneumonitis from sarcoidosis. These two markers were tested in a retrospective study on the diagnostic accuracy of bronchoalveolar lavage fluid data in discriminating sarcoidosis from a diverse population of patients investigated for possible diffuse parenchymal lung disease. We measured diagnostic accuracy as the area under the receiver operating characteristic curve (ROC curve). The area under the ROC curve for the prediction model with and without fractions of NKT cells and HLA-DR^+ $CD8^+$ T cells was 0.937 and 0.898, respectively ($p=0.008$). Thus, addition of these two markers increased the diagnostic accuracy of bronchoalveolar lavage fluid data in sarcoidosis.

In the third paper we found that fractions of regulatory (Fox$P3^+$ $CD4^+$) T cells and Th17 cells were lower in sarcoidosis compared to healthy controls (median: 3.4% versus 5.3%, $p=0.017$ and 2.5% versus 4.5%, $p=0.01$). Interestingly, the fraction of Th17 cells also producing the Th1 cytokine IFN-γ was more than two-fold greater in sarcoidosis (median: 72.4% versus 31%, $p=0.0005$), and this fraction was highly correlated with fractions of Th1 cells. Moreover, the fraction of Th17 cells producing IFN-γ was positively correlated with severity of sarcoidosis, measured as radiologic stage ($n=23$, $rho=0.45$, $p=0.03$).

In conclusion, these studies provide evidence that lymphocyte phenotypes can be used as diagnostic markers in sarcoidosis and possibly hypersensitivity pneumonitis. Furthermore, we found that the Th1 immune response in sarcoidosis is correlated with increased expression of IFN-γ in Th17 cells. This may be an indication of a Th17-Th1 drift in sarcoidosis, due to an intense Th1 response.

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Since the 1970's, studies on immune cells in bronchoalveolar lavage fluid (BALF) have had an immense effect on our understanding of the immune processes in normal and diseased lungs, and particularly so in sarcoidosis, an inflammatory granulomatous disease affecting the lungs. Research on sarcoidosis has a long tradition in Scandinavia since its initial description in 1899 [1].

The airways and the lungs contain the greatest epithelial surface of the body, estimated to $120m^2$ [2]. The scavenger cells of the lungs, the alveolar macrophages (AM) are specialized in removing foreign particles and microbes from the alveoli while suppressing unnecessary inflammation that may cause disruption of the respiratory membrane and compromise gas exchange[3]. Peptides from microbes are presented by dendritic cells (DC) or AM to cells of the adaptive immune system in pulmonary and mediastinal lymph nodes, and antigen-specific lymphocytes can be recruited to the lungs during an inflammatory response.

The cellular profile and lymphocyte phenotypes in BALF may guide in the diagnosis of some diffuse parenchymal lung diseases (DPLD). However, the overlaps between various DPLD are considerable, and the debate on the usefulness of cellular investigations in BALF is still on going[4]. Modern flow cytometry enables increasingly complex profiling of the surface markers and intracellular protein expression in immune cells, providing a key tool in research on lung immunity both in health and disease.

In the present project, we use flow cytometry to study BALF T cell phenotypes and cellular profiles both to explore the immune processes in inflammatory lung diseases and as diagnostic markers, focusing on sarcoidosis.

1.2 DIFFUSE PARENCHYMAL LUNG DISEASE

DPLD [also called interstitial lung disease(ILD)] is a heterogeneous group of more than 150 disease entities[5], characterized by acute or chronic bilateral parenchymal tissue inflammation or fibrosis, and without evidence of infection or neoplasia [6]. Throughout the last century, our understanding of the pathogenesis of DPLDs has increased rapidly, and a system of classification of these diseases has been made [7, 8]. The current classification of DPLD consists of disorders of known cause (including DPLD associated with drugs, radiation therapy or collagen vascular disease, hypersensitivity pneumonitis or pneumoconiosis) or unknown cause. DPLD of unknown cause is subdivided into the groups of idiopathic interstitial pneumonias, granulomatous DPLD (sarcoidosis) and a final group of other forms of DPLD (including eosinophilic pneumonia and pulmonary Langerhans' cell histocytosis)[7]. Much attention has been paid to the idiopathic interstitial pneumonias, which are: idiopathic pulmonary fibrosis, idiopathic nonspecific interstitial pneumonia, respiratory bronchiolitis-interstitial lung disease, desquamative interstitial pneumonia, cryptogenic organizing pneumonia, acute interstitial pneumonia, idiopathic lymphoid interstitial pneumonia, idiopathic pleuroparenchymal fibroelastosis and unclassifiable idiopathic interstitial pneumonia[8].

Patients usually present with complaints of dyspnea on exertion or at rest. Cough is a common symptom as well. Symptoms and signs of extrapulmonary disease are important, as they may provide clues to an underlying disease, such as rheumatoid arthritis or inflammatory bowel disease. Thoracic imaging shows bilateral pulmonary infiltrates, and biopsy specimens show an accumulation of inflammatory immune cells, sometimes with abnormal extracellular matrix. BAL cellular analysis is a useful adjunct in patients with suspected DPLD.

A multidisciplinary approach is recommended to obtain a diagnosis of the idiopathic interstitial pneumonias, as the patterns seen on high-resolution CT and lung biopsy specimens are overlapping between the different entities. Idiopathic interstitial pneumonias are frequently confused with hypersensitivity pneumonitis [8], and more specific diagnostic markers are sought after.

In the following sections, we present a more detailed description of sarcoidosis and hypersensitivity pneumonitis.

1.3 SARCOIDOSIS

Sarcoidosis is a multisystem inflammatory disorder of unknown etiology, affecting the lungs and intrathoracic lymph nodes in the majority of cases. The term ‘sarcoidosis’ is derived from a case report from 1899 by the Norwegian dermatologist Cæsar Boeck, who described the histologic appearance of a skin lesion with similarities to sarcoma, in an article titled "Multiple benign sarcoid of the skin" [1]. Patients often present in adulthood, with hilar lymphadenopathy and pulmonary infiltration, and sometimes eye and skin lesions[9], although almost any organ may be affected. The highest incidence of sarcoidosis is reported to be in northern European countries (5 to 40 cases per 100,000) [10] and amongst African Americans in United States (35.5 cases per 100,000) [9, 11], and the latter have more skin, liver and eye involvement, a more chronic course and worse prognosis[12].

The most common symptom at presentation is persistent cough, and patients often have constitutional symptoms such as fatigue, low-grade fever or weight loss[13]. An incidental abnormal chest radiograph in an asymptomatic patient is also a frequent presentation[10]. Lung function test may show a volume restriction or an airflow limitation [13, 14]; the latter may indicate airway involvement [15, 16]. A reduced carbon monoxide diffusing capacity is a common finding. The typical high-resolution computed tomography (HRCT) features of sarcoidosis are bilateral hilar lymph node enlargement with a perilymphatic micronodular pattern and upper lobe predominance. It has been estimated that 95 % of patients with sarcoidosis have bilateral hilar lymph node enlargement [17]. Sarcoid granulomas are seen as micronodular lesions, and may coalesce over time, forming larger nodules[18].

The granulomas of sarcoidosis are well-defined interstitial collections of giant cells, epithelioid histiocytes, and chronic inflammatory cells, with a lymphatic distribution (centrilobular in the bronchovascular bundle, interlobular septa and in the pleura). The granulomas are mostly non-necrotizing, and concentric hyaline fibrosis is often seen around the granulomas, while the interstitium, unlike in hypersensitivity pneumonitis (HP), does not show signs of inflammation apart from the granulomas[9]. Unfortunately, the granulomas in sarcoidosis do not have any unique histologic features that discriminate them from granulomas of other causes[10].

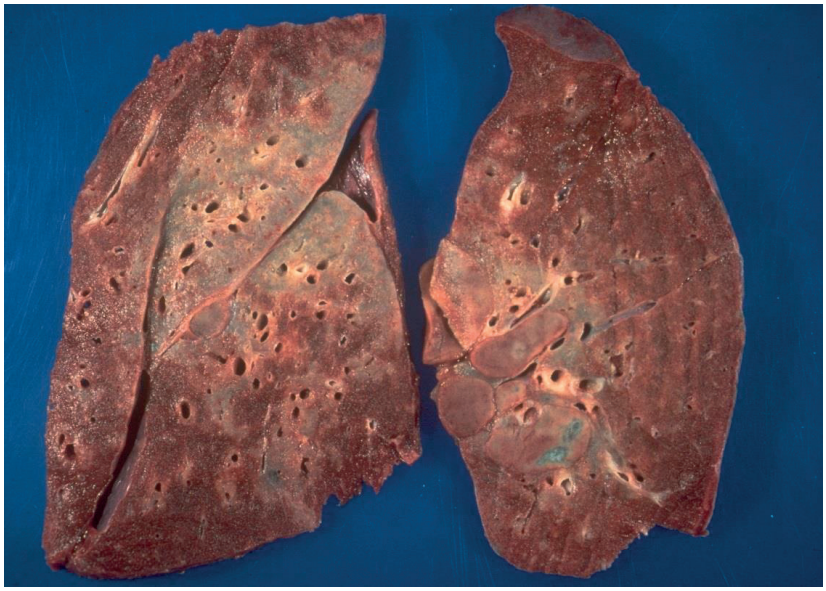


Figure 1: Lung granulomas

The large areas of consolidation represent confluent granulomas without significant fibrosis. Involved hilar lymph nodes are markedly enlarged (Yale Rosen, via Wikimedia Commons).

Sarcoidosis is characterized by increased fraction and number of IFN-γ-producing T helper cells (Th1 cells) in inflamed tissue [19, 20], commonly the lungs and lymphatics. The inciting antigen in sarcoidosis remains unknown. According to a current hypothesis, a persistent and poorly degradable antigen provokes a Th1 type immune reaction. An inhaled antigen is suspected [21, 22], and both mycobacterial proteins and DNA from Proprionibacterium acnes have been identified as possible candidate antigens[23]. The causative antigen(s) may not be viable bacteria, as patients with sarcoidosis do not demonstrate any increased risk of mycobacterial disease or other opportunistic infections while receiving immunosuppressive therapies [24], and patients with sarcoidosis who acquire HIV do not demonstrate progression of their underlying sarcoidosis[25]. Conversely, sarcoidosis has been reported in patients with HIV infections receiving anti-retroviral therapy, possibly reflecting an immune reconstitution inflammatory syndrome [26-28], and in patients receiving biologic agents that promote a Th1 response, i.e. interferon treatment [29]. Interestingly, bone marrow and heart transplants from patients with sarcoidosis have caused granulomatous inflammation in recipients[24]. A

possible explanation for this may be that the eliciting antigen persists in phagosomes, resisting degradation.

An increased incidence among monozygotic twins compared with non-twin siblings, as well as variation in disease incidence and disease course between different ethnic groups suggest genetic influence on the pathogenesis of sarcoidosis [9]. HLA class II genes have been associated with both disease susceptibility and disease phenotypes of sarcoidosis, indicating a link to antigen presentation to $CD4^+$ T cells, via binding of the TCR to antigen-loaded HLA class-II molecules[30]. Moreover, environmental exposures may produce a granulomatous pulmonary disease similar to sarcoidosis. This was demonstrated in a study on New York City Fire Department rescue workers during the first years after World Trade Center attack in 2001, where an increased incidence of sarcoidosis or a sarcoid-like granulomatous pulmonary disease was found[31]. Chronic beryllium disease may give a granulomatous disease of lung and skin with high degree of similarities to sarcoidosis. This is a hypersensitivity reaction in beryllium-exposed individuals, and reports have suggested that many patients with chronic beryllium disease may be misdiagnosed as sarcoidosis[32].

1.3.1 Diagnosis of sarcoidosis

The diagnosis of sarcoidosis relies on the demonstration of non-caseating granulomas in tissue biopsy or aspirate from lymph node, and exclusion of other causes of granulomatous inflammation[33]. The clinical and radiological presentation must also be consistent with sarcoidosis, although some patients have atypical findings at presentation, sometimes without signs of pulmonary involvement. Thus, sarcoidosis is per definition of unknown etiology, excluding similar diseases with known etiology.

The demonstration of granulomas is not always feasible, due to suboptimal sensitivity of transbronchial or bronchial mucosa biopsies, patient preferences or individual cost-benefit considerations. In certain subgroups of patients, with presentation suggestive of an excellent prognosis, such as Löfgren's syndrome or Heerfordt's syndrome (uveoparotid fever), biopsies are not considered necessary according to a recent review[13].

Several other granulomatous diseases may mimic sarcoidosis, such as silicosis, tuberculosis, berylliosis[13] or hypersensitivity pneumonitis[34, 35]. The use of BAL cellular differentials and lymphocyte phenotypes may be helpful both in identifying patients with an immune cell

profile not consistent with sarcoidosis, and to strengthen the diagnosis in cases where biopsy specimens fail to confirm granulomas.

1.4 HYPERSENSITIVITY PNEUMONITIS

HP is a syndrome of diffuse and predominantly mononuclear cell inflammation of the small airways and lung parenchyma [36, 37]. The inflammation is an immune response to an inhaled antigen in predisposed individuals[38], and over 300 antigens, primarily organic in nature, have been identified. The disease was described as early as 1713 by Ranazzini da Capri, in an influential work on early occupational medicine, in which he stated that 'Almost all who make a living by sifting or measuring grain are short of breath and cachectic and rarely reach old age'[36].

Although the list of possible causes of HP is extensive, data from a tertiary care referral center in the Midwest region of the United States suggest that most cases are caused by avian antigens, mycobacterium avium complex in hot tub water, farmer's lung or household mold exposure [39]. In that study the offending exposure was not identified in 25% of the cases.

HP is commonly classified as acute, subacute or chronic disease, although the usefulness of this is debated[40]. Most patients present with symptoms of dyspnea and cough. In the acute form, patients often experience a flu-like syndrome 4-12 hours after high level exposure, and the offending antigen may be easier to identify than in the chronic form, which often results from continuous, low-level exposure[37, 41].

On HRCT thorax, subacute HP often presents with patchy or diffuse ground-glass opacities, poorly defined centrilobular nodules, mosaic attenuation or air trapping, while reticulation due to fibrosis or honeycombing may be seen in patients with chronical disease, often with subpleural sparing [42]. Histologically, the classical triad of cellular bronchiolitis, bronchiolocentric lymphocytic interstitial pneumonitis and noncaseating granulomas is seen in approximately 75% of cases. Areas of organizing pneumonia may also be seen [41, 42]. The granulomas in HP are commonly characterized as small or loosely formed, although patients with hot tub lung may have well-formed granulomas[37].

Patterns on HRCT and pathology specimens from the lung in chronic HP may closely mimic those of idiopathic NSIP or IPF [42, 43]. In the updated classification for the idiopathic interstitial pneumonias, it was stated that a multidisciplinary discussion is particularly important in distinguishing HP from NSIP [8]. Although a set of major and minor criteria for

the diagnosis of HP has been proposed [44], there is no universally accepted definition of the disease.

It is still unclear why only a few of those exposed to an antigen develop disease, and why some patients heal, while others develop fibrosis[36]. HP is commonly mentioned amongst the differential diagnosis in patients with suspected DPLD, and more specific markers of the disease are wanted.

1.5 BRONCHOALVEOLAR LAVAGE

Bronchoalveolar lavage was introduced as a research tool by Reynolds and Newball in 1974 [45]. During the next few years the procedure rapidly gained acceptance and expectations for BAL as a diagnostic tool were optimistic[46]. In recent years, the clinical utility of BAL fluid cell analysis in patients with suspected DPLD has been a matter of debate and controversy [4, 6]. In the American Thoracic Society clinical practice guideline from 2012 concerning the use of BAL cellular analysis in DPLD [6], it is stated that BAL cellular analysis including total and differential counts may be a useful adjunct in the diagnostic evaluation of patients with a suspected DPLD. Lymphocyte subset analysis is not recommended as a routine analysis, but could be performed if a lymphocytic disease is suspected. On the other hand, the role of BAL in respiratory research is unquestionable, and a large part of current knowledge regarding inflammatory lung diseases has its origin in research on BALF cells[47]. BAL is considered a safe and well tolerated procedure, and can be performed even in critically ill patients with adult respiratory distress syndrome [48].

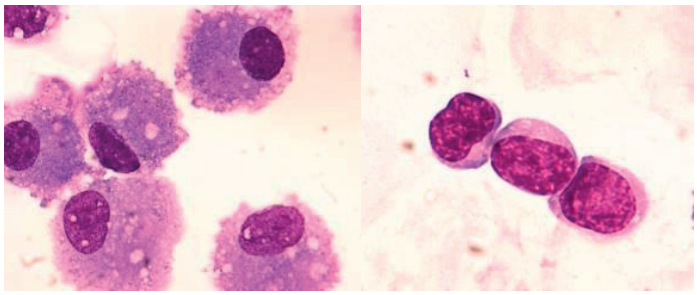


Figure 2: Bronchoalveolar lavage fluid cells

May-Grünwald Giemsa stain. Alveolar macrophages (picture to the left) and three mature lymphocytes (picture to the right). Magnification: 1000x. Source: BAL Atlas: © ild care foundation.

The BAL procedure is described elsewhere[49]. In brief, a flexible fiberoptic bronchoscope is introduced via the mouth or nose, and advanced as far as possible into the distal airways to a wedged position, where several aliquots of sterile fluid, commonly normal saline, are instilled and aspirated through the suction channel of the bronchoscope. The first aliquot of the BALF contains more cells from the distal airways, while the subsequent aliquots are thought to represent a higher relative fraction of cells from the alveoli. BALF differential cell counts are

commonly performed on cytospin preparations stained with May Grünwald/Giemsa and can be a useful supplement in the diagnostic work-up of DPLD[46].

1.5.1 BALF findings in sarcoidosis and hypersensitivity pneumonitis

The total cell count and relative and absolute number of lymphocytes are commonly increased in sarcoidosis and HP [50]. Whereas 10-15% of patients with sarcoidosis do not have a BAL lymphocytosis at diagnosis, a BAL lymphocyte fraction of <30% makes the diagnosis of HP uncertain [6, 51], and the diagnosis of HP is excluded with a normal BAL [41]. The lymphocytic cellular pattern in sarcoidosis is dominated by $CD4^+$ T cells typically with a CD4/CD8 ratio >3.5 [33]. However, this is not a sensitive marker, as the CD4/CD8-ratio is normal or even below normal in ~50% of the patients [52]. In addition, the ratio may be increased in normal elderly subjects [53]. The BAL lymphocytosis in HP is often dominated by $CD8^+$ T cells, resulting in an inverted CD4/CD8-ratio, together with an increase in relative numbers of mast cells and neutrophils, and the presence of foamy alveolar macrophages[54]. However, considerable overlaps are seen between these two diseases. The CD4/CD8 ratio is sometimes increased in HP, and lymphocyte phenotypes may vary with the causative antigens [54-56].

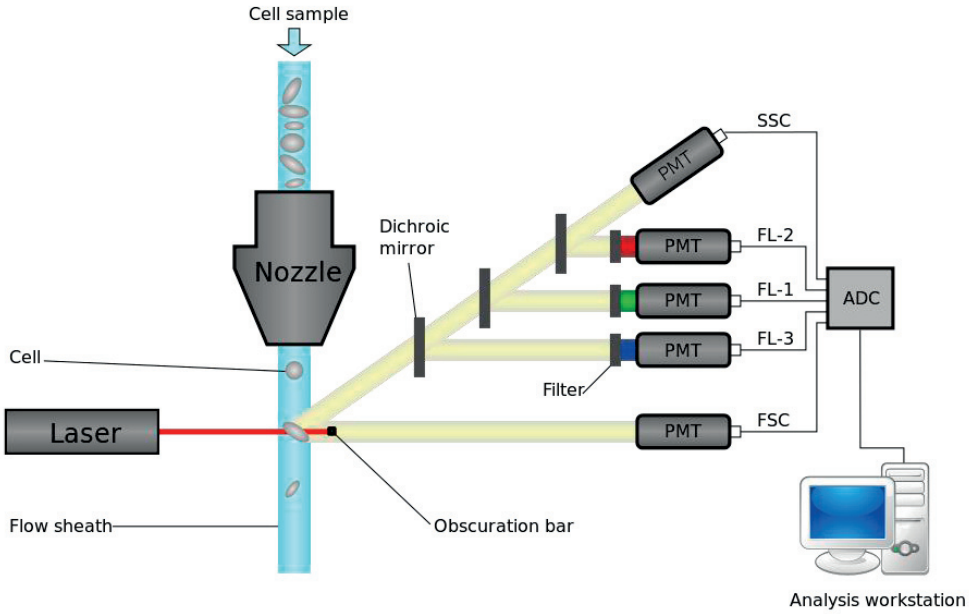
Activated BALF lymphocytes in both sarcoidosis and HP express human leucocyte antigen (HLA)-DR [57]. HLA-DR expression on $CD8^+$ lymphocytes has been reported to be lower in sarcoidosis than in HP[58], indicating that this may be a useful marker in discriminating sarcoidosis from other DPLD. Other lymphocyte subsets can also be identified by immunophenotyping with flow cytometry or immunocytochemistry. One of these is the Natural Killer T (NKT) cells, which express CD56 and/or CD16, in addition to the T cell receptor (TCR). In BALF, a lower fraction of NKT cells has been found in sarcoidosis compared to HP [59].

Studies on BAL cells have added immensely to our understanding of the immunopathogenesis of sarcoidosis [6, 60, 61], and constitute a safe and well tolerated way to obtain immune cells representative of the inflammatory cells found in histological sections of lung biopsy specimens[62]. While much of our knowledge on T cell immunology in the lung is derived from studies in mice, it is a growing awareness that more emphasis on studies in human immune cells is needed to realize the potential benefits of T cell immunology to human health[63].

1.6 FLOW CYTOMETRY

Flow cytometry (FC) is the *sine qua non* (without which, nothing) of the modern immunologist's toolbox[64]. For detecting antigens expressed intracellularly or on the cell surface the technique utilizes the binding of specific monoclonal antibodies conjugated to different light-emitting fluorochromes. The advances in both fluorescent molecules and modern instrument hardware have made 15-18 color immunophenotyping possible, at speeds up to 10000 cells per second. Such high-dimensional, high throughput immunophenotyping provides a tool for both broad and in-depth characterization of the cells of the human immune system[65]. In addition, the technique allows for sorting of live cell populations on the basis of fluorescence and light scatter properties.

A schematic view on the principles of flow cytometry is presented in figure 1.



Figur 3: Principles of flow cytometry

Schematic diagram of a flow cytometer. PMT: photomultiplier tubes; ADC: analogue-to-digital converter. "Cytometer" by Kierano - Own work. Licensed under Creative Commons Attribution 3.0 via Wikimedia Commons.

In FC, the cells are focused into a narrow stream by a rapidly moving sheet fluid, so that they pass the laser beams in 'single file' (figure 3). The light from the laser beams hitting the cells is scattered in the forward or sideward direction (Forward Scatter, FSc and Side Scatter, SSc). FSc is an indirect measure of cell size, whereas SSc is related to the intracellular complexity of the cell, i.e. granularity. Fluorescently tagged antibodies bound to antigens on the cell surface are excited by laser beams at particular wavelengths when the cell is passing by, and emitted light from the fluorochromes is detected by photomultiplier tube fluorescence detectors. A particular fluorochrome is excited by and emits light within a given range of wavelengths. A series of dichroic mirrors and filters split the emitted light, so that each detector receives light within a particular range of wavelengths[64]. The light generates a voltage pulse in the detector, which is recorded, and data from all detectors are integrated. Light emitted from a particular fluorochrome consists of a range of wavelengths and some light will spill over into other detectors. This spectral overlap is one of the problems with polychromatic flow cytometry. Spectral overlap is dealt with in a computational process termed 'compensation', which is basically to subtract estimated spectral overlap of light recorded in one detector, from recorded signals in other detectors. Individual cells are thus characterized by their antigens detected by light scatter properties and emitted light from up to 18 different fluorescent and specific antibodies.

Intracellular cytokine staining in FC has made it possible to study the cytokines produced by the individual cells after stimulation in a short-term incubation. A protein secretion inhibitor is added to secure retention of the cytokines in the cells[66]. This method allows analyzes of a large number of cellular functions, including T helper cell subsets, markers of cell viability, proliferation or activation states.

1.6.1 Flow cytometry on BALF cells

FC has provided an important tool in respiratory research, both in health and disease, such as DPLD and sarcoidosis. AM represent the most frequent cell population in the BALF under normal condition, often ~90% of the cells.

The human AM can be identified by their surface expression of CD11c[67], CD14 and MHC class II and the size and granularity result in high light scatter properties. Due to their high intracellular content of flavo- and metalloproteins, AM display high degrees of autofluorescence, i.e. they emit light without being labeled by an antibody-fluorochrome

conjugate. When excited by the standard argon laser at a wavelength of 488 nm, the autofluorescence of AM peaks at 540 nm, which is close to the wavelength emitted by standard fluorochromes like FITC (525 nm) or PE (575 nm)[68]. This must be taken into account when designing a panel of antibody-fluorochrome conjugates for immunophenotyping of BALF cells. The light scatter properties of BALF cells, contaminating erythrocytes and debris are often overlapping, making delineation of lymphocytes by FSc and SSc more difficult than in peripheral blood[69]. The lack of standardized protocols or guidelines for FC of BALF cells has been a problem, and makes comparison between studies difficult[69]. A panel of monoclonal antibodies for identification of leukocyte populations in BALF has been proposed [70].

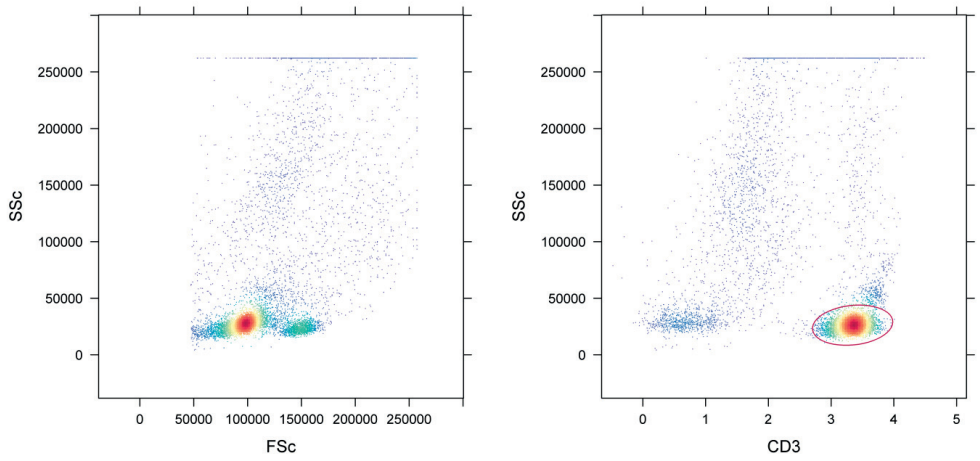


Figure 4: Gating and visual display of flow cytometry data

Flow cytometry of bronchoalveolar lavage fluid cells from one patient with sarcoidosis. In the first diagram, the forward (FSc) and sidewise (SSc) light scatter properties of the cells are displayed in a scatter-plot. The density of cells is coded by color. In the second diagram, $CD3^+$ cells are marked by a red elliptical ring. These cells are T cells. For the purpose of this example, the gate is automatically set by a software package in R/Bioconductor (See section 7: Future aspects).

1.7 THE IMMUNE SYSTEM IN THE LUNG

Traditionally, the immune system has been classified into the innate and the adaptive immune systems. These two systems differ in the specificity of the immune response and the ability to generate immunological memory.

1.7.1 Innate immunity

The term 'innate immune system' refers to the cells and effector mechanisms of our immune system that are the oldest, in evolutionary terms, and constitute a system for pathogen detection based on molecular patterns shared by many pathogens, and conserved in the evolution. The anatomy of the nose and airways, the tight junctions of the epithelial surfaces, mucociliary escalator and multiple microbicidal proteins of the airway epithelial fluid are important parts of the innate immune system of the lungs as well. An innate immune response does not generate immunologic memory, and cannot recognize foreign molecules that do not bind to cellular pattern recognizing receptors (PRR), but the response can be immediate, thus providing a way to generate inflammation and recruit phagocytes rapidly to counteract a potential threat. The interplay between the two legs of the immune system is complex, and the division into innate and adaptive immunity may be artificial.

PAMP and PRR

Pathogen associated molecular patterns (PAMP) are expressed on the surface of bacteria, viruses, fungi or other microbes. PAMP are recognized by PRR expressed intracellularly or on the surface of various cells in the lungs, such as epithelial cells, dendritic cells and macrophages. Key examples of cell surface and endosomal PRR are the Toll-like receptors (TLR), of which 10 are so far identified in humans, so named due to similarities to the 'toll'-gene identified in the Drosophilia in 1985. TLRs 1,2,4,5 and 6 are expressed on the cell surface, while TLRs 3,7,8 and 9 are expressed in the intracellular compartments. TLR form homo- and heterodimers with specificity towards a multitude of PAMP, as well as danger-associated molecular patterns (DAMP) such as heat shock proteins. An example is TLR2, which is expressed widely on AM, DC, lunge epithelial cells and granulocytes [71]. TLR2 recognizes many bacterial, fungal and viral substances, including the mycobacterial protein

Lipomannan. Activation of TLR2 signals induces production of the Th1-driving cytokine IL-12 in human macrophages through the adaptor protein MyD88 signaling pathway[72].

Alveolar macrophages

During the first few days after birth, the lungs and airways are colonized by AM derived from fetal monocytes. This cell population develop functional characteristics as an adaptive response to cues in the alveolar microenvironment, such as the high oxygen tension and surfactants[73], and is maintained by self-renewal throughout life [74]. Lung macrophages may be classified as interstitial macrophages, monocyte-derived AM or resident AM, although interstitial macrophages are not readily retrieved by BAL [75]. The majority of studies on lung derived macrophages do not differentiate between alveolar and airway macrophage populations.

AM have been described as “the masters of contradictory function” [75]. In the steady-state, they act as scavenger cells, continuously patrolling and clearing the alveoli and airways for debris, apoptotic cells, inhaled foreign particles or microbes and pulmonary surfactant. The importance of this function is demonstrated in patients with pulmonary alveolar proteinosis, a disease caused by autoantibodies towards the granulocyte-monocyte colony-stimulating factor (GM-CSF). These patients have a decrease in number and function of AM, resulting in accumulation of surfactant in the alveoli and compromised gas-exchange[76]. Another critical function of AM is to tonically suppress the development of inflammation and immune responses towards inhaled particles and microbes. Interactions with regulatory ligands on epithelial cells or microbes transmit inhibitory signals to the AM through CD200R, TREM2, SIRPα, mannose receptor, MARCO and TGF-βR. Under normal conditions, AM produce TGF-β and retinoic acid, suppressing T cell activation and skewing the T cell development towards regulatory T cells. Due to the lack of co-stimulatory molecules AM function weakly as antigen presenting cells (APC).

However, several mechanisms can override the default anti-inflammatory state of AM. In settings with destruction of the airway epithelium, the expression of regulatory ligands may be interrupted. Similarly, necrosis, but not apoptosis, liberates pro-inflammatory damage-associated cellular constituents [75]. In these settings AM may respond to antigens with production of pro-inflammatory cytokines, chemokines and lipid metabolites such as IL-8,

macrophage inflammatory protein (MIP)-1α, TNF-α, IL-12 or IL-4 and IL-13. In addition, circulating blood monocytes are recruited to the lungs under inflammatory conditions, and differentiate into inflammatory macrophages[73].

Dendritic cells

Much of our knowledge on DC is based on studies on mice [73]. There are several subsets of DC in the lung and airways. DC provide an important link between the innate and the adaptive immune system, and are pivotal in priming the response of naïve lymphocytes to their specific antigen in peripheral lymphatic tissue. Interestingly, some authors argue that DC are in fact cells of the broader mononuclear phagocyte system, without any unique adaptation for antigen presentation that is not shared by macrophages [67].

Epithelial cells

Whereas the conducting airways of the lower respiratory tract are lined with columnar, ciliated epithelial cells, the epithelial cells of the respiratory bronchioles and alveoli are flattened, non-ciliated type 1 pneumocytes. Scattered among these cells in the alveoli are surfactant-producing type 2 pneumocytes [73], and dendritic cells with extensions protruding between the cells into the lumen are embedded within the epithelium. The epithelial cells are important contributors in the homeostatic control of the mucosa. They express PRR [77, 78] and can thus sense the presence of microbes [79], and can be induced to produce pro-inflammatory mediators in response to potential threats [80]. Mediators released by epithelial cells may activate and skew the dendritic cell cytokine production and thus the adaptive immune response [81]. In one study, an essential role for TLR-signaling in epithelial cells in control of pseudomonas aeruginosa pneumonia was suggested [82].

Granulocytes

Neutrophils are short-lived phagocytic bone-marrow derived leucocytes that circulate in the bloodstream, and serve as the immediate effector arm of the innate immune system[73]. They are recruited to the lung by chemotactic factors including leukotriene B_4 and IL-8 produced

by activated AM and epithelial cells [83], and slowed down in the capillary bed, due to the small cross-capillary diameter[84]. Neutrophils express TLR on their surface (lacking only TLR3 and 7), and are able to sense the presence of various PAMP [85]. In the air spaces, neutrophils phagocyte bacteria and fungi that have been opsonized by complement and immunoglobulins, and the microbes are killed in the protected environment of the phagolysosome [73]. They are also able to project uncoiled nuclear DNA into the surrounding environment as a mean to entrap extracellular bacteria in neutrophil extracellular traps (NET) [86]. In addition, neutrophils can produce IFN-γ, TNF-α, and the chemoattractant CXCL-10 to recruit and modulate activation of NK cells and Th1 cells [87]. *Defensins* produced by neutrophils may also contribute to the modulation of the adaptive immune response via a modulatory effect on epithelial cells, T cells and DC [88]. Moreover, the neutrophils produce mediators with anti-inflammatory and pro-resolution properties, providing an important interface between innate and adaptive immunity [89].

During the resolution of inflammation, neutrophils undergoing programmed cell death (apoptosis) are cleared by macrophages that produce anti-inflammatory cytokines (IL-10 and TGF-β), resulting in dampening of the inflammation. The balance between apoptosis and necrosis must be tightly regulated, as intracellular content from necrotic cells are recognized as DAMP or *alarmins* by AM, causing the release of pro-inflammatory signals [90]. In the adult respiratory distress syndrome (ARDS), neutrophils accumulate in the lung microvasculature, interstitium and alveolar space, with disruption of the endothelial-epithelial barrier, alveolar damage and pulmonary edema [91]. While the role of neutrophils in the pathogenesis in ARDS is still unclear, the reactive oxygen species and other factors released by neutrophils may result in tissue damage [92].

Another granulocyte subset is the eosinophils. These cells are crucial in the killing of parasites, and control mechanisms associated with asthma and allergy. They are also capable of antigen presentation to T cells [93].

1.7.2 Adaptive immunity

In addition to being highly specific towards the pathogen that induces an adaptive immune response, the adaptive immune system has the ability to generate immunological memory[94], features that are essential for maintaining immunity to ubiquitous pathogens [95]. The

induction of a primary specific immune response takes 7-10 days, while a secondary specific immune response can be mounted rapidly, due to the presence of memory cells.

The lung in healthy individuals contains a small number of lymphocytes, and mostly these cells are resident effector-memory T cells, that do not circulate in the peripheral blood [95]. In BALF from healthy individuals, less than 10-15% of the cells are lymphocytes [96]. The adaptive immune response constitute an interplay of T and B cells with cells of the innate immune system, and cells capable of antigen presentation in the context of MHC class 1 or 2 play an important role.

B cells

B cells, so named because of their site of maturation in birds, the **b**ursa of Fabricius, are lymphocytes generated in the bone marrow capable of producing immunoglobulins that can recognize almost any foreign antigen that poses a threat to the individual. The enormous variety of antigen specificity of the immunoglobulins is generated in a process of genetic recombination of gene segments, combinatorial diversity of heavy and light chain matching, and somatic mutation of rearranged genes[96]. These cells are important in the protection against extracellular bacteria, such as streptococcus pneumonia. To be activated a naïve B cell that binds to its antigen is in general dependent on signals from an activated T helper cell that recognize the same antigen.

In the lung, B cells constitute a small fraction of the lymphocytes, with diverse functions, serving roles ranging from immunoglobulin production, antigen-presentation and as sources of both inflammatory and regulatory cytokines [97, 98]. Lung B cells produce immunoglobulins of IgA, IgG or IgM subclasses. IgA antibodies in bronchial secretions bind antigens and facilitate their elimination[99].

$CD8^+$ (Cytolytic) T cells

Cytolytic $CD8^+$ T cells are important for recognizing and killing virus-infected cells, and major producers of the cytokines IFN-γ, TNF-α and IL-2 [100]. These cells recognize peptides from viral antigens presented in the context of MHC class I, and are able to kill infected cells directly through several mechanisms. Furthermore, antigen-specific $CD8^+$ T

cells are able to recognize and kill cells infected with mycobacterium tuberculosis, and provide IFN-γ and TNF-α which are crucial for macrophage activation[100].

$CD4^+$ T cells

In contrast to cytolytic $CD8^+$ T cells, most $CD4^+$ T cells have helper functions, and are essential for the generation of an efficient antibody response. The critical role of T helper cells in lung immunity is clearly demonstrated by the high incidence of pulmonary infections in patients suffering from infection with human immunodeficiency virus[101]. After activation in the lymph node, $CD4^+$ T cells may evolve into one of the three major T helper cell subsets [96]. Th1 cells produce IL-2, IFN-γ and TNF-α, and are essential in the cell-mediated immune responses against virus infected cells or intracellular bacteria. Th2 cells produce IL-4, IL-5 and IL-13, and are important in the humoral immune response, as well as immunity towards parasitic infections. Th2 cells have also been implicated in atopic and allergic diseases, and play an important role in the immunopathogenesis of asthma. Recently, a subset of T helper cells has been discovered that is characterized by its production of the proinflammatory cytokine IL-17. Yet another subset of $CD4^+$ T cells, although not strictly a T helper cell is the regulatory T cells, identified by intracellular expression of the transcription factor FoxP3. These two subsets of $CD4^+$ T cells have altered the traditional dichotomy of Th1 and Th2 cells, and expanded our understanding of immune regulation in health and disease[102-105]. In the following sections, we will describe Th17 cells and regulatory T cells in more detail.

Th17 cells

Th17 cells have an important role in host immunity towards pathogens not adequately taken care of by Th1 or Th2 responses, such as extracellular bacteria, fungi and some intracellular bacteria requiring a strong inflammatory response [106]. The essential role of Th17 cells in lung immunity is clearly demonstrated in patients with hyperimmunoglobulin E syndrome, where mutations targeting the STAT3 pathway lead to impaired Th17 cell differentiation and function. These patients suffer from recurrent and often severe respiratory infections, particularly staphylococcal and fungal, with cutaneous cold abscesses and eczema [107, 108]. Th17 cells secrete chemokines that attract neutrophils, and several cytokines are varyingly

expressed, including IL-22, IL-17F, TNF-α, GM-CSF and IFN-γ or IL-10. IL-23 secreted by DC and AM induces production of IFN-γ by Th17 cells. These cells are reported to be highly inflammatory and pathogenic in certain settings [109].

Candidate sarcoidosis antigens Mycobacterium tuberculosis and Proprionibacterium acnes may both elicit strong Th17 responses [106], although Th17 cells seem to be dispensable for protective immunity towards mycobacterial infection [110]. On the other hand, IL-17 recruit Th1 cells to the lungs [106], and is required for proper formation of granulomas[111]. In one interesting study on mice infected with the intracellular pathogen Francisella tularensis, IL-17 were reported to induce IL-12 production in lung DC and macrophages, thereby regulating the Th1 response, and were required for optimal Th1 immunity [112].

Regulatory T cells

Regulatory T cells are characterized by expression of the transcription factor FoxP3, and have a pivotal role in maintaining immune homeostasis and preventing autoimmunity [113, 114]. Their importance is demonstrated by the immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which is a severe autoimmune disorder caused by mutations in the FoxP3 gene [115]. Since their discovery in 2003 [116, 117], a variety of subsets of regulatory T cells have been identified [118]. Apart from the traditional division into thymus-derived *natural* regulatory T cells (nTreg) and *induced* (also called *peripheral*) regulatory T cells (iTreg), recent studies have revealed an additional level of complexity. Regulatory T cells express different homing receptors in different anatomical locations, and under the influence of inflammatory cues, and can be divided into several distinct subsets, with unique functional properties [118]. Much of the current knowledge on regulatory T cells is generated from mouse studies.

1.7.3 The mucosal immune system

The mucosa of the lungs contains AM and DC within and beneath the surface epithelium [119]. Several other cell populations are also present, such as small numbers of mast cells and granulocytes, and T cells of effector memory phenotype. The T cell receptor repertoire of these cells is unknown [119]. During recent years, the emerging concept of the mucosa associated lymphoid tissue (MALT) has increased our insight in how the immune system

accomplishes the maintenance of tolerance towards commensal bacteria and inhaled or ingested antigens while providing protective immunity against potential harmful microbes. The immune system in the lung should be evaluated in this context, and the evolution of the adaptive immune system may be a fruitful starting-point. The immune cells of the MALT may be key players in the pathology of many chronic diseases, such as asthma, inflammatory bowel disease, inflammatory lung disease and COPD.

The evolution of adaptive immunity

The mucosal immune system contains the lymphoid tissues associated with the mucosal surfaces on the body, holding approximately three-quarters of all lymphocytes in healthy individuals. It has been proposed that this may have been the first adaptive immune system to evolve in vertebrates, possibly to deal with the vast number of commensal bacteria on the mucosal surfaces that co-evolved with the vertebrates [99]. Lymphocyte subsets seem to have evolved before the division of the vertebrates into the jawless (agnathans) and jawed (gnathostome) lineages, more than 500 million years ago, predating the evolution of both the thymus and spleen. These ancestral T and B cells had mechanisms of generation of cell surface receptor diversity through some process of somatic gene rearrangement, although the adaptive immune system based on the major histocompatibility complex (MHC) and the recombination-activating gene (RAG) recombinase enzymes, with T cell receptors (TCR) and immunoglobolins (Ig) is unique to the jawed vertebrates (examples are sharks, birds and mammals). Common to all the jawed vertebrates is also the thymus, a lymphoid organ derived from the embryonic intestine, thought to have co-evolved with the adaptive immune system, and the development of highly diverse antigen receptors (i.e TCR and Ig), which created the need for a system of negative selection of immune cells to maintain self tolerance[120]. Inducible regulatory T cells and Th17 cells may have evolved as gut mucosal immune cells with a crucial role in maintaining the immune tolerance towards non-pathogenic commensal bacteria, while simultaneously providing an efficient and rapid mechanism for protective immunity towards potential threats [121]. In fact, it has been speculated that these cells appeared before other $CD4^+$ T cell subsets, Th1 and Th2. An IL-17 ortologue has been found in activated lamprey lymphocytes, suggesting that ancestral Th17 cells appeared early in the evolution of adaptive immunity [122].

Mucosal tolerance

Subtypes of MALT exist in several tissues containing an epithelial surface, such as the gut-associated lymphoid tissue, and similarly the bronchus-associated lymphoid tissue (BALT), which is found in the larger airways of humans during childhood[119]. Naïve T cells from the thymus that encounter their antigens in the mucosa are activated. These cells leave the mucosa through the lymphatics, differentiate into effector T cells and recirculate in the bloodstream, before they reenter the mucosa due to their expression of homing factors to the tissue in which they were primed with antigen. The cytokine micro-environment during initial activation of a T cell determines the direction of the immune response to that particular antigen, mainly conferred by DC. Different populations of antigen presenting cells, including various subtypes of DC as well as AM orchestrate the immune reaction by initiating and controlling the activation of antigen-specific T cells [123]. In resting conditions, the DC in the mucosa of the respiratory system are biased towards tolerance, and produce IL-10 in response to antigen uptake, preventing the development of pro-inflammatory T cells and generating induced regulatory T cells expressing FoxP3. Contrary to this, the activation of DC by PRR such as TLRs from potential harmful microbes may induce IL-12 production by DC [99]. Interestingly, in Crohn's disease, a granulomatous inflammatory bowel disease, there seem to be a break-down of tolerance towards commensal bacteria in the intestine, and consequently an intense inflammatory T cell immune response in the gut mucosa[99].

Induction of mucosal tolerance is affected by the route, dose and duration of antigen exposure [124]. It is conceivable that these aspects of antigen exposure may be of importance in lung inflammatory diseases as well.

1.7.4 The immunopathology of sarcoidosis

The immunologic response of sarcoidosis has been reviewed by Zissel et al.[23]. Sarcoidosis is considered as an exaggerated immune response to so far unidentified antigens. BALF shows high numbers of activated alveolar macrophages and T cells, whereas in peripheral blood lymphopenia is a common finding and T cells express activation markers to a much lesser degree [58].

Alveolar macrophages and Th1cells

The core of sarcoidosis immunopathogenesis is the presentation of antigen(s) by host macrophages in the context of major histocompatibility complex (MHC) class II alleles, which are then recognized by antigen-specific $CD4^+$ T cells[9]. Several sequential immunological events are involved [23]. Antigen(s) seem to enter the host by inhalation[125], and are phagocytosed by APC, predominantly AM or dendritic cells [22]. The pathogenesis seems to involve the interplay of antigen, HLA class II and T-cell receptors [126]. It is possible that the triggering antigen varies depending on ethnicity, geographic location and individual genetic background [22], and different HLA- antigens lead to different disease courses. AM in sarcoidosis have excellent APC capacities, due to up-regulated expression of HLA class II and co-stimulatory molecules [23], and display a proinflammatory activation state and increased expression of adhesion molecules.

PAMP of microbial antigens can trigger or amplify inflammation[13]. Moreover, serum amyloid A in patients with sarcoidosis interact with TLR2 and enhance the production of TNF-α, IL-18 and IL-10 (figure 5) [127]. AM produce chemokines including RANTES, mcp-1, MIP-1α and IL-8, and the proinflammatory cytokines IL-1 and IL-6 and the NF-κB family of transcription factors are also up-regulated in sarcoidosis. TNF-α promote microbe killing and IL-1β promote granuloma formation[23]. Importantly, IL-12 produced by AM induce and maintain the differentiation of Th1 cells, and failure to produce sufficient IL-12 may result in incomplete Th1 differentiation[23].

T cells in sarcoidosis exhibit a restricted T cell receptor repertoire, shown to be consistent with oligoclonal expansion, strongly suggesting an antigen-specific response[24]. The Th1 transcription factor STAT1 is up-regulated, and most $CD4^+$ T cells produce IFN-γ, which activates AM and enhance phagocytosis and oxidant production, and IL-2. IL-15 and TNF-α

from AM in synergy with IL-2 stimulate T cell proliferation[9], granuloma formation and maintenance. In addition, the Bcl-2 family of genes are up-regulated in sarcoidosis, consistent with a prosurvival and anti-apoptotic profile for activated T cells [23].

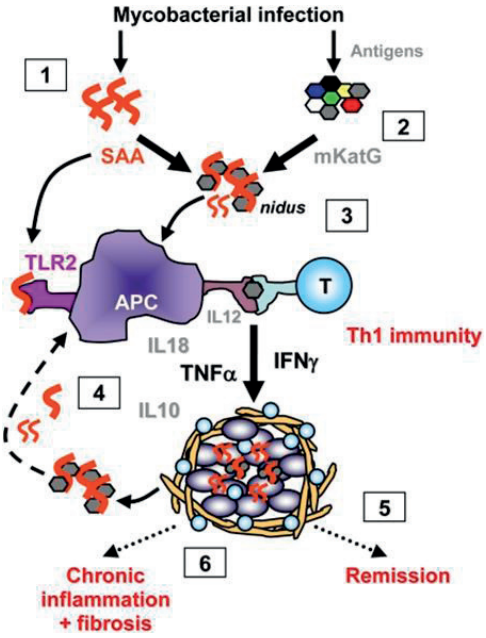


Figure 5: Hypothesis to the pathobiology of sarcoidosis.

In the acute-phase response towards the offending microbe (according to this hypothesis a mycobacterial infection) serum amyloid A (SAA) is induced (1). Mycobacterial proteins (mKatG) (2) are trapped within aggregates of misfolded SAA that serve as a nidus for granuloma formation (3). SAA can be released from graunulomas, leading to an amplification of the Th1-response through interactions with Toll-like receptor (TLR)2 (4). The granulomatous inflammation results in remission after successful clearance of the inciting antigen and SAA (5), or chronic inflammation and fibrosis (6).

Granulomas

The generation of granulomas is considered to be an immune response to foreign material or microbes that are poorly degradable or persisting, as a way of preventing antigen dissemination and further tissue damage[24]. AM play a key role in the control of granulomatous inflammation, and is the cell type that interact most frequently with both the infectious agent and other immune cells within the granulomas[128]. In the granulomas of tuberculosis, cells and mediators that promote bacteria killing must be controlled to prevent tissue damage. AM and regulatory T cells exerting anti-inflammatory effects seem to be beneficial to the control of infection, possibly via regulatory effects on effector T cells [128]. Within the sarcoidosis granulomas $CD4^+$ T cells are scattered, while $CD8^+$ T cells and B cells can be seen in the periphery.

Other immune cells

The role of IL-17 producing T helper cells and FoxP3$^+$ CD4$^+$ T cells in sarcoidosis has not been fully clarified[19]. Increased Th17 cell fractions in BALF and the granulomas on tissue specimens have been reported [129]. Cell subsets producing TNF-α or IFN-γ in addition to IL-17 have been found in inflamed tissue in various conditions[130-132], and these cells are believed to have distinct effector functions[133]. Peripheral blood Th17 cells in sarcoidosis were reported to contain an augmented fraction of IFN-γ$^+$ cells [134]. The reports on regulatory T cells in sarcoidosis are differing. Augmented numbers of FoxP3$^+$ CD4$^+$ T cells are found in and around granulomas [135], while in BALF both increased [136] and decreased frequencies [137] have been reported. Regulatory T cells and Th17 cells share developmental and functional links [121, 138], and in some autoimmune diseases an imbalance of these cell subsets has been suggested [139-142].

Less attention has been offered to the cytolytic CD8$^+$ T cells. These cells release TNF, IFN-γ and IL-2, adding to the overall Th1 cytokine release in sarcoidosis. In addition, CD1d-restricted NKT cells, a subset with immunoregulatory properties has been reported in diminished numbers in patients with sarcoidosis without Lõfgren's syndrome [143].

Resolution or persistence

Resolution is thought to be the result of a successful immune response, with removal of the eliciting agent and a subsequent down-regulation of the immune processes involved by TGF-β and regulatory T cells. However, the contribution of regulatory T cells in sarcoidosis is equivocal. In theory these cells may dampen the inflammatory response, which could be considered beneficial during the resolution phase. On the other hand, fibrotic sarcoidosis may also be the result of an insufficient Th1 immune response. Incomplete removal of the eliciting antigen may result in persistence of the granulomas, and ongoing granuloma formation [23].

Mechanisms leading to fibrotic lung disease might include generation of a pro-fibrotic alveolar macrophage phenotype, M2 AM, possibly promoted by IL-10 and/or IL-13 [19]. M2 AM release the pro-fibrotic chemokine CCL18. The lack of a sufficient Th1 or M1 activation may result in a shift from classically to alternatively activated AM (M2), and might be responsible for the fibrotic outcome in sarcoidosis[23]. To increase our understanding of the processes leading to fibrotic sarcoidosis is a key priority[19].

2 AIMS

2.1 OVERALL AIMS

To investigate and identify BALF profiles and lymphocyte phenotypes that can increase the diagnostic accuracy of BALF in sarcoidosis and HP.

To explore the frequency and balance of regulatory T cells and Th17 cells in sarcoidosis and healthy control subjects.

2.2 SPECIFIC AIMS FOR THE INDIVIDUAL PAPERS

Paper 1

To compare fractions of BALF HLA-DR$^+$ CD8$^+$ T cells and NKT cells between patients with sarcoidosis and HP, to identify typical BALF cellular profiles and phenotypes that may be used to discriminate HP and sarcoidosis.

Paper 2

To evaluate whether the inclusion of the lymphocyte subsets HLA-DR$^+$CD8$^+$ T cells and NKT cells increases the diagnostic accuracy of BALF investigations in a diagnostic model of sarcoidosis.

Paper 3

To investigate the fractions of FoxP3$^+$ CD4$^+$ T cells, Th1, Th17, and IFN-γ^+ Th17 cells in BALF in patients with sarcoidosis compared to healthy controls, and to assess the hypothesis that the balance between these subsets is skewed. To correlate the frequencies of these subsets with the radiological stage in patients.

3 GENERAL MATERIAL AND METHODS

3.1 STUDY POPULATION

For all the papers the diagnosis of sarcoidosis was considered certain if clinical presentation and thoracic imaging were consistent with pulmonary sarcoidosis and there was non-caseating granulomas in endobronchial or transbronchial biopsy specimens or from endobronchial ultrasound-guided transbronchial needle aspirations of enlarged hilar or mediastinal lymph nodes [33]. Histological demonstration of granulomas was not required for patients presenting with Löfgren's syndrome, defined as bilateral hilar lymphadenopathy with fever, erythema nodosum and/or ankle arthritis. Patients on systemic corticosteroids at the time of bronchoalveolar lavage were not included. Patients were stratified by findings on chest radiograph at presentation into stages 0-4, according to Scadding[144]. Exclusion based on missing data, sarcoidosis with concomitant cancer, immunosuppressive therapy or uncertain diagnosis was decided without knowledge of the results of the flow cytometry analyses. More detailed descriptions of the inclusion and exclusion criteria are provided in the individual papers.

Paper 1 and 2

The study population was generated from 226 patients investigated with flow cytometric analysis of BALF samples in our clinic between September 2007 and June 2010, where DPLD was a possible differential diagnosis after initial clinical and radiological evaluation. After investigative work-up, patients were grouped on the basis of the diagnosis reached by the attending respiratory physician into sarcoidosis (N=76) and non-sarcoidosis. Patients with a clinical diagnosis of sarcoidosis who did not fulfill the diagnostic criteria described above for sarcoidosis were excluded, yielding a study-population of sarcoidosis (N=51) and non-sarcoidosis (N=132) for paper 2. Eleven patients presented with Löfgren's syndrome.

As some time had elapsed before data analysis for the study in paper 1, we were able to extend the inclusion period for this study until September 2012. From this cohort 146 patients were given a diagnosis of sarcoidosis or HP. Eighty-tree patients with sarcoidosis and 10 patients with HP fulfilled the respective diagnostic criteria, and 53 patients were excluded (uncertain diagnosis: N=35; active cancer: N=7; missing data: N=1; immunosuppression:

N=10). Sixteen patients presented with Löfgren's syndrome. Patients with HP had at least 4 of the major criteria and 2 of the minor criteria as suggested by Schuyler et al.[44].

Paper 3

For the third paper, patients investigated with bronchoalveolar lavage between November 2010 and September 2012 for possible DPLD were eligible for inclusion in the study if they had a BALF lymphocyte fraction greater than 5% of the leucocytes and were not receiving systemic therapy with corticosteroid or methotrexate. We included 30 patients with sarcoidosis and 18 patients with other DPLDs. Five patients presented with Löfgren's syndrome. Investigation of intracellular expression of IL-17A and IFN-γ was performed in 23 patients with sarcoidosis (3 patients with Löfgren's syndrome) and 11 patients with other DPLD.

Eight male and 7 female healthy non-smoking control subjects with no history of allergy, asthma or other lung diseases were recruited by advertising on the hospital web-site. All had normal chest radiography and CO diffusing capacity. The FEV1/FVC ratio was below 0.7 in one subject (0.67). A second subject had a FEV1 of 76 % of predicted value.

For papers 1 and 2, the regional ethics committee (REC Central, Norway) did not consider the study to require ethical approval, because it was regarded to be a routine clinical quality control investigation and in accordance with the amended Declaration of Helsinki (Ref. no.: 2009/909-2). For paper 3, written informed consent was obtained from all subjects, and the study was approved by the Regional Ethics Committee (Ref.nr.: 2010/1939-4).

3.2 BRONCHOALVEOLAR LAVAGE

BAL was performed in accordance with recommendations [49]. The lavage site was guided by evidence of parenchymal pathology on HRCT, and if indifferent, lavage was performed in the right middle lobe. Local anesthesia with lignocaine and intravenous sedation with midazolam and alfentanyl was given, and with the bronchoscope in a wedged position in a segmental bronchus, 2-3 aliquots of 60 ml phosphate-buffered saline were instilled, and retrieved by applying gentle suction. The first fraction was used for microbiological analyses when appropriate, while the second and third fractions were pooled and filtered through nylon gauze (paper 1and 2) or a Falcon Cell strainer with 100 μm nylon mesh (paper 3) and stored at 4° C until processing.

Total and Differential Cell Counts.

The total cell count was made by ADVIA 120 Hematology System, and differential cell counts on a minimum of 300 cells were performed on Cytospin slides stained with May-Grünwald/Giemsa.

3.3 MITOGEN STIMULATION OF LUNG IMMUNE CELLS (PAPER 3)

BALF cells were stimulated within 4 hours of collection and fixed according to the protocol in the Human Th17/Treg phenotyping kit (BD Biosciences). Firstly, the cells were incubated for 5 hours in a medium of Roswell Park Memorial Institute medium (RPMI) with 10 % Fetal calf serum (FCS) with addition of the mitogens ionomycin at 1µg/ml and phorbol-12-myristate-13-acetate (PMA) at 50 ng/ml. A protein transport inhibitor was added to prevent protein transport to the extracellular space. Cells were also cultured in medium without mitogens, as negative control. Secondly, the cells were fixed using the BD FoxP3 buffer set. Lastly, the cells were suspended in a medium of 90% FCS and 10% dimethyl sulfoxide and stored for <6 months at −80° C until batch processing.

3.4 FLOW CYTOMETRY

Flow cytometry was performed with a FACS Canto I flow cytometer with FACS DIVA software. A minimum of 10.000 cells or 50 000 cells were analyzed in tubes investigating only surface antigens or intracellular antigens, respectively.

For paper 1 and 2, tubes containing 0.5-1.0 x 10^6 BALF cells per tube were incubated for 15 minutes with antibodies against surface antigens, and the cells were then washed twice in phosphate buffered saline with 0.1 % bovine serum albumin.

For paper 3, unstimulated cells were incubated with permeabilization buffer (BD Biosciences), and then stained with antibodies to surface antigens and intracellular $FoxP3^+$. The mitogen-stimulated cells and unstimulated controls, were thawed, permeabilized and then stained with antibodies to surface antigens and intracellular IL-17 and IFN-γ.

Details of the antibody panels are listed in Table 1.

Table 1: Flow cytometry antibody panel

Tube	Antibody conjugates					
	FITC	**PE**	**PerCP-Cy5.5**	**PE-Cy7**	**APC**	**APC-Cy7**
Paper 1 and 2						
1	Isotype control	Isotype control	Isotype control		Isotype control	
2	CD8	CD4	HLA-DR		CD3	
3	CD3	CD16 and CD56	CD45		CD19	
Paper 3						
1	Isotype control	Isotype control	Isotype control	Isotype control	Isotype control	CD45
2	CD8	CD4	HLA-DR		CD3	
3	CD4	FoxP3	CD8	CD27	CD39	CD25
4	CD4	IL-17A	CD8	IFN-γ	FoxP3	CD3

FITC: Fluorescein isothiocyanate, PE: phycoerythrobilin, PerCP-Cy5.5: Peridinin chlorophyll protein with cyanine dye (Cy5.5), APC: Allophycocyanin.

Gating

Lymphocytes were identified by their low side- and forward scatter.

For paper 1 and 2, lymphocytes were also confirmed to be $CD45^+$ (tube 3). Gates discriminating HLA-DR^+ and HLA-DR^- lymphocytes were generated from the isotype control tube. NKT cells, in this study defined as CD16/$CD56^+$ $CD3^+$ lymphocytes, were gated by visual comparison to the dominating population of CD16/$CD56^-$ $CD3^+$ lymphocytes.

For paper 3, regulatory T cells were defined as $FoxP3^+$ $CD4^+$ lymphocytes (tube 3). In the tube with mitogen-stimulated cells T cells were identified as $CD3^+$ lymphocytes and further gated by expression of CD4 and CD8 into the two main subsets. Gating of $CD4^+$ T cell subsets is shown in figure 5. Unstimulated cells were used as negative controls.

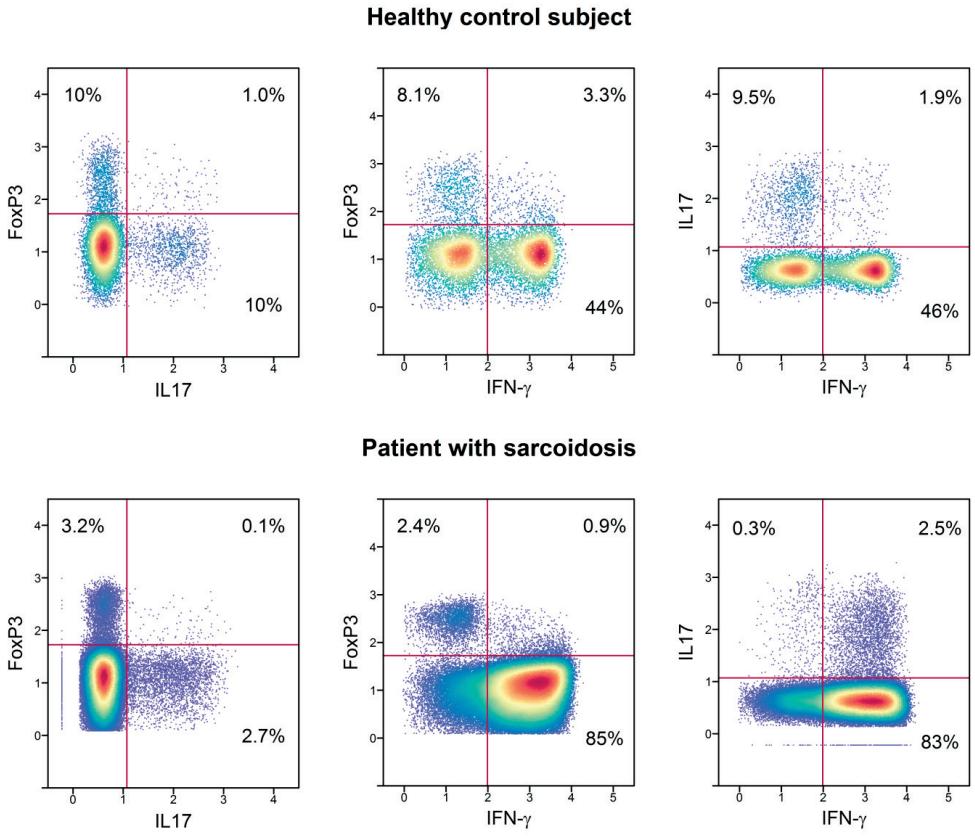


Figure 6: Regulatory T cells, Th1, Th17 and IFN-γ^+ Th17 cells in bronchoalveolar lavage fluid

Gating of $CD4^+$ T cell subsets in a healthy control subject (upper panel) and a patient with sarcoidosis (lower panel). The T cell subsets are defined as follows: Th1 cells: IFN-γ^+ $CD4^+$; Th17 cells: $IL17^+$ $CD4^+$; regulatory T cells: $FoxP3^+$ $CD4^+$ and IFN-γ^+ Th17 cells: IFN-γ^+ IL-17^+ $CD4^+$. Gates for IL17 and IFN-γ were set according to unstimulated $CD4^+$ T cells.

3.5 STATISTICAL ANALYSIS

Group comparison of continuous data was done with Kruskal-Wallis test in papers 1 and 3, and Mann-Whitney U test in paper 2. If a significant difference was found with Kruskal-Wallis test, post hoc analyses were performed with Mann-Whitney U test (Bonferroni correction for multiple comparisons was deployed in paper 1). Categorical data were analyzed with Fishers exact test in paper 1 and Pearson chi-squared test in papers 2 and 3. In paper 3, correlation was investigated with Spearman's rank correlation test. A p-value of < 0.05 was considered to be statistically significant.

Statistical analyses were done in R: A Language and Environment for Statistical computing (R Core Team, R Foundation for Statistical Computing, Vienna, Austria).

In paper 2, we evaluated the diagnostic accuracy of BALF investigations in a diagnostic model of sarcoidosis with and without the inclusion of the BALF lymphocyte subsets HLA-DR^+CD8^+ T cells and NKT cells. Essentially, we sought to design a prediction model with which clinicians can calculate the probability of sarcoidosis based on variables obtained by BALF investigation.

In our prediction model, we used age, sex, total cell concentration, eosinophils and neutrophils in differential counts and proportion of lymphocytes[145] and CD4/CD8-ratio from flow cytometry analyses, based on expected clinical relevance of these variables [46, 57, 146]. Model selection from these candidate variables was done with backwards stepwise elimination based on the Akaike information criterion[147]. The variables neutrophils and total cell concentration did not significantly improve model fit, and were excluded from the model.

With the knowledge of the final diagnoses in the individual patients in the study population (sarcoidosis or non-sarcoidosis), binary logistic regression was used to estimate a function for the likelihood ratio (LR) of sarcoidosis, based on the variables in our prediction model. LR is the probability of seeing a certain data set in a patient with a given disease divided by the probability of seeing the same data set in a patient without the disease[148]. For the individual patients, the posttest probability of sarcoidosis could then be calculated as:

$(\mathrm{LR} \times \text{pretest probability}) / (\mathrm{LR} \times \text{pretest probability} + 1 - \text{pretest probability})$. The pretest probability was defined as the prevalence of sarcoidosis in the study population (51/183).

Sarcoidosis often presents in younger adults, but a second peak in incidence rates is seen in older women[149]. Thus, the probability of sarcoidosis may not vary linearly with the variable 'age', and similar relations may be found in other variables as well. Fractional polynomials, a tool in logistic regression modeling, was used to find the simplest (if any) non-linear transformation of continuous variables that could increase model fit [147], and by transformation of the variables age and proportion of lymphocytes model fit was significantly increased.

The posttest probability of sarcoidosis for each individual patient was then calculated from the resulting prediction model (the basic model), and from a final model also including the variables HLA-DR^+CD8^+ T cells and fraction of NKT cells. The diagnostic accuracy of the models can be estimated as the area under the Receiver operating characteristic (ROC) curves [150, 151], which are plots of sensitivity versus 1-specificity at different thresholds for a positive test result, and represent a measure of the diagnostic accuracy of a test.

In lack of a new dataset to validate the model, we performed an internal validation, in which we randomly selected half of the patients in the study population for estimation of the model coefficients, and then tested the diagnostic accuracy of the resulting model in the other half [152, 153]. This procedure was repeated 1000 times in both prediction models, and the median value of the area under the ROC curve with 95% confidence interval was calculated for each model, representing a more robust estimate of the diagnostic accuracy of the models. The logistic regression models were compared with the likelihood-ratio test, and a non-parametric approach to the analysis of areas under correlated ROC curves described by DeLong et al [154] was used to compare the areas under the ROC curves.

4 MAIN RESULTS AND DISCUSSION

4.1 BALF LYPHOCYTE PHENOTYPES IN SARCOIDOSIS AND HP (PAPER 1)

We investigated BALF cellular profiles and T cell phenotypes in patients with a confident diagnosis of sarcoidosis (N=83) and HP (N=10), in search of diagnostic markers. In our laboratory, BALF lymphocyte phenotyping has included HLADR expression and identification of NK cells (to count for non-T non-B cell lymphocytes), thereby also providing quantification of T cells expressing NK cell markers (termed NKT cells).

Lymphocytes and eosinophils fractions in BALF were higher in patients with HP compared to sarcoidosis [median (IQR): 69 (66-74) % versus 31 (21-52) %, p=0.0001 and 4 (1-7) % versus 0 (0-1) %, p=0.008], while fractions of alveolar macrophages were markedly higher in patients with sarcoidosis compared to HP [57 (39-75) % versus 21 (15-28) %, p<0.0001]. The expression of HLADR on $CD4^+$ T cells was higher in patients with HP compared to sarcoidosis [89 (81-93) % versus 69 (60-79) %, p= 0.0002]. Both NKT cells and the fraction of $HLADR^+$ $CD8^+$ T cells were strikingly augmented in patients with HP compared to patients with sarcoidosis and HC, while the CD4/CD8-ratio was higher in sarcoidosis (figure 6). The combination of high fractions of NKT cells and HLA-DR^+ $CD8^+$ T cells seem to discriminate patients with HP from sarcoidosis very well, as shown in figure 7d. A typical example of flow cytometry dot plot for expression of NK cell markers on T cells and histograms denoting $HLADR^+$ populations of $CD4^+$ and $CD8^+$ T cells in a patient with HP is given in figure 7e.

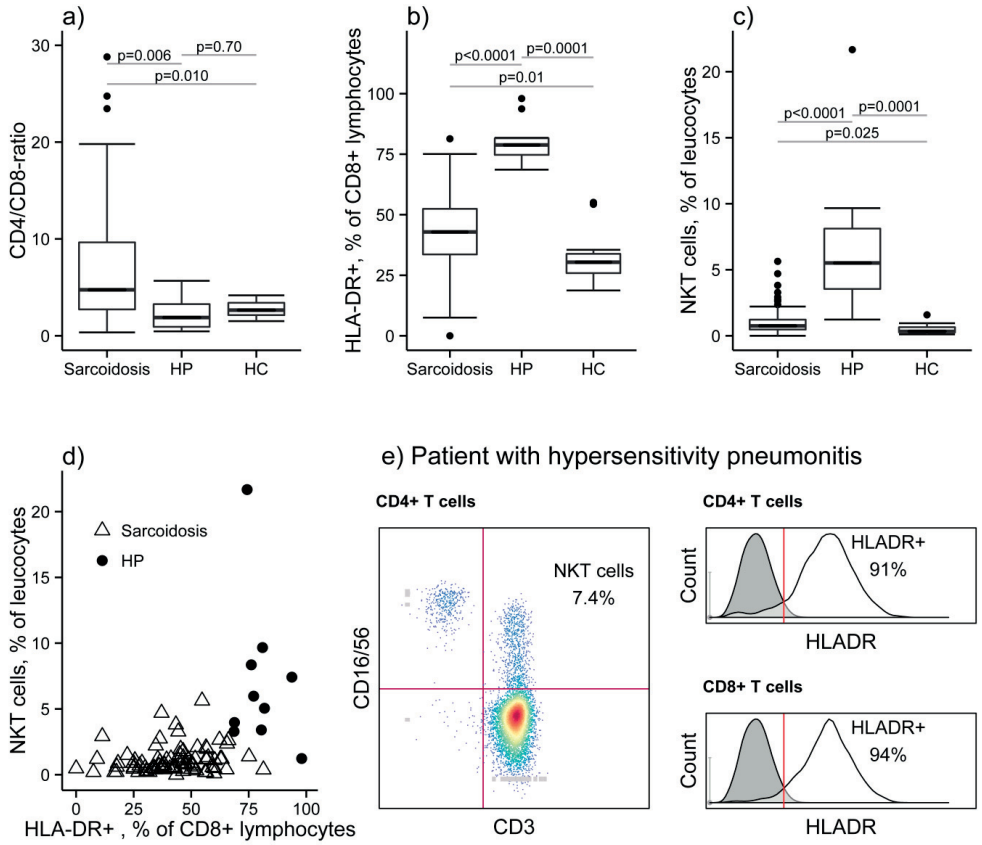


Figure 7: NKT cells and HLADR$^+$ CD8$^+$ T cells in BALF

Panel a-c): CD4/CD8- ratio, fractions of HLADR$^+$ CD8$^+$ T cells and NKT cells in BALF in patients with sarcoidosis, hypersensitivity pneumonitis (HP) and healthy control subjects (HC). d) Scatterplot of fractions of NKT cells and HLADR$^+$ CD8$^+$ T cells in individual patients. In this plot these two markers used in conjunction seem to discriminate between the two diagnoses. e) Example of flow cytometry diagrams from a representative patient with hypersensitivity pneumonitis.

Our findings of higher NKT cell fraction of BALF lymphocytes in HP is in agreement with a study by Korosec et al., in which BALF NKT cells from patients with HP (N=17) and sarcoidosis (N=57) was investigated [59]. They reported NKT cells as fractions of lymphocytes, while we reported NKT cells in fractions of leucocytes, to minimize the impact of noise in the flow cytometry gates in patients with low lymphocyte counts. However, recalculation of the NKT cell fractions as fractions of lymphocytes did not alter the principal findings. Also consistent with the previous study, we did not find significant difference in the frequency of traditional NK cells (CD3$^-$CD16/56$^+$ lymphocytes). In a recent study increased

fractions of NKT cells was found in patients with COP, but not in HP, compared to controls [155]. However, the control group in that study was generated from patients referred for investigations for chronic cough. We also provide a comparison with NKT cell fractions in HC, which are lower than in sarcoidosis (see figure 6c). NKT cells were for the purpose of this study defined as CD16/56$^+$ CD3$^+$ lymphocytes, in line with the initial description of T cells expressing NK cell markers. In the study by Korosec et al., this cell subset was identified as mainly CD8$^+$, and expressing unbiased αβ-TCR, while only a small percentage of NKT cells expressed the invariant CD1d-restricted TCR[59]. Accordingly, NKT cells as defined in both studies chiefly correspond to cells that are recently termed NKT-like cells[156]. These cells might vary widely in function, and are reported to produce IFN-γ, and not IL-4[156], and may include conventional T cells with upregulated NK cell markers[157]. It is conceivable that these cells are not functionally linked to the CD1d-dependent NKT cells. One study on human CD8$^+$ T cells expressing NK cell markers CD56 and/or CD57 denoted that these cells produced large amounts of IFN-γ when stimulated with IL-2, IL-12 and IL-15, and displayed anti-tumor and cytotoxic activity dependent on the perforin/granzyme pathway[158].

The increased expression of HLADR in CD8$^+$ T cells in HP observed in the current study is also in line with a previous report[58]. In that study, expression of several activation markers was investigated in BALF T cell subsets in patients with sarcoidosis, HP and HC. Lymphocytes in BALF from both patients and HC were found to display higher expression of markers of activation (as reported by others[159]), including HLA-DR, compared to lymphocytes in peripheral blood. Expression of HLA-DR was higher in CD8$^+$ T cells in BALF from patients with HP compared to sarcoidosis. We found no correlation of HLA-DR expression on CD8$^+$ T cells to radiologic stage in sarcoidosis, as they reported.

HLA-DR is a MHC class II molecule and has an important function on the cell surface of antigen presenting cells and B cells presenting antigens to naïve T cells. HLA-DR has been known to be expressed on activated T cells for several decades, perhaps somewhat surprising, as T cells do not normally function as antigen presenting cells. In this context, HLA-DR is thought to play a role in regulating or limiting a specific T cell response [160]. Cross-binding of HLA-DR molecules induce protein tyrosine phosphorylation and increased cytoplasmic second messengers, and upregulate expression of adhesion molecules [160]. Increased expression of HLA-DR on CD8$^+$ T cells has been observed in autoimmune diseases, human immunodeficiency virus infections and in normal aging [161]. Activated T cells in Th1-responses produce IFN-γ, and are essential in granuloma formation, a characteristic of both

HP and sarcoidosis[162]. Activated $CD8^+$ T cells are cytolytic, and are crucial in the host defense towards intracellular pathogens, and in killing tumor cells. The reasons for the expansion of cells of this phenotype in HP are unclear, and although many kinds of fungi and bacteria have been identified as environmental agents in HP [37], they are not present as virulent pathogens requiring a cytolytic immune response. Thus, a further exploration of the function, phenotype and cytokine profile of these cells in HP may be justified.

We are not aware of any prior reports on the use of these two markers in discriminating HP from sarcoidosis. In the present study, the number of patients with HP is not sufficient to investigate the diagnostic accuracy of HLADR status on $CD8^+$ T cells and fractions of NKT cells in HP. Furthermore, this combination of lymphocyte phenotypes may not be specific for the diagnosis HP. Increased NKT cell fractions have been reported in COP, and these lymphocyte phenotypes should also be studied in patients with other differential diagnoses to HP, such as NSIP, DPLD associated with medication or rheumatic disease or infectious diseases mimicking HP. However, as demonstrated in figure 6d, the combination of high fraction of NKT cells and a high degree of expression of HLADR on $CD8^+$ T cells are strongly suggestive of HP rather than sarcoidosis, and if a patient has BALF findings not in agreement with the suspected diagnosis, a careful reevaluation may be justified.

4.2 BALF IN DIAGNOSIS OF SARCOIDOSIS (PAPER 2)

In our clinic there has been a tradition for the use of BALF investigations in research (for example [163, 164]) and in the diagnostic work-up of sarcoidosis, HP and suspected DPLD. Lymphocyte phenotyping with flow cytometry was performed with four colours from September 2007, and extended to six colours in October 2010. Due to the controversy of the usefulness of BALF investigations in DPLD, and lymphocyte phenotyping in particular, we designed a study on the diagnostic accuracy of BALF in sarcoidosis, by far the most common entity of DPLD in our clinic. We also included the patterns of HLADR expression and fractions of NKT cells presented in paper 1, to see if these lymphocyte phenotypes could increase the diagnostic accuracy of BALF in sarcoidosis.

Table 2: Differential counts and lymphocyte phenotypes

Characteristic		Sarcoidosis (N=51)	Non-sarcoidosis (N=132)	P
Differential cell count	Total cell count (x10^6/ml)	0.19 (0.13-0.29)	0.26 (0.16-0.47)	0.002
	Neutrophils (%)	3 (1-10)	7 (2-32)	0.001
	Eosinophils (%)	0 (0-1)	1 (0-4)	0.015
Flow cytometry	Lymphocytes, (%)	37.9 (20.5-57.0)	13.6 (5.5-46.9)	<0.0001
	CD4/CD8 ratio	3.8 (2.1-8.9)	1.5 (0.7-2.8)	<0.0001
	NKT cells (%)	0.27 (0.12-0.56)	0.10 (0.01-0.62)	0.007
	HLA-DR+ CD4+ T cells (% of CD4+)	65.6 (59.1-77.1)	71.7 (54.0-83.3)	0.238
	HLA-DR+ CD8+ T cells (% of CD4+)	37.1 (27.6—48.8)	50.0 (33.3-71.6)	0.0003

Data presented as median (IQR). Numbers represent fractions of leucocytes, unless otherwise stated.

Patients with sarcoidosis had higher fractions of lymphocytes and CD4/CD8-ratio compared to non-sarcoidosis patients, while neutrophils, eosinophils and HLA-DR^+ fractions of $CD8^+$ T cells were higher in the latter group. Although NKT cell fractions were marginally higher in patients with sarcoidosis, this was not so in patients with elevated lymphocyte fractions. In patients with lymphocyte fraction above 15 %, NKT cell fractions were lower in sarcoidosis (median: 0.31 versus 0.73, p=0.004).

Interestingly, among 18 patients with NKT cell fractions above 2.0% of the leucocytes, five patients had HP, three had lymphoma/chemotherapy and/or pneumocystis jirovecii (one of which had HIV and pneumocystis infection), one had methotrexate-induced DPLD, one had cytomegalovirus pneumonia, one had sarcoidosis, two had cancer and four patients had another entity of DPLD. The presence of NKT cells, which are commonly thought to have high anti-viral and anti-tumor activity in patients with cancer or intracellular infections, is not surprising. Patients with suspected DPLD associated with drugs (chemotherapy or methotrexate) also had high expression of HLADR on $CD8^+$ T cells, similar to patients with HP (see section 4.1). This may reflect the case that drug-induced DPLD in some cases may be hypersensitivity reactions[165].

Lymphocyte phenotypes increase diagnostic accuracy of BALF in sarcoidosis

As described in section 3.5, we used logistic regression to estimate a function for the likelihood ratio of sarcoidosis based on selected BALF variables, age and sex. The aim was to design a prediction model to be used as an adjunct in diagnosing DPLD and sarcoidosis, and evaluate the diagnostic accuracy of this model. Selection of variables in a prediction model is performed with the aim to identify good predictors of an outcome or a diagnosis (in this case sarcoidosis). In general, the simpler model may be the better in clinical use, however the model structure and number of variables are less critical in a prediction model compared to an explanatory model[166]. Even so, knowledge of the subject should guide variable selection[147]. The resulting model (basic model) was compared with a final model also including NKT cell fraction and HLA-DR^+ fraction of $CD8^+$ T cells. The resulting function for LR for the final model was:

$$LR = e^{\left[\begin{array}{l} 14.919+(0.011 \times Lymphocytes)-\left(1.298 \times (Lymphocytes/10)^{(-2)}\right)+(0.060 \times CD4/CD8)-(0.126 \times Eosinophils)- \\ (0.174 \times Age)-\left(0.589 \times (Age/100)^{(-2)}\right)+(0.806 \times Sex)-\left(0.049 \times HLADR^{+}CD8^{+}\ T\ cells\right)-(1.035 \times NKT\ cell\ fraction) \end{array}\right]}$$

Knowing LR, the predicted probabilities of sarcoidosis for the individual patients were calculated by the formula shown in section 3.5. The results are displayed in figure 7a. ROC curves were generated and area under the curve calculated to assess the diagnostic accuracy of both models (figure 7b).

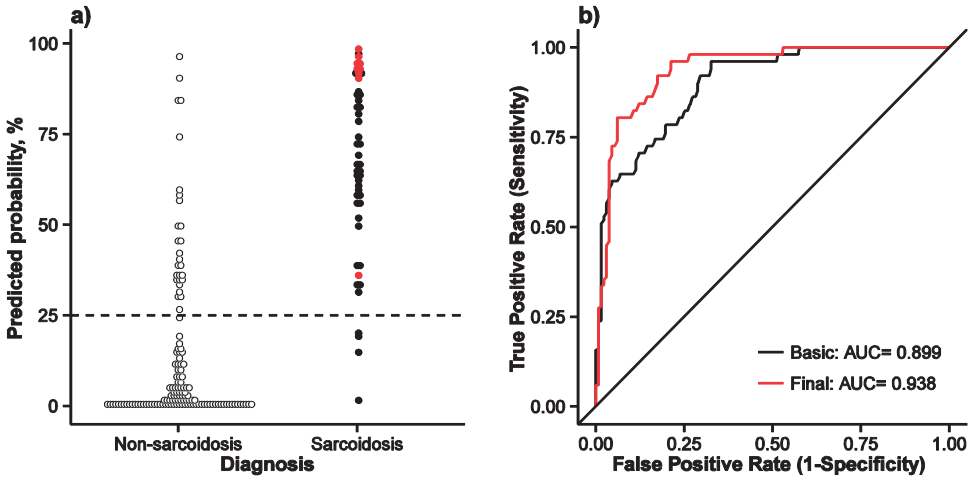


Figure 8: Predicting sarcoidosis from bronchoalveolar lavage fluid data

Panel a): Predicted probability of sarcoidosis using the final model for the individual patients in the study population, grouped according to the true diagnosis. Patients with Löfgren's syndrome are represented by red dots. The dashed horizontal line demarks a cutoff of 25% for discriminating a positive versus negative test. This cutoff would have yielded 4 false negative and 27 false positive patients. b) ROC curves for the basic (black line) and final (red line) model.

The area under the ROC curve for the final model was significantly higher compared to the basic model: 0.937 (95% CI: 0.902-0.973) versus 0.898 (0.851-0.945), p = 0.008, see figure 7b, and had significantly better fit to the data ($p<0.0001$, likelihood ratio test). Thus, the addition of NKT cell fractions and $HLADR^+$ fraction of $CD8^+$ T cells increases the diagnostic accuracy of BALF in sarcoidosis.

Assessing the diagnostic accuracy of a given test critically depends on the selection of the study population. Patients with sarcoidosis (N=51) were confirmed to fulfill diagnostic criteria. In contrast to this, the non-sarcoidosis group (N=132) also included patients without a confident diagnosis, as a diagnostic model should be able to discriminate patients with sarcoidosis from these patients as well. In this study we retrospectively included patients with suspected sarcoidosis or DPLD *prior* to BALF investigations. An improvement of the study design might be to include prospectively only patients with suspected or possible *sarcoidosis*. However, to do this retrospectively without introducing biased patient selection may prove challenging. The common failure to reach a confident diagnosis in patients with DPLD is an obstacle, and excluding patients with uncertain diagnoses also means excluding patients in

which supplementary diagnostic tools such as BALF investigations are most needed. Conversely, it is possible that patients with sarcoidosis included in paper 2 (and patients with HP in paper 1) may be those with more typical presentation, clinical and radiological findings, and easily detectable granulomas. Details on the heterogeneous group of non-sarcoidosis patients are given in paper 2.

The preferred study design to investigate the diagnostic accuracy of two prediction models would be to select the variables and estimate model coefficients in one half of the study population and test the performance of the models in the other half. Any assessment of retrospective fit 'uses the data twice'[166], and may overestimate the diagnostic accuracy [167]. However, the number of patients is a limiting factor in a logistic regression model, and we did not have a sufficient number of patients to split the dataset in two. The approach of repeated random subsampling is a method of internal validation, and provides a more realistic estimate of the diagnostic accuracy. The median area under ROC curve after 1000 repeated random subsampling was 0.898 versus 0.866 in the final and basic models, respectively. Nevertheless, these corrected estimates are also biased by the selection of variables in the model, which was based on data from all patients. Thus, an external validation of our model in a prospective setting could further strengthen our results.

Logistic regression has been used previously in studies on BALF in diagnosis of sarcoidosis and other DPLD [168-170]. These studies conclude that BALF investigations are helpful in discriminating sarcoidosis from other DPLDs or infectious disorders. The two studies by Drent et al. are restricted to patients with confirmed diagnoses of sarcoidosis, HP or idiopathic pulmonary fibrosis, and in the later study [169] extended to include patients with infectious disorders. It is uncertain how well these results translate to a population of *suspected* DPLD or sarcoidosis, which is a more common clinical situation. The other study [168] investigates BALF lymphocyte fractions and CD4/CD8-ratio. In our study, two additional lymphocyte subsets are added in the model, significantly increasing diagnostic accuracy.

Some patients have been wrongly classified by the final prediction model, particularly 4 patients with sarcoidosis, who had a predicted probability of less than 25%, and 5 patients who did not have sarcoidosis, and was scored by the prediction model as having >70% probability of sarcoidosis. Two of the former patients had lymphocyte counts below 10%, whereas two in the latter group had CD4/CD8-ratio above 10, indicating that the presence of particular BALF findings may have strong influence on the predictions by our model.

This study establishes that BALF investigations including lymphocyte phenotyping can be used as a supplementary diagnostic tool in sarcoidosis, and indicate that similar prediction models based on BALF data may be developed for other diagnoses, such as HP. As demonstrated in paper 1, studies on immune profiles in BALF from patients with DPLD and HC provide insights into the immune processes, and may possibly reveal clues to the immunopathology that can be modified by therapy.

4.3 $CD4^+$ T CELL SUBSETS IN SARCOIDOSIS (PAPER 3)

In sarcoidosis, IFN-γ^+ $CD4^+$ T cells and Th1-related cytokines IFN-γ, IL-2 and TNF-α are elevated in BALF, as well as the anti-inflammatory cytokine IL-10. Subgroups of patients with good prognosis tend to exhibit a less pronounced Th1-response [171].

BALF Regulatory T cells are decreased in sarcoidosis

Regulatory T cells, characterized by expression of the transcription factor FoxP3, produce IL-10, and are essential for immunoregulation at mucosal tissues such as the lungs[118]. We investigated expression of FoxP3 in BALF $CD4^+$ T cells in patients with sarcoidosis (N=30), other DPLD (N=18) and HC (N=15), and found significantly lower fractions in sarcoidosis, compared to other DPLD or HC (median: 3.4% versus 7.1%, p=0.001 and versus 5.3%, p=0.017, figure 10a). These results are in agreement with two other studies, in Swedish [137] and Chinese patients[172], but at variance with a report from France [135]. In the latter report, regulatory T cells were defined as $CD25^{bright}$ $CD4^+$ T cells, and FoxP3 expression was confirmed in the majority of these cells by a PCR-approach, making direct comparison less relevant. CD25 is the α-chain of the IL-2 receptor, and is also intermediately expressed by the majority of non-regulatory human memory $CD4^+$ T cells [173].

In sarcoidosis, peripheral blood lymphopenia and anergy to particular antigens such as tuberculin protein are common [174]. Lymphocyte depletion due to increased infiltration of lymphocytes in target organ may explain both phenomena, although anti-proliferative activity exerted by peripheral regulatory T cells has also been suggested to explain the state of anergy [135]. Similarly, $FoxP3^+$ $CD4^+$ T cells are reported to be augmented and the CD4/CD8-ratio lower in enlarged mediastinal lymph nodes compared to BALF. However, the immune process in sarcoidosis is believed to be primarily located to the airways and alveoli, possibly as a response to an inhaled antigen[125]. This is also consistent with the bronchovascular distribution of the lung noduli often seen on HRCT.

Interestingly, regulatory T cell populations may contract due to IL-2 deficiency under specific inflammatory conditions[175], such as Toxoplasma gondii infection, where an excessive IFN-γ-production leads to IL-2 shutdown. Similarly, both Th17-responses and IL-27, a cytokine thought to play a role in the initiation and resolution of a Th1-response, downregulate the

regulatory T cell pool via effects on IL-2[176]. It is conceivable that some of these mechanisms might contribute to the decreased fraction of BALF regulatory T cells observed in the current study. Figure 8 illustrates how the amount of IL-2 is a limiting factor controlling regulatory T cell homeostasis during infection.

Recent studies have explored the phenotypes and function of subsets of FoxP3$^+$ regulatory T cells in non-lymphoid tissues, and subsets specialized in regulating Th1, Th2 or Th17 polarized immune responses have been found [177]. These studies serve as an interesting background for our study on regulatory T cell phenotypes in the lung/mucosal system of the respiratory tract, both in the context of regulatory T cells in non-lymphoid tissues and under inflammatory conditions (the profound Th1-polarized immune response of sarcoidosis).

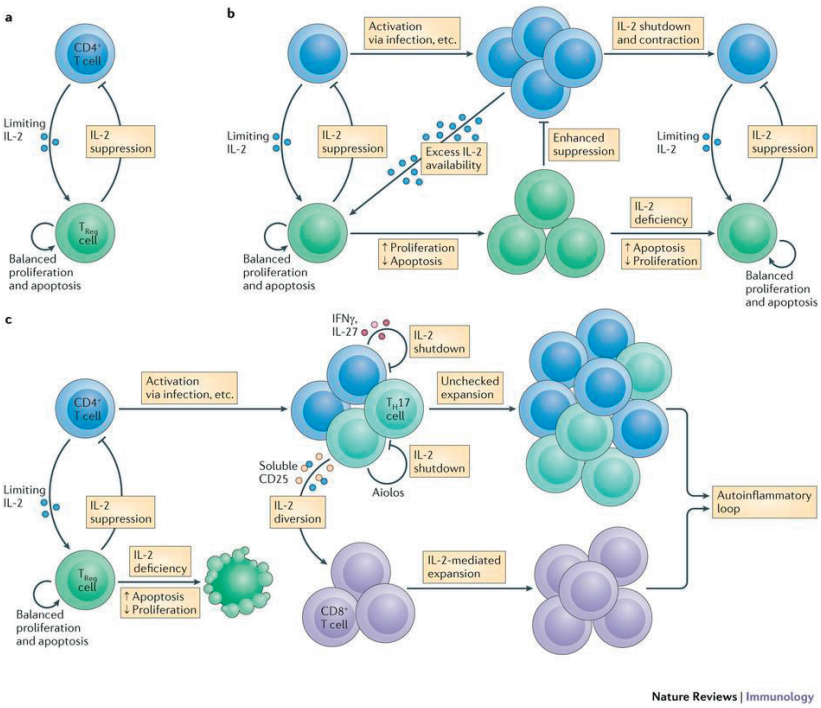


Figure 9: Disordered regulatory T cell homeostasis during infection

In normal homeostasis a limited amount of IL-2 produced by non-regulatory CD4$^+$ T cells controls the balance between proliferation and apoptosis in the population of regulatory T cells (a). During inflammation, the proliferation of conventional CD4$^+$ T cells is accompanied by excess production of IL-2, allowing a balanced expansion, and subsequently contraction of the population of regulatory T cells (b). Under some conditions, deficiency of IL-2 may limit the population of regulatory T cells, allowing an unchecked expansion of conventional CD4$^+$ or CD8$^+$ T cells (c). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology [175], copyright (2014).

We did not find any correlation between the frequency of FoxP3$^+$ CD4$^+$ T cells and radiological stage or Löfgren's syndrome in our material. However, only five patients presented with Löfgren's syndrome, and four patients had radiologic stage 3 or 4.

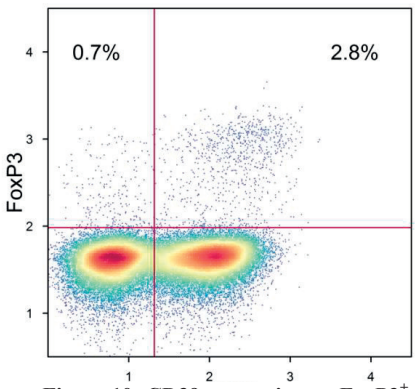


Figure 10: CD39 expression on FoxP3$^+$ CD4$^+$ T cells

A typical patient with sarcoidosis. Eighty-one % of the FoxP3$^+$ CD4$^+$ T cells expressed the surface marker CD39.

CD27, a tumor necrosis factor receptor superfamily member, plays a role in maintaining T cell survival, and is transiently expressed on T effector cells during activation. The majority of FoxP3$^+$ CD4$^+$ T cells in all groups expressed CD27 [median (IQR): 87 (79-95) %], confirming that CD27 may serve as a surface marker of regulatory T cells in BALF, as suggested by others [178]. The ectonucleotidase CD39 is highly expressed on regulatory T cells, and serves as an effector mechanism by hydrolysis of extracellular ATP, but is also expressed by non-regulatory T cells[179]. We found expression of CD39 on the majority of BALF FoxP3$^+$ CD4$^+$ T cells [median (IQR): 66 (44-80) %], but there was no significant difference between the patients and controls.

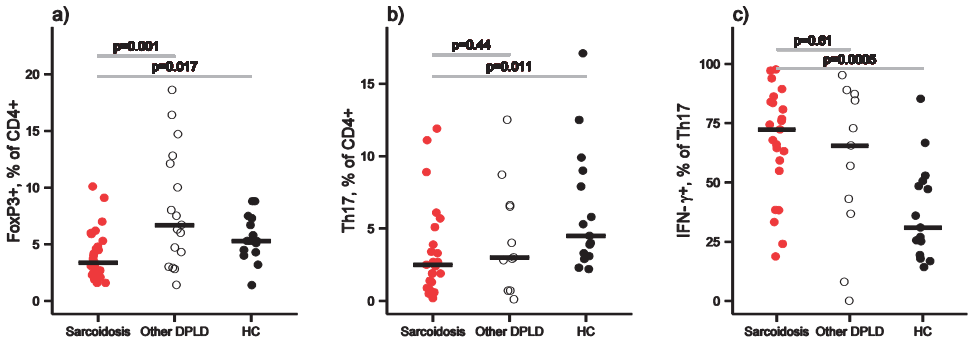


Figure 11: Frequency of CD4$^+$ T cell subsets in bronchoalveolar lavage fluid

FoxP3$^+$ CD4$^+$ T cells, Th17 and IFN-γ$^+$ Th17 cells in patients with sarcoidosis, other diffuse parenchymal lung disease (DPLD) and healthy controls (HC).

Th1, Th17 and IFN-γ^+ Th17 and cells in sarcoidosis

In patients with sarcoidosis, other DPLD and in HC, the majority of BALF $CD4^+$ T cells were Th1 cells (IFN-γ^+) (median: 75.5 %, 62.1 % and 65.7 %, respectively), and although the median fraction of Th1 cells was higher in patients with sarcoidosis, this difference failed to reach statistical significance. More than 80 % of $CD8^+$ T cells produced IFN-γ in all groups. When testing for production of the cytokine IL-17 (Th17 cells) after culture of the cells in medium with mitogen, we found markedly lower fraction of Th17 cells in patients with sarcoidosis compared to HC (2.5 versus 4.5 % of $CD4^+$ BALF T cells, p=0.011). This is consistent with one study from Sweden, in which gene expression of IL-17A in sorted $CD4^+$ BALF T cells was found to be lower in patients with sarcoidosis than in HC [180]. On the other hand, several previous studies have found increased frequencies of Th17 cells in BALF and peripheral blood in sarcoidosis [129, 134, 172, 181]. Methodological differences such as stimulation protocol, gating of IL-17^+ from IL-17^- cells and the ability to obtain clean gates on $CD4^+$ T cells may explain some of the variance between the studies (autofluorescent AM polluting the lymphocyte- or the $CD4^+$ T cell gate may influence the fractions of cells counted as IL-17^+). Importantly, characteristics of the study population, including genetic background, could probably explain differing results. Accordingly, our results are in line with the study from Sweden, but at variance with the studies on Chinese and American patients.

In patients with sarcoidosis the majority of Th17 cells also produced IFN-γ (median: 72.4% of Th17 cells), while in BALF from HC, only median: 32% of Th17 cells produced IFN-γ (p=0.0005). This is consistent with recent reports on Th17 cells in BALF [181, 182] and peripheral blood [134, 181] in patients with sarcoidosis.

Since their discovery a decade ago, the notion of Th17 cells as a stable, pro-inflammatory T helper cell subset has been challenged. Lately, it has been increasingly appreciated that Th17 cells are a heterogeneous population with both pro- and anti-inflammatory effector functions and remarkable plasticity [183, 184]. Cytokine production by human Th17 cells is extensively regulated in tissue microenvironment and by commensal microbiota[185]. Two major subsets of Th17 cells have been described, with different homing receptors, one of which has the ability to produce IFN-γ[183]. In one study, Th17 cells induced by Candida albicans also produced the Th1 cytokine IFN-γ, whereas Th17 cells induced by Staphylococcus aureus produced IL-10, and not IFN-γ[133]. Furthermore, in a study utilizing a mouse model system with fate mapping of Th17 cells, IL-17 expression was rapidly lost during inflammatory

immune responses in vivo, and a substantial proportion of these cells instead produced IFN-γ[186]. Accordingly, a substantial number of human Th17 cells seem to acquire Th1-associated features such as expression of T-bet and IFN-γ in inflamed tissues, eventually differentiating to a Th1-like effector progeny, via a Th17-Th1 'phenotypic drift'[183]. IL-12 and IFN-γ induce the shift of Th17 cells into IFN-γ^+ Th17 cells and subsequently IL17$^-$IFN-γ^+ Th1-like cells (non-classical Th1 cells) [187, 188].

In the present study, IFN-γ^+ fractions of Th17 cells were highly correlated with Th1 cells in patients with sarcoidosis (N=23, rho=0.64, p=0.001), other DPLD (N=11, rho=0.7, p=0.02), healthy controls (N=15, rho=0.91, p<0.00001) and in patients and HC combined (N=49, rho=0.75, p<0.0001) (figure 11). In sarcoid granulomas, AM produce the Th1-driving cytokine IL-12 in an inflammatory feedback loop with IFN-γ from Th1 cells. In addition, AMs produce TNF-α and IL-1β. Hence, the observation that the majority of Th17 cells in the present study also produce IFN-γ is fully consistent with current knowledge on the plasticity of Th17 cells. Similarly, the correlation of IFN-γ^+ fractions of Th17 cells and Th1 cells may be a reflection of the concentration of Th1-driving cytokines in the microenvironment of the Th17 cells. This effect is also seen in non-sarcoidosis patients and HC, and may not be specific to the immune response in sarcoidosis. Moreover, as we found a decreased frequency of Th17 cells in sarcoidosis, it is tempting to speculate that this could be a consequence of the 'phenotypic drift' of Th17 cells into Th1-like cells. In addition, the rarity of Th17 cells at inflammatory sites may also be explained by their impaired IL-2 production in response to TCR signaling, limiting their expansion [189, 190].

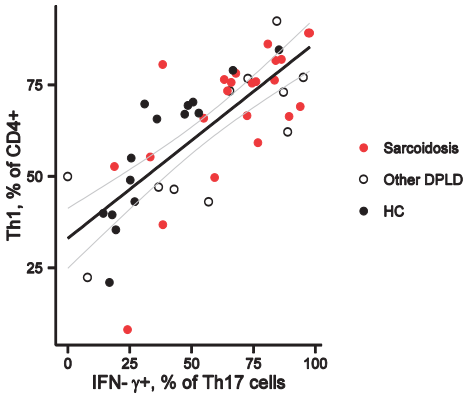


Figure 12: The fraction of Th1 cells was highly correlated with fractions of Th17 cells producing IFN-γ. HC: Healthy control. DPLD: Diffuse parenchymal lung disease.

The question remains, however: Are Th17, IFN-γ^+ Th17 or Th1-like cells pathogenic? Th17 cells have been associated with several human diseases with suspected autoimmune etiology, including rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis and psoriasis [106, 191, 192]. IFN-γ^+ Th17 have been reported to be pathogenic in multiple sclerosis and

mice with experimental autoimmune encephalomyelitis [131, 132]. However, the emerging picture of Th17 cells and their pathogenic potential is complex, and depending on the different experimental model or the inflammatory disorder Th1, Th17, IFN-γ^+ Th17 or Th1-like cells may all be involved in tissue damage[189]. In the present study, fractions of IFN-γ^+ Th17 cells increased with radiologic stage (rho=0.45, p=0.03). This may be a result of a more Th1-polarized cytokine milieu on Th17 cells, as reduced expression of Th1-cytokines has previously been reported in patient subgroups with particularly good prognosis[171]. Still, this does not preclude the possibility that IFN-γ^+ Th17 cells or their progeny are pathogenic, and the contribution of these cells in the immunopathology of sarcoidosis is yet unresolved. An interesting observation in this respect is that TNF-α inhibitors, which have been used in sarcoidosis refractory to other treatments, may inhibit the transition of Th17 cells to IFN-γ^+ Th17 or Th1-like cells, as Th17 cells express TNF-α receptor II [189].

The balance between CD4$^+$ T cell subsets in sarcoidosis

An increased ratio of Th17 to FoxP3$^+$ CD4$^+$ T cells has been reported in sarcoidosis[172]. This was not confirmed in our study, although the ratio of Th1/ FoxP3$^+$ CD4$^+$ T cells was higher in sarcoidosis compared to other DPLD and compared to HC [median (IQR): 21.7 (14.4-35.2) versus 6.2 (2.8-13.5), p=0.002, and versus 9.5 (8.9-13-6), p=0.009]. Thus, the expansion of Th1 cells in sarcoidosis is not accompanied by a proportional expansion of FoxP3$^+$ CD4$^+$ T cells, possibly due to deficiency of IL-2 (see section on regulatory T cells). Similarly, the ratio of IFN-γ^+ Th17 to FoxP3$^+$ CD4$^+$ T cells was considerably increased in sarcoidosis compared to other DPLD and compared to HC [median (IQR): 16.5 (8.9-48.3) versus 5.9 (2.8-11.1), p=0.005, and versus 4.9 (3.3-8.4), p=0.0004]. This ratio conveys the information that the combination of high fractions of INF-γ^+ Th17 cells and low frequency of FoxP3$^+$ CD4$^+$ T cells is typical of sarcoidosis. Patients with other DPLD tend to have higher fractions of FoxP3$^+$ CD4$^+$ T cells. Accordingly, the balance of CD4$^+$ T cell subsets is altered in sarcoidosis compared to other DPLD and HCs.

We found no correlation between fractions of Th1, Th17 or FoxP3$^+$ CD4$^+$ T cells and either sarcoidosis stage according to Scadding, or Löfgren's syndrome. However, the number of patients with Löfgren's syndrome or stage 3 or 4 sarcoidosis according to Scadding's classification is too low in the present study to exclude the possibility that such correlations exist.

6 CONCLUSIONS

Lymphocyte phenotypes NKT cells and $HLADR^+$ $CD8^+$ T cells are augmented in BALF from patients with HP. These markers provide a possible tool for discrimination of patients with HP from sarcoidosis. The presence of NKT cells in HP may provide clues to the immune response in HP.

The use of BALF immune cell profiles and lymphocyte phenotypes may be used to predict the probability of sarcoidosis in the individual patients, yielding high diagnostic accuracy in our study population. The addition of lymphocyte phenotypes NKT cells and $HLADR^+$ fractions of $CD8^+$ T cells increases the diagnostic accuracy of BALF investigations in sarcoidosis.

BALF Regulatory T cells and Th17 cells are both decreased in sarcoidosis. The majority of Th17 cells also produced IFN-γ, whereas only 1/3 of Th17 cells in HCs produced IFN-γ. This fraction was highly correlated with fractions of Th1 cells in all groups, indicating that a Th1-polarized immune response induce IFN-γ in BALF Th17 cells, or alternatively a common factor induce both a Th1 response and IFN-γ in BALF Th17 cells. The fraction of Th17 cells producing IFN-γ is correlated with severity of sarcoidosis in terms of radiological stage. Furthermore, the expansion of Th1 cells in sarcoidosis was not accompanied by a comparable expansion of $FoxP3^+$ $CD4^+$ T cells.

These studies provide evidence of altered cellular immune responses in sarcoidosis and HP that can be used as diagnostic markers, and may contribute to our knowledge of the immunopathology of the disorders.

7 FUTURE ASPECTS

The use of BALF in a predictive, diagnostic model, discussed in paper 2, makes an inspiring base for future studies. In the future, we might be able to 'fingerprint' the immune profiles in sarcoidosis and HP, possibly eliminating the need for transbronchial or transthoracal biopsies. To achieve this, a more comprehensive profiling of immune cell phenotypes in a larger number of patients is needed.

The study of complex cellular systems, such as T cell functional responses in pathological conditions, will be aided by the recent advancements in deep profiling techniques[193], such as polychromatic FC, and the feasibility of high dimensional, high throughput single cell analysis[194]. The recent increments in numbers of parameters measured in FC has introduced a need for analysis platforms that are able to handle the complexity of n-dimensional datasets, as well as enable human-readable graphical visualizations of the data[193, 195]. An interesting example on this is the Bioconductor project (www.bioconductor.org) which provides open software for computational biology and bioinformatics in the R environment [196], including tools for gating and analysis of polychromatic FC and graphical visualization of complex data. Automated flow cytometry data analysis techniques have recently been reviewed [197].

In a future study the number of parameters analyzed in FC could be increased (or alternatively, mass cytometry could be used), to provide an in-depth phenotyping of both lymphocytes and AM in BALF from patients with sarcoidosis and other DPLD. An enhanced predictive model discriminating patients with sarcoidosis could then be developed, and the diagnostic accuracy tested prospectively, in a new cohort of patients. Parallel to this, an algorithm for automated flow cytometry data analysis could be generated and tested. We could provide a comparison of manual and automated gating of BALF cells.

A future study on the NKT cells (or NKT-like cells) seen in BALF from patients with HP, and in some other diseases (paper 1 and 2) may be justified. This cell population could be sorted and cultured, and the phenotype, cytokine profile and functional characteristics could be explored.

Similarly, deep profiling and functional studies of T cells subsets in the lung in sarcoidosis, utilizing FC with increasing number of colors, mass cytometry or single cell PCR may

provide insight into the nature and function of regulatory T cells and Th17 in sarcoidosis and quantify the Th17 derived Th1-like cell population.

Ultimately, such studies may identify patterns that are linked to the development of fibrotic sarcoidosis, and possibly identify molecular targets for treatment.

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SARCOIDOSIS VASCULITIS AND DIFFUSE LUNG DISEASES 2014; 31; 316-324

Activated CD8+ T cells and natural killer T cells in bronchoalveolar lavage fluid in hypersensitivity pneumonitis and sarcoidosis

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Abstract. *Background:* Sarcoidosis and hypersensitivity pneumonitis are diffuse parenchymal lung diseases characterized by formation of non-caseating granulomas with a bronchocentric distribution. Analysis of the white blood cell differential profile in bronchoalveolar lavage fluid can be a useful supplement in the diagnostic work-up. *Objective:* Diagnostic markers that can improve the discrimination of sarcoidosis and hypersensitivity pneumonitis are wanted. *Methods:* Bronchoalveolar lavage fluid fractions of $CD4^+$ and $CD8^+$ T cells expressing the activation marker HLA-DR and fractions of natural killer T cells determined by flow cytometry were investigated in sarcoidosis (N=83), hypersensitivity pneumonitis (N=10) and healthy control subjects (N=15). *Results:* In hypersensitivity pneumonitis, natural killer T cell fractions were over 7-fold greater [median (IQR): 5.5% (3.5-8.1) versus 0.7% (0.5-1.2), p<0.0001], and HLA-DR^+ fractions of $CD8^+$ lymphocytes were almost two fold greater [median (IQR): 79% (75-82) versus 43% (34-52), p<0.0001] than in sarcoidosis. In healthy control subjects, natural killer T cell fractions of leucocytes and HLA-DR^+ fractions of $CD8^+$ lymphocytes were lower [median (IQR): 0.3% (0.3-0.6) and 30% (26-34), p=0.02 and p=0.01 compared to sarcoidosis]. The combined use of these two markers seems to discriminate the diseases very well. *Conclusion:* This study suggests a role for the bronchoalveolar lavage fluid lymphocyte subsets HLA-DR^+ $CD8^+$ T cells and natural killer T cells in the diagnostic work up of sarcoidosis and hypersensitivity pneumonitis. *(Sarcoidosis Vasc Diffuse Lung Dis 2014; 31: 316-324)*

Key words: BALF, hypersensitivity pneumonitis, harcoidosis, NKT cells, HLA-DR, lymphocyte subsets

Abbreviations:
BALF: Bronchoalveolar lavage fluid, DPLD: diffuse parenchymal lung diseases, HP: hypersensitivity pneumonitis, HRCT: High Resolution Computed Tomography, HLA: Human leucocyte antigen, NKT cell: Natural killer T cell, TCR: T cell receptor, IQR: Inter quartile range.

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Introduction

Sarcoidosis and hypersensitivity pneumonitis (HP) are diffuse parenchymal lung diseases (DPLD) characterized by formation of non-caseating granulomas with a bronchocentric distribution. In HP, the inflammation is an immune response to an inhaled antigen in predisposed individuals (1), and over 300 antigens, predominantly organic in nature, have been identified. In contrast, the inciting antigen in sarcoidosis has not yet been identified(2), but an inhaled antigen is suspected (3, 4).

Analysis of the white blood cell differential profile in bronchoalveolar lavage fluid (BALF) can be a

useful supplement in the diagnostic work-up of DPLD (5). Although not diagnostic, relative and absolute number of lymphocytes are increased in sarcoidosis and HP (6). The lymphocytic alveolitis in sarcoidosis is dominated by $CD4^+$ T cells with an increased CD4/CD8 ratio. In contrast, the alveolitis in HP is often dominated by $CD8^+$ T cells, resulting in an inverted CD4/CD8-ratio, together with an increase in relative numbers of mast cells, neutrophils and foamy alveolar macrophages(7). However, considerable overlaps are seen between the diseases, as the CD4/CD8 ratio can also be increased in HP, and different causes of HP may cause different BALF cellular patterns (7, 8)

Lymphocyte subsets in the BALF may be identified and quantified by the use of monoclonal antibodies and flow cytometry. In sarcoidosis and HP, there is an increased expression of the activation marker HLA-DR on T-lymphocytes in the BAL fluid. HLA-DR expression is increased in $CD4^+$ T cells in both diseases (9, 10), and is lower in $CD8^+$ T cells in sarcoidosis than in HP (10, 11). Natural Killer T (NKT) cells, a subset of T cells expressing the NK cell markers CD56 and/or CD16, are increased in HP(12). As far as we are aware, the combined use of these subsets has not been reported previously in HP and we report our findings on these two lymphocyte subsets in sarcoidosis, HP and healthy controls.

Materials and methods

Study population

Between September 2007 and September 2012, bronchoscopy and flow cytometric analysis of BALF samples were performed in all patients investigated for suspicion of DPLD in our institution. Of these patients, 146 were given a clinical diagnosis of sarcoidosis or HP, and were eligible for inclusion in the study. Patients on systemic corticosteroid or other immunosuppressive treatment or active cancer at the time of bronchoalveolar lavage were excluded from the study. In addition, patients who did not meet the diagnostic criteria for sarcoidosis and HP were excluded. The final diagnoses of the included patients were sarcoidosis (N=83) and HP (N=10) (figure 1). Sixteen patients presented with Löfgren's syndrome.

Healthy control subjects (N=15) with no known history of asthma or allergy were recruited by advertisement on the hospital web-site. All subjects had normal CO diffusing capacity. The FEV1/FVC ratio was below 0.7 in one subject (0.67), a second subject had a FEV1 of 76% of predicted value and a third subject was on anti-hypertensive therapy.

Demographics of the study population are presented in table 1.

The Regional Ethics Committee assessed that use of patient data did not require ethical approval as it was considered to be a quality control of a routine clinical investigation (Ref.nr.: 2009/909-2). Ethical approval (Ref.nr.: 2010/1939-4) and written informed consent were obtained for bronchoscopy with bronchoalveolar lavage in control subjects.

Diagnostic criteria

The diagnostic criteria of sarcoidosis were a clinical and radiological pattern consistent with sarcoidosis, presence of non-caseating granulomas on biopsy, and exclusion of other known causes of granulomatous diseases(13). Histological demonstration of granuloma was not required for patients with classic features of Löfgren's syndrome, defined as fever, erythema nodosum and/or ankle arthritis together with bilateral hilar lymphadenopathy.

Patients with HP had at least 4 of the major criteria and 2 of the minor criteria as suggested by Schuyler et al. (14). The major criteria are: symptoms

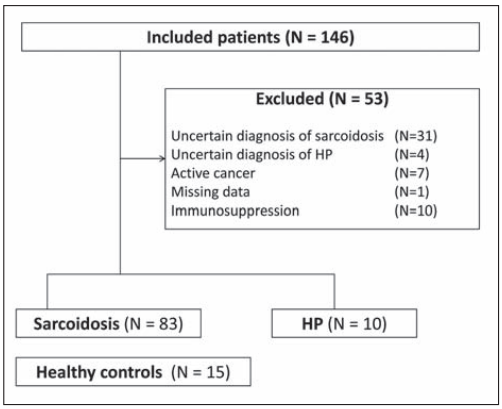

Fig. 1. Study population. HP: Hypersensitivity pneumonitis

Table 1. Demographics and BAL differential cell counts of study population

	Sarcoidosis (N=83)	HP (N=10)	HC (N=15)	P
Age, years*	48 (18-77)	54 (39-72)	35 (21-53)	0.003
Sex (male/female)	61/22	6/4	8/7	0.22
Smoker/non-smoker	6/76	0/10	0/15	0.77
Total cell count (x 10^6/ml)	0.19 (0.14-0.29)	0.39 (0.25-0.53)	0.14 (0.12-0.17)	0.0009
Lymphocytes (%)	31 (21-52)	69 (66-74)	14 (10-25)	<0.0001
Neutrophils (%)	2 (1-6)	5 (3-6)	4 (2-5)	0.35
Eosinophils (%)	0 (0-1)	4 (1-7)	0 (0-0)	0.0009
Alveolar macroph. (%)	57 (39-75)	21 (15-28)	81 (69-85)	<0.0001

Fisher exact test was used for categorical variables and Kruskal-Wallis test for continuous variables. *Data shown as median (range). All other data are shown as median (IQR). HP: Hypersensitivity pneumonitis. HC: Healthy control subjects

compatible with HP, serologic evidence or a history of antigen exposure, chest radiograph or High Resolution Computed Tomography (HRCT) findings compatible with HP, lymphocytosis in the BALF, compatible histologic findings on lung biopsy and reproduction of symptoms after exposure to the antigenic environment. The minor criteria are: bibasilar rales, decreased diffusing capacity and arterial hypoxemia.

BAL procedure and flow cytometry

Bronchoscopy with bronchoalveolar lavage was performed in accordance with recommendations(15), as previously described(16). In brief, lavage was done with 2-3 aliquots of 60 ml phosphate buffered saline (Hospital pharmacy, Haukeland University Hospital, Bergen, Norway) under local anaesthesia with lignocaine and intravenous sedation with midazolam and alfentanyl. The first fraction was used for microbiological or cytological analyses, and the remainder of the lavage fluid was pooled, filtered through nylon gauze and kept at 4° C until processing.

The total cell count was made by ADVIA 120 Hematology System (Siemens AG, Erlangen, Germany). Differential cell counts of a minimum of 300 cells were performed on cytospin preparations stained with May Grünwald/Giemsa.

Cell pellets were prepared by centrifugation for 5 min at 1500 rpm. Tubes containing 0.5-1.0 x 10^6 cells per tube were incubated for 15 minutes with selected antibodies as detailed in table 2. The cells were then washed twice in 2 ml phosphate buffered saline containing 0.1% bovine serum albumin (Dulbecco A, Sigma-Aldrich, St. Louis, USA).

Table 2. Antibody panel

Tube	Antibody conjugates			
	FITC	PE	PerCP-Cy5.5	APC
1	Isotype control[1]	Isotype control[1]	Isotype control[2]	Isotype control[1]
2	CD8[1]	CD4[1]	HLA-DR[2]	CD3[1]
3	CD3[3]	CD16[3] and CD56[3]	CD45[3]	CD19[3]

[1] Multimix, Dako Denmark AS, Glostrup Denmark, [2] BD Biosciences, San Jose, USA, [3]MultiTEST™, BD Biosciences. FITC: Fluorescein isothiocyanate, PE: phycoerythrobilin, PerCP-Cy5.5: Peridinin chlorophyll protein with cyanine dye (Cy5.5), APC: Allophycocyanin

Flow cytometry of a minimum of 10.000 cells was performed with a FACS Canto I flow cytometer with FACS DIVA software (BD Biosciences, Mountain view, CA, USA). Lymphocytes were identified as $CD45^+$ cells with low side and forward scatter. An isotype control tube was used to set the gates to discriminate $HLA\text{-}DR^+$ and $HLA\text{-}DR^-$ lymphocytes. NKT cells were defined as $CD3^+$ lymphocytes with expression of CD16/CD56, and the gates were set by visual comparison to the dominating population of $CD16/CD56^-$ T cells. In October 2010, the tube for identification of NKT cells was changed in our laboratory, and NKT cells in subsequent patients (Sarcoidosis: N=30, HP: N=4, HC: N=15) were defined as $CD3^+$ lymphocytes with expression of CD56 (CD16 was omitted from the antibody panel). Gating strategy is presented in figure 2.

Statistical methods

Group comparison of continuous data was done with Kruskal-Wallis test. Post hoc analyses were performed with pairwise Wilcoxon rank sum test and

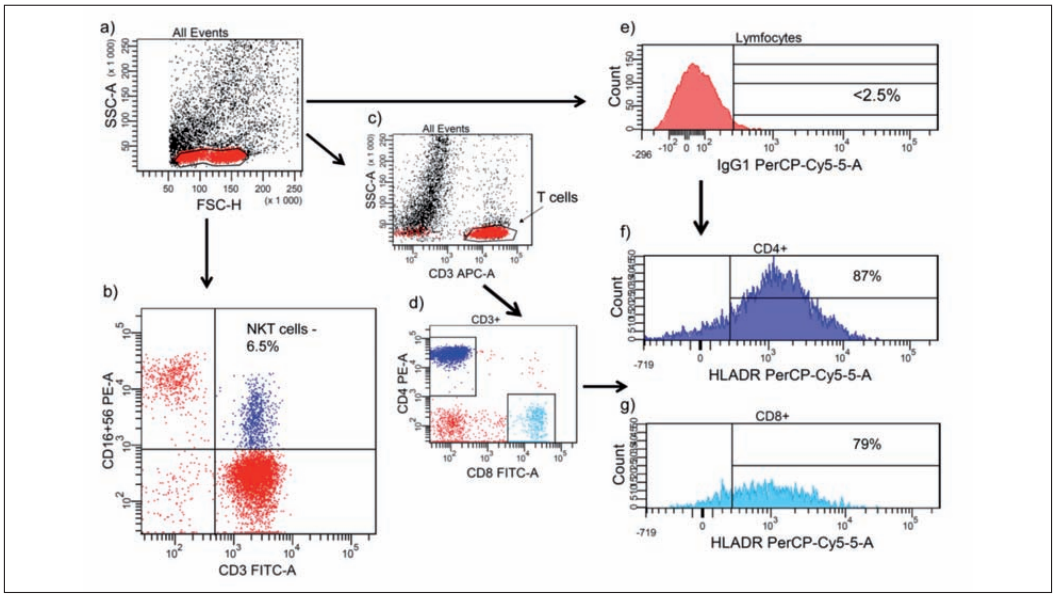

Fig. 2. Gating strategy. The example is a typical HP patient. a) Lymphocytes are gated by their low forward and side scatter signal. b) From the lymphocytes, the T-cells, NKT cells and NK cells (CD3-CD16/56+) are gated by expression of CD3 and CD16/CD56. Numbers represent fractions of leucocytes (CD45+ BALF cells). c) T cells are gated as $CD3^+$ lymphocytes. d) CD4+ and CD8+ T cells. e) In the isotype control tube, the gates for HLA-DR were set so that > 97.5% of the lymphocytes was assigned negative. f) and g) HLA-DR-gates from e) were used to discriminate HLA-DR^+ CD4+ and CD8+ T cells. In October 2010, the tube for identification of NKT cells was changed in our laboratory, and NKT cells in subsequent patients (Sarcoidosis: N=30, HP: N=4, HC: N=15) were defined as $CD3^+$ lymphocytes with expression of CD56 (CD16 was omitted from the antibody panel).

the Bonferroni correction for multiple comparisons. Categorical data were analyzed with Fishers exact test. A p value of < 0.05 was considered to be statistically significant. Statistical analyses were done in R: A Language and Environment for Statistical computing (R Core Team, R Foundation for Statistical Computing, Vienna, Austria).

Results

In HP, the BALF NKT cell fraction was over 7-fold and 18 -fold greater, respectively, than in sarcoidosis and controls [median (IQR): 5.5% (3.5-8.1) versus 0.7% (0.5-1.2), p<0.0001, and versus 0.3% (0.3-0.6), p=0.0001]. The HLA-DR^+ fraction of $CD8^+$ lymphocytes were almost two- fold and 2.5-fold greater, respectively, than in sarcoidosis and controls [median (IQR): 79% (75-82) versus 43% (34-52), p<0.0001, and versus 30% (26-34), p=0.0001]. HLA-DR $CD4^+$ lymphocyte fraction was almost one and a half- fold and two- fold greater, respectively, than in sarcoidosis and controls [median (IQR): 89% (81-93) versus 69% (60-79), p=0.0002, and versus 45% (36-57), p=0.0001] (Figure 3).

Typical patterns of NKT cells and HLA-DR^+ $CD8^+$ cells in HP and sarcoidosis are shown in figure 4. The difference in HLA-DR expression on $CD4^+$ T cells in HP compared to sarcoidosis patients was less striking in comparison to that on $CD8^+$ cells (figure 5b). The combination of high fractions of NKT cells and HLA-DR^+ $CD8^+$ T cells was more suggestive of HP than sarcoidosis (figure 5).

The NKT cell fraction and HLA-DR^+ fraction of $CD8^+$ and $CD4^+$ lymphocytes were higher in sarcoidosis compared to controls (p=0.02, p=0.01 and p < 0.0001).

As expected, the CD4/CD8- ratio was higher in sarcoidosis compared to HP and healthy controls (p=0.006 and p=0.010) (Fig. 3).

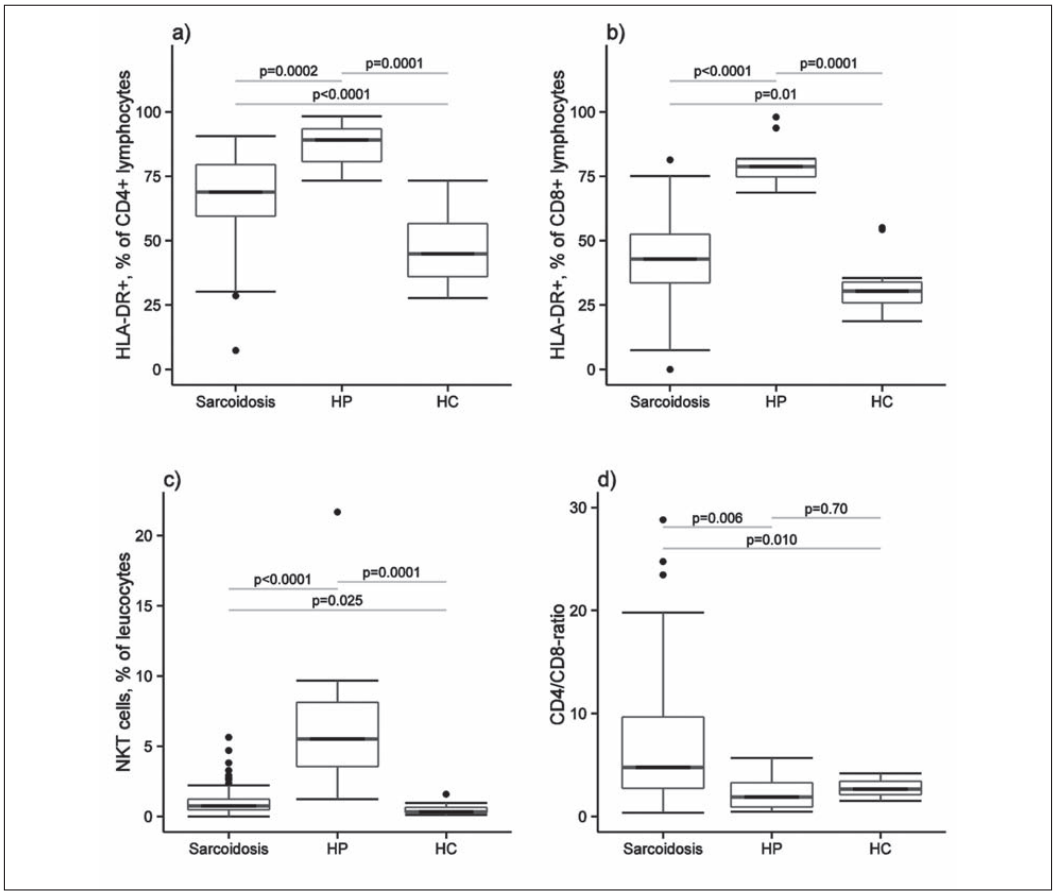

Fig. 3. Higher fractions of NKT cells and HLA-DR$^+$ CD4$^+$ and HLA-DR$^+$ CD8$^+$ T cells in hypersensitivity pneumonitis than in sarcoidosis and healthy controls. a) HLA-DR expression on CD4$^+$ was higher in sarcoidosis than healthy control subjects, while HP patients had highest expression of HLA-DR on CD4$^+$ T cells of all groups. b) HLA-DR$^+$ fractions of CD8$^+$ T cells, and c) fraction of NKT cells were highly significant raised in HP versus sarcoidosis and HC, and significantly higher in sarcoidosis compared to healthy control subjects. d) HP patients have lower CD4/CD8-ratio than patients with sarcoidosis. Kruskal-Wallis with post hoc multiple comparison and p-value adjustment with Bonferroni correction. HP: Hypersensitivity pneumonitis; HC: Healthy control subjects.

Differential cell counts in BALF are presented in table 1. BALF lymphocyte fractions were more than two-fold greater in patients with HP compared to sarcoidosis (p=0.0001). Fractions of eosinophils were higher in HP patients (p=0.008), and fractions of alveolar macrophages were almost threefold greater in patients with sarcoidosis compared to HP (p<0.0001). There was a strong trend towards higher total cell count in HP compared to sarcoidosis, although this was not significant after multiple comparisons correction (p=0.052).

Discussion

This study investigated the HLA-DR$^+$ fractions of CD8$^+$ and CD4$^+$ T cells and fractions of NKT cells in BALF in patients with sarcoidosis, HP and

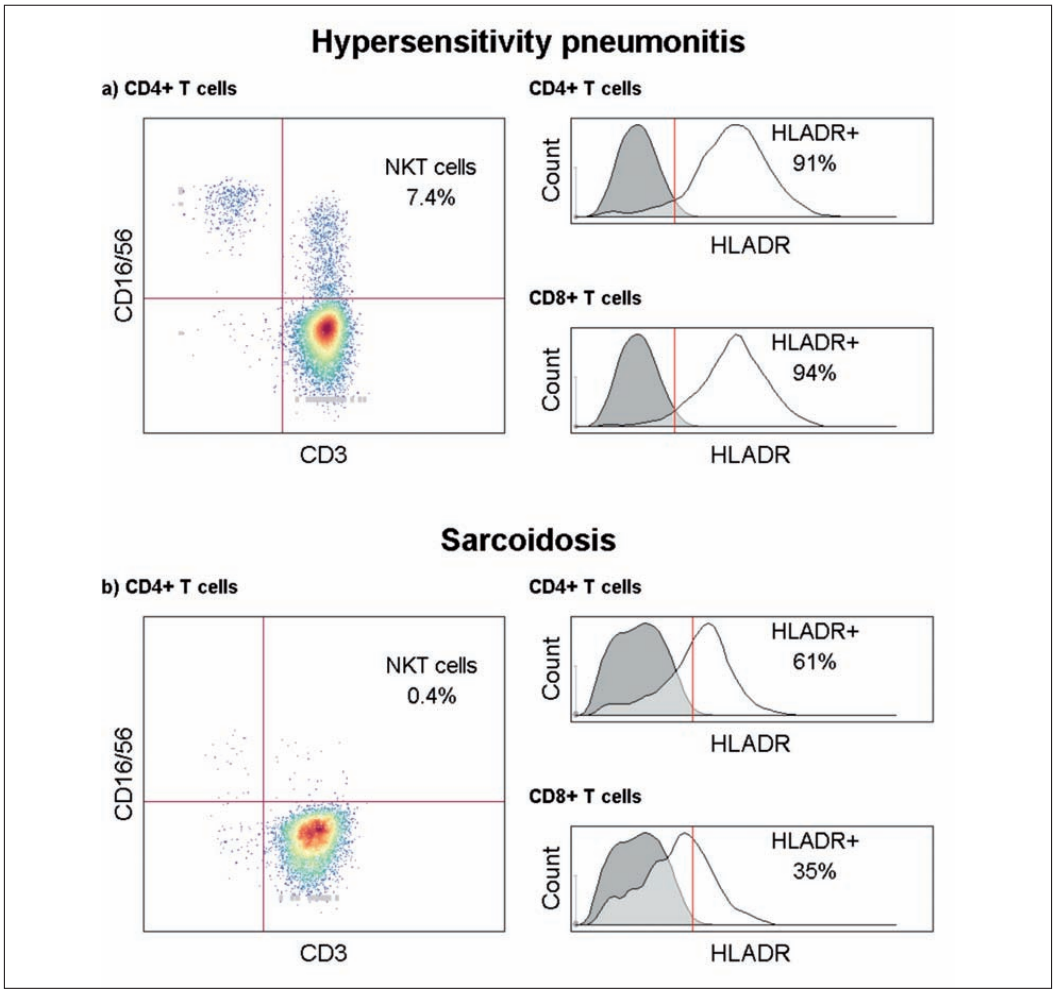

Fig. 4. Flow cytometry scatter plots and histograms in Hypersensitivity pneumonitis and Sarcoidosis. Scatterplot of NKT cells and histograms displaying HLA-DR$^+$ CD4$^+$ and CD8$^+$ lymphocytes for a) a typical hypersensitivity pneumonitis patient, and b) a typical sarcoidosis patient. NKT cells are displayed in fraction of leucocytes. HLA-DR$^+$ CD4$^+$ and CD8$^+$ T cells are fractions of CD4$^+$ and CD8$^+$ T cells, respectively.

healthy control subjects. In addition to a decrease in the CD4/CD8 ratio and an increase in lymphocytes and eosinophils, HLA-DR CD8$^+$ and NKT lymphocyte subsets were augmented in HP compared to sarcoidosis patients. To our knowledge, these subsets have not previously been investigated simultaneously. Together, fractions of HLA-DR$^+$ CD8$^+$ T cells and NKT cells in BALF seem to discriminate very well between HP and sarcoidosis, suggesting that these markers may be a valuable tool in the diagnosis of HP and sarcoidosis. Fractions of HLA-DR$^+$ CD4$^+$ T cells, on the other hand, may be less useful in discriminating HP from sarcoidosis.

The combined use of NKT cells and HLA-DR expression on CD8$^+$ T cells in the BALF as a diagnostic tool in HP has not been addressed in prior

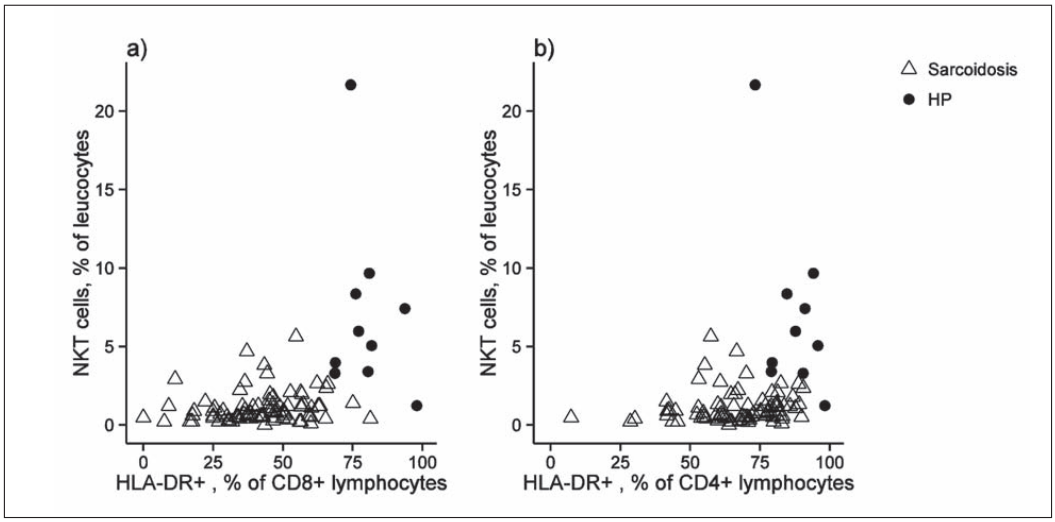

Fig. 5. Fractions of HLA-DR$^+$ CD8$^+$ T cells and NKT cells discriminate patients with hypersensitivity pneumonitis from patients with sarcoidosis. Scatterplot of a) HLADR$^+$CD8$^+$ lymphocytes, and b) HLADR$^+$CD4$^+$ lymphocytes versus NKT cell fraction grouped for patients with hypersensitivity pneumonitis (HP) or sarcoidosis.

studies. We previously reported that the use of these markers improve diagnostic accuracy in sarcoidosis(16). A greater fraction of NKT cells in HP than in sarcoidosis has been previously reported in a study by Korosec et al. (12) who like us defined NKT cells as CD3$^+$CD16/56$^+$ lymphocytes. In that study, NKT cells are reported as a proportion of lymphocytes, while we found it more suitable to report NKT cells as a fraction of leucocytes. In patients with low lymphocyte fractions, noise in the flow cytometry gates has a greater influence on the NKT cell fractions, if reported as fractions of the lymphocytes. When recalculated as fractions of lymphocytes, we found a median of 2.0% NKT cells in patients with sarcoidosis and 7.6% in patients with HP (p=0.0004), while they reported a median fraction of NKT cells of 3% and 11% in sarcoidosis and HP.

Higher fractions of HLA-DR$^+$ CD8$^+$ T cells in HP compared to sarcoidosis, has been reported in one study(11). The difference in HLADR$^+$ fractions of CD8$^+$ T cells in HP compared to sarcoidosis reported by Heron et al. was less pronounced (median 96% versus 80%), although the principle findings were similar. Interestingly, they reported a higher fraction of HLA-DR$^+$ CD8$^+$ T cells in patients with sarcoidosis and parenchymal involvement (radiologic stage II and higher) versus patients without parenchymal involvement (median: 93% versus 77%), while this was not found in our study (median: 47% versus 42%, p=0.20). Dissimilarities in the patient selection, anti-HLA-DR antibodies and flow cytometry set-up may account for the variations.

Our study has limitations. Firstly, the number of patients with suspected HP is small in our clinic, and only 10 patients did fulfill the diagnostic criteria for HP. The frequent inability to make a certain diagnosis of a specific DPLD, may lead to a tendency that only the typical patients with HP are correctly diagnosed, while less typical patients more often are classified as unspecified DPLD. Secondly, our data are insufficient to estimate the diagnostic accuracy of NKT cell and HLA-DR$^+$CD8$^+$ lymphocyte fractions as a test to discriminate HP from sarcoidosis. This would have required a greater number of patients with HP. However, a visual display of these two markers in combination, as shown in figure 5a, suggests that they may discriminate patients with HP from sarcoidosis. Finally, due to a change in the antibody panel in October 2010, NKT cells were de-

fined as T cells expressing CD56 rather than CD16/CD56 in a subset of the patients. This may have resulted in lower fractions of NKT cells in these patients and healthy control subjects. However, we found no significant difference between NKT cell fractions in sarcoidosis or HP patients investigated with the former versus the newer antibody panel (p=0.60 and p=0.48), whereas the NKT cell fractions were significantly different in HP compared to sarcoidosis also in the subgroup of patients analyzed with the former antibody panel and in sarcoidosis compared to healthy controls analyzed with the newer antibody panel (data not shown). These results show that the change of antibody panel has not influenced our principle findings. In addition, Korosec et al. reported that NKT cells in patients with HP are mainly $CD56^+$ (12), and thus would stain as positive also with the newer antibody panel.

NKT cells are a heterogeneous population of cells with elements of both the innate and the adaptive immune system, capable of rapid responses to antigens with cytotoxic NK cell activity and production of Th1 or Th2 cytokines. They are classified according to their TCR, which can be invariant (CD1d-restricted invariant NKT cells) or have normal variability in antigen specificity. $CD8^+CD56^+$ T cells with an αβ TCR have been implicated in antitumor immunity(17). The contribution of the various NKT cell subsets and activated T cells to the pathogenesis of either sarcoidosis or HP is not yet elucidated. In HP, most of the NKT cells in BALF have αβ TCR(12), and are $CD8^+CD56^+$, capable of both antigen specific and NK-like cytolytic activities(18). On the other hand, deficiencies and impaired INF-γ production of CD1d restricted invariant NKT cells in blood and BALF have been reported in sarcoidosis(19, 20). In this study, the NKT cells are defined as $CD16/CD56^+$ $CD3^+$ lymphocytes. These cells are strikingly increased in patients with HP compared to sarcoidosis and HC, indicating a role of these cells in the immunopathology of HP.

HLA-DR expression on T cells is a well known activation marker, and may play a role in regulating or limiting a specific T cell response to an antigen(21). The fraction of $HLA\text{-}DR^+$ $CD8^+$ lymphocytes increases in some autoimmune diseases, as well as with ageing, possibly as a part of normal immunoregulation(22), although its exact role is unclear. Activated $CD4^+$ T cells of Th1 type produce INF-γ, and is essential for the formation of granulomas in sarcoidosis(23). The finding of increased expression of HLA-DR on $CD4^+$ lymphocytes in sarcoidosis may reflect the prominent role of activated Th1 cells. In contrast, the $HLA\text{-}DR^+$ $CD8^+$ T cells may be involved in the immunology of HP. It may seem surprising that cytolytic T cells exhibit strong activation in HP. The most important biological function of cytolytic T cells is to kill infected cells and cancer cells. Infection is not a central element in HP even though many kinds of fungi and bacteria have been identified as important sensitizing environmental agents. Contrary to this, helper T cells are central both in activating allergen-clearing macrophages and in the production of allergen-specific antibodies, which are among the important characteristics of the disease.

Identifying exposure to an antigen known to cause HP is often difficult, and surgical lung biopsy may be necessary to confirm the diagnosis. However, surgical lung biopsy may not be achievable in all patients, due to procedure related risks, patient preferences or the severity of the disease. Therefore, our demonstration of a BALF lymphocyte subset pattern that may discriminate HP from sarcoidosis may be relevant and helpful in this clinical situation. In addition, the considerable overlap in CD4/CD8 ratio seen in patients with HP and sarcoidosis in the present study emphasize the need for more accurate diagnostic markers.

In conclusion, our study suggests a role for the combined use of NKT cell and $HLA\text{-}DR^+CD8^+$ T cell fractions in BALF as diagnostic markers in HP and sarcoidosis. However, further studies with a greater number of patients with HP are necessary to evaluate the diagnostic accuracy of these lymphocyte subsets. In addition, functional studies on NKT cells and $HLA\text{-}DR^+CD8^+$ T cells in BALF may provide important clues to our understanding of the immunopathogenesis of both diseases.

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Activated CD8$^+$ T Cells and NKT Cells in BAL Fluid Improve Diagnostic Accuracy in Sarcoidosis

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Abstract
Purpose The clinical diagnosis of pulmonary sarcoidosis is based on the presence of noncaseating granulomas in an appropriate clinical setting with either bilateral hilar adenopathy and/or parenchymal infiltrates. Lymphocytosis with an increased CD4/CD8 T cell ratio in bronchoalveolar lavage fluid is supportive. We evaluated the diagnostic accuracy of a predictive binary logistic regression model in sarcoidosis based on sex, age, and bronchoalveolar lavage fluid cell profile with and without the inclusion of HLA-DR$^+$ CD8$^+$ T cells and natural killer T-cell fractions.
Methods A retrospective analysis of differential cell counts and lymphocyte phenotypes by flow cytometry in bronchoalveolar lavage was performed in 183 patients investigated for possible diffuse parenchymal lung disease. A logistic regression model with age, sex, lymphocyte fraction, eosinophils, and CD4/CD8 ratio in bronchoalveolar lavage fluid (basic model) was compared with a final model, which also included fractions of HLA-DR$^+$ CD8$^+$ T cells and natural killer T cells. Diagnostic accuracy of the two models was assessed by receiver operating characteristic (ROC) curves.
Results The area under the ROC curve for the basic and final model was 0.898 [95 % confidence interval (CI) 0.852–0.945] and 0.937 (95 % CI 0.902–0.972), respectively, $p = 0.008$.
Conclusions Assessment of HLA-DR$^+$ CD8$^+$ T cell and natural killer T-cell fractions may improve diagnostic accuracy and further strengthen the importance of bronchoalveolar lavage in the diagnostic workup of sarcoidosis.

Keywords Sarcoidosis · Lymphocyte subsets · Bronchoalveolar lavage fluid · Predicitive model · Logistic regression · NKT cells · HLA-DR

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Introduction

Sarcoidosis is a multisystem inflammatory disorder of unknown aetiology. The lung is the most commonly affected organ, with heterogeneous involvement of the bronchovascular and subpleural parenchyma and intrathoracic lymph nodes. The diagnosis of pulmonary sarcoidosis is based on the finding of noncaseating granulomas in either bronchial or transbronchial biopsies or transbronchial needle aspirates of enlarged lymph nodes, in an appropriate clinical setting and in the absence of other known causes of granulomatous disease.

Analysis of bronchoalveolar lavage fluid (BALF) for total and differential counts of inflammatory cells and the CD4/CD8 ratio has been considered a useful supplement in the diagnostic workup of selected diffuse parenchymal lung diseases (DPLD) [1], such as sarcoidosis [2], hypersensitivity pneumonitis (HP) [3, 4], and eosinophilic pneumonia [5]. In sarcoidosis, the characteristic findings in BALF are a T-cell–dominated lymphocytosis and a CD4/CD8 ratio >3.5 [6]. However, the latter can be inverted [7] and in elderly subjects also normally increased [8]. The $CD4^+$ T cells express the human leucocyte antigen activation marker (HLA-DR) [2]. However, HLA-DR expression on $CD8^+$ lymphocytes is lower in sarcoidosis than in HP [9], suggesting that this may be useful in differentiating sarcoidosis from other DPLD. Another lymphocyte subset of interest is the natural killer T (NKT) cells, which express CD56 and/or CD16, as well as the T-cell receptor (TCR). The proportion of NKT cells in BALF has been found to be lower in sarcoidosis than in HP [10].

The purpose of this retrospective study was to evaluate the diagnostic accuracy of BALF in sarcoidosis in a population of suspected DPLD after initial clinical evaluation, using a logistic regression model with and without $HLA\text{-}DR^+$ $CD8^+$ lymphocytes and NKT cells in order to evaluate whether the inclusion of these two lymphocyte subsets increases diagnostic accuracy.

Materials and Methods

Study Population

All patients investigated with bronchoscopy and flow cytometric analysis of BALF samples between September 2007 and June 2010 were considered eligible for inclusion in the study if a DPLD was a possible differential diagnosis after initial clinical evaluation and high-resolution computed tomography (HRCT) thorax and not receiving treatment with systemic corticosteroids at the time of lavage. Patients with missing data or technical error in flow cytometry were excluded. The remaining patients were then grouped on the basis of the diagnosis reached by the attending respiratory physician after investigative workup into sarcoidosis ($N = 76$) and nonsarcoidosis ($N = 132$). For the purpose of this study, 51 patients fulfilled the following diagnostic criteria for sarcoidosis: a clinical and radiological pattern consistent with sarcoidosis, presence of noncaseating granulomas on endobronchial (4 patients) or transbronchial (38 patients) biopsy or endobronchial ultrasound-guided fine needle aspiration (4 patients) of mediastinal or hilar nodes, and exclusion of other known causes of granulomatous disease [6]. In one patient, granulomas were found only in extrapulmonary tissue (liver biopsy and bone marrow). The presence of granulomas was not required in 11 of these patients with Löfgren's syndrome, defined as fever, erythema nodosum, and/or ankle arthritis together with bilateral hilar lymphadenopathy. Patients were stratified by findings on chest radiograph at presentation into stages 0–4, according to Scadding [11]. 21 patients who did not fulfill these criteria and four additional patients with sarcoidosis and active cancer were excluded (Fig. 1).

The regional ethics committee (REC Central, Norway) did not consider the study to require ethical approval, because it was regarded to be a routine clinical quality control investigation and in accordance with the amended Declaration of Helsinki (Ref. no.: 2009/909-2).

BAL Procedure

Fiberoptic bronchoscopy was performed in accordance with established recommendations [12]. The bronchoscope was wedged in a segmental bronchus with radiological evidence of parenchymal changes or in the medial segmental bronchus of the right middle lobe. See supplementary material 1 for details on BAL procedure and total and differential leucocyte count.

Flow Cytometry

The samples were analysed using a FACS Canto I flow cytometer, with FACS DIVA software (BD Biosciences, Mountain View, CA), with a panel of antibodies in a four-colour setup, as listed in Table 1. Data from a minimum of 10,000 cells were recorded for each tube. Standardized gating was performed without any clinical information about the individual patients. Gating strategy is displayed in Fig. 2.

Statistical Methods

Binary logistic regression was used to estimate a function for the likelihood ratio (LR) of sarcoidosis. LR is the probability of seeing a certain data set in a patient

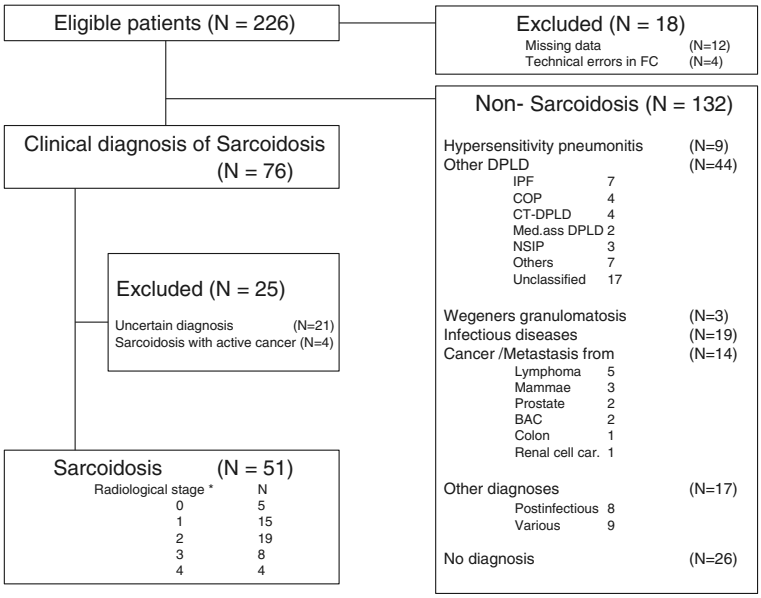

Fig. 1 Flowchart of included and excluded patients. Overview of the patients included and excluded in the study. *FC* flow cytometry; *DPLD* diffuse parenchymal lung disease; *IPF* idiopathic lung fibrosis; *COP* cryptogenic organizing pneumonia; *CT-DPLD* connective-tissue disease-associated DPLD; *Med.ass DPLD* medication associated DPLD; *NSIP* nonspecific interstitial pneumonia; *BAC* bronchoalveolar carcinoma; *Renal cell car*. renal cell carcinoma. *Asterisk* indicates radiological stage according to Scadding [11]

Table 1 Flow cytometry antibody panel

Tube	Antibody conjugates			
	FITC	PE	PerCP-Cy5.5	APC
1	Isotype control[a]	Isotype control[a]	Isotype control[b]	Isotype control[a]
2	CD8[a]	CD4[a]	HLA-DR[b]	CD3[a]
3	CD3[c]	CD16[c] and CD56[c]	CD45[c]	CD19[c]

FITC fluorescein isothiocyanate; *PE* phycoerythrobilin; *PerCP-Cy5.5* peridinin chlorophyll protein with cyanine dye (Cy5.5); *APC* allophycocyanin

[a] Multimix, Dako Denmark AS Glostrup Denmark

[b] BD Biosciences, San Jose, CA, USA

[c] MultiTEST^{TM}, BD Biosciences

with a given disease divided by the probability of seeing the same data set in a patient without the disease [13]. Knowing LR, the posttest probability of sarcoidosis can be calculated as:$(\text{LR} \times \text{pretest probability})/(\text{LR} \times \text{pretest probability} + 1 - \text{pretest probability})$. The pretest probability was defined as the prevalence of sarcoidosis in the study population (51/183).

The prediction model was generated using age, sex, total cell concentration, eosinophils, and neutrophils in differential counts, proportion of lymphocytes [14], and CD4/CD8-ratio from flow cytometry in BALF as variables. The selection of variables was based on expected clinical relevance [2, 5, 15]. Fractional polynomials, a tool in logistic regression modeling, was used to find the simplest nonlinear transformation (if any) of continuous variables [16]. Model fit was increased significantly by transformation of the variables age and proportion of lymphocytes. Variables were then eliminated with backwards stepwise elimination based on the Akaike information criterion [16]. Model fit was not significantly decreased by elimination of neutrophils and total cell concentration, resulting in the basic prediction model. The final prediction model was generated using the variables in the basic prediction model, as well as HLA-DR$^+$ CD8$^+$ T cells and fraction of NKT cell.

The LR and estimated posttest probability of sarcoidosis for each individual patient was calculated from the basic and final prediction models. The diagnostic accuracy of the basic and final models was estimated as the area under the receiver operating characteristic (ROC) curve [17, 18]. As estimation of the model coefficients and calculation of the area under the ROC curve in the same cohort of patients may give an overtly optimistic estimate of the diagnostic accuracy [19], and as we lacked a new cohort for model validation, we randomly selected data from half of the patients for estimation of the model coefficients, and tested the diagnostic accuracy of the resulting model in the other half [20, 21].This procedure was repeated 1,000 times in both prediction models. The median value of the area under the ROC curve with 95 % confidence interval was calculated for each model.

Mann–Whitney *U* was used for group comparison of continuous variables. Categorical data were analyzed with Pearson chi squared test and differences between the

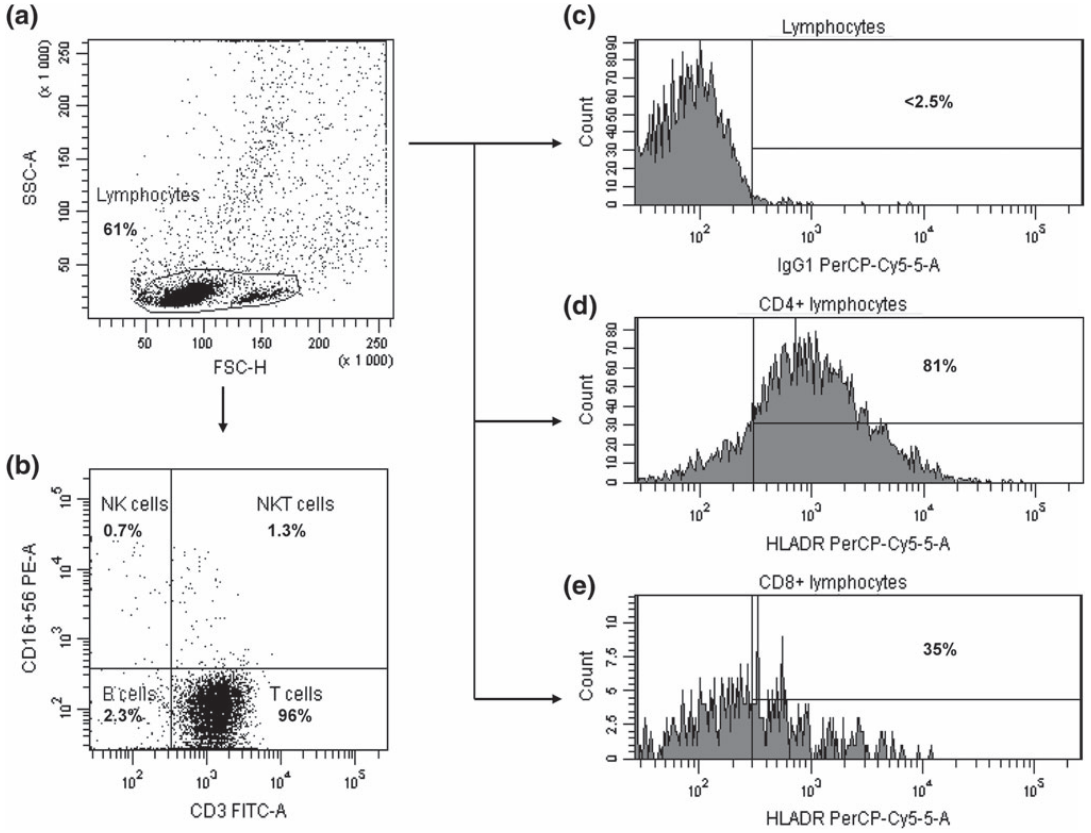

Fig. 2 Gating strategy. **a** Lymphocytes were gated by their scatter signal. **b** T cells and NKT cells were gated by their expression of CD3 (CD3 is part of the TCR complex) and CD16/CD56. Lymphocytes were confirmed to be CD45 positive and NKT cells were calculated as proportion of leucocytes (all CD45-positive cells, not shown). **c** Isotype control gating of lymphocytes. **d** and **e** HLA-DR$^+$ fractions of lymphocytes for CD4$^+$ and CD8$^+$ T cells, respectively. The case displayed is a patient with sarcoidosis

logistic regression models with the likelihood-ratio test. The area under the ROC curves was compared with the method described by DeLong et al. [22]. R version 2.15.2 [R Core Team (2012) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria] was used for statistical analyses. p value <0.05 was considered to be statistically significant.

Results

BALF Cellular Profiles

The basic characteristics of the study population and data on differential cell counts and lymphocyte immunophenotyping are presented in Table 2. Lymphocyte fraction and CD4/CD8-ratio were almost threefold and 2.5-fold greater in sarcoidosis than in nonsarcoidosis patients. Neutrophils, eosinophils, and fraction of HLA-DR$^+$ CD8$^+$ lymphocytes were significantly lower, and the fraction of NKT cells was significantly higher in sarcoidosis patients.

In the subgroup of 44 sarcoidosis and 60 nonsarcoidosis patients with BALF lymphocyte fraction above 15 %, the median (IQR) NKT cell fraction was lower in sarcoidosis than in non-sarcoidosis patients [0.31 (0.20–0.61) vs. 0.73 (0.25–2.14), $p = 0.004$]. Sarcoidosis patients were characterized by the combination of high lymphocyte fraction, a relatively low NKT cell fraction and a low fraction of CD8$^+$ T cells expressing HLA-DR (Fig. 3).

HLADR$^+$ fractions of CD8$^+$ T cells and fractions of NKT cells were not significantly different in sarcoidosis stages according to Scadding's classification (HLADR$^+$CD8$^+$ T-cell fraction: $p = 0.45$; NKT cell fraction: $p = 0.82$; Kruskal–Wallis test).

Table 2 Characteristics of the study population

Characteristic	Sarcoidosis ($N = 51$)	Nonsarcoidosis ($N = 132$)	p
Age (year)*	48 (27–77)	63 (7–85)	<0.0001
Sex (male/female)	36/15	70/62	0.044
Smoker/nonsmoker	5/46	27/103	0.088
Differential cell count			
Total cell count (x10^6/ml)	0.19 (0.13–0.29)	0.26 (0.16–0.47)	0.002
Lymphocytes (%)	29 (17–51)	12 (5–41)	<0.0001
Neutrophils (%)	3 (1–10)	7 (2–32)	0.001
Eosinophils (%)	0 (0–1)	1 (0–4)	0.015
Alveolar macrophages (%)	54(39–75)	57(26–79)	0.559
Flow cytometry			
Lymphocytes (%)	37.9 (20.5–57.0)	13.6 (5.5–46.9)	<0.0001
T cells (% of lymphocytes)	88.4 (79.6–95.1)	81.9 (67.8–89.6)	0.0001
CD4/CD8 ratio	3.8 (2.1–8.9)	1.5 (0.7–2.8)	<0.0001
NKT cells (%)	0.27 (0.12–0.56)	0.10 (0.01–0.62)	0.007
HLA-DR+ CD4+ T cells (% of CD4+ T cells)	65.6 (59.1–77.1)	71.7 (54.0–83.3)	0.238
HLA-DR+ CD8+ T cells (% of CD8+ T cells)	37.1 (27.6–48.8)	50.0 (33.3–71.6)	0.0003

* Data presented as median (range). BALF data presented as median (IQR). Numbers represent fractions of leucocytes, unless otherwise stated

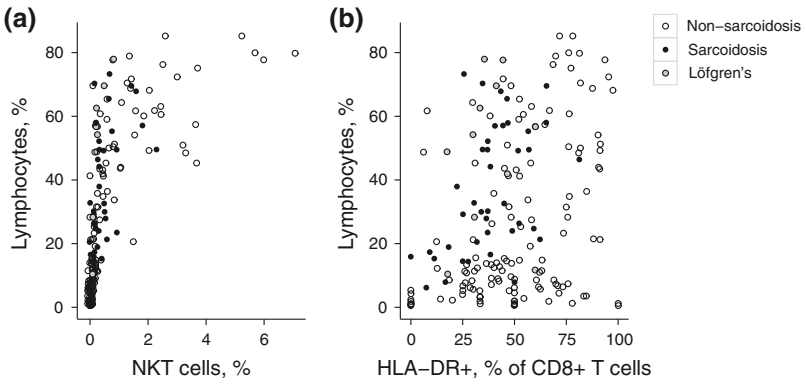

Fig. 3 NKT cells and HLA-DR+CD8+ T cells in sarcoidosis, Løfgren's syndrome, and nonsarcoidosis. Scatterplot of lymphocyte fraction versus NKT cells (**a**) and HLA-DR^+ fraction of $CD8^+$ T cells (**b**). *NKT cell* natural killer T cell

Logistic Regression Models

Covariates and coefficients for the basic and final logistic regression models are presented in Table 3.

The LR function for the final model is:

$$\begin{aligned}\mathrm{LR} = \exp\,(&14.919 + (0.011 \times \text{lymphocytes}) - (1.298 \\ &\times (\text{lymphocytes}/10)^{(-2)}) + (0.060 \times \text{CD4/CD8}) \\ &- (0.126 \times \text{eosinophils}) - (0.174 \times \text{age}) - (0.589 \\ &\times (\text{Age}/100)^{(-2)}) + (0.806 \times \text{sex}) - (0.049 \\ &\times \text{HLADR} + \text{CD8} + \text{T cells}) - (1.035 \\ &\times \text{NKT cell fraction})\end{aligned}$$

A scatterplot of the predicted probabilities for sarcoidosis using the final model for the individual patients grouped according to their diagnoses is displayed in Fig. 4.

ROC Curves

ROC curves based on the basic and final regression models are presented in Fig. 5. The area under the curve (95 % CI) for the final model was 0.937 (0.902–0.973) and the basic model was 0.898 (0.851–0.945), $p = 0.008$. The median area under the curves (95 % CI for the median) after repeated random subsampling were 0.898 (0.896–0.9) versus 0.866 (0.863–0.868) in the final and basic models, respectively.

Discussion

In this study, we evaluated the diagnostic accuracy of two predictive regression models in sarcoidosis diagnosis, with

Table 3 Logistic regression models

Covariate	Basic model		Final model	
	Coefficient	*p* value	Coefficient	*p* value
Lymphocytes (%)	−0.031	0.021	0.011	0.551
(Lymphocytes/10)$^{-2}$	−1.338	0.017	−1.298	0.021
CD4/CD8-ratio	0.131	0.018	0.060	0.32
Eosinophils	−0.19	0.019	−0.126	0.15
Age	−0.15	<0.0001	−0.174	<0.0001
(Age/100)$^{-2}$	−0.47	0.003	−0.589	0.003
Sex (male)	1.017	0.027	0.806	0.11
NKT cells (%)			−1.035	0.028
HLA-DR+ CD8+ (%)			−0.049	0.002
Constant (adjusted)[a]	11.511	0.0001	14.919	<0.0001

Likelihood-ratio test (basic vs. final): $p < 0.0001$. Overview of the coefficients in the basic and final model

[a] The Constant is adjusted for prevalence of disease [13] by the formula Constant (adjusted) = Constant + log (number of nonsarcoidosis patients/number of sarcoidosis patients)

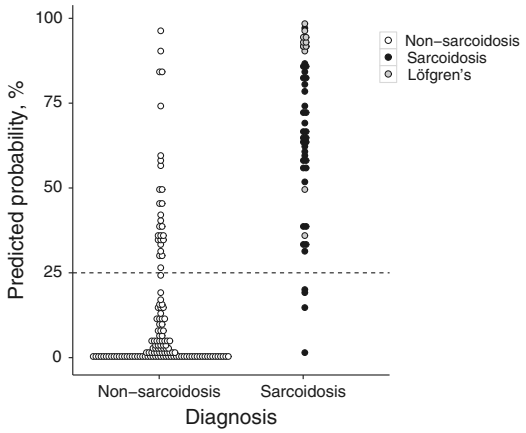

Fig. 4 Ability of the final logistic regression model to predict sarcoidosis. The final model's ability to predict sarcoidosis displayed as a scatterplot of predicted probabilities (the calculated probability of sarcoidosis for the 183 individual patients). The sensitivity and specificity of the test will vary depending on the cutoff value for a positive test. *Grey circle* represent predictions for patients with Löfgren's syndrome. The *dashed line* on 25 % predicted probability yields a sensitivity (true positives/sarcoidosis patients) of 47/51 = 0.922 and a specificity (true negatives/nonsarcoidosis patients) of 105/132 = 0.795

lymphocytes, eosinophils, and CD4/CD8-ratio in BALF, age, sex, the activation marker HLA-DR on $CD8^+$ lymphocytes and the fraction of NKT cells in BALF as covariates. The diagnostic accuracy of the basic predictive model with eosinophils and CD4/CD8 in BALF, age, and

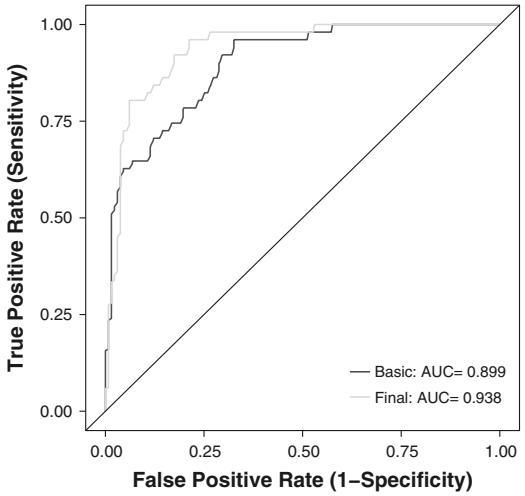

Fig. 5 ROC curves for basic and final prediction model. The area *under* the *curve* for the *black curve* is a measure of the diagnostic accuracy of the basic model in discriminating sarcoidosis from nonsarcoidosis patients. The area *under* the *curve* increases by adding NKT cells and HLA-DR^+CD8^+ lymphocytes fractions as variables in the model, as represented by the *grey line* (final model). *ROC* receiver operating characteristic; *AUC* area under curve

sex is improved by the addition of HLA-DR on $CD8^+$ lymphocytes and the fraction of NKT cells to the model. These findings may be useful in cases where histologic confirmation cannot be obtained.

The final model's area under the ROC curve after cross-validation of 0.898 indicates that these diagnostic markers may be of value in the diagnostic workup of sarcoidosis. Our findings suggest that there is role for lymphocyte subset analyses in sarcoidosis diagnostics, which is in contrast to recent recommendations [23]. Although marginally higher in sarcoidosis patients compared with nonsarcoidosis patients, BALF NKT cell fractions are lower in sarcoidosis than in nonsarcoidosis patients with lymphocyte fractions in BALF in excess of 15 %. From a clinical point of view, the combination of elevated lymphocyte fraction and relatively low fractions of NKT cell and $HLADR^+$ $CD8^+$ T cell seem to discriminate patients with sarcoidosis fairly well from other DPLDs. In contrast, the combination of elevated lymphocyte fraction and high fraction of NKT cells may be more suggestive of another DPLD, such as HP than of sarcoidosis. In our population, only 4 of 51 patients with sarcoidosis had an NKT cell fraction >1 % of the BALF leucocytes. Similarly, only one sarcoidosis patient had $HLADR^+$ $CD8^+$ T-cell fraction >66 %. If the NKT cell or $HLADR^+$ $CD8^+$ T-cell fraction is higher than that usually seen in sarcoidosis, the clinician should be alert to the possibility for other diagnoses than

sarcoidosis. The prediction model can be developed easily to clinical application for an individual patient, by calculating LR and posttest probability of sarcoidosis from the formula specified in the methods section.

Logistic regression has been used previously in evaluation of BALF profiles in diagnosis of sarcoidosis and other DPLDs [15, 24–26] or sputum-negative tuberculosis [27]. A computer program based on logistic regression in a predictive model has been developed by Drent et al. [24] as a diagnostic tool to discriminate the most common DPLDs. Our study differed from those studies in two respects. First, the regression model in the studies by Drent et al. [24] was initially restricted to a selection of patients with sarcoidosis, HP, or idiopathic pulmonary fibrosis and was later extended to include infectious disorders [26]. In our study, we included all patients where sarcoidosis or another DPLD was considered as a differential diagnosis. This may be closer to the clinical situation, where sarcoidosis may be considered as a differential diagnosis in many patients with diffuse interstitial infiltrates on HRCT. Second, the variables selected in the models are different between the studies. Our study also included lymphocyte subsets, which significantly increased model fit in our data. Many previous BALF studies were conducted before the implementation of HRCT as a routine investigation [23] in suspected DPLD and may be difficult to translate to the current clinical situation.

In the present study, we compared the expression of HLA-DR on BALF $CD8^+$ T cells and fraction of NKT cells in sarcoidosis and nonsarcoidosis patients. These T-cell subsets have been investigated in two other studies of DPLDs [9, 10]. In those studies, comparisons were made between patients with sarcoidosis and HP, with a lower fraction of BALF HLA-DR^+ $CD8^+$ lymphocytes [9] and a lower fraction of NKT cells [10] in sarcoidosis. Our results for HLA-DR^+ $CD8^+$ lymphocytes and for NKT cells in sarcoidosis and nonsarcoidosis patients with lymphocyte fractions in excess of 15 % are consistent with those studies. However, to our knowledge the utility of both markers in the diagnosis of sarcoidosis has not been evaluated in previous studies.

Our study has several limitations. One such limitation is related to the frequent inability to make a definite diagnosis of a DPLD. There were 21 patients with a clinical diagnosis of sarcoidosis in the patient record, which were excluded from the study because they did not fulfill the diagnostic criteria. Of these patients, 10 had probable sarcoidosis with typical clinical and radiological findings but no verified granulomas on tissue samples, and 11 had either atypical clinical course of the disease or atypical radiological presentation. Three patients in the latter group also had a diagnosis of Crohn's disease, and one patient had terminal renal failure. Applying the final model, the median (range) predicted probability of sarcoidosis was: 75.6 % (1.9–95.8) in the former group and 25 % (0.1–87.3) in the latter group. Using a cutoff of 25 % predicted probability, as suggested by the ROC curves, would support a diagnosis of sarcoidosis in 9 of 10 in the former group and 5 of 11 patients in the latter group. As these patients have unconfirmed diagnoses, it is conceivable that some may have been wrongly classified. Despite this, reanalysis of the dataset with inclusion of these patients does not alter the principal findings (data not shown). Second, our material included patients with Löfgren's syndrome who at presentation have a high pretest probability of sarcoidosis and thus may have had a substantial impact on the coefficients in the models. However, the findings are not essentially altered on reanalysis of the dataset with the exclusion of these patients [AUC (95 % CI) for the final and basic models were 0.926 (0.885–0.967) and 0.881 (0.827–0.934), respectively, $p = 0.02$]. Third, a high number of variables per case may increase the risk of overfitting the regression model. This is especially so if the goal is to gain insight into the relationship between the predictors and the outcome in an explanatory model. Because the main goal of the present study was to identify potential predictors of a diagnosis, the model structure and number of variables are less critical [16, 28]. Finally, and not least, our study is retrospective and the models lack external validation. The median area under the curves of repeated random subsampling gives a more realistic estimate of the diagnostic accuracy than merely ROC curve analysis of the same dataset used for model estimation. However, in a different clinical population with a different spectrum of disease and where the laboratory analyses are done differently, the diagnostic accuracy may be different. Thus, a prospective study that addresses the utility of our model as a diagnostic tool in sarcoidosis would further strengthen the validity of our results.

The role of NKT cells and activated $CD8^+$ T cells in sarcoidosis pathophysiology is not yet clear. A deficiency of a NKT cell subset in sarcoidosis has been reported [29]. Conversely, the high fractions of NKT cells in some patients in the nonsarcoidosis group (Fig. 3) may reflect involvement of NKT cells in the immunopathology of specific DPLDs, such as HP [10], as NKT cells contain subsets with both regulatory and cytotoxic functions, as well as antitumor activity [30]. HLA-DR expression on T cells may play a role in regulating or limiting a specific T-cell response to an antigen [31]. The fraction of HLA-DR^+ $CD8^+$ lymphocytes increases in some autoimmune diseases, possibly as a part of normal immunoregulation [32], although its exact role is unclear.

In conclusion, the diagnostic accuracy of BALF cell profiles in sarcoidosis is good and can be increased by adding the NKT cell fraction and HLA-DR expression on $CD8^+$ lymphocytes, thus leading to a better discrimination between sarcoidosis and nonsarcoidosis. These findings

should be validated in other centers, preferably in a prospective setting.

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Conflict of interest None.

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Research Article

Bronchoalveolar Lavage Fluid IFN-γ^+ Th17 Cells and Regulatory T Cells in Pulmonary Sarcoidosis

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In sarcoidosis, increased Th17 cell fractions have been reported in bronchoalveolar lavage fluid, and elevated numbers of Th17 cells producing IFN-γ have been observed in peripheral blood. The balance between Th1, Th17, and FoxP3$^+$ CD4$^+$ T cell subsets in sarcoidosis remains unclear. Bronchoalveolar lavage fluid cells, from 30 patients with sarcoidosis, 18 patients with other diffuse parenchymal lung diseases, and 15 healthy controls, were investigated with flow cytometry for intracellular expression of FoxP3. In a subset of the patients, expression of the cytokines IL17A and IFN-γ was investigated. The fractions of FoxP3$^+$ CD4$^+$ T cells and Th17 cells were both lower in sarcoidosis compared to controls ($P = 0.017$ and $P = 0.011$, resp.). The proportion of Th17 cells positive for IFN-γ was greater in sarcoidosis than controls (median 72.4% versus 31%, $P = 0.0005$) and increased with radiologic stage ($N = 23$, rho = 0.45, and $P = 0.03$). IFN-γ^+ Th17 cells were highly correlated with Th1 cells ($N = 23$, rho = 0.64, and $P = 0.001$), and the ratio of IFN-γ^+ Th17/FoxP3$^+$ CD4$^+$ T cells was prominently increased in sarcoidosis. IFN-γ^+ Th17 cells may represent a pathogenic subset of Th17 cells, yet their expression of IFN-γ could be a consequence of a Th1-polarized cytokine milieu. Our results indicate a possible immune cell imbalance in sarcoidosis.

1. Introduction

Sarcoidosis is a granulomatous disease with a predilection for the lungs and lymphatic tissue and is characterized by increased fractions and number of IFN-γ-producing T helper cells (Th1 cells) in inflamed tissue [1, 2]. A current hypothesis about the etiology is that an inhaled antigen, which may be persistent and poorly degradable, provokes a Th1 type immune reaction. Both mycobacterial proteins and DNA from *Propionibacterium acnes* have been identified as possible candidate antigens [3].

The discovery of the CD4$^+$ T cell subsets regulatory T cells and later Th17 cells has modified the traditional concept of Th1- or Th2-polarized adaptive immune responses [4–7]. Whereas regulatory T cells, which are characterized by expression of the transcription factor FoxP3, have a pivotal role in maintaining immune homeostasis and preventing autoimmunity [8, 9]; Th17 cells produce the potent proinflammatory cytokine IL-17 and have a crucial role in host immunity towards extracellular bacterial and fungal pathogens [10]. Both Th17 cells and FoxP3$^+$ CD4$^+$ T cells have been implicated in various human diseases with suspected autoimmune etiology, such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and psoriasis [10–12]. Intriguingly, the putative sarcoidosis antigens *Mycobacterium tuberculosis* and *Propionibacterium acnes* have both been reported to trigger strong Th17 responses [10]. Furthermore, Th17 cells recruit Th1 cells to the lungs during a mycobacterial infection and are required for proper formation of granulomas [13].

Increased Th17 cell fractions in peripheral blood and bronchoalveolar lavage fluid (BALF), and surrounding the central core of the granuloma on tissue specimens have been reported in sarcoidosis [14]. Within the Th17 cell population, there are subsets secreting different cytokines, including TNF-α and IFN-γ, with distinct effector functions [15]. IFN-γ^+ Th17 cells have been observed in elevated numbers in inflamed tissue in various conditions in man and mice [16–18]. In sarcoidosis, peripheral blood Th17 cells were reported to contain an increased fraction of IFN-γ^+ cells compared to controls, although the IFN-γ median fluorescent intensity of these cells was decreased [19].

Reports on regulatory T cells in sarcoidosis are conflicting. FoxP3$^+$ CD4$^+$ T cells are present in increased numbers in and around granulomas [20]. However, in BALF both increased [21] and decreased frequencies [22] have been reported. Interestingly, an imbalance of the regulatory T cells and the proinflammatory Th17 cells may contribute to the pathophysiology of autoimmune diseases [23–26]. These two CD4$^+$ T cell subsets share common promoting factors and chemokine receptors that constitute developmental and functional links [27, 28].

In this study, we investigated the proportion of CD4$^+$ T cell subsets expressing FoxP3 and, upon stimulation, IL17 or IFN-γ in patients with sarcoidosis, other DPLDs, and healthy control subjects. The aim of the study was to investigate the fractions of FoxP3$^+$ CD4$^+$ T cells, Th1, Th17, and IFN-γ^+ Th17 cells in BALF and to assess the hypothesis about an imbalance between these subsets in sarcoidosis. We also investigated if these phenotypes correlated with the radiological staging of the sarcoidosis.

2. Materials and Methods

2.1. Study Population. Patients who underwent diagnostic workup with bronchoscopy and bronchoalveolar lavage in our clinic between November 2010 and September 2012 for possible diffuse parenchymal lung disease (DPLD) were eligible for inclusion in the study if they had a BALF lymphocyte fraction greater than 5% of the leucocytes and were not receiving systemic therapy with corticosteroid or methotrexate. We included 30 patients with sarcoidosis and 18 patients with other DPLDs (hypersensitivity pneumonitis: $N = 5$; idiopathic pulmonary fibrosis: $N = 2$; nonspecific interstitial pneumonia: $N = 1$; connective tissue disease or medication-associated lung disease: $N = 2$; pneumoconiosis: $N = 1$; unspecified DPLD: $N = 7$). Patients with a concluding non-DPLD clinical diagnosis were not included. For this study, the diagnosis of sarcoidosis was considered certain if clinical presentation and thoracic imaging were consistent with pulmonary sarcoidosis and there were noncaseating granulomas in endobronchial or transbronchial biopsy specimens or from endobronchial ultrasound transbronchial aspirations of enlarged hilar or mediastinal lymph nodes [29]. Histological demonstration of granuloma was not required for patients with classic features of Löfgren's syndrome, defined as bilateral hilar lymphadenopathy with fever, erythema nodosum, and/or ankle arthritis. There were 3, 14, 9, 2, and 2 sarcoidosis patients with radiological staging 0, 1, 2, 3, and 4, respectively, according to Scadding [30], and 5 patients presented with Löfgren's syndrome.

Investigation of intracellular expression of IL-17A and IFN-γ after mitogen stimulation was performed in a subgroup of the patients: sarcoidosis: $N = 23$ (3 patients with Löfgren's syndrome); other DPLDs: $N = 11$ (hypersensitivity pneumonitis: $N = 3$; idiopathic pulmonary fibrosis: $N = 1$; connective tissue disease or medication associated lung disease: $N = 2$; unspecified DPLD: $N = 5$).

Eight male and 7 female healthy control subjects were recruited by advertising on the hospital website. All were nonsmokers with no history of allergy, asthma, or other lung diseases, and had normal chest radiography. All healthy control subjects and all patients except two were Caucasians of Scandinavian descent. Characteristics of the study population are displayed in Table 1.

Written informed consent was obtained from all subjects, and the study was approved by the Regional Ethics Committee (Ref.nr.: 2010/1939-4).

2.2. BAL Procedure. BAL was performed as previously described [31]. The choice of the lavage site was guided by the location of parenchymal pathology on high resolution computed tomography (HRCT), or if indifferent, the right middle lobe was chosen. With the bronchoscope in a wedged position in a segmental bronchus, 2-3 aliquots of 60 mL phosphate-buffered saline (hospital pharmacy at Haukeland University Hospital, Bergen, Norway) were instilled and retrieved by applying gentle suction. The second and third fractions of BALF were pooled and filtered through a Falcon Cell strainer with 100 μm nylon mesh (Becton, Dickinson and Company (BD) Biosciences, Mountain view, CA) and stored at 4°C until processing within 4 hours of collection.

2.3. Total and Differential Cell Counts. ADVIA 120 Hematology System (Siemens AG, Erlangen, Germany) was used for determination of total cell count. Smears were prepared by cytocentrifugation (Hettich Universal 320, DJB Labcare Ltd., Buckinghamshire, England) and stained with May Grünwald/Giemsa. Differential cell counts were done on a minimum of 300 cells.

2.4. Mitogen Stimulation. The cells were processed according to the recommendations in the human Th17/Treg phenotyping kit (BD Biosciences) (see supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/438070; tube 4). Briefly, 1-2 $\times 10^6$ cells were incubated at 37°C and 5% CO_2 for 5 hours in Roswell Park Memorial Institute medium (RPMI) with 10% fetal calf serum (FCS) with and without phorbol-12-myristate-13-acetate (PMA) at 50 ng/mL and ionomycin 1 μg/mL, in the presence of GolgiStop, a protein transport inhibitor (BD Biosciences). The cells were fixed by BD FoxP3 buffer set, buffer A (BD Biosciences), and stored for <6 months in a medium of 90% FCS and 10% dimethyl sulfoxide (DMSO) at −80°C until batch processing.

Table 1: Basic characteristics of the study population.

Characteristic	Sarcoidosis ($N = 30$)	Other DPLDs ($N = 18$)	Healthy controls ($N = 15$)	P^*
Age, years (range)	46 (37–56)$^{\#\#}$	63 (48–72)§§	35 (30–49)	0.03
Sex (male/female)	23/7	11/7	8/7	0.25
Smokers (current/former/never)	1/9/20	4/7/7	0/3/12	0.03**
FEV1, percentage of predicted	80 (68–90)	75 (63–88)§§	101 (95–104)	<0.001
FEV1/FVC	0.78 (0.73–0.83)$^{\#}$	0.82 (0.78–0.88)	0.85 (0.81–0.86)	0.03
TLCO, percentage of predicted	92 (77–102)$^{\#\#}$	57 (53–69)§§	103 (98–109)	0.01

Data are shown as median (IQR) unless otherwise stated. $^*P$ values represent pairwise comparison between Sarcoidosis and healthy controls with Mann-Whitney U test conducted if significant differences were found with Kruskal-Wallis test. $^{\S\S}P < 0.01$ compared to healthy controls. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to other DPLD. $^{**}P$ value from chi-squared test. DPLD: diffuse parenchymal lung disease; FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; TLCO: transfer factor of the lung for carbon monoxide.

2.5. Antibody Staining and Flow Cytometry. For surface antigen staining, BALF was centrifuged for 5 min at 500 ×g, and the cell pellet incubated for 15 min with the antibodies as listed (supplementary Table 1, tubes 1-2). For detection of FoxP3$^+$ CD4$^+$ T cells, the unstimulated cells were incubated with permeabilization buffer (BD Biosciences) and then stained with antibodies to surface and intracellular antigens (supplementary Table 1, tube 3). In the mitogen-stimulated samples, the thawed cells were incubated with permeabilization buffer (BD Biosciences) and then stained with antibodies to surface and intracellular antigens (supplementary Table 1, tube 4). Unstimulated cells were used as negative controls for gating.

A minimum of 10 000 and 50 000 cells were analysed for surface and intracellular antigens, respectively, using a FACS Canto I flow cytometer (BD Biosciences), with FACS DIVA software (BD Biosciences). Lymphocytes were identified by their low side scatter (SSc) and forward scatter (FSc), and T cells were identified as CD3$^+$ lymphocytes. T cells were further gated by expression of CD4 and CD8 into the two main subsets, CD4$^+$ and CD8$^+$ (cytotoxic) T cells. Gating of CD4$^+$ T cell subsets is displayed in Figure 1.

2.6. Statistical Methods. Group comparison for continuous and categorical data was done by Kruskal-Wallis test and Pearson chi-squared test, respectively. Pairwise comparison was done with Mann-Whitney U test if a significant difference was found with Kruskal-Wallis test. Spearman's rank correlation test was used for investigating correlation. Statistical analyses were done in R, a language and environment for statistical computing (R Core Team, R Foundation for Statistical Computing, Vienna, Austria). A P value of < 0.05 was considered to be statistically significant.

3. Results

3.1. FoxP3$^+$ CD4$^+$ T Cells. The fraction of FoxP3$^+$ CD4$^+$ T cells in BALF was significantly lower in patients with sarcoidosis compared to other DPLDs and HCs (median (IQR): 3.4% (2.1–4.8) versus 7.1% (4.4–12.6), $P = 0.001$ and versus 5.3% (4.4–7.0), $P = 0.017$) (Figure 2(a)).

The majority of FoxP3$^+$ CD4$^+$ T cells expressed CD27 and CD39 in all groups, with no significant differences between sarcoidosis and healthy controls ($P = 0.063$ and $P = 0.51$, resp.) (Table 2). Expression of CD27 and CD39 on FoxP3$^+$ CD4$^+$ T cells was higher in other DPLDs compared to sarcoidosis ($P = 0.01$ and $P = 0.03$, resp.), and in other DPLDs compared to healthy controls ($P = 0.001$ and $P = 0.02$, resp.).

3.2. Th17, IFN-γ^+ Th17, and Th1 Cells. Compared to healthy control subjects, the Th17 cell fraction of CD4$^+$ T cells was 45% lower ($P = 0.011$) and the proportion of Th17 cells positive for IFN-γ was over twofold greater ($P = 0.0005$) in sarcoidosis (Figure 2 and Table 3). These fractions were not significantly different between patients with sarcoidosis and other DPLDs. Fractions of Th1 (IFN-γ^+ CD4$^+$) cells were not significantly different between any of the groups.

Flow cytometry dot plots with a display of FoxP3$^+$ CD4$^+$ T cells, Th1, Th17, and IFN-γ^+ Th17 cells from a representative sarcoidosis and control subject are shown in Figure 1.

The fraction of IFN-γ^+ Th17 cells increased with radiologic stage of sarcoidosis ($n = 23$, rho = 0.45, and $P = 0.03$) (Figure 3).

3.3. The Relationship between Th1, Th17, IFN-γ^+ Th17, and FoxP3$^+$ CD4$^+$ T Cell Subsets in Sarcoidosis. In patients with sarcoidosis, fractions of Th17 cells were moderately correlated to fractions of Th1 cells ($N = 23$, rho = 0.53, and $P = 0.009$), while we found no significant correlation between Th17 cells and FoxP3$^+$ T helper cells ($N = 23$, rho = 0.25, and $P = 0.26$) or between Th1 cells and FoxP3$^+$ T helper cells ($N = 23$, rho = −0.25, and $P = 0.26$).

IFN-γ^+ fractions of Th17 cells were highly correlated with Th1 cells in patients with sarcoidosis ($N = 23$, rho = 0.64, and $P = 0.001$). This correlation was also found in an analysis of all patients and healthy controls combined ($N = 48$, rho = 0.75, $P < 0.0001$) (Figure 4(a)), and in the other individual groups (healthy controls: $N = 15$, rho = 0.91, and $P < 0.00001$ and other DPLDs: $N = 11$, rho = 0.7, and $P = 0.02$). The ratio of Th17/FoxP3$^+$ CD4$^+$ T cells was not significantly different between the groups ($P = 0.09$, Kruskal-Wallis test), while the ratio of Th1/FoxP3$^+$ CD4$^+$ T cells was higher in

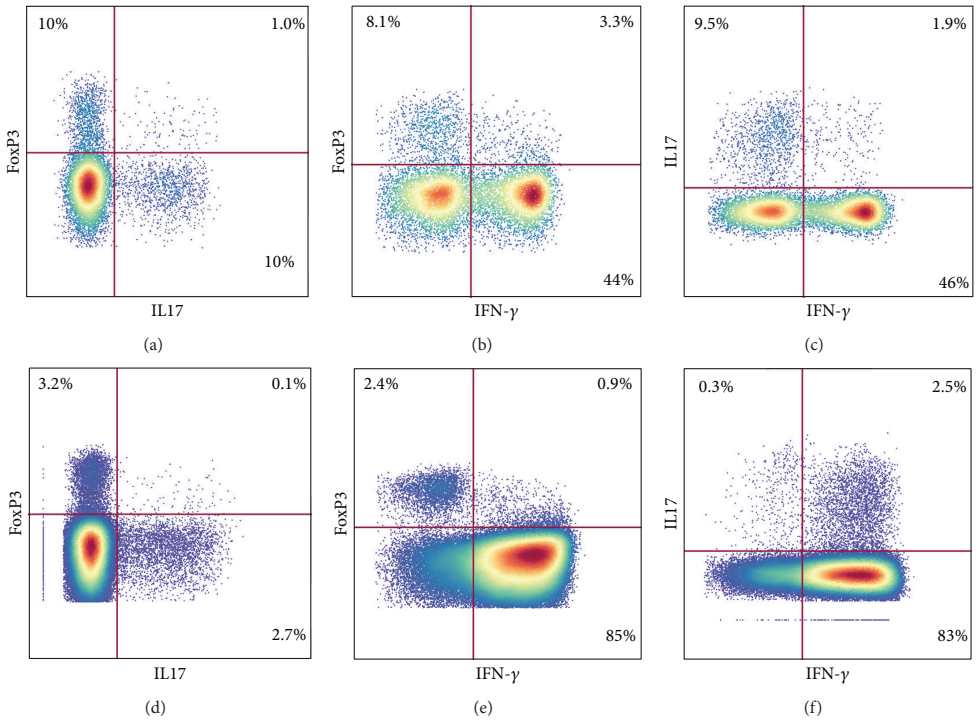

Figure 1: T helper cells in bronchoalveolar lavage fluid: regulatory T cells, Th1, Th17, and IFN-γ^+ Th17 cells. Flow cytometry dot plots of bronchoalveolar lavage fluid $CD4^+$ T cells gated by expression of FoxP3, IL17 (synonymous to IL-17A), and IFN-γ from a healthy control subject ((a)–(c)) and a patient with sarcoidosis ((d)–(f)). The T cell subsets are defined as follows: Th1 cells: IFN-γ^+ $CD4^+$; Th17 cells: $IL17^+$ $CD4^+$; regulatory T cells: $FoxP3^+$ $CD4^+$ and IFN-γ^+; Th17 cells: IFN-γ^+ IL-17^+ $CD4^+$. Thus, the Th17 cells include the cells in both upper quadrants of plots (c) and (f). The patient and healthy control are representative examples. Gates for IL17 and IFN-γ were set according to unstimulated $CD4^+$ T cells. Very few cells express intracellular IL-17 or IFN-γ prior to stimulation (median 0.0% in fractions of $CD4^+$ T cells for both IL-17 and IFN-γ).

Table 2: BALF lymphocyte subsets assessed by flow cytometry.

Characteristic	Sarcoidosis ($N = 30$)	Other DPLDs ($N = 18$)	Healthy controls ($N = 15$)	P^*
$CD3^+$ T cells (% of lymphocytes)	96.2 (95.3–97.5)$^{\#\#}$	92.3 (87.0–94.6)	92.9 (89.7–94.6)	<0.0001
CD4/CD8 ratio	6.1 (4.1–10.8)$^{\#\#}$	2.0 (0.5–3.3)	2.6 (2.1–3.4)	<0.0001
HLA-DR^+ (% of $CD4^+$ T cells)	75.1 (60.9–80.7)	76.1 (70.2–84.4)$^{\$\$}$	44.9 (36.1–56.7)	<0.0001
HLA-DR^+ (% of $CD8^+$ T cells)	47.3 (42.2–56.3)$^{\#\#}$	70.0 (58.6–78.1)$^{\$\$}$	30.4 (25.9–33.9)	<0.0001
$FoxP3^+$ (% of $CD4^+$ T cells)	3.4 (2.1–4.8)$^{\#\#}$	7.1 (4.4–12.6)	5.3 (4.4–7.0)	0.02
$CD39^+$ (% of $FoxP3^+$ $CD4^+$ T cells)	61 (34–78)$^{\#}$	80 (68–84)$^{\$}$	55 (45–66)	0.51
$CD27^+$ (% of $FoxP3^+$ $CD4^+$ T cells)	86 (80–93)$^{\#}$	95 (87–97)$^{\$\$}$	78 (72–86)	0.06

Data are shown as median (IQR). $^*P$ values represent pairwise comparison between sarcoidosis and healthy controls with Mann-Whitney U test conducted if significant differences were found with Kruskal-Wallis test. $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ compared to healthy controls. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to other DPLD.

sarcoidosis compared to other DPLDs and compared to HC (median (IQR): 21.7 (14.4–35.2) versus 6.2 (2.8–13.5), $P = 0.002$, and versus 9.5 (8.9–13-6), $P = 0.009$). The ratio of IFN-γ^+ Th17/$FoxP3^+$ $CD4^+$ T cells was considerably increased in sarcoidosis compared to other DPLDs or HC (Figure 4(b)).

The lymphocyte fraction was 2.5-fold greater in patients with sarcoidosis compared to HC (Table 4). BALF lymphocyte subset fractions in the study groups are summarized in Table 2. The CD4/CD8 ratio, HLA-DR^+ $CD4^+$, and HLA-DR^+ $CD8^+$ T cells were significantly higher in patients with

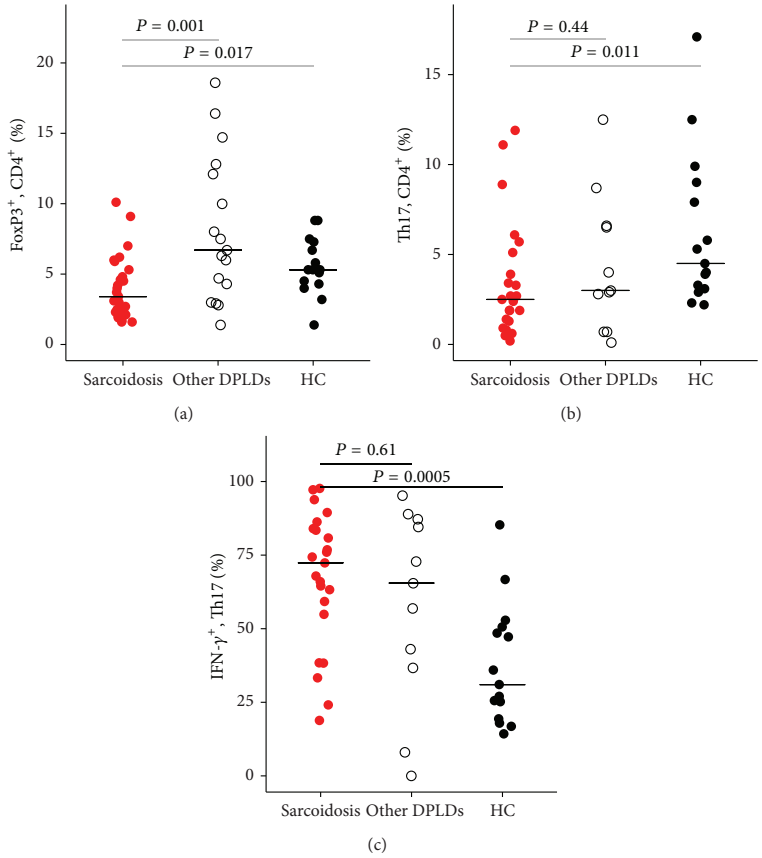

Figure 2: Bronchoalveolar lavage fluid fractions of FoxP3$^+$ CD4$^+$ T cells, Th17, and IFN-γ^+Th17 in sarcoidosis, other DPLDs, and healthy control subjects. (a) Lower fractions of regulatory T cells, defined as FoxP3$^+$ CD4$^+$ T cells, in sarcoidosis compared to other DPLDs and HC. (b) Lower Th17 cell fractions of CD4$^+$ T cells in sarcoidosis compared to HC (median: 2.5% versus 4.5%, $P = 0.011$). (c) Higher IFN-γ^+ fraction of Th17 cells in sarcoidosis compared to HC (median: 72.4% versus 31%, $P = 0.0005$). Pairwise comparisons were done with Mann-Whitney U test. DPLD: Diffuse parenchymal lung disease; HC: healthy controls.

sarcoidosis compared to HC ($P < 0.0001$, $P < 0.0001$, and $P < 0.0001$, resp.). Expression of HLA-DR on CD8$^+$ T cells was higher in other DPLDs compared to healthy controls and sarcoidosis ($P = 0.0001$ and $P = 0.0008$, resp.) and on CD4$^+$ T cells compared to healthy controls ($P = 0.0002$).

4. Discussion

In this study, IFN-γ expression in Th17 cells was over twofold greater in sarcoidosis than in healthy controls and increased with the radiologic stage of sarcoidosis. Fractions of BALF FoxP3$^+$ CD4$^+$ T cells and Th17 cells were lower in sarcoidosis compared to healthy controls. Furthermore, we found a high degree of correlation between fractions of IFN-γ^+ Th17 and Th1 cells, and the ratio of IFN-γ^+ Th17/FoxP3$^+$ CD4$^+$ T cells was prominently increased in sarcoidosis compared to other DPLDs or HCs. This may indicate a strong Th1 immune response in sarcoidosis patients resulting in deviation of CD4$^+$ T cell differentiation from FoxP3$^+$ CD4$^+$ T cells and plain Th17 cells into IFN-γ^+ Th17 and Th1 cells.

To our knowledge, investigation of BALF IFN-γ^+ Th17 cells in sarcoidosis is limited to one study by ten Berge et al. [32]. In that study expression of IFN-γ was reported in a large fraction of BALF Th17 cells in 5 patients. Our finding, stating that the majority of BALF Th17 cells in sarcoidosis are IFN-γ^+, is in line with reports on Th17 cells in peripheral blood from patients with sarcoidosis [19, 32]. In the study by Richmond et al., peripheral blood IFN-γ^+ Th17 cells of sarcoidosis patients had significantly lower mean fluorescence intensity for IFN-γ compared to cells from control subjects, indicating a reduced capacity in these cells to produce IFN-γ [19]. In sarcoidosis, T cells exhibit a more differentiated phenotype in the affected organ [33, 34]. Thus, the immune process in

Table 3: BALF lymphocyte subsets assessed by flow cytometry of mitogen stimulated cells.

Mitogen stimulated cells	Sarcoidosis ($N = 23$)	Other DPLDs ($N = 11$)	Healthy controls ($N = 15$)	P^*
Th1 (IFN-γ^+, % of $CD4^+$ T cells)	75.5 (62.6–79.4)	62.1 (46.7–75.0)	65.7 (41.5–69.6)	n.s
Th17 cells (% of $CD4^+$ T cells)	2.5 (1.4–4.5)	3.0 (1.8–6.6)	4.5 (3.2–8.5)	0.01
IFN-γ^+ Th17 (% of Th17)	72.4 (57.1–83.8)	65.5 (39.9–85.9)	31.0 (22.3–49.6)	0.0005
IFN-γ^+ $CD8^+$ T cells (% of $CD8^+$)	83.4 (69.3–87.9)	83.1 (76.4–94.3)	85.5 (77.5–89.9)	n.s
$IL17^+$ $CD8^+$ T cells (% of $CD8^+$)	0.8 (0.5–2.4)	0.5 (0.3–1.4)	1.1 (0.9–1.6)	n.s

Data are shown as median (IQR). $^*P$ values represent pairwise comparison between sarcoidosis and healthy controls with Mann-Whitney U test conducted if significant differences were found with Kruskal-Wallis test. Pairwise comparisons between sarcoidosis and other DPLD or other DPLD and healthy controls showed no significant difference. n.s: nonsignificant (Kruskal-Wallis test).

Table 4: BALF cellular differential counts assessed by microscopy.

	Sarcoidosis ($N = 30$)	Other DPLDs ($N = 17$)	Healthy controls ($N = 14$)	P^*
Total cell concentration (10^6/mL)	19 (15–28)	26 (11–29)	14 (12–17)	0.01
Lymphocytes (%)	35 (24–53)	34 (14–48)$^{\$}$	14 (10–25)	0.001
Neutrophils (%)	2 (1–4)$^{\#\#}$	6 (2–9)	3 (2–5)	0.15
Mast cells (per 10 squares)	4 (2–9)$^{\#\#}$	21 (8–63)$^{\$\$}$	2 (0–5)	0.04
Eosinophils (%)	0 (0-1)$^{\#\#}$	3 (1–9)$^{\$\$}$	0 (0-0)	0.14
Alveolar macrophages (%)	60 (45–73)	47 (26–74)$^{\$\$}$	81 (69–85)	0.001

Data are shown as median (IQR). $^*P$ values represent pairwise comparison between sarcoidosis and healthy controls with Mann-Whitney U test conducted if significant differences were found with Kruskal-Wallis test. $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ compared to healthy controls. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to other DPLD. Differential counts were missing for one patient with other diffuse parenchymal lung disease (DPLD) and one healthy control.

sarcoidosis may be better characterized in studies on BALF cells.

Fractions of BALF Th17 cells were lower in sarcoidosis patients compared to controls in the present study. This is consistent with another study in which IL-17A mRNA expression in sorted $CD4^+$ T cells was investigated [35], but at variance with other studies that detected intracellular expression of IL-17A by flow cytometry [14, 19, 32], several possible explanations may account for the different results. The gating of $IL17^+$ from $IL17^-$ $CD4^+$ T cells may differ between the studies. We used unstimulated cells from the individual patients to set the gates, which we think may be an appropriate way to delineate the $IL17^+$ from the $IL17^-$ populations. Importantly, differences in genetic background may influence the sarcoidosis immune process. Our results are similar to those of the study from Sweden [35], but at variance with the American study [19], where approximately half of the patients had Afro-American background, and 14/37 patients had sarcoidosis of the skin. In contrast, all of our patients had pulmonary sarcoidosis. In addition, Löfgren's syndrome is more frequent in sarcoidosis in Scandinavia.

In the present study, the $FoxP3^+$ fractions of $CD4^+$ T cells in sarcoidosis were lower than in HC. This is in agreement with one previous study, which reported decreased frequency of $FoxP3^+$ $CD4^+$ T cells in BALF in patients compared to HC [22], with relative fractions resembling our results. In that study FoxP3 mRNA expression was also investigated and found to be lower in BALF $CD4^+$ T cells from patients compared to HC. In another study, regulatory T cells in BALF and peripheral blood were found to be higher in patients with sarcoidosis than in control subjects [20]. However, they defined regulatory T cells as $CD25^{bright}$ $CD4^+$ T cells and confirmed the expression of FoxP3 by, on average, 86.5% of these cells by a single cell PCR approach. Later, the same group reported increased fractions of $CD45RA^-FoxP3^{bright}$ $CD4^+$ T cells in peripheral blood and $CD4^+$ $FoxP3^+$ cells in lymph nodes and granulomas of patients with active sarcoidosis [36]. The definition of regulatory T cells as $FoxP3^{bright}$ $CD4^+$ T cells is also different than in the present study, where regulatory T cells are defined as $FoxP3^+$ $CD4^+$ T cells, making direct comparison less relevant. As documented by Darlington et al., $FoxP3^+$ $CD4^+$ T cells are augmented in lymph nodes compared to BALF [37], thus our finding of lower fractions of regulatory T cells in BALF in sarcoidosis may not be inconsistent with increased fractions in lymph nodes and granulomas.

IFN-γ^+ Th17 cells may represent a pathogenic subset of Th17 cells in some autoimmune diseases [17, 18]. In one study, human *Candida albicans*-specific but not *Staphylococcus aureus-specific* Th17 cells were demonstrated to produce IFN-γ upon stimulation [15]. During the last few years it has become increasingly clear that there exists plasticity in the differentiation of T helper cells [38]. IL-12 and IFN-γ influence Th17 cells to change towards IFN-γ^+ Th17 cells or Th1-like cells [16, 39]. In a study on Th17 cells recovered from diseased gut areas in colitis patients, a median of ~ 40% of the Th17 cells was also IFN-γ^+ [40]. In sarcoidosis,

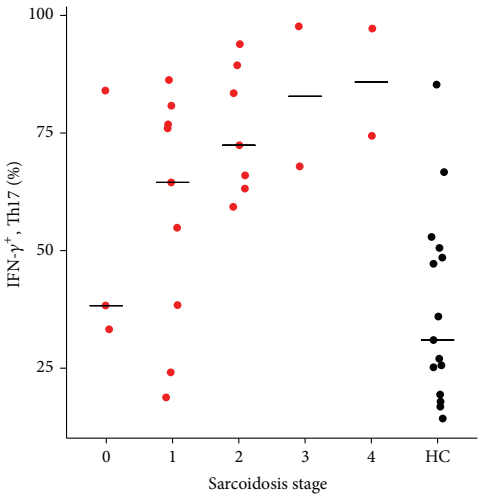

Figure 3: Fractions of IFN-γ^+ Th17 cells increase with radiological stage in sarcoidosis. The relationship of IFN-γ^+ Th17 cells and radiological stage in patients with sarcoidosis ($N = 23$) and healthy control subjects ($N = 15$). Fractions of IFN-γ^+ Th17 cells increase with advancing stage ($N = 23$, Spearman's rho = 0.454, and $P = 0.03$). Bars represent median. HC: Healthy control subjects.

macrophages and Th1-cells are enriched in the granulomatous lung tissue, and the dominating cytokines in the feedback loop between these cells are IL-12 and IFN-γ. Accordingly, the reduced Th17 and increased IFN-γ^+ Th17 cells that we detected in BALF from the sarcoidosis patients might be a result of dominating Th1-driving transcription factors and cytokines. In Figure 4(a) we show that in BALF from patients and HC there seems to be a clear-cut and highly significant linear correlation between Th1 cells as a fraction of CD4$^+$ T cells and IFN-γ^+ Th17 cells as a fraction of Th17 cells. This might favor an idea of a common mechanism in switching of Th17 towards IFN-γ^+ Th17 and expansion of Th1 cells. Conversely, it has recently been demonstrated in experiments with mice, in whom the Th1-driving transcription factors had been knocked out in T cells, that such naïve T cells can still develop into pathogenic IFN-γ^+ Th17 cells and that IL-23 is essential for differentiation and expansion of these cells [18]. It may thus seem to be, at least in mice, a way of differentiation for IFN-γ^+ Th17 cells independent of the IFN-γ driving transcription factors. The observation that the relative frequency of IFN-γ^+ Th17 cells is higher in patients with advanced sarcoidosis stage (Figure 3) might be an indication that these cells possibly have a deleterious effect in sarcoidosis or might just be the consequence of a more dominating and pathogenic Th1 immune reaction.

The ratio between IFN-γ^+ Th17 cells and FoxP3$^+$ CD4$^+$ T cells is markedly raised in patients with sarcoidosis in our study. Many studies have shown a reciprocal relationship between Th17 and regulatory T cells [26], and autoimmune diseases often result from an imbalance in these CD4$^+$ T cell subsets [41]. An increased ratio between IFN-γ^+ Th17 cells and FoxP3$^+$ CD4$^+$ T cells may represent a corresponding feature of sarcoidosis. A recent study reported an increased Th17/regulatory T cell ratio in peripheral blood and BALF in sarcoidosis compared to healthy controls [42]. This was not found in our study, and fractions of Th17 cell in BALF were decreased in our study and increased in their study. Both genetic background and environmental exposures may differ between a Scandinavian and a Chinese population of patients, possibly accounting for some of these observed differences.

In the present study, CD4$^+$ T cells from BALF are studied, which are more directly involved in the immunologic process of sarcoidosis than peripheral blood cells. However, there are some limitations to the study. In our clinic, the number of patients with other DPLD diagnoses is small, and patients consist of many different entities. A comparison with a sufficient number of patients with other granulomatous lung diseases would be of interest. Furthermore, the number of sarcoidosis patients in different radiological stages is small, limiting the use of our data in investigating differences between patients in different stages of the disease.

In conclusion, we found that the majority of BALF Th17 cells in sarcoidosis are IFN-γ^+, and the fraction of IFN-γ^+ Th17 cells was highly correlated with the fraction of Th1 cells. Fractions of both Th17 cells and FoxP3$^+$ CD4$^+$ T cells were lower in sarcoidosis than in HC, and we found a highly elevated ratio between IFN-γ^+ fractions of Th17 cells and FoxP3$^+$ CD4$^+$ T cells, possibly indicating an immune cell imbalance. Despite the explanation offered in this paper that the generation of IFN-γ^+ Th17 cells may be a direct consequence of a Th1-polarized cytokine milieu on Th17 cells, they may still represent a pathogenic subset of Th17 cells, and further characterization of these cells may provide a target for therapy and provide additional clues to the complex immunopathology of sarcoidosis. Functional studies on these cells and investigations of these cells in patients with different disease course are warranted.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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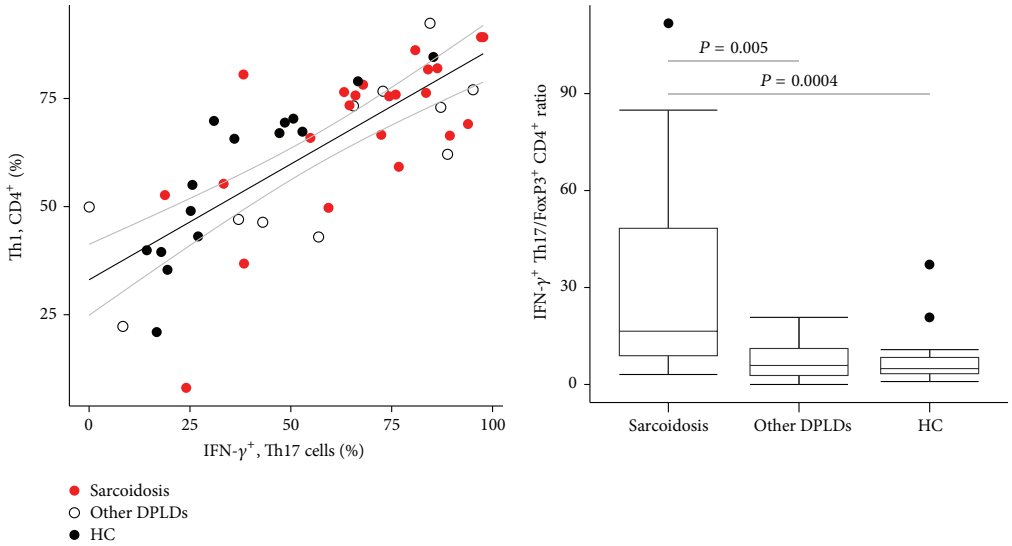

Figure 4: The relationship of Th1, IFN-γ^+ Th17, and FoxP3$^+$ CD4$^+$ T cells in bronchoalveolar lavage fluid. (a) There was a highly significant correlation between fractions of IFN-γ^+ Th17 cells and Th1 cells (IFN-γ^+ CD4$^+$) (n = 48, Spearman's rho = 0.75, and $P < 0.0001$). Black and grey lines represent linear regression lines with 95% CI. (b) The IFN-γ^+ Th17/FoxP3$^+$ CD4$^+$ T cell ratio was markedly elevated in patients with sarcoidosis compared to other DPLDs or healthy control subjects. DPLD: diffuse parenchymal lung disease; HC: healthy control subjects.

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