Dendritic Cells and T Lymphocytes in the Pathogenesis of Pulmonary Sarcoidosis

Bregje ten Berge

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	Prof.dr. B.N. Lambrecht
Overige leden:	Prof.dr H.C. Hoogsteden
	Prof.dr. J.C. Grutters
	Prof.dr. M. Drent
Copromotor:	Dr. B. van den Blink

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Introduction to Pulmonary Sarcoidosis

1. PULMONARY SARCOIDOSIS

1.1 Definition, epidemiology and etiology of sarcoidosis

The history of sarcoidosis begins in 1899 when the Norwegian dermatologist Ceasar Boeck described nodular skin lesions characterized by epitheloid cells and a few giant cells as multiple benign sarcoid of the skin [1]. Now, many years later, a lot more is known about sarcoidosis. The definition of sarcoidosis is described in the American Thoracic Society statement on sarcoidosis in 1999: sarcoidosis is regarded as a multisystem disorder of unknown cause, commonly affecting young and middle aged adults, with bilateral hilar adenopathy, pulmonary infiltrates, eye and skin lesions. Also other organs like the liver, the spleen, lymph nodes, salivary glands, the heart, muscles, bones and the nervous system can be involved. The diagnosis is established when clinical and radiological findings are supported by histological evidence of non-caseating epitheloid cell granulomas. Granulomas caused by other diseases need to be excluded [2, 3].

Sarcoidosis affects people of all racial and ethnic groups and occurs at all ages although it usually develops before the age of 50 years, with a peak incidence at 20-39 years[4]. The incidence of sarcoidosis varies widely all over the world with the highest incidence in the northern European countries [5]. There is a predispondance of sarcoidosis in females. In America, the incidence of sarcoidosis in Afro-Americans is roughly three times higher compared to white americans [4]. In black Americans the peak incidence occurs later in live and sarcoidosis is more often chronic and fatal [6].

Unfortunately, to date the etiology of sarcoidosis remains unknown, despite all research performed so far. Because sarcoidosis involves the skin, the eyes and the lungs, search for environmental causes is focussed on airborne antigens. Many investigations have been performed and associations were made with irritants in rural settings [7], exposure to anorganic particles [8], and occupational exposition in the U.S. Navy and fire fighting [9, 10]. With the use of polymerase chain reaction techniques, associations of sarcoidosis with mycobacterial and propionibacterial DNA have been found [11]. Recently Mycobactrium tuberculosis katG has been identified as a possible risk factor of sarcoidosis [12]. So multiple risk factors were reported for sarcoidosis, but it seems credible that sarcoidosis is the end result of immune responses to different environment stimuli.

Beside the search for an environmental cause, epidemiological data and the fact that siblings of patients with sarcoidosis are at increased risk for the disease, points to a genetic predisposition to sarcoidosis. The strongest associations are found in the HLA-DR region, and recent studies show specific HLA-DR subtypes are more associated with typical disease phenotype rather than disease susceptibility [13, 14]. Other recent studies show associations with apoptotic genes like ANXA11 and sarcoidosis [15].

Beryllium has been known to cause chronic beryllium disease (CBD), a debilitating and potentially fatal granulomatous disease that mainly affects the lungs. Chronic beryllium disease can take more than 30 years to develop, but it can also present within 3 months after initial exposure. Pathological and clinical features of sarcoidosis and chronic beryllium disease are often indistinguishable. Beryllium sensitisation can occur within 2 months after exposure. Differential diagnosis between sarcoidosis and CBD can be made with the beryllium lymphocyte-proliferation test (BeLPT) [16].

1.2 Clinical presentation and diagnosis of sarcoidosis

Sarcoidosis is a multiorgan disorder and symptoms are depending on the ethnicity, duration of illness, site and extent of organ involvement and the activity of the granulomatous process. Non-specific constitutional symptoms of fatigue, fever, malaise and weight loss occur in about one third of all the patients [6]. Often sarcoidosis comes first to attention when abnormalities are detected on a chest X-ray during a routine screening examination. In more than 90% of patients pulmonary involvement in seen leading to symptoms such as dyspnoea, cough, vague chest discomfort and wheezing. Clubbing, haemoptysis and crackles are rarely found in sarcoidosis [6].

Chest radiographs in sarcoidosis have been classified in four different stages (see Figure 1) [17]. Stage 1 is defined as bilateral hilar lymphadenopathy without pulmonary infiltrates. Stage 2 is a bilateral hilar lymphadenopathy with pulmonary infiltrates. Stage 3 is characterized by infiltrations in the lung alone, whereas in stage 4 fibrotic bands, bul-

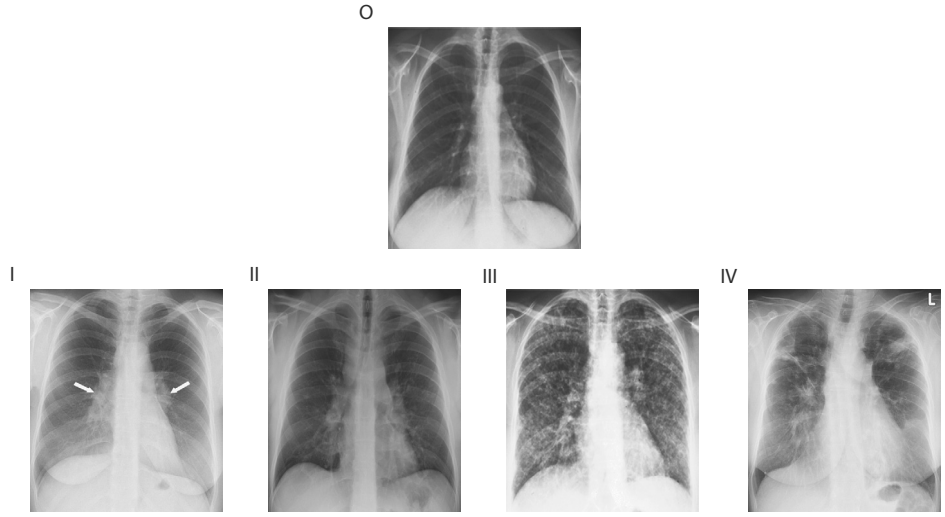

Figure 1. Chest X rays showing the different stages of sarcoidosis. Stage 0: normal chets x ray. Stage 1: bihilar lymphadenopathy. Stage 2: bihilar lymphadenopathy and reticulonodular infiltrates. Stage 3: reticulonodular infiltrates. Stage 4: fibrocystic sarcoidosis typically with upward hilar retraction, cystic and bullous changes (with acknowledgement to M. Drent)

lae, hilar retraction, bronchiectasis and diaphragmatic tenting are present. Importantly, these stages only represent radiologic findings and do not indicate disease chronicity or correlate with changes in pulmonary function [18]. About two third of the patients have airflow limitation at presentation and spirometry usually indicates restrictive ventilatory disfunction with reduced forced vital capacity (FVC) and reduced expiratory volume in 1 second (FEV1) [6]. Pulmonary lesions can resolve spontaneously, but can also result in pulmonary fibrosis with pulmonary hypertension as a major complication [19, 20].

Besides the lungs, organs often involved in sarcoidosis include the skin, with different lesion like lupus pernio or erythema nodosum, the eyes, the heart, the bones and joints, the liver, the spleen, the nervous system and the kidney, associated with hypercalciemic renal disease [2]. Involvement of these organs results in different lesions and a variable clinical presentation in patients. Some extra-pulmonary manifestations such as skin lesions can be mild and are not harmful, but others, particularly cardiac or neurosarcoidosis, can be life-treating.

Löfgren's syndrome is an acute form of sarcoidosis presenting with arthritis, erythema nodosum and bilateral hilar adenopathy occuring in 9-34% of all patients.[21] Löfgren's syndrome presents differently in men and women. Erythema nodosum is observed predominantly in women and marked ankle periarticular inflammation or arthritis without erythema nodosum is more common in men [22, 23]. Two thirds of patients with sarcoidosis generally have a spontaneous remission within a decade after diagnosis with few or no consequences. In most of the patients remission occurs within 3 years. One third of the patients have relenting disease leading to significant organ impairment. Recurrence after one or more years is uncommon but recurrent disease may develop at any age in any organ. Less than 5% of patients die from sarcoidosis, whereby death is usually the result of pulmonary fibrosis with respiratory failure or cardiac or neurologic involvement [24]. Severe pulmonary hypertension (PH) is an important complication of pulmonary fibrosis in stage IV sarcoidosis. PH is very resistant to treatment and often lung transplantation (single or double lung) or heart-lung transplantation is the only option [25, 26].

In the past the Kveim-Silzbach test was used for the diagnosis of sarcoidosis [27]. This test is performed by injecting homogenate of human sarcoid tissue extract intradermally. 4weeks later the papula that has developed at the side of injection is biopsied and shows granuloma formation in the skin. This test is now no longer used because injection of human material implicates many constraints.

Nowadays the diagnosis is established on the basis of compatible clinical and radiological findings together with histological evidence of non-caseating granuloma in one or more organs. Differential diagnosis should be made with mycobacterium tuberculosis infection, CBD, lymphoma, aspergillosis and hypersensitivity pneumonitis [3, 16, 28]. In Löfgren's syndrome the diagnosis can be made based on clinical findings even without a biopsy [24]. When it is not possible to obtain material for histological evidence analysis

of the lymphocytes in a bronchoalveolar lavage (BAL, see Figure 2) can also point to the diagnosis of sarcoidosis. In sarcoidosis a high number of lymphocytes is found in the BAL, with an increased ratio of CD4/CD8 cells (>3,5) [3].

Angiotensine converting enzyme (ACE) is produced by sarcoidal granulomas, and ACE levels are elevated in 60% of patients with sarcoidosis. ACE levels are influenced by ACE gene polymorphisms. Measurement of ACE levels lacks sensitivity and specificity to use as diagnostic tool but ACE levels can be used to improve interpretation of clinical signs [29, 30].

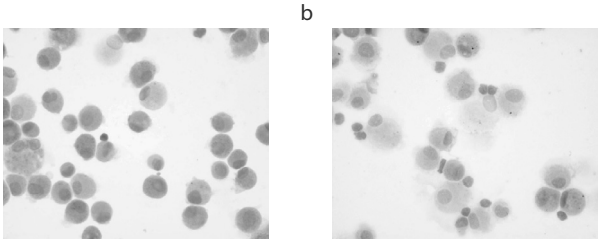

Figure 2. Broncho alveolar lavage cells showing an increased number of lymphocytes typical for sarcoidosis (b) compared to healthy control lavage (a).

1.3 Immunopathogenesis

Histologically sarcoidosis is characterized by the presence of granulomas in the involved organs (See Figure 3). Granulomas in sarcoidosis are non-caseating, well circumscribed and consist of epitheloid cells, small mononuclear cells and Langerhans-type giant cells. A rim of lymphocytes surrounds the sarcoid granuloma. Epitheloid cells are monocyte-derived cells attracted to the side of inflammation. Giant cells are large cells, containing many nuclei and are derived by fusion of epitheloid cells. Within the giant cells, three types of inclusion antibodies can be found, Schaumann, asteroid and residual bodies [31]. Differential diagnosis should be made with other granulomatous diseases of the lung like tuberculosis, aspergillosis and hypersensitivity pneumonitis. Granulomata should be carefully evaluated for necrosis, the distribution patterns along the lymphatic routes, the presence of fibrosis or an associated inflammatory infiltrate [28].

Clinically sarcoidosis is characterized by peripheral blood T cell lymphopenia, accompanied by cutaneous anergy to tuberculin and other skin tests. In the BAL increased numbers of $CD3^{+}CD4+$ T cells are found, resulting in an increased CD4/CD8 ratio. Lung T cells in sarcoidosis patients are highly activated, expressing the IL-2 receptor (CD25), CD69 and CD26 [32, 33]. When BAL $CD4^{+}$ and $CD8^{+}$ T cells were stimulated in vitro, Wahlstrom et al. [34] showed at single cell level that these cells secreted high levels the cytokines interferon gamma (INF-γ), interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF-α), which are known as typical T helper 1 (Th1) cytokines (see below). In the BAL

fluid of sarcoidosis patients and at sites of inflammation also increased levels of Th1 cytokines are seen [35, 36].

Sarcoidosis is a Th1-mediated disease in which activated and cytokine producing T cells play a major role in the pathogenesis [37]. To become activated, T cells need to engage the proper major histocompatibility complex (MHC) – peptide antigen complex, together with correct co-stimulatory molecules presented by antigen presenting cells (APCs). In the lung, both macrophages and dendritic cells present antigens to lymphocytes, of which the the dendritic cell is the most potent APC and will be discussed below.

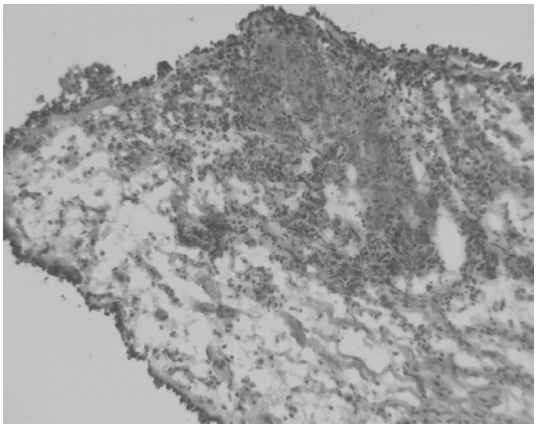

Figure 3. Lung biopsy of a sarcoidosis patient stained with CD11c revealing a granuloma typical for sarcoidosis.

2. DENDRITIC CELLS

2.1 Dendritic cell subsets in the lung

Dendritic cells (DCs) are professional APC that are present throughout the body, but in higher numbers at sides of high antigen exposure. DCs are present in a so-called immature state, specially equipped to capture different antigens, but still unable to stimulate naive T cells. DCs in the lung were first described by Sertl and collegues[38]. They described DCs in the trachea, bronchi, alveoli and visceral pleura of mouse and human pulmonary tissue. These cells were positive for HLA-DR and acted as potent T cell stimulators in vitro. Different cell characteristics and immunological markers on DCs were used over the years to identify and isolate DCs from the human long. Recently detailed phenotyping of human pulmonary DCs was performed. In human BAL fluid three types of DCs were detected. Myeloid DC type 1 ($CD11c^{+}CD1c+$), myeloid DC type 2 ($CD11c^{+}BDCA\text{-}3^{+}$) and plasmacytoid DCs ($CD11c^{-}BDCA\text{-}2^{+}$)[39]. DCs use toll like receptors (TLR) to detect the presence of infection. TLRs are a type of pattern recognition recep-

tor and recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs). TLR ligands include e.g. bacterial cell-surface lipopolysaccharides (LPS), proteins such as flagellin from bacterial flagella, double-stranded RNA of viruses, unmethylated CpG of bacterial and viral DNA. Myeloid DC type 1 and myeloid DC type 2 express mRNA transcripts for TLR1, TLR2, TLR3, TLR4, TLR6 en TLR8. In response to TLR2 and TLR4 ligands, mDC type 1 and mDC type 2 release proinflammatory cytokines (TNFα, IL-1β, IL-6 and IL-8). When stimulated with TLR3 ligands (recognizing viral double stranded RNA) only type 1 mDCs produce proinflammatory cytokines. Plasmacytoid DCs express TLR 7 and TLR 9 (recognizing single-stranded RNA and bacterial, non-methylated CpG) and release proinflammatory cytokines in response to imiquimod, and TNFα and to CpG oligonucleotides. Furthermore, the different DC populations were tested *in vitro* for their T cell stimulatory capacity to purified allogeneic T cells. mDC type 1 are strong inducers of T cell proliferation, while pDCs hardly induce any proliferation. mDCs type 2 have an intermediate T-cell stimulatory capacity [40].

2.2 Dendritic cell activation in the lung

In a resting state in the lung resident DCs are present, but when inflammation in the lung occurs, DCs and monocytes are attracted from to blood into the lung. Differentiation of monocytes into DCs in the lung requires IL-15, produced by airway epithelial cells [41-44].

In the lung DCs are present as a superficial submucosal network. Mouse studies with DCs in the gut show that DCs are able to sample the lumen by opening the tight junctions of the epithelium to stretch their dendrites into the lumen [45]. Probably in the lung the same mechanism occurs to pick up antigen from the endobronchial lumen. The uptake of antigen by DCs occurs in two different ways, by macropinocytosis for soluble antigens [46] and with C-type Lectin molecules that act as pathogen recognition receptors on the membrane of DCs [47]. Captured antigens are stored and processed in endocytotic vesicles for presentation on MCH class II (HLA-DR). After having taken up the antigen, DCs will mature, whereby the skills to capture antigens rapidly decline and the MHC class II complexes and expression of co-stimulatory markers are upregulated on the cell membrane. Mature DCs are characterized by high expression of MCH class II [48] and membrane expression of co-stimulatory markers, such as CD80, CD86, CD83 and CD40. These co-stimulatory markers are necessary for T cell activation because they need to bind with their complementary receptors on the T cell membrane. Furthermore DCs express the chemokine receptor CCR7, which allows them to migrate to the draining lymph nodes of the lung [49, 50].

In the draining lymph nodes DCs will fully mature and activate the present naive $CD4^+$ T cells starting the immune reaction against the invading antigens in the lung. DCs do

not only activate T cells, they can also tolerate T cells to self-antigens, to prevent autoimmune reactions. Depending on the recognition of self or non-self signals in the presence or absence of danger signals, DC antigen uptake and presentation leads to activation or downregulation of the T cell response [51]. Moreover, DCs can influence the polarization of naive T cells into Th1, Th2, Th17 or T regulatory direction depending on the type of microorganism that is recognized and the tissue factors present on the site of activation (See Figure 4). This allows DCs not only to initiate active immune responses, but also to control and dampen these immune responses, and to prevent unwanted responses against self-antigens.

In sarcoidosis skin lesions increased DC numbers are found, located around the granulomata and in close contact with $CD3^+$ T lymphocytes [52-54]. In blood of sarcoidosis patients normal and decreased levels of dendritic cells were reported [54, 55], but isolated blood DCs or DCs cultured from blood monocytes seem to have a impaired capacity to stimulate T cells [56]. In BAL fluid, similar numbers of DCs were reported for healthy controls and for sarcoidosis patients but DCs show a different phenotype [57]. The role of DCs in the lung, in particular in various interstitial lung diseases including sarcoidosis is introduced and discussed in detail in chapter 2 of this thesis.

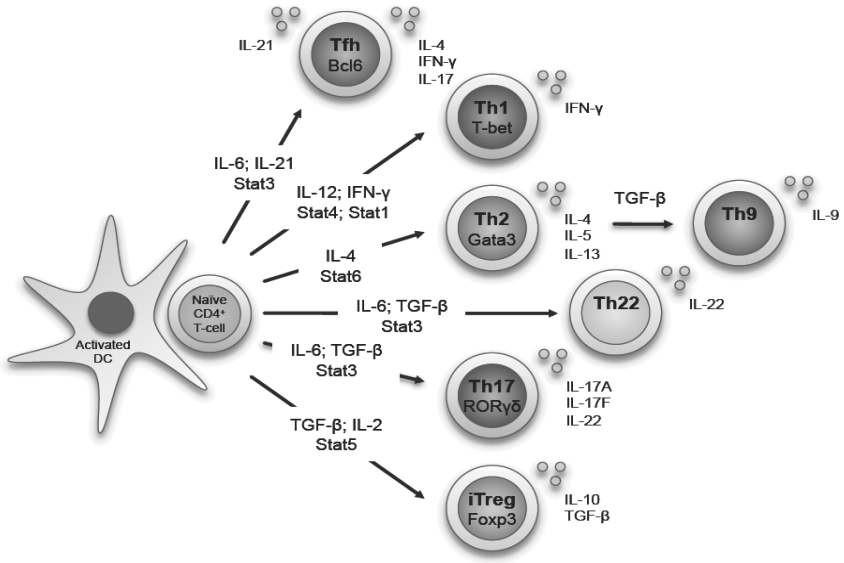

Figure 4. Schematic presentation of differentiation into various subsets of T cells upon DC-mediated activation. Taken from: C. Ribeiro de Almeida and R.W. Hendriks, The zinc-finger DNA-binding protein CTCF: a crucial regulator of gene expression in T lymphocytes. In: R. Ciofani and L. Makrlík (Editors) *Zinc Fingers: Structure, Properties and Applications*. Nova Science Publishers, Inc. Hauppauge NY, USA (in press).

3. T LYMPHOCYTES

3.1 T helper cell differentiation

Years ago Mosmann and Coffman [58] proposed that T helper cells could be divided into two distinct subsets, the Th1 and Th2 cells, characterized by distinct cytokine profiles and effector function. Th1 cells, producing IFNγ and lymphotoxin-α, are associated with the elimination of intracellular pathogens. Two major signalling pathways facilitate Th1 development, one involving IL-12/STAT4 and the other involving IFNγ/STAT1/T-bet. Th2 cells, producing IL-4, IL-5 and IL-13, are critically important for the eradication of parasitic worms, but are also implicated in allergic responses, in particular asthma [59]. Th2 differentiation is dependent on IL-4-induced activation of STAT6, leading to expression of the zinc-finger transcriptional regulator GATA3. GATA3 auto-activates its own expression and increases the accessibility of the Th2 cytokine cluster containing the genes coding for IL-4, IL-5 and IL-13. Furthermore, GATA3 suppresses Th1 development by downregulating the expression of STAT4 and IL-12R β2 chain. Only if T-bet is sufficiently induced in naive T cells, such GATA3 suppression is counteracted, permitting Th1 differentiation to occur (See Figure 4).

Next to Th1 and Th2 cells, regulatory T cells have been defined as $CD4^+$ characterized by high expression of the IL-2αR CD25 and the transcription factor Forkhead boxP3 (FoxP3), development of which is dependent on TGFβ (see Figure 4) [60].

More recently the discovery of the IL-17 family of cytokines and the analysis of IL-23 mediated effector function on T cells suggested the existence of an additional subset of Th cells that produce the proinflammatory cytokine IL-17 and for that reason were called Th17 cells. IL-17 is involved in the control of a wide range of infections at mucosal surfaces and has been implicated in the pathogenesis of autoimmune diseases, including experimental autoimmune encephalomyelitis, rheumatoid arthritis, autoimmune myocarditis and psoriasis (reviewed in [61-63]).

3.2 Th17 cells and their cytokines

Initial studies identifying Th17 cell biology were performed in mice and focused on factors required for initiation and differentiation of the Th17 cell function. To become Th17 cells, naive $CD4^+$ cells need to be activated through the T cell receptor in the presence of TGFβ and IL-6, which leads to the expression of the transcription factor RORγt (See Figure 4). TGFβ, IL-6 and RORγt drive $CD4^+$ naive T cells towards the Th17 lineage. The effects of IL-6 on Th17 differentiation are mediated by the transcription factor STAT3 [64]. In humans not only TGFβ and IL-6 are important for Th17 differentiation but also IL-1β, produced by APCs, seems to play an important role in the induction of RORγt [64].

Like IL-12 is important for the initiation of Th1 cells, IL-23 is specifically and primarily involved in the induction of Th17 cells. IL-23 and IL-12 are produced in the innate im-

mune response by APCs such as DCs and macrophages. IL-12 and IL-23 are members of the same family of cytokines. They both share a common p40 chain, linked to p35 for IL-12 or to p19 for IL-23. IL-23 acts by activating the IL-23 receptor. Neutralization studies with human naive T cells and studies in which different combinations of recombinant cytokines were added show that IL-1, TFG-β, IL-23 and IL-6 were necessary for IL-17 production by T cells [65].

Th17 cells are also capable of expressing TNFα, IL-6, IL-22, IL-21 as well as IL-26. However, because other T cell subsets are also able to produce these cytokines, it is currently unclear whether they should be included as a part of their defining cytokine production profile [66]. In addition to IL-17A, IL-17 family members also include IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. Of all these cytokines IL-17A and IL-17F share the greatest homology and are regulated by similar mechanisms [67]. IL-17A is most extensively studied and is often referred as IL-17. IL-17 has divers biological functions, it recruits neutrophils to sites of inflammation [68], it acts on macrophages to promote their recruitment and survival [69], and it stimulates the production of proinflammatory cytokines by a variety of immune and non immune cells [70]. Recently, it was discovered that IL-17 enhances the capacity of human $CD4^+$ T cells to produce IL-2 and the proliferation of T cells and Tregs[71].

IL-22, a cytokine belonging to the IL-10 family is produced by human Th17 cells and Th1 cells but in lower amount. Studies with neutralization of TGFβ and IL-6 show that in human naive T cells the production of IL-17, IL-22 and IFN-y is regulated differently. IL-22 is a mediator of mucosal host defence, it enhances wound repair, and it is crucial for epithelial barrier integrity.

Th17 cells in humans are only recently the subject of research so the function of Th17 cells in the human immunopathology is not clear. Only a limited number of recent studies have addressed the role of Th17 cells in cystic fibrosis, rheumatoid arthritis, multiple sclerosis [72-78] and no data have been reported about the role of Th17 in sarcoidosis. In another granulomatous disease, tuberculosis, IL-17 seems to play an important role after infection. Quickly after infection γδ T cells mainly produce IL-17. IL-17 production by $CD4^+$ T cells is also required, not only to clear the primary infection but also to establish effective memory responses [79].

3.3 Regulatory T cells

The ability to distinguish between self and non-self is an important hallmark of the immune system. The ability to make this discrimination allows the immune system to inhibit autoimmune responses but at the same time initiate effective responses against microbial antigens [80]. The state of unresponsiveness to self-antigens is maintained by different mechanisms. Besides elimination of autoreactive T cells in the thymus, called central tolerance, potential auto reactive T cells, that have escaped thymic selection, are

disabled in the periphery, called peripheral tolerance. One of the cells that can actively contribute to suppressing the response of the immune system to self or other antigens are the regulatory T cells (Tregs) [81]. Tregs suppress the activity of effector T cells (Teff) and other immune cells as a physiologic mechanism of immune regulation. Two different subsets of Tregs can be distinguished, Tregs derived and programmed in the thymus (naturally occurring or innate Tregs) and Tregs generated from naive $CD4^+$ T cells in the periphery, named adaptive Tregs [60]. Both Tregs are $CD4^+$ and characterized by high expression of IL-2R/CD25 and the transcription factor Forkhead boxP3 (FoxP3) [60].

A trademark of Tregs is their lack of proliferative response upon T cell receptor (TCR) activation or stimulation with mitogenic antibodies. Thus these cells show an anergic phenotype [82]. However, Tregs have to be activated by their TCR to exert their inhibitory function and when Tregs are activated, they inhibit other T cells in an antigen non-specific way [83].

Three mechanisms have been suggested to be involved in Treg mediated suppression. First, Tregs repress the cytologic functions of Teff by disturbance of the IL-2 homeostasis through inhibition of IL-2 production by Teff cells [84] and by rapid consumption of the IL-2 produced by Teff cells[85]. IL-2 depletion impairs the cytologic responses of Teff by inducing anergy in $CD4^+$ and $CD8^+$ T cells after TCR stimulation [84]. The second mechanism is the secretion of IL-10 and TGFβ by Tregs [86]. The third mechanism is the direct cytotoxic effect of Tregs on Teff, B-cells, and monocytes, which is dependent on cell-cell contact. Innate Tregs predominantly use perforin/granzym for direct killing [87, 88], whereas in adaptive Tregs this mechanism is downregulated and FasL is the predominant cytotoxic molecule inducing apoptosis in the target cells [85, 89]. In addition the deprivation of IL-2 makes Teff also more sensitive for apoptosis [90].

Recently new insights show that human FoxP3-expressing T cells in isolated $CD4^+$ T cells are a phenotypically and functionally heterogeneous population. Discrimination can be made between (I) $CD45RA^+FoxP3^{low}$ naive or resting Tregs which do actively proliferate after TCR stimulation and exert suppression of Teff during and after proliferation and (II) $CD45RA\text{-}FoxP3^{high}$ memory or activated Tregs showing a high Teff suppression capacity, but die quickly after activation. Upon activation, resting or naive Tregs convert into memory/activated Tregs and only a small fraction of the activated/memory Tregs did develop from $Foxp3\text{-}CD4^+$ non-Treg cells *in vivo*[91].

In sarcoidosis Tregs are found in and around kidney and lymph node granulomas. They express Ki-67, indicating that they proliferate [92]. In blood and BAL of sarcoidosis patients different results have been reported. Idahli *et al.* [93] found a decreased expression of Foxp3 mRNA in $CD4^+$ BAL T cells and a decreased number of $Foxp3^+CD4^+$ T cells in the BAL and blood of patients. In contrast, Miyara *et al.* [94] found an increased number of $CD4^+CD25^{bright}$ Tregs in blood and BAL. Taflin *et al.* reported an increase of CD45RA-Tregs and to a lesser extend an increase of $CD45^+$ Tregs in the blood of sarcoidosis patients

[92]. A recent study showed an increase in activated/memory Tregs and a decrease of resting/naive Tregs in the blood of sarcoidosis patients [91].

Functional tests with Tregs from sarcoidosis patients revealed a decreased ability to suppress the TNFα and IFNγ production by Teff [94]. Furthermore, in an *in vitro* model of granuloma formation, using BCG-coated beads, depletion of the $CD25^+$ fraction as a mechanism to depelete Treg from the $CD4^+$ T cell fraction, did not affect granuloma growth rates [92]. Activated $CD7^-$ Tregs from BALfluid of sarcoidosis patients show decreased suppressive activity in vitro [95].

Taken together, these reports show conflicting data regarding numbers of Tregs, but nevertheless point to impaired Treg function in sarcoidosis.

4. AIM OF THIS THESIS

We hypothesized that defective DC-T cell interaction is the basis of the immunopathology in sarcoidosis, whereby aberrant dendritic cell activation, defective regulatory T cell function and the effector functions of Th1 and Th17 cells could contribute to the pathogenesis.

First a general overview of DC function in interstitial lung diseases is given (**Chapter 2**). In order to study the role of DCs that are present in the lung in detail, we aimed to develop a method to purify these cells for *in vitro* studies. **Chapter 3** describes a method for isolation of myeloid en plasmacytoid dendritic cells of human bronchoalveolar lavage fluid and evaluates the isolated DCs by immunohistochemistry and functional tests in *in vitro* co-culture experiments with $CD4^+$ naive T cells.

Because of the conflicting data in the current literature on DC numbers and activity in various compartments analysed in sarcoidosis, an important aim of this thesis was to functionally analyse DC subsets in sarcoidosis. **Chapter 4** evaluates the number and phenotype of myeloid en plasmacytoid dendritic cells in bronchoalveolar lavage, peripheral blood and pulmonary mucosal biopsies of sarcoidosis patients. Furthermore, myeloid DCs were isolated from bronchoalveolar lavage, cultured from peripheral blood, tested for their function *in vitro*, and compared with healthy control DCs.

Since, knowledge on the possible involvement of the Th17 subset in sarcoidosis was lacking, we aimed to analyse the expression of Th17-specific cytokines, in particular IL-17A and IL-22 in sarcoidosis. In **Chapter 5** a detailed analysis of the expression of IL-17A and IL-22 as well as other pro-inflammatory cytokines in peripheral blood T cells is presented, together with studies on bronchoalveolar lavage and pulmonary mucosal biopsies of sarcoidosis patients and healthy controls. Our findings prove evidence for the involvement of the Th17 subset.

Another key objective was to characterize Treg function in sarcoidosis. As described in **Chapter 6**, the number of regulatory T cells was measured in blood and bronchoaveolar lavage of sarcoidosis patients and healthy controls. The number and location of regulatory T cells in the pulmonary mucosal biopsies was assessed and Tregs were purified from peripheral blood and funcionally investigated *in vitro*.

Taken together, these studies provided evidence for defective DC-T cell interaction in sarcoidosis, which is discussed in **Chapter 7**.

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Human pulmonary dendritic cells in interstitial lung disease

Bregje ten Berge[1], Bernt van den Blink[1] and Bart N. Lambrecht[1,2]

(1) Department of Pulmonary Medicine, Erasmus MC, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

(2) Laboratory of Immunoregulation and Mucosal Immunology, MRBI, Department of Respiratory Diseases, University Hospital Gent, De Pintelaan 185, B9000 Ghent, Belgium

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ABSTRACT

Dendritic cells (DCs) are professional antigen presenting cells. They are present at sites of high antigen exposure. After capturing an antigen they mature and migrate to the draining lymph nodes were they can initiate an immune response by stimulation of naive T cells and inducing polarization to different T helper subsets. In the human lung of healthy volunteers, three distinct DC populations can be recognized, namely mDC type 1, mDC type2 and pDCs, each with different expression of receptors, different T cell stimulative capacities and different cytokine production upon stimulation. In sarcoidosis, DCs can be found to reside in the lymphocyte-rich layer surrounding the granuloma where they make contact with lymphocytes. In sarcoidosis, there is no consensus regarding the number of circulating peripheral blood DCs, but the T cell stimulative function of blood mDCs is decreased compared to controls, despite the normal expression of costimulatory molecules by DCs. In BAL, CD1a+ mDCs are increased and they express a more mature phenotype. In lung fibrosis, increased numbers of mature and immature DCs are found in lung biopsies with high expression of chemokines and receptors suggesting DC recruitment from the peripheral blood. In BAL of IPF patients, the mDCs show a more immature phenotype compared to healthy controls. In pulmonary Langerhans cell histiocytosis (PLCH) inflammation with accumulation of LCs is seen in the lung of smokers. This accumulation can be caused by local proliferation of LCs, by enhanced recruitment to the lung or by increased levels of hematopoeitic growth factors for LCs.

The past few years much work has been done on DCs and interstitial lung diseases, but detailed information about the function of DCs is still lacking en needs to be elucidated in the near future. This is necessary before DCs can be identified as possible targets for treatment.

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1. INTRODUCTION : DENDRITIC CELLS AND INTERSTITIAL LUNG DISEASES

DCs are professional antigen presenting cells (APCs) that are specialized to initiate primary immune responses in the T and B cell compartment [1] and are the bridge between innate and adapative immunity. In most tissues, DCs are present in a so-called immature state, unable to stimulate T cells but extremely well equipped to recognize and capture antigens through expression of pattern recognition receptors and endocytosis receptors. When the DC has captured an antigen, it can also receive signals to mature when the antigen encountered is part of a microbe or is encountered in the context of tissue damage or inflammation. In this process of maturation, the capacity to capture antigens is rapidly lost while the expression of MHC class II complexes and expression of costimulatory molecules are rapidly upregulated. During the process of maturation, DCs travel to the lymphoid tissues such as the lymph nodes draining the site of antigen exposure, where they will complete their maturation process and start the communication with cells of the adaptive immune response such as B cells and T cells. DCs present their antigen to naïve CD4 and CD8 T cells inducing a strong T-cell response. [1]. DCs do not only activate T cells, they can also tolerize T cells to self-antigens, to prevent autoimmune reactions. Depending on the recognition of self-signals or non-self-signals in the presence or absence of danger signals, DC antigen uptake and presentation leads to activation or down regulation of the T cell response. [2] Moreover DCs can influence the polarization of the naïve T-cell into Th1, Th2, Th17 or T regulatory direction depending on the type of microorganism that is recognized and the tissue factors present on the site of activation.[3] This enables DCs not only to initiate active immune responses, but also to control and dampen these immune responses, and to prevent unwanted responses against self-antigens. Not surprisingly, these cells play a predominant role in various lung diseases where the adaptive immune response plays an important role such as asthma, COPD and interstitial lung diseases. Many experimental and pre-clinical work has addressed the role of DCs in asthma and COPD [4, 5]. However, less is known about the role of DCs in insterstitial lung diseases like sarcoidosis, lung fibrosis or Langerhans' cell histiocytosis.

Sarcoidosis is a heterogeneous disorder affecting people worldwide. The causing antigen is yet unknown, but seasonal clustering and the clustering of disease in individuals who share environments are reasons to assume that an infective agent, or a triggering antigen are involved in the pathogenesis of this disease. Also a genetic susceptibility has been suspected in the pathogenesis of sarcoidosis, based on the observation that there is a variable prevalence in different races and ethnic backgrounds, and the clustering of sarcoidosis in families, and the given association of particular HLA haplotypes with disease prevalence. [6] It is most likely that the combination of environmental exposure on the right genetic background predisposes individuals to sarcoidosis . The

granulomas seen in sarcoidosis are generally non-necrotizing and consist of epitheloid cells surrounded by a peripheral rim or collar of lymphocytes. Multinucleate giant cells and inclusions can be seen in the granuloma. The cellular infiltrates present at the site of disease activity consist predominantly of Th1 lymphocytes. In most cases of sarcoidosis the granulomas will resolve but in 10-30% the lungs undergo progressive fibrosis which can result in end stage fibrotic lung.[7]

Many other immune-mediated diseases can be the cause of fibrosis in the lung, and often these form part of a systemic auto-immune disorder like systemic lupus erythematosus, rheumatoid arthritis or scleroderma, or are associated with chronic hypersensitivity reactions to inhaled antigens, like in the case of extrinsic allergic alveolitis. Often however, the lung fibrosis is idiopathic. In lung fibrosis, different histological patterns exist with different cellular infiltrates and types of fibrotic lesions. Idiopathic pulmonary fibrosis (IPF) is a progressive form of severe fibrosis that consists mainly of a histological pattern of usual interstitional pneumonia (UIP), characterized by little inflammation, epithelial changes and temporal and spatial heterogeneity. The characteristic lesions consist of fibroblast foci, which represents small islands or channels of young myofibroblasts surrounded by a disrupted basement membrane and hyperplastic type II alveolar epithelial cells. Another major part of pulmonary fibrosis consists of a histological pattern of non-specific interstitial pneumonia (NSIP), characterized by more uniform lesions of neutrophilic and lymphocytic inflammation, and more generalized fibrosis. [8] This review will be limited to these two types of lung fibrosis as the other ones like desquamative interstitial pneumonia (DIP) and lymphocytic interstitial pneumonitis (LIP) are more rare and studies about DCs are lacking.

The accumulation of Langerhans cells (LCs), a subtype of DCs, in the lung is the characteristic feature of pulmonary Langerhans cell histiocytosis (PLCH). The exact cause is still unknown but PLCH occurs mostly in young (20-40y) smokers. The activated LCs will form granuloma in the distal bronchiole walls. Other inflammatory cells are also present and depending on the stage of the disease less or more fibrosis is seen in the pulmonary tissue. [9]

We here summarize what is known about human DCs in healthy pulmonary tissue and when sarcoidosis, pulmonary fibrosis or Langerhans cell histiocytosis is present.

2. DENDRITIC CELL SUBSETS IN THE HUMAN LUNG

Sertl and colleagues were the first to describe the presence of DCs in the trachea, bronchi, alveoli and visceral pleura of mouse and human pulmonary tissue. In human, these cells were shown to highly express HLA-DR. These DCs did show a typical dendritic morphology but lacked the Birbeck granulae, typically described in skin LCs. *In vitro*, these DCs

acted as potent T cell stimulators [10]. Subsequent studies have further refined the way by which lung DCs can be identified and purified. For identification of human pulmonary DCs, first transient adherence to plastic after overnight culture was used to separate bronchoalveolar lavage (BAL) cells into an adherent and a non-adherent population. The non-adherent population was subsequently divided into a low- and high-density fraction. The low-density fraction contained cells with a typical DC-like morphology and induced a strong allogeneic T cell response [11]. Van Haarst et al. and Nicod et al. used flowcytometry to separate the low autofluorescent fraction of the BAL cells containing pulmonary DCs from the high autofluorescent macrophages. These low autofluorescent DCs expressed LC markers CD1a and S100 and also exhibited potent T-cell stimulatory capacities [12]. More detailed identification and phenotyping of human pulmonary DCs was performed since the advent of blood DC markers became widely available. In BAL, three types of DC populations were detected. Myeloid DC type 1 (CD11c+/CD1c+), myeloid DC type 2 (CD11c+/BDCA-3+) and plasmacytoid DCs (CD11c-/BDCA-2+). It is questionable however whether the definition "myeloid" should still be maintained given the resemblance of mDC1 and mDC2 to the mouse lung conventional (c)DCs. These mDC and pDC populations grossly correspond to equivalent populations in the mouse, where mDC1 would correspond to the CD11b+CD103-cDC subset, the mDC2 to the CD11b-CD103+langerin+ cDCs, and human pDCs correspond to murine pDCs [13] Unfortunately, only the total DC population was tested for immunostimulative capacity, without separation into subpopulations, so the individual function of the human pulmonary DC populations available through BAL remains unclear [14]. Another way to obtain human pulmonary DCs is to isolate them from lung resection tissue through enzymatic digestion or mechanical disruption of lung tissue. In lung tissue, the same previously described DC subsets are present [15]. More recently, when lung tissue of COPD patients was assessed by flowcytometry two segregated subsets of tissue resident pulmonary myeloid DCs were identified in single cell suspensions as a langerin+ LDC and a DC-SIGN+ interstitial-type DC (intDC) population. LDC partially expressed the markers CD1a and BDCA-1, which are also present on their known blood precursors. It is likey that these langerin+ DCs represent the murine CD103+CD11b- cDC subset. In contrast, intDC did not express langerin, CD1a or BDCA-1, but were more closely related to monocytes [16] . The equivalent population in the mouse would be a monocyte derived inflammatory type DC that was also recently shown to express DC-SIGN [17].

2.1 Dendritic cells in the human lung express pattern recognition receptors

DC use toll like receptors (TLR) to detect the presence of infection or tissue damage [18]. TLRs belong to a group of highly conserved pattern recognition receptors (PPRs) which recognize pathogen associated molecular patterns (PAMPs), unique molecular microbial signatures on different pathogens [19, 20]. Recognition of PAMPS by TLR results in

the activation of two major pathways. The MyD88 dependent pathway and the TolI/IL-1R domain-containing adaptor-inducing IFN-β (TRIF)-dependent pathway. The MyD88 dependent pathway involves the production of IL-6 and tumor necrosis factor (TNF)-α and the TRIF depending pathway activated by TLR2 and TLR 4 leads to the production of Type 1 interferons [21]. Myeloid DC type 1 and mDC type 2 express mRNA transcripts for TLR1, TLR2, TLR3, TLR4, TLR6 en TLR8. In response to TLR2 and TLR4 ligands mDC type 1 and mDC type 2 release proinflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) When stimulated with TLR 3 ligands only type 1 mDC produce proinflammatory cytokines. Plasmacytoid DC express TLR 7 and TLR 9 and release proinflammatory cytokines in respons to imiquimod, and IFN-a in respons to CPG oligonucleotides.

The T-cell stimulatory capacity of the different DC populations was also evaluated by mixed leukocyte reactions of purified DCs incubated with allogeneic T cells. mDC type 1 are strong inducers of T cell proliferation, while pDC hardly induce any proliferation. mDC type 2 have in intermediate T-cell stimulatory capacity [22].

3. DC RECRUITMENT TO THE LUNG

3.1 Animal studies for steady state and inflammation-induced recruitment

In mouse and rat models, much work has been done to unveil the recruitment of DCs to the lung. Even in the absence of inflammation, DCs or their precursors are constantly recruited from the blood to the lung provided by a steady state bone marrow output [23]. Studies of CFSE stained airway DCs have shown that the turnover rate of these cells depend on their anatomic localization and their surface markers. The turnover of airway DCs in the upper airway is much greater than in the lung parenchyma [24], and when an antagonist to the chemokine receptors CCR1 and CCR5 is used, baseline numbers of rat tracheal DCs are reduced with 50% [25]. Assuming that this antagonist was fully effective, these data suggest that at least one other pathway is involved in DC recruitment under steady state condition. This pathway can involve the CX3CR1 chemokine receptor. This receptor is highly expressed on a population of monocytes that are recruited to non-inflamed tissues, including the lung. In CX3CR1 deficient mice, the monocyte recruitment to non-inflamed tissue is markedly reduced [26]. As monocytes can differentiate into DCs the recruitment of monocytes by the CX3CR1 receptor can contribute to the maintenance of DC homeostasis in the lung.

The rate of DC accumulation in the lung increases markedly after challenge with heat-killed bacteria [27]. However unlike a wave of incoming neutrophils, which rapidly move towards the airway lumen, DCs remain in the epithelium. This recruitment of DCs into the respiratory system is an universal feature after local challenge, not only with heat killed bacteria, but also after challenge with other bacterial, viral and soluble protein

antigens [28]. Again it seems that this recruitment is supported by an increased influx of DC precursors from the bone marrow, that reach the lung in a monocyte-like stage and give rise to monocyte-derived inflammatory DCs [29].

The precise chemokine signals through which DCs reach the lung upon conditions of inflammation is a matter of debate. A CCR1/CCR5 antagonist blocks even a higher proportion of bacteria-induced DC recruitment to the lung, than that seen in steady state conditions, implying an important role for this chemokine receptor pair [25]. Also an in vitro study shows that freshly isolated respiratory mucosal DC respond to different CC chemokines, (MCP-1, -4, RANTES and eotaxin), complementary cleavage products and N-formyl-leucine-phenylalanine [28]. Besides challenge by viruses or bacteria also antigenic challenge to the lungs causes influx of DCs. When a challenge with sheep red blood cells is given in the lungs, CCR2 directs DC precursors from the blood to the lung interstitium, whereas CCR6 directs transit of DCs from the interstitium to the airway[30]. A very elegant study using mixed bone marrow chimeras, van Robaeys et al demonstrated that the predominant chemokine attracking DCs into the airway compartments was CCR2,and not CCR6 or CCR5 [31]

The precise role of the CX3CR1 receptor in lung DC recruitment following inflammation is currently unknown. After challenging mouse lungs with cigarette smoke as a model for chronic obstructive pulmonary disease, CR3CL1 is upregulated in the lungs. As CX3CL1 is the ligand for CX3CR1, present on many inflammatory cells like monocytes, it is likely that this pathway could also contribute to recruitment of inflammatory type DCs to the lung [32].

3.2 Recruitment of dendritic cells to human lung

In humans less is known about the DC recruitment to the lungs. Freshly isolated human pulmonary immature DCs were found to highly express both the chemokine receptors CCR1 and CCR5 [33]. When endotoxin is inhaled by healthy volunteers, 6 hours later an upregulation of DC maturation markers (HLA-DR, CD80 and CD86) on monocytes in induced induced sputum is seen, and a discrete population of mature DCs were observed [34]. When endotoxin is instilled into a lung segment of healthy volunteers and saline in a control segment, endotoxin instillation resulted in a focal inflammatory response with increased monocytes in the brochoalveolar lavage after 24-48 hours. These recruited monocytes can be precursors for DCs in the lung [35] because Regamey and colleagues show that IL-15 produced by airway epithelial cells induces the differentiation and maturation of monoctyes into mature and functional DCs [36]. When asthmatic patients were challenged with allergen and mucosal biopsies were taken 4-5 hours after allergen challenge, a dramatic increase in the number of CD1c+HLA-DR+ DCs was seen in the lamina propria of the biopsies, compared to baseline values. The rapid accumulation of these cells suggests a direct recruitment of these DCs from peripheral blood [37].

Bratke and colleagues performed segmental allergen challenges during bronchoscopy in patients diagnosed with asthma. After 10 min and 24 hours, patients underwent a BAL in the challenged and the control segment. In the BALF, they analyzed the type and number of DCs. After 24 hours (but not 10 min) there was a markedly increase of both pDCs and mDCs compared to the control segment for mDC and pDC. The percentage increase was higher for the pDCs than the mDCs, which resulted in a higher pDC/mDC ratio. In the peripheral blood was a decrease visible of mDCs and a trend to a decrease for pDCs [38]. The same phenomenon was seen when smokers were challenged to a 4 hour period of cigarette smoke. Also here there was a strong increase in mDC number in BALF and a concomitant decrease in mDC number in blood following the acute smoke exposure. In addition, acute smoke exposure led to an increase in the expression of the surface molecules BDCA-1 and -4 and a decrease in the expression of the lung homing receptor, CCR5, on mDCs in BALF [39]. In emphysema, not only did the tobacco smoke increases mDC recruitment to the lung, the lung mDCs also induced T helper 1 and T helper 17 responses in CD4 T cells, induced interleukin-17A production, and enhanced secretion of CCL20, a chemoattractant for DCs. So mDCs initiate their own recruitment via IL-17 secretion by activated Th17 cells [40].

Recently it was shown that the activation of DCs is influenced by components of the complement pathway. A study to in vitro the role of complement factor C1q, produced and accumulated at inflammatory sites, on the recruitment of DCs to the site of inflammation showed that C1q mediates the chemotaxis and transendothelial migration of immature moDCs. C1q also enhances the chemotaxis of mature DCs to CCL19 by upregulation of CCR7 expression on DCs [41]. Several subsets of human DCs express many of the components of complement. Moreover human DCs have receptors known to detect the biologically active peptides C3a and C5a which serve in immune adhesion. The human DC surface is characterized by membrane bound regulators of complement activation, which are also known to participate in intracellular signaling. [42]

Together, these results show that after endotoxin, antigen or allergen challenge in the lung, DCs are recruited as part of a generalized inflammatory response. The exact stage of development (monocyte versus dedicated DC precursor or circulating blood mDC) in which DCs are recruited remains an issue of debate. Monocytes can develop into DCs in the local lung environment and IL-15 seems to be involved in this process, although clearly many other differentiation factors like GM-CSF and Flt3L could be involved.

4. ANTIGEN UPTAKE IN THE LUNG AND MIGRATION TO THE LYMPH NODES

4.1 Antigen uptake by dendritic cells in the lung

The most important function of DCs is the capture and delivery of antigens to local lymphoid tissues, where DCs can present the antigens to naive T-cells and induce T-cell proliferation and differentiation. Airway DC are localized in a superficial dense submucosal network [10]. A study from Rescigno and colleagues showed that in mice DC cells in the submucosal layer of the gut, open the tight junctions between the epithelial cells, send dendrites outside the epithelium and directly sample bacteria. Because DCs express tight junction proteins, the integrity of the epithelial is preserved [43]. Similar conclusions were reached in lung DCs in the mouse [44, 45]. More recently, it was shown that in an *in vitro* model of the human airway wall, macrophages and DCs express tight junction proteins and exchange particles [46]. DCs can capture antigen via at least two mechanisms. First, DCs use macropinocytosis for continuous uptake of large amounts of soluble antigens. Second, they express receptors of the C-type lectin family [47]. These transmembrane sugar-binding proteins act as pathogen recognition receptors (PPRs), recognizing carbohydrate motifs present on the surface of several microbial organisms. In addition they deliver captured antigens to endocytotic vesicles for further processing and presentation by MHC class II [48].

On human pulmonary DCs several C-type lectin receptors have been described. Chocand and colleagues described the presence of efficient mannose receptors on pulmonary DCs, so pulmonary DCs have a high endocytotic activity for mannosylated antigens [33]. In vitro cultured monocyte derived DC expressed mannose receptors that could efficiently take up Derp1, a house dust mite allergen [49]. And another C-type lectin receptor, DC-SIGN on human monocyte-derived (mo-) DCs is the main target for *Mycobacterium tuberculosis* to enter the DC [50]. DEC-205 is another type I C-type lectin receptor present on human pDCs and mDCs. DEC -205 mediates antigen uptake and presentation by both resting and activated human pDCs. DEC-205 does not affect TLR-induced expression of co-stimulatory molecules, but clearly impairs TLR-induced IFN-α secretion. DEC-205-targeted mature DCs (mDCs) presented the antigen more efficiently than peptide-pulsed DCs [51, 52].

4.2 Migration to the lymph nodes

4.2.1 Mouse studies

After capturing antigen by DCs, the DCs will migrate to the regional lymph node were they can induce T cell responses. In mice, this migration is most commonly studied by the use of carboxyfluorescin succinimidyl ester (CFSE). When CFSE is delivered to the trachea, cells exposed to the airway will take up the dye en retain it for a few days. So a differentiation can be made between resident and migrated cells in the airway. One

day after CFSE treatment approximately 2% of cells within the draining lymph nodes are CFSE+. These CFSE DCs have a higher expression of CD40, CD80, CD86 and MHC class II than the resident DCs in the lymph node that do not contain CFSE [24]. Uncertain in these studies is whether DCs that migrate to the lymph nodes did take up antigens or not. Vermaelen and colleagues studied this and used fluorescent labeled proteins (fluorescein isothiocynate-conjugated ovalbumin) to demonstrate that DCs take up antigens in the airway and subsequently migrate to the draining lymph nodes. The antigen bearing cells were found to be CD11c med-hi / MHC class II hi, whereas DCs with lower levels of MHC-class II contained no antigen [53]. This pulmonary DC trafficking is dependent on CCR7 receptor expression. CCR7 deficient mice, but not wild type mice, failed to migrate to the draining lymph nodes after airway exposure to fluorescent labeled ovalbumin [54]. This finding is consistent with the work of Jakubzick who showed that mice lacking the chemokine ligands of CCR7 have a reduced capacity to transport latex particles from the lung to the draining lymph nodes [55].

4.2.2 Human studies

In human, detailed information about the trafficking of DCs to the draining lymph nodes is lacking. In vitro, using a transendothelial trafficking model, is shown that a CD16+ subpopulation of monocytes is predisposed to become migratory DCs. These DCs migrate across endothelium in the abluminal-to-luminal direction, reminiscent of the migration into lymphatic vessels [56]. Until now there is no data available concerning the migration of DCs in vivo in human. However, Hammad et al. showed that in a humanized SCID model, the migration of human mo-DCs to the draining mediastinal lymph nodes could be blocked by antibodies to CCL19 and CCL21, implying the use of the CCR7 receptor for human DC migration to the lung lymph nodes in vivo [57]New information of human lung DC migration in vivo can be obtained by the use of indium-oxide labeled DCs and the use of positron emission tomography like recently published by Prince and colleagues in a study with immunotherapy in multiple myeloma patients. Labeled DCs can be followed when they migrate to the lymph nodes and other organs like the lungs after subcutaneous or intravenous injection [58].

5. INDUCTION OF T-CELL RESPONSE IN THE DRAINING LYMPH NODES

5.1 Induction of T cell responses in the lymph nodes

After capturing antigens in the airway and the migration to the lymph nodes, DCs will perform their last and most important task. They upregulate MHC class II, present the captured antigens to T cells and stimulate the T cells to perform an adequate T cell response. In different studies to the activation and function of DCs in mouse models,

multiple factors were identified to influence this process. Several chemical mediators that are produced as part of the acute inflammatory response like neuropeptides, prostaglandins, complement factors, ATP, uric acid, and other chemical mediators can influence this process profoundly [4] As only a few examples, NO2 exposure in a mouse model of allergic inflammation caused pulmonary CD11c+ cells to acquire a phenotype capable of increased antigen uptake, migration to the draining lymph node, expression of MHCII and co-stimulatory molecules required to activate naïve T cells, and secretion of polarizing cytokines to shape a Th2/Th17 response [59]. Vascular endothelial growth factor (VEGF) present in the murine lung predisposed the lung to inflammation by activating local DC. It regulates lung mDC expression of innate immunity effector molecules and promotes a Th2 type of response [60]. Furthermore histamine influences DCs to enhance Th2 dependent asthma-like inflammation in the lungs [61]. Not only chemical mediators released by the acute inflammatory response, but also other inflammatory or structural cells present in the surroundings of the DCs, will influence the function of DCs. For example, using a series of bone marrow chimeric mice, Hammad et al. showed clearly that airway epithelial cells responded to inhaled LPS and allergens, and were necessary to stimulate the function and migratory behaviour of local intrapulmonary DCs, causing them to polarize to a default Th2 inducing phenotype through secretion of TSLP, IL-25, IL-33 and GM-CSF [62]. Local dendritic cells can also be influenced by regulatory T cells, who diminish dendritic cell function [63, 64].

Upon arrival in the lymph nodes, DCs determine the outcome of the T cell response (See figure 1). The T cell response is divided into Th1, Th2 regulatory T cells (Treg) and Th17 response. DCs are primed for the expression of the T cell polarizing signal. Various DC-derived molecules have been identified. For a Th1 response DC produce interleukin 12, Type 1 interferons (IFNs) and express intracellular adhesion molecule 1 (ICAM1). A Th1 response is characterized by the production of IFN-y and TNF by T cells, is generated after DC exposure to viruses or bacteria, and is crucial for the control of intracellular pathogens. Also the delayed hypersensitivity reaction is a Th1 response [3, 65].

A Th2 response mainly occurs after contact of the DC with helmints or allergens. The Th2 polarizing signals expressed by DC are OX40 ligand (OX40L) [66] and Notch ligand Jagged [67]. In a Th2 response T cells produce interleukin-4, interleukin-5 and interleukin-13 resulting in mast cell and eosinophil accumulation and IgE production. Studies in the mouse have revealed that DCs are sufficient and required for induction of a pulmonary Th2 response to allergens like house dust mite, and often these DCs collaborate with basophils that provide the initial source of polarizing IL-4 for Th0 cells to switch to Th2-type immunity [68]

Th17 cells are more recently recognized as the third effector T cell subset in addition to Th1 and Th2 helper T cell lineages. Th17 cells produce IL-17A, IL-17F and IL-22. Naïve

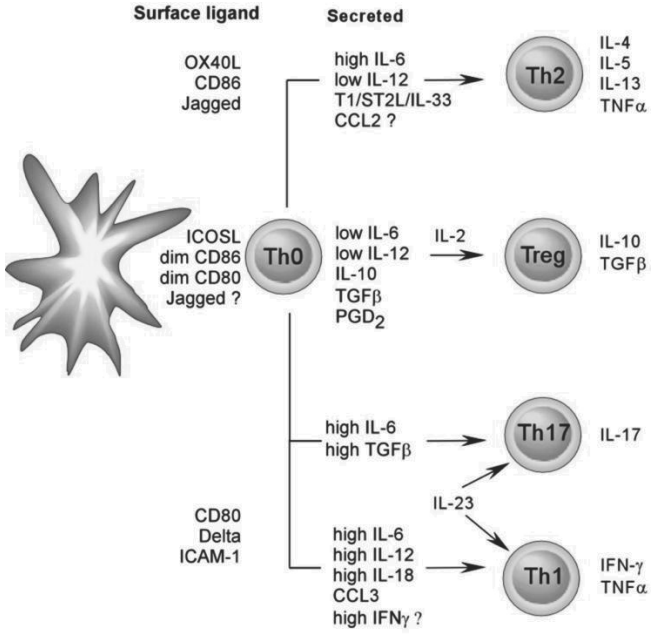

Figure 1. DCs are primed for the expression of the T cell polarizing signal. Depending on the surface ligands expressed on the DCs, and the cytokines secreted by the DCs, DCs determine the outcome of the T cell response.

CD4+ T cells are stimulated with IL-6 produced by DCs and TGF (mouse) or IL-1 (human) to up regulate the IL-23 receptor and the specific transcription regulator RORyt as a commitment factor for the Th-17 lineage. DC's activated by TLR-2 ligands or lipopolysaccharide (LPS) produce IL-23 which is now regarded as a factor for the maintaining and stabilizing of the Th17 phenotype [69]. Th17 cells are now clearly implicated in several models of human inflammatory disorders. In the lung IL-17 promotes the recruitment and expansion of neutrophils [70]. In a mouse model of allergic airway inflammation, systemic blockade of IL-17 inhibits the allergen induced accumulation in the airway [71]. In bleomycin induced lung injury γδ IL-17 producing T cells are necessary for an organized inflammatory response and tissue repair. Different cells present in the lungs like mast cells and fibroblasts can initiate a Th17 phenotype by dendritic cells. Fibroblasts act on DCs to increase IL-23 expression and thereby promoting the expansion of Th17 cells [72]. Mast cell activated DCs express higher levels of co-stimulatory molecules, and modulate DCs to initiate Th1 and Th17 responses [73]. Not only myeloid DCs, but also human plasmacytoid dendritic cells can initiate CD4+ T cells to become Th17 cells *in vitro* [74] .

The last main response is the induction of tolerogenic or regulatory T cells producing immunosuppressive cytokines like IL-10 and TGF-β. This is probably the most prevalent

response in steady state conditions when inert antigens within the airways generally invoke tolerogenic responses [75] .

5.2 Induction of peripheral tolerance by DCs in the lung

DCs are important in inducing peripheral tolerance, which is critical in avoiding pathologic reactions to self-antigens or harmless foreign antigens like harmless allergens. Traditionally it was thought that DCs that induce a tolerogenic response were semi mature DCs carrying a low dose of antigen. These DCs do not secrete proinflammatory cytokines and fail to stimulate the corresponding T cells. However accumulating evidence now indicates that antigen specific tolerance is not simply caused by the absence of an immune response, but an active process requiring T cell proliferation and differentiation [76]. An important way for dendritic cells to induce peripheral tolerance is the induction of regulatory T cells [77]. A subset of murine DCs exposed to respiratory antigens produces transiently the anti-inflammatory cytokine IL-10 and induces regulatory T-cells via the ICOS-ICOS-ligand pathway leading to peripheral tolerance [78]. Recently the expression of CTLA-4 is found on human dendritic cells, and these DCs show less maturation and are supposed to have a regulatory function but further research is nessecary [79]. Respiratory tolerance also depends on the presence of pDCs in the lung. pDCs have the capacity to drive tolerogenic functions. For example, pDCs express high levels of ICOS and induce the proliferation of immunosuppressive regulatory T cells [80, 81]. The induction of a tolerogenic response by dendritic cells depends on the type of antigen and certain environmental factors present in the surroundings of the DCs. Multiple environmental factors have been described until now to influence the in vitro human DC response to a tolerogenic one, like 1,25 dihydroxy vitamin-D [82], neutrophil elastase [83], fungal proteases [84], estriol,[85] and also influence of regulatory T cells can induce tolerogenic DCs[86].

5.3 Antigen uptake and presentation to T cells by human lung DCs

Human pulmonary DCs are thought to present captured antigens to naïve T cells in the lymph nodes in the same way as mice DCs do, but detailed study is lacking. The scarce information we have of human DCs stimulating naïve T cells has been obtained from *in vitro* studies using mostly monocyte-derived DCs form the peripheral blood. These monocyte-derived DCs are pulsed with different antigens, mimicking the antigen contact of the DCs in the lung. Subsequently the effect of the antigens that are typically found in the lungs, or enter the body through the lungs, on the maturation status and the ability to induce T cell proliferation and polarization is assessed.

When DCs are matured with CD40L in the presence of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) they show enhanced IL-10 production and primed naïve T cells into IL-10 producing T cell without Th1 or Th2 bias [87]. When DCs are co cultured with

CD45RO- T cells in medium of measles virus or measles vaccine infected DCs, DCs in the measles vaccine medium generate a strong Th1 differentiation while DCs cultured with wild type measles virus medium generated a mixed Th0, Th1 response [88]. Antigens activate DCs and induce T cell polarization, but also the different donors of the DCs can influence the outcome of the T cell stimulation. Holden and colleagues show in a mixed leukocyte reaction where they used enriched peripheral blood DCs from atopic and non-atopic donors were stimulated with the contact sensitizer 2,4-dinitrochlorobenzene (DNBC) and the respiratory sensitizer Trimellitic anhydride (TMA). Subsequently the DCs were incubated with freshly isolated PBMC's from a different donor. Non-atopic donor DCs induce the production of Th1 cytokines IL-12 and IFN-γ when stimulated with DNCB and IL-13 when stimulated with TMA. In contrast the atopic donor DCs failed to induce divergent T cell responses after sensitizer treatment [89].

6. DENDRITIC CELLS IN SARCOIDOSIS

Sarcoidosis is a heterogenic multisystem disease characterized by the histological finding of non-caseating granulomas in affected organs. A causative antigen is lacking, sarcoidosis appears in many different forms and there is no satisfactory animal model available. This makes it difficult to unveil the immunopathogenesis of sarcoidosis.

Clinically, sarcoidosis is associated with a peripheral blood T-cell lymphopenia [90, 91] and cutaneous anergy to tuberculin and other skin tests [92]. In contrast to the peripheral lymphopenia, pulmonary sarcoidosis is often characterized by increased level of CD3+ CD4+ T cells in the bronchial lavage resulting in a CD4+/CD8+ ratio greater than 3,5:1 [93, 94]. These pulmonary T cells in sarcoidosis express activation markers on their cell surface like IL-2R (CD25), CD69 and CD26 [95, 96] and spontaneously produce potent Th1 cytokines like IL-2 and IFN-γ [97, 98]. Walhstrom and colleagues showed using intracellular staining after in vitro stimulation with PMA/ionomycin the cytokine production of T cells at single cell level. Both CD4 and CD8 bronchoalveolar lavage T cells secrete high levels of IFN-γ, IL-2 and TNF-α. IL-4 production was seen, but at a lower level compared to peripheral blood CD4+ cells of sarcoidosis patients [99]. The activation of the T cells is the result of interaction of the TCR receptor with an appropriate antigen loaded on the major histocompatibility complex on APCs. This activation is followed by an oligoclonal expansion of T cells, in the case that a specific antigen is recognized. Moller et al showed greatly expanded numbers of V-β8+ T cells in both lung and blood mononuclear cells in a subgroup of sarcoidosis patients wit acute presentation and favourable prognosis (Löfgren syndrome)[100]. These findings are characteristic for a classic antigen driven immune response. An important goal in the future will therefore be to isolate and define the sarcoidosis antigen in the MHC-II complex.

DCs are potent APCs that are capable of inducing the Th1 inflammation typically found in sarcoidosis. The study of DCs in sarcoidosis started with analyzing biopsies of skin, lymph node and lung. Maceira and colleagues defined DCs as S-100 protein-positive cells with a large irregular nucleus and branching cytoplasm without Birbeck granules. They stated that the increased number of DCs in sarcoidosis skin granulomas compared with foreign body granulomas suggests they act as assescory cells in eliciting the granulomatous response [101]. This high number of DCs was confirmed by Munro who showed that both LCs (CD1a+) as interstitial (RFD1+) DCs were easily identified in skin biopsies. In lymph nodes and lung biopsies however, DCs were sparse [102] . More recently, biopsies of skin and lymph node of sarcoidosis patients were stained with Fascin (also known as p55, a mature DC- specific marker), HLA-DR and DC-Lamp (a specific DC marker). In the lymphocyte-rich rim surrounding granulomas, a considerable number of Fascin+ cells were detected. These cells were also HLA-DR positive. Double staining with Fascin and DC-Lamp showed positive cells with typical DC projections in the lymphocyte rim. In a double staining of Fascin and CD3 they observed that many CD3+ cells were in contact with Fascin positive mononuclear cells suggesting DC interact functionally with T-cells in the granulomatous lesions of sarcoidosis patients [103] . In granulomatous lesions in muscles of sarcoidosis patients, CD1a+ mDCs and BDCA-2+ pDCs were increased, with increased expression of the CD40/CD40L system [104] .

For the study of sarcoid granulomas also Kveim reagens has been used to induce granulomas experimentally. Kveim reagens is an extract of human sarcoid spleen, and was used previously as a diagnostic skin test for sarcoidosis. When Kveim reagens is injected intracutaneously in a patient with sarcoidosis, inflammation results in the development of a papula at the site of injection after 3 to 6 weeks. The papula shows classic non-caseating granulomatous inflammation typical of sarcoid granulomas. Recently Kveim reagens gains interest in finding a transmissible antigen in sarcoidosis. A study by Song and colleagues identified *Mycobacterium tuberculosis* catalase-peroxidase (mKatG) as a tissue antigen and target of the adaptive immune response in sarcoidosis [105]. Recently also the T cells responses of sarcoidosis patients to mKatG have been assessed and they fit the profile expected for reaction to a pathogenic antigen [106] .

When the early inflammatory response in the skin biopsy (48 hours after Kveim injection) is assessed, small juxtacapillary clumps of CD1a+ Langerhans cells and interstitial DCs (RFD1+), are seen. This feature is seen both in sarcoidosis patients and healthy controls. Probably the difference in antigen response occurs later in the inflammatory response [107]. When S-100 protein positive DCs are evaluated in histologically positive Kveim skin biopsies, S-100 positive epidermal DCs were 2-fold higher than in foreign body reactions [108] .

When DC number in the peripheral blood of sarcoidosis patients was assessed, conflicting results have been reported. Ota found “myeloid” (lin-, HLA-DR+. CD11c+) and

"lymphoid" DC (lin-, HLA-DR+, CD11c^{dim}, most likely representing pDCs) numbers were decreased in the peripheral blood of sarcoidosis patients compared to healthy controls [103]. Contrasting these reports, another more recent study by Mathew found similar numbers of myeloid DCs (lin-, HLA-DR+. CD11c+) and pDCs (lin-, HLA-DR+, CD123+) compared to healthy controls [109]. The mDCs are similarly defined, so that will not be the reason for this difference found, but the lympoid DCs do not match the plasmacytoid DCs as not all lympoid DCs are CD123 $^{+}$ (unpublished observations) .

Besides evaluating the number of DCs in the peripheral blood of sarcoidosis patient, also the function of DCs has been assessed. When isolated mDCs from the peripheral blood were incubated with T cells in an allogeneic mixed leukocyte reaction (MLR), mDCs from sarcoidosis patients had significant decreased ability to stimulate T cells compared to healthy controls. The T cell function of sarcoidosis patients remains intact when tested in an MLR with allogenic LPS stimulated DCs. The attenuated function of sarcoid-derived mDCs in the allogeneic MLR was not associated with lack of phenotypic maturation. Sarcoidosis mDCs showed significantly increased expression of CD40, but other maturation markers, CD83, CD86, CD80 were comparably up regulated. No significant correlations were observed between mDC marker expression and their ability to drive allogeneic T cell responses. [109]. In contrast to this study Kulakova et al showed that in an allogenic MLR with monocyte derived DCs cultured from peripheral blood of sarcoidosis patients or healthy controls and peripheral blood mononuclear cells (PBMCs) of a single donor, no significant differences in T cell stimulation could be found. When monocyte decrived DCs from a single donor stimulated PBMCs of patients and healthy controls, PBMCs of patients showed a decreased response, probably by an increased number of regulatory T cells in the PBMC fraction. They found markedly depressed proliferation kinetics in autologous DC-peripheral blood mononuclear cell (PBMC) co-cultures from sarcoid patients compared to normal [110].

When pulmonary pDCs and mDCs obtained from bronchoalveolar lavage were analyzed. The total numbers of pDCs and mDCs did not differ between sarcoidosis patients and healthy controls. However there was a significant increase in the number of CD1a negative mDCs in the BAL of sarcoidosis patient compared to controls. In addition there was altered expression of costimolatory markers on mDCs of sarcoidosis patients. Increased CD80, and decreased CD86. No differences in chemokine expression (CCL20, potent chemotactic factor for mDCs), and CXCL10, (a potent chemotactic factor for pDCs) in lung tissues were seen compared to healthy control lung tissue [111] .

In summary studies in the past few years show conflicting data concerning DCs in sarcoidosis. It is hard to conclude whether the number of DCs in biopsies of skin, lymph nodes or lung tissue are elevated or decreased, because of the different definitions and stainings used to identify the DCs. But DCs are present in the sarcoid granulomas mostly in the surrounding lymphocyte layer and seem to interact with the lymphocytes pres-

ent. In blood there is also no consensus about the number of mDCs and pDCs. In the BAL there are more CD1a+ mDCs (LCs ?) and the mDCs seem to be more mature than DCs of healthy controls. This observation is done by only one group but it fits the general function of DCs to initiate a T cell response in reaction to an antigenic stimulus. In sarcoidosis lung, a Th1 lymphocytosis is seen and this could be the result of antigen presentation by mature DCs seen in the sarcoidosis lung. Functional analysis of DCs from the peripheral blood shows conflicting results, and more research is necessary to conclude if DCs are responsible for the anergy seen in sarcoidosis and to define the exact role of DCs in the pathogenesis of sarcoidosis. Also more research is needed to study if DCs are involved in the expansion of Tregs that is commonly seen in the lungs and granulomas of sarcoidosis patients, and is responsible for the immune paradox of sarcoidosis [112].

7. DENDRITIC CELLS IN LUNG FIBROSIS

7.1 Dendritic cells in idiopathic pulmonary fibrosis (IPF) / usual interstitial pneumonia (UIP)

IPF is a relatively rare disease carrying a bad prognosis. In contrast to other interstitial lung diseases associated with lung fibrosis caused by drugs or underlying systemic diseases, the cause of IPF is unknown. Diagnosis can be made by a surgical biopsy showing a UIP pattern.

A UIP pattern typically demonstrates a heterogeneous appearance of normal lung tissue alternating with areas of peripheral fibrosis, little interstitial inflammation and honeycombing. The inflammation seen in UIP is typically mild, with some lymphocytes and plasma cells. Dense relatively acellular collagen bundles are seen with a higher magnification, and are mixed with areas of young fibrosis. On the border of normal and fibrotic lung, fibroblastic foci are seen. Fibroblastic foci are collections of fibroblasts/myofibroblasts and assumed to be the active inflammatory lesions of UIP. The presence of these fibroblastic foci is an important prognostic factor in UIP [113].

The pathogenesis of IPF is very complex. Different studies show multiple gene mutations associated with IPF reflecting a possible predisposition to develop IPF and the progression of IPF. The inflammation starts with a lung injury caused by different provoking factors like particles, auto-immune events, viruses or chemicals followed by a range of molecular abnormalities in the coagulation-, (anti-)oxidant-, and Th1/Th2-cascades and by inflammatory cells and fibrocytes. This will lead to an imbalance of the pro-and anti-fibrotic pathways resulting in excessive extracellular matrix deposition and fibrosis [114].

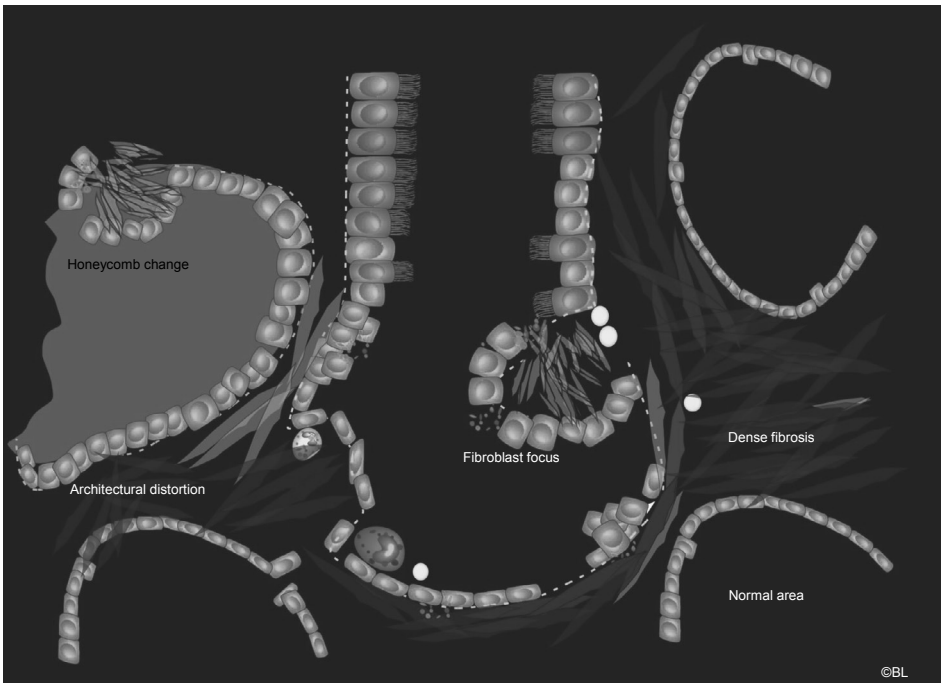

Figure 2. A UIP pattern typically demonstrates a heterogeneous appearance of normal lung tissue alternating with areas of peripheral fibrosis, little interstitial inflammation and honeycombing. On the border of normal and fibrotic lung, fibroblastic foci are seen. Fibroblastic foci are collections of fibroblasts/myofibroblasts and assumed to be the active inflammatory lesions of UIP.

Studies using various cytokine deficient mice showed that the development of fibrosis in the lung is strongly linked with a Th2 response, involving IL-4, IL-5 and IL-13. Th2 cytokines in the lungs strongly promote the mechanisms of wound healing. However if the stimulus persists, the end result of persistent healing will be fibrotic tissue remodeling with collagen deposition. Th1 cytokines IFN-γ and IL-12 can inhibit the profibrotic cytokine TGF-β and IL-13, and can indirectly inhibit collagen synthesis by fibroblasts [115]. When IL 12 p40-/- mouse were treated with bleomycin intratracheal as a model for IPF, they show decreased pulmonary inflammation and increased fibrosis [116]. In a bleomycin mouse model of fibrosis, increased numbers of CD11c+MHCII+ DCs, including CD11b(hi) monocyte-derived inflammatory DCs, infiltrated the lung of treated animals during the fibrotic phase of the response to bleomycin. These DCs were mature DCs expressing CD40, CD86, and CD83. They were associated with increased numbers of recently activated memory T cells, suggesting DC are the key pro-inflammatory cells initiating fibrosis in the lung [117].

The first data reported about human pulmonary DCs in IPF was a study in which lung biopsies were stained for the DC marker S100. Cells positive for S100 with a dendritic cell appearance, were absent in all cases of IPF [118]. More recently however, Marchal-

Somme and colleagues assessed the formation of secondary lymphoid tissue in the lung of IPF patients. In this lymphoid tissue, clusters of T and B cells were observed, and in all fibrotic biopsies the T cells aggregates were infiltrated with mature DCs expressing CD40, CD86, CD83 and DC-Lamp. There was a positive correlation between the numbers of mature DCs and the intensity of the lymphocytic infiltrate. The lymphocytes were positive for CCL19 and the DCs were strongly positive for CCR7 suggesting recruitment via CCR7-CCL19 interaction. The blood vessels around the lymphoid tissue were positive for CCL21, suggesting recruitment of CCR7+ cells from the blood. The T cells in the lymphoid aggregate were mostly CD40L positive, and therefore were likely to interact with CD40 on the DCs [119]. Interestingly CD40/CD40L interaction was essential in the pathogenesis of an autoimmune model of lung fibrosis [120]. Lung tissue of IPF patients is also heavily infiltrated by immature DCs (CD1a+, CD1c+ and DC-SIGN+) and whereas CD1a+ and CD1c+ DCs were essentially distributed in areas of type II alveolar epithelial cell hyperplasia, DC-SIGN+, CD68- DCs were scattered in the less cellular areas of established fibrosis and around blood cells. In fibroblast foci no DCs (mature or immature) were seen. ICAM-2 expression (endothelial cells of blood vessels) -the ligand for DC-SIGN and involved in DC trafficking-, and ICAM-3 expression (T cell aggregates, allowing immunologic synapse formation in lymphoid organs through DC-SIGN) were seen in the fibrotic lung tissue. In general altered epithelial cells and fibroblasts in IPF expressed all chemokines necessary for the recruitment DCs, suggesting the recruitment of DCs to the lung in IPF is a chemokine driven process [121]. When DCs in the BAL of IPF patients were assessed, normal percentages of pDCs and mDCs are found but the mDCs were not as mature (CD83) as mDCs of healthy controls [111].

7.2 Nonspecific interstitial pneumonia (NSIP)

NSIP is the other major form of fibrotic lung disorder besides UIP/IPF. The cause of NSIP is either idiopathic or caused by an underlying disease (eg Collagen vascular disease, hypersensitivity pneumonitis, infection, drug induced or immunodeficiency). Lung biopsies may show predominantly interstitial inflammation or fibrosis or a combination of inflammation and fibrosis. The inflammation is usually mild with lymphocytes and a few plasma cells. Comparable to IFP, in NSIP also lymphoid aggregates are present. There is a widespread increase of altered alveolar epithelial cells, and fibroblasts/myofibroblasts. Fibroblastic foci are absent in NSIP. In contrast to IPF where almost no S100+ DCs were recognized, S100 positive DC were observed in all the cases of NSIP. These S100+ DCs were also positive for HLA-DR but not for CD1a. CD8 and CD4 positive lymphocytes were infiltrated diffusely around the DCs [122]. Marchal-Somme and colleagues who examined DC in lung biopsies of IPF/UIP and NSIP showed that in contrast to the previous study, DC type and distribution pattern in the lung of NSIP resembled that of IPF. In NSIP also mature DCs (CD83, CD86 or DC-Lamp+) were detected infiltrating the lymphoid

follicles. The lung was massively infiltrated with immature DCs and the chemokine expression pattern also resembled IPF [121].

In conclusion S100+ DC in biopsies of IPF are rather absent, but in NSIP increased numbers of S100+ DCs are present. Despite this contradiction two excellent recent studies by the same group show that immature and mature DCs are both present in IPF and NSIP lung tissue with the same distribution pattern. Immature and mature DCs have a distinct localization. In fibroblastic foci, assumed to be the active lesions of IPF, no DCs are seen. The expression of chemokines in the fibrotic tissue and chemokine receptors on DCs suggest active recruitment of DCs to the lung in IPF and NSIP. In IPF lung mDCs from BAL were not as mature as healthy control. Unfortunately no studies are performed yet to study the function of dendritic cells in IPF and NSIP and hopefully this will elucidate the role of DC more in the future.

8. PULMONARY LANGERHANS CELL HISTIOCYTOSIS

The characteristic feature of Langerhans cell histiocytosis is accumulation of LCs, CD1a+ positive histiocytes of DC lineage, derived from CD34+ progenitors from the bone marrow, in one or several organs. Pulmonary Langerhans cell histiocytosis (PLCH) is an interstitial lung disease occurring predominantly in adult cigarette smokers [123, 124]. Clinically PLCH is uncommon, comprising approximately 5% of all interstitial lung disease cases generally occurring in middle-aged men and women [125, 126] .

PLCH is typically diagnosed with an open lung biopsy. The appearance of the biopsies varies according to the disease stage and progression. Early lesions show multiple stellate nodules ranging from less than 1 cm to 2 cm. The nodules are cellular and consist of a variable mix of LCs, lymphocytes, eosinophils and plasma cells, with a background of generally mild fibrosis. When the disease progresses, more and more fibrosis is seen. The cellularity decreases and only a few LCs and eosinophils are seen in the nodules. Finally the nodules will confluence and large cysts will be formatted, with in some cases end stage fibrosis and honeycombing [127]. LCs in the lesions are usually immunopositive with CD1a, Langerin, E-cadherin, S-100 and contain Birbeck granules [9] .

The accumulation of LCs could occur either by local proliferation that even can be clonal [128], but the proliferation rate of LCs is slow [129]. LC lesions are often spontaneously remissive, and in end stage lesions LCs are even virtually absent. Alternatively LC lesions could grow by continuous recruitment of LCs or precursors from the blood. LCs express the CCR6 marker and its ligand CCL20. This could work as a autocrine mechanism of recruiting and retention of LCs in the granuloma [130] .

Pulmonary LCs in PLCH are mature with high expression of CD80 and CD86 [131]. This activated state of the LC is supported by the local presence of dendritic cell activating cytokine IL-1β and low levels of IL-10. Also an increase in GM-CSF production have been documented [132]. In contrary to these results, LCs in skin and bone lesions in PLCH express an immature phenotype with low levels of CD83 and CD86 and weak T-cell stimulatory capacity. This immature phenotype is also confirmed by a high expression of CCR6 and numerous IL-10 producing cells in the direct tissue environment [133]. Futhermore analyzing lung lesions of PLCH patients showed the presence of many cytotoxic T cells in the lesions and a small population of non-Langerhans monocytotic cells, with an unknown function at this moment [134].

The cytokines in the tissue environment of LC lesions or serum of patients with PLCH can favour the development of PLCH. The increased GM-CSF production in pulmonary lesions as mentioned above, together with increased TNF-α production in extrapulmonary lesions [135] has been described as critical factors for the hematopoeitic development of LCs [136]. In the serum of patients elevated levels of Flt3-L and stem cell factor (SCF) have been found [137], both hematopoeitic growth factors shown to sustain long term expansion of primitive DC precursors [138].

PLCH is a complex disease. Despite different studies who support a role for local proliferation, enhanced recruitment of LCs, and elevated levels of hematopoeitic growth factors for LCs, the exact cause of the accumulation of LCs is still unclear and more study needs to be done.

9. CONCLUSION

Numerous studies in T cell mediated lung diseases like asthma, COPD, sarcoidosis, and fibrotic lung disease now support the concept that DCs play an important role in disease pathogenesis. This concept is furthermore supported by the numerous studies in mouse models of these diseases where DCs can be eliminated or administered and where clear contributions to disease pathogenesis have been demonstrated. By necessity, the studies on lung DCs in human lung disease have been mainly descriptive. Further understanding of these cells in human disease await the further development of drugs that selectively inhibit the function of DCs.

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A novel method for isolating dendritic cells from human bronchoalveolar lavage fluid

Ten Berge B.[1], Muskens F.[1], KleinJan A.[1], Hammad, H.[2], Hoogsteden H.C[1], Lambrecht B.N[12]*, Van den Blink B.[1]*

[1] *Department of Pulmonary Medicine, Erasmus MC, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands*

[2] *Department of Respiratory Diseases, Laboratory of Mucosal Immunology, MRBI, University Hospital Gent, De Pintelaan 185, B9000 Ghent, Belgium*

** These authors shared the supervision of this study.*

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ABSTRACT

Background: Dendritic cells (DCs) play a pivotal role in linking the innate and adaptive immune response and have been implicated in a variety of pulmonary diseases. Currently, studies on the role of DCs are limited by difficulties in isolating DCs from the lung. Surgical lung specimens are not readily available and purification of DCs from digested lung tissue is likely to induce phenotypical and functional changes. DCs obtained from the alveolar spaces are thought to represent the local microenvironment and can be obtained using minimally invasive techniques. We developed a novel method of isolating DCs from bronchoalveolar lavage (BAL) fluid

Methods: After removal of macrophages, the remaining BAL cells were stained with a lineage mix (CD3-, CD14-, CD16-, CD19-, CD56-FITC), CD11c and HLA-DR and sorted with a FACS ARIA. DAPI was used as a dead-live marker. mDCs were low autofluorescent, lineage mix negative, $CD11c^+$ and $HLA\text{-}DR^+$ cells. pDCs were $CD11c^-$ but $CD123^+$. Morphological assessment of sorted mDC's and pDCs was performed. Sorted mDCs were tested in a mixed leukocyte reaction (MLR) with naïve $CD4^+$ T cells and evaluated for T cell differentiation and cytokine production. With confocal microscopy DC-T cell interaction was assessed.

Results: Using our sorting strategy, mDCs and pDCs, with a high purity upon FACS analysis of the sorted fraction, were obtained. These cells showed the morphological characteristics of DCs. Most importantly, mDCs were able to induce T cell proliferation and differentiation in a MLR, and interact with T cells as assessed by confocal microscopy. These results indicate the presence of functional DCs. Freezing and thawing of the BAL cells did not affect phenotype or T cell stimulatory capacity of the isolated DCs.

Conclusion: Using a novel sorting strategy, functional mDCs can be isolated from BAL fluid, enabling detailed study in pulmonary disease.

1. INTRODUCTION

Dendritic cells (DCs) are antigen presenting cells (APCs) with an important capacity to induce primary immune responses. DCs recognize different invading pathogens and transfer this information to the cells of the adaptive immune system. DCs are very important in the initiation and modulation of the immune system response to "danger" signals [1] and play a central role in directing effector T cells into a T helper-1 (Th1) or a T helper-2 (Th2) phenotype in response to variable microbiological antigens and cytokines. [2, 3]

In the lung DCs are present in the bronchial epithelium as a superficial airway network. In absence of inflammation DCs are distributed with a density of several hundred cells per square millimeter. The lung parenchyma contains even more DCs mostly found in the septa. [4]

Depending on the use of different DC-markers, 2 or 3 different populations of DCs can be recognized in the human lung. According to Masten and colleagues, in the lung myeloid dendritic cells (mDC) ($CD1c^+$, $CD11c^+$, $CD14^-$, $HLA\text{-}DR^+$) and plasmacytoid dendritic cells (pDC) ($CD123^+$, $CD11c^-$, $CD14^-$, $HLA\text{-}DR^+$) can be documented. [5] Demedts divided the DC population in the lung according to blood dendritic cell antigen (BDCA) markers into mDC type 1 ($CD11c^+$/$BDCA\text{-}1^+$), mDC type 2 ($CD11c^+$/$BDCA\text{-}3^+$) and pDCs ($CD11c^-$/$BDCA\text{-}2^+$)[6] The three types of dendritic cells show different cytokine profiles and different T cell stimulatory capacities when stimulated with toll like receptor (TLR) ligands, implicating different roles for mDCs and pDCs in the human lung. [6]

Important murine findings have increased the interest in the role of pulmonary DCs in human disease, but research into pulmonary DCs in humans is still hampered by difficulties in isolating DCs. Frequently, the studied pulmonary DCs are purified from lung tissue fragments obtained from patients undergoing a lobectomia or pneumectomia. [5-7] This implicates limited availability of material, surgical lung specimens are not readily available and surgery cannot be scheduled for research purposes, making isolation of DC's from resected lung fragments unpractical. Furthermore, these fragments are from or adjacent to diseased lung, with unpredictable effects on the DCs. Finally, isolation of DCs from lung tissue requires digestion of the tissue with enzymes which may induce phenotypic and functional changes to the DCs

Isolation of DCs from bronchoalveolar lavage (BAL) fluid seems an attractive alternative to study DCs in different lung diseases. BAL is a minimally invasive procedure that instills and subsequently recovers lavage fluid and its components from the alveolar space. It gives an *in situ* specimen from affected lung tissue and airway and can help to understand the immunopathogenesis of many pulmonary diseases. [8] A small number of DCs can be detected in BAL fluid. [9] However, BAL procedures on patients are

often unscheduled, which makes collecting series of specimens, isolating DC's and the planning of further experiments difficult and time-consuming. Freezing BAL fluid cells until further use could solve this problem. There are no data available on the effect of freezing on human pulmonary DC function but freezing mouse DCs does not induce any phenotypic or functional changes on DCs [10]

The problem with the current available methods thus lies in defining and isolating a pure population of DCs. To date, no satisfying method is available for isolating DCs with a high degree of purity from readily available human pulmonary material in a practical manner, while avoiding contamination of alveolar macrophages. Developing such a method would allow for detailed study of the role of DCs in the immunopathogenesis of pulmonary diseases.

The purpose of this investigation was to develop such a method. To this end, we isolated DCs from BAL fluid using a flowcytometric sorting strategy. DCs isolated from freshly obtained BAL fluid were compared to those isolated from previously frozen BAL fluid cells. DCs were assessed for morphology, phenotype and function. In a mixed leukocyte reaction, DC-induced T cell activation, proliferation and polarization was measured and DC-T cell interactions were assessed using live confocal imaging.

2. MATERIALS AND METHODS

2.1 Patients

After informed consent, 10 patients, suspected for pulmonary sarcoidosis, underwent fibre-optic bronchoscopy. The protocol was approved by the Medical Ethical Committee of the Erasmus University, Rotterdam.

2.2 Collecting BAL cells and storage

BAL was performed with a flexible fibre-optic bronchoscope (Olympus) placed in the right middle lobe in wedge position. Four aliquots of 50 ml saline were instilled and subsequently gently aspirated. BAL fluid was collected in siliconized bottles to prevent cell adherence and kept at 4 °C. BAL fluid was filtered through a 100µm cell strainer (BD Biosciences) and centrifuged for 7 min at 450g at 4°C. The supernatant was removed and the BAL fluid cells were counted and subsequently frozen in 1 ml RPMI 1640 (Gibco), 10% Fetal Calf Serum (FCS, Sigma), 10% Dimethyl sulphoxide Hybri-Max (DMSO, Sigma) in a cryovial using a 5100 Cryo 1°C Freezing Container (Nalgene) to -80°C. Afterwards the cells were stored at -150°C.

2.3 Isolation of BALF DCs

The BAL fluid cells were quickly defrosted at 37 °C. 5 ml RPMI 1640 with 20% FCS was added and the cells were centrifuged for 7 min at 450g at 4°C. The BAL fluid cells were counted and resuspended in culture medium (RPMI 1640, 10% FCS and gentamycin (Gibco (50 ug/ml final concentration)) to 1 x 10^6 cells / ml. The cells were incubated in a plastic petri dish for 1 hr at 37 °C to deplete macrophages, adhered to the petri dish. After 1 hr the non-adhered cells were collected washing the petri dishes 3 times with warm RPMI 1640 (37°C). After centrifugation the non-adhered BAL fluid cell fraction was resuspended in FACS buffer (PBS supplemented with 0.25% Bovine serum albumine (BSA), 0.5mM ethylenediaminetetraacetic acid (EDTA) and 0.05% NaN3) supplemented with 1% heat inactivated human serum and subsequently stained with the following antibodies: CD3 Fitc (UCHT1), CD14 Fitc (61D3), CD54 Fitc (MEM188) (all eBioscience), CD16 Fitc (3G8), CD19 Fitc (HIB19), CD11c Pe-Cy5 (B-ly6), HLA-DR PE-Cy7 (L243) (all BD Biosciences), and CD123 APC (AC145, Miltenyi). 4',6-diamidino-2-phenylindole (DAPI, Molecular probes) was used as a dead-live marker in the violet channel. DCs were sorted using a FACS ARIA (BD Biosciences with Diva software, for analysis FlowJo software Tree Star. Inc, was used). PDCs and mDCs were recognized based on scatter characteristics and expression of different markers. After sorting the purity of the cell fraction was measured on the FACS ARIA . A short overview of this protocol is shown in table 1. To test whether freezing of mDCs affects their phenotype, the expression of the surface molecule markers CD80 and CD86 was measured by flowcytometry on mDCs from a fresh aliquot and on mDCs from a frozen and thawed aliquot of the same BAL fluid sample.

2.4 Isolation of peripheral blood naïve T cells and labeling with CFSE

One buffy coat (Sanquin, Amsterdam, The Netherlands) was used to create a batch of naïve T cells, which were used for all the experiments. The buffy coat was diluted 1:4 with RPMI 1640, and centrifuged over a Ficoll-Paque (GE Healthcare) density gradient for 20 min, 1200g, at room temperature. The mononuclear cells at the interface were collected and washed in RPMI 1640. To isolate naïve $CD4^+$ T cells a commercially available kit (Miltenyi, 130-091-894) was used according to manufacturers' instructions. All steps were performed with MACS buffer (PBS supplemented with 5mM EDTA and 1% BSA) at 4°C. In brief: All cells, except $CD4^+$ naïve T cells, were indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies (CD8, CD14, CD16, CD19, CD36, CD45RO, CD123, TCRγ/δ and Glycophorin A) followed by incubation with anti-biotin microbeads. Isolation of pure naïve T cells was achieved by depletion of the magnetically labeled non- $CD4^+$ naïve T cells.

For fluorescent cell labeling with Carboxyfluorescein succinimidyl ester (CFSE), cells were washed twice with serum free medium and labeled in a final concentration of 5 µM CFSE (Molecular Probes) for 10 min in serum-free medium at 37°C. Adding excess ice-cold culture medium stopped the reaction.

Tabel 1. Protocol for dendritic cell isolation from bronchoalveolar lavage fluid

Collecting cells and storage				
1	Filter BAL fluid through a 100 µm gauze			
2	Centrifuge the BAL fluid at 450 g for 7 min at 4°C			
3	Remove the supernatant. Cells can be used for sorting directly or frozen for storage			
	Freezing: resuspend BAL cells in 1ml RPMI + 10% FCS + 10% DMSO and freeze in a cryovial to –80°C			
	Storage: at –150°C			
	Defrosting: defrost cells quickly at 37°C, add 5 ml RPMI with 20% FCS and centrifuge the cells at 450g for 7min at 4°C			
Adherence step				
1	Resuspend BAL cells in culture medium to 1x10e6 cells/ml			
2	Incubate BAL cells in a petridish (10x10e6 cells/dish) for 1 hour at 37°C			
3	Collect the non-adhere cell fraction by washing 3 times with warm (37°C) culture medium			
4	Centrifuge the BAL cells at 450g for 7 min at 4°C and remove the supernatant			
Staining BAL cells and sorting				
1	Stain the BAL cells with fluorescent labeled antibodies			
	FITC	lin mix	CD3	dilution 1/50
			CD14	dilution 1/20
			CD16	dilution 1/20
			CD19	dilution 1/80
			CD56	dilution 1/20
	Pe-Cy5	CD11c		dilution 1/20
	APC	CD123		dilution 1/50
	Pe-cy7	HLA-DR		dilution 1/100
2	Sort the DCs, for gating strategy see figure 2			
3	Sorted DCs are collected in 1 ml filtered FCS. Fill the tubes after sort completely with PBS and centrifuge at 450 g for 15 min at 4°C. Remove the supernatant, count the DCs and use them for further experiments			

2.5 Immunocytochemistry

After sorting, the cells were resuspended in culture medium. Lipopolysaccharide (LPS, E.coli strain 026:B6, Sigma) at a final concentration of 10µg/ml was added and the cells were incubated at 37°C for 30 min. Cytospins were prepared by spinning the cell suspension on object slides for 3 min at 400g (Cytofuge, Nordic immunological laboratories, Tilburg, The Netherlands) Object slides were dried overnight and frozen at -80°C until staining. After defrosting the cytospins, cells were acetone fixed for 5 seconds, incubated with normal goat serum (Sanquin) for 10 minutes and subsequently incubated with anti-HLA-DR (1E5, Sanquin) for 60 min. After washing with PBS cytospins were in-

cubated with goat anti mouse biotin (Biogenics) for 30 min and washed again, followed by an incubation step with streptavidin-peroxidase (Biogenics) for 30 min. The staining was developed with H2O2-activated-3,3'-diaminobezidine for 10 min (DAB, Sigma). For nucleus staining, the cells were shortly incubated with Gill's heamatoxylin.

2.6 Mixed leukocyte reaction

Peripheral blood naïve T cells were co-cultured with isolated mDCs from the BALF with a DC:T cell ratio of 1:20 in culture medium in 96 wells round bottom plates for 5 days at 37°C. At day 5 3H-thymidine (0.5 µCi/well, Packard, cat nr 6004052 2.15x10^6dpm 1995) was added to the cells. After 16 hours cells were harvested and proliferation of the cells was determined by scintillation counting (Top count model B9912/VI, Packard Bioscience Benelux). At day 5 T cells were also stained with the following antibodies CD4 PE-Cy5 (RPA-T4, eBioscience), CD45RO PE (UCHI-1), CD25 FITC (2A3) (BD Biosciences), CCR4(205410), CXCR3 PE (49801) (R&D), and fluorescence was measured using a LSR II (BD Biosciences).

To test whether freezing of dendritic cells affects their ability to induce T cell proliferation, mDCs were isolated from an aliquot of freshly obtained BAL fluid cells and compared to the isolated mDCs from a frozen and thawed aliquot from the same BAL. mDCs were incubated with CFSE labeled $CD4^+$ naïve T cells with or without LPS stimulation at a final concentration of 10 µg/ml and after 4 days proliferation of T cells was measured by flowcytometry.

2.7 Cytokine measurement

Cytokine measurement was performed on day 5 culture supernatant using Luminex 100 system. The human 25-plex cytokine kit (Invitrogen) was used according to the manufacturers protocol. Standard curves for each cytokine (in duplicate) were generated using the reference cytokine concentrations supplied in this kit. The assay was performed in a 96-well filter plate. All incubation steps were performed at room temperature and in the dark to protect the beads from light. In addition the plates were shaken while incubating. In brief: polystyrene beads with different red and infrared dyes, coated with antibodies against human cytokines, were incubated with 25 µl of supernatant diluted 1:1 in incubation buffer for 3 hours. After washing with PBS 0.05% Tween, the beads were incubated with Biotinylated-antibodies diluted in Biotin-diluent for one hour. To remove the excess of antibodies, the plate was washed again with PBS, 0.05% Tween. The last step involved incubation with streptavidin-PE in streptavidin-PE diluent for one hour. After washing, the beads (minimum of 50 beads per cytokine) were analyzed in the Luminex 100 (Luminex, Austin, USA) instrument. This monitored the spectral properties of the beads while simultaneously measuring the amount of fluorescence associated

with PE. Raw data (mean fluorescence intensity, MFI) were analyzed using StarStation software (Applied Cytometric Systems, Dinnington, UK).

2.8 Confocal microscopic analysis

Confocal analysis was performed on DCs sorted from the BAL fluid.

mDCs stained with HLA-DR APC (clone G46-6 (L243), BD biosciences) were placed on Poly-L-Lysine-coated slides, naïve $CD4^+$ T cells stained with CD4 FITC (clone RPA-T4, BD biosciences) were added and cells were imaged on a LSM-510 confocal laser microscope (ZEISS). Images were processed using Imaris software (Bitplane)

2.9 Statistics

Results were compared using a two-tailed Mann Whitney U test. Differences were considered significant at $P<0.05$

3. RESULTS

3.1 Isolating dendritic cells from bronchoalveolar lavage fluid

Previously, the auto fluorescent characteristics of BAL fluid cells have been used to separate the T cell stimulatory fraction from the suppressive macrophages. Subdividing BAL cells according to their autofluorescent properties yields a high autofluorescent fraction (HAF), characteristically consisting of macrophages, and a low autofluorescent fraction (LAF) that has clear T cell stimulatory properties [11, 12], suggestive of the presence of DC's. However, the LAF contains a mixed cell population, as evident from cell morphology on a cytospin from sorted LAF (figure 1). This is a mixed population, with in theory containing lymphocytes, monocytes, eosinophils, NK-cells, B-cells and DCs as these cells have all been described in the BAL fluid. [13, 14]

We set out to isolate DCs from BAL cells using a modified strategy. First, in order to get rid of the bulk of macrophages, cells were allowed to adhere for one hour to a culture dish. Nonadherent cells were stained and subsequently sorted using a flowcytometric sorter. A FITC-labeled lineage mix, containing antibodies against CD3 (T cells,) CD14 (monocytes), CD16 (monocytes, NK cells), CD19 (B cells) and CD56 (NK cells) was used to stain the lymphocytes, B-cells, NK cells, neutrophils and eosinophils in the LAF fraction. We sorted living (DAPI negative), low autofluorescent, lineage mix-negative, HLA-DR positive and CD11c positive cells as mDCs. The CD11c negative, but CD123 positive cells were sorted as pDCs (figure 2).

To validate the purity of the DCs, the sorted cells were analyzed again using the identical flowcytrometric gating strategy. In our hands the purity of the sorted cells was approximately 94% (range 91.7-96.6%) (Figure 3)

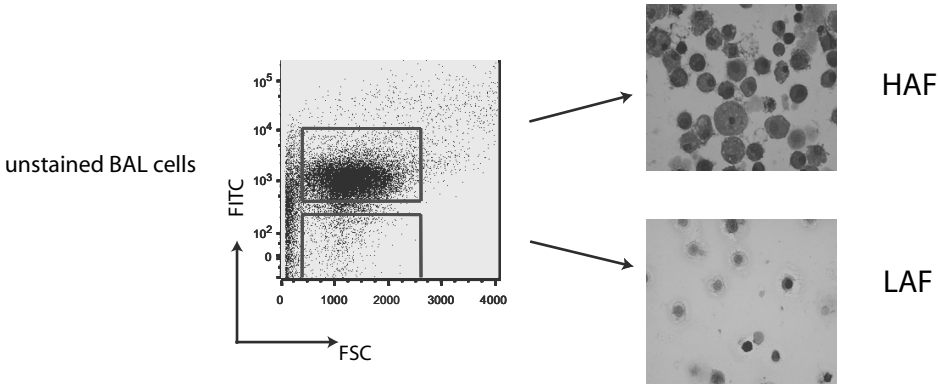

Figure 1. Unstained bronchoalveolar cells can be separated according to their autofluorescence. When unstained BAL fluid cells are measured on a flowcytometer the macrophages are highly autofluorescent and appear to be positive in the FITC channel. The high autofluorescent fraction (HAF) and the low autofluorecenst (LAF) fraction can be sorted and morphological assessed on cytospins. The HAF almost exclusively consist of macrophages, the LAF fraction however is a heterogeneous population. Cytospins were stained with Gills' heamatoxillin and pictures are made with a 20X magnification.

3.2 Morphology of the isolated DCs

After ensuring that our sorting strategy enables isolation of mDCs and pDCs with a high degree of purity, we asked ourselves the question whether these cells also bear the morphological characteristics of DCs. Cytospins of sorted mDCs and pDCs were stained with an anti-HLA-DR antibody. Figure 2 shows representative examples of mDCs and a pDC after sorting.

The mDCs have an irregular cell membrane with blebs caused by retracted dendrites. On the cell membrane long typical tunneling nanotubules characteristic for DCs can be seen. The nucleus has a lobular to kidney-shaped form depending on the maturation status of the DC. The nucleus shows also an irregular stained aspect, a so called chess-board pattern. In the cytoplasm near the nucleus, a dark HLA-DR+ spot can be seen, with an intensity varying between the DCs, matching the acidic compartment in the cell filled with HLA-DR+ complexes [15]

The pDC shows a more round appearance with a smooth membrane without blebs or nanotubules and with a round irregular stained nucleus, just like plasma cells. The dark spot, marking the acidic compartment in the cytoplasm, is also visible.

3.3 Effects of freezing a BAL sample on function and phenotype of isolated myeloid dendritic cells

To analyze the effect of freezing on the phenotype and function of BAL mDCs, we divided a BAL fluid sample in two portions. Half of the cells was first frozen as described in the methods section, subsequently defrosted and used for mDC isolation and subsequent phenotype and function analysis. The other half of the cells was used directly mDC isola-

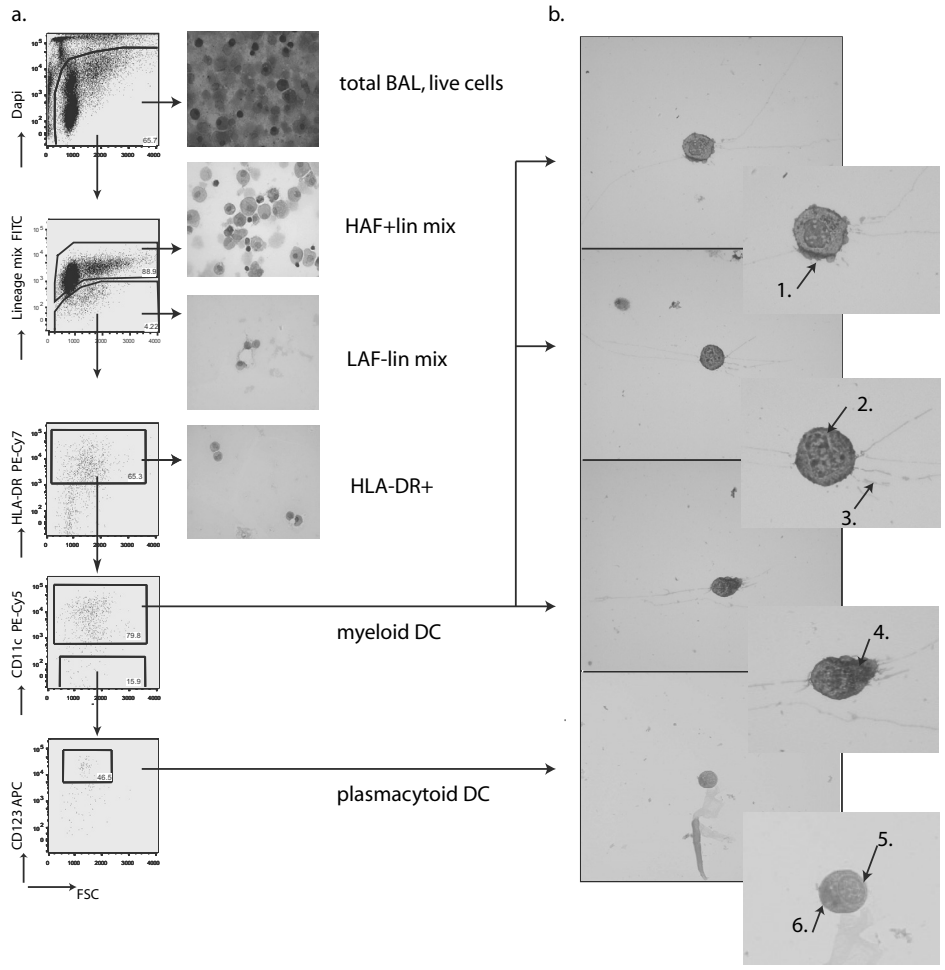

Figure 2.

(a) Sorting strategy for isolating DCs. Dead cells were removed from the total fraction of the BAL cells using DAPI as a dead-live marker. The DAPI negative cells are expressed in the FITC channel were the HAF fraction and the lineage ($CD3^+$, $CD14^+$, $CD16^+$, $CD19^+$ and $CD56^+$) positive cells were excluded. The LAF fraction without the lineage mix is expressed in the Pe-Cy7 channel. Cells are subsequently selected for HLA-DR expression and the expression of CD11c. The CD11c positive cells are the mDCs and the CD11c negative cells, positive for CD123, are the pDCs. Cytospins of all the different fractions show with every step a more pure population. The cytopsins are stained with Gills' heamatoxillin and pictures are made with a 20X magnification.

(b) *Morphological assessment of sorted mDCs and pDCs.* Cytospins stained with HLA-DR in a 40X magnification. mDCs show blebs on their membrane (1), long HLA-DR positive nanotubules (2), a lobular and irregular stained nucleus (3) and a dark spot (4) in the cytoplasm matching the acidic compartment were the HLA-DR complexes are stored. pDCs have a more round aspect with a smooth membrane surface and a round irregular stained nucleus (5). Also here a dark spot (6) in the cytoplasm can be recognized.

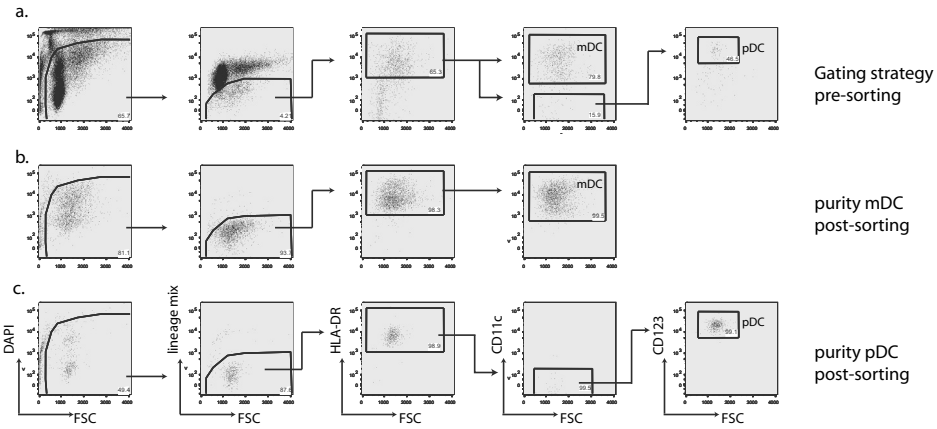

Figure 3. Purity of sorted mDCs and pDCs.
(a) The DCs were sorted by the strategy as shown in figure 2. The sorted fraction was analyzed using the same gating strategy for mDC
(b) and pDCs
(c). The purity of the sorted fraction reached approximately 94% (range 91,6-96,6%)

tion and subsequent phenotype and function analysis. The phenotype of BAL mDCs was assessed according to expression of the surface molecules CD80 and CD86. As shown in figure 4a, the surface molecule expression of CD80 and CD86 on fresh or thawed mDCs is identical. When fresh and thawed mDCs were isolated from BAL fluid, both mDC samples were analyzed in a MLR with CFSE-labeled naïve CD4 T cells with or without extra mDC stimulation with LPS. As shown in figure 4b, when unstimulated isolated BAL mDCs were incubated with CD4 naïve T cells, the freshly obtained mDCs induced a T cell proliferation of 6.28 percent of the total CD4 T cells population. The thawed mDCs induced a T cell proliferation of 7.19 percent. When the isolated mDCs were extra stimulated with LPS during incubation with the T cells, the freshly obtained mDCs induced proliferation in 11 percent of total CD4 T cells, and the thawed mDCs induced a T cell proliferation of 11.4 percent. So mDCs do not show an altered phenotype or altered T cell activating capacity after a freeze-thaw cycle.

3.4 Function of the isolated myeloid dendritic cells: mDC-T cell interactions

After establishing that our method yields cells that have phenotypic and morphological characteristics typical of DC subsets, we set out to investigate the functionality of the sorted population. Using confocal microscopy, we studied T cell – mDC contact dynamics, measuring lymphocyte adhesion to isolated mDCs under flow conditions. As described more extensively in the methods section, LPS pulsed mDCs were allowed to adhere to a culture dish, while T cells were added under convective flow conditions. Using confocal microscopy, mDC-T cell interactions were examined. In figure 5 we show an example of

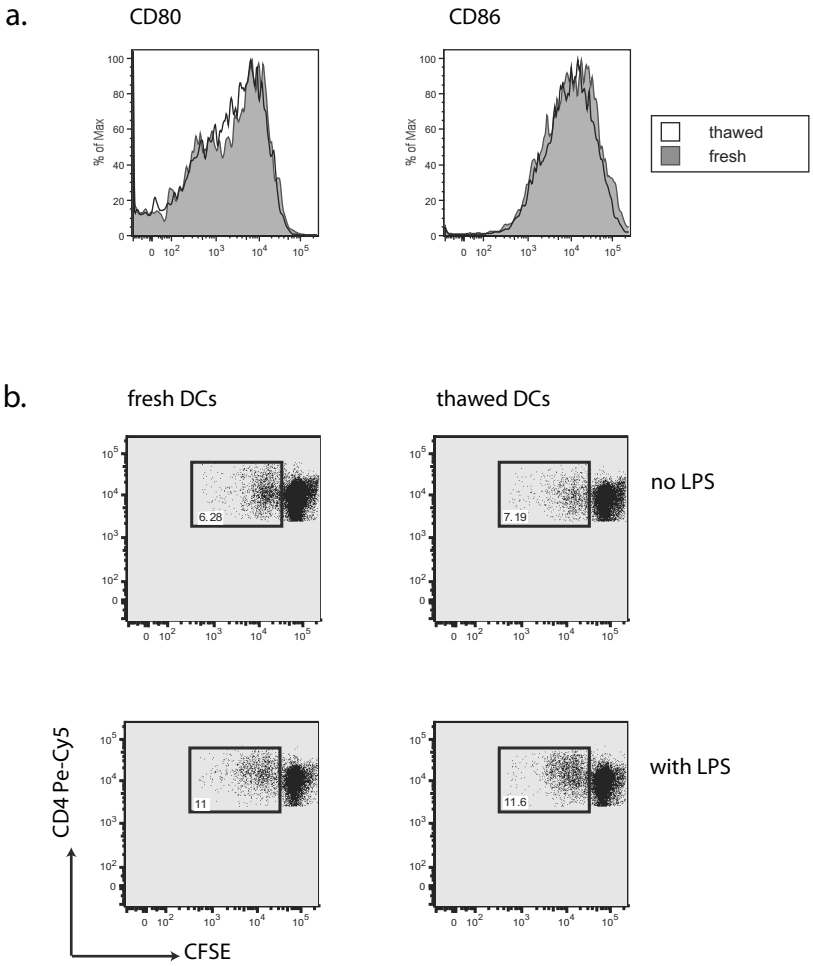

Figure 4. Influence of freezing on the phenotype and the T cell activating capacity of BAL mDCs. mDCs from an aliquot of freshly obtained BAL fluid cells were compared to those from a frozen and thawed aliquot from the same BAL. (a) mDCs were gated as described earlier and expression of surface molecules CD80 and CD86 on mDCs is shown in histograms (b) Isolated mDCs were incubated with CFSE labeled CD4 + naïve T cells, with or without LPS stimulation. T-cell proliferation was measured, using flowcytometry, after 4 days.

a $CD4^+$ T cell transiently interacting with a dendritic cell. The T-cell moves towards the mDC, rolls over the cell membrane and interacts with the mDC for approximately 620 seconds before it moves away. Measurements of different transient mDC-T cell contacts shows a contact duration varying between 100-760 seconds. (Figure 5b) This result is comparable with earlier desbribed mDC-T cell contact times in mice. In mice mDC-T cell contacts in the presence of antigen can be divided into transient contacts with duration of approximately 3-11 minutes and stable contacts with duration up to several hours.

[16] These results show that our isolated mDCs are functionally active and able to interact with naive T cells

Figure 5.
a) Imaging of mDC-T cell interactions. Confocal microscopy images with a 120 sec interval of a purple stained HLA-DR$^+$ mDC interacting with green labeled CD4$^+$ T cells. The yellow arrow points to a green T-cell, that is moving towards the purple stained mDC. The T cell interacts with the mDC while rolling over the mDC membrane for approximately 620 sec, before the T cell moves away again.
b) mDC-T cell interaction duration. Measurement of different transient mDC-T cell contact times as assessed by confocal microscopy.

3.5 Myeloid dendritic cells isolated from BALF stimulate CD4+ T cells

In order to investigate whether interaction of the isolated mDC's with CD4$^+$ T cells leads to T cell activation, we co-cultured the mDCs with allogenic T cells and subsequently analyzed the T cell proliferation, expression of several T cell markers and cytokine production by activated T cells in the supernatant. (Figure 6)

Analyzing the day 5 culture supernatant of the T cells, Figure 6a shows that sorted mDC-stimulated T cells produced significantly more IFN-gamma, IL-13, TNF-α and IL-5 compared to unstimulated T cells. The production of IL-4 was also elevated but not statistically significant. mDC-stimulated T cells show a higher proliferation compared to unstimulated T cells as measured by 3H Thymidine incorporation (figure 6b).

T cells stimulated by mDCs, demonstrate after co-culture an increased expression of the activation marker CD25 compared to unstimulated T cells. A part of the T cells differentiate into memory T cells expressing CD45RO on their membrane. Moreover T cells show Th1/Th2 polarization by expression of CCR4 and CXCR3 (figure 6c and 6d).

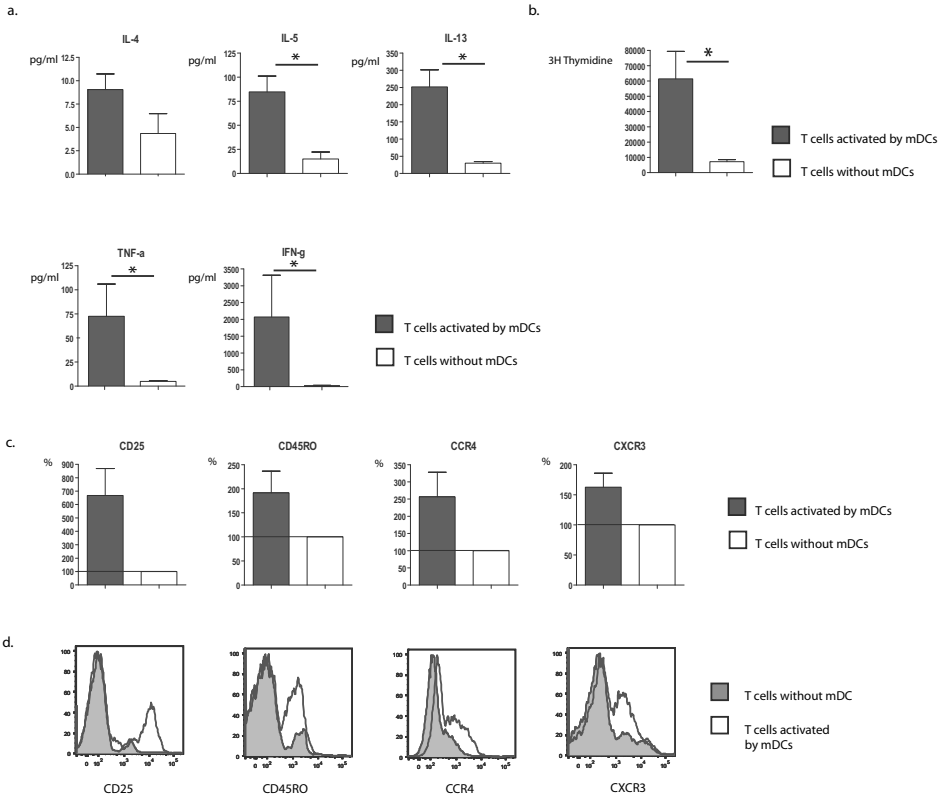

Figure 6. CD4+ T cell activation by isolated BAL mDCs. mDCs isolated from BAL were cultured for five days in a MLR with $CD4^+$ naïve T-cells from an allogeneic donor. As a control we used CD4 naïve T cells cultured for 5 days without adding mDCs. After 5 days the T cells were analyzed using flowcytometry for activation and differentiation markers. (a) With Luminex the cytokines in the culture supernatant were measured. (b) The proliferation of the T cells was measured by 3H-Thymidine incorporation. (c) percentage of upregulation of CD25, CD45RO, CCR4 and CXCR3 on activated T cells. (d) expression of CD25, CD45RO, CCR4 and CXCR3 on the activated T cells compared to the unstimulated T cells.

4. DISCUSSION

DCs are professional antigen-presenting cells that play a central role in the lung's immune defense and homeostasis. As a consequence, there is considerable interest in the role of DCs in human pulmonary disease. So far however, research has been hampered by the absence of a practical method of obtaining functional human pulmonary DCs.

Earlier studies used lung tissue from patients who underwent a lobectomy or pneumectomy for various reasons, but mostly lung cancer, to obtain DCs.(5, 6, 7) This approach has several limitations. First of all, the obtained tissue is adjacent to a diseased part of

the lung, which may influence the DCs. Secondly, to obtain DCs from lung tissue a digestion step is needed. This digestion step is likely to induce phenotypical or functional changes to the cells, with an unknown effect on the outcome of subsequent functional tests. Thirdly, the need for resection material limits the number of different diseases that can be studied: most pulmonary diseases do not need invasive surgery for diagnosis or treatment.

Using digested lung tissue, several pulmonary dendritic cell types were indentified. Masten et al identified mDCs in digested lung resection tissue as $CD1c^+$, $CD11c^+$, $CD14^-$, $HLA\text{-}DR^+$ and pDC as $CD123^+$, $CD11c^-$,$CD14^-$,$HLA\text{-}DR^+$. A population enriched for mDCs ($CD11c^+$, $CD14^-$, $lineage^-$ LAF cells) was shown to be much more potent in stimulating an allo-reaction than a monocyte enriched ($CD11c^+$, $CD14^+$, $lineage^-$, LAF cells) population. The mDC enriched population expressed higher co-stimulatory markers than a pDC enriched population ($CD11c^-$,$CD14^-$,$CD123^+$, lineage-) and responded with cytokine production to TLR4, whereas the pDC enriched population responded only to TLR7 stimulation with IFN-a production. [5] These data suggest that DCs can induce an allo-reaction and that in the lung several different types of DCs respond differently to TLR stimulation. However, enriched populations were tested and the degree of purity of the DC fraction is uncertain, making this method less suitable for studying the exact role of DCs in lung diseases. Demedts et al isolated three types of DCs from resected lung fragments using BDCA markers. They used anti-CD3, -CD11b and –CD16 beads to deplete lymphocytes, monocytes/macrophages and NK-cells from lung cell suspension, and subsequently CD4+ beads to enrich the BDCA+ cells because all BDCA+ cells express also CD4. After this enrichment procedure, cells were stained and sorted by flowcytometry for the low autofluorescent, CD3 negative, CD19 negative and BDCA-positive fractions, which resulted in a $BDCA^+$ lung population of +/- 90% purity. mDC type 1 ($CD11c^+$/$BDCA\text{-}1^+$) showed potent T cell-stimulatory capacities, mDC type 2 ($CD11c^+$/$BDCA\text{-}3^+$) exhibited an intermediate capacity to initiate T cells proliferation and pDCs ($CD11c^-$/$BDCA\text{-}2^+$) induced hardly any T cell proliferation. [6] This study shows a method for isolating DCs with a fair degree of purity. However, the effect of harvesting DCs from lung fragments, as used in this study, is unknown.

BAL is a minimal invasive and low risk method to obtain pulmonary cells from the lung. The cells collected in the BAL fluid and are suggested to be representative for the interstitial and alveolar compartment of the lung. [8] Havenith and colleagues used a special method to enlarge the relative number of DCs in the BAL fluid cell population by depleting other cells using transient adherence to plastic and density gradient separation, resulting in a population of 7-8% typical DC. This cell population demonstrated the capacity to induce T cell proliferation. [15] Van Haarst and colleagues separated BAL fluid cells with flowcytometry, into high auto fluorescent alveolar macrophages (AM)

with T-cell suppressive properties and in low auto fluorescent cells with powerful T-cell stimulating capacities. The low auto fluorescent fraction (LAF) shows features of DCs but does contain other cells as well.[12] Nicod and colleagues concluded that the LAF measured by flowcytometry of loosely adherent mononuclear cell fractions of BAL fluid cells, was very potent in stimulating mixed leukocyte reactions. [11]

Purification of DCs from the LAF fraction used to be difficult by the lack of DC specific markers. $CD1a^+$ DCs were isolated from the LAF fraction of BAL fluid cells, but this $CD1a^+$ population represents only a part of the total lung DC population. In addition the long overnight culture step to isolate transiently adherend mononuclear cells, as a step to enrich for dendritic cells, is also likely to induce phenotypical and functional changes to the DCs.[12]

Another more recently described method for isolating BAL fluid DC by Tsoumakidou et al [17] used immunomagnetic separation with BDCA-beads. Mononuclear cells from BAL fluid obtained by Ficol plaque density gradient centrifugation, were magnetically labeled for monocyte, T- and B-cell markers and these cells were depleted. The unlabeled cell fraction was subsequently labeled with BDCA-1 en BDCA-3 for mDC and BDCA-2 and -4 for pDC to positively select the DCs from the BAL fluid. The main disadvantage of this method is the labeling of the DCs with magnetic beads. There is a possibility that they can alter the functional properties of the DC. When the labeled DC were cocultured with naïve $CD4^+$ T cells, the number of activated cytokine producing T cells was low, despite the known stimulatory capacity of DCs. There are two possible explanations for this observation. First the function of the DC may be altered by the labeling with magnetic beads. A second explanation may be that the purity of the isolated DCs is not optimal with this method.

With our method the cells are sorted by flowcytometry, which implicates only labeling with FACS antibodies. FACS antibodies are much smaller than beads which makes is unlikely that they interfere with the function of the sorted cells. After sorting, the purity of the sorted fraction was high and the morphology showed nicely all typical characteristics know to be distinctive for mDCs and pDCs. The mDCs isolated with our method are capable of contacting with naïve T cells as recorded by confocal microscopy. When incubated with allogenic $CD4^+$naïve T cells, these mDCs demonstrate potent stimulatory capacity of T cells. The stimulated T cells produced significant higher amounts of cytokines than unstimulated T cells and expressed markers of activation, differentiation and polarization. Furthermore, our procedure is practical to use, because BAL fluid cells can be frozen until the isolation of DCs, without measurable interference on mDC phenotype and function. This makes our method suitable for testing more samples of patient BAL fluid cells at the same time.

There are certain limitations to our study. First, the percentage of DCs in the BAL fluid is low, so a fair amount of BAL fluid is necessary to obtain enough cells for function testing. This makes our technique unsuitable for pediatric patients. For mDCs this method will provide enough cells for further functional testing, but for pDCs on the other hand this method is limited. The percentage of pDCs in the BAL fluid is extremely low, as allready mentioned by Lommatzsch et al [18] and in our hands, after sorting and counting the cells, there was usually not enough material left for functional testing. Second, the percentage of DCs differs between different subjects, and different pathologies [18], with our method the exact percentages of pDCs and mDCs in the BAL fluid cannot be assessed due the removal of a variable amount of macrophages by the adherence step before sorting and counting. Diseases with low numbers of mDCs and pDCs in the BAL fluid will not be suitable for testing.

Third, the procedure is time consuming, expensive and requires the use of a FACS.

In conclusion, we have developed a new protocol for the isolation of human DCs from BAL fluid. With this practical method, functional properties of pulmonary DCs can be revealed in different diseases. This method can help clarifying the immunopathology of different pulmonary diseases in the near future.

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Evidence for local dendritic cell activation in pulmonary sarcoidosis

Ten Berge B.[1], KleinJan A.[1], Muskens F.[1], Hammad, H.[2], Hoogsteden H.C.[1], Hendriks R.W.[1] Lambrecht B.N.[1,2*], Van den Blink B.[1*]

[1] Department of Pulmonary Medicine, Erasmus MC, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

[2] Department of Respiratory Diseases, Laboratory of Mucosal Immunology, MRBI, University Hospital Gent, De Pintelaan 185, B9000 Ghent, Belgium

** These authors shared the supervision of this study*

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ABSTRACT

Background: Sarcoidosis is a granulomatous disease characterized by a seemingly exaggerated immune response against a difficult to discern antigen. Dendritic cells (DCs) are pivotal antigen presenting cells thought to play an important role in the pathogenesis. Paradoxically, decreased DC immune reactivity was reported in blood samples from pulmonary sarcoidosis patients. However, functional data on lung DCs in sarcoidosis are lacking. We hypothesized that at the site of disease DCs are mature, immunocompetent and involved in granuloma formation.

Methods: We characterized myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) in broncho-alveolar lavage (BAL) and blood from newly diagnosed, untreated pulmonary sarcoidosis patients and healthy controls. We used 9-color flow cytometry to analyze expression of the cell surface markers CD40, CD80, CD83, CD86 and CD208 (DC-LAMP). DCs, isolated from BAL using flowcytometric sorting (mDCs) or cultured from monocytes (mo-DCs), were functionally assessed in a mixed leukocyte reaction with naïve allogeneic CD4+ T cells. Using Immunohistochemistry, location and activation status of $CD11c^+$DCs was assessed in mucosal airway biopsies.

Results: mDCs in BAL, but not in blood, from sarcoidosis patients were increased in number and maturation status, as evidenced by significantly increased CD86, CD40 and DC-LAMP expression, when compared with mDCs from healthy controls. mDCs purified from BAL of sarcoidosis patients induced T cell proliferation and differentiation and did not show diminished immune reactivity. Mo-DCs from patients induced increased TNFα release in co-cultures with naïve allogeneic $CD4^+$ T cells. Finally, immunohistochemical analyses revealed increased numbers of mature $CD86^+$ DCs in granuloma-containing airway mucosal biopsies from sarcoidosis patients.

Conclusion: Taken together, these findings implicate increased local DC activation in granuloma formation or maintenance in pulmonary sarcoidosis.

1. INTRODUCTION

Sarcoidosis is a systemic disease characterized by the presence of noncaseating granulomas in involved organs, affecting the lung in more than 90% of patients [1-2]. The granulomatous reaction occurs in the absence of a clearly defined immunological target. However, a reaction to an unidentified antigen is suspected [3]. An antigen-driven pathogenesis is supported by disease-associated polymorphisms in genes encoding antigen recognizing or antigen presenting molecules such as Toll-like receptors and MHC class II [4]. Epidemiological and experimental data are suggestive of airborne or infectious antigens, in particular mycobacterial peptides, but attempts to link sarcoidosis to a causative pathogen are difficult and remain controversial [5-7]. Increased numbers of $CD4^+$ T cells in the broncho-alveolar lavage (BAL) fluid are a further hallmark of disease [3-4]. Increased proportions of oligoclonal $CD4^+$ T cells were found in the BAL from patients with sarcoidosis, consistent with a MHC-restricted antigen-driven process [8-9]. Both granuloma formation and T cell alveolitis have been characterized as Th-1 responses [3-4, 10-12]. These data have led to the hypothesis that sarcoidosis emerges from an exaggerated Th1 immune response upon presentation of an unidentified antigen by an antigen presenting cell (APC).

Past studies on APCs involved in pulmonary sarcoidosis focused on alveolar macrophages [8-9, 13]. However, in recent years it has become clear that dendritic cells (DCs) are the key APCs in the lung, responsible for presentation of antigen in draining lymph nodes, inducing T cell activation and proliferation [14-15]. Models of granulomatous disease in response to mycobacterial antigens showed that DCs contribute to granuloma formation [16-18]. We recently found that pulmonary granuloma formation is dependent on the presence of DCs and DC-induced T cell proliferation in draining lymph nodes [19]. These data suggest that DCs are pivotal mediators in the pathogenesis of sarcoidosis. Indeed, DCs were observed in skin, lymph node and lung lesions from sarcoidosis patients [14, 20]. Lymph node granulomas contained many mature DCs expressing the lysosome-associated membrane glycoprotein DC-LAMP, which is induced upon DC maturation [21]. These DC-$LAMP^+$ DC were typically located in the lymphocyte layer of granulomas and adjacent to $CD3^+$ T cells, suggesting functional DC-T cell interaction [21]. In muscular sarcoidosis, recruitment of mDCs and upregulation of the CD40/CD40L system in affected muscles suggested that mDCs would be involved in granulomatous inflammation through antigen presentation in a Th1 immune milieu [22]. However, there is debate about the number and function of DCs in pulmonary sarcoidosis: numbers of myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) in peripheral blood of pulmonary sarcoidosis patients were reported to be either normal or reduced [21, 23]. On the other hand, proportions of pDC and mDC in the BAL of sarcoidosis patients were reported to be similar and increased, respectively, when compared with healthy controls [24]. Also,

decreased proportions of BAL mDCs were found positive for CD83 and CD86, suggesting an immature phenotype of these cells [24-25]. Furthermore, peripheral blood mDCs and *in vitro* differentiated monocyte-derived DCs (mo-DCs) from sarcoidosis patients demonstrated either a decreased or a normal ability to stimulate T cells in co-culture experiments [23, 26]. These data have led to the prevailing opinion that in pulmonary sarcoidosis, DCs are immature and anergic in the lung [25]. Thus, the exaggerated immune response in pulmonary sarcoidosis is paradoxically associated with DCs displaying diminished immunoreactivity. Studies into this area have been hampered by technical difficulties in isolating functionally active DCs with a high degree of purity from the site of active disease. It therefore remained unclear whether pulmonary DCs are functionally different in sarcoidosis.

In this study, we set out to investigate whether local DCs are functionally different in patients with pulmonary sarcoidosis, employing our recently developed cell sorting strategy to isolate functionally active DCs from BAL fluid [27]. We found that at the site of disease, more and mature DCs are present and that both granuloma formation and T cell alveolitis are associated with enhanced DC activation. Furthermore, DCs from patients with sarcoidosis display intrinsic properties associated with induction of an exaggerated immune response.

2. MATERIALS AND METHODS

2.1 Patients and healthy control subjects

After informed consent, 37 patients and 33 healthy volunteers underwent fibre-optic bronchoscopy. The protocol was approved by the Medical Ethical Committee of the Erasmus MC in Rotterdam. The patient group (mean age: 40.1 y) consisted of 16 males and 21 females (8 smokers) with newly diagnosed sarcoidosis stage I (19 patients) or stage II (18 patients) disease. The diagnosis of sarcoidosis was made according to the guidelines of the ATS/ERS/WASOG statement on sarcoidosis [2]. None of the patients were on corticosteroid or immunosuppressive drugs at the time of diagnosis and sampling. Healthy controls had a normal pulmonary function test and chest X-ray and did not have a history of pulmonary disease. Healthy controls had a male/female ratio of 11/22 (3 smokers) and the mean age was 22.7 y.

2.2 Collection of BAL, peripheral blood cells and mucosal biopsies

BAL was performed with a flexible fibre-optic bronchoscope (Olympus) placed in the right middle lobe in wedge position. Four aliquots of 50 ml saline were instilled and subsequently gently aspirated. BAL fluid was collected in siliconized bottles to prevent cell adherence and kept at 4°C. BAL fluid was filtered through a 100 µm cell strainer (BD

Biosciences) and centrifuged for 7 min at 450g at 4 °C. Supernatants were aliquoted for ELISA. A portion of BAL fluid cells were counted and used directly to assess DC number and phenotype. Remaining BAL cells were frozen in 1 ml RPMI 1640 (Gibco), 10% FCS, 10% DMSO in a cryovial using a 5100 Cryo 1°C Freezing Container (Nalgene) to -80°C and stored at -150 °C. Peripheral blood mononuclear cells (PBMCs) were collected as described before and used directly to assess DC number and phenotype or frozen as described above [27]. Mucosal biopsies were frozen in Tissue Tek, O.C.T. compound, (Sakura Finetek Europe) and stored at -80°C.

2.3 Flow cytometric analysis and sorting

PBMCs, BAL cells or cultured DCs were incubated in FACS buffer (PBS supplemented with 0.25% BSA, 0.5mM EDTA and 0.05% NaN3 and stained with the following antibodies: FITC-conjugated anti-CD3 (UCHT1), anti-CD14 (61D3), anti-CD56 (MEM188), anti-CD4 PE-Cy5 (RPA-T4), anti-CD123-PE (6H6), anti-FoxP3-APC, all from eBiosciences, FITC-conjugated anti-CD16 (3G8), anti-CD19 (HIB19) and anti-CD25 (2A3), PE-conjugated or biotinylated anti-CD80 (L307.4), anti-CD86 (2331(FUN-1)) or CD45RO (UCHI-1), APC-conjugated anