# Immunological markers of cell activation in sarcoidosis

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# Immunological markers of cell activation in sarcoidosis

Immunologische merkers van cel activatie in sarcoïdose
(met een samenvatting in het Nederlands)

Proefschrift

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*Voor Toddy Heron-Muller (1940-2009)*

# Content

# 1

# General introduction

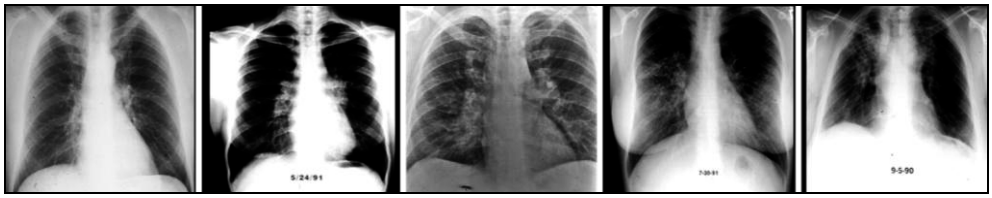
# 1. SARCOIDOSIS

Sarcoidosis is a systemic granulomatous disease with unknown cause. Both disease onset as well as disease course may vary greatly and depend on the organs involved and the severity and duration of the inflammation. Non-caseating granulomas, a hallmark of sarcoidosis, can be found in every organ but are most often found in the lungs.
The following descriptive definition is provided in the American Thoracic Society/European Respiratory Society/World Association for Sarcoidosis and Other Granulomatous Disorders Statement on Sarcoidosis in 1999[1]: "Sarcoidosis is a multisystem disorder of unknown cause. It commonly affects young and middle-aged adults and frequently presents with bilateral hilar adenopathy, pulmonary infiltration, ocular and skin lesions. The liver, spleen, lymph nodes, salivary glands, heart, nervous system, muscles, bones, and other organs may also be involved. The diagnosis is established when clinicoradiographic findings are supported by histologic evidence of non-caseating epithelioid cell granulomas. Granulomas of unknown causes and local sarcoid reactions must be excluded. Frequently observed immunologic features are depression of cutaneous delayed-type hypersensitivity and a heightened $T_{Helper}1$ ($T_H1$) immune response at sites of disease. Circulating immunecomplexes along with signs of B cell hyperactivity may also be found. The course and prognosis may correlate with the mode of the onset, and the extent of the disease. An acute onset with erythema nodosum or asymptomatic bilateral hilar adenopathy usually heralds a self-limiting course, whereas an insidious onset, especially with multiple extrapulmonary lesions, may be followed by relentless, progressively fibrosis of the lungs and other organs."

## 1.1 Clinical presentation

Sarcoidosis commonly presents with symptoms such as fatigue, fever, and malaise or weight loss. Lung involvement occurs over 90% of patients with sarcoidosis and many patients have respiratory symptoms, including cough, dyspnea and chest pain[2]. In the majority of sarcoidosis patients the disease undergoes spontaneous remission. Approximately 10% of the sarcoidosis cases develop parenchymal fibrosis[3]. Fibrosis of the lung parenchyma is associated with poor pulmonary function and an unfavorable prognosis with increased morbidity and mortality[4].

The stage of pulmonary involvement is based upon the chest radiograph and is classified according to the Scadding criteria (Figure 1)[1].



**A** **B** **C** **D** **E**

**Figure 1** Chest radiographic staging in pulmonary sarcoidosis. Stage 0, normal chest radiographic findings (A); stage I, bilateral hilar adenopathy with normal lung parenchyma (B); stage II, bilateral hilar adenopathy with pulmonary infiltrates (C); stage III, pulmonary infiltrates without hilar adenopathy (D); stage IV, pulmonary fibrosis (E).

The most prominent sites of extrapulmonary disease include the skin, eyes, reticuloendothelial system, musculoskeletal system, exocrine glands, heart, kidney, and central nervous system. Extrapulmonary manifestations vary on the basis of sex, age at presentation, and ethnicity. Acute sarcoidosis or Löfgren's syndrome is accompanied by erythema nodosum[5], a harmless skin condition, while neurosarcoidosis or myocardial sarcoidosis may be life threatening[6,7]. Uveitis, an ophthalmic complication of sarcoidosis, might impair vision, sometimes leading to blindness[8].
Disease presentation, severity and prognosis vary greatly and depend on age, sex, ethnicity, type of organs involved as well as the severity and/or duration of the inflammation.

## 1.2 Pathogenesis

Antigen presentation and recognition in the draining lymph nodes results in cellular immunity against the unidentified antigen and initially leads to a $CD4^+$ T cell alveolitis. Ongoing presentation of antigens by macrophages to $T_H1$ lymphocytes and failure to eliminate antigenic stimuli such as foreign antibodies or microorganisms leads to the formation of granuloma, the pathological hallmark of sarcoidosis[9]. Stimulation and activation of macrophages and $CD4^+$ T cells induces the production of IL-2, IFN-γ and TNF-α. These cytokines are considered pivotal for granuloma formation[10,11]. The granuloma is a focal, chronic inflammatory reaction formed by the accumulation of epithelial cells, monocytes, lymphocytes, macrophages and fibroblasts (Figure 2). Multinucleated giant

cells are frequently found among the epithelial cells within the granuloma follicle[12]. The sarcoid granuloma can resolve without residual manifestations of previous inflammation or become fibrotic lesions with the resultant development of parenchymal fibrosis.
Granuloma formation, not only a feature of sarcoidosis, is found in lungs of patients with other chronic interstitial lung diseases, *e.g.* hypersensitivity pneumonitis, berylliosis and pulmonary Langerhans cell histiocytosis[13].

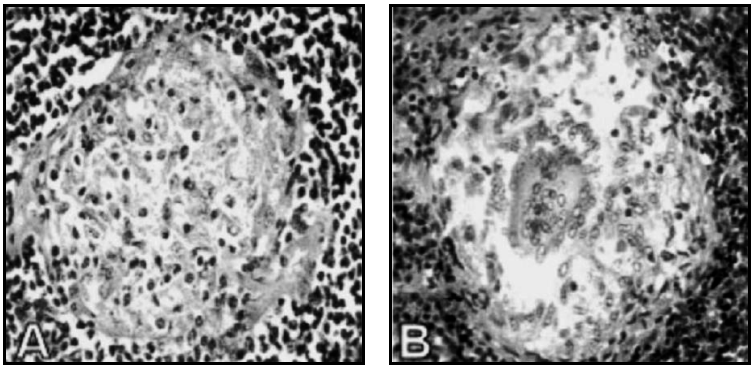



**Figure 2** Non-caseating granuloma (A) Non-caseating granuloma with giant cell (B).

## 1.3 Disease parameters

Several molecular biomarkers and clinical parameters are used to assess sarcoidosis disease activity (ACE, sIL-2R) or disease severity (x-thorax, lung function, HR-CT scan). Others may yield prognostic value for progressive disease (x-thorax) or discriminate between sarcoidosis and other interstitial lung diseases (bronchoalveolar lavage fluid (BALF) $CD4^+/CD8^+$ ratio, BALF cell profiles).

In the absence of a known causative agent, however, sarcoidosis remains a diagnosis of exclusion. The differential diagnose of sarcoidosis is extensive, including infectious diseases (e.g. tuberculosis), granulomatous diseases associated with exposure to inorganic or organic agents (e.g. hypersensitivity pneumonitis, chronic beryllium disease, drug-induced pneumonitis), autoimmune disorders (e.g. Wegener's granulomatosis, Churg-Strauss vasculitis) and malignancies (e.g. lymphomas, tumour-related granuloma)[14-16].
A lymphocytic alveolitis with a BALF $CD4^+/CD8^+$ ratio > 3.5 is consistent with pulmonary sarcoidosis. However, the finding of a $CD4^+$ lymphocytosis in BAL is neither specific nor sensitive for the diagnosis of sarcoidosis[17-19]. Interpretation of BALF cell differentials shows that no single cell but rather a cell profile or a combination of several features

discriminates sarcoidosis from other interstitial lung diseases. Mildly elevated total cell counts with a predominance of lymphocytes, normal eosinophil and neutrophil percentages, and the lack of plasma cells and ''foamy'' alveolar macrophages characterizes sarcoidosis[20]. The expression of the integrin CD103 on BALF $CD4^+$ lymphocytes has been shown to be a promising candidate for a new diagnostic marker for sarcoidosis[21].

Angiotensin-converting enzyme levels (ACE) in serum and serum soluble IL-2R (CD25) levels are used as markers of disease activity, where serum ACE levels reflect the granuloma burden and serum sIL-2R levels reflect mainly the activity of the T cell component[22,23]. However, the added value of ACE levels in the diagnosis and follow-up of sarcoidosis is still subject to debate. Other than a marker for the presence of granulomatous disease and its activity, ACE lacks disease specificity and does not have predictive value for the course of sarcoidosis[23]. Despite its shortcomings, ACE is mostly used in the assessment and follow-up of sarcoidosis[24].

Genotype corrected ACE values may improve the interpretation of ACE levels significantly as demonstrated by Kruit et al. They introduced ACE I/D corrected Z-scores for ACE activity[25]. The dimensionless quantity *Z* represents the distance between the raw score and the population mean in units of standard deviation. This approach may be a pragmatic way for reporting analyte activity based on genotype-corrected reference intervals.

Grutters et al. demonstrated a positive correlation between serum sIL-2R levels and absolute BALF $CD4^+$ cells in sarcoidosis patients measured at presentation[22]. This is corroborative to the concept of sIL-2R as an index for T cell activation, and supports the use of serum sIL-2R as marker for the intensity of the T cell alveolitis in sarcoidosis. In contrast, Müller-Quernheim and colleagues did not find significant correlations between serum sIL-2R levels and BALF $CD4^+$ cells[26]. Activated T cells in other involved organs and shedding of CD25 by alveolar macrophages may diminish correlations between BALF $CD4^+$ cells and serum sIL-2R levels[27]. Other serum biomarkers for sarcoidosis disease activity have been investigated. Serum lysozyme, neopterin, $beta_2$-microglobulin, and adenosine deaminase however, have not proven to be superior to ACE as markers for disease activity in several studies[28-33]. In contrast, Prior et al. found neopterin the most useful marker for overall disease activity and Wetzel et al. demonstrated that in comparison to sACE, sIL-2R and serum neopterin, measurement of serum adenosine deaminase indicated a greater discrimination between active and inactive disease[34,35].

Chest radiography and respiratory function test are still the best indices of lung dysfunction and failure, despite their inability to predict the disease course of sarcoidosis. Pulmonary function testing usually shows restriction, but sarcoidosis is one of a handful of

lung diseases that can have mixed obstructive and restrictive physiology. Disparities between radiographic staging and lung function parameters have been reported. Fibrotic changes might be reflected by lung function but may be too small to be discerned on chest x-ray[36,37]. High resolution CT-scan may be superior to chest x-ray in discriminating subtle changes in lung parenchyma or development of fibrotic lesions.

Radiographic staging at presentation itself has prognostic value for pulmonary disease outcome. Patients presenting with stage I are more likely to have normal x-ray at pulmonary disease outcome than patients presenting with stage II or III. Interestingly, patients presenting with stage III either persist in stage III or progress to stage IV, or improve towards stage 0 at pulmonary disease outcome. It remains to be determined whether radiographic stages reflect successive processes in sarcoid inflammation.

The challenge is to optimize existing and to find novel, better and easier to perform biomarkers. Chest radiography and respiratory function tests still remain the standard against which novel disease markers are assessed.

# 2. IMMUNE CELLS IN SARCOIDOSIS

Most clinical manifestations of interstitial lung diseases are secondary to the direct effect of the accumulation of activated immune cells in involved tissues, notably T cells, macrophages, or neutrophils[38,39]. T lymphocytes are important mediators of adaptive immune responses and are vital for host defense against infection. Although important for immunity, aberrant accumulation of T cells in the lung is seen in numerous noninfectious pulmonary inflammatory diseases such as sarcoidosis, where T lymphocytes in the lung are believed to orchestrate an abnormal inflammatory process.

## 2.1 T lymphocytes

Mature T cells recognize and respond to the antigen/MHC complex through their antigen-specific receptors (TCR). The most immediate consequence of TCR activation is the initiation of signaling pathways including induction of specific protein tyrosine kinases (PTKs), breakdown of phosphatidylinositol 4,5-biphosphate (PIP2), activation of protein kinase C (PKC) and elevation of intracellular calcium ion concentration. These early events are transmitted to the nucleus and result in: (1) clonal expansion of T cells, (2) up-regulation of activation markers on the cell surface, (3) differentiation into effector cells, (4) induction of cytotoxicity or cytokine secretion and (5) induction of apoptosis.

Activation markers were initially described on lymphocytes stimulated *in vitro* with mitogens and antigen. Early activation markers such as CD25 and CD69 appear within hours of stimulation, in contrast to very late activation markers (VLA-1, VLA-4) which are expressed after days to weeks of stimulation[40,41]. In addition, T cell activation solely through the T cell receptor results in a nonresponsive state (anergy) in which T cells are refractory to restimulation. Coligation of other cell surface receptor provides additional signals required for anergy avoidance and productive T cell activation. The costimulatory molecule CD28 is the most important of many costimulatory molecules and preferentially binds CD80 expressed on antigen presenting cells[42].

Upon antigenic encounter and specific cytokine signals naïve $CD4^+$ T cells differentiate into $T_H1$, $T_H2$ and $T_H$-17 subsets and induces their effector functions (Figure 3)[43,44]. Specific transcription factors regulate $T_{Helper}$ cell differentiation[45-47] and these specific subsets induce immunity in a variety of immune reactions. $T_H1$ cells secrete large amounts of IFN-γ and lymphotoxin, and are responsible for clearing of intracellular pathogens by activating macrophages[48]. $T_H2$ cells predominantly release IL-4, IL-5 and IL-13 and mediate immunity

against extracellular pathogens[49]. IL-4 and IL-5 production by $T_H2$ cells can activate mast cells and eosinophils leading to atopy and allergic reactions. Interestingly, the cytokines of a particular $T_H$ subtype are able to further promote the expansion of that subtype population while simultaneously inhibiting the development of the other subset.
On the other hand, regulatory T cells (naturally occurring $Foxp3^+$ Tregs, $T_H3$, and IL-10-producing Tr1 cells) are induced by indicated cytokines (predominantly TGF-β) and regulate $T_H$ effector (Teff) cells. Dysregulation of $T_H$ pathways may be harmful to the host and often leads to immune pathology and tissue destruction during infections or autoimmune reaction[50].

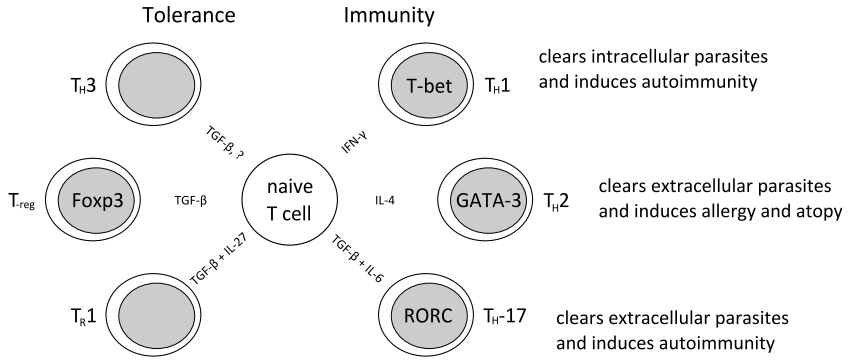



**Figure 3** T cell subsets: axis of immunity and immune tolerance.

The lung has a large network of antigen-presenting cells and lymphatics that aid in antigen presentation to T cells in associated lymphoid tissue located within the lung parenchyma and mediastinum. In addition, the adult lung contains T cells residing in the alveoli, airway (bronchial) lumen, intraepithelial layer, submucosa, and interstitium[51].
In sarcoidosis a distinct compartmentalization to the lungs of $CD4^+CD45RO^+$ (memory) T cells[52], spontaneously releasing IL-2[53] and expressing the activation marker, human leukocyte antigen (HLA)-DR[54], was found already in the early 1980s through the use of bronchoscopy and bronchoalveolar lavage. Of patients with active pulmonary sarcoidosis the equivalent of $25 \times 10^6$ T cells can be recovered from BAL. The typical $T_H1$ response in acute sarcoidosis has been demonstrated mainly by increased mRNA and protein levels of $T_H1$ cytokines (TNF-α, IFN-γ and IL-2) and chemokines (IL-8, IP-10 and RANTES) in bronchoalveolar lavage fluid (BALF) and BALF cells[53,55-63]. A switch to $T_H2$ cells may occur in patients with progressive sarcoidosis who evolve toward lung fibrosis[64]. In these patients, lung T cells release $T_H2$ cytokines, including IL-4. IL-4 stimulates the production of extracellular matrix proteins and is a chemoattractant for fibroblasts (Figure 4). Recently,

Rosenbaum and colleagues compared gene expression profiles in peripheral blood from patients with sarcoidosis to controls and found that several transcripts associated with interferon and STAT-1 (up-regulates T-bet, a master transcription factor required for $T_H1$ differentiation and development) were up-regulated[65]. Lung and lymph node analyses also showed dramatic increases in STAT-1 and STAT-1-regulated chemokines. This report demonstrated at transcription factor level that sarcoidosis is a $T_H1$ immune mediated disease. However, expression of specific $T_{Helper}$ cell lineage transcription factors in different sarcoidosis phenotypes (progressive disease, fibrotic phenotype) remains to be determined.

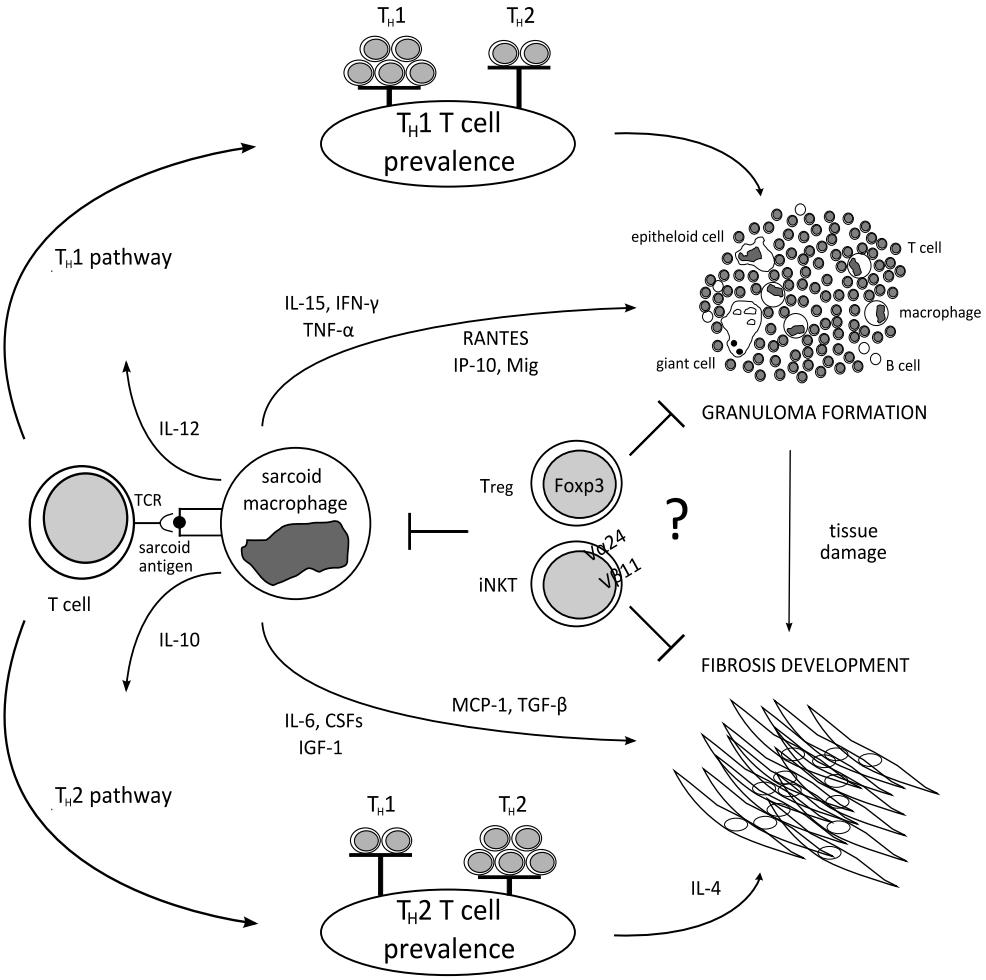



**Figure 4** Sarcoidosis is associated with a typical $T_H1$ response. A switch towards a $T_H2$ response might occur with persisting antigen presentation, ultimately leading to development of fibrosis. The exact role of T cells with regulatory properties ($Foxp3^+$ cells, iNKT cells) remains unclear.

The characteristic finding of lung accumulated $CD4^+$ T cells and the resulting increase of the BALF $CD4^+/CD8^+$ ratio has come to be a clinically important marker of the disease[14]. The $CD4^+/CD8^+$ ratio however is specific nor sensitive for sarcoidosis[17,18]. Further characterization of the involved cells, stratified by clinical phenotypes, might improve the understanding of the immune process and may be a tool for clinical evaluation of sarcoidosis.

Wahlström et al have shown differential expression of activation markers between blood and BALF lymphocytes in pulmonary sarcoidosis[66]. Highly activated T cells were found in BALF of patients, in particular $CD4^+$ T cells that have a preferential usage of the TCR AV2S3 gene segment[67,68]. Moreover, enhanced expression of costimulatory molecule CD28 on BALF T cells was demonstrated by Agostini et al. This associated with increased expression of activation markers CD69 and CD103[69]. In addition, increased expression of very late antigen-1 (VLA-1), VLA-4 and VLA-5 has been demonstrated on tissue-infiltrating T cells from the lungs of sarcoidosis patients, especially in patients with active disease[70,71].

**Intraepithelial lymphocytes (IEL)**

Damage to the lung mucosal epithelium plays a crucial role in the pathophysiology of interstitial lung diseases. The mucosal epithelium represents a unique lymphoid compartment containing a distinct population of lymphocytes, the intraepithelial lymphocytes (IEL). In the intestine, a substantial fraction of IEL expresses γδ T cell receptors. Although γδ T cells are commonly found in murine lung, almost all human bronchial IEL express αβ T cell receptors[72]. As with other lymphocytes, IEL may use a distinct profile of cell surface molecules to localize to or anchor in the epithelium. Lung epithelial cells express E-cadherin at the basolateral side which serves as a ligand for the $\alpha_E\beta_7$/CD103$\beta_7$ integrin complex. This integrin is expressed mainly by cells of the T lymphocyte lineage within mucosal tissues of gut, urogenital tract and lung[73-76]. In contrast to the T cell integrins LFA-1 and VLA-4, CD103 is poorly expressed by peripheral blood T cells. In humans, CD103 expression is confined to a small subset (< 2%) of total circulating T lymphocytes[77]. After T cells have migrated to mucosal tissues, CD103 expression is induced and maintained by TGF-β, produced mainly by epithelial cells[78].

The function of IEL is unknown but IEL may respond to antigens at mucosal sites and perform effector functions such as cytolysis of infected or transformed epithelial cells, production of cytokines that alter epithelial cell growth and differentiation, or induction of tolerance. As each of these potential functions requires a strong cell-cell interaction, it is possible that IEL use $\alpha_E\beta_7$ as adhesion molecule to maximize their responsiveness[72,73]. Interestingly, Schlickum et al. recently reported that binding of CD103 to E-cadherin

determines shape and locomotion of $CD103^+$ cells[79], consistent with the function of CD103 as the receptor mediating retention and/or interaction between IEL and mucosal epithelial cells. Moreover, a blocking antibody, M290, to the $\alpha_E$ subunit was shown to prevent or ameliorate immunisation induced colitis in IL-2 deficient mice. The effect was attributed to reduced retention of $CD4^+$ T cells in the mucosa[80]. In line with these observations, Sharma and colleagues showed that development of inflammation in skin and lungs in Scurfy mice correlated with CD103 expression on $CD4^+$ T cells. These data demonstrate that, in murine models, interaction between $CD103^+CD4^+$ T cells and E-cadherin$^+$ target organs plays a role in the inflammation process and might contribute directly to T cell mediated damage of epithelial cells[81].

Notable, CD103 expression is thought to identify peripheral tissue $Foxp3^+$ regulatory T cells (Tregs)[82]. CD103 single positive cells showed strong suppressive potential *in vitro* and *in vivo* in murine disease models[82,83] and in humans[84] and expression of CD103 may contribute to their regulatory function by interacting with inflamed lung epithelium.

In fibrotic lung diseases increased expression of the integrin CD103 on BALF T lymphocytes was found by Lohmeyer and coworkers[85] and $CD103^+CD4^+$ BALF cells were characterized in different interstitial lung diseases by Braun et al[86]. Low CD103 expression on BALF $CD4^+$ T cells was indicative for the diagnosis of pulmonary sarcoidosis versus other interstitial lung diseases in a study by Kolopp-Sarda et al[21].

Interestingly, a recently published report underscores the role of integrin expression on T cells in pulmonary inflammation. Luzina et al. demonstrated that the expression of integrins αVβ3 and/or αVβ5 on pulmonary T lymphocytes may regulate the extent of lymphocytic infiltration and the degree of pulmonary fibrosis and that such expression may be a driving force contributing to prolonged T lymphocyte infiltration and/or connective tissue accumulation in the lungs[87].

**Regulatory T cells**

Tolerance to self antigens is an active process that has a central component and a peripheral component. Central tolerance involves the deletion of autoreactive clones during thymocyte development, whereas peripheral tolerance is achieved largely through three mechanisms: clonal deletion, anergy, and suppression. Of these three mechanisms, only suppression has a dedicated set of T cells generated for the specific purpose of controlling the responses of other T cells. This set T cells, referred to as regulatory T cells, is actually comprised of several subsets, and can be broadly subdivided into two groups: (i) cells that originate from the thymus, referred to as 'naturally occurring Tregs' (nTregs), and (ii) Tregs that have been induced in the periphery, also called 'adaptive Tregs'. Natural

occurring regulatory T cells are characterized by the expression of CD25 and the forkhead family transcription factor Foxp3 (forkhead box P3). Once activated, nTregs are able to suppress T cell proliferation and cytokine production as well as antigen-presenting cell function. Foxp3-expressing $CD4^+$ regulatory T cells play a major role in the control of immune responses against self and exogenous antigens[88,89]. Recently, Ono et al. found that Foxp3 physically interacts with the transcription factor acute myeloid leukemia 1/Runt-related transcription factor 1 (AML1/Runx1), thereby preventing IL-2 and interferon (IFN)-γ production by Tregs while inducing Treg-cell-associated molecules and suppressive activity[90].

Deficient nTregs, functional or in number, have been reported in different lung diseases[91-93]. Contrasting results have been found in sarcoidosis. $Foxp3^+$ $T_{reg}$ cells in active sarcoidosis have been found increased but functionally impaired and unable to suppress granuloma formation *in vitro*[94]. In contrast, Idali and colleagues showed decreased expression of regulatory T cell genes in peripheral blood and lungs of sarcoidosis patients[95].

**iNKT cells**

Invariant natural killer T (iNKT) cells are cells of the innate immune system that influence $T_{Helper}$ cell differentiation. iNKT cells are a CD1d-restricted T cell population that expresses an invariant T cell receptor α-chain by using a Vα14-Jα18 rearrangement in mice and a Vα24-Jα18 rearrangement in humans. The presentation of pathogen-derived or endogenous glycolipid antigens by CD1d-expressing antigen-presenting cells (APCs) elicits the iNKT cells response and can support $T_H1$ responses by activating NK cells which secrete IFN-γ, thus facilitating dendritic cell (DC) maturation and IL-12 production[96]. iNKT cells may favor $T_H2$ cell differentiation by producing IL-4 and IL-13 under different inflammatory conditions[97,98].

Invariant NKT cells are associated with asthma[99], with tuberculosis[100] and with COPD in mice[101]. Several groups have published on iNKT cells in pulmonary sarcoidosis[102-104]. Kobayashi and colleagues found a reduction in both Vα24 NKT cell numbers and IFN-γ production in peripheral blood mononuclear cells (PBMC) and pulmonary granuloma lesions of non-remitting, but not remitting, sarcoidosis patients. Their results suggested a role for Vα24 NKT cells in the disease progression of sarcoidosis[103]. Consistent with Kobayashi et al., Ho and colleagues showed that CD1d-restricted NKT cells were absent or greatly reduced in peripheral blood from all patients with sarcoidosis, except those with Löfgren's syndrome[102]. The authors concluded that loss of CD1d immune regulation could explain the amplified and persistent T cell activity that characterizes sarcoidosis. Regarding NKT cells in granulomas of patients with sarcoidosis, the results are conflicting[102-104].

Non-remitting sarcoidosis may be associated with inappropriate iNKT cell numbers and function. Insufficient iNKT cell response to Mycobacteria leading to a dysfunctional $T_H1$ response might explain persistent T cell activity sarcoidosis.
Despite the interesting results found in various lung diseases, iNKT cells were not investigated in lungs of 'true' healthy controls.

## 2.2 NK cells

Natural killer (NK) cells are effector lymphocytes of the innate immune system. They are involved in the continuous surveillances and early defenses against "nonself" allogenic or autologous cells that underwent cell transformation induced by pathogen infection or tumor proliferation. In order to distinguish normal cells from stressed or aberrant cells, NK cells express a variety of activating and inhibitory receptors that regulate their cytotoxicity. The inhibitory receptors mainly focus on major histocompatibility complex (MHC) class I molecules and fall into different families. The killer cell immunoglobulin-like receptors (KIRs) or leukocyte immunoglobulin-like receptors primarily recognize human leukocyte antigen (HLA)-A, -B, -C or -G molecules, whereas the C-type lectin-like receptor dimer CD94/NKG2A binds HLA-E when loaded with HLA class I-derived peptides[105]. As well as having a cytotoxic function, NK cells have the capacity to secrete several cytokines and chemokines that are important in the induction of antigen-specific immune response and in the maturation of professional antigen-presenting cells such as dendritic cells[105].
In sarcoidosis patients, an accumulation of $CD56^{bright}CD94^{high}KIR^{low}$ NK cells with the capacity to produce large amounts of cytokines such as IFN-γ and TNF-α but to have less cytotoxic activity, was found in BALF compared to PB and compared to BALF of healthy controls[106]. These NK cells may contribute to the local ongoing immune response by secreting $T_H1$ cytokines, which exaggerate the inflammatory response in the lower respiratory tract of sarcoidosis patients[107]. In addition, Agostini et al. demonstrated an increased *in vitro* cytotoxic activity by NK cells in peripheral blood of patients with active sarcoidosis. In contrast, the small proportion of NK cells found in BALF was almost inactive[108]. Finally, Tutor-Ureta et al. showed that increased percentages of NK cells in the bronchoalveolar lavage fluid from patients with sarcoidosis are associated with a poor outcome and a higher probability to need steroids treatment[109].

## 2.3 Monocytes/macrophages

Activation of monocyte/alveolar macrophage cell lineage is thought to play an important role in the pathogenesis of sarcoidosis. The central core of the sarcoid granuloma is made up of monocyte/macrophages in various states of activation, epithelioid cells and multinucleated giant cells. Recruitment of circulating monocytes to the local inflammatory lesion is regulated by chemotactic and activating factors actively secreted by tissues involved with sarcoidosis. Monocyte chemoattractant protein-1 (MCP-1), monocyte inflammatory protein-1α (MIP-1α), and RANTES have been detected in the tissues and serum of patients with sarcoidosis, suggesting that monocytes are attracted by these relevant chemotactic stimuli[110-112]. In addition, macrophages in sarcoid lesions express the calcium-binding protein calgranulin B (S100A9), an antigen shared by granulocytes and circulating monocytes but only by a minimal proportion of tissue macrophages. This observation suggests that macrophages in sarcoid granuloma are recruited from circulating monocytes[10].

Proinflammatory monocytes are activated circulating monocytes that might be related to the initial events that lead to granuloma formation. Proinflammatory monocytes co-express CD14, CD16 and CD69. CD14 is part of the TLR4 membrane receptor complex and expressed on all monocytes. Peripheral blood CD16 (a low affinity Fc-γ type III receptor) positive monocytes have been shown to expand in different pathological conditions, such as cancer, asthma, sepsis, human immunodeficiency virus infection, and AIDS progression (recently reviewed in ref.[113]) suggesting an activated state of the monocytes. Okamoto et al.[114] have shown increased percentages of circulating CD16 positive monocytes in sarcoidosis. In contrast to $CD14^{++}CD16^{-}$ monocytes, $CD14^{+}CD16^{+}$ cells are known to produce high levels of TNF-α and show higher antigen-presenting capacities[115]. Interestingly, Landsman et al. showed that murine lung macrophages exclusively generate from $Gr\text{-}1^{low}CX3CR1^{high}$ blood monocytes[116], the equivalent of $CD14^{+}CD16^{+}$ monocytes in humans and the presence of this distinct monocyte phenotype in newly diagnosed sarcoidosis might suggest they are involved in the formation of lung granulomas.

In sarcoidosis, alveolar macrophages reveal an altered phenotype, enabling these cells to present antigen with high efficiency. Several groups found that alveolar macrophages from patients with active sarcoidosis, and not from patients with inactive disease, disclosed an increased antigen presenting capacity compared with control alveolar macrophages[117-120]. Furthermore, these findings are corroborated by the fact that there is an increased expression of various costimulatory molecules on alveolar macrophages from

patients with sarcoidosis. These include CD154 (ligand for CD40), CD72 (ligand for CD5), CD80, CD86 (both ligands for CD28), and CD153 (CD30L). In addition, molecules also involved in cell adhesion, such as CD54 (ICAM), CD11a, CD11b, and CD11c have been shown to be involved in antigen presentation and are increased in sarcoidosis[66,69,121,122].
Regarding the role of dendritic cells in sarcoidosis, few data are available. Munro et al. showed that dendritic cells are primarily located in the paracortical zone and not in the hilar lymph nodes or in lesions of lung parenchyma[123]. Consistent, Gibejova et al. could not detect CCL20 mRNA (LARC, MIP-3α) in BAL cells from patients with sarcoidosis, a chemokine attracting immature dendritic cells[124]. In contrast, in extra pulmonary sarcoidosis such as in skin, dendritic cells (interdigitating cells and Langerhans' cells) were consistently associated with granulomas.

## 2.4 Granulocytes

In other interstitial lung disorders, such as idiopathic pulmonary fibrosis (IPF), a marked increase in polymorphonuclear neutrophils (PMNs) and/or eosinophils was reported to adversely affect prognosis, whereas elevated lymphocyte counts were found to be more likely to be associated with a good response to corticosteroid treatment. In sarcoidosis, elevated neutrophil and eosinophil counts in BALF are associated with poor prognosis[19,20,109,125,126]. Moreover, peripheral blood eosinophilia occurs frequently in sarcoidosis. However, there appears to be no association between peripheral blood eosinophil count and presence of lung tissue eosinophils[127]. The exact role of neutrophils and eosinophils in the pathogenesis and prognosis of patients suffering from sarcoidosis needs further investigation. In this regard Fortunati et al. compared the BALF neutrophil phenotype between healthy controls and sarcoidosis patients. They demonstrated a similar activated neutrophil phenotype in the lung of normal individuals and patients. Their finding indicated that homing of neutrophils to the lung *per se* leaded to this phenotype and did not necessarily reflect inflammation in the tissue[128].

**BALF cell profiles: normal values**

BALF cell counts usually show variations in the white blood cell count differential that differ from patterns found in normal subjects. These patterns tend to reflect inflammatory cell profiles in affected lung tissues[129].

In 1974, the first paper detailing BAL dealt with normal values, as the authors selected normal subjects and patients undergoing fibreoptic bronchoscopy (FOB) for ‘‘evaluation of intrathoracic lesions’’. True healthy controls were included in the study of van den Bosch and colleagues performed in our hospital in 1983[130].

In the ongoing pursuit to characterize normal lung values, a group of healthy individuals underwent bronchoalveolar lavage in St. Antonius Hospital in 2007-2009. The materials of this study are used as control samples in scientific research including for chapters of this thesis. The appendix presents normal values of peripheral blood and bronchoalveolar lavage samples of 45 non-atopic healthy subjects between 18-65 years.

# 3. AIM OF THE THESIS

The underlying hypothesis of this thesis is that markers of cell activation are a useful tool for diagnostic and prognostic evaluation, and for determination of disease activity or severity in sarcoidosis. Furthermore, we hypothesized that genetic variation in genes encoding proteins related to cell activation bear influence on sarcoidosis disease susceptibility or disease course.

# 4. OUTLINE OF THE THESIS

**Chapter 2** describes the expression of different activation markers on T lymphocytes subsets in different ILD and in healthy controls. The ability of distinct expression profiles of cell activation markers to discriminate between different clinical phenotypes of sarcoidosis patients (according to chest radiography at presentation) is evaluated. The proportion of blood $CD8^{+}CD28^{null}$ cells in sarcoidosis might be a new biomarker for sarcoidosis disease severity.

In **chapter 3**, a novel diagnostic marker for sarcoidosis is evaluated. The expression of the integrin CD103 is analyzed in BALF samples from patients with different ILD. A combination of the relative PB/BAL $CD4^{+}/CD8^{+}$ ratio and the $CD103^{+}CD4^{+}/CD4^{+}$ ratio is proposed as a diagnostic marker for pulmonary sarcoidosis.

**Chapter 4** presents the analysis of a novel sarcoidosis susceptibility gene in Dutch sarcoidosis patients: the *ITGAE* gene, encoding the $\alpha_E$ unit CD103. Different pulmonary disease phenotypes (according to pulmonary disease outcome) are compared in terms of SNP frequency distribution. Functional effects of the promoter SNP associated with sarcoidosis are analyzed comparing messenger RNA and protein levels in the genetic variants.

**Chapter 5** evaluates the differences in activation status between CD103 positive and CD103 negative BALF lymphocytes. BALF lymphocytes of healthy controls and sarcoidosis patients with different chest radiographic stages are analyzed. The percentage of BALF CD103 positive lymphocytes at presentation may be prognostic for parenchymal involvement after > 4 years follow-up.

**Chapter 6** demonstrates a highly activated monocyte phenotype in sarcoidosis patients compared to healthy subjects. Eight sarcoidosis patients were included in follow-up analysis. The added value of monocyte activation is illustrated by comparison with well established markers for disease activity.

In **chapter 7**, a susceptibility gene for sarcoid inflammation is discussed. Genotype frequencies of six SNPs in the gene encoding for IL7Rα (*IL7R*) are compared between Dutch sarcoidos*e*s patients and healthy subjects.

**Chapter 8** presents a review of regulatory T cell subsets in sarcoidosis and introduces new data of iNKT and Foxp3$^{+}$ Treg cells from peripheral blood and BALF of healthy subjects.

**Chapter 9** Summary and concluding remarks.

**Appendix** presents new normal values of cell differentiation and phenotypic characterization of BALF and blood samples from healthy individuals who underwent BAL in 2007-2009 in St. Antonius Hospital.

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# 2

## T cell activation profiles in different granulomatous interstitial lung diseases – a role for $CD8^{+}CD28^{null}$ cells?

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## Abstract

Lymphocytes play a crucial role in lung inflammation. Different interstitial lung diseases may show distinct lymphocyte activation profiles. The aim of this study was to examine the expression of a variety of activation markers on T lymphocyte subsets from blood and bronchoalveolar lavage fluid (BALF) of patients with different granulomatous interstitial lung diseases and healthy controls.

Bronchoalveolar lavage cells and blood cells from 23 sarcoidosis patients, 7 patients with hypersensitivity pneumonitis, and 24 healthy controls were analyzed. Lymphocyte activation status was determined by flow cytometry. Lymphocytes were stained with antibodies against CD3, CD4, CD8, CD25, CD28, CD69, VLA-1, VLA-4 and HLA-DR.

In general, CD28, CD69 and VLA-1 expression on BALF $CD4^+$ lymphocytes and HLA-DR expression on BALF $CD8^+$ lymphocytes was different in patients with hypersensitivity pneumonitis and sarcoidosis patients with parenchymal involvement. This BALF lymphocyte phenotype correlated with Dl*co* values across ILD ($R^2$ = 0.48, p = 0.0002). In sarcoidosis patients, $CD8^+CD28^{null}$ blood lymphocytes correlated with lower Dl*co* values (r = -0.66, p = 0.004), chronic BALF lymphocyte activation phenotype ($R^2$ = 0.65, p < 0.0001), radiographic staging (stage I vs. II and higher, p = 0.006) and with the need for corticosteroid treatment (p = 0.001).

Higher expression of CD69, VLA-1 and HLA-DR and lower expression of CD28 on BALF lymphocytes suggests prolonged stimulation and chronic lymphocyte activation in patients with ILD. In sarcoidosis, blood $CD8^+CD28^{null}$ cells might be a new biomarker for disease severity but needs further investigation.

## Introduction

Many acute and chronic lung disorders with variable degrees of pulmonary inflammation and fibrosis are collectively referred to as interstitial lung diseases (ILD). The major abnormality in ILD is disruption of the lung parenchyma, progression of the diseases results in impaired oxygen transfer and scarring within the lung. Interstitial lung diseases are classified into 4 categories: (1) ILD of known association, (2) granulomatous ILD, (3) other and often rare ILD, and (4) the idiopathic interstitial pneumonias (IIPs). The most common ILD in the western world is sarcoidosis, with an annual incidence of 10 to 25 per 100,000 persons in Western Europe and the United States[1,2].

Sarcoidosis is a multi-system granulomatous disease characterized by an accumulation of $T_{Helper}1$ ($T_H1$) lymphocytes in the affected organs, commonly the lung. The disease course is variable. Sarcoidosis is often acute and self-limiting; in some individuals it becomes chronic, occasionally flaring up over a period of many years[3]. The majority of sarcoidosis patients undergo spontaneous remission; approximately 10-15 percent of the sarcoidosis cases develop parenchymal fibrosis[4].

Another common granulomatous ILD is hypersensitivity pneumonitis (HP). HP is due to a combined type III allergic reaction with the formation of precipitins and a type IV lymphocyte reaction with a granulomatous inflammation. HP is pathologically characterized by non-caseating granulomas in the interstitium, bronchiolitis, and organizing pneumonia, with or without interstitial fibrosis[5].

Most clinical manifestations of ILD are secondary to the direct effect of the accumulation of activated immune cells in involved tissues, notably T cells, macrophages, or neutrophils[6,7]. Activated T lymphocytes play an important role in the pathophysiology of ILD[6]. T lymphocyte activation involves sequential expression of different, well-defined activation markers. For instance, CD25 and CD69 are expressed *in vitro* within 24 hours after stimulation while very late antigen-1 (VLA-1) and very late antigen-4 (VLA-4) can be detected after several days to weeks[8-10]. CD69 is an early membrane receptor transiently expressed upon lymphocyte activation and selectively expressed in chronic inflammatory infiltrates and at sites of active immune responses *in vivo*[11]. CD28 is a co-stimulatory molecule and constitutional expressed by T cells. Wahlström et al. have shown differential expression of activation markers between blood and BALF lymphocytes in pulmonary sarcoidosis[12]. Highly activated T cells were found in BALF of patients, in particular $CD4^{+}$ T cells that have a preferential usage of the TCR AV2S3 gene segment[13,14]. In addition, increased expression of very late antigen-1 (VLA-1), VLA-4 and VLA-5 has been demonstrated on tissue-infiltrating T cells from the lungs of sarcoidosis patients, especially in patients with active disease[15,16]. Moreover, we and others found high percentages of

chronically activated BALF CD103$^{+}$ intraepithelial lymphocytes (IEL) in fibrotic lung diseases[8,17,18]. However, a detailed comparison of lymphocyte activation profiles between different ILD has not been made yet.

In order to better characterize the lymphocyte phenotype in sarcoidosis and hypersensitivity pneumonitis we examined the expression of a comprehensive set of activation markers on T lymphocyte subsets obtained by bronchoalveolar lavage and in peripheral blood. Correlations of T lymphocyte marker profiles with lung function parameters and radiographic staging were analyzed to understand the relation between lymphocyte activation phenotype and disease severity.

## Subjects & Methods

*Subjects*

*Sarcoidosis (SARC) patients.* Twenty-three patients presenting to our department because of symptomatic sarcoidosis with > 15% lymphocytes in bronchoalveolar lavage were included in this study (median age, 42 [range: 24-65]; male/female, 16/7). The diagnosis of sarcoidosis was established on the basis of clinical findings and histological evidence of non-caseating epithelioid granulomas and after exclusion of other known causes of granulomatosis in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis[19]. In 6 patients, the diagnosis was made without biopsy proof because these patients presented with the classic symptoms of Löfgren's syndrome, namely fever, erythema nodosum, arthralgia and bilateral hilar lymphadenopathy. All patients with Löfgren's syndrome were confirmed as TB-negative. All patients were steroid naïve at time of inclusion in the study. Pulmonary disease severity at presentation was evaluated by chest radiography. In brief, this comprises five stages: stage 0, normal, stage I, bilateral hilar lymphadenopathy (BHL), stage II, BHL and parenchymal infiltration, stage III, parenchymal infiltration without BHL, stage IV, irreversible fibrosis with loss of lung volume. Distribution of chest radiographic stages at presentation showed that 14 patients presented with stage I (including 6 patients diagnosed with Löfgren's syndrome), 4 patients presented with stage II, 4 patients presented with stage III, and 1 patient presented with stage IV. For further analysis, patients were divided into a group presented without parenchymal involvement (radiographic stage I, n = 14) and a group presented with parenchymal involvement (radiographic stage II and higher, n = 9). Of 21 patients, data of corticosteroid treatment within 2 years after presentation were available. Seven

patients received no medication while 14 patients needed corticosteroids within 2 years after presentation (Table 4)[29].

*Hypersensitivity pneumonitis (HP) patients.* Seven patients presenting to our department because of symptomatic hypersensitivity pneumonitis were included in this study (median age, 64 [range: 41-75]; male/female, 4/3). All of them experienced respiratory symptoms including dyspnoea or cough, with or without systemic symptoms such as fever and arthralgia after exposure to birds. Additionally, they all had precipitating antibodies to birds and a lymphocytic alveolitis, *i.e*. > 15% lymphocytes in BALF. None of the patients received corticosteroids, nor had they within the previous three months.

*Healthy controls.* Twenty-four healthy subjects were included in this study (mean age, 30 [range 20-64]; male/female, 9/15). All healthy controls had normal chest radiography, normal lung function ($FEV_1$, FVC) and none had any sign of pulmonary disease.

The clinical and demographic data of the study subjects are summarized in Table 1.

Verbal and written consent was obtained from all subjects, and authorization was given by the Ethics Committee of the St. Antonius Hospital, Nieuwegein.

*Pulmonary function tests*

Pulmonary function tests were performed at presentation. Forced vital capacity (FVC), forced expiratory volume in 1 second ($FEV_1$) and carbon monoxide diffusing lung capacity (D*lco*) were used to assess the presence of lung function impairment at presentation of the disease. All lung function parameters are expressed as percent predicted values. FVC and $FEV_1$ were calculated from volumes in liters and adjusted to BTPS (body temperature, ambient pressure, saturated with water vapor).

*Bronchoalveolar lavage*

BAL was performed during flexible fiberoptic bronchoscopy at the time of the diagnosis according to standardized and validated procedure previously described[18]. The procedure involved detailed explanation to the patient, premedication (0.5 mg atropine s.c., 20 mg codeine p.o.), and local anesthesia of the larynx and lower airways (0.5% tetracaine in the oropharynx, 8 cc 0.5% tetracaine in lower airways). Transcutaneous oxygen saturation was monitored continuously by oximeter with a finger probe. BAL was performed, preferably in the right middle lobe, with four 50-ml aliquots of sterile isotonic saline solution (37 °C). The aspirated lavage fluid from the first 50-ml aliquot was kept apart and excluded from further analysis. The BAL fluid (BALF) recovered from the three subsequent aliquots was collected in a siliconized specimen trap and kept on ice. BALF was filtered through nylon gauze and centrifuged (10 min at 400 x g at 4 °C).

The cell pellet was washed twice, counted, and resuspended in minimal essential medium/RPMI 1640 (Gibco; Grand Island, NY), supplemented with 0.5% bovine serum albumin (Organon; Teknika; Boxtel, The Netherlands). Cells were counted in a Bürker chamber. Cell viability was determined by Trypan blue exclusion. Smears for cell differentiation were prepared by cytocentrifugation (Shandon; Runcorn, UK). Cell differentiation was performed on cytospinslide after staining with May-Grünwald-Giemsa (Merck; Darmstadt, Germany), at least 2x 500 cells were counted.

*Flow cytometry*

Fifty µl of ethylenediamine tetra acetic acid (EDTA) blood and 50 µl BALF (at least 500.000 cells) were prepared for four-color flow cytometry. BALF and blood samples were incubated for 15 min at room temperature with 10 µl of the following mAbs: anti-CD3 FITC, anti-CD4 PerCP-Cy5.5 and anti-CD8 APC combined with either anti-CD25 PE, anti-CD28 PE, anti-CD69 PE, anti-VLA-1 PE, anti-VLA-4 PE or anti-HLA-DR PE, all from BD Biosciences (Alphen aan den Rijn, The Netherlands). Immunofluoresence was measured by flow cytometry (FACSCalibur, Becton Dickinson, Alphen aan den Rijn, The Netherlands). Lymphocytes were defined based on FSC/SSC characteristics and expression of the activation markers was evaluated on $CD4^+$ and $CD8^+$ T cells in BALF and PB from HC and ILD patients (Figure 1). Isotype-matched negative control antibodies stained less than 1% of $CD4^+$ and $CD8^+$ BALF and PB cells, and were used to set markers to delineate positive and negative cells. At least 10,000 lymphocyte events were analyzed with FlowJo software (Tree Star, Inc., Ashland, USA).

*Statistical analysis*

Data are expressed as median ± range or as stated otherwise. Multiple groups were compared performing Kruskal-Wallis with post-hoc test by Dunn's. The Mann-Whitney U test was used to compare BALF and PB cell populations. Correlations between different variables were determined using the Spearman rank coefficient. To analyse correlations between an expression profile of multiple activation markers and other parameters, individual values of the different activation markers were ranked and the sum of ranks was correlated to lung function or the percentage of PB $CD8^+CD28^{null}$ cells. Fisher's exact test was used to test the prognostic value of $CD8^+CD28^{null}$ cells for the need for medication. Considering multiple comparisons, a p value of less than 0.01 was regarded as significant. The statistical evaluation of our data was performed using SPSS 15.0 (SPSS Inc; Chicago, IL, USA) and Graphpad Prism v.5 (Graphpad Software, Inc., San Diego, USA) software packages.

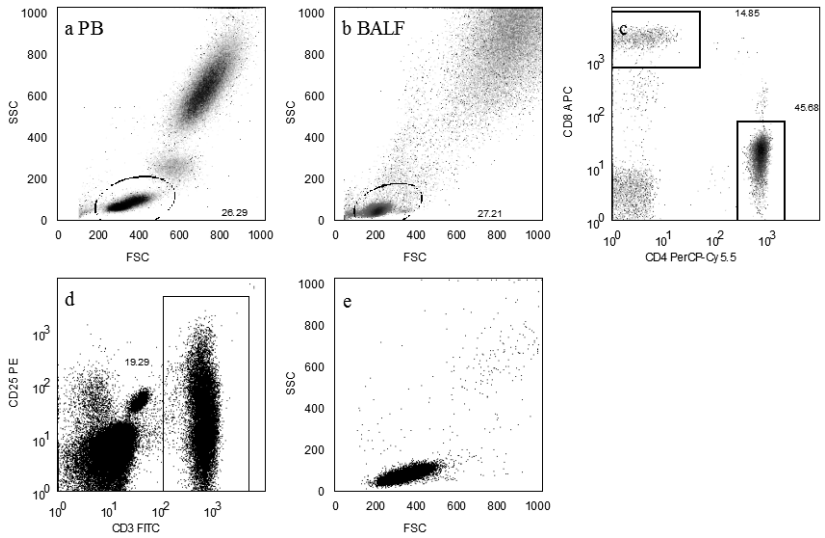


**Figure 1** Gating strategy. The expression of activation markers was analyzed on $CD4^{+}$ and $CD8^{+}$ cells within the lymphocyte gate (panel a, b and c). Back gating of gated cell populations double stained for TCR marker CD3 and an activation marker into the SSC-FSC plot, showed that the gated cells fell within the SSC-FSC lymphocyte gate hence no cells were omitted using the original lymphocyte gate (panel d and e).

**Table 1** Characteristics of the study population

| Characteristics | SARC (*n* = 23) | HP (*n* = 7) | HC (*n* = 24) |
|---|---|---|---|
| Age,[a] yr | 41.2 [24 - 65] | 60.3 [41 - 75] | 30.0 [20 - 64] |
| Male/female gender[b] | 16/7 | 4/3 | 9/15 |
| Smoker/non-smoker | 1/19 | 1/5 | 7/17 |
| *Lung function parameters* | | | |
| $FEV_1$ % pred (*n* = 18/6/6/21) | 93 (59-115)[d] | 87 (52-142)[d] | 107 (79-130) |
| FVC % pred (*n* = 18/6/6/21) | 97 (65-117)[d] | 108 (56-134)[e] | 110 (81-135) |
| Dl*co* % pred (*n* = 18/6/5) | 82 (44-105)[f] | 48 (24-83) | ND |
| Chest radiographic stages I/II/III/IV | 14/4/4/1 | | |
| Löfgren's syndrome | 6 | | |
| *Organ involvement*[b c] | | | |
| Pulmonary involvement | 100 (17) | | |
| Extrapulmonary involvement | 35 (6) | | |
| Kidney | 24 (4) | | |
| Skin | 12 (2) | | |
| Extrathoracic lymph node | 6 (1) | | |
| Neurologic | 6 (1) | | |
| Eyes | 6 (1) | | |
| Parotid/salivary | 6 (1) | | |
| Muscles | 6 (1) | | |
| Bone/joints | 6 (1) | | |

SARC, sarcoidosis; HP, hypersensitivity pneumonitis; HC, healthy controls. ND, not determined. [a]Values are given as the mean [range]. [b]Values are given as % (No.).[c]Based on the ACCESS assessment instrument[20]. Organ involvement data are from non-Löfgren's sarcoidosis patients (n = 17).
[d]Different from HC, $p < 0.001$
[e]Different from HC, $p < 0.01$
[f]SARC vs. HP, $p = 0.0007$

## Results

Total and differential cell counts for BALF, PB and $CD4^+/CD8^+$ ratios are presented in Table 2.

*Expression of activation markers on lymphocytes from BALF vs. PB*

In BALF, highly activated T lymphocyte subsets were found both in ILD patients and in healthy controls. CD69, VLA-1 and HLA-DR expression was higher on $CD4^+$ and $CD8^+$ BALF cells compared to corresponding PB lymphocytes ($p < 0.0001$). VLA-4 was increased only on BALF $CD4^+$ cells ($p < 0.0001$). $CD4^+CD25^+$ cells were lower in BALF vs. PB in SARC ($p < 0.0001$). In contrast, in healthy controls CD25 positive cells were higher in $CD8^+$ BALF cells ($p < 0.0001$). Finally, CD28 expression was decreased on BALF vs. PB lymphocytes in HC ($p < 0.0001$).

**Table 2** Total and differential cell counts for bronchoalveolar lavage fluid (BALF) and peripheral blood (PB) from patients and controls

| Characteristics | | SARC (*n* = 23) | HP (*n* = 7) | HC (*n* = 24) |
|---|---|---|---|---|
| Total BALF cells x $10^4$/ml | | 31.1 (9.9-65.0)[a] | 26.2 (5.1-102.9)[c] | 10.1 (4.3-16.3) |
| *Cell types: % of total BALF cells : x $10^4$/ml* | | | | |
| Alveolar macrophages | % | 65.2 (13.4-84.6)[a] | 67.6 (8.5-79.7)[a] | 87.1 (56.3-96.8) |
| | x $10^4$/ml | 16.0 (6.8-34.5)[a] | 9.8 (4.0-21.2) | 8.2 (3.9-15.8) |
| Lymphocytes | % | 47.0 (15.0-83.0)[a] | 37.0 (31.0-80.0)[a] | 9.6 (2.3-39.9) |
| | x $10^4$/ml | 11.2 (1.5-54.0)[a] | 9.9 (1.8-82.3)[a] | 0.9 (0.3-5.4) |
| Neutrophils | % | 1.1 (0.1-2.4)[c] | 1.9 (0.5-4.7) | 1.6 (0.1-8.1) |
| | x $10^4$/ml | 0.3 (0.02-1.0) | 0.8 (0.07-2.0) | 0.2 (0.0-0.8) |
| Eosinophils | % | 0.5 (0.1-4.1) | 0.7 (0.1-3.0) | 0.3 (0.0-4.1) |
| | x $10^4$/ml | 0.1 (0.01-0.7)[b] | 0.3 (0.01-0.9)[b] | 0.03 (0.0-0.4) |
| BALF CD4/CD8 cell ratio | | 3.6 (0.5-25.2)[a] | 0.8 (0.6-6.3) | 1.1 (0.2-11.0) |
| *Cell types: % of total PBL cells : x $10^6$/ml* | | | | |
| Total PB cells x $10^6$/ml | | 5.6 (3.2-14.4)[d] | 7.7 (6.5-11.2) | 5.8 (3.2-9.0)§ |
| Lymphocytes | % | 23.0 (11.0-44.0)[a] | 25.0 (20.0-36.0) | 33.5 (20.6-49.9) |
| | x $10^6$/ml | 1.3 (0.4-2.5) | 1.8 (1.4-3.2)[e] | 1.9 (1.3-2.8)[e] |
| PB CD4/CD8 cell ratio | | 1.2 (0.4-3.3)[c] | 2.0 (0.6-4.0) | 1.8 (0.9-5.8) |

SARC, sarcoidosis; HP, hypersensitivity pneumonitis; HC, healthy controls; BALF, bronchoalveolar lavage; PB, peripheral blood leukocytes. Numbers are presented as median (range).
Significantly different from HC; [a]$p < 0.001$, [b]$p < 0.01$, [c]$p < 0.05$
Significantly different from HP; [d]$p < 0.05$
Significantly different from SARC; [e]$p < 0.01$

*Expression of activation markers on BALF lymphocytes in ILD patients and healthy controls*

Comparisons of BALF cells between groups showed significantly higher expression of CD25 (IL-2 recepter alpha chain) on BALF $CD4^+$ and $CD8^+$ cells in HC vs. SARC. Moreover, the costimulatory molecule CD28 was expressed by a lower percentage of BALF $CD4^+$ lymphocytes in HP vs. HC and SARC. Increased expression of CD69 and VLA-1 was found on BALF $CD4^+$ lymphocytes in HP vs. HC and SARC patients. HLA-DR expression was increased in HP compared to SARC patients on BALF $CD8^+$ lymphocytes (Table 3).

**Table 3** Expression of activation markers on BALF and PB $CD4^+$ and $CD8^+$ lymphocytes from ILD patients and healthy controls

| | SARC (*n* = 23) | HP (*n* = 7) | HC (*n* = 24) | SARC (*n* = 23) | HP (*n* = 7) | HC( *n* = 24) |
|---|---|---|---|---|---|---|
| Markers | Bronchoalveolar lavage cells | | | Peripheral blood cells | | |
| | | | $CD4^+$ lymphocytes | | | |
| CD25 | 17.6 (7.5-39.5) | 19.7 (15.6-75.4) | 48.8 (28.5-75.0)[a] | 52.4 (22.7-74.9) | 58.6 (44.0-63.5) | 44.4 (30.8-81.6) |
| CD28 | 97.4 (73.9-99.9)[b] | 57.3 (38.9-93.1) | 94.4 (80.4-99.4)[b] | 99.4 (73.6-100) | 95.9 (90.5-99.8) | 99.9 (80.5-100)[a b] |
| CD69 | 69.8 (37.1-94.8)[b] | 89.4 (80.5-95.7) | 66.7 (44.8-79.3)[b] | 2.7 (0.3-13.8) | 1.2 (0.8-4.8) | 2.2 (1.2-16.5) |
| VLA-1 | 25.6 (7.7-71.5)[b] | 55.9 (35.5-69.4) | 27.9 (11.3-54.2)[b] | 5.5 (1.8-17.2) | 5.0 (3.1-12.5) | 2.9 (0.6-17.5)[a] |
| VLA-4 | 99.2 (97.3-99.8) | 99.6 (97.2-99.9) | ND | 86.7 (66.7-93.5) | 86.2 (78.8-89.1) | ND |
| HLA-DR | 93.4 (84.5-98.7) | 93.9 (91.7-97.4) | ND | 19.0 (7.2-64.7) | 15.6 (10.6-21.4) | ND |
| | | | $CD8^+$ lymphocytes | | | |
| CD25 | 6.1 (1.7-20.5) | 7.5 (5.3-46.1) | 19.3 (7.8-40.3)[a] | 8.4 (1.3-26.1) | 11.8 (4.4-22.2) | 7.1 (3.5-29.5) |
| CD28 | 57.0 (27.5-85.2) | 24.1 (8.9-76.7) | 64.2 (39.3-80.1) | 60.2 (10.0-96.2) | 69.5 (19.3-100) | 87.6 (62.3-96.4)[a] |
| CD69 | 86.4 (37.0-97.3) | 97.3 (79.8-98.8) | 85.1 (75.0-94.2) | 5.1 (0-21.2) | 1.0 (0.3-3.3)[a] | 2.9 (1.6-23.1) |
| VLA-1 | 75.6 (28.3-96.3) | 94.9 (68.9-97.2) | 80.5 (53.6-96.0) | 5.1 (0.6-36.8) | 5.4 (1.8-10.3) | 3.3 (0.9-29.8) |
| VLA-4 | 99.7 (97.2-100) | 99.9 (99.2-100) | ND | 98.4 (93.6-100) | 99.0 (98.8-99.4) | ND |
| HLA-DR | 80.4 (61.3-98.8)[b] | 96.4 (89.0-99.1) | ND | 35.3 (9.9-91.8) | 43.4 (22.7-64.0) | ND |

SARC, sarcoidosis; HP, hypersensitivity pneumonitis; HC, healthy controls; ND, not determined. Numbers are expressed as median (range).

[a]Significant difference ($p < 0.01$), compared to SARC

[b]Significant difference ($p < 0.01$), compared to HP

*Sarcoidosis clinical phenotypes*

Analysis of different clinical sarcoidosis phenotypes showed a significantly higher proportion of BALF $CD4^+$ lymphocytes expressing VLA-1 (35.9 [14.6-71.5] vs. 16.4 [7.7-34.2], $p < 0.01$) and more BALF $CD8^+$ cells expressing HLA-DR (93.0 [77.0-98.8] vs. 77.3 [61.3-95.6], $p < 0.01$) in patients with parenchymal involvement (radiographic stage II and higher) vs. patients without parenchymal involvement (radiographic stage I).

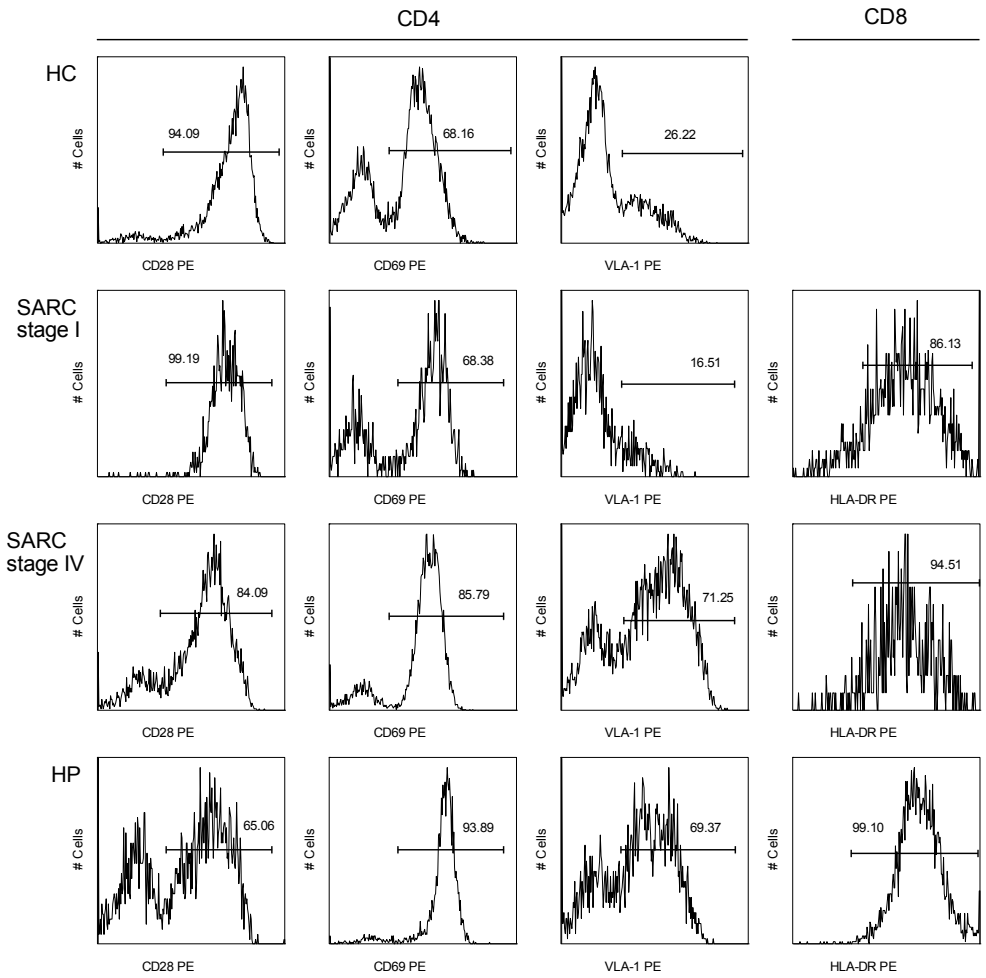


**Figure 2** Fluorescence histograms presenting CD28, CD69 and VLA-1 expression on BALF $CD4^+$ cells and HLA-DR expression on BALF $CD8^+$ cells from one representative HC, SARC stage I, SARC stage IV and HP subject.

Figure 2 illustrates a flow cytometric analysis of the chronicly activated BALF lymphocytes from one HC, SARC stage I, SARC stage IV and HP subject.

To analyse a combination of BALF $CD4^+$ and $CD8^+$ lymphocyte phenotypes, individual values of the different activation markers were ranked and the sum of ranks was compared between ILD. ANOVA revealed the highest sum for HP and the lowest sum for SARC without parenchymal involvement (radiographic stage I) ($p < 0.0001$) (Figure 3).

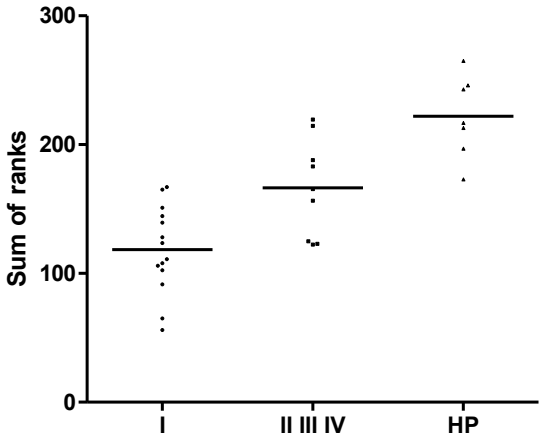


**Figure 3** Scatter diagram illustrating the sum of ranks of CD25, CD28, CD69, VLA-1 and HLA-DR expression on BALF $CD4^{+}$ lymphocytes and BALF $CD8^{+}$ lymphocytes in sarcoidosis patients without parenchymal involvement (radiographic stage I), sarcoidosis patients with parenchymal involvement (radiographic stage II and higher) and patients with hypersensitivity pneumonitis. VLA-4 expression was 100% in both BALF lymphocyte subsets in all patients, hence VLA-4 expression was excluded from sum of rank analysis (all subjects would have been assigned the same rank number, i.e. 1).

Analysis of absolute cell numbers revealed no differences between groups (data not shown). Separation of Löfgren's patients from stage I group did not reveal significant differences between Löfgren's patients and sarcoidosis patients with radiographic stage I for all comparisons (data not shown). No major differences were found between sexes or smokers and non-smokers for all comparisons in patients and controls (data not shown). Moreover, multivariate regression analysis of normally distributed markers controlled for age as a covariate and gender as fixed factor, did not significantly alter the results (data not shown).

*Correlation between BALF T lymphocyte phenotype and lung function tests in ILD*

Next, we investigated whether the presence of activated T lymphocytes in BALF correlated with lungfunction parameters in ILD. Dl*co* is the best descriptor for lung function and decreases with increasing parenchymal involvement. The combination of CD28, CD69 and VLA-1 expression on BALF $CD4^{+}$ lymphocytes and CD28 and HLA-DR expression on BALF $CD8^{+}$ lymphocytes showed a positive correlation with Dl*co* values, superior to the individual significant correlations ($R^2 = 0.48$, $p = 0.0002$).

*Expression of activation markers on PB lymphocytes from ILD patients and healthy controls*

In peripheral blood, minor differences were found between SARC, HP and HC comparing the expression of the activation markers on $CD4^{+}$ cells and $CD8^{+}$ cells (Table 3). Notably,

the percentage of CD28 positive $CD8^+$ cells was lower in both ILD compared to healthy controls, in particular in sarcoidosis patients (Table 3).

*Correlation between blood $CD8^+CD28^{null}$ cells and parameters of disease severity in sarcoidosis*

In sarcoidosis patients, significant correlations were found between the percentage of peripheral blood $CD8^+CD28^{null}$ cells (Figure 4) and Dl*co* values (r = -0.66 (CI: -0.87 - -0.26), p = 0.004) (Figure 5). Moreover, the percentage of blood $CD8^+CD28^{null}$ lymphocytes was increased in sarcoidosis patients with parenchymal abnormalities, compared to sarcoidosis patients without parenchymal abnormalities (p = 0.006) (Figure 5, inlay) and strongly correlated with BALF $CD4^+CD28^+$, $CD4^+VLA1^+$, $CD8^+CD25^+$, $CD8^+VLA\text{-}1^+$ and $CD8^+HLA\text{-}DR^+$ lymphocytes (sum of ranks, $R^2 = 0.65$, $p < 0.0001$) (Figure 6). In addition, the proportion of blood $CD8^+CD28^{null}$ cells revealed prognostic value for the need for corticosteroid treatment within 2 years after presentation (Figure 7). Seven out of ten patients with higher percentages of $CD8^+CD28^{null}$ cells (> 50%) needed corticosteroid treatment within 2 years after presentation compared to none of the eleven patients with lower $CD8^+CD28^{null}$ percentages (< 50%) (p = 0.001). Individual $CD8^+CD28^{null}$ levels and values of disease severity parameters are summarized in Table 4. Finally, the presence of blood $CD8^+CD28^{null}$ cells in sarcoidosis did not correlate with extrapulmonary organ involvement (data not shown).

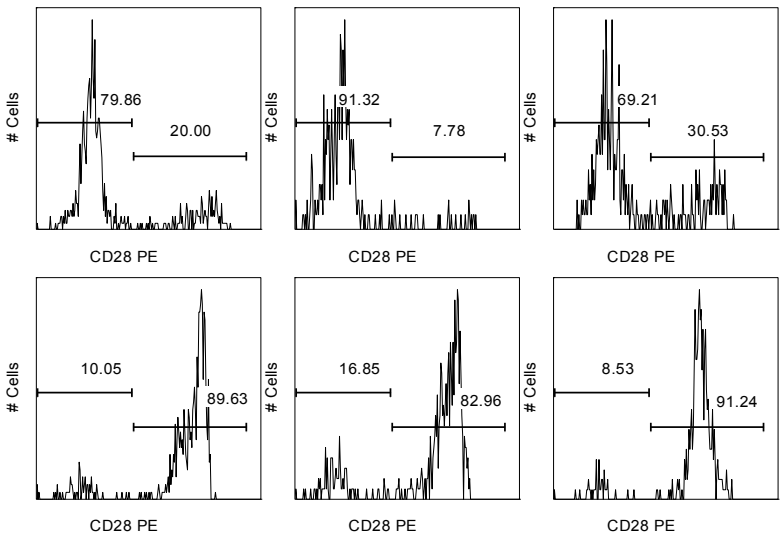


**Figure 4** Fluorescence histograms showing blood $CD8^+CD28^{null}$ and $CD8^+CD28^+$ cell populations in sarcoidosis patients (upper row) and healthy controls (lower row).

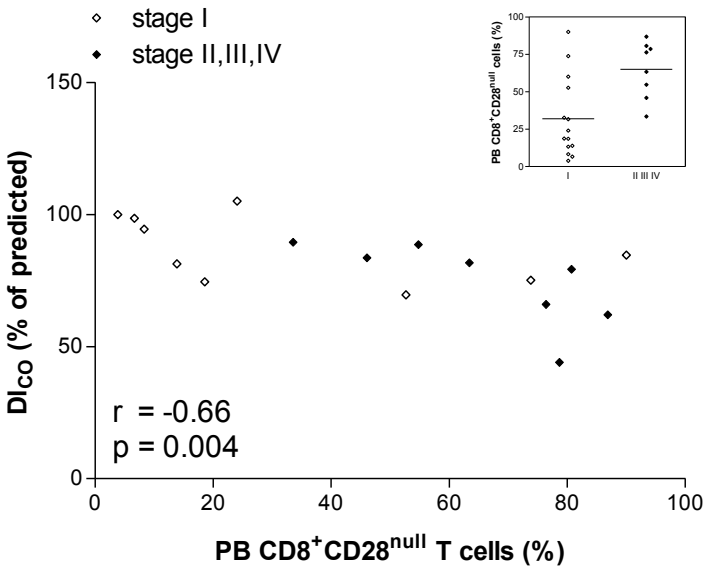


**Figure 5** Scatter diagram illustrating the correlation between the percentage of PB $CD8^{+}CD28^{null}$ T-cells and diffusion capacity (D*lco*) in 17 sarcoidosis patients. Inlay presents the percentage of PB $CD8^{+}CD28^{null}$ T-cells between sarcoidosis patients without parenchymal involvement, radiographic stage I (n = 14) and with parenchymal involvement, radiographic stage II and higher (n = 9) at presentation.

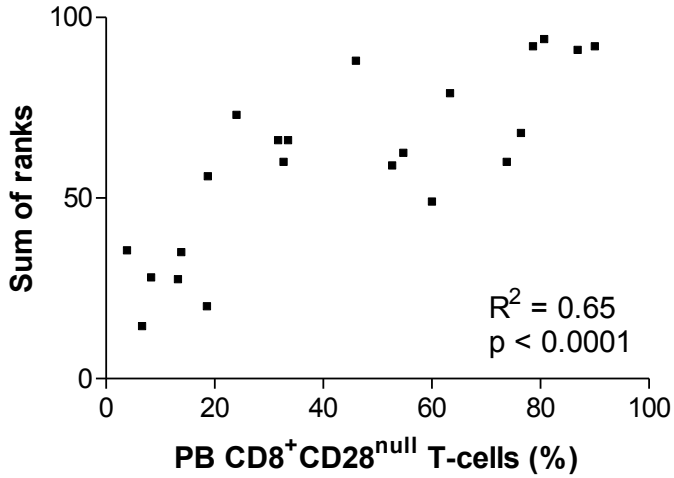


**Figure 6** Scatter diagram illustrating the correlation between the percentage of PB $CD8^{+}CD28^{null}$ T cells and the sum of ranks of the individual significant correlations with the percentage of BALF $CD4^{+}CD28^{+}$, $CD4^{+}VLA1^{+}$, $CD8^{+}CD25^{+}$, $CD8^{+}VLA\text{-}1^{+}$ and $CD8^{+}HLA\text{-}DR^{+}$ lymphocytes from sarcoidosis patients.

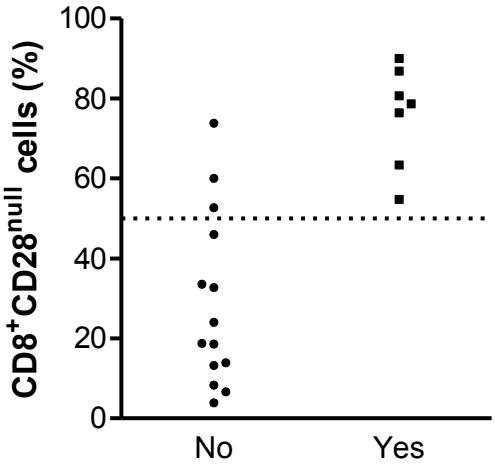


**Figure 7** Scatter plot of the percentage of PB $CD8^{+}CD28^{null}$ T cells in sarcoidosis patients (n = 21) at presentation and the need for corticosteroid treatment within 2 years after presentation. The dotted line indicates 50% level. Ten patients had $CD8^{+}CD28^{null}$ values above 50%. Of these patients, 7 needed medication. None of the patients with $CD8^{+}CD28^{null}$ values lower than 50% received corticosteroids within 2 years after presentation. Fisher's exact test: p = 0.001.

**Table 4** Parameters of disease severity and percentages of $CD8^{+}CD28^{null}$ cells in sarcoidosis patients

| Patient | Chest x-ray | D*lco* (% pred.) | Corticosteroid therapy (< 2years) | BAL T lymphocyte phenotype (SOR) | Peripheral blood $CD8^{+}CD28^{null}$ cells (%) |
|---|---|---|---|---|---|
| ILD50 | I | 100 | No | 35.5 | 3.9 |
| ILD51 | I | 99 | No | 14.5 | 6.6 |
| ILD31 | I | 94 | No | 28 | 8.3 |
| ILD39, Löfgren | I | ND | No | 27.5 | 13.3 |
| ILD20 | I | 81 | No | 35 | 13.9 |
| ILD17, Löfgren | I | 75 | No | 20 | 18.6 |
| ILD37 | I | ND | No | 56 | 18.8 |
| ILD22, Löfgren | I | 105 | No | 73 | 24.1 |
| ILD14 | I | ND | Unknown | 66 | 31.7 |
| ILD09 | I | ND | No | 60 | 32.7 |
| ILD08 | II | 90 | No | 66 | 33.5 |
| ILD13 | IV | 84 | No | 88 | 46.0 |
| ILD11, Löfgren | I | 70 | No | 59 | 52.7 |
| ILD47 | III | 89 | P | 62.5 | 54.7 |
| ILD25, Löfgren | I | ND | No | 49 | 60.1 |
| ILD24 | III | 82 | P + M | 79 | 63.4 |
| ILD26, Löfgren | I | 75 | No | 60 | 73.8 |
| ILD10 | II | 66 | P + I | 68 | 76.4 |
| ILD32 | II | 44 | P + M | 92 | 78.7 |
| ILD27 | III | 79 | P | 94 | 80.7 |
| ILD15 | II | 62 | P + M | 91 | 86.8 |
| ILD42 | I | 85 | P + M + I | 92 | 90.0 |

P, prednisone/prednisolone; M, methatroxate; I, infliximab; ND, not determined; SOR, sum of ranks.

## Discussion

This study showed compartmentalization of activated lymphocytes in the lungs of patients with different granulomatous interstitial lung diseases and healthy subjects. Early (CD69) as well as late (HLA-DR, VLA-1 and VLA-4) activation markers were found in a much higher frequency on BALF lymphocyte subsets than on blood lymphocyte subsets in all groups. In SARC and HP patients, increased expression of CD69, VLA-1 and decreased expression of CD28 on BALF $CD4^+$ cells and increased expression of HLA-DR on BALF $CD8^+$ cells correlated with the extend of parenchymal involvement and decreased Dl*co* values. Corresponding peripheral blood lymphocytes in ILD patients hardly expressed an activated phenotype, a finding which is consistent with other studies[12,14,16]. However, we found a remarkable loss of CD28 expression on PB $CD8^+$ T cells in particular in sarcoidosis patients. Interestingly, in sarcoidosis, the increased proportion of peripheral blood $CD8^+CD28^{null}$ T cells significantly correlated with Dl*co* values, radiographic staging and with BALF lymphocytes with a chronically activated phenotype (sum of ranks). Consistent with the observed correlations between blood $CD8^+CD28^{null}$ cells and parameters of disease severity, the proportion of $CD8^+CD28^{null}$ cells at presentation had prognostic value for the need for corticosteroid medication within 2 years after presentation. Loss of CD28 expression on T cells from sarcoidosis patients has been described but mainly concerned expansion of $CD4^+CD28^{null}$ cells[12,14,21].

The loss of CD28 is associated with chronic inflammation upon exposure to inflammatory cytokines and in the context of chronic antigenic exposure[22,23]. $CD8^+CD28^{null}$ cells are MHC class I restricted and function in an antigen-dependent manner. They might exert their immunosuppressive effect by rendering APC unable to initiate and support $T_{Helper}$ cell activation and growth[24]. On the other hand, $CD8^+CD28^{null}$ cells might exert their cytotoxic T lymphocyte (CTL) effector cell activity by production of granzymes, perforins, TNF-α and IFN-γ[25,26]. Loss of CD28 may not merely indicate chronic inflammation, but is also the result of aging of the host. $CD8^+$ cells show a higher susceptibility to age-dependent functional and phenotypic changes than $CD4^+$ cells[27] and the percentage of peripheral blood $CD8^+CD28^{null}$ cells has been shown to positively correlate with age and with a decrease in $CD4^+/CD8^+$ ratio[28]. However, univariate analysis of variance revealed no effect of age on the relationship between $CD8^+CD28^{null}$ cells and Dl*co* values, radiologic stages or BALF lymphocyte phenotype in sarcoidosis (data not shown). The functional significance of circulating $CD8^+CD28^{null}$ cells in sarcoidosis remains to be determined. The correlations with indicators of disease severity suggest that measuring the percentage of peripheral blood $CD8^+CD28^{null}$ cells might be used as a marker for the amount of lung parenchymal

infiltration in sarcoidosis. This hypothesis undoubtedly needs to be validated in a larger patient cohort including follow-up measurements.
BALF cells from patients with HP showed the most chronic activated phenotype compared to controls and compared to sarcoidosis patients. Higher expression of early (CD69) and late (VLA-1, HLA-DR) activation markers and lower expression of co stimulatory molecule CD28 was found on BALF lymphocytes. This suggests continuous lymphocyte activation, consistent with prolonged local antigen exposure in HP.

## Conclusion

This study shows that a specific phenotype of chronically activated BALF lymphocyte subsets was found in sarcoidosis and hypersensitivity pneumonitis patients and correlated with the extend of parenchymal involvement and lower lung function. In sarcoidosis, the blood $CD8^{+}CD28^{null}$ subset may be a new biomarker for disease severity. Further research is necessary to confirm this hypothesis and to understand its role in sarcoidosis disease progression.

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# 3

## Evaluation of CD103 as a cellular marker for the diagnosis of pulmonary sarcoidosis

*Clin Immunol 2008; 126(3):338-344*

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## Abstract

A high $CD4^+/CD8^+$ ratio in bronchoalveolar lavage fluid is indicative for the diagnosis pulmonary sarcoidosis but this ratio only does not fully discriminate pulmonary sarcoidosis from other interstitial lung diseases. Recently, the integrin CD103 has been implicated in the diagnostic evaluation of sarcoidosis. CD103 is expressed on intra-epithelial lymphocytes in mucosal areas, including bronchi, and is possibly involved in the retention of lymphocytes to the mucosa. The Dutch BAL working party initiated an investigation to evaluate the diagnostic value of relative number of CD103 expressing $CD4^+$ T lymphocytes in the BAL fluid of patients with a variety of interstitial lung diseases. The expression of CD103 was examined on bronchoalveolar lavage cells from 119 patients including 56 patients with pulmonary sarcoidosis. We redefined criteria for alveolar $CD4^+$ T cell lymphocytosis and for the relative enumeration of CD103 expressing $CD4^+$ T lymphocytes in the BAL fluid. Our data demonstrate that the combined use of the $CD103^+CD4^+/CD4^+$ ratio (< 0.2) and the BAL $CD4^+/CD8^+$ ratio (> 3) or the relative alveolitis $CD4^+/CD8^+$ BAL/PB ratio (> 2) provides a specific tool for discriminating sarcoidosis, also without a clear $CD4^+$ alveolitis, from other interstitial lung diseases.

## Introduction

Interstitial lung diseases (ILD) are a heterogeneous group of pulmonary disorders, comprising over 100 different members that are classified together because of similar clinical, radiographic, physiologic, or pathologic manifestations. The major abnormality in ILD is disruption of the lung parenchyma. When the lung is injured, epithelial cells are damaged and basement membranes may lose their integrity, heralding the appearance of a variety of inflammatory cells, regenerating type II pneumocytes, and increasing the expression of extracellular matrix components[1-3].

The most common ILD in the western world is sarcoidosis, with an annual incidence of between 10 and 25 per 100,000 persons in Western Europe and the United States[4,5].

Sarcoidosis is a chronic systemic inflammatory disorder of unknown origin characterized by the accumulation of macrophages and in particular $CD4^+$ T lymphocytes in the involved organs, most frequently the lung, ultimately leading to the formation of non-caseating granulomas. Since the key pathologic finding in sarcoidosis is non-caseating granulomas, the diagnosis should be confirmed by a biopsy whenever possible – except in patients with a typical presentation of a Löfgren's syndrome. Sarcoidosis commonly affects young adults. Because there is spatial, seasonal, and occupational clustering, it is generally believed that the disease is triggered by environmental agents[6].

In the absence of a known causative agent, however, sarcoidosis remains a diagnosis of exclusion. The differential diagnose of sarcoidosis is extensive, including infectious diseases (e.g. tuberculosis), granulomatous diseases associated with exposure to inorganic or organic agents (e.g. hypersensitivity pneumonitis, chronic beryllium disease, drug-induced pneumonitis), autoimmune disorders (e.g. Wegener's granulomatosis, Churg-Strauss vasculitis) and malignancies (e.g. lymphomas, tumour-related granuloma)[7-10].

In sarcoidosis, the lungs are frequently affected and bronchoalveolar lavage (BAL) is an important diagnostic tool to sample cells at the site of inflammation. A lymphocytic alveolitis with a $CD4^+/CD8^+$ ratio > 3.5 is consistent with pulmonary sarcoidosis. However, the finding of a $CD4^+$ lymphocytosis in BAL is neither specific nor sensitive for the diagnosis of sarcoidosis[9,11-13]. Therefore it is of interest to investigate additional cellular markers of the $CD4^+$ T lymphocytes to specify the characteristics of this cell population in pulmonary sarcoidosis.

The expression of the $\alpha_E\beta_7$/CD103$\beta_7$ integrin (CD103) has been related to the retention of intraepithelial lymphocytes (IEL) in mucosal tissues of gut, urogenital tract and lung. CD103 mediates binding to E-cadherin at the basolateral side of the epithelium and is expressed by lymphocytes within the bronchial epithelium[14-16], by some alveolar wall

lymphocytes and by T lymphocytes in the bronchoalveolar fluid[17,18]. It has been shown that the relative amount of CD103-expressing T cells in the bronchoalveolar lavage fluid differs in patients with ILD, depending on the type of disease. This variation is predominantly seen in the $CD4^+$ T cell population: patients with idiopathic pulmonary fibrosis (IPF) and hypersensitivity pneumonitis (HP) have a significantly higher proportion of $CD4^+$ T cells expressing CD103 compared to patients with sarcoidosis[19,20]. This is corroborative to the concept that lymphocytosis in HP results from the local expansion of mucosal lymphocytes while lymphocytosis in pulmonary sarcoidosis is the result of lymphocytes of non-mucosal origin. The absence of CD103 on $CD4^+$ lymphocytes in the BAL fluid of sarcoidosis patients is consistent with a peripheral origin of these cells. Decreased levels of peripheral $CD4^+$ lymphocytes with increased levels of BAL $CD4^+$ lymphocytes have been described in sarcoidosis[21] and suggest a redistribution from the peripheral blood and compartmentalization in the lung[22].

Kolopp-Sarda and colleagues investigated the expression of the integrin CD103 on BAL lymphocytes and proposed diagnostic criteria to discriminate sarcoidosis from other ILDs[19]. However, their criteria exclude sarcoidosis patients with a low $CD4^+/CD8^+$ ratio (< 2.5) in BAL, whereas such patients may comprise a substantial part of the sarcoidosis patients (see for example Kantrow et al.[12]).

The Dutch BAL working party initiated an investigation to evaluate the diagnostic value of relative number of CD103 expressing $CD4^+$ T lymphocytes in the BAL fluid of patients with a variety of interstitial lung diseases. We redefined criteria for alveolar $CD4^+$ T cell lymphocytosis and for the relative enumeration of CD103 expressing $CD4^+$ T lymphocytes in the BAL fluid. Our results indicate that the combination of $CD103^+CD4^+/CD4^+$ ratio with an absolute or relative (to peripheral blood) $CD4^+/CD8^+$ ratio in BAL is a specific parameter for the diagnosis of pulmonary sarcoidosis, also in sarcoidosis patients without an apparent $CD4^+$ alveolitis. In comparison with the criteria used by Kolopp-Sarda et al., analysis of the BAL using our renewed criteria increases the sensitivity with an equal specificity, predicting the correct group of sarcoidosis patients.

## Subjects & Methods

*Patients*

The diagnoses of the patients are listed in Table 1. In all cases, the diagnosis of sarcoidosis was confirmed by a biopsy obtained from the lung showing non-caseating epithelioid

granulomas and after exclusion of other known causes of granulomatosis in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis[23]. In one patient, the diagnosis was made without biopsy because this patient presented with the classic symptoms of Löfgren's syndrome (i.e. fever, erythema nodosum, arthralgia and bilateral hilar lymphadenopathy). Other diagnoses were made according to standard criteria. None of the patients received corticosteroids at time of the diagnostic work up. Five hospitals participated in this multicenter study.
For validation, an additional cohort of 39 patients (St. Antonius Hospital: 26 sarcoidosis patients and 13 patients with other ILD) was used (Table 2).

*Material*
Patient material (BAL fluid and blood) was collected during regular diagnostic work up. Only patients with alveolar lymphocytosis, defined by ≥ 10% lymphocytes in bronchoalveolar lavage, were included.

*Bronchoalveolar Lavage*
BAL - according to the Dutch BAL working party protocol - was performed during flexible fiberoptic bronchoscopy at the time of the diagnosis[24]. BAL was performed, preferably in the right middle lobe, with four 50-mL aliquots of sterile isotonic saline solution. The aspirated lavage fluid from the first 50-mL aliquot was kept apart and excluded from further analysis. The BAL fluid (BALF) recovered from the three subsequent aliquots was pooled in a siliconized specimen trap and kept on ice. BALF was filtered through nylon gauze and centrifuged (10 min at 400 x g at 4 °C).
The cell pellet was washed twice, counted, and resuspended in minimal essential medium/RPMI 1640 (Gibco; Grand Island, NY), supplemented with 0.5% bovine serum albumin (Organon; Teknika; Boxtel, the Netherlands). Cells were counted in a Bürker chamber. Cell viability was determined by Trypan blue exclusion. Smears for cell differentiation were prepared by cytocentrifugation (Shandon; Runcorn, UK). After staining with May-Grünwald-Giemsa (Merck; Darmstadt, Germany), at least 2x 500 cells were counted. BAL cells were processed in the participating laboratories mentioned at the end of the article. All contributing hospitals participated in the Dutch BAL Quality Control Survey.

*Flow cytometry*
Flowcytometry was performed according to standard protocols. Shortly, BALF cells were stained with fluorochrome labelled monoclonal antibodies to CD45, CD3, CD4, CD8 and

CD103. Three to four-color flow cytometric analysis was performed on flow cytometer (Becton Dickinson/Beckman Coulter).

*Statistics*

Data are expressed as means ± SEM and Student's t-test or Mann-Whitney test were used to test statistical difference between groups.

The optimal cut-off point (for the predictor) coincided with the point on the ROC curve where the sensitivity equals specificity. Statistical analyses were performed using the Statistical Package for Social Science for Windows (SPSS; Chicago, IL, USA) and Graphpad Prism 3.00 (San Diego, CA, USA). Additionally, the Hanley test was used to compare areas under the ROC curve[25]. Statistical significance was denoted by a value of $p < 0.05$ for all tests performed.

**Table 1** Diagnosis and number of patients analyzed

| Diagnosis | No. of patients |
|---|---|
| Sarcoidosis (biopt proven) | 55 |
| Löfgren's syndrome | 1 |
| total sarcoidosis | 56 |
| Hypersensitivity pneumonitis | 22 |
| IPF | 8 |
| Other interstitial pneumonia | 3 |
| Infection | 13 |
| TBC | 4 |
| Systemic disease | 8 |
| Malignancy | 6 |
| NHL | 1 |
| MM | 1 |
| CLL | 1 |
| Other | 3 |
| total other ILD | 63 |

IPF, Idiopathic Pulmonary Fibrosis; NHL, non-Hodgkin Lymphoma; MM, Multiple Myeloma; CLL, Chronic Lymphocytic Leukemia; TBC, Tuberculosis.

**Table 2** Diagnosis and number of patients in the validation cohort

| Diagnosis | No. of patients |
|---|---|
| Sarcoidosis (biopt proven) | 20 |
| Löfgren's syndrome | 6 |
| total sarcoidosis | 26 |
| Hypersensitivity pneumonitis | 7 |
| IPF | 1 |
| Other interstitial pneumonia | 4 |
| Infection | 1 |
| TBC | 1 |
| total other ILD | 13 |

IPF, Idiopathic Pulmonary Fibrosis; TBC, Tuberculosis.

## Results

*Expression of cell surface markers*

A significant higher $CD4^+/CD8^+$ ratio in BAL fluid (mean ± SEM) was found for sarcoidosis patients (7.9 ± 0.9, n = 56) compared to patients with other ILD (2.3 ± 0.3, n = 63, $p < 0.0001$, Figure 1). The proportion of $CD103^+$ lymphocytes (mean ± SEM) of total BAL lymphocytes was significantly lower in sarcoidosis patients (23.5 ± 2.0, n = 56) compared to patients with other ILD (48.4 ± 2.8, n = 63, $p < 0.0001$) (data not shown).

Figure 2 presents the $CD103^+CD4^+/CD4^+$ ratio, introduced in the present study, reflecting the relative number of CD103 expressing T lymphocytes within the $CD4^+$ subpopulation. Sarcoidosis patients showed a significantly lower $CD103^+CD4^+/CD4^+$ ratio compared to patients with other ILD (0.16 ± 0.02 vs. 0.4 ± 0.03, $p < 0.0001$).

ROC curves were used to evaluate the discriminative value of the BAL $CD4^+/CD8^+$ ratio and the $CD103^+CD4^+/CD4^+$ ratio for sarcoidosis. The differences between the AUCs did not reach statistical significance (AUC $CD4^+/CD8^+$: 0.81, CI 0.73 – 0.88; $CD103^+CD4^+/CD4^+$: 0.79, CI 0.71 – 0.86).

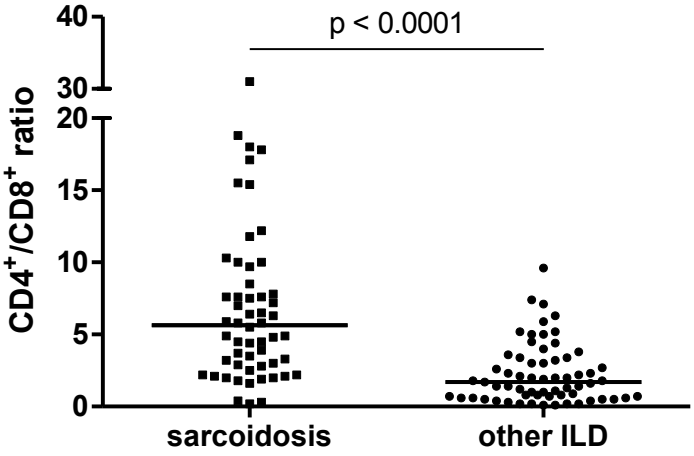


**Figure 1** Scatterplot illustrating individual $CD4^+/CD8^+$ ratios in BAL from sarcoidosis patients (n = 56) and patients with other ILD (n = 63). The horizontal line represents the median value.

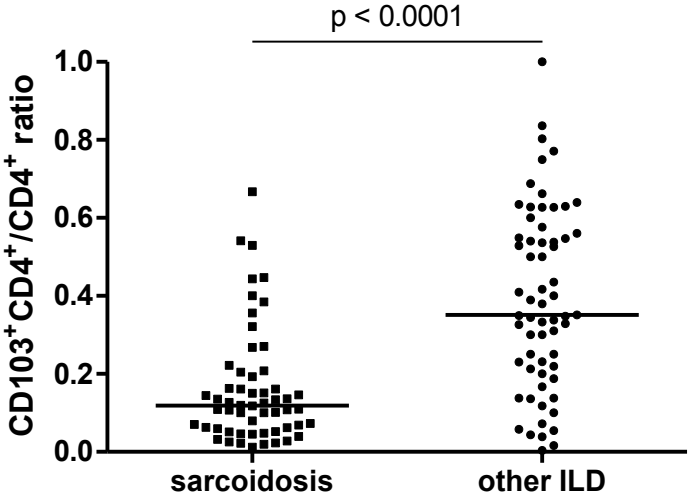


**Figure 2** Scatterplot illustrating individual $CD103^+CD4^+/CD4^+$ ratios in BAL from sarcoidosis patients (n = 56) and patients with other ILD (n = 63). The horizontal line represents the median value.

*Cut-off levels*

A BAL $CD4^+/CD8^+$ ratio > 3.5 has been used by several investigators to define $CD4^+$ alveolitis[13,26]. However, a BAL with a $CD4^+/CD8^+$ ratio within normal range may exhibit a relative $CD4^+$ lymphocytosis when compared to the peripheral blood $CD4^+/CD8^+$ ratio. Therefore, we defined the term 'relative $CD4^+$ alveolitis' by comparing the $CD4^+/CD8^+$ ratio in BAL to the $CD4^+/CD8^+$ ratio in peripheral blood. Cut-off levels were set at that point

where sensitivity equals specificity and were being determined for i) the $CD103^+CD4^+/CD4^+$ ratio in BAL fluid at 0.2 (Figure 3 A), ii) the $CD4^+/CD8^+$ ratio in BAL fluid at 3 (Figure 3 B), and iii) the relative alveolitis $CD4^+/CD8^+$ (BAL/PB) ratio at 2 (Figure 3 C).

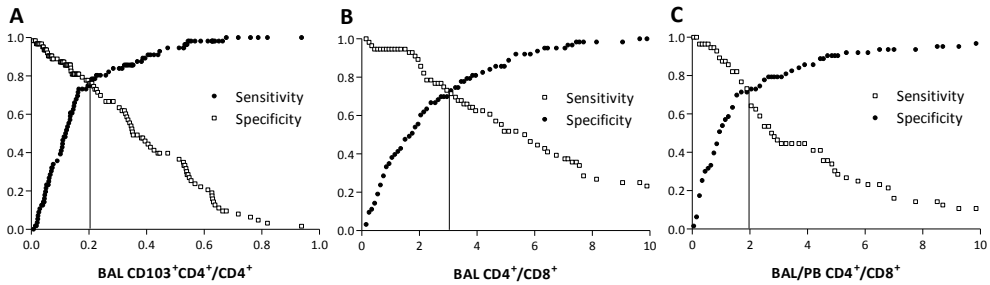


**Figure 3 A, B, C** Graphic presentation of cut-off values for the different parameters in our patient cohort. The cut-off value is determined at the point where sensitivity equals specificity, denoted by the vertical line.

*Specificity and sensitivity of combined criteria*

In the study of Kolopp-Sarda et al. all sarcoidosis patients were characterized by a BAL $CD4^+/CD8^+$ ratio $\geq$ 2.5, in addition to confirmed pulmonary sarcoidosis according to clinical, radiographic and pathological criteria. However, our confirmed sarcoidosis population included 12 out 56 (21%) patients with a $CD4^+/CD8^+$ ratio < 2.5. Apparently, $CD4^+$ alveolitis defined by a BAL $CD4^+/CD8^+$ ratio $\geq$ 2.5 is not an accurate diagnostic criterion.

When using the criteria proposed by Kolopp-Sarda et al.: $CD103^+/CD4^+$ ratio, (cut-off point 0.31) combined with $CD4^+/CD8^+$ ratio ($\geq$ 2.5), the sensitivity of predicting the correct group was only 57% (32/56), with a specificity of 90% (57/63).

As shown in Table 3, using the criteria proposed in this study for the $CD103^+CD4^+/CD4^+$ ratio combined with either $CD4^+/CD8^+$ ratio in BAL or the relative $CD4^+/CD8^+$ ratio (if $CD4^+/CD8^+$ ratio in BAL < 3), sensitivity increases to 66% (37/56) and specificity equals the specificity found with the previous proposed criteria: 89% (56/63).

Validation of the proposed combined criteria in an additional cohort of 39 patients confirmed the increased sensitivity of our new criteria compared to criteria used by Kolopp-Sarda et al. (54% vs. 35%) while the probability of correctly predicting the group with other ILD was similar (Table 4).

**Table 3** Cut-off values, sensitivity, specificity, PPV and NPV for different parameters in patient cohort assembled by the Dutch BAL working party (n = 119; 56 sarcoidosis, 63 other ILD)

| Patient cohort (n = 119) | Selected cut-off | Sensitivity, % | Specificity, % | PPV, % | NPV, % |
|---|---|---|---|---|---|
| [a]BAL $CD4^+/CD8^+$ | 2.5 | 73 | 67 | 66 | 74 |
| [a]BAL $CD4^+/CD8^+$ and $CD103^+/CD4^+$ | 2.5 and 0.31 | 57 | 91 | 84 | 70 |
| BAL $CD4^+/CD8^+$ | 3 | 68 | 73 | 69 | 72 |
| BAL $CD4^+/CD8^+$ or $CD4^+/CD8^+$ BAL/PB and $CD103^+CD4^+/CD4^+$ | 3 or 2 and 0.2 | 66 | 89 | 82 | 74 |

[a]Values according criteria from Kolopp-Sarda et al.

**Table 4** Cut-off values, sensitivity, specificity, PPV and NPV for different parameters in validation cohort from St. Antonius Hospital. (n = 39; 26 sarcoidosis, 13 other ILDs)

| Validation cohort (n = 39) | Selected cut-off | Sensitivity, % | Specificity, % | PPV, % | NPV, % |
|---|---|---|---|---|---|
| [a]BAL $CD4^+/CD8^+$ | 2.5 | 65 | 54 | 74 | 44 |
| [a]BAL $CD4^+/CD8^+$ and $CD103^+/CD4^+$ | 2.5 and 0.31 | 35 | 92 | 90 | 41 |
| BAL $CD4^+/CD8^+$ | 3 | 62 | 85 | 92 | 79 |
| BAL $CD4^+/CD8^+$ or $CD4^+/CD8^+$ BAL/PB and $CD103^+CD4^+/CD4^+$ | 3 or 2 and 0.2 | 54 | 92 | 93 | 50 |

[a]Values according criteria from Kolopp-Sarda et al.

## Discussion

Sarcoidosis is characterized by an alveolar $CD4^+$ lymphocytosis. This study demonstrates that the percentage of alveolar $CD4^+$ T-lymphocytes expressing the integrin CD103 in patients with pulmonary sarcoidosis is significantly different from patients with other ILDs. This result suggests that the $CD103^+CD4^+/CD4^+$ ratio in BAL is an additional cellular marker for pulmonary sarcoidosis. Secondly, as some sarcoidosis patients without an apparent $CD4^+$ alveolitis show a $CD4^+$ depletion in the peripheral blood, we introduce the relative $CD4^+/CD8^+$ ratio, which compares the BAL $CD4^+/CD8^+$ ratio to the $CD4^+/CD8^+$ ratio in peripheral blood. As a diagnostic marker, a high relative $CD4^+/CD8^+$ ratio without a clear $CD4^+$ alveolitis ($CD4^+/CD8^+$ ratio BAL < 3) is considered equivalent to a high $CD4^+/CD8^+$ ratio

in BAL. This is consistent with the concept that there is an accumulation of peripheral blood $CD4^+$ lymphocytes in the lungs of sarcoidosis patients[22,27,28].

Initially, Kolopp-Sarda et al. described the $CD103^+/CD4^+$ ratio in sarcoidosis patients and found that when combined with $CD4^+/CD8^+$ ratio $\geq$ 2.5 in BAL, the sensitivity for the diagnosis pulmonary sarcoidosis was 96%. However, the population studied by Kolopp-Sarda et al. did not encompass pulmonary sarcoidosis patients with a BAL $CD4^+/CD8^+$ ratio < 2.5. Consistent with a study by Kantrow et al.[12] describing the highly variable $CD4^+/CD8^+$ ratio in BAL fluid in biopsy-proven sarcoidosis, our cohort includes 12 out of 56 sarcoidosis patients with a $CD4^+/CD8^+$ ratio < 2.5. Including these patients and using the criteria proposed by Kolopp-Sarda et al., the sensitivity of their diagnostic test decreases considerably in our cohort (Table 3). The diagnosis and the lymphocytic characteristics of the non-sarcoidosis patients also fulfilling our proposed criteria for sarcoidosis are shown in table 5. These patients have been diagnosed otherwise during the work up, since sarcoidosis can only be diagnosed after exclusion of these diseases.

A further advantage of our proposed method is the application of the ratio $CD103^+CD4^+/CD4^+$ rather than the ratio $CD103^+$total lymphocytes/$CD4^+$. It is known that in different lung diseases the majority of $CD8^+$ lymphocytes co-express CD103[20,29,30], while the effector cells that are involved in the $T_H1$ immune response in sarcoidosis are primarily $CD4^+$ T lymphocytes. The relative absence of CD103 expression on $CD4^+$ T lymphocytes of patients with pulmonary sarcoidosis is corroborative to the concept of influx of $CD4^+$ T lymphocytes from the peripheral blood to the lung compared to local expansion of $CD103^+$ lymphocytes in other ILD. Interestingly, in sarcoidosis patients with different radiographic stages different numbers of $CD4^+$ T lymphocytes expressing CD103 have been described. Analysis of our data showed that patients with radiographic stage II/III/IV have a significantly higher proportion of $CD4^+$ T lymphocytes expressing CD103 (Figure 4) than patients with radiographic stage I (including Löfgren's syndrome).

Similarly, Lohmeyer et al. found significantly higher proportions of $CD4^+$ T lymphocytes co-expressing CD103 in sarcoidosis patients with radiographic stage II/III compared to radiographic stage I (including Löfgren's syndrome), suggesting that the analysis of this integrin might be helpful to discriminate between acute self-remitting forms of alveolitis and chronic manifestations with tendency towards fibrosis[20]. The latter is in accordance with Braun et al.[18], who suggest that CD103 positive cells are long-lived, terminal differentiated effector cells and are involved in fibrogenic inflammation. Interestingly, a recent study by Kruit et al. revealed that genetic variation in the gene that encodes for TGF-β3 is associated with pulmonary fibrosis in sarcoidosis patients. Isoforms 1 and 2 of the TGF-β family are generally described as having a profibrotic nature, whilst TGF-β3

shows a more diverse character which may either sustain or resolve the progression of fibrosis[31].

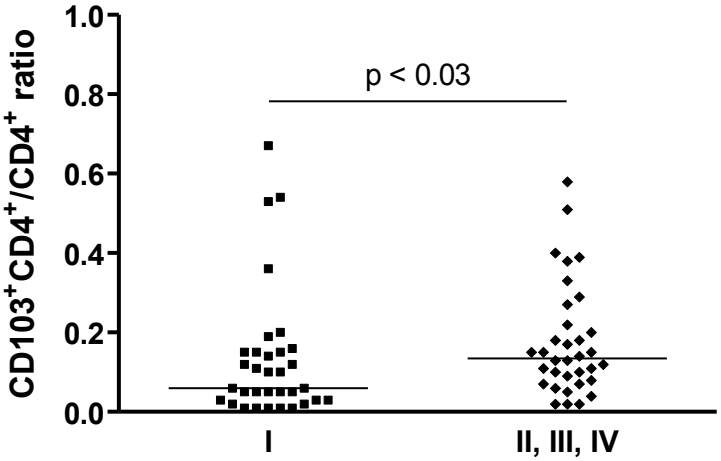


**Figure 4** Scatterplot illustrating individual $CD103^+CD4^+/CD4^+$ ratios in BAL from sarcoidosis patients with radiographic stage I (including Löfgren's syndrome; n = 33) versus sarcoidosis patients with radiographic stage II, III, IV (n = 34). The horizontal line represents the median value.

TGF-β is the only known stimulus that can induce CD103 expression[32]. Although speculative, an altered balance between all three TGF-β isoforms may have a direct effect on remodeling of injured tissue and an indirect effect on lymphocyte retention by increased induction of CD103 expression and could be unfavorable to the outcome of sarcoidosis.

Recently, Baker and colleagues suggested that dysregulation in the TGF-β signaling pathway in colorectal neoplasms plays a role in promoting the retention of lymphocytes within the malignant epithelium[33], underscoring the entwined relationship between TGF-β production, CD103 expression and lymphocyte retention.

Finally, lack of CD103 expression might have implications for regulatory T cell function in sarcoidosis considering recent findings of increased CD103 expression on inflammation seeking $Foxp3^+$ regulatory T cells[34,35].

**Table 5** Diagnoses of patients falsely identified as positive for sarcoidosis when applying the newly proposed criteria only

| | | $CD4^+$ lymphocytosis | | |
|---|---|---|---|---|
| | $CD103^+CD4^+/CD4^+$ | BAL $CD4^+/CD8^+$ | relative $CD4^+/CD8^+$ | Diagnosis |
| 1. | 0.016 | 4.4 | | TBC |
| 2. | 0.039 | 3.8 | | tumour |
| 3. | 0.044 | 3.4 | | Infect eci |
| 4. | 0.072 | 5.9 | | Bronchitis |
| 5. | 0.188 | 4.0 | | MM |
| 6. | 0.200 | 5.0 | | IPF, without biopt |
| 7. | 0.004 | 2.2 | 11.0 | NHL |
| 8. | 0.055 | 2.3 | 4.6 | chemical pneumonitis |

IPF, Idiopathic Pulmonary Fibrosis; NHL, non-Hodgkin Lymphoma; MM, Multiple Myeloma; TBC, Tuberculosis

## Conclusion

This study shows that determination of CD103 expression on BAL $CD4^+$ T lymphocytes is of additional value in the diagnostic work-up of patients with suspected pulmonary sarcoidosis. The use of the relative $CD4^+/CD8^+$ ratio prevents missing those sarcoidosis patients without an absolute $CD4^+$ alveolitis, thereby underscoring the limited diagnostic accuracy of the $CD4^+/CD8^+$ ratio of BAL T lymphocytes as the only parameter. Our data demonstrate that the combined use of the $CD103^+CD4^+/CD4^+$ ratio (cut-off: 0.2) and the BAL $CD4^+/CD8^+$ ratio (cut-off: 3) or the relative alveolitis $CD4^+/CD8^+$ BAL/PB ratio (cut-off: 2) provides a specific tool for discriminating sarcoidosis from other ILD.

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# 4

## Effect of variation in *ITGAE* on risk of sarcoidosis, CD103 expression, and chest radiography

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## Abstract

The integrin $\alpha_E\beta_7$ is believed to play a key role in retention of lymphocytes in mucosal tissues of gut, urogenital tract and lung. Five common single nucleotide polymorphisms spanning *ITGAE*, the gene encoding the $\alpha_E$ (CD103) unit, were genotyped in 556 sarcoidosis patients and 465 controls. The -1088 A/G polymorphism was associated with sarcoidosis ($p = 0.004$). An increased risk of disease was found for homozygous carriers of the A allele vs. carriers of the G allele ($p = 0.001$, odds ratio = 1.63 [1.22-2.17]). Analysis of lymphocytes from bronchoalveolar lavage and *in vitro* functional tests showed higher percentages of $CD103^+CD4^+$ T cells for the sarcoidosis risk genotype. Radiographic staging at disease outcome revealed prevalence of -1088 AA genotype in patients with fibrosis ($p = 0.01$). A higher proportion of $CD103^+CD4^+$ T cells and *ITGAE* -1088 AA genotype might be associated with fibrosis formation in pulmonary sarcoidosis.

## Introduction

Sarcoidosis is a systemic disorder characterized by the formation of non-caseating granulomas, with the lungs, lymph nodes and skin being the most frequently affected organs (recently reviewed in Iannuzzi et al.[1]). The involved organs show a fierce influx of $CD4^+$ $T_{Helper}1$ lymphocytes. Although granulomas may often resolve spontaneously, pulmonary fibrosis occurs in 10 to 15% of patients[2]. The pathogenesis of pulmonary fibrosis in sarcoidosis remains uncertain but carriage of TGF-β3 risk alleles[3], production of CCL18 chemokine by alveolar macrophages[4], and the production of $T_{Helper}2$ lymphocyte associated cytokines[5] have been reported to enhance progression towards fibrosis.

Tissue-specific localization of T cells is guided by differential expression of cytokines and chemokines and their receptors[6]. In addition, a complex array of adhesion molecules[7] expressed by T cells and counter-receptors on the vascular endothelium, extracellular matrix and the epithelium is thought to mediate T cell migration and retention[8]. Recent studies have identified several of the specific molecular interactions that mediate lymphocyte trafficking and retention. The $\alpha_E\beta_7$ integrin is hardly expressed by peripheral blood lymphocytes (< 2%) but expression by T cells occurs in almost all mucosal epithelial sites[9,10]. The only known ligand for $\alpha_E\beta_7$ is E-cadherin, a homophilic cell adhesion molecule that is expressed on the basolateral side of epithelial cells and in adherens junctions. This integrin is believed to play a key role in retention of intraepithelial lymphocytes (IELs) in mucosal tissues of gut, urogenital tract and lung[11-13]. Binding of $\alpha_E\beta_7$ to E-cadherin provides a costimulatory signal for T cell proliferation, and has recently been found to determine ligand directed shape and locomotion of $\alpha_E\beta_7^+$ cells [14], suggesting a mechanism that could promote the survival and proliferation of $\alpha_E\beta_7^+$ IELs. The alpha unit of $\alpha_E\beta_7$, CD103, is expressed by lymphocytes within the bronchial epithelium, by some alveolar wall lymphocytes and by T lymphocytes in the bronchoalveolar lavage fluid[12,15].

Genetic variation in genes encoding for adhesion molecules may predispose to chronic inflammatory disease. The gene encoding integrin $\alpha_4$ (*ITGA4*), has been implicated in disease susceptibility to multiple sclerosis[16,17]. and *ITGAM,* encoding the α chain of the integrin $\alpha_M\beta_2$, has been found to predispose to SLE[18,19]. In addition, experiments with $\alpha_E^{-/-}$ mice suggest that $\alpha_E$ deficiency may be a genetic factor predisposing to inflammatory skin disease[20,21].

The gene *ITGAE*, encoding the $\alpha_E$ unit CD103, is located on chr17p13.3-p13.2, and is approximately 87 kb in length. To date no studies have been published examining genotypic differences in *ITGAE* between sarcoidosis patients and controls. We genotyped five common SNPs belonging to the *ITGAE* gene region and studied possible functional consequences of the genotypic variation.

## Subjects and Methods

*Subjects*

Case subjects included 556 unrelated Dutch Caucasian sarcoidosis patients (male/female; 290/266). The diagnosis sarcoidosis was established after exclusion of other known causes of granulomatosis in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis[22]. Eighty-one patients presented with the classic symptoms of Löfgren's syndrome, namely fever, erythema nodosum, arthralgia and bilateral hilar lymphadenopathy. The control subjects comprised 466 healthy, unrelated Dutch Caucasian employees of the St. Antonius Hospital and blood donor samples from the blood bank Utrecht, the Netherlands. Thirty-nine individuals included in the control group underwent bronchoalveolar lavage and donated blood for functional assays. Verbal and written consent was obtained from all subjects, and authorization was given by the Ethics Committee of the St. Antonius Hospital, Nieuwegein.

*Radiographic staging*

Chest radiographs were assessed in consensus by chest physicians specialized in diffuse lung diseases to determine disease severity using standard radiographic staging for sarcoidosis, classified according to the Scadding criteria[23]. Radiographic data at presentation were available of 197 sarcoidosis patients. Radiographic data at disease outcome, defined by chest x-ray after a minimum of 4-years follow-up, were available of 311 patients. Patients presenting with pulmonary fibrosis or showing pulmonary fibrosis on radiography at follow-up < 4-years, were classified as stage IV at disease outcome considering the irreversibility of fibrotic scarring.

*Pulmonary function tests*

Pulmonary function tests were performed at presentation. Vital capacity (inspiratory) (iVC), forced expiratory volume in 1 second ($FEV_1$) and carbon monoxide diffusing lung capacity (D*lco*) were used to assess the presence of lung function impairment at presentation of the disease. All lung function parameters are expressed as percent predicted values. IVC and $FEV_1$ were calculated from volumes in liters and adjusted to BTPS (body temperature, ambient pressure, saturated with water vapor).

*Bronchoalveolar lavage*

Bronchoalveolar lavage (BAL) was performed during flexible fiberoptic bronchoscopy at the time of diagnosis according to standardized and validated procedure[24]. BAL data were obtained from 72 patients and 39 healthy subjects. BAL was performed, preferably in the

right middle lobe, with four 50-ml aliquots of sterile isotonic saline solution (37 °C). The aspirated lavage fluid from the first 50-ml aliquot was kept apart and excluded from further analysis. The BAL fluid (BALF) recovered from the three subsequent aliquots was collected in a siliconized specimen trap and kept on ice. BALF was filtered through nylon gauze and centrifuged (10 min at 400 x g at 4 °C). The cell pellet was washed twice, counted, and resuspended in minimal essential medium/RPMI 1640 (Gibco; Grand Island, NY), supplemented with 0.5% bovine serum albumin (Organon; Teknika; Boxtel, the Netherlands). Cells were counted in a Bürker chamber. Cell viability was determined by Trypan blue exclusion.

*Analysis of ITGAE single nucleotide polymorphisms*

DNA was extracted from whole blood samples and SNPs were analyzed on a custom Illumina goldengate bead SNP assay that was performed in accordance with the manufacturer's recommendations. Five SNPs in the *ITGAE* gene were selected (Figure 1). From these five SNPs, two SNPs were manually chosen, located at the following gene positions (relative from ATG): -1088 A/G (rs2891, promoter), and 71603 C/T (rs1716, Arg950Trp, exon 24). Three haplotype tagging SNPs were selected using the tagger program on the genomic region of *ITGAE* on genome build 35, the CEU HapMap analysis panel and preferential picking of SNPs with a minimum allele frequency of 25% and using the pairwise tagging option with an $r^2$ threshold for SNPs > 0.8[25]. The three haplotype tagging SNPs were located at positions (relative from ATG): 33555 T/G (rs6502743), 66485 T/C (rs178323) and 86141 A/G (rs1367950).


**Figure 1** The $\alpha_E$ gene. Location of *ITGAE* SNPs (described in Subjects and Methods) and organization of intron/exon boundaries. Dark bars represent exons.

*CD103 surface expression ex vivo*

CD103 surface expression was measured on T lymphocytes in bronchoalveolar lavage fluid (BALF) from healthy subjects and from sarcoidosis patients at disease presentation. Fifty µl BALF, containing at least $1 \times 10^5$ cells, was incubated for 15 min at room temperature with 10 µl of the following mAbs: anti-CD3 PE, anti-CD4 PerCP, anti-CD8 APC and anti-CD103

FITC. Immunofluoresence was measured by flow cytometry (FACS-Calibur, Becton Dickinson, Alphen aan den Rijn, The Netherlands) and data were analyzed with FlowJo software (Tree Star, Inc., Ashland, USA). During each experiment a total of $5 \times 10^4$ cells were analyzed. The lymphocyte population was selected based on forward scatter–side scatter (FSC–SSC) characteristics.

*Functional assays in vitro*

Functional assays were performed with Peripheral Blood Mononuclear Cells (PBMCs) from 17 healthy donors selected on basis of -1088 genotype (5 AA, 6 AG, 6 GG). PBMCs were isolated from heparinized venous blood using Ficoll-Paque density gradient centrifugation and cryopreserved until further analysis. After carefully thawing, cells were seeded on 24-well plates (750 µl per well, $2 \times 10^6$ cells/ml). Part of the cells was used to measure CD103 expression immediately after thawing. PBMCs were stimulated with anti-CD3/CD28 (Sanquin M1654 en M1650, diluted 1:10000) and recombinant human TGF-β1 (CHO cell derived, 5 ng/ml) (PeproTech EC Ltd, London, UK). All stimulations were performed in a final volume of 750 µl RPMI-1640 (GIBCO, Breda, The Netherlands) containing 20% heat-inactivated fetalclone-I (hyclone) and penicillin/streptomycin (100U/ml, 100µg/ml) (GIBCO, Breda, The Netherlands). After 1 and 4 days of incubation at 37 °C in humidified air containing 5% CO2, cells were harvested and stored for later RNA analysis; 50 µl cell suspension was incubated for 15 min. at room temperature with 10 µl of the following mAbs: anti-CD3 PE, anti-CD4 PerCP, anti-CD8 APC and anti-CD103 FITC, all from BD Biosciences (Alphen aan den Rijn, The Netherlands).

The lymphocyte population was selected based on forward scatter–side scatter (FSC–SSC) characteristics. Immunofluoresence was measured by flow cytometry (FACS-Calibur, Becton Dickinson, Alphen aan den Rijn, The Netherlands) and data were analyzed with FlowJo software (Tree Star, Inc., Ashland, USA). At least $5 \times 10^4$ cells were analyzed.

Figure 2 illustrates a representative flow cytometric analysis of CD103 expression on BALF lymphocyte subsets from a sarcoidosis patient and a healthy control (Figures 2*A* and 2*B*), and on lymphocyte subsets from *in vitro* stimulated PBMCs (Figure 2*C*).

*RT-PCR*

The expression of CD103 mRNA was analyzed by quantitative RT-PCR amplification. Total RNA was isolated from cultured PBMC using de RNeasy microkit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. RNA concentration and purity was determined using the NanoDrop. 0.2 µg RNA was used for first-strand cDNA synthesis using the i-script cDNA synthesis kit (Biorad, Veenendaal, The Netherlands). The obtained

cDNA was diluted 1/10 with water of which 4 µl was used for amplification in a reaction volume of 20 µl. Primer sets were purchased from Sigma. The PCR was performed with the $RT^2$ Real-Time™ SYBR Green PCR master mix (SA-Biosciences, Frederick, USA) according to the manufacturer's protocol. Samples were amplified using a Biorad MyiQ real time PCR detection system for 40 cycles (10 sec at 95 ˚C, 20 sec at 61 ˚C and 25 sec at 72 ˚C). The copy number of the CD103 gene was normalized by the housekeeping gene β-actin, and is presented as the number of transcripts per 1 copy of β-actin.

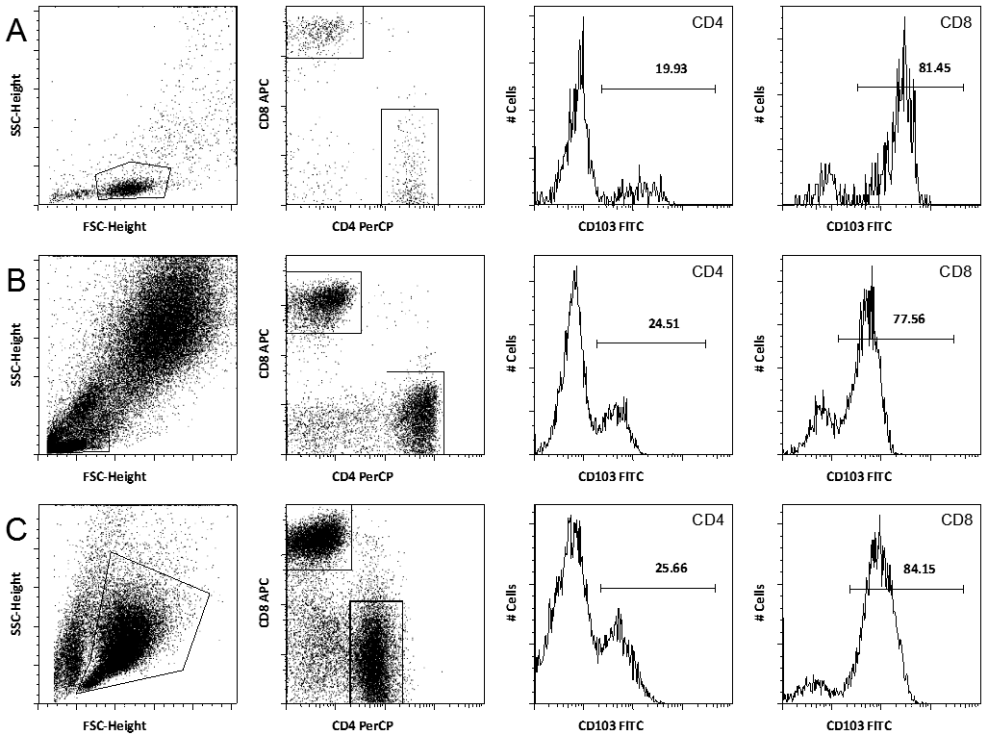


**Figure 2** Representative flow cytometric analysis of CD103 expression on BALF lymphocyte subsets from a sarcoidosis patient (*A*), on BALF lymphocyte subsets from a healthy control (*B*) and on lymphocyte subsets from *in vitro* stimulated PBMCs (*C*). Numbers indicate the percentage of CD103 positive cells.

Statistical analysis

*Single SNP analysis*. Allele and genotype frequencies were calculated for each locus and tested for Hardy–Weinberg equilibrium (HWE) in controls. Case-control association studies were analyzed by $\chi^2$ test using 2 x 3 and 2 x 2 contingency tables of genotype and allele frequencies, respectively. Odds ratios and confidence intervals (CIs) were calculated with an online tool, available at http://ihg2.helmholtz-muenchen.de/ihg/snps.html.

For SNP association analysis the significance threshold was set after accounting for multiple comparisons using a Bonferroni correction for the effective number of independent SNPs ($P_{corrected}$: 0.05/5 SNPs = 0.01)[26,27].
Population-specific Linkage Disequilibrium (LD) block structure was examined using Haploview[28]. We used Lewontin's D' statistic and $r^2$ as measures of LD strength[29]. Individual haplotypes were inferred using PHASEv2 software[30].
ANOVA or Student's *t*-test was used to test differences between genotypes comparing CD103 mRNA, CD103 protein expression or lung function parameters.
A p value < 0.05 was considered significant.

## Results

*ITGAE SNP disease association analysis*

Table 1 summarizes the genotype and allele frequencies of the investigated *ITGAE* SNPs (Figure 1) in sarcoidosis patients and controls. Genotype data from all populations conformed to Hardy-Weinberg equilibrium. No linkage disequilibrium was observed between the 5 SNPs (D' < 0.55, $r^2$ < 0.18) (Figure 3).
The most significant disease association was with *ITGAE* -1088 A/G ($p_{allele}$ = 0.004, $p_{corrected}$ = 0.02; odds ratio (OR) = 1.31, 95% confidence interval (CI) = 1.09-1.57). The second significant associated SNP was *ITGAE* 86141 ($p_{allele}$ = 0.008, $p_{corrected}$ = 0.04; OR = 1.27 (1.07-1.53). For both SNPs, the recessive coding resulted in the best model conferring an increased risk of disease for homozygous carriage of the A allele at *ITGAE* -1088 and homozygous carriage of the G allele at *ITGAE* 86141 (p = 0.001, $\chi^2$ = 10.7, OR = 1.63 (1.22-2.17) and p = 0.003, $\chi^2$ = 8.7, OR = 1.56 (1.16-2.09), respectively).
The -1088 A/G and the 86141 A/G frequencies did not differ between patients with Löfgren's syndrome and controls (p = 0.43 and p = 0.15, respectively).
The remaining three *ITGAE* polymorphism frequencies did not differ between the total patient population and controls.
Phase reconstruction of individual haplotypes from genotypes at sites -1088 and 86141 revealed arbitrary haplotype assignment in 23% of the individuals (probability of haplotype assignment < 0.8). This is caused by the low D' (0.04) and $r^2$ (0) values between the two sites (Figure 3).
After permutation analysis (100,000 permutations) the single marker comparison at -1088 remained significant (-1088 A allele, $p_{permutated}$ = 0.02; 86141 G allele, $p_{permutated}$ = 0.05).
Further data analysis was carried under recessive gene model for site -1088 comparing AA homozygous individuals with individuals carrying either AG or GG.

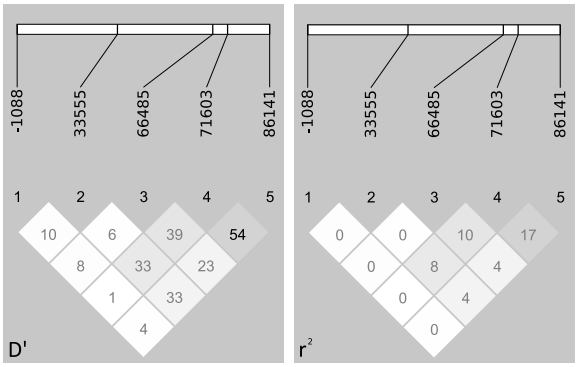


**Figure 3** Pair-wise linkage disequilibrium values of *ITGAE* SNPs in a Dutch population. Values of the pair-wise D' (×100) and $r^2$ (×100) are shown in blocks.

**Table 1** *ITGAE* genotype and allele frequencies in Dutch Caucasian sarcoidosis patients and controls

| *ITGAE* SNP | Genotype and allele | Controls (*n* = 466) | Sarcoidosis (*n* = 465) | Löfgren's (*n* = 81) |
|---|---|---|---|---|
| Promoter; -1088 (A/G) | AA | 0.22 (102) | 0.31 (146) | 0.25 (20) |
| | AG | 0.53 (246) | 0.47 (220) | 0.50 (41) |
| | GG | 0.25 (117) | 0.22 (99) | 0.25 (20) |
| | A | 0.48 (450) | 0.55 (513)[a] | 0.50 (81) |
| | G | 0.52 (480) | 0.45 (419) | 0.50 (81) |
| Intron 2; 33555 (G/T) | GG | 0.52 (244) | 0.47 (221) | 0.51 (41) |
| | GT | 0.39 (179) | 0.45 (209) | 0.41 (33) |
| | TT | 0.09 (40) | 0.08 (36) | 0.09 (7) |
| | G | 0.72 (667) | 0.70 (651) | 0.71 (115) |
| | T | 0.28 (259) | 0.30 (281) | 0.29 (47) |
| Intron 21; 66485 (C/T) | CC | 0.26 (119) | 0.30 (142) | 0.30 (24) |
| | CT | 0.52 (242) | 0.51 (236) | 0.52 (42) |
| | TT | 0.22 (104) | 0.19 (89) | 0.19 (15) |
| | C | 0.52 (480) | 0.56 (520) | 0.56 (90) |
| | T | 0.48 (450) | 0.44 (414) | 0.44 (72) |
| Exon 24; 71603 (C/T) Arg950Trp | CC | 0.41 (192) | 0.42 (195) | 0.43 (35) |
| | CT | 0.47 (218) | 0.43 (201) | 0.43 (35) |
| | TT | 0.12 (55) | 0.15 (70) | 0.14 (11) |
| | C | 0.65 (602) | 0.63 (591) | 0.65 (105) |
| | T | 0.35 (328) | 0.37 (341) | 0.35 (57) |
| Intron 30; 86141 (A/G) | AA | 0.27 (125) | 0.23 (108) | 0.22 (18) |
| | AG | 0.51 (237) | 0.46 (215) | 0.48 (39) |
| | GG | 0.22 (103) | 0.31 (143) | 0.30 (24) |
| | A | 0.52 (487) | 0.46 (432) | 0.46 (75) |
| | G | 0.48 (443) | 0.54 (502)[b] | 0.54 (87) |

Data are provided as genotype and allele frequencies with absolute numbers in parentheses.
[a]Sarcoidosis vs. controls: p = 0.004, $p_c$ = 0.02, $\chi^2$ = 8.3, OR = 1.31, 95% CI: 1.09 - 1.57 (df = 1)
[b]Sarcoidosis vs. controls: p = 0.008, $p_c$ = 0.04, $\chi^2$ = 7.0, OR = 1.27, 95% CI: 1.07 - 1.53 (df = 1)

*ITGAE polymorphisms and CD103 expression on BALF lymphocytes*

*Healthy subjects.* CD103 expression was measured on BALF lymphocytes of 30 healthy subjects. In accordance with the recessive model, we compared CD103 expression between *ITGAE* -1088 AA vs. AG + GG genotypes. CD103 expression per cell (Median Fluorescence Intensity, MFI) and the percentage of CD103 positive lymphocytes were not significantly different between the two groups (data not shown). Separate analysis of BALF $CD8^+$ and $CD4^+$ T cell subsets revealed higher CD103 expression (MFI, median [range]) on $CD8^+CD103^+$ T lymphocytes (165 [89 - 290] vs. 92 [61 - 197], $p < 0.0001$), however there was no genotypic effect (data not shown).

*Sarcoidosis patients.* In sarcoidosis patients however, a significant difference was found between *ITGAE* -1088 AA vs. AG + GG genotypes comparing the percentage of BALF $CD103^+CD4^+$ lymphocytes (median [range], AA: 13.4 [1.6-49.3] vs. AG + GG: 5.0 [0.7-40.3], $p = 0.017$) and the $CD103^+CD4^+/CD4^+$ ratio (AA: 19 [2-62] vs. AG + GG: 9 [1-61], $p = 0.017$) (Figures 4*B* and 4*D*).

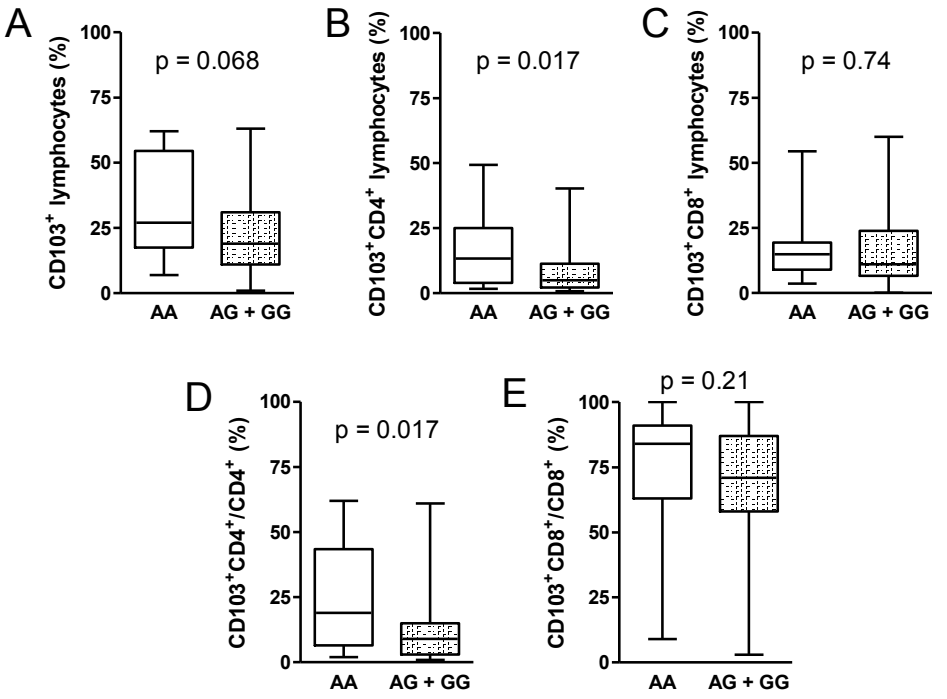


**Figure 4** Boxplots illustrating the *ITGAE* promoter -1088 genotypes and the percentage of $CD103^+$, $CD103^+CD4^+$ and $CD103^+CD8^+$ BALF lymphocytes (*A-C*) and the $CD103^+CD4^+/CD4^+$ and $CD103^+CD8^+/CD8^+$ ratios (*D* and *E*) in sarcoidosis patients (AA: *n* = 21, AG + GG: *n* = 51). Box whiskers indicate the 25$^{th}$ and 75$^{th}$ percentiles (*box*), median (*horizontal bar*), and the minimum and maximum values (*vertical topped bars*).

*Influence of ITGAE promoter polymorphism -1088 A/G on CD103 expression in vitro*

Genetic variance in promoter regions may influence gene expression levels.

PBMCs from 17 healthy subjects were cultured and stimulated with anti-CD3/CD28 and TGF-β1 (5 ng/ml) for 4 days. CD103 mRNA expression induced by TGF-β1 after 24 hours, stratified according to genotype at position -1088 is shown in Figure 5. Basal CD103 mRNA expression levels, corrected for β-actin mRNA expression, suggested that *ITGAE* was expressed higher in AA individuals than in individuals with AG + GG genotypes, but the results were not conclusive owing to large inter-individual variation (median [range]; 0.034 [0.017-0.046] vs. 0.018 [0.009-0.056], p = 0.23). Figure 6 illustrates CD103 protein expression on T cells for AA vs. AG + GG after four days stimulation. Both the percentage of $CD103^+CD4^+$ lymphocytes (Figure 6*A*) and the proportion of $CD4^+$ cells positive for CD103 expression ($CD103^+CD4^+/CD4^+$ ratio, Figures 6*B* and 7) were higher in AA vs. AG + GG (13.5 [12.5-19.5] vs. 11.7 [6.1-13.8], p = 0.009 and 28.8 [21.0-32.8] vs. 21.4 [13.8-29,0], p = 0.01, respectively). No differences were observed between genotypes comparing CD103 expression on $CD8^+$ cells after four days of stimulation (data not shown).

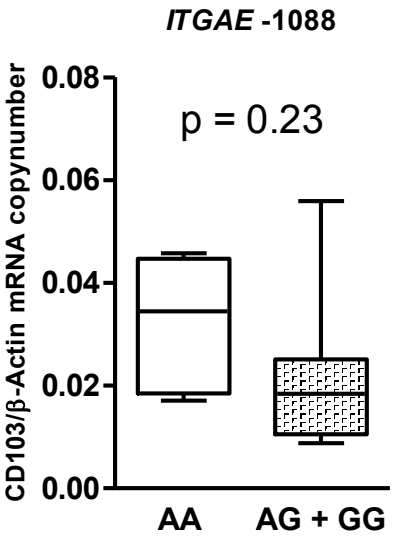


**Figure 5** Boxplot illustrating the *ITGAE* promoter -1088 AA (*n* = 5) vs. AG + GG (*n* = 12) genotypes and CD103 mRNA expression after stimulation of PBMC with TGF-β1 for 24 hours. Box whiskers indicate the 25$^{th}$ and 75$^{th}$ percentiles (*box*), median (*horizontal bar*), and the minimum and maximum values (*vertical topped bars*).

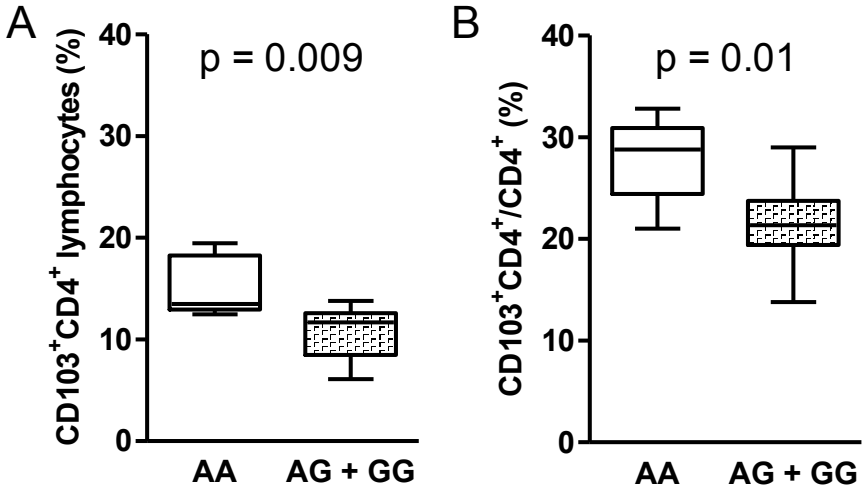


**Figure 6** Boxplots illustrating the *ITGAE* promoter -1088 AA (*n* = 5) vs. AG + GG (*n* = 12) genotypes and the percentage of $CD103^+CD4^+$ lymphocytes (*A*) and the $CD103^+CD4^+/CD4^+$ ratio (*B*) from *in vitro* stimulated PBMC. Box whiskers indicate the 25th and 75th percentiles (*box*), median (*horizontal bar*), and the minimum and maximum values (*vertical topped bars*).

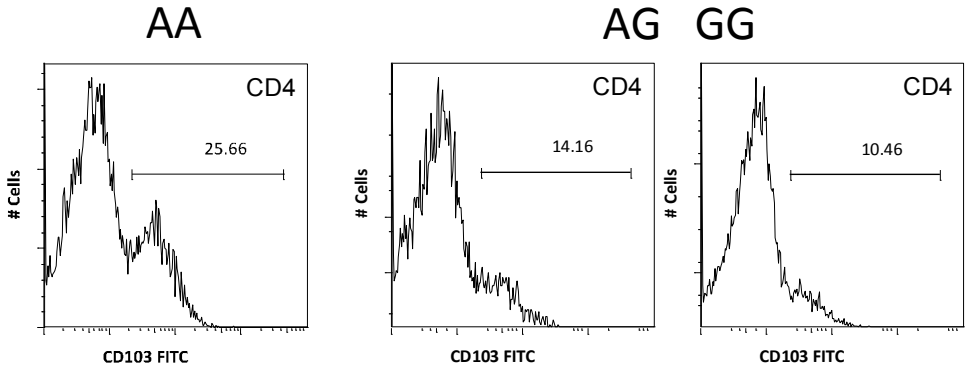


**Figure 7** Representative flow cytometric analysis of CD103 expression on $CD4^+$ cells from *in vitro* stimulated PBMCs with *ITGAE* -1088 AA vs. AG and GG genotypes. Numbers indicate the percentage of CD103 positive cells.

*Correlation of ITGAE promoter polymorphism -1088 A/G with clinical parameters in sarcoidosis patients*

We examined the correlation of -1088 A/G *ITGAE* with lung function parameters in sarcoidosis patients measured at presentation. Neither $FEV_1$ nor iVC or Dl*co* values were significantly different when grouped according to -1088 AA vs. AG + GG genotypes (data not shown).

Radiographic staging at presentation failed to show an association with *ITGAE* AA vs. AG + GG genotypes (data not shown). Genotype frequencies were compared between patients

with normal pulmonary disease outcome (0) or chest x-ray stage I, II, III or IV after a minimum of 4 years follow-up.

The most significant difference at pulmonary disease outcome was found between patients with normalized chest radiography (*n* = 146) and patients with fibrosis (stage IV, *n* = 70) comparing AA vs. AG + GG genotype frequencies (p = 0.012, $\chi^2$ = 6.3, OR = 2.16 (1.18-3.97)). Compared to controls, the frequency of -1088 AA genotype in patients with fibrosis was also higher (0.41 vs. 0.22, p = 4.1 × $10^{-4}$, $\chi^2$ = 12.5, OR = 2.52 (1.49-4.3)) but not in patients with normalized chest radiography (0.25 vs. 0.22, p= 0.49).

## Discussion

This study is the first to describe the genetic variation of the *ITGAE* gene, encoding for the alpha chain of the $\alpha_E\beta_7$ integrin, in sarcoidosis patients. We demonstrated that a promoter polymorphic site at position -1088 A/G is a susceptibility locus for sarcoidosis. The magnitude of the odds ratio was comparable with odds ratios reported for genetic associations of related integrins with other (chronic) inflammatory diseases[16,18]. Our data implicate the *ITGAE* pathway in the pathogenesis and pulmonary disease outcome of sarcoidosis.

*ITGAE* -1088 AA genotypes consistently showed significantly higher values of CD103 expression on BALF $CD4^+$ lymphocytes *in vivo* and on $CD4^+$ cells *in vitro* after stimulation with anti-CD3/CD28 and TGF-β1. Moreover, -1088 *ITGAE* AA genotype was more prevalent in patients with pulmonary fibrosis at disease outcome vs. patients with normalized chest radiography.

*ITGAE* genotype or allele frequencies revealed no association with Löfgren's syndrome, supporting the concept of Löfgren's syndrome as a genetically distinct disease entity, different from sarcoidosis. Recently, integrins have been an area of interest in (chronic) inflammatory diseases such as multiple sclerosis[16,17] and SLE[18,19]. In sarcoidosis, increased expression of integrins VLA-4 and VLA-5 on BALF lymphocytes has been reported by Berlin et al.[31], and increased expression of $\beta_1$ and $\beta_2$-integrins on peripheral blood monocytes and alveolar macrophages has been found in patients with active sarcoidosis compared to patients with inactive disease[32-34]. Interestingly, radiographic staging showed that parenchymal involvement (stages II and higher) correlated with a significantly higher percentage of CD103 positive $CD4^+$ lymphocytes[35,36].

The two associated *ITGAE* SNPs, approximately 87 kb apart, were completely out of LD (D' = 0.04, $r^2$ = 0). The promoter -1088 A/G SNP might be the causal marker by influencing

CD103 expression levels, and confers the loss of a *cap* site in -1088 G. Robinson and colleagues tested human *ITGAE* promoter constructs in mouse T cell lines and found that the region -516 bp to -1096 bp was required for responsiveness to PMA/ionomycin[37]. The intron 86141 A/G SNP may confer functionality by linkage with a regulatory 3' UTR site or other unknown markers which affect the quality or quantity of the gene product.
The percentage of $CD103^+CD4^+$ BALF lymphocytes was found to be higher in -1088 homozygous AA sarcoidosis patients. To test genotypic effects of the *ITGAE* -1088 variants, functional *in vitro* experiments were performed. CD103 expression induced by stimulating PBMCs with TGF-β1 and anti-CD3/CD28 revealed increased percentage of $CD103^+CD4^+$ lymphocytes for the AA genotype. CD103 mRNA expression did not reach significance between genotypes which might be explained by the preferential CD103 expression on $CD8^+$ lymphocytes[38]. When multiplied by the ratio of CD103 positive $CD4^+$ and $CD8^+$ lymphocytes, CD103 mRNA expression indeed was significantly higher in AA vs. AG + GG genotypes (data not shown). Measuring mRNA expression in sorted $CD4^+$ and $CD8^+$ populations might have shown amplification of the genotype effect in $CD4^+$ cells and not in $CD8^+$ cells.

Altered CD103 expression may lead to an altered intraepithelial lymphocyte (IEL) localization, shape, locomotion and binding to E-cadherin[14,39] which is likely to modulate functional responses of both IEL and epithelial cells[40]. $CD103^+$ lymphocytes are long-lived, chronically activated T lymphocytes phenotypically characterized by expression of chronic activation markers[41]. These cells express a unique $T_H2$ cytokine pattern with emphasis on IL-13[42], which is classified as having pro-fibrotic properties and mediates induction of TGF-β1 dependent tissue fibrosis[43,44]. It has been shown that some of the major effects of IL-4 and IL-13 on the airway involve direct effects on epithelial cells[45,46]. Taken together, antigen exposure and the presence of TGFβ-1 may enhance CD103 expression on $CD4^+$ cells in AA individuals. The localization near and interaction of these cells with lung mucosal epithelia might increase the risk of interstitial damage and ultimately of fibrosis formation in pulmonary sarcoidosis.
Our data showed that carriage of the *ITGAE* -1088 AA genotype predisposes to sarcoidosis. However, it is plausible that additional triggers are required for the development and progression of sarcoidosis, which is consistent with a complex disease model in which multiple genes and environmental factors contribute to the phenotype. Since our data are from Caucasian patients, it would be interesting to see whether the same genetic predisposition exists in patients of African descent. Further research is necessary to confirm *ITGAE* as a fibrosis gene in sarcoidosis and to investigate the effect of CD103

functionality (expression or its binding to E-cadherin) in relation to parenchymal damage or fibrogenic inflammation.

## Conclusion

We identified a sarcoidosis susceptibility locus, *ITGAE*, and showed that the -1088 AA genotype correlated with increased $CD103^{+}CD4^{+}$ BALF lymphocytes and fibrosis on chest x-ray at disease outcome. Functional studies revealed that variation at position -1088 A/G correlated with higher CD103 expression on $CD4^{+}$ cells. Our data implicate the $\alpha_E\beta_7$ integrin in the pathophysiology of pulmonary sarcoidosis.

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# 5

## Increased CD103$^{+}$ lymphocytes in lungs of sarcoidosis patients with parenchymal involvement

*Submitted*

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## Abstract

Intraepithelial lymphocytes (IEL)s are present near all mucosal areas. These cells express CD103 which binds E-cadherin on epithelial cells. IELs are thought to be involved in the first line of defence against pathogens, in close relation with epithelial cells.

We examined the activation status of CD103 positive lymphocytes from bronchoalveolar lavage fluid (BALF). BAL was performed on 23 patients presenting with active pulmonary sarcoidosis and 14 healthy subjects. Expression of CD4, CD8, CD25, CD28, CD69, VLA-1 and CD103 on BALF lymphocytes was determined by flow cytometry.

$CD103^+$ lymphocytes were highly activated and expressed a chronic activation profile compared to non-mucosal lymphocytes. The chronic activated phenotype expressed on $CD103^+$ lymphocytes was characterized by $CD28^{dim}$, $CD69^{high}$ and $VLA\text{-}1^{high}$, and was similar in healthy controls and sarcoidosis patients with and without parenchymal involvement on chest radiography. In twenty sarcoidosis patients, the percentage of $CD103^+$ BALF lymphocytes at presentation revealed a high prognostic value for parenchymal involvement at pulmonary disease outcome after 4-year follow-up (area under the ROC curve, 0.91).

Lung $CD103^+$ lymphocytes were highly activated and expressed a chronic activation profile compared to non-mucosal lymphocytes in healthy controls and in sarcoidosis patients. The proportion of CD103 positive BALF lymphocytes at presentation might be used as a new prognostic biomarker for parenchymal involvement at pulmonary disease outcome in sarcoidosis.

## Introduction

Sarcoidosis is a systemic disorder characterized by the formation of non-caseating granulomas, with the lungs, lymph nodes and skin being the most affected (recently reviewed in[1]). The involved organs are invaded mainly by CD4$^+$ $T_{Helper}1$ ($T_H1$) lymphocytes. Although granulomas may often resolve spontaneously, pulmonary fibrosis occurs in 10 to 15% of patients with sarcoidosis. The factors which contribute to the development of pulmonary fibrosis in sarcoidosis remain uncertain but carriage of transforming growth factor β3 (TGF-β3) risk alleles[2], production of CCL18 chemokine by alveolar macrophages[3] and a shift towards $T_{Helper}2$ cell ($T_H2$) response and the production of $T_H2$ associated cytokines[4] may enhance progression with fibrotic development and damage to the lung epithelium and parenchyma.

The mucosal epithelium represents a unique lymphoid compartment containing a distinct population of lymphocytes, the intraepithelial lymphocytes (IEL). The $\alpha_E\beta_7$/CD103$\beta_7$ integrin is regarded as the receptor mediating retention of these lymphocytes in mucosal tissues of gut, urogenital tract and lung. CD103$\beta_7$ binds to E-cadherin at the basolateral side of the epithelium[5,6] and determines ligand directed shape and locomotion of CD103$^+$ cells[7]. CD103 is expressed by lymphocytes within the bronchial epithelium, by some alveolar lymphocytes and by T-lymphocytes in the bronchoalveolar fluid[8]. Epithelial cells produce TGF-β that promotes expression of lymphocyte CD103 and the long term survival of IELs that may function as a pool of memory cells primed to respond rapidly to antigens[9]. Patients with interstitial lung diseases show an aberrant pattern of CD103 expression on lymphocytes from bronchoalveolar lavage depending on the type of disease. Patients with idiopathic pulmonary fibrosis (IPF) and hypersensitivity pneumonitis (HP) have a significantly higher proportion of CD4$^+$ T cells bearing CD103 compared to patients with sarcoidosis, while most of the CD8$^+$ T cells express this integrin independently of the disease of the lung[10,11]. Furthermore, Braun and colleagues[12] reported that in fibrotic lung diseases the CD103$^+$ population displayed a distinct phenotype of continuously activated long-living cells characterized as CD103$^+$CD25$^{dim}$CD27$^-$CD28$^{low}$CD69$^+$VLA-1$^+$. In addition to TGF-β1, T cell receptor mediated signals are needed to maintain CD103 expression[13]. Taken together, these findings suggest a distinctive role for CD103$^+$ lymphocytes in interstitial lung disease associated with fibrosis. However, little is known about the phenotype of CD103$^+$ lymphocytes in sarcoidosis, especially in the acute/non-fibrotic disease phenotype, or in healthy subjects for that matter.

We hypothesized that the expression of activation markers on CD103$^+$ lymphocytes in the acute, self-limiting alveolitis in sarcoidosis may be different from CD103$^+$ lymphocytes in

sarcoidosis patients presenting with chronic manifestations with a tendency towards fibrosis. In addition, we investigated the prognostic value of the percentage of $CD103^+$ BALF lymphocytes at presentation for pulmonary disease outcome after 4-year follow-up.

## Subjects & Methods

*Subjects*

Twenty-three consecutive patients, all Caucasian, presenting to our department because of symptomatic sarcoidosis and who had ≥ 15% lymphocytes in bronchoalveolar lavage, a criterion for active pulmonary alveolitis, were included in this study. Table 1 summarizes the clinical characteristics of the patient group. The diagnosis of sarcoidosis was established on the basis of clinical findings and histological evidence of non-caseating epithelioid granulomas and after exclusion of other known causes of granulomatosis in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis[14]. In 6 patients, the diagnosis was made without biopsy proof because these patients presented with the classic symptoms of Löfgren's syndrome, namely fever, erythema nodosum, arthralgia and bilateral hilar lymphadenopathy. All patients were steroid naïve at time of inclusion in the study. Pulmonary disease severity at presentation was evaluated by chest radiography. In brief, severity score comprises five stages: stage 0, normal, stage I, bilateral hilar lymphadenopathy (BHL), stage II, BHL and parenchymal infiltration, stage III, parenchymal infiltration without BHL, stage IV, irreversible fibrosis with loss of lung volume. Chest radiographic staging at presentation showed that 14 patients presented with stage I (including 6 patients diagnosed with Löfgren's syndrome), 4 patients presented with stage II, 4 patients presented with stage III and 1 patient presented with stage IV. For further analysis, patients were divided into a group presented without parenchymal involvement (stage I, n = 14) and a group presented with parenchymal involvement (stage II and higher, n = 9). In addition, 14 randomly selected healthy controls were included in the study (Table 1).

A separate patient group was included for analysis of the prognostic value of the percentage of $CD103^+$ BALF lymphocytes and consisted of 20 sarcoidosis patients presenting with lymphadenopathy on chest x-ray (radiographic stage I or II). The percentage of $CD103^+$ BALF lymphocytes and radiographic staging were assessed at time of diagnosis. The diagnosis of sarcoidosis was established as described above. All patients were steroid naïve at time of diagnosis. Pulmonary disease outcome was defined by radiography after minimal 4-year follow-up.

Verbal and written consent was obtained from all subjects, and authorization was given by the Medical-Ethical Committee of the St. Antonius Hospital, Nieuwegein.

*Bronchoalveolar lavage*

BAL was performed during flexible fiberoptic bronchoscopy at the time of the diagnosis according to standardized and validated procedure[15]. The procedure involved detailed explanation to the patient, premedication (0.5 milligram (mg) atropine s.c., 20 mg codeine p.o.), and local anesthesia of the larynx and lower airways (0.5% tetracaine in the oropharynx, 8 cc 0.5% tetracaine in lower airways). BAL was performed, preferably in the right middle lobe, with four 50-ml aliquots of sterile isotonic saline solution (37 °C). The aspirated lavage fluid from the first 50-ml aliquot was kept apart and excluded from further analysis. The BAL fluid (BALF) recovered from the three subsequent aliquots was collected in a siliconized specimen trap and kept on ice. BALF was filtered through nylon gauze and centrifuged (10 min at 400 x g at 4 °C).

The cell pellet was washed twice, counted, and resuspended in minimal essential medium/RPMI 1640 (Gibco; Grand Island, NY), supplemented with 0.5% bovine serum albumin (Organon Teknika; Boxtel, the Netherlands). Cells were counted in a Bürker chamber. Cell viability was determined by Trypan blue exclusion. Smears for cell differentiation were prepared by cytocentrifugation (Shandon; Runcorn, UK). Cell differentiation was performed on cytospinslides after staining with May-Grünwald-Giemsa (Merck; Darmstadt, Germany), at least 2x 500 cells were counted.

*Flow cytometry*

Fifty microliter (µl) BALF (at least 500,000 cells) were incubated for 15 min at room temperature with 10 µl of the following monoclonal antibodies: anti-CD103 fluorescein isothiocyanate (FITC), anti-CD25 phycoerythrin (PE), anti-CD28 PE, anti-CD69 PE, anti-VLA-1 PE, anti-CD4 peridinin-chlorophyll-protein (PerCP) and anti-CD8 allophycocyanin (APC), all from BD Biosciences (San Jose, CA, USA). Immunofluoresence was measured by flow cytometry (FACS-Calibur, Becton Dickinson, San Jose, CA, USA). Lymphocytes were defined based on forward-scatter and side-scatter characteristics, and analyzed for expression of the activation markers. A total of at least 10,000 total events were analyzed with FlowJo software (Tree Star, Inc., Ashland, Oregon).

*Statistics*

Data are expressed as mean ± standard deviation or as stated otherwise. Multiple groups were compared performing one-way ANOVA with post hoc test by Newman-Keuls. Paired

t-test was used comparing $CD103^-$ and $CD103^+$ cell populations. ROC curve analysis was used to test the prognostic value of BALF $CD103^+$ cells for evolution towards radiographic stage III or IV at pulmonary disease outcome. Considering multiple comparisons, a p value of less than 0.01 was regarded as significant. The statistical evaluation of our data was performed using SPSS 15.0 (SPSS Inc; Chicago, IL, USA) and Graphpad Prism v.5 (Graphpad Software, Inc., San Diego, USA) software packages.

**Table 1** Characteristics of study subjects

| | Sarcoidosis | Healthy controls |
|---|---|---|
| Subjects (*n*) | 23 | 14 |
| Age (years)[a] | 41.2 [24-65][c] | 21.2 [20-26] |
| Chest X-ray stages, I/II/III/IV (*n*) | 14/4/4/1 | NA |
| Löfgren's syndrome (*n*) | 6 | |
| BALF[b] | | |
| Cellular concentration (x $10^4$ cells/ml) | 31.9 ± 17.5[c] | 11.4 ± 5.8 |
| Lymphocytes (%) | 46.5 ± 20.5[c] | 10.0 ± 6.7 |
| Lung function parameters[a] | | |
| $FEV_1$ % pred (*n* = 18/14) | 91 [59-115][d] | 107 [84-121] |
| FVC % pred (*n* = 18/14) | 95 [65-116][e] | 111 [80-128] |
| Dl*co* % pred (*n* = 18/14) | 80 [44-105] | NA |

[a]Values are given as the mean [range]. [b]Values are expressed as mean ± S.D.
[c]Significantly different from healthy controls ($p < 0.0001$)
[d]Significantly different from healthy controls ($p = 0.0009$)
[e]Significantly different from healthy controls ($p = 0.001$)

## Results

*Frequency of $CD103^+$ lymphocyte subpopulations in the lung*

The integrin CD103 is expressed on a fraction of lymphocytes in the lung. We determined the percentage of BALF lymphocytes co-expressing CD103 and CD4 or CD8 (Figure 1). Significant differences in CD103 positive lymphocyte subpopulations were found between healthy subjects and patients with different sarcoidosis phenotypes defined by chest radiography. Patients with parenchymal involvement (stages II and higher) showed a significantly higher percentage (mean ± S.D.) of $CD103^+CD4^+$ lymphocytes in BALF (14.2 ± 11.4) compared to patients without parenchymal involvement (stage I and Löfgren's patients) (4.9 ± 3.3, $p < 0.01$) and healthy subjects (5.8 ± 2.8, $p < 0.01$). In contrast, the percentage of $CD103^+CD8^+$ lymphocytes was higher in healthy subjects (40.6 ± 13.1) compared to sarcoidosis patients with parenchymal involvement (17.9 ± 14.9, $p < 0.001$) and compared to sarcoidosis patients without parenchymal involvement (10.1 ± 9.9, $p < 0.001$) (Figure 2).

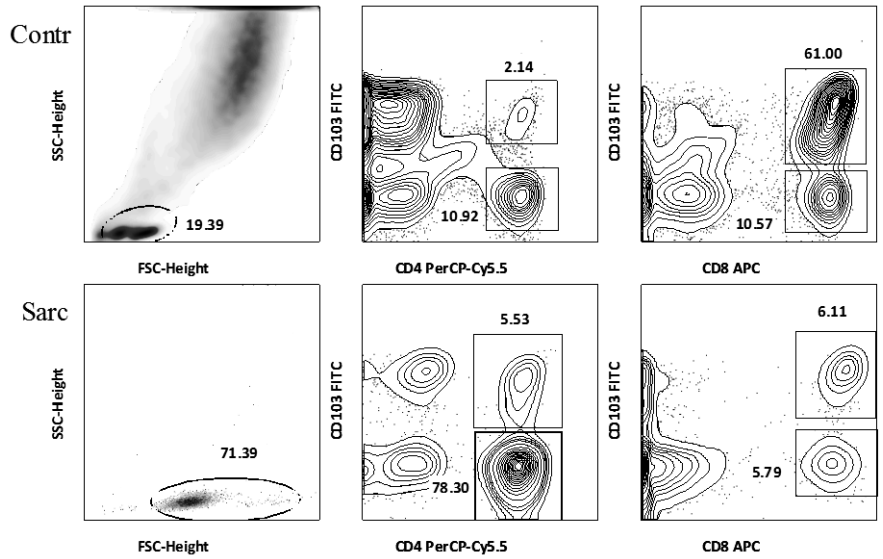


**Figure 1** Representative flow cytometric analysis of co-expression of CD103 and CD4, and co-expression of CD103 and CD8 on BALF lymphocytes (defined by FSC-SSC characteristics) from a control subject and sarcoidosis patient.

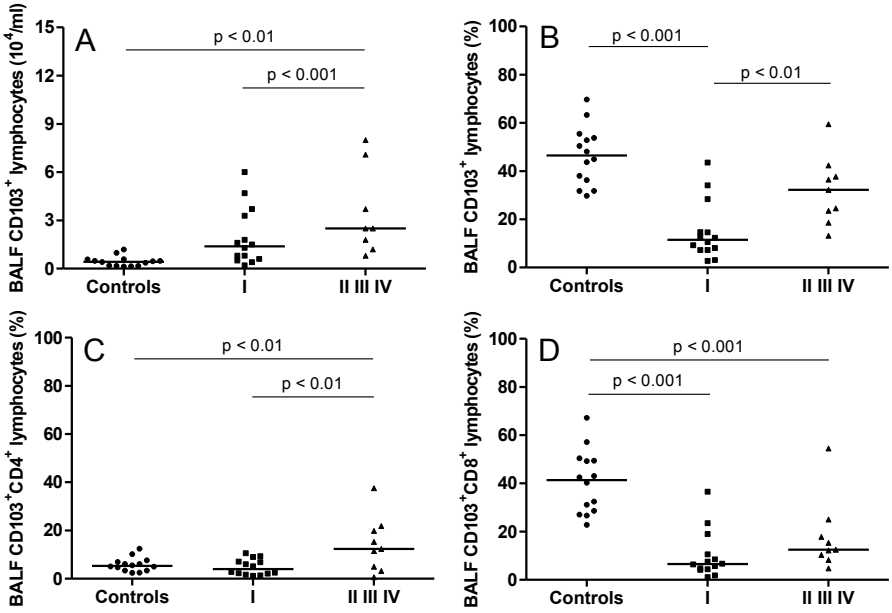


**Figure 2** Percentages of (A) CD103$^+$, (B) CD103$^+$CD4$^+$ and (C) CD103$^+$CD8$^+$ BALF lymphocytes in healthy subjects (n = 14), sarcoidosis patients without parenchymal involvement (n = 14) and sarcoidosis patients with parenchymal involvement (n = 9). The horizontal bars represent median values.

*Expression of activation markers on BALF CD103$^+$ and CD103$^-$ cells*

In order to study potential differences in activation status between CD103$^-$CD4$^+$ and CD103$^+$CD4$^+$ cells, the expression of CD25, CD28, CD69 and VLA-1 was analyzed. CD103$^+$ cells showed higher expression of CD69 and VLA-1 and lower expression CD28. The differences between these two lymphocyte populations were found consistently in healthy subjects and in sarcoidosis patients, irrespective of the clinical phenotype. The data are summarized in Table 2. The expression of CD25 (IL-2 receptor) differed significantly between CD103$^+$CD4$^+$ and CD103$^-$CD4$^+$ lymphocytes only in healthy controls. The expression of activation markers on the CD8$^+$ subset revealed similar differences between CD103$^-$CD8$^+$ and CD103$^+$CD8$^+$ populations (Table 3). A representative flow cytometric analysis of the aforementioned activation markers expressed by CD103$^+$CD4$^+$, CD103$^-$CD4$^+$, CD103$^+$CD8$^+$ and CD103$^-$CD8$^+$ BALF lymphocytes is shown in Figure 3.

**Table 2** Expression of activation markers on the surface of CD103$^-$CD4$^+$ and CD103$^+$CD4$^+$ lymphocytes in the bronchoalveolar lavage of sarcoidosis patients and healthy subjects

| | CD103-CD4+ lymphocytes ± S.D. | | | CD103+CD4+ lymphocytes ± S.D. | | |
|---|---|---|---|---|---|---|
| Marker | Controls | Stage I | Stage II, III, IV | Controls | Stage I | Stage II, III, IV |
| CD25 | 60.4 ± 12.2[b] | 19.7 ± 9.1 | 23.7 ± 10.0 | 29.2 ± 17.7[a] | 22.3 ± 15.8 | 11.1 ± 8.0 |
| CD28 | 98.2 ± 1.3 | 98.7 ± 1.8 | 96.2 ± 3.1 | 79.2 ± 9.5[a] | 74.1 ± 18.7[a] | 67.9 ± 17.1[a] |
| CD69 | 56.3 ± 11.5 | 60.6 ± 15.4 | 74.7 ± 16.8 | 92.5 ± 4.3[a] | 92.4 ± 5.7[a] | 94.3 ± 6.1[a] |
| VLA-1 | 18.8 ± 7.5 | 17.1 ± 8.7 | 33.5 ± 14.1[c] | 57.8 ± 9.5[a] | 61.3 ± 15.7[a] | 73.9 ± 10.0[a] |

The data of all groups were normally distributed. Differences in expression between CD103$^-$CD4$^+$ and CD103$^+$CD4$^+$ lymphocytes were statistically analyzed by Paired t test.

[a] $p < 0.001$, compared to CD103$^-$CD4$^+$ lymphocytes

[b] $p < 0.001$, compared to stage I and stage II, III, IV within CD103$^-$CD4$^+$ subpopulation

[c] $p < 0.01$, compared to controls and stage I within CD103$^-$CD4$^+$ subpopulation

**Table 3** Expression of activation markers on the surface of CD103$^-$CD8$^+$ and CD103$^+$CD8$^+$ lymphocytes in the bronchoalveolar lavage of sarcoidosis patients and healthy subjects

| | CD103-CD8+ lymphocytes ± S.D. | | | CD103+CD8+ lymphocytes ± S.D. | | |
|---|---|---|---|---|---|---|
| Marker | Controls | Stage I | Stage II, III, IV | Controls | Stage I | Stage II, III, IV |
| CD25 | 23.8 ± 12.3[b] | 11.7 ± 7.3 | 7.4 ± 4.9 | 25.0 ± 19.4 | 9.9 ± 6.6 | 8.4 ± 6.5 |
| CD28 | 86.4 ± 5.9 | 80.1 ± 10.1 | 80.7 ± 7.4 | 52.9 ± 10.8[a] | 48.9 ± 20.0[a] | 48.7 ± 18.0[a] |
| CD69 | 63.5 ± 8.7 | 65.9 ± 21.8 | 78.7 ± 11.6 | 92.4 ± 3.4[a] | 91.9 ± 6.5[a] | 96.1 ± 2.9[a] |
| VLA-1 | 49.7 ± 18.2 | 48.8 ± 20.8 | 68.8 ± 10.5 | 89.2 ± 5.8[a] | 86.0 ± 9.8[a] | 91.6 ± 4.7[a] |

The data of all groups were normally distributed. Differences in expression between CD103$^-$CD8$^+$ and CD103$^+$CD8$^+$ lymphocytes were statistically analyzed by Paired t test.

[a] $p < 0.001$, compared to CD103$^-$CD8$^+$ lymphocytes

[b] $p < 0.01$, compared to stage I and stage II, III, IV within CD103$^-$CD8$^+$ subpopulation

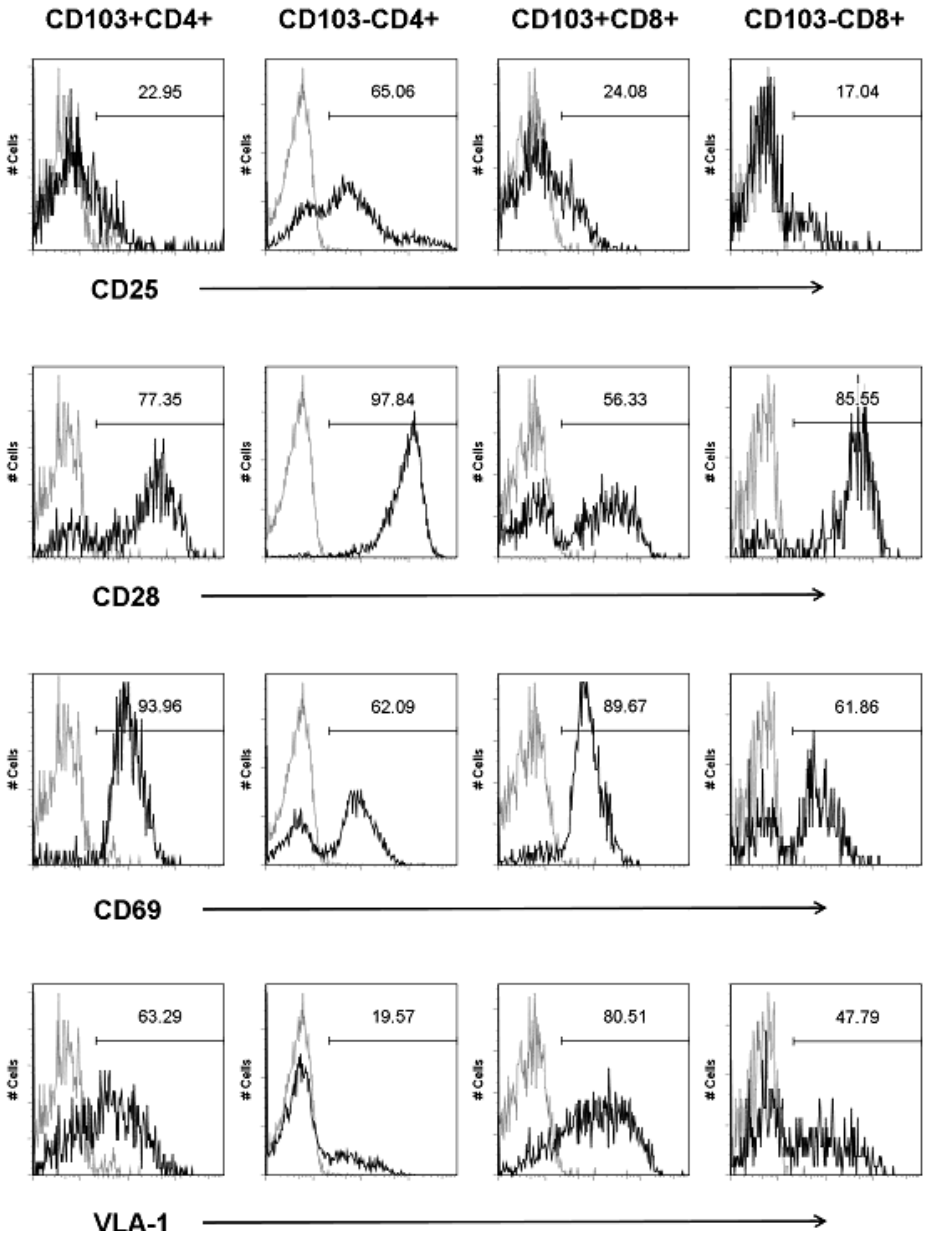


**Figure 3** Cell surface expression of mouse IgG1 isotype, CD25, CD28, CD69 and VLA-1 on gated CD103$^{+}$CD4$^{+}$, CD103$^{-}$CD4$^{+}$, CD103$^{+}$CD8$^{+}$ and CD103$^{-}$CD8$^{+}$ BALF lymphocytes, respectively. Numbers indicate the frequency of cells positive for activation markers in each gated population. The differences between CD103$^{+}$ and CD103$^{-}$ cells are similar for CD4$^{+}$ and CD8$^{+}$ lymphocyte subsets.

*Prognostic value of CD103$^+$ BALF lymphocytes*

We found increased chronic activated CD103 positive BALF lymphocytes in sarcoidosis patients, the highest levels in patients presenting with parenchymal abnormalities.

Next, we sought to determine whether the percentage of CD103 positive BALF lymphocytes might have prognostic value for pulmonary disease outcome, independent of chest x-ray at presentation. In a separate group of twenty sarcoidosis patients who presented with lymphadenopathy on chest x-ray (stage I or II), CD103 expression on BALF cells at presentation and radiographic staging after a minimum of 4-year follow-up were available. The proportion of CD103$^+$ BALF lymphocytes was higher in those patients showing radiographic evolution to stage III and IV compared to patients who stayed in stage I or II or evolved to stage 0, I or II (45.6 ± 11.6 vs. 20.8 ± 14.1, p = 0.0004). ROC curve analysis showed an area under the curve of 0.91 (p = 0.002) (Figure 4A and 4B). The highest likelihood ratio of 8.2 was found at a cut-off level of 27% CD103$^+$ BALF lymphocytes and was accompanied by a sensitivity and specificity of respectively 91% (CI: 59-100) and 89% (CI: 52-100) for the prognosis stage III or IV on chest x-ray at pulmonary disease outcome. The use of corticosteroids did not differ between patients who progressed to stage III or IV and patients who stayed in or evolved to stage 0, I or II (data not shown).

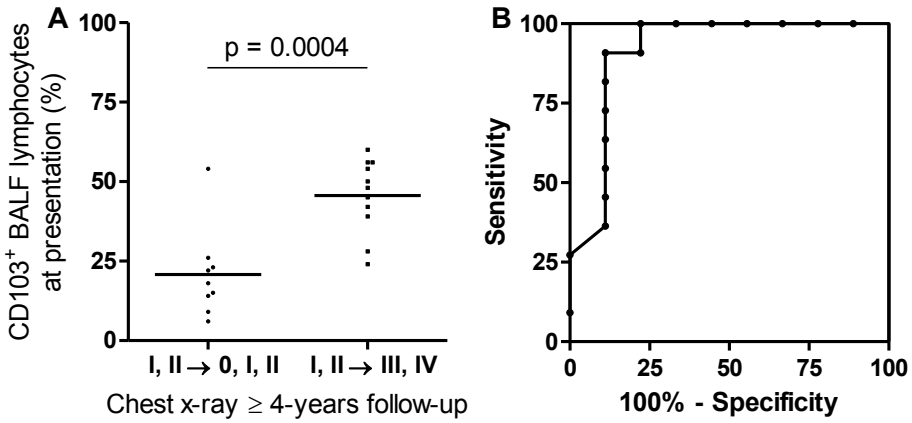


**Figure 4** The percentage of CD103$^+$ BALF lymphocytes in patients presenting with radiographic stage I or II showed prognostic value for radiographic evolution to stage III and IV after a minimum of 4-year follow-up (A). ROC curve analysis revealed an area under the curve of 0.91 (p = 0.002) (B).

## Discussion

The aim of this study was to correlate the activation status of lymphocytes from BALF of patients with active sarcoidosis to clinical progression of the disease. In particular, we focused on BALF lymphocytes that express CD103. The integrin CD103 is typically expressed on mucosal lymphocytes. Recent experiments with CD103 deficient mice suggest that the integrin plays a role in retention of T lymphocytes in or near the mucosal epithelia, interacting with E-cadherin[16].

For the first time, we described a prognostic BALF biomarker for sarcoidosis disease evolution, independent of chest radiography. In a subset of sarcoidosis patients presenting with lymphadenopathy on chest x-ray, the proportion of CD103 positive BALF lymphocytes (both within CD4 and CD8 subsets, data not shown) was increased in patients showing radiographic evolution to stage III and IV versus stage 0, I and II. Therefore, the percentage of CD103$^+$ BALF lymphocytes might be used as a prognostic biomarker for the progression of sarcoidosis to the form with parenchymal involvement. This finding undoubtedly needs to be verified in a much larger patient cohort. Using more accurate high resolution computed tomography (HR-CT) to examine the presence of parenchymal abnormalities or fibrotic lesions in the lung, may improve the prognostic value of the percentage of CD103$^+$ BALF lymphocytes.

We demonstrated that both CD103$^-$ and CD103$^+$ lymphocytes from the lung were highly activated CD69 and VLA-1 positive lymphocytes. However, the CD103$^+$ lymphocytes consistently showed a distinct phenotype of chronic activated T cells. Regardless which subject group or T lymphocyte subset was analyzed, BALF CD103$^+$ lymphocytes expressed significantly less CD28 while expressing significantly more CD69 and VLA-1 compared to CD103$^-$ lymphocytes. CD25 expression was relatively high on CD103$^-$CD4$^+$ lymphocytes of healthy subjects compared to sarcoidosis patients. This might be explained by the vast expansion or influx of activated CD4$^+$ lymphocytes in sarcoidosis patients and the subsequent down modulation or shedding of the IL-2 receptor. In agreement, soluble IL-2R measurements reflect T cell activation and are used to assess disease activity in sarcoidosis[17,18].

Our data showed that both BALF CD103$^+$CD4$^+$ and CD103$^+$CD8$^+$ lymphocytes have a distinct phenotype of continuously stimulated and prolonged activated T lymphocytes. The CD103$^+$CD28$^{dim}$CD69$^{high}$VLA-1$^{high}$ phenotype might reflect their role as first line of defence in the lung[12]. Intriguingly, CD103$^+$ lymphocytes express a unique $T_H2$ cytokine pattern with emphasis on IL-13[19], which is classified as having pro-fibrotic properties and mediates induction of TGF-β1 dependent tissue fibrosis[20]. A switch towards a $T_H2$ response when the $T_H1$ response fails to clear the antigen is considered beneficial in terms of

eliminating the antigen more effectively but subsequently may activate the fibrotic pathway[21].

Corroborative to a fibrogenic role of CD103 positive lymphocytes, Luzina et al. reported recently that, based on observations in patients with diffuse parenchymal lung disease, in an animal model of pulmonary fibrosis, and in *in vitro* experiments the expression of integrins αVβ3 and/or αVβ5 on pulmonary T lymphocytes may regulate the extent of lymphocytic infiltration and the degree of pulmonary fibrosis. Whereas T lymphocytes that do not express integrins may be not involved in the fibrotic regulation process[22].

Moreover, natural occurring regulatory T cells (Treg) are responsible for limiting pathological or physiological immune responses and may play a role in dampening of the $T_H1$ response[23]. CD103 has been put forward as a regulatory T cell marker in peripheral tissue[19,24,25]. It has to be determined whether in sarcoidosis and in healthy subjects the $CD103^+$ subpopulation is indeed a regulatory T cell subset, and whether these cells express Foxp3, the transcription factor that best identifies regulatory T cells[26]. Functional tests with lung $CD103^+$ cells of sarcoidosis patients and healthy subjects may provide answers to these questions.

Speculative, increased amount of chronic activated CD103 positive lymphocytes early in disease may be the result of already damaged lung epithelium or may cause damage to the lung epithelium itself. Either way, this might ultimately lead to parenchymal abnormalities in sarcoidosis patients. Immunohistochemical staining of lung biopsies with anti-CD103 antibodies is required to the proof the localisation of $CD103^+$ lymphocytes adjacent to the epithelium.

## Conclusion

The results described in this chapter demonstrate that lung mucosal lymphocytes are highly activated and express a chronic activation profile compared to non-mucosal BALF lymphocytes. The exact role of $CD103^+$ lymphocytes in sarcoidosis has yet to be elucidated but our data showed that the percentage of $CD103^+$ BALF lymphocytes might be used as a prognostic biomarker for the progression of sarcoidosis to the form with parenchymal involvement. Additional studies using larger groups of sarcoidosis patients are needed to confirm this hypothesis.

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# 6

## Increased expression of CD16, CD69 and VLA-1 on blood monocytes in active sarcoidosis

*Chest 2008; 134(5):1001-1008*

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## Abstract

Different types of immune cells are involved in the formation of granulomas, a hallmark of pulmonary sarcoidosis. Proinflammatory monocytes are activated circulating monocytes thought to be related to the initial events of granuloma formation. We tested the hypothesis that peripheral blood monocytes in patients with active pulmonary sarcoidosis have an activated phenotype, and secondly, that measuring this activation status can provide a new tool for monitoring disease activity. Blood was collected of 23 steroid naive patients presenting with pulmonary sarcoidosis and 10 healthy controls. Expression of CD16 (Fc-$\gamma$ type III receptor), CD69 (a general activation marker of cells of the haematopoietic lineage), and the integrin VLA-1 (upon interaction with ECM compounds, mediates cell adhesion) was measured by flow cytometry. Percentages of monocytes expressing CD16, CD69 and VLA-1 in patients versus controls were 54.6 +/- 4.2 versus 12.2 +/- 2.4 ($p < 0.0001$), 84.5 +/- 3.4 versus 8.6 +/- 3.3 ($p < 0.0001$) and 64.0 +/- 4.3 versus 11.2 +/- 2.3 ($p < 0.0001$), respectively. Moreover, the $CD69^{+}VLA\text{-}1^{+}$ monocyte subset, abundantly present at disease presentation, was found to decrease to normal levels during follow-up with disease remission. Peripheral blood monocytes from patients with pulmonary sarcoidosis show a highly activated phenotype. Phenotyping circulating monocytes might be a promising tool for monitoring sarcoidosis disease activity but needs further investigation.

## Introduction

Sarcoidosis is a systemic, granulomatous disease of unknown origin, primarily affecting the lungs. The disease is characterized by a mononuclear cell alveolitis dominated by activated $CD4^+$ (helper/inducer) T cells and macrophages. The coordinated interplay between these cells leads to the characteristic formation of non-caseating granulomas and, in a subgroup of patients, to fibrosis and permanently impaired lung function[1,2]. Proinflammatory monocytes are activated circulating monocytes that might be related to the initial events that lead to granuloma formation. Proinflammatory monocytes co-express CD14, CD16 and CD69. CD14 is part of the TLR4 membrane receptor complex and expressed on all monocytes. Peripheral blood CD16 (a low affinity Fc-γ type III receptor) positive monocytes have been shown to expand in different pathological conditions, such as cancer, asthma, sepsis, human immunodeficiency virus infection, and AIDS progression (recently reviewed in ref.[3]). CD69 is a general activation marker of cells of the haematopoietic lineage and has been identified as the earliest activation marker on the surface of cytokine- or mitogen activated lymphocytes[4].

Recent studies suggest that interactions between leukocyte-associated integrins and the interstitial matrix may promote the migration and/or activation of extravasated leukocytes (e.g., T cells and monocytes) within the perivascular compartment[5,6]. Consistent with the initial description of $\alpha_1\beta_1$ as very late antigen, in the immune system, VLA-1 is expressed on T cells, natural killer (NK) cells, NKT cells, and macrophages after (long-term) activation by antigen, superantigens, or cytokines[7]. Expression of VLA-1 has been demonstrated on tissue-infiltrating T cells from a variety of chronic inflammatory settings, including the rheumatoid synovium of arthritis patients[8] and the lungs of sarcoidosis patients[9]. Moreover, increased expression of $\beta_2$-integrins on peripheral blood monocytes and alveolar macrophages has been reported in patients with active sarcoidosis compared to patients with inactive disease[10,11] and on peripheral blood monocytes in patients with rheumatoid arthritis[12].

Differences in phenotype of peripheral blood monocytes in patients with pulmonary sarcoidosis have been described, although not extensively[10,13,14]. We hypothesized that considering the systemic character of the disease, peripheral blood monocytes in patients with active pulmonary sarcoidosis are activated and show a proinflammatory phenotype.

The aim of our study was to study the expression of activation markers CD16, CD69 and VLA-1 on monocytes in patients presenting with sarcoidosis and compare the results with clinical phenotypes and conventional biomarkers of sarcoidosis such as angiotensin-converting enzyme levels (ACE) in serum and serum soluble IL-2R levels. Both are well-

recognized markers of disease activity[15,16], where serum ACE levels reflect the granuloma burden and serum sIL-2R levels reflect mainly the activity of the T cell component. In extension, we measured the activation status of blood monocytes in a subgroup of the patients showing disease remission.

## Subjects & Methods

*Subjects*

Twenty-three consecutive patients presenting to our department because of symptomatic sarcoidosis and ≥ 15% lymphocytes in bronchoalveolar lavage, a criterion for active pulmonary alveolitis, were included in this study. Table 1 summarizes the clinical characteristics of the patient group. The diagnosis of sarcoidosis was established on the basis of clinical findings and histological evidence of non-caseating epithelioid granulomas and after exclusion of other known causes of granulomatosis in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis[17]. In 6 patients, the diagnosis was made without biopsy proof because these patients presented with the classic symptoms of Löfgren's syndrome, namely fever, erythema nodosum, arthralgia and bilateral hilar lymphadenopathy. All patients were steroid naïve at time of inclusion in the study. Pulmonary disease severity at presentation was evaluated by chest radiography. In brief, this comprises five stages: stage 0, normal, stage I, bilateral hilar lymphadenopathy (BHL), stage II, BHL and parenchymal infiltration, stage III, parenchymal infiltration without BHL, stage IV, irreversible fibrosis with loss of lung volume. Distribution of chest radiographic stages at presentation showed that 14 patients presented with stage I (including 6 patients diagnosed with Löfgren's syndrome), 4 patients presented with stage II, 4 patients presented with stage III, and 1 patient presented with stage IV. For further analysis, patients were divided into a group presented with lymphadenopathy (radiographic stage I and II, n = 18) and without lymphadenopathy (radiographic stage III and IV, n = 5). For further analysis, patients were divided into a group presented with lymphadenopathy (radiographic stage I and II, n = 18) and without lymphadenopathy (radiographic stage III and IV, n = 5). In addition, 10 randomly selected healthy controls (employees from St Antonius Hospital) were included in the study (mean age [range], 31.3 [23 - 45]; male/female, 6/4). The institutional review board approved the study, and all subjects gave informed written consent.

*Follow-up study*

Follow-up data were available in eight patients. During routine follow-up examination of the patients, peripheral blood was collected for quantification of sACE and sIL-2R levels and immuno-phenotyping of the monocytes with the aforementioned antibodies. The mean time interval between initial presentation and follow-up was 23 ± 4.7 months. Four patients received prednisone medication between disease presentation and follow-up.

*Flow cytometry*

Fifty µl of ethylenediamine tetra acetic acid (EDTA) blood was incubated for 15 min at room temperature with 10 µl of the following mAbs: anti-CD14 FITC, anti-CD16 PE, anti-CD45 PerCP, anti-CD69 APC and anti-VLA-1 PE, all from BD Biosciences (Alphen aan de Rijn, The Netherlands). Immunofluoresence was measured by flow cytometry (FACSCalibur, Becton Dickinson, Alphen aan den Rijn, The Netherlands). $CD14^+$ monocytes were defined within SSC/$CD45^+$ population, and analyzed for expression of the putative activation markers (Figure 1). A total of 10,000 total events were analyzed with FlowJo software (Tree Star, Inc., Ashland, Oregon). The results are presented as percentage of the CD14 positive population.

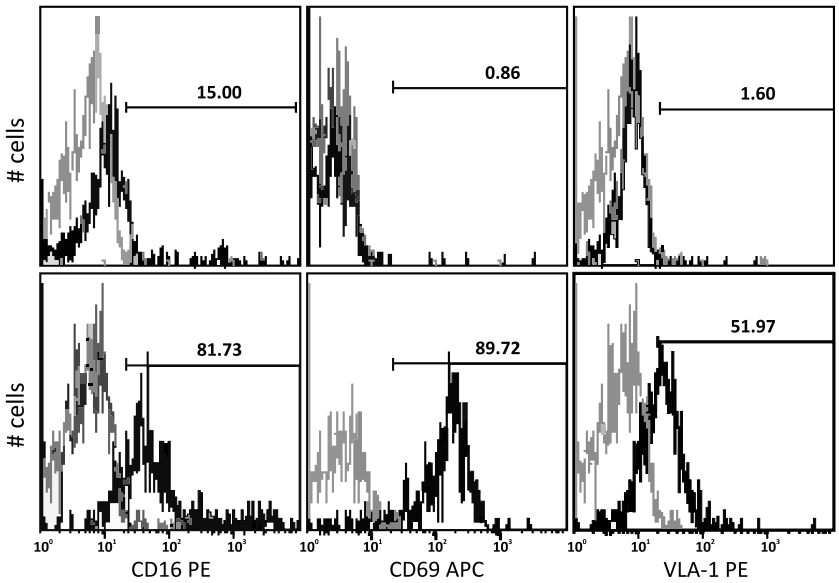


**Figure 1** Representative flow cytometric analysis of control staining (grey lines), CD16, CD69 and VLA-1 staining (black lines) gated on $CD14^+$ population. Upper row represents a healthy control; lower row shows a sarcoidosis patient. The numbers indicate the percentage of positive cells.

*Assay for IL-2R in serum*

Serum IL-2R was quantitatively determined using enzyme-linked immunosorbent assay (AMDS Benelux, Malden, The Netherlands). We used the normal range limited by an upper reference level given by the manufacturer: 710 U/ml.

*Assay for ACE in serum*

Quantification of ACE activity was measured in lithium heparin plasma using the Bühlmann ACE kinetic test, according to previously described methods (Bühlmann Laboratories AG, Switzerland)[18]. Conventional reference interval: 9-70 U/l (calculated as -1.96 and +1.96 SD in our healthy population). Fifteen patients were genotyped for ACE I/D polymorphisms as described by Kruit et al[18].

*Statistical analysis*

The statistical evaluation of our data was performed using Graphpad Prism v.3 (Graphpad Software, Inc., San Diego, CA, USA) software package. Data analysis was carried out by Student's *t*-test and Paired *t*-test for matched pairs. Correlation analysis was performed using a Pearson test. All results are presented as mean ± standard error of the mean. Statistical significance was associated with p values less than 0.05.

## Results

Flow cytometric analysis of peripheral blood monocytes

Analysis of $CD14^+$ monocytes gated within $CD45^+$ leukocytes revealed no significant differences between groups in percentages of monocytes (Table 2). Notably, the side scatter pattern of the monocytes differed between patients and controls. The monocyte populations from patients showed an increased and more diverse Side-light Scatter Characteristics (SSC), compatible with a granular appearance and an activated state. Although no difference in frequencies of $CD14^+$ monocytes were observed, a significant elevated Median Fluorescence Intensity (MFI) for CD14 was found in sarcoidosis patients (2103.0 ± 108.1) when compared to controls (902.0 ± 55.1) ($p < 0.0001$) (Table 2).

We investigated whether the percentage of monocytes expressing CD16 was different between sarcoidosis patients and controls. An increased percentage of monocytes expressing CD16 was found in patients compared to healthy controls. The mean percentage was 54.6 ± 4.2 for sarcoidosis patients vs. 12.2 ± 2.4 for controls ($p < 0.0001$). Furthermore, we found a significant increase in the percentage of monocytes expressing CD69 and VLA-1 in patients compared to healthy controls.

**Table 1** Characteristics of sarcoidosis patient population

| Variables | Data |
|---|---|
| Patients | 23 |
| Age,[a] yr | 41.2 [24 - 65] |
| Male/female gender[b] | 70/30 (16/7) |
| *Lung function parameters*[a] | |
| $FEV_1$ % pred (*n* = 18) | 91 [59-115] |
| FVC % pred (*n* = 18) | 95 [65-116] |
| Dl*co* % pred (*n* = 18) | 80 [44-105] |
| Chest radiographic stages | 14/4/4/1 |
| Löfgren's syndrome | 6 |
| *Organ involvement*[bc] | |
| Pulmonary involvement | 100 (17) |
| Extrapulmonary involvement | 35 (6) |
| Kidney | 24 (4) |
| Skin | 12 (2) |
| Extrathoracic lymph node | 6 (1) |
| Neurologic | 6 (1) |
| Eyes | 6 (1) |
| Parotid/salivary | 6 (1) |
| Muscles | 6 (1) |
| Bone/joints | 6 (1) |

[a]Values are given as the mean [range].
[b]Values are given as % (No.).
[c]Based on the ACCESS assessment instrument[19]. Organ involvement data are from non-Löfgren's sarcoidosis patients (n = 17).

The mean percentage of $CD69^+$ and $VLA\text{-}1^+$ monocytes was respectively 84.5 ± 3.4 for sarcoidosis patients vs. 8.6 ± 3.3 for controls ($p < 0.0001$) and 64.0 ± 4.3 vs. 11.2 ± 2.3 ($p < 0.0001$) (Figure 2). As shown in Table 2, increased frequency paralleled increased median fluorescence intensity of total monocyte population. Remarkably, no expression of the activation markers (CD69, VLA-1) was observed on circulating lymphocytes (data not shown).

Comparison of different clinical phenotypes, patients with lymphadenopathy (Löfgren's, chest radiographic stage I, II) versus patients with only parenchymal abnormalities (radiographic stage III, IV), showed no significant differences between the percentage of monocytes for any surface antigen tested or for the median fluorescence intensity of the antigens (Table 2). No significant differences were observed between the clinical phenotypes comparing absolute monocyte numbers (data not shown).

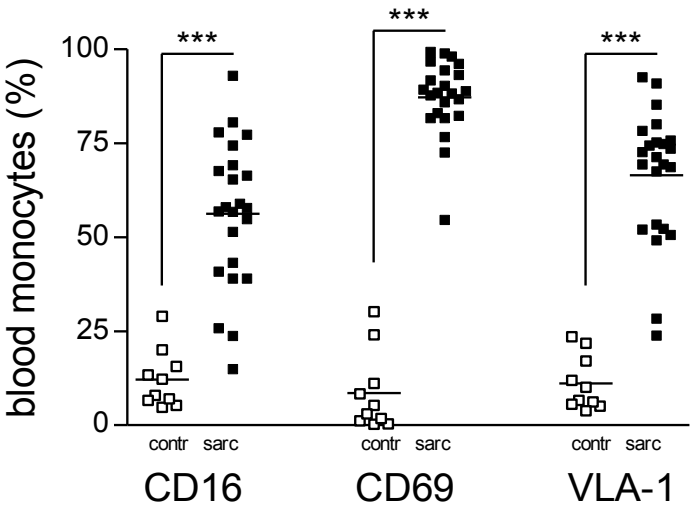


**Figure 2** Percentages of CD16, CD69 and VLA-1 positive peripheral blood monocytes of 10 healthy controls and 23 untreated patients with active pulmonary sarcoidosis. Values are presented as percentage of $CD14^+$ cells. Horizontal bars indicate mean values. Student's *t*-test (***, $p < 0.0001$).

**Table 2** Immunophenotyping of peripheral blood monocytes in patients with sarcoidosis and healthy controls

| | Controls | Sarcoidosis total | Lymphadenopathy (Löfgren's, I, II) | No lymphadenopathy (III, IV) |
|---|---|---|---|---|
| Subjects (*n*) | 10 | 23 | 18 | 5 |
| $CD14^+$ (% $CD45^+$) | 7.6 ± 0.5 | 7.0 ± 0.4 | 7.0 ± 0.5 | 7.0 ± 0.9 |
| $CD16^+$ (% $CD14^+$) | 12.2 ± 2.4 | 56.2 ± 4.1[a] | 55.7 ± 5.2 | 58.5 ± 2.6 |
| $CD69^+$ (% $CD14^+$) | 8.6 ± 3.3 | 87.3 ± 2.1[a] | 86.2 ± 2.5 | 90.9 ± 2.7 |
| VLA-$1^+$ (% $CD14^+$) | 11.2 ± 2.3 | 66.5 ± 3.6[a] | 64.5 ± 4.6 | 73.6 ± 4.4 |
| CD14 (MFI) | 902.0 ± 55.1 | 2141.0 ± 105.5[a] | 2069.0 ± 113.0 | 2402.0 ± 253.9 |
| CD16 (MFI) | 12.2 ± 1.2 | 35.7 ± 3.7[a] | 36.4 ± 4.7 | 33.3 ± 1.1 |
| CD69 (MFI) | 4.6 ± 1.2 | 125.3 ± 16.0[a] | 120.3 ± 18.9 | 143.2 ± 29.2 |
| VLA-1 (MFI) | 9.1 ± 0.6 | 29.4 ± 2.4[a] | 28.7 ± 2.7 | 32.2 ± 5.1 |

Values are means +/- SEM. MFI = Median Fluorescence Intensity of total monocyte population (units).
[a]Significantly different from healthy control subjects ($p < 0.0001$)

*Correlations between expression of different monocyte surface molecules*

Strong correlations were found between the percentage of monocytes expressing CD16 and CD69 ($r = 0.79$, $p < 0.0001$), CD16 and VLA-1 ($r = 0.66$, $p = 0.0006$) and CD69 and VLA-1 ($r = 0.79$, $p < 0.0001$). Moreover, the MFI of these markers as well as the percentage of positive cells correlated positively with the MFI of CD14 expression and with absolute

monocyte numbers (Table 3). Finally, a positive correlation was observed between granularity (side light scatter) of monocytes and the percentage of the $CD69^+VLA\text{-}1^+$ monocyte subset in patients with active disease (r = 0.66, p = 0.0007) (Figure 3).

**Table 3** Observed correlations between the expression of CD16, CD69 and VLA-1 and absolute $CD14^+$ cells or MFI of CD14 expression on monocytes of sarcoidosis patients

| | $CD14^+$ cells in blood[a] | | MFI CD14 on monocytes | |
|---|---|---|---|---|
| monocyte surface markers | r | p | r | p |
| % $CD16^+$ | 0.43 | 0.04[b] | 0.60 | 0.002[b] |
| % $CD69^+$ | 0.28 | 0.18 | 0.60 | 0.002[b] |
| % $VLA\text{-}1^+$ | 0.41 | 0.052 | 0.46 | 0.03[b] |
| % $CD69^+VLA\text{-}1^+$ | 0.41 | 0.055 | 0.52 | 0.01[b] |
| MFI CD16 | 0.63 | 0.001[b] | 0.48 | 0.02[b] |
| MFI CD69 | 0.25 | 0.24 | 0.52 | 0.01[b] |
| MFI VLA-1 | 0.48 | 0.02[b] | 0.29 | 0.18 |

MFI: Median Fluorescence Intensity
[a]absolute numbers
[b]$p < 0.05$

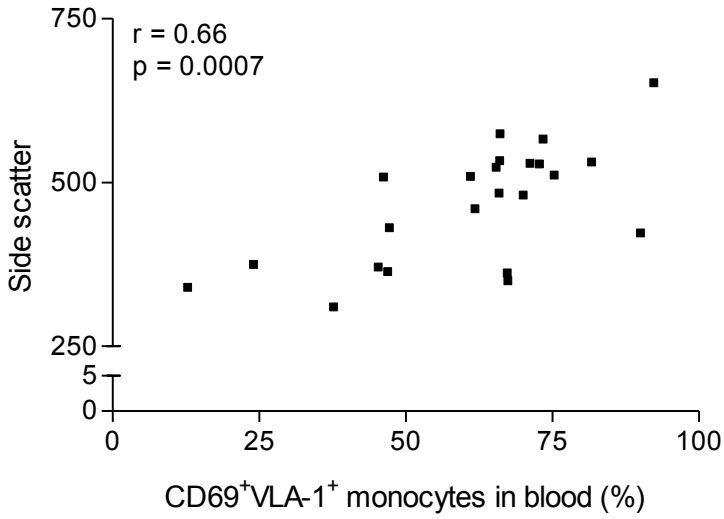


**Figure 3** Correlation between percentage of $CD69^+VLA\text{-}1^+$ monocyte subset and side light scatter of peripheral blood monocytes in 23 untreated patients with active pulmonary sarcoidosis. A significant, positive linear correlation was observed (r = 0.66, $p < 0.0007$).

*Correlation between activated monocyte phenotype and sIL-2R or sACE levels in serum*

To further understand the association of activated monocytes with disease activity, we examined the correlation between the percentage of $CD16^+$, $CD69^+$ and VLA-$1^+$ monocytes and serum levels of IL-2R and genotype corrected serum ACE levels. No significant correlations were found between the percentage or absolute numbers of $CD16^+$, CD69 and VLA-$1^+$ monocytes and serum ACE or serum sIL-2R levels.

Assessment of disease activity at presentation based on serum ACE and serum sIL-2R measurements showed that only 10/20 = 50% of the patients had elevated levels of both biomarkers, conventionally a strong indication for active disease. Of note, 3/20 = 15% of the patients showed normal values of both biomarkers and this would not indicate active disease (missing data on 3 patients: 2 patients showed normal values of one biomarker while the value of the other biomarker was not determined and in one patient with Löfgren's disease, both biomarkers were not measured). In contrast, all patients showed expansion of this monocyte subset compared to controls. The mean percentage of the monocyte subset expressing both CD69 and VLA-1 was respectively 61.2 ± 4.0 in patients and 2.1 ± 0.6 in controls.

*Follow-up study*

From the initial 23 patients, 8 patients were included in a follow-up study. Four patients were immunophenotyped with antibodies for CD16 and CD69 only. In the 8 individuals tested longitudinally, there was a marked contraction of the initially expanded percentage of activated monocytes and almost returned to normal levels upon clinical remission of disease.

The percentage of monocytes positive for CD16, CD69 and VLA-1 in these patients decreased from respectively 62.8 ± 5.6 to 34.7 ± 4.0 ($n = 8$, $p = 0.005$), 91.5 ± 1.3 to 15.4 ± 4 ($n = 8$, $p < 0.0001$) and 71.7 ± 8.4 to 15.1 ± 5.0 ($n = 4$, $p = 0.008$) upon disease remission (Figure 4). Moreover, the MFI of CD14 expression decreased from 2300 ± 147 to 1136 ± 73 ($p < 0.0001$). No significant differences were found between patients with either self-remitting disease or induced remission upon immunosuppressive treatment. Interestingly, forward scatter and side scatter characteristics of the monocyte population of patients in disease remission were now similar to controls.

At follow-up, 7 out of 7 patients had normal values of serum sACE (in one patient sACE was not measured). In 6 out of 8 patients normal levels of serum sIL-2R were measured, while 2 out of 8 patients showed lower serum sIL-2R levels (Table 4).

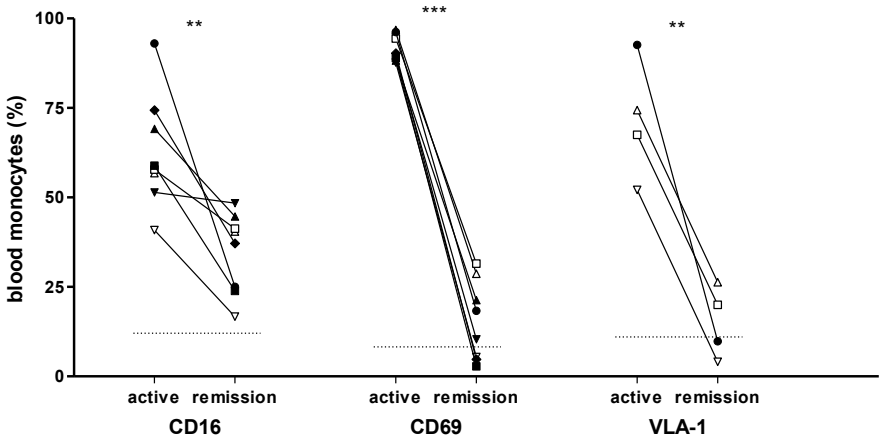


**Figure 4.** Longitudinal monitoring of activated monocytes in patients with pulmonary sarcoidosis (mean time interval between initial presentation and follow-up was 23 ± 4.7 months). Values are presented as percentage of $CD14^+$ cells. The horizontal dotted lines show the average values in healthy controls (n = 10). Identical symbols represent the same patient. Statistical comparisons were made using the Paired *t*-test (**, $p < 0.01$; ***, $p < 0.001$).

**Table 4** Clinical data of patients included in follow-up group

| Patient visit | Therapy | Thorax/clinic[a] | sACE (U/l) | sIL-2R (U/ml) | $CD14^+CD69^+VLA\text{-}1^+$ (%) |
|---|---|---|---|---|---|
| First visit | - | II | - | 549 | 71.2 |
| + 29 months | | remission | 56 | 609 | 11.6 |
| First visit | Prednison | IV | 39 | 280 | 61.0 |
| + 28 months | | remission | 53 | 373 | 14.4 |
| First visit | - | Löfgren's | 77 ↑ | 762 ↑ | 92.3 |
| + 25 months | | remission | 66 | 392 | 5.5 |
| First visit | Prednison | III | 115 ↑ | 2528 ↑ | 89.3 |
| + 22 months | | remission | - | 390 | 10.4[b] |
| First visit | - | Löfgren's | 71 ↑ | 390 | 46.9 |
| + 25 months | | remission | 48 | 204 | 2.9 |
| First visit | Prednison | III | 71 ↑ | 2441 ↑ | 88.9 |
| + 20 months | | remission | 50 | 974 ↑ | 2.8[b] |
| First visit | Prednison | II | 194 ↑ | 1351 ↑ | 88.3 |
| + 20 months | | remission | 51 | 272 | 21.3[b] |
| First visit | - | I | 45 | 1368 ↑ | 90.3 |
| + 15 months | | remission | 58 | 1162 ↑ | 4.8[b] |

[a]Pulmonary disease severity at presentation was evaluated by chest radiography (first visit), and clinic as assessed at follow-up. Disease remission was evaluated by patient's complaints i.e. fatigue, cough, fever, shortage of breath; lung function test, laboratory test (sACE, sIL-2R) and deterioration of chest radiography. Serum sACE and sIL-2R levels were considered elevated (↑) as described in Methods. The activated monocyte subset expressing both CD69 and VLA-1 is presented as percentage of $CD14^+$ cells; in four[(b)] patients only CD69 expression was measured at follow-up and was compared with CD69 expression at first presentation.

## Discussion

Activation of monocyte/alveolar macrophage cell lineage is thought to play an important role in the pathogenesis of sarcoidosis. The present study showed activated peripheral blood monocytes in patients with active pulmonary sarcoidosis, characterized by elevated expression of CD14, CD16, CD69 and VLA-1. The correlations observed between the cells expressing these markers strongly suggest that these cells are Proinflammatory monocytes.

Our data in survey suggest that alteration of disease activity in sarcoidosis might be monitored by measuring the CD16, CD69 and VLA-1 expression on $CD14^+$ blood monocytes of patients; Follow-up data of eight patients showing self-remitting disease or induced remission upon immunosuppressive treatment showed that the percentage of monocytes positive for CD16, CD69 and VLA-1 decreased to almost normal values while MFI values returned to normal.

Considering the observed correlations, measuring only CD69 might be sufficient for monitoring disease activity. Obviously, this has to be demonstrated in a larger patient group with regular follow-up measurements and shorter time intervals.

The recovery to 'normal' monocyte phenotype seems not to be related to treatment with corticosteroid. Moreover, the genes *FCGR3* (CD16), *CD69* and *ITGA1* (VLA-1) were not amongst the genes in monocytes reported to be regulated by glucocorticoids[20].

The absence of significant correlations with serum sACE (even with genotype correction) or serum sIL-2R levels can be explained by the fact that both markers are not directly related to blood monocyte activation but depend on leakage to blood from granuloma formation (sACE) and activation of mainly T lymphocytes and to some extend on activation of (alveolar) macrophages (sIL-2R)[21]. These processes may not necessarily be present simultaneously and therefore no correlation should be expected. However, the finding that 15% of the patients showed normal values of serum sACE and serum sIL-2R but increased percentage of activated monocytes at disease presentation, suggests that phenotyping monocytes may be more sensitive in assessing sarcoidosis disease activity.

For the first time, we show a significantly higher percentage of monocytes expressing VLA-1 in sarcoidosis patients compared to healthy controls. The $\alpha_1\beta_1$ integrin interacts with (type IV) collagen molecules in the extra cellular matrix (ECM), via the extracellular domain of the $\alpha_1$ subunit. The importance of monocytes and ECM in the inflammatory cascade is underscored by recently published gene expression data demonstrating that integrin-mediated attachment of monocytes to ECM results in increased expression of numerous inflammatory and immune response genes important in promoting cell

recruitment and activation[6]. Recently, Krieglstein et al. demonstrated that blockade and/or genetic deletion of the alpha chain of VLA-1 in a dextran sulfate sodium (DSS) colitis model resulted in decreased numbers and activation state of monocytes infiltrating the lamina propria in mice[22]. A similar protective effect of anti-$\alpha_1$ mAb was previously seen in experimental arthritis and antigen-induced airway responses[23,24]. Furthermore, Rubio et al. demonstrated that IFN-γ, a $T_H1$ cytokine elevated in BAL lymphocytes in sarcoidosis[25], specifically induced VLA-1 expression on monocytes[26]. In agreement with our findings, previous reports have shown increased expression of $\beta_2$-integrins on peripheral blood monocytes and alveolar macrophages in patients with active sarcoidosis compared to patients with inactive disease, suggesting another integrin-regulated step in the monocyte differentiation and activation cascade[10,11]. Taken together, up-regulation of VLA-1 expression might suggest a crucial role in monocyte recruitment to the lung and continuation of the inflammatory response in sarcoidosis.

Up-regulation of CD14 expression on circulating monocytes is known to parallel the maturation of monocytes to macrophages[27]. In the present study we found a twofold increase in the MFI of CD14 expression on blood monocytes from patients compared to controls. Moreover, monocytes from sarcoidosis patients were found to express significantly more $CD16^+$ within the $CD14^+$ monocyte population compared to healthy subjects. This is consistent with Okamoto et al.[13] and other studies finding increased co-expression of CD16 on monocytes in other systemic inflammatory disorders, suggesting an activated state of the monocytes[3,28]. In contrast to $CD14^{++}CD16^-$ monocytes, $CD14^+CD16^+$ cells are known to produce high levels of TNF-α and show higher antigen-presenting capacities[29]. Interestingly, Landsman et al. showed that murine lung macrophages exclusively generate from $Gr\text{-}1^{low}CX3CR1^{high}$ blood monocytes[30], the equivalent of $CD14^+CD16^+$ monocytes in humans and the presence of this distinct monocyte phenotype in newly diagnosed sarcoidosis might suggest they are involved in the formation of lung granulomas.

In this study we also found an elevated percentage of $CD14^+CD69^+$ monocytes in sarcoidosis patients compared to healthy controls. CD69 is thought to be a pluripotent signaling molecule expressed on the surface of a number of activated leukocytes including B, T, and NK cells, monocytes, neutrophils and platelets and acts as a signal transducer molecule. Crosslinking of CD69 receptors on monocytes induces extracellular calcium influx, the secretion of inflammatory mediators, such as prostaglandins $E_2$ and $F_1$ and leukotriene $B_4$, and increased production of nitric oxide (NO)[31]. Several reports describe the release of these inflammatory mediators by alveolar macrophages in pulmonary sarcoidosis suggesting they may play a part in modulating granulomatous lung

inflammation[32-34]. Increased expression of activation markers on peripheral blood monocytes was found in patients with other interstitial lung diseases (interstitial pneumonia, hypersensitivity pneumonitis), suggesting this phenomenon is not specific for sarcoidosis (data not shown).

Monocytes with a high side-light scatter value (granularity) are the most activated (Figure 3) and an intriguing next research step would be to sort, culture and stimulate this specific monocyte subset and measure cytokine release to prove the inflammatory characteristics of these cells.

## Conclusion

In conclusion, these data demonstrate that circulating monocytes in patients with active sarcoidosis exhibit an activated phenotype. The distribution as well as the intensity of the expression of CD16, CD69 and VLA-1 on peripheral blood monocytes is markedly increased in active disease and returns to normal values upon disease remission. Phenotyping peripheral blood monocytes may serve as a new tool for monitoring disease activity in patients with pulmonary sarcoidosis. Further research with a larger patient group, including regular follow-up of patients, is necessary to confirm this hypothesis.

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## Variation in *IL7R* predisposes to sarcoid inflammation

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## Abstract

Sarcoidosis is a chronic granulomatous disorder characterized by a massive influx of $T_H1$ lymphocytes. Both naïve and memory T cells express high levels of IL7Rα, encoded by the *IL7R* gene. The purpose of this study was to investigate the role of the *IL7R* gene region in susceptibility to sarcoidosis. Six common SNPs spanning *IL7R* were genotyped and analyzed in 475 sarcoidosis patients and 465 healthy controls. Replication of one significant associated SNP was done in 188 independent sarcoidosis patients, 121 controls and 139 patients with Löfgren's disease. The rs10213865 SNP was associated with sarcoidosis ($p = 0.008$), and *in silico* analysis showed a complete linkage ($r^2 = 1$, $D' = 1$) with a functional nonsynonymous coding SNP in exon 6 (rs6897932, T244I). Combined analysis of 663 individuals with sarcoidosis and 586 controls (homozygous carriers of risk allele, $p = 5 \times 10^{-4}$, odds ratio = 1.49 (1.19-1.86)) provided a strong statistical support for a genuine association of *IL7R* with sarcoidosis risk.

In addition, we report the same trend between variation in the *IL7R* gene and patients with Löfgren's disease, suggesting that variation in *IL7R* may confer general risk for developing granulomatous lung disease.

## Introduction

Sarcoidosis is a chronic, often disabling disease, affecting 20-50 in 100,000 people in most western countries. The inflammatory lesions typical of sarcoidosis show a massive $T_H1$ lymphocyte influx. Disease susceptibility and progression is thought to depend partly on genetic factors[1-4]. Of these genetic factors, only the HLA gene complex has been repeatedly confirmed to be associated with sarcoidosis in multiple populations of multiple ethnicities. Polymorphisms in a number of non-HLA genes have been reported to be associated with sarcoidosis, but so far confirmation has been difficult (recently reviewed by Grunewald[5]).

IL7Rα (also known as CD127), encoded by the gene *IL7R*, is a member of the type I cytokine receptor family and forms a receptor complex with the common cytokine receptor gamma chain ($\gamma_c$ or CD132) for which IL-7 is the ligand, which is produced by stromal cells in lymphoid tissues. Importantly, IL7Rα is a key functional marker of the early heterogeneity observed in effector T cells[6-8]. Both naive and memory T cells express high levels of IL7Rα, and IL-7 is required for their homeostasis[9]. Activation of T cells results in down-regulation of IL7Rα within 48 h after antigenic stimulation, but there is re-expression on the resting memory pool during the course of an immune response. Kaech et al. demonstrated that expression of IL7Rα on a small numbers of $CD8^+$ effector cells identifies that subset which is destined to differentiate into true memory cells[7]. Other reports have demonstrated an important role for IL-7 in the generation of $CD4^+$ memory cells as well[10]. Basal levels of IL-7 are sufficient to maintain survival of the $CD8^+$ memory pool and may contribute to survival of the $CD4^+$ memory pool[11]. Interestingly, IL-7R signaling has an immunomodulatory role in dendritic cells, especially in the context of thymic stromal lymphopoietin (TSLP), which also signals through the IL7Rα in a heterodimeric complex via dimerization with the product of the cytokine receptor-like factor 2 gene (*CRLF2*) to form the TSLP receptor (TSLPR). TSLP is homologous to IL-7 and is produced by cells of epithelial origin in the thymus, lung, gut and skin[12]. As well as an indirect effect of TSLP-activated DCs on $CD4^+$ T cell homeostasis[13], and T regulatory cell development in the thymus[14], Rochman and colleagues recently demonstrated a direct effect of TSLP on activated $CD4^+$ cells[15].

The gene *IL7R* is located on chromosome 5p13, a region linked with multiple sclerosis[16], asthma[17] and type 1 diabetes[18]. To date no studies have been published examining genotypic differences in *IL7Rα* between sarcoidosis patients and controls. We genotyped six common single-nucleotide polymorphisms (SNPs) spanning *IL7R*, including the 5' promoter, a SNP in intron 5, a nonsynonymous SNP in exon 8 and 3 haplotype tagging

SNPs. Moreover, correlations with disease activity parameters, lung function parameters and bronchoalveolar lavage cell counts were analyzed.

## Subjects & Methods

*Subjects*

*Original cohort.* 475 unrelated Dutch Caucasian sarcoidosis patients were included in this study (male/female; 212/263). The diagnosis sarcoidosis was established when clinical findings were supported by histological evidence and after exclusion of other known causes of granulomatosis in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis[19]. The control subjects comprised 465 healthy, Dutch Caucasian employees of the St. Antonius Hospital and blood samples from Sanquin blood bank in the Netherlands. Thirty-nine individuals included in the control group underwent bronchoalveolar lavage. Verbal and written consent was obtained from all subjects, and authorization was given by the Ethics Committee of the St. Antonius Hospital, Nieuwegein.

*Validation cohort.* To replicate the results, we included an independent case-control data set of sarcoidosis patients presented to multiple hospitals (within a 30 kilometer radius of St. Antonius Hospital) in the Netherlands in 2008 (188 cases and 121 controls).
In order to test whether the genetic association was specific for sarcoidosis, 139 patients with the classic symptoms of Löfgren's syndrome, namely fever, erythema nodosum, arthralgia and bilateral hilar lymphadenopathy, were included. For these patients the diagnosis was made without biopsy proof.

*Radiographic staging*

Chest radiographs were assessed in consensus by chest physicians specialized in diffuse lung diseases to determine disease severity using standard radiographic staging for sarcoidosis, classified according to the Scadding criteria[20]. Radiographic data at presentation were available for 191 sarcoidosis patients. Radiographic data at pulmonary disease outcome, defined by chest x-ray after a minimum of 4-years follow-up, were available for 309 patients. Patients presenting with pulmonary fibrosis or showing pulmonary fibrosis on chest radiography at follow-up < 4-years, were classified as stage IV at disease outcome considering the irreversibility of fibrotic scarring.

*Pulmonary function tests*

Pulmonary function tests were performed at presentation. Vital capacity (inspiratory) (iVC), forced expiratory volume in 1 second ($FEV_1$) and carbon monoxide diffusing lung capacity (Dl*co*) were used to assess the presence of lung function impairment at presentation of the disease. All lung function parameters are expressed as percent predicted values. IVC and $FEV_1$ were calculated from volumes in liters and adjusted to BTPS (body temperature, ambient pressure, saturated with water vapor).

*Bronchoalveolar lavage*

Bronchoalveolar lavage was performed during flexible fiberoptic bronchoscopy at the time of the diagnosis according to standardized and validated procedure previously described[21]. Bronchoalveolar lavage fluid was filtered through nylon gauze and centrifuged (10 min at 400 x g at 4 °C). The cell pellet was washed twice, counted, and resuspended in minimal essential medium/RPMI 1640 (Gibco; Grand Island, NY), supplemented with 0.5% bovine serum albumin (Organon; Teknika; Boxtel, The Netherlands). Cells were counted in a Bürker chamber. Cell viability was determined by Trypan blue exclusion. Smears for cell differentiation were prepared by cytocentrifugation (Shandon; Runcorn, UK). Cell differentiation was performed on cytospinslide after staining with May-Grünwald-Giemsa (Merck; Darmstadt, Germany), at least 2x 500 cells were counted.

*Assay for IL-2R in serum*

Serum IL-2R was quantitatively determined using enzyme-linked immunosorbent assay (AMDS Benelux, Malden, The Netherlands). We used the normal range limited by an upper reference level given by the manufacturer: 710 U/ml.

*Assay for ACE in serum*

Quantification of ACE activity was measured in lithium heparin plasma using the Bühlmann ACE kinetic test, according to previously described methods (Bühlmann Laboratories AG, Switzerland)[22]. Serum ACE concentrations are influenced by an insertion/deletion (I/D) polymorphism of the ACE gene. The D allele of this polymorphism is linked with raised serum ACE (sACE) levels. In our analysis we used the Z-score based on ACE I/D-corrected reference intervals (II: 9 – 43, ID: 14 – 62, DD: 24 – 82)[22].

*Analysis of IL7R genetic single nucleotide polymorphisms*

DNA was extracted from whole blood samples and SNPs were analyzed on a custom Illumina goldengate bead SNP assay that was performed in accordance with the

manufacturer's recommendations (Illumina Inc, San Diego, USA). Six SNPs in the *IL7R* gene were selected (Table 1). Three haplotype tagging SNPs were selected using the tagger program entering the genomic region of *IL7R* on genome build 35, using the CEU HapMap analysis panel and preferential picking of SNPs with a minimum allele frequency of 25% and high Illumina design scores under the pairwise tagging options with an $r^2$ threshold for SNPs > 0.8[23].

*Flow cytometry*

*IL7Rα surface expression ex vivo.* IL7Rα surface expression was measured on T lymphocytes from peripheral blood and from bronchoalveolar lavage fluid (BALF) of healthy subjects. Fifty µl PB or BALF, containing at least 100,000 cells, was incubated for 15 min at room temperature with 10 µl of the following mAbs: anti-CD3 PE, anti-CD4 PerCP, anti-CD8 APC and anti-CD127 FITC. The lymphocyte population was selected based on forward scatter–side scatter (FSC–SSC) characteristics. Immunofluoresence was measured by flow cytometry (FACS-Calibur, Becton Dickinson, Alphen aan den Rijn, The Netherlands). Fluorescence stability was checked by weekly calibration of the flow cytometer during the time of the experiment. Data were analyzed with FlowJo software (Tree Star, Inc., Ashland, USA). During each experiment a total of 50,000 cells were counted.

*Statistical analysis*

*Single SNP analysis*. Allele and genotype frequencies were calculated for each locus and tested for Hardy-Weinberg equilibrium (HWE) in controls. Case-control association studies were analyzed by $\chi^2$ test using 2 x 3 and 2 x 2 contingency tables of genotype and allele frequencies, respectively. Odds ratios (OR) and confidence intervals (CI) were calculated with an online tool, available at http://ihg2.helmholtz-muenchen.de/ihg/snps.html.

The significance threshold was set after accounting for multiple comparisons using a Bonferroni correction for the effective number of independent SNPs proposed by Li and Ji[24]. Due to linkage disequilibrium, the effective number of SNPs was 4.001 for *IL7R* (6 genotyped SNPs), resulting in an adjusted significance threshold of 0.05/4.001 = 0.0127 (available at http://gump.qimr.edu.au/general/daleN/matSpD/).

*Haplotype analysis.* Haplotype frequencies and tests of haplotype association were determined using Haploview version 4.0[25]. Individual haplotypes were inferred using PHASEv2 software[26].

ANOVA was used to test differences between *IL7R* genotypes and IL7Rα expression. A p value < 0.05 was considered significant.

## Results

*Distribution of IL7R gene variation in sarcoidosis patients and healthy controls*

Table 1 summarizes the allele frequencies of the investigated *IL7R* SNPs in sarcoidosis patients and controls. The SNPs did not deviate from Hardy-Weinberg equilibrium in controls. Linkage disequilibrium was observed between positions rs1353252 and rs1389832 ($r^2$ = 0.99, D' = 1) and nearly complete linkage disequilibrium between rs1494554 and rs3194051 ($r^2$ = 0.97, D' = 0.99) (Figure 1). When comparing sarcoidosis patients with controls, the frequency of allele A at rs10213865 was higher in sarcoidosis patients (0.75 vs. 0.69, $\chi^2$ = 7.1, p = 0.008, $p_{corrected}$ ($p_c$) = 0.03, odds ratio (OR) = 1.32 (1.08-1.62], d.f. = 1). Similarly, rs1353252 was significant associated with sarcoidosis but did not withstand Bonferroni correction for the effective number of independent SNPs ($\chi^2$ = 5.0, p = 0.03, $p_c$ = 0.10, d.f. = 1). At position rs10213865, the recessive model (AA vs. AC + CC) fitted best and conferred an increased risk of disease for homozygous carriers of the A allele (0.57 vs. 0.48, $\chi^2$ = 8.5, p = 0.004, OR = 1.47 (1.14-1.91)).

After permutation analysis (10,000 permutations) the above single marker comparison remained significant (rs10213865 A allele; $p_{permutated}$ = 0.035). Constructed haplotypes were completely defined by unique SNPs, hence haplotype analyses for *IL7R* did not provide any additional information beyond individual SNP analyses.

The remaining *IL7R* polymorphism frequencies did not differ significantly between sarcoidosis patients and controls (Table 1).

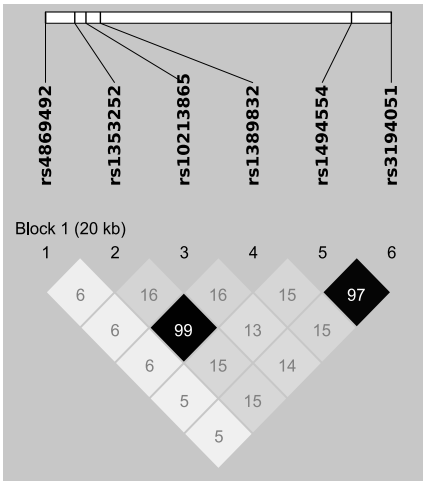


**Figure 1** Pair-wise linkage disequilibrium values of *IL7R* single-nucleotide polymorphisms in a Dutch population. Values of the pair-wise $r^2$ (×100) are shown in blocks.

**Table 1** Analysis of *IL7R* SNPs in 475 individuals with sarcoidosis and 465 controls (original cohort)

| | | | | | | | P value[c] |
|---|---|---|---|---|---|---|---|
| SNP | Allele | Frequency in individuals with sarcoidosis | Frequency in controls | Genotype | Frequency in individuals with sarcoidosis | Frequency in controls | Original cohort |
| rs4869492 | C | 0.87 | 0.86 | CC | 0.749 | 0.744 | 0.75 [NS][d] |
| | T | 0.13 | 0.14 | CT | 0.234 | 0.234 | |
| | | | | TT | 0.017 | 0.022 | |
| rs1353252[a] | G | 0.69 | 0.73 | GG | 0.499 | 0.538 | 0.03 [NS] |
| | C | 0.31 | 0.27 | CG | 0.373 | 0.389 | |
| | | | | GG | 0.128 | 0.073 | |
| rs10213865 | A | 0.75 | 0.69 | AA | 0.571 | 0.475 | 0.008 [0.03] |
| | C | 0.25 | 0.31 | AC | 0.359 | 0.440 | |
| | | | | CC | 0.070 | 0.085 | |
| rs1494554[b] | A | 0.73 | 0.72 | AA | 0.552 | 0.532 | 0.74 [NS] |
| | C | 0.27 | 0.28 | AC | 0.362 | 0.384 | |
| | | | | CC | 0.082 | 0.084 | |

[a]In linkage disequilibrium with rs1389832 ($r^2$ = 0.99, D' = 1)
[b]In linkage disequilibrium with rs3194051 ($r^2$ = 0.97, D' = 0.99)
[c]Allele frequency differences. [d]P values corrected for the number of independent SNPs are shown between brackets ($p_{corrected}$). NS = nonsignificant.

Rs10213865 did not reveal any functional motif and therefore, linkage analysis of populations from European descent deposited at the NCBI SNP population databank was performed for our set of SNPs and any SNPs showing functional variation in dbSNP. Complete linkage disequilibrium (LD) ($r^2$ = 1, D' = 1) was observed between rs10213865 and rs6897932, a functional nonsynonymous coding SNP in exon 6 (T244I). The threonine residue (T244) was found to be highly conserved among mammalian species. In rodents, threonine is replaced by amino acid serine, both belonging to the group of amino acids with uncharged polar side chains (Table 2).

**Table 2** Part of the amino acid composition of *IL7R*

| | | | |
|---|---|---|---|
| 235 - SGEMDPILL | T | ISILSFFSV - 253 | Human |
| SGEMDPILL | T | ISILSFFSV | Chimpanzee |
| ---MDPVLL | T | ISILSFFSV | Dog |
| SGETDPVLL | T | ISILSFFSV | Cow |
| SGGWDPVLP | S | VTILSLFSV | Mouse |
| SGGWDPVLP | S | IILLSLFSM | Rat |

*Replication of the rs10213865 effect*

To replicate the SNP association, we genotyped an independent case-control data set of patients presenting in multiple hospitals in The Netherlands in 2008 (188 cases and 121 controls). The frequency of allele A at rs10213865 was similar to the frequency of allele A in the original cohort and higher in sarcoidosis patients (0.76 vs. 0.67, p = 0.01). Combined analysis of 663 independent individuals with sarcoidosis and 586 controls (p = 0.0005, OR = 1.49 (1.19-1.86) for recessive genotype coding) provided a strong statistical support for a genuine association of *IL7R* with sarcoidosis risk (Table 3).

**Table 3** Combined analysis for recessive coding of genotypes at rs10213865 in 663 individuals with sarcoidosis and 586 controls

| | | Odds ratio (95% c.i.)[a] | | | P value | | |
|---|---|---|---|---|---|---|---|
| | Genotype | Original | Validation | Combined | Original | Validation | Combined |
| rs10213865 | AA vs. AC + CC | 1.47 (1.14-1.91) | 1.55 (0.99-2.41) | 1.49 (1.19-1.86) | 0.004 | 0.055 | 0.0005 |

[a]c.i. = confidence interval

Furthermore, to test whether the association with *IL7R* was specific for sarcoidosis we genotyped 139 patients with Löfgren's syndrome considered as a clinically and genetically distinct granulomatous lung disease. In patients with Löfgren's syndrome, the frequency of allele A at rs10213865 revealed the same distribution as was found in sarcoidosis patients. However, due to the low number of patients no significance was reached when compared with controls (0.74 vs. 0.69, p = 0.09) (Table 4).

**Table 4** Replication of finding in original cohort with a validation cohort of 188 individuals with sarcoidosis and 139 patients with Löfgren's syndrome

| | | Sarcoidosis | | Controls | | Löfgren's | | Sarcoidosis | | Controls | | Löfgren's |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SNP | Allele | Or | Val | Or | Val | | Genotype | Or | Val | Or | Val | |
| rs10213865 | A | 0.75[a] | 0.76[b] | 0.69 | 0.67 | 0.74[c] | AA | 0.571 | 0.573 | 0.475 | 0.465 | 0.561 |
| | C | 0.25 | 0.24 | 0.31 | 0.33 | 0.26 | AC | 0.359 | 0.369 | 0.440 | 0.402 | 0.360 |
| | | | | | | | CC | 0.070 | 0.058 | 0.085 | 0.133 | 0.079 |

Or = Original cohort, Val = Validation cohort

[a]Sarcoidosis vs. controls: p = 0.008 ($p_c$ = 0.03)

[b]Sarcoidosis vs. controls: p = 0.01

[c]Löfgren's vs. controls (combined): p = 0.09

*IL7Rα expression on peripheral blood mononuclear cells and BALF lymphocytes*

To explore the biological relevance of the *IL7R* rs10213865 genotype variation, we measured expression patterns in parallel samples of peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage fluid (BALF) from healthy subjects. Analysis of membrane bound IL7Rα expression in relation to the genotype of the sarcoidoses risk-modifying SNP rs10213865 revealed no significant difference between *IL7R* genotypes and IL7Rα median fluorescence intensity (MFI) on PB or BALF lymphocytes (data not shown). Neither did we observe significant differences between rs10213865 AA vs. AC + CC genotypes comparing the percentages or absolute numbers of PB and BALF lymphocytes, $CD4^+$ lymphocytes and $CD4^+/CD8^+$ ratios (data not shown).

*Correlation between IL7R gene variation and clinical parameters in sarcoidosis patients*

We examined the influence of the associated *IL7R* locus on lung function parameters in sarcoidosis patients measured at presentation. When DL*co* values of patients (*n* = 75) were grouped according to AA vs. AC + CC genotypes, the results were (mean ± SEM): AA (80.2 ± 2.8) vs. AC + CC (86.7 ± 3.0), p = 0.12. Similarly, both iVC and $FEV_1$ values were lower in homozygous AA individuals without reaching significance (data not shown).

Moreover, no significant differences were observed between rs10213865 AA vs. AC + CC genotypes comparing the percentages or absolute numbers of PB and BALF lymphocytes, $CD4^+$ lymphocytes, $CD4^+/CD8^+$ ratios and serum sIL-2R and sACE (corrected for ACE I/D genotypes) values measured at presentation (Figure 2).

No correlation was observed with radiographic staging at presentation (data not shown). Analysis of pulmonary disease outcome, defined by radiographic staging after minimal 4-year follow-up, revealed no association with *IL7R* rs10213865 genotypes (Table 5).

These data suggest no contribution of *IL7R* variation to disease severity or activity in clinical overt disease.

**Table 5** *IL7R* rs10213865 genotypes in Dutch sarcoidosis patients in relation to radiographic staging at pulmonary disease outcome

| rs10213865 | Radiographic staging after minimal 4-years follow-up | | | | |
|---|---|---|---|---|---|
| | 0 | I | II | III | IV |
| AA | 84 (0.48) | 14 (0.08) | 14 (0.08) | 21 (0.12) | 44 (0.25) |
| AC + CC | 56 (0.45) | 15 (0.12) | 12 (0.10) | 17 (0.14) | 25 (0.20) |

Data are provided as absolute numbers with genotype frequencies in parentheses.

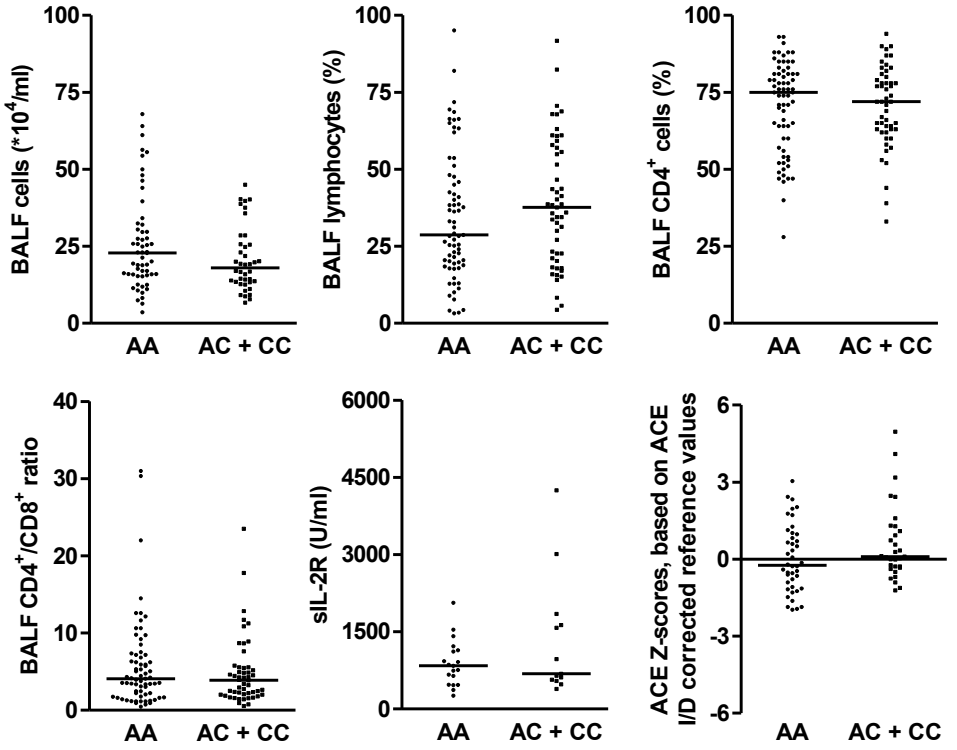


**Figure 2** Median absolute BALF cells, percentage of BALF lymphocytes, percentage of BALF $CD4^+$ cells, BALF $CD4^+/CD8^+$ ratios, serum sIL-2R values and ACE Z-scores (based on ACE I/D corrected reference intervals) for individuals carrying *IL7R* rs10213865 AA vs. AC + CC genotypes. None of the parameters were significantly different between genotypes.

## Discussion

In this paper we described an association between an intron SNP in the *IL7R* gene and sarcoidosis. The novel finding was replicated in a validation cohort revealing the same differences in allele frequencies between sarcoidosis patients and controls. The intron SNP at position rs10213865 was in complete linkage disequilibrium with a functional nonsynonymous coding SNP in exon 6 (rs6897932, T244I), leading to an altered membrane bound/soluble IL7Rα ratio[27]. Gregory et al. demonstrated that transcripts containing the C allele at position rs6897932 resulted in a twofold increase in the skipping of exon 6 by disrupting an exonic splicing silencer compared to transcripts with the T allele, and simultaneously produced less membrane-bound IL7Rα protein leading to a further increase of the soluble form of IL7Rα[27]. This is likely to affect the dynamics of IL-7 or TSLP

signaling network in multiple cell types[8,28] and may have a direct impact on the pathogenesis of sarcoidosis.

The frequency of rs10213865 was equally distributed in patients with Löfgren's disease, although limited numbers prevented it to reach significance. This might suggest that the sarcoidosis associated allele confers a general risk to inflammatory granulomatous lung disease. Genetic variation in other genes may confer susceptibility to different clinical sarcoidosis phenotypes as has been reported previously[29-34]. Our results implicate the IL7Rα-related immune response pathway in the etiology of sarcoidosis.
Variation in *IL7R* might confer susceptibility to sarcoidosis in early stages of the response to the 'sarcoidosis antigen' when compromised IL-7 signalling may reduce initial immune activation. Persistence of the antigen may lead to clinical overt disease and the influence of *IL7R* polymorphism might be diminished. This could explain the lack of correlation between *IL7R* rs10213865 genotypes and clinical parameters measured at presentation or radiography after minimal 4-years follow-up.

In healthy controls, no correlation was found between sarcoidosis risk genotype and IL7Rα expression on PB or BALF T lymphocytes, although we cannot exclude the possibility that soluble IL7Rα adhered to the cell membrane which may have increased fluorescence intensity values. Decreased expression in the BALF compartment would have supported a simplistic interpretation of recently activated lymphocytes that have migrated to the lung and subsequently have down regulated IL7Rα.

The mechanism behind the importance of the IL7-IL7Rα ligand-receptor pair in sarcoidosis needs to be further investigated. Reduced IL-7 signaling due to increased production of soluble IL7Rα in sarcoidosis patients may have marked consequences for immune activity, favouring less T cell proliferation and survival. Immune response to bacteria[35] may be compromised because of reduced B and T cell differentiation. Alternatively, impaired regulatory T cell function may lead to chronic immune activation and related tissue damage, and may play a role in granuloma evolution[36]. Low expression of IL7Rα by $CD4^+CD25^+$ T cells correlates with high expression of the nuclear transcription factor Foxp3, and has recently been identified as an important characteristic of peripheral regulatory T cells[37]. Both IL-7 and TSLP signaling are required for normal development of the $T_{reg}$ cell lineage[38] It had previously been reported that Foxp3 positive cells required a $\gamma_c$ stimulus in addition to the IL-2 receptor[39]. A recent study by Mazzucchelli et al. implicated

IL7Rα as providing both a $\gamma_c$ stimulus, and when paired with the TSLPR, a $\gamma_c$-independent stimulus[40].

$Foxp3^+$ $T_{reg}$ cells in active sarcoidosis have been found increased but functionally impaired and unable to suppress granuloma formation *in vitro*[36]. In contrast, Idali and colleagues showed decreased expression of regulatory T cell genes in peripheral blood and lungs of sarcoidosis patients[41]. Competition for scarce IL-7 between cell types may result in reduced survival of protective cells in sarcoidoses, such as regulatory T cells. In particular the role of TSLP in sarcoidosis produced by epithelial cells warrants further research. TSLP activates $CD11c^+$ dendritic cells and results in their $T_H2$ cytokine production[42], reduction in levels of the TSLP receptor may cause a $T_H1$ skew in sarcoidosis.

## Conclusion

This study is the first to deliver compelling evidence that polymorphisms in *IL7R* contribute to the non-HLA genetic risk in sarcoidosis, demonstrating a role for this protein in the pathophysiology of the disease. As the risk genotype is quite common in the general population, with a frequency of 0.48 in individuals of European descent, it is plausible that additional triggers are required for the development and progression of sarcoidosis, which is consistent with a complex disease model in which multiple genes and environmental factors contribute to the phenotype.

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# 8

## Regulatory T cell subsets in sarcoidosis patients and healthy controls

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## Abstract

Sarcoidosis is a chronic granulomatous disorder characterized by a massive influx of $T_H1$ lymphocytes. The immune response in sarcoidosis may develop into a chronic, excessive inflammatory reaction partly due to imbalanced regulation. Several lymphocyte populations with immune-regulatory properties have been described in sarcoidosis. However, data on regulatory T cells subsets from lungs of healthy subjects however is sparse. This study presents new data on iNKT cells and $Foxp3^+$ cells from blood and bronchoalveolar lavage fluid from 'true' healthy subjects. Furthermore, current data on these regulatory T cells subsets in sarcoidosis is reviewed.

## Introduction

Sarcoidosis is a systemic disorder characterized by the formation of non-caseating granulomas, with the lungs, lymph nodes and skin being the most affected organs (recently reviewed in Iannuzzi et al.[1]). The involved organs show a fierce influx of $CD4^+$ $T_{Helper}1$ ($T_H1$) lymphocytes. Although granulomas may often resolve spontaneously, pulmonary fibrosis occurs in 10 to 15% of patients with sarcoidosis[2]. The pathogenesis of pulmonary fibrosis in sarcoidosis remains uncertain but carriage of TGF-β3 risk alleles[3], production of CCL18 chemokine by alveolar macrophages[4], and a shift towards $T_{Helper}2$ cell ($T_H2$) response and the production of $T_H2$ associated cytokines[5] have been reported to enhance progression with fibrotic development.

The immune response in sarcoidosis may develop into a chronic, excessive inflammatory reaction partly due to imbalanced regulation. Several lymphocyte populations with immune-regulatory properties have been described in sarcoidosis[6,7].

The aim of this study was to present new data of regulatory T cell subsets from peripheral blood and lungs of healthy subjects, and secondly, to review the current knowledge of regulatory T cell populations in sarcoidosis.

## Subjects & Methods

Thirty-three healthy subjects underwent bronchoalveolar lavage and were included in this study.

Verbal and written consent was obtained from all subjects, and authorization was given by the Ethics Committee of the St. Antonius Hospital, Nieuwegein.

*Bronchoalveolar lavage*

BAL was performed during flexible fiberoptic bronchoscopy at the time of the diagnosis according to standardized and validated procedure[8]. BAL data were investigated of 33 healthy subjects. The procedure involved detailed explanation to the patient, premedication (0.5 mg atropine s.c., 20 mg codeine p.o.), and local anesthesia of the larynx and lower airways (0.5% tetracaine in the oropharynx, 8 cc 0.5% tetracaine in lower airways). BAL was performed, preferably in the right middle lobe, with four 50-ml aliquots of sterile isotonic saline solution (37 °C). The aspirated lavage fluid from the first 50-ml aliquot was kept apart and excluded from further analysis. The BAL fluid (BALF) recovered from the three subsequent aliquots was collected in a siliconized specimen trap and kept on ice. BALF was filtered through nylon gauze and centrifuged (10 min at 400 x g at 4 °C).

The cell pellet was washed twice, counted, and resuspended in minimal essential medium/RPMI 1640 (Gibco; Grand Island, NY), supplemented with 0.5% bovine serum albumin (Organon; Teknika; Boxtel, the Netherlands). Cells were counted in a Bürker chamber. Cell viability was determined by Trypan blue exclusion.

*Flow cytometry*

Fifty µl of ethylenediamine tetra acetic acid (EDTA) blood and 50 µl BALF (at least $5 \times 10^5$ cells) were incubated for 15 min at room temperature with 10 µl of the following mAbs: anti-CD3 FITC, anti-CD4 PerCP, anti-CD8 APC, anti-CD25 PE, anti-CTLA-4 PE, anti-CD127 PE, anti-Foxp3 APC, anti-CD56 APC, anti-Vα24 PE and anti-Vβ11 FITC (all from BD Biosciences, Alphen aan den Rijn, The Netherlands). Immunofluoresence was measured by flow cytometry (FACSCalibur, Becton Dickinson, Alphen aan den Rijn, The Netherlands). Lymphocytes were defined based on FSC/SSC characteristics, and analyzed for expression of the putative markers. A total of at least $3 \times 10^5$ total events were analyzed with FlowJo software (Tree Star, Inc., Ashland, Oregon).

*Statistical analysis*

The Mann-Whitney U test was used to compare BALF and PB cell populations. Student's t-test was used to compare $Foxp3^+$ and $Foxp3^-$ populations. Statistical significance was associated with p values less than 0.05. The statistical evaluation of our data was performed using SPSS 15.0 (SPSS Inc; Chicago, IL, USA) and Graphpad Prism v.5 (Graphpad Software, Inc., San Diego, USA) software packages.

**Regulatory T cell subsets**

*Invariant Natural Killer T cells*

Invariant Natural Killer T cells (iNKT cells) are a subpopulation of innate-like T lymphocytes that express an invariant T cell Ag receptor α-chain. iNKT cells are characterized by the co-expression of a TCR along with typical cell surface receptors for NK cells. The invariant TCR α-chains have a skewed association with TCR Vβ8.2 in the mouse and the homologous Vβ11 chain in the human (Figure 1)[9]. Like conventional T cells, iNKT cells comprise distinct functional subsets. Notably, $NK1.1^{pos}$ iNKT cells produce IL-4 and IFN-γ, whereas $NK1.1^{neg}$ iNKT cells produce IL-17 and IL-21[10,11]. The presentation of pathogen-derived or endogenous glycolipid antigens by CD1d-expressing antigen-presenting cells (APCs) elicits the iNKT cells response and can support $T_H1$ responses by activating NK cells which secrete IFN-γ, thus facilitating dendritic cell (DC) maturation and IL-12 production[9]. iNKT cells may favor $T_H2$ cell differentiation by producing IL-4 and IL-13 under different inflammatory

conditions[12,13]. Their murine Vα14-Jα281 counterparts have been shown to be necessary for the development of the granulomatous lesions that follow the injection of deproteinized mycobacterial cell walls.

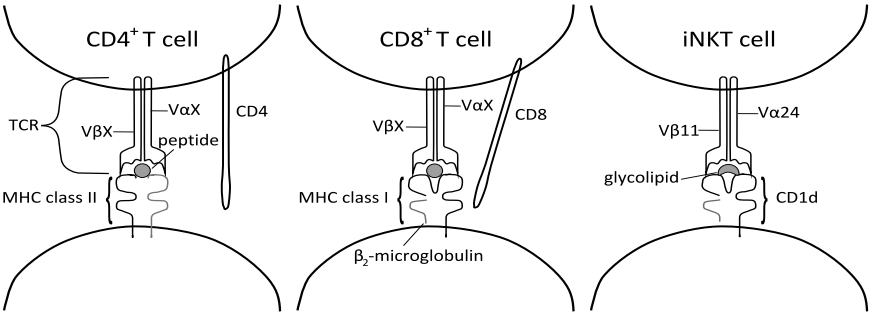


**Figure 1** Schematic representation of $CD4^+$, $CD8^+$ and invariant NKT cell receptors and their antigen presenting counterparts respectively MHC class II, MHC class I and CD1d molecules.

Invariant NKT cells are associated with asthma but results are controversial[14], with tuberculosis[15] and with COPD in mice[16]. Several groups have published on iNKT cells in pulmonary sarcoidosis[6,17]. Moreover, Mempel et al investigated granulomas in cutaneous sarcoidosis and leprosy[18].

Kobayashi and colleagues found a reduction in both Vα24 NKT cell numbers and IFN-γ production in PBMC and pulmonary granuloma lesions of non-remitting, but not remitting, sarcoidosis patients. Their results suggested a role for Vα24 NKT cells in the disease progression of sarcoidosis. Consistent with Kobayashi et al., Ho and colleagues showed that CD1d-restricted NKT cells were absent or greatly reduced in peripheral blood from all patients with sarcoidosis, except those with Löfgren's syndrome. The deficiency was found in both acute and resolved disease and was unrelated to systemic corticosteroid therapy. No difference was observed in the proportion of CD1d-restricted NKT cells between peripheral blood and lungs in patients, suggesting that the peripheral-blood deficiency was not due to sequestration of these cells in the lungs. In contrast with Kobayashi et al., NKT cells were not observed in mediastinal lymph nodes or granulomatous lesions. CD1d expression on antigen-presenting cells of patients was normal, thus the deficiency of CD1d-restricted NKT cells was not explained by abnormal CD1d expression. The authors concluded that loss of CD1d immune regulation could explain the amplified and persistent T cell activity that characterizes sarcoidosis. In contrast with Kobayashi et al. but consistent with Ho et al., Mempel and colleagues did not find classical Vα24-Jα18 NKT cells in cutaneous sarcoidosis lesions and concluded that mycobacteria do not contribute to the

progression of cutaneous sarcoidosis. In conclusion, non-remitting sarcoidosis may be associated with inappropriate iNKT cell numbers and function. Insufficient iNKT cell response to Mycobacteria leading to a dysfunctional $T_H1$ response might explain persistent T cell activity sarcoidosis.

Despite the interesting results found in various lung diseases, iNKT cells were not investigated in lungs of true healthy controls. Recently, we examined blood and bronchoalveolar lavage samples from 16 healthy subjects, using Vα24 and Vβ11 monoclonal antibodies. Invariant natural killer T cells constituted less than 1% of the $CD3^+$ cells, partly expressing natural killer-cell marker CD56 (Table 1)[19]. We found no compartmentalization of invariant natural killer T cells in the lungs of healthy subjects (Table 1). Our data provide unique normal values of iNKT cells in BAL fluid of healthy subjects and add to the growing body of evidence that iNKT cells are found in low numbers in human airways in health and disease.

**Table 1** iNKT cells in specimens of 16 healthy subjects

| | Invariant Natural Killer T Cells | |
|---|---|---|
| Variable | Peripheral Blood | Bronchoalveolar Lavage Fluid |
| Gated on $CD3^+$ cells | | |
| Median | 0.08 | 0.04 |
| Interquartile range | [0.04 - 0.23] | [0 - 0.23] |
| Gated on $CD3^+CD56^+$ cells | | |
| Median | 0.03 | 0.02 |
| Interquartile range | [0.01 - 0.06] | [0 - 0.06] |

*$Foxp3^+$ regulatory T cells*

Tolerance to self antigens is an active process that has a central component and a peripheral component. Central tolerance involves the deletion of autoreactive clones during thymocyte development, whereas peripheral tolerance is achieved largely through three mechanisms: clonal deletion, anergy, and suppression. Of these three mechanisms, only suppression has a dedicated set of T cells generated for the specific purpose of controlling the responses of other T cells. This set T cells, referred to as regulatory T cells, is actually comprised of several subsets, and can be broadly subdivided into two groups: (i) cells that originate from the thymus, referred to as 'naturally occurring Tregs' (nTregs), and (ii) Tregs that have been induced in the periphery, also called 'adaptive Tregs'. Natural

occurring regulatory T cells are characterized by the expression of CD25 and the forkhead family transcription factor Foxp3 (forkhead box p3). Once activated, nTregs are able to suppress T cell proliferation and cytokine production as well as antigen-presenting cell function. Foxp3-expressing CD4 regulatory T cells play a major role in the control of immune responses against self and exogenous antigens[20,21]. Recently, Ono et al. found that Foxp3 physically interacts with the transcription factor acute myeloid leukaemia 1/Runt-related transcription factor 1 (AML1/Runx1), thereby preventing IL-2 and interferon (IFN)-γ production by Tregs while inducing Treg-cell-associated molecules and suppressive activity[22].

Although several other markers have been identified in human and/or murine $CD4^+CD25^+$ Tregs, such as cytotoxic T lymphocyte activation antigen-4 (CTLA-4)[23], the glucocorticoid induced tumour necrosis factor receptor (TNFR) family related gene (GITR)[24], integrin $\alpha_E\beta_7$ (CD103)[25], and the absence of CD127[26], Foxp3 represents the most specific marker for naturally occurring Tregs available to date. It is, however, increasingly apparent that not all Foxp3 human T cells correspond to Tregs[27,28].

Deficient nTregs, functional or in number, have been reported in different lung diseases[29-31]. $Foxp3^+$ $T_{reg}$ cells in active sarcoidosis have been found increased but functionally impaired and unable to suppress granuloma formation *in vitro*[32]. The findings of Taflin et al. showed that active sarcoidosis was characterized by an amplification of peripheral memory phenotype $CD4^+CD45RA^-Foxp3^{bright}$ Tregs and further showed a high prevalence of memory Tregs within the granulomas present in nonlymphoid organs such as the kidney. Moreover, these Treg cells proliferated within diseased organ granulomas during sarcoidosis. *In vitro* granuloma formation however, was suppressed by Tregs from healthy controls but not by Tregs from sarcoidosis patients. These results suggest a defect in the suppressive function of Tregs on autologous granulomas in sarcoidosis and are consistent with the observation that Treg cells from sarcoidosis patients are not able to completely inhibit the secretion of IFN-γ and of TNF-α by effector T cells[7].

In contrast, Idali and colleagues showed decreased expression of regulatory T cell genes in peripheral blood and lungs of sarcoidosis patients[33]. The relative mRNA expression of Foxp3 was decreased in BALF $CD4^+$ T cells from patients versus healthy controls. Similarly, mRNA expression of regulatory T cell associated cytokines, IL-10 and CCR2 was decreased in patients versus healthy controls. The frequency of Foxp3 expressing cells in both BALF $CD4^+$ T cells and blood $CD4^+$ T cells of sarcoidosis patients was significantly lower compared with controls. Both groups concluded that, although based on contrasting results, a deficiency in regulatory T cell function may enhance the development of sarcoidosis. It

remains to be determined whether this suppression defect is a cause or consequence of the disease.

Foxp3$^+$ $T_{reg}$ cells from lungs of healthy subjects have hardly been described. We stained peripheral blood and BALF cells from healthy subjects with antibodies against CD4, CD25, CTLA-4, CD103, CD127 and Foxp3 (Figure 2). The frequency of Foxp3$^+$ cells in peripheral blood and bronchoalveolar lavage fluid was analyzed and the expression of CD25 and CTLA-4 was compared between Foxp3$^+$ and Foxp3$^-$ cell populations.
Foxp3 expression per cell was higher on CD4$^+$ lymphocytes (median [range]) from BALF compared to PB (145 [43-791] vs. 29 [8-77], $p < 0.0001$). Moreover, the percentage of CD4$^+$ cells positive for Foxp3 expression was higher in BALF samples versus PB samples (10.1 [4.2-18.9] vs. 7.4 [2.3-12.6], $p < 0.0001$) (Figure 3).

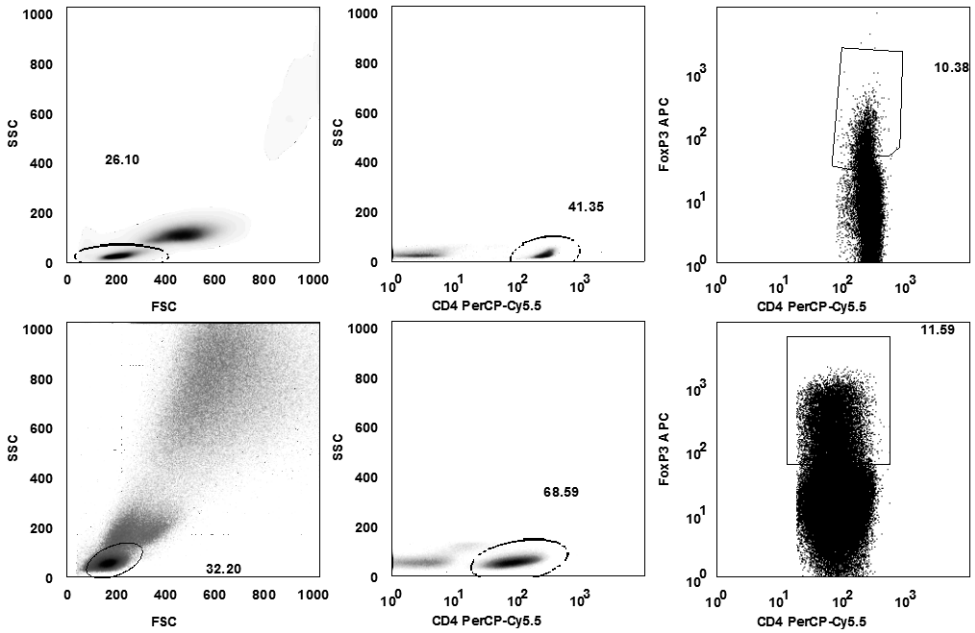


**Figure 2** Gating strategy for Foxp3$^+$ cells from peripheral blood (upper row) and bronchoalveolar lavage (lower row) samples.

In both blood and BALF samples, the expression of CD25 and CTLA-4 was higher on Foxp3$^+$ cells compared to Foxp3$^-$ cells ($p < 0.0001$ for all comparisons). In BALF, CTLA-4 expression was more pronounced than in peripheral blood samples (Figure 4).

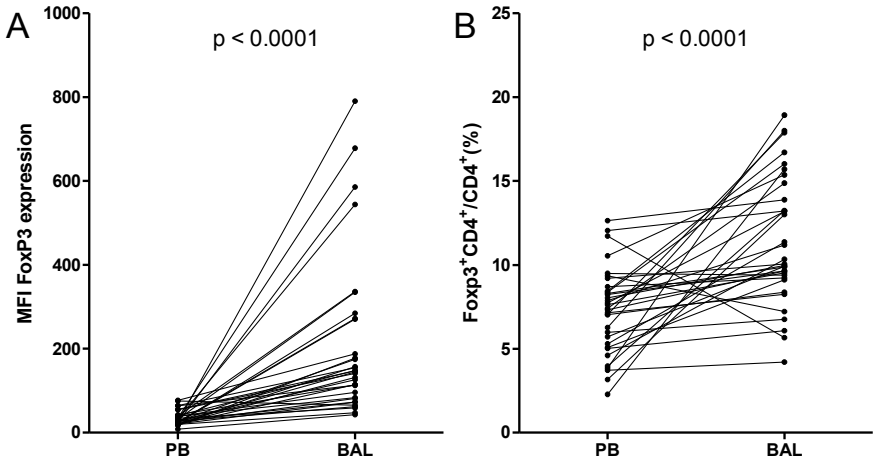


**Figure 3** Foxp3 median fluorescence intensity on $CD4^+$ cells (MFI) (A) and the proportion of $Foxp3^+$ positive $CD4^+$ cells (B) were significantly higher in BALF than in peripheral blood samples from 33 healthy subjects.

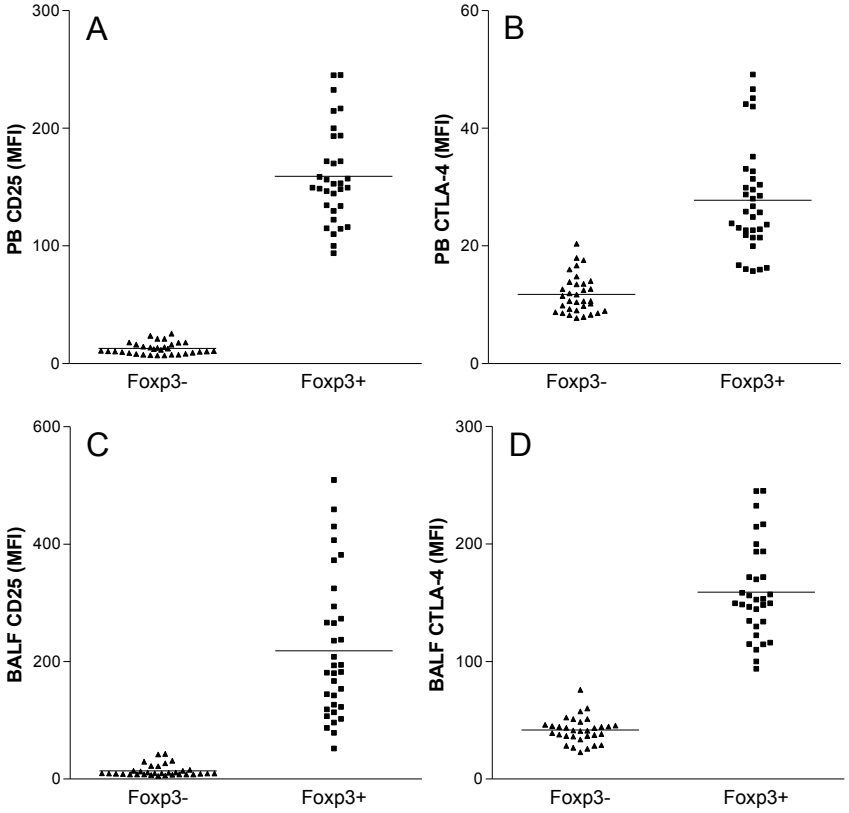


**Figure 4** Scatter plots illustrating CD25 and CTLA-4 expression on $Foxp3^-$ and $Foxp3^+$ T cell subsets from peripheral blood (A and B) and bronchoalveolar lavage fluid (C and D).

One of the best characterized examples of a cell surface marker on Treg cells is the integrin $\alpha_E\beta_7$ (CD103), which binds E-cadherin. It is typically expressed on 20-30% of Foxp3$^+$ cells in secondary lymphoid organs and on a higher percentage of Treg cells in tissues such as the skin and lamina propria of the gut. The functional relevance of CD103 expression in the Treg cell population is highlighted by the greater potential of Treg cells to access and be retained in peripheral tissues during infection or acute inflammatory insults. We found no co-expression of Foxp3 and CD103 on BALF lymphocytes from healthy controls (Figure 5). Whether in sarcoidosis, under inflammatory conditions, BALF lymphocytes co-express Foxp3 and CD103 remains to be determined.

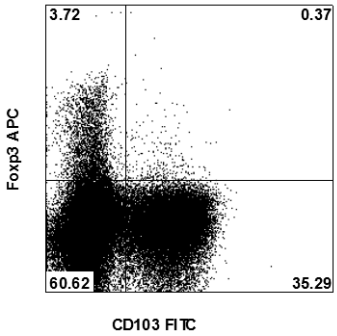


**Figure 5** Representative flow cytometric analysis of CD103 and Foxp3 co-expression on BALF lymphocytes from healthy subjects.

Finally, low CD127 (IL7Rα) expression on lymphocytes has recently been proposed as a surface marker identifying Foxp3$^+$ cells[26,34]. In peripheral blood of healthy subjects, CD127$^{low}$CD25$^+$ CD4$^+$ lymphocytes were found to be a discrete cell population and gated CD4$^+$Foxp3$^+$ cells were primarily detected within the CD127$^{low}$CD25$^+$ gate (Figure 6a, c and d). In addition, they strongly correlated with blood CD4$^+$Foxp3$^+$ lymphocytes ($r^2 = 0.63$, $p < 0.0001$). In BALF lymphocytes however, the CD127$^{low}$CD25$^+$ population was less unambiguous. When gated CD4$^+$Foxp3$^+$ cells were analyzed in the CD127/CD25 plot, an overlap was visible with the CD127$^-$CD25$^-$ subset (Figure 6e, g and h). Vice versa, when the CD4$^+$Foxp3$^+$ dot plot was overlaid with gated CD127$^-$CD25$^+$ cells, a significant number of lymphocytes was found to be Foxp3 negative (Figure 6f). The latter was also observed in blood lymphocytes but not as pronounced as compared to BALF cells (Figure 6b). Identifying or isolating Treg cells from bronchoalveolar lavage fluid on the basis of CD127$^-$ and CD25$^+$ expression, may exclude Foxp3 positive cells with low CD25 expression and on at the same time dilute the Treg population with Foxp3 negative lymphocytes.

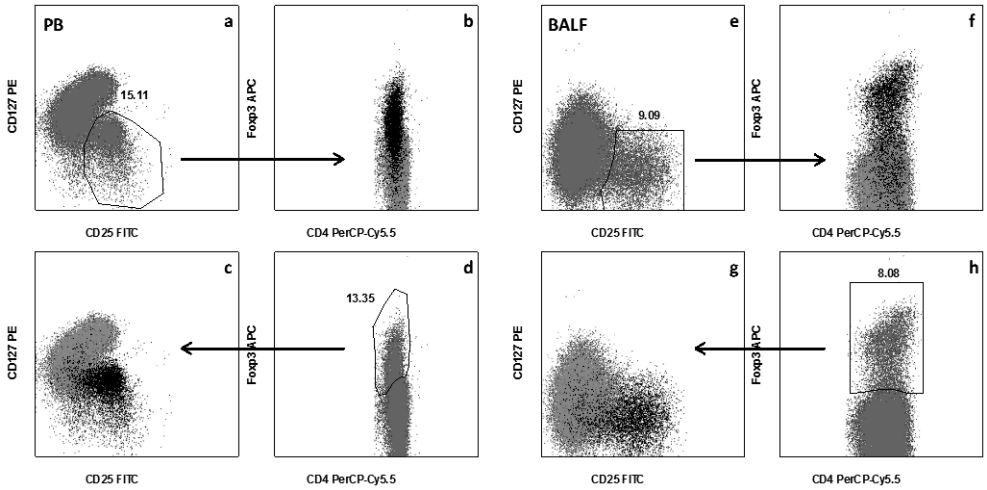


**Figure 6** Flow cytometric analysis of peripheral blood $CD4^+$ cells illustrating gated $CD127^{low}CD25^+$ cells (a) depicted in CD4/Foxp3 plot (b), and gated $CD4^+Foxp3^+$ cells (d) depicted in CD127/CD25 plot (g). Comparable analysis of BALF $CD4^+$ cells is presented in figures e-h.

## Discussion

This study is one of the first to explore Foxp3 positive cells in lungs of healthy subjects. Consistent with Idali et al.[33], the percentage of Foxp3 expressing $CD4^+$ T cells and the Foxp3 expression per cell were higher in BALF versus blood. Furthermore, iNKT cell numbers from lungs of 'true' healthy subjects have not been published before. Both types of regulatory T cells have been investigated in sarcoidosis but data are far from conclusive. $Foxp3^+$ cells from sarcoidosis patients seem to be functional inadequate to suppress $T_{effector}$ cell cytokine production and granuloma formation *in vitro* despite an accumulation of Treg cells in blood and involved organs in *vivo*[7,32].

Several murine studies have suggested that the suppressive function of Tregs depends both on the inflammatory context of their own state of activation and on the level of activation of effector T cells[35,36]. Billiard et al.[37] have shown *in vivo* in the mouse that strong activation of effector cells renders them resistant to the suppressive action of Tregs. More generally, the inflammatory context seems to be determinant in the inhibition of the suppressive function of Tregs, notably through Toll-like receptor pathways. Sutmuller et al.[38] have shown an *in vitro* expansion of Tregs having altered suppressive function in response to the Toll-like receptor 2 ligand (Pam3cys) in a murine model. Interestingly, Veltkamp et al. found that a specific genotype in TLR-2 gene, -16934 AA, was prevalent in patients with chronic sarcoidosis compared to patients with acute/self remitting disease. *In vitro* TLR-2 stimulation with Pam3Cys revealed increased IL-6 cytokine release by PBMC with the -16934 AA genotype. IL-6, known for making T cells

insensitive for suppression by regulatory T cells[39], are also correlated with persisting disease activity[40,41]. They speculated that patients with the -16934 AA genotype have increased risk of developing chronic disease[42].

Genetic variation in Treg genes specifically up- or down regulated in Foxp3$^+$ $T_{reg}$ cells[43] might render Treg cells more susceptible to inappropriate response in early disease stages[44] or leave Treg cells susceptible to exhaustion in chronic inflammatory context[38].

The apparent defect in immunoregulation observed in sarcoidosis could result both from resistance of effector cells due to their strong activation status and from chronic inflammation-related (exhausted) Treg impairment. During an immune response, Foxp3$^+$ T cells become hypersensitive to and depend on the IL-2 produced by activated effector T cells[45]. This hypersensitivity is achieved by the activity of Foxp3 in maintaining high levels of CD25, the high-affinity receptor for IL-2. The negative feedback loop ensures that T cell activation promotes the expression of IL-2 which drives both the effector response to clear the antigen and the Foxp3$^+$ regulatory T cell response to control the effector T cells. During a chronic immune response, in which effector T cells are incapable of clearing the stimulus, the negative feedback loop can be broken by the activation of the programmed cell death 1 (PD-1) receptor pathway[46]. PD-1 is an inhibitory CD28 family member. It is expected that activation of PD-1 signalling allows effector T cells to maintain a chronic level of activation without promoting the regulatory T cell silencing pathway, in effect breaking the negative feedback loop by short-circuiting the IL-2 sensitivity of Foxp3$^+$ T cells. This mechanism might explain regulatory T cell dysfunction in sarcoidosis and may lead to chronic disease with fibrosis formation.

## Conclusion

In conclusion, we characterized iNKT and Foxp3$^+$ cells in blood and lungs of healthy subjects. It remains to be determined whether Foxp3 expression in T lymphocytes from healthy lungs is locally induced or whether these Foxp3$^+$ cells indeed are lineage specific, thymus derived regulatory T cells. In sarcoidosis it would be interesting to investigate whether Foxp3$^+$ cells differ between progressive and self remitting disease, and what their role is in formation, persistence, and resolution of granulomas *in vivo* in involved organs. Moreover, it remains to be shown whether Tregs would be primarily defective in sarcoidosis, or only “exhausted” by repetitive and chronic stimuli. To address these issues, among other things, “criss-cross” experiments need to be performed. Foxp3$^+$ cells from lungs of sarcoidosis patients are tested on healthy donors’ responder cells, and vice versa. Finally, the exact role of natural killer T cells in sarcoidosis remains undefined.

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# 9

**Summary and concluding remarks**

## Summary

The purpose of this thesis was to evaluate the usefulness of markers of cell activation for diagnosis, prognosis and assessment of disease activity and severity in pulmonary sarcoidosis (chapter 2, 3, 5 and 6). Furthermore, polymorphisms in genes encoding proteins related to cell activation were investigated for their influence on disease susceptibility or disease course (chapter 4 and 7).

**Chapter 2** describes the expression of different activation markers on T lymphocytes subsets in different granulomatous ILD and in healthy individuals. The ability of distinct expression profiles of cell activation markers to discriminate between different clinical phenotypes of sarcoidosis patients (according to chest radiography at presentation) is evaluated. Highly activated T cells were found in bronchoalveolar lavage fluid in both ILD patients but also in healthy subjects. In ILD patients, the expression on BALF cells of CD28, CD69 and VLA-1 combined, correlated negatively with lung function, an indicator for disease severity. In sarcoidosis, the proportion of blood $CD8^{+}CD28^{null}$ cells negatively correlated with lung function and chest radiography staging and was associated with chronic activated BALF lymphocyte phenotype. The finding of a specific peripheral blood lymphocyte phenotype which correlates with different clinical and radiological indicators of disease severity is of interest. The results may prompt further research to evaluate the value of $CD8^{+}CD28^{null}$ cells as biomarker for disease severity in pulmonary sarcoidosis. Moreover, the functional properties of the $CD8^{+}CD28^{null}$ cells in blood of sarcoidosis patients should be investigated and compared with those of healthy controls.

A lymphocytic alveolitis with a $CD4^{+}/CD8^{+}$ ratio > 3.5 is consistent with pulmonary sarcoidosis. However, a $CD4^{+}$ lymphocytosis in BAL is neither a specific nor a sensitive marker supportive for the diagnosis of sarcoidosis. In **chapter 3**, a novel diagnostic marker for sarcoidosis is evaluated. The expression of the integrin CD103 is analyzed in BALF samples from patients with different ILD. Patients with sarcoidosis showed a significantly lower proportion BALF $CD4^{+}$ lymphocytes expressing CD103 as compared to patients with other ILD. This is corroborative to the concept that the lymphocytosis in, for example patients with hypersensitivity pneumonitis, results from the local expansion of mucosal lymphocytes while lymphocytosis in pulmonary sarcoidosis is the result of lymphocytes of non-mucosal origin.
A combination of both markers (relative BAL/PB $CD4^{+}/CD8^{+}$ ratio and the $CD103^{+}CD4^{+}/CD4^{+}$ ratio) is proposed as a diagnostic marker for pulmonary sarcoidosis.

**Chapter 5** evaluates the prognostic value of $CD103^+$ BALF lymphocytes and describes the differences in activation status CD103 positive T lymphocytes, compared to CD103 negative cells. BALF cells of healthy controls and sarcoidosis patients with different chest radiographic stages were analyzed. Although in a relatively small group, the percentage of CD103 positive lymphocytes, determined at time of diagnosis, revealed a high prognostic value for developing parenchymal abnormalities at pulmonary disease outcome. Interestingly, this was independent of radiographic stage at presentation. $CD103^+$ lymphocytes expressed more chronic activation markers ($CD28^{null}$, CD69 and VLA-1) than $CD103^-$ lymphocytes. An increased number of CD103 positive cells might be the result of local, chronic stimulation near mucosal epithelia. Our findings suggest that these cells are involved in the progression of pulmonary sarcoidosis.

**Chapter 6** demonstrates a highly activated monocyte phenotype in sarcoidosis patients compared to healthy subjects. The added value of determination of monocyte activation is illustrated by the fact that 15% of the sarcoidosis patients had normal values of serum sACE and serum sIL-2R but increased percentage of activated monocytes at disease presentation. It is conceivable that the circulating monocytes are the actual progenitors of the lung macrophages involved in granuloma formation. Future research should be directed to monitor these monocytes to find out whether they actually enter the lung and participate in the sarcoid inflammation.

**Chapter 4** presents the analysis of a novel sarcoidosis susceptibility gene in Dutch sarcoidosis patients: the *ITGAE* gene, encoding the $\alpha_E$ unit CD103. Different pulmonary disease phenotypes (according to pulmonary disease outcome) are compared in terms of SNP frequency distribution of the *ITGAE* gene. The functional effects of a SNP in the promoter region of *the ITGAE* gene, associated with sarcoidosis, are analyzed by comparing RNA and protein levels in the genetic variants. Patients carrying *ITGAE* -1088 AA genotype had higher proportions of BALF $CD103^+CD4^+$ lymphocytes and were, clinically, more susceptible for developing fibrotic lesions at pulmonary disease outcome. After *in vitro* activation of PBMC, higher percentages of $CD103^+CD4^+$ lymphocytes were found in the *ITGAE* -1088 AA group. These results implicate that the $\alpha_E\beta_7$ integrin may be involved in the pathogenesis and disease progression of sarcoidosis.

A second gene involved in the clinical expression of sarcoidosis is the *IL7R* gene (**chapter 7**). Genotype frequencies of six SNPs in the gene encoding for IL7Rα are compared between Dutch sarcoidosis patients and healthy subjects. An intron SNP was associated

with sarcoidosis and revealed a similar trend in patients with Löfgren's disease. *In silico* analysis showed a complete linkage between the intron SNP and a functional SNP in exon 6. The function of the amino acid change (T244I) has recently been described and the allele associated with sarcoidosis leads to a twofold increase in the skipping of exon 6 by disrupting an exonic splicing silencer compared to transcripts with the other allele, and simultaneously produces less membrane-bound IL7Rα protein. Carriage of the sarcoidosis risk genotype might result in a decreased ratio of membrane bound and soluble IL7Rα, leading to impaired IL-7 signaling. It is conceivable that compromised IL-7 signaling may reduce initial immune activation, leading to persistence of the putative antigen(s) and subsequent development of sarcoid granuloma.

The activated T lymphocytes and activated monocytes which are found in sarcoidosis may result from inadequate regulatory capacity of the immune system. Regulatory T lymphocytes play a central role in this respect and these cells are described in **Chapter 8**. The presence of different types of regulatory T lymphocytes in healthy subjects is presented and data from sarcoidosis patients is reviewed. Invariant NKT cells and Foxp3 positive T cells have both potent immune regulatory properties. In literature, results regarding both cell types in sarcoidosis are contradictory and not conclusive. Data on $Foxp3^+$ cells and iNKT cells from lungs of healthy subjects are scarce. We found that in BALF of healthy subjects both $Foxp3^+$ cells and iNKT cells are demonstrable. Invariant NKT numbers were found in equally low numbers as described in sarcoidosis. BALF $Foxp3^+$ cell numbers from healthy subjects however, were found higher compared to sarcoidosis in one study and lower compared to sarcoidosis in another study. Whether the excessive T cell and monocyte activation in active sarcoidosis is caused by abnormal immunoregulatory capacity locally in the lung remains undefined.

## Concluding remarks

Phenotyping immune cells by measuring expression of activation markers on the cell surface gives direct information on the cells which take part in the immune response in sarcoidosis. Instead of relying on analyte levels derived from complex processes (sACE reflecting granuloma formation or sIL-2R reflecting the activity of the T cell component), or on low resolution chest radiography, using immune cell phenotype as biomarker may be more precise and might improve sensitivity and specificity of determination of disease activity or assessment of disease severity.

Sarcoidosis is a complex disease thought to develop in the context of exposure to unknown triggers from the environment, on the background of a spectrum of genetic polymorphisms that may influence susceptibility to disease. In the last couple of years many associations have been reported between polymorphisms in various genes and susceptibility to sarcoidosis. Chapter 4 and 7 describe two novel susceptibility genes for sarcoidosis. *ITGAE* encodes the α chain for the integrin $\alpha_E\beta_7$, a protein which accounts for the interaction between T cells and epithelial cells. The data suggest an important role for the interaction between T cells and epithelium in development of sarcoidosis. These chronic activated effector T cells, illustrated in chapter 5, may be important as first line of defense but might also be harmful to an already compromised epithelium. Consistent, increased percentages of CD103 positive BALF cells at time of diagnosis showed prognostic value for development of parenchymal infiltrates at pulmonary disease outcome independent of chest radiography at presentation (chapter 5). Recently, Luzina et al. reported that based on observations in patients with diffuse parenchymal lung disease, in an animal model of pulmonary fibrosis, and in *in vitro* experiments the expression of integrins αVβ3 and/or αVβ5 on pulmonary T lymphocytes may regulate the extent of lymphocytic infiltration and the degree of pulmonary fibrosis. Whereas T lymphocytes that do not express integrins may be not involved in the fibrotic regulation process[1]. Based on our findings we conclude that $\alpha_E\beta_7$ is an additional integrin that, when expressed by pulmonary T lymphocytes may be a driving force contributing to prolonged T lymphocyte retention or accumulation at the mucosal epithelium in the lungs of sarcoidosis patients. To proof the intraepithelial localization of $CD103^+$ lymphocytes, lung epithelium tissue has to be stained with anti-CD103 antibodies. Investigating the functional properties of and the interaction between $CD103^+CD4^+$ effector T cells and lung epithelial cells may shed light on their role in epithelial damage and increased susceptibility for developing parenchymal abnormalities in sarcoidosis.

IL7Rα (CD127) is a functional key marker of the early heterogeneity observed in effector T cells. Both naive and memory T cells express high levels of IL7Rα, and IL-7 is required for their homeostasis. Activation of T cells results in down-regulation of IL7Rα within 48 h after antigenic stimulation, but there is re-expression on the resting memory pool during the course of an immune response. One intron SNP in the gene encoding the IL-7 receptor, *IL7R*, is associated with sarcoidosis (chapter 7). This SNP is in complete linkage with a functional SNP leading to an altered membrane bound/soluble IL7Rα ratio. IL-7 signaling leads to activation of STAT-1, the master transcription factor for development of $T_H1$ mediated immune responses. Compromised IL-7 signaling may contribute to reduced

initial immune activation, leading to persistence of the antigen and subsequent development of sarcoid granuloma.

Chapter 2 and 6 describe the research on the activation status of lymphocytes and monocytes in patients with different granulomatous interstitial lung diseases and healthy individuals. We have assessed this issue by determination of activation marker profiles on cells from blood and bronchoalveolar lavage fluid. The results in chapter 2 suggest the use of $CD8^{+}CD28^{null}$ cells as marker for disease severity in sarcoidosis. The correlations between $CD8^{+}CD28^{null}$ cells with indicators of disease severity are suggestive of a functional role of this subset of T lymphocytes for progression of the disease. Loss of CD28 expression occurs when lymphocytes are continuously activated by chronic stimulation. On the other hand, $CD8^{+}CD28^{null}$ cells might be harmful by production of granzymes, perforins, TNF-α and IFN-γ. Either way, monitoring CD28 expression on blood lymphocytes gives direct information of their presence in sarcoidosis patients. Functional tests with blood $CD8^{+}CD28^{null}$ cells from sarcoidosis patients are pivotal for characterizing the properties of these cells in the disease. Stimulation assays for example with bacterial pathogens *Propionibacterium acnes* and *Mycobacterium tuberculosis*, both have been leading suspects as the cause of sarcoidosis, may elucidate the function of this specific memory T cell population in sarcoidosis and why $CD8^{+}CD28^{null}$ cells correlate with indicators of disease severity.

Next to differences in the activation state of lymphocytes, also peripheral blood monocytes in sarcoidosis have an activated phenotype. The activation status of blood monocytes (described in chapter 6) might be a valuable additional disease activity marker in sarcoidosis. The results showed that 15% of the patients had normal values of serum sACE and serum sIL-2R but increased percentage of activated monocytes at disease presentation, suggesting that phenotyping monocytes may be more sensitive in assessing sarcoidosis disease activity. Unlike the histologic evidence of non-caseating epithelioid cell granuloma as gold standard for the diagnosis of sarcoidosis, there is no gold standard to define disease activity in sarcoidosis. *In vivo* 'granuloma activity' might be the best parameter of disease activity. Molecular imaging by $^{18}$F fluorodeoxyglucose ($^{18}$F-FDG) PET depicts metabolic activity in tissue and showed promising sensitivity in detecting metabolic 'granuloma activity' in newly diagnosed sarcoidosis patients[2]. It would be interesting to investigate whether $CD69^{+}VLA\text{-}1^{+}$ monocytes, possibly progenitors of macrophages involved in granuloma formation, remain superior to sACE and sIL-2R when analyzing correlations with serial volumetric measurements of $^{18}$F-FDG PET activity in sarcoidosis patients.

In chapter 3, a new diagnostic marker for pulmonary sarcoidosis is introduced. The $CD103^+CD4^+/CD4^+$ ratio, combined with a relative BAL/PB $CD4^+/CD8^+$ ratio, discriminates pulmonary sarcoidosis from other interstitial lung diseases. This ratio presents the proportion of $CD4^+$ effector T cells that express the integrin CD103 for retention at the mucosal epithelium. In sarcoidosis, the ratio is lower compared to the ratio in other ILD but does not differ from normal values. The use of the relative BALF/PB $CD4^+/CD8^+$ ratio prevents missing those sarcoidosis patients without an absolute $CD4^+$ alveolitis, thereby underscoring the limited diagnostic accuracy of the $CD4^+/CD8^+$ ratio of BAL T lymphocytes as the only parameter.

Due to the relatively small patients groups, additional research is needed to verify our findings and to evaluate the utility of the disease markers for clinical management of the patients. In this regard, CD69VLA-1 positive blood monocytes are being evaluated as a general activation marker for interstitial lung diseases in our hospital. Preliminary results in 250 samples from patients with different ILD show high numbers of activated monocytes in patients with sarcoidosis, hypersensitivity pneumonitis and interstitial pneumonia. Although the highest percentages are observed in sarcoidosis, increased proportions of activated monocytes in peripheral blood is not sarcoidosis specific.
The relevance of the percentage of CD103 positive BALF lymphocytes as prognostic marker for developing parenchymal abnormalities, needs to be validated in an even higher number of patients and awaits follow-up (> 4 years) chest radiography data. The use of HRCT scans will enable a more detailed characterization of the phenotypical differences between the types or extent of parenchymal abnormalities/pulmonary fibrosis and may improve the prognostic value of the percentage of $CD103^+$ BALF lymphocytes. In addition, it may specify the correlation between the severity of affected lung tissue and the presence of blood $CD8^+CD28^{null}$ cells.

Due to the increased possibilities to characterize the cellular and protein composition of BALF, the normal values of these novel parameters are mostly lacking for historical controls. We therefore set out to determine the normal values for cellular subsets and a broad range of protein biomarkers in a group of healthy subjects who underwent bronchoalveolar lavage in St. Antonius Hospital in 2007-2009. This enabled us to define unique blood and corresponding lung control values of the experimental parameters described in the different chapters.

*Future directions*

As described in this thesis, the phenotype of the different cells in peripheral blood as well as the lung is well characterized in sarcoidosis patients, and in healthy subjects for that matter. Unanswered questions are, how these cells dictate disease? What is their mode of action in progressive disease? How do these cells direct fibrosis formation? Which mechanisms are involved in the regulation of the effector cells? Generally, sarcoidosis is considered a $T_H1$ mediated immune disease. On transcription factor level, only one recently published article has confirmed this conviction in patients with acute sarcoidosis[3]. As pointed out in the introduction, other T cell subsets may be involved especially in progressive or chronic disease. Recent advances suggest that in chronic inflammation, a distinct lineage of $T_H$-17 cells may play a crucial role. $T_H$-17 cells express a unique transcription factor, ROR-c, which induces transcription of the *IL-17* gene in naive helper T cells and is required for the development of IL-17 producing cells in the presence of IL-21 and TGF-β or IL-6, or IL-6 and IL-23[4,5]. Interestingly, $T_H$-17 cell differentiation is inhibited by TGF-β induced Foxp3 by antagonizing ROR-c function[6]. Whether $T_H$-17 cells are involved in sarcoidosis or in a specific sarcoidosis phenotype will certainly be determined in the near future. Even more recent, $T_H$-22 cells (IL-22 producing T helper cells), become the latest addition to diversity of $CD4^+$ $T_H$ cell subsets[7]. This new subset of human T cells, is shown to be distinct from $T_H1$, $T_H2$ and $T_H$-17 cells, and is characterized by the expression skin-homing chemokine receptors and to produce IL-22, TNF-α and IL-13 but not IL-17 or IFN-γ. In sarcoidosis, $T_H$-22 cells might be relevant in a subset of patients suffering from skin abnormalities.

Finally, future genetic research may not only focus on analysis of polymorphisms but also on the methylation status of decisive genes. Molecular mechanisms of epigenetic imprinting include selective demethylation of CpG motifs and histone modifications as shown for cytokine genes[8-10]. Notably, demethylation at a highly conserved region within the human *Foxp3* gene (Treg-specific demethylated region, TSDR[11]) was found to be restricted to Treg when tested against all major peripheral blood cell types and a selection of non-blood cells. In addition, it was also observed that *Foxp3* TSDR demethylation occurred only in natural Treg, but not in recently activated effector T cells transiently expressing Foxp3[11-14]. The latter is especially relevant in TGF-β rich conditions like lung epithelia. These data indicate that epigenetic modifications in the *Foxp3* TSDR serve as a valuable marker for the identification of T cells with a stable Treg phenotype and might help to clarify whether Treg cells in sarcoidosis are primarily defective, or “exhausted” by repetitive and chronic stimuli.

Which cell is determinative in pulmonary sarcoidosis? Analogue to a recent article concerning Crohn's disease[15], sarcoidosis might be a primary inborn immunodeficiency of macrophages. These errors may result in impaired attraction of granulocytes to the lung epithelium, causing impaired clearance of intruding bacteria, thereby precipitating the formation of granulomas. The alveolar macrophage has not been discussed here but may serve as basis for a next thesis 'sarcoidosis revisited'.

This thesis however, may stimulate others to use cell activation profiles as means to determine the disease status in patients with sarcoidosis. Table 1 provides a straightforward protocol for the use of activation markers in sarcoidosis.

**Table 1** Recommendations for the use of cell activation markers

| | Marker | Cells |
|---|---|---|
| Diagnosis | $CD103^{+}CD4^{+}/CD4^{+}$ | BALF lymphocytes |
| Activity | $CD69^{+}VLA\text{-}1^{+}$ | PB monocytes |
| Severity | $CD8^{+}CD28^{null}$ | PB lymphocytes |
| Prognosis | $CD103^{+}$ | BALF lymphocytes |

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# Appendix

Bronchoalveolar lavage (BAL) is widely accepted as diagnostic tool in interstitial lung diseases. BAL differential cell counts can provide important information that supports the diagnosis of specific ILD[1,2]. BAL is a useful tool not only to express the complex pathogenic mechanisms of these ILD but also in excluding other diagnoses and causes of alveolitis, and to document specific exposures, such as the identification of asbestos bodies (ABs) in asbestosis or the proliferative response of BAL lymphocytes to beryllium in chronic beryllium disease (CBD)[3]. Moreover, BAL may be used to investigate inflammatory parameters in infection, neoplasms, exposure to toxic substances, asthma and chronic obstructive pulmonary disease (COPD)[4] and it is useful in monitoring the lung allograft and in evaluating pediatric lung disease. Additionally, BAL can be diagnostic *per se* in few rare disorders, *e.g.* increased BAL $CD1^+$ cells for histiocytosis X[5]. BAL differential cell counts usually show variations that differ from patterns found in normal subjects. These patterns tend to reflect inflammatory cell profiles in affected lung tissues[6].

In 1974, the first paper detailing BAL dealt with normal values, as the authors selected normal subjects and patients undergoing fibreoptic bronchoscopy (FOB) for ''evaluation of intrathoracic lesions''. True healthy subjects were included in the study of van den Bosch and colleagues performed in our hospital in 1983[7]. Many groups have been investigating BAL in healthy subjects since then, recently reviewed by Balbi et al[8]. Studies concerning BAL in healthy subjects often reveal shortcomings: small groups, young age group, no atopy status[8]. We performed bronchoalveolar lavage in 45 non-atopic healthy subjects between 18-65 years. BAL was performed according to standardized and validated procedure previously described[1]. Differential cell counts and lymphocyte subpopulations (T cell subsets, B cells and NK cells) were measured by flow cytometry in peripheral blood and bronchoalveolar lavage. Moreover, expression of the integrin CD103 on BALF lymphocytes, used as a new diagnostic marker in interstitial lung diseases[1], was analyzed. Table 1 and 2 present the peripheral blood and bronchoalveolar lavage data obtained from 45 non-atopic healthy subjects.

**Table 1** Differential cell counts, normal values in adults (18-65 years)[a]

| Non-smokers | Peripheral blood | | Bronchoalveolar lavage | | |
|---|---|---|---|---|---|
| n = 30 | #[b] | % | % | #[c] | |
| | | | 58 [44-72] | | Recovery |
| | | | | 9.8 [1.7-36.6] | Cell yield ($\times 10^6$) |
| Leukocytes | 5.4 [3.2-12.9] | | | 10.1 [1.7-34.9] | Cells/ml |
| | | | 95 [88-99] | | Vitality |
| Monocytes | 0.4 [0.2-1.0] | 7.4 [4.6-13.4] | 86.0 [56.3-96.3] | 7.5 [1.5-28.3] | Macrophages |
| Lymphocytes | 1.6 [1.2-3.1] | 34.1 [20.2-44.7] | 11.7 [2.7-39.9] | 1.0 [0.1-6.1] | Lymphocytes |
| Neutrophils | 2.9 [1.4-8.2] | 54.6 [43.4-69.3] | 1.2 [0-5.8] | 0.1 [0-0.6] | Neutrophils |
| Eosinophils | 0.14 [0.03-0.95] | 2.5 [0.9-16.4] | 0.3 [0-4.3] | 0.03 [0-0.44] | Eosinophils |
| Basophils | 0.03 [0-0.11] | 0.6 [0 -2.2] | 0 [0-0.4] | 0 [0-0.04] | Basophils |
| | | | 0 | 0 | Plasma cells |

| Smokers | Peripheral blood | | Bronchoalveolar lavage | | |
|---|---|---|---|---|---|
| n = 15 | #[b] | % | % | #[c] | |
| | | | 55 [35-65] | | Recovery |
| | | | | 19.7 [6.3-44.5] | Cell yield ($\times 10^6$) |
| Leukocytes | 7.5 [4.1-10.3] | | | 19.7 [9.5-46.4] | Cells/ml |
| | | | 95 [86-99] | | Vitality |
| Monocytes | 0.5 [0.4-1.0] | 7.6 [6.1-11.9] | 96.1 [78.5-98.6] | 19.0 [8.1-45.5] | Macrophages |
| Lymphocytes | 2.0 [1.5-2.8] | 30.9 [14.6-47.0] | 2.2 [0.2-17.4] | 0.4 [0.04-2.1] | Lymphocytes |
| Neutrophils | 4.4 [1.6-7.4] | 60.5 [38.0-71.9] | 1.5 [0.4-8.1] | 0.2 [0.1-0.8] | Neutrophils |
| Eosinophils | 0.19 [0.06-0.32] | 2.3 [1.2-4.8] | 0.3 [0.1-1.0] | 0.05 [0.01-0.27] | Eosinophils |
| Basophils | 0.03 [0.02-0.07] | 0.4 [0.3-1.0] | 0 [0-0.2] | 0 [0-0.08] | Basophils |
| | | | 0 | 0 | Plasma cells |

[a]Healthy subjects were non-atopic (total serum IgE ≤ 200 kU/l and allergen-specif IgE (Phadiatop®) ≤ 2.5). Values are expressed as median [range].
[b]$\times 10^6$ cells/ml.
[c]$\times 10^4$ cells/ml.

**Table 2** Lymphocyte subpopulations, normal values in adults (18-65 years)

| Non-smokers | Peripheral blood | | Bronchoalveolar lavage | | |
|---|---|---|---|---|---|
| n = 30 | #[a] | % | % | #[b] | |
| $CD3^+$ | 1.3 [0.8-2.3] | 77 [58-87] | 90 [81-99] | 0.9 [0.1-5.6] | $CD3^+$ |
| $CD4^+$ | 0.8 [0.5-1.6] | 48 [31-70] | 50 [18-88] | 0.4 [0.1-3.7] | $CD4^+$ |
| $CD8^+$ | 0.4 [0.2-0.9] | 25 [12-39] | 34 [8-78] | 0.4 [0.02-2.7] | $CD8^+$ |
| $CD4^+/CD8^+$ | | 1.9 [0.8-5.8] | 1.5 [0.2-11.0] | | $CD4^+/CD8^+$ |
| $CD19^+$ | 0.2 [0.1-0.5] | 12 [5-25] | 2 [0.3-12] | 0.01 [0.001-0.24] | $CD19^+$ |
| $CD3^-CD16^+CD56^+$ | 0.2 [0.1-0.4] | 10 [4-20] | 4 [1-12] | 0.03 [0.003-0.27] | $CD3^-CD16^+CD56^+$ |
| | | | 36 [14-76] | 0.36 [0.03-2.61] | $CD103^+$ |
| | | | 0.18 [0.03-0.33] | | $CD103^+CD4^+/CD4^+$ |
| | | | 0.9 [0.2-6.7] | | BAL/PB $CD4^+/CD8^+$ |

| Smokers | Peripheral blood | | Bronchoalveolar lavage | | |
|---|---|---|---|---|---|
| n = 15 | #[b] | % | % | #[c] | |
| $CD3^+$ | 1.5 [1.3-2.2] | 77 [65-85] | 89 [81-98] | 0.5 [0.03-2.0] | $CD3^+$ |
| $CD4^+$ | 1.0 [0.5-1.3] | 45 [35-65] | 43 [8-51] | 0.2 [0.01-0.6] | $CD4^+$ |
| $CD8^+$ | 0.5 [0.2-0.8] | 25 [12-48] | 43 [28-67] | 0.2 [0.02-1.2] | $CD8^+$ |
| $CD4^+/CD8^+$ | | 1.9 [0.7-5.4] | 1.1 [0.1-1.8] | | $CD4^+/CD8^+$ |
| $CD19^+$ | 0.3 [0.1-0.5] | 13 [7-25] | 1 [0.1-6] | 0.004 [0-0.08] | $CD19^+$ |
| $CD3^-CD16^+CD56^+$ | 0.2 [0.1-0.3] | 8 [4-14] | 5 [1-12] | 0.03 [0.005-0.1] | $CD3^-CD16^+CD56^+$ |
| | | | 44 [31-86] | 0.29 [0.02-1.20] | $CD103^+$ |
| | | | 0.21 [0.06-0.62] | | $CD103^+CD4^+/CD4^+$ |
| | | | 0.4 [0.1-2.5] | | BAL/PB $CD4^+/CD8^+$ |

Values are expressed as median [range].
[a] $\times 10^6$ cells/ml.
[b] $\times 10^4$ cells/ml.

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# Nederlandse samenvatting

## Immunologische merkers van cel activatie in sarcoïdose

De ziekte sarcoïdose kenmerkt zich door opeenhoping van ontstekingscellen tot zogenaamde granulomen. Deze granulomen ontstaan om de trigger, ofwel het antigeen, van de ziekte te isoleren en op te ruimen. Hoewel de meeste granulomen weer verdwijnen, zal een deel leiden tot chronische ontsteking met een risico op schade van het long parenchym en littekenvorming (fibrose). De immuun respons in sarcoïdose bestaat voornamelijk uit T lymfocyten die een cellulaire afweer reactie in gang zetten ($T_{Helper}$ 1 response). De T cellen die een rol spelen in de afweer reactie brengen eiwitten tot expressie die van belang zijn voor bijvoorbeeld het uit de bloedbaan treden van de cellen om in het longweefsel te komen. Andere tot expressie gebrachte eiwitten hebben specifiek te maken met herkenning van antigenen of met receptor functie voor signaal stoffen. De mate van expressie van deze verschillende eiwitten geeft aan hoe actief de cellen op dat moment zijn.
In dit proefschrift is onderzocht of het meten van de activatie status van de betrokken immuuncellen mogelijk gebruikt kan worden als biomerker voor het bepalen van de activiteit dan wel de ernst van de ziekte. Bovendien is gezocht naar variatie in genen die bij de activatie van immuuncellen een rol spelen. Deze genetische variatie, single nucleaire polymorfismen (SNP's), kunnen leiden tot veranderingen in de structuur van een eiwit waardoor de functie van het eiwit gestoord raakt of de expressie verhoogd of verlaagd is. Indien aanwezig kunnen SNP's een individu vatbaarder maken voor een ziekte of de ziekte kan een ander klinisch beloop hebben.

*Hoofdstuk 2: T cel activatie profielen in verschillende granulomateuze interstitiële longziekten – een rol voor $CD8^{+}CD28^{null}$ cellen?*
In dit hoofdstuk wordt de expressie van activatie merkers op lymfocyten onderzocht in patiënten met verschillende granulomateuze interstitiële longziekten. Van deze patiënten en van gezonde mensen is zowel perifeer bloed als bronchoalveolaire lavage vloeistof onderzocht. De drie groepen, sarcoïdose patiënten, patiënten met hypersensitieve pneumonitis en gezonde mensen vertoonden zeer geactiveerde lymfocyten uit de lavage vloeistof in tegenstelling tot lymfocyten uit perifeer bloed. Er bleek een sterke negatieve correlatie tussen diffusie capaciteit, een maat voor longfunctie, en het geactiveerde lymfocyten fenotype uit de long in de patiënten met interstitiële longziekten. In sarcoïdose werd een verschil gevonden tussen patiënten die zich presenteerden zonder en met parenchymale afwijkingen op de thoraxfoto. Patiënten met parenchymale afwijkingen hadden een verhoogd percentage bloed lymfocyten dat het costimulatoire molecuul CD28

niet tot expressie bracht. Deze cellen correleerden bovendien sterk met verminderde diffusie capaciteit en met geactiveerde lymfocyt fenotype uit de long. De correlaties met indicatoren van ziekte ernst suggereren dat dit bloed lymfocyt fenotype zou kunnen fungeren als nieuwe biomerker voor ziekte ernst in sarcoïdose. Vervolg studies met meer patiënten inclusief follow-up metingen kunnen de waarde van deze merker bewijzen.

*Hoofdstuk 3: Evaluatie van CD103 als diagnostische merker voor pulmonale sarcoïdose.*
Binnen de diagnostiek van de interstitiële longziekten wordt de verhouding tussen $CD4^+$ en $CD8^+$ lymfocyten uit de lavage gebruikt om onderscheid te maken tussen sarcoïdose en andere interstitiële longziekten zoals idiopathische pulmonale fibrose of hypersensitieve pneumonitis. Deze parameter is echter niet heel specifiek of sensitief voor sarcoïdose. Van het eiwit CD103, een integrine dat onder andere door lymfocyten op de celwand tot expressie wordt gebracht en bindt aan E-cadherine op epitheelcellen, is bekend dat het verlaagd is in sarcoïdose in vergelijking met fibrotische longziekten. De binding tussen CD103 en E-cadherine zorgt voor retentie van de lymfocyten bij de mucosale epitheel cellaag. In samenwerking met verschillende ziekenhuizen in Nederland is in lavage monsters van patiënten met verschillende interstitiële longziekten de CD103 expressie gemeten. De resultaten toonden aan dat de $CD103^+CD4^+/CD4^+$ ratio in de lavage in combinatie met lavage/bloed $CD4^+/CD8^+$ ratio gebruikt kan worden om sarcoïdose te onderscheiden van andere interstitiële longziekten. Dit is een verfijning en vernieuwing van gangbare markers binnen de diagnostiek van interstitiële longziekten.

*Hoofdstuk 4: Effect van variatie in ITGAE op het risico op sarcoïdose, CD103 expressie, en thorax radiologie.*
In dit hoofdstuk worden SNP's in het *ITGAE* gen onderzocht, het gen dat codeert voor de alfa keten van $\alpha_E\beta_7$ ofwel de integrine $CD103\beta_7$. Verder is gekeken naar variatie in *ITGAE* en de ontwikkeling van fibrose als gevolg van sarcoïdose. De aanwezigheid van T cellen bij het epitheel kan wijzen op een eerstelijns verdedigings functie tegen mucosale antigenen. Het eiwit CD103 is betrokken bij de binding van lymfocyten aan het long epitheel en dit heeft mogelijk invloed op de ontwikkeling en ziekte beloop van sarcoïdose. Wellicht dat genetische variatie in het *ITGAE* gen hierbij een rol speelt.
De frequentie van een promoter polymorfisme in het *ITGAE* gen bleek significant hoger in sarcoïdose patiënten. Patiënten met het AA genotype op plek -1088 hadden bovendien meer $CD4^+CD103^+$ lymfocyten in de lavage vloeistof en het AA genotype kwam significant vaker voor in de groep patiënten die bij pulmonale ziekte uitkomst fibrose vorming liet zien op longfoto versus patiënten die geen afwijkingen meer vertoonden op longfoto.

Functionele *in vitro* testen met bloed cellen toonden aan dat na 4 dagen kweken een hoger percentage T cellen met het genotype *ITGAE* -1088 AA CD103 positief waren. De resultaten toonden aan dat het *ITGAE* -1088 sarcoïdose risico genotype een effect had zowel *in vivo* als *in vitro* en suggereert betrokkenheid van CD103 integrine signalering in de ontwikkeling van sarcoïdose en mogelijk van fibrose vorming als gevolg van sarcoïdose.

*Hoofdstuk 5: Verhoogde CD103$^+$ lymfocyten in longen van sarcoïdose patiënten met parenchymale afwijkingen.*

De CD103 positieve lymfocyten uit de long spoeling van zowel sarcoïdose patiënten alsook van gezonde mensen vertoonden een specifiek fenotype ten opzichte van de niet mucosale of CD103 negatieve lymfocyten. Bovendien gold dit voor beide lymfocyten subsets, CD4$^+$ en CD8$^+$ cellen. Het fenotype van deze cellen werd gekenmerkt door expressie van eiwitten die het gevolg zijn van chronische activatie van deze lymfocyten.

Langdurige binding aan epitheelcellen en chronische activatie van de mucosale effector T cellen kan mogelijk schade veroorzaken aan het longepitheel en zodoende bijdrage aan de ontwikkeling van parenchymale afwijkingen in de long. Niet alleen werd bij sarcoïdose patiënten die zich presenteerden met parenchymale afwijkingen op de long foto een verhoogd percentage CD103$^+$ lymfocyten gevonden, het percentage CD103$^+$ lymfocyten bleek ook een prognostische waarde te hebben voor het ontwikkelen van stadium III of IV op de thoraxfoto na minimaal 4 jaar follow-up. Het laatste is bijzonder omdat dit ook onafhankelijk bleek van het stadium van de long foto bij presentatie; de analyse werd alleen uitgevoerd op sarcoïdose patiënten met vergrote klieren op de long foto, stadium I of II. Een verhoogd percentage CD103$^+$ lymfocyten in de long kan mogelijk een aanwijzing zijn voor een al aangetast longepitheel of tot vroege epitheelschade schade leiden. Deze bevinding zal moeten worden bevestigd in een veel groter cohort sarcoïdose patiënten alvorens CD103 expressie op lymfocyten uit de long spoeling als klinisch relevante prognostische merker gebruikt kan worden.

*Hoofdstuk 6: Verhoogde expressie van CD16, CD69 en VLA-1 op bloed monocyten in actieve sarcoïdose.*

Sarcoïdose is een systemische ziekte met een (chronisch) inflammatoire reactie op een nog onbekend antigeen. De ziekte kenmerkt zich door de aanwezigheid van ontstekingshaarden in de aangedane organen (granulomen). De reactie manifesteert zich vooral in de longen. Een belangrijke vraag is hoe actief de ziekte is in patiënten bij presentatie en bij follow-up. Bekende parameters voor het bepalen van ziekte activiteit zijn serum sIL-2R, een maat voor lymfocyten activatie en serum sACE, een maat voor

'granuloom lading'. Deze parameters zijn echter niet sensitief en niet specifiek voor sarcoïdose. Het onderzoek beschreven in dit hoofdstuk heeft zich toegespitst op de monocyte, een witte bloedcel en voorloper van de macrofaag die in granulomen een belangrijke rol heeft in het presenteren van het antigeen aan lymfocyten. De hypothese: monocyten in bloed van sarcoïdose patiënten hebben een geactiveerd fenotype en geactiveerde monocyten weerspiegelen ziekte activiteit. Geactiveerde monocyten brengen specifieke receptoren op de celwand tot expressie die gemeten kunnen worden. Monocyten van sarcoïdose patiënten vertoonden een meer geactiveerd fenotype dan monocyten van gezonde controles. Deze waarden daalden naar normale waarden nadat de ziekte in remissie was gekomen. In 15% van de patiënten zou bij presentatie noch de sIL-2R, noch de ACE concentratie actieve ziekte aangeven, terwijl geactiveerde monocyten in alle 23 patiënten aangetoond zijn.
Het meten van de tot expressie gebrachte receptoren, is een directe en gevoelige methode om de activatie status van monocyten te meten. Hoe actiever de monocyte, hoe actiever de immuun response en dus mogelijk een maat voor (sarcoïdose) ziekte activiteit.

*Hoofdstuk 7: Variatie in IL7R predisponeert voor sarcoïde ontsteking.*
*IL7R* is het gen dat codeert voor de interleukine 7 (IL-7) receptor. IL-7 is een sleutel eiwit in de homeostase van naïeve en 'memory' T lymfocyten. De verhouding tussen membraan gebonden en ongebonden IL-7R, en daarmee de mate van IL-7 signaal transductie, wordt bepaald door variatie (T244I) in het coderende deel van het *IL7R* gen. De genetische variatie van *IL7R* werd onderzocht in gezonde mensen en patiënten met sarcoïdose. De frequentie van genetische variatie in het eerste intron van *IL7R*, met dezelfde variatie als het T244I polymorfisme, bleek significant geassocieerd met sarcoïdose. Dit effect werd gerepliceerd in een validatie cohort. Bovendien werden, als granulomateuze controle ziekte, 139 patiënten met Löfgren syndroom onderzocht. Ook in deze patiënten groep kwam het AA genotype van het intron polymorfisme vaker voor. Sarcoïdose patiënten met het AA genotype hadden bij presentatie van de ziekte geen verhoogde ziekte activiteit merkers of verhoogde immuun cel aantallen in de lavage vloeistof ten opzichte van patiënten met de AG en GG genotypen. De resultaten wijzen erop dat IL-7 signalering mogelijk betrokken is bij de vroege ontwikkeling van granulomateuze long aandoeningen. Toekomstige studies moeten uitwijzen of IL-7 signalering in sarcoïdose inderdaad andere activiteit vertoond.

*Hoofdstuk 8: Regulatoire T cel subpopulaties in gezonde mensen en sarcoïdose patiënten.*
Persistente antigeen presentatie kan leiden tot chronische ontsteking en uiteindelijk tot destructie van longweefsel in sarcoïdose. De balans tussen de beschermende en de pathologische effecten van de immuunrespons in sarcoïdose bepaalt een goede prognose, bijvoorbeeld in patiënten met het syndroom van Löfgren, of heeft een chronische manifestatie tot gevolg met ernstige long fibrose en afname van longfunctie.
Een unieke populatie witte bloedcellen, regulatoire T cellen, zijn verantwoordelijk voor de suppressie van cellulaire immuunresponsen. Naast cel-intrinsieke perifere tolerantie mechanismen spelen deze Treg cellen, gekenmerkt door de expressie van de transcriptie factor Foxp3, een onontbeerlijke rol in het onderhouden van natuurlijke tolerantie, in het afwenden van autoimmuun reacties alsook het controleren van ontstekingsreacties. Een ander type T cel met immuun regulatoire eigenschappen is de invariant Natural Killer T cel (iNKT cel). iNKT cellen worden gekenmerkt door een T cel receptor met Vα24 en Vβ11 ketens. Deze invariante receptor bindt aan CD1d moleculen beladen met glycolipide antigenen, tot expressie gebracht door antigeen presenterende cellen. Over het fenotype van beide type cellen uit longen van gezonde mensen is echter weinig bekend. In dit hoofdstuk wordt nieuwe data gepresenteerd over de frequentie en fenotype van iNKT en $Foxp3^+$ regulatoire lymfocyten populaties in perifeer bloed en uniek, uit de longen van gezonde mensen. Voorts wordt een overzicht gegeven van de huidige kennis over deze cellen in sarcoïdose patiënten.

*Conclusie*
Het doel van dit proefschrift was te onderzoeken of de activatie status van betrokken immuun cellen bruikbaar is in de diagnostiek van sarcoïdose. Voorts is onderzocht of variatie in genen die coderen voor eiwitten gerelateerd aan cel activatie, invloed heeft op de vatbaarheid voor het krijgen van sarcoïdose of op het ziekte verloop.
Het bepalen van expressie patronen van activatie eiwitten op het oppervlak van cellen geeft directe informatie over de cellen die deel nemen aan de immuun reactie in sarcoïdose. Dit in plaats van afhankelijk te zijn van indirect parameters die hele, complexe processen weerspiegelen, zoals serum ACE waarden die granuloom belading reflecteren of serum IL-2R waarden die de activiteit van de T cel component vertegenwoordigt of de long foto met relatief lage resolutie. Gebruik maken van het immuun cel fenotype als biomerker is mogelijk preciezer en kan de sensitiviteit en specificiteit verhogen van het bepalen van ziekte ernst danwel ziekte activiteit.
Sarcoïdose is een complexe ziekte waarvan gedacht wordt dat die zich ontwikkelt in de context van relevante blootstelling in mensen met specifieke genetische achtergrond. In

de laatste jaren zijn meerdere associaties beschreven tussen variatie in verscheidene genen en de vatbaarheid voor sarcoïdose. Hoofdstuk 4 en 7 in dit proefschrift beschrijven twee nieuwe genen die predisponeren voor sarcoïdose. Het gen *ITGAE* codeert voor de α keten van het integrine $\alpha_E\beta_7$ (CD103), een eiwit dat verantwoordelijk is voor de interactie tussen T lymfocyten en epitheel cellen. De resultaten geven inzicht in de rol van T cellen nabij het epitheel. Deze chronisch geactiveerde effector T cellen, geïllustreerd in hoofdstuk 5, kunnen belangrijk zijn als eerstelijns verdediging maar zijn mogelijk zelf ook schadelijk voor een al aangetast epitheel. In samenhang hiermee, verhoogde percentages CD103 positieve lymfocyten uit de lavage op het moment van diagnose stelling vertoonden prognostische waarde voor het ontwikkelen van afwijkingen aan het long parenchym of voor het ontwikkelen van fibrose (littekenvorming) ten gevolge van het niet op een andere wijze oplossen van granulomateuze ontsteking.
Het gebruik van HRCT scans in plaats van thoraxfoto's maakt een nauwkeuriger beschrijving mogelijk van de verschillende typen en de uitgebreidheid van parenchymale afwijkingen of pulmonale fibrose. Mogelijk verbetert dit de prognostische waarde van het percentage $CD103^+$ lymfocyten. Voor het bewijzen van de intra epitheliale lokalisatie van $CD103^+$ lymfocyten bij mucosale long epithelia moet long weefsel worden aangekleurd met antilichamen tegen CD103. Onderzoek naar functionele eigenschappen van en de interactie tussen $CD103^+CD4^+$ effector T cellen en long epitheel cellen is van belang om de rol van deze cellen in de ontwikkeling van parenchymale afwijkingen in sarcoïdose op te helderen.
Het tweede nieuwe gen dat predisponeert voor sarcoïdose is het *IL7R* gen dat codeert voor IL7Rα (CD127), de receptor voor interleukine 7 (IL-7). IL-7 is een sleutel cytokine nodig voor de homeostase van T cellen met immunologisch geheugen ofwel 'memory' T cellen. Het allel geassocieerd met sarcoïdose heeft een bewezen functioneel effect op de verhouding tussen membraan gebonden IL-7 receptoren en IL-7 receptoren die vrij voorkomen in het serum. Het homozygote AA genotype zorgt voor een lagere ratio en dus een verminderde IL-7 signalering. Verminderde IL-7 signalering reduceert mogelijk vroege immuun activatie. Mogelijk leidt dit tot persisterende antigenen met als gevolg ontwikkeling van sarcoïde granulomen.
De activatie status van lymfocyten en monocyten in patiënten met verschillende interstitiële longziekten en gezonde mensen wordt beschreven in hoofdstuk 2 en 6. Door middel van het meten van de expressie patronen van activatie eiwitten op leukocyten in bloed en lavage vloeistof is onderzocht of deze onderscheidend zijn voor een specifieke interstitiële longziekte, of voor een typerend klinisch sarcoïdose fenotype, de ernst van de ziekte weergeven of de ziekte activiteit weerspiegelen. De resultaten in hoofdstuk 2

suggereren dat het voorkomen van $CD8^{+}CD28^{nul}$ cellen in bloed van sarcoïdose patiënten te maken heeft met de ernst van de ziekte. Niet alleen correleerde deze marker met verschillende indicatoren voor ziekte ernst, ook voorspelde de $CD8^{+}CD28^{nul}$ cel frequentie het gebruik van corticosteroïden binnen twee jaar na presentatie van de ziekte. Mogelijk is dit een waardevolle biomerker voor het monitoren van ziekte ernst, vooral als deze meerdere andere testen kan vervangen. Hiervoor is nodig dat een groot cohort goed omschreven sarcoïdose patiënten langere tijd wordt vervolgd en seriële metingen moeten uitwijzen of deze hypothese stand houdt. Functionele experimenten met $CD8^{+}CD28^{nul}$ cellen van patiënten zullen moeten uitwijzen waarom deze cellen in sarcoïdose patiënten voorkomen en of ze bijdragen aan of een gevolg zijn van de ziekte.

Een biomerker voor ziekte activiteit in sarcoïdose is vaak een onderwerp van debat. In de tijd zijn verschillende biomerkers de revue gepasseerd, echter geen heeft bewezen superieur te zijn aan de meest gebruikte, maar weinig sensitieve of specifieke biomerker voor sarcoïdose ziekte activiteit namelijk serum ACE. Circulerende monocyten zijn mogelijk voorlopers van alveolaire macrofagen betrokken bij granuloom formatie. Hoofdstuk 6 laat zien dat de het meten van specifieke activatie moleculen op monocyten gevoeliger is voor het aantonen van klinisch actieve ziekte dan serum ACE of serum IL-2R waarden. Wederom zal vervolg onderzoek in een grotere groep patiënten met verschillende interstitiële longziekten moeten uitwijzen of dit fenomeen specifiek is voor sarcoïdose en hoe goed geactiveerde monocyten correleren met ziekte remissie.

In tegenstelling tot de gouden standaard voor de diagnose sarcoïdose, een histologisch bewijs van niet-verkazende granulomen, is er geen gouden standaard om ziekte activiteit te definiëren. *In vivo* 'granuloom activiteit' is misschien de beste parameter van ziekte activiteit. Hiervoor kunnen volumetrische metingen van $^{18}$F-FDG PET activiteit in sarcoïdose patiënten, een maat voor metabole activiteit in weefsel, een uitkomst zijn. Huidige en nieuwe biomerkers voor ziekte activiteit kunnen dan hier tegen worden afgezet.

Ten slotte, CD103 expressie op lymfocyten heeft ook een diagnostische waarde. Het aandeel $CD4^{+}$ lymfocyten dat ook CD103 tot expressie brengt is in patiënten met andere interstitiële longziekten hoog ten opzichte van waarden gevonden in de lavage vloeistof van sarcoïdose patiënten. Of ten opzichte van de normaal waarden in gezonde mensen. Het meten van deze ratio in de lavage vloeistof in combinatie met een relatieve lavage/bloed $CD4^{+}/CD8^{+}$ ratio, is een aanscherping van de huidige diagnostische biomerker voor sarcoïdose, de $CD4^{+}/CD8^{+}$ ratio in de lavage vloeistof.

De resultaten beschreven in dit proefschrift laten zien dat het gebruik van immuun cel fenotype als biomerker voor de ziekte sarcoïdose veelbelovend is. Hopelijk zet dit anderen aan tot verder onderzoek naar het gebruik van deze biomerkers in de behandeling van sarcoïdose patiënten en vult fundamenteel onderzoek de gaten in de kennis over de rol van de diverse witte bloed cellen in de ontwikkeling van pulmonale sarcoïdose.

## List of publications

Michiel Heron, Jan C. Grutters, Anke M. E. Claessen, Daniëlle Hijdra, and Jules M. M. van den Bosch. Increased $CD103^+$ lymphocytes in lungs of sarcoidosis patients with parenchymal involvement. *Submitted.*

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## Curriculum vitae

Michiel Heron was born September 20, 1973 in Bath, Great Britain. After secondary education at Koningin Wilhelmina College in Culemborg, he started with Biology at Utrecht University in 1998. In 2003, Michiel obtained his Master's degree (MSc) in Biology with a specialty in cell biology and immunology.

Before commencing his doctoral studies in March 2005 with Prof. dr. Jules M.M. van den Bosch, Dr. Jan C. Grutters and Dr. Anke M.E. Claessen at the St. Antonius Hospital in Nieuwegein, he worked as a employee at the department of radiology at the St. Antonius Hospital. Michiel is currently working as researcher at the center for Interstitial Lung diseases (cIL) with Prof. dr. Jules M.M. van den Bosch.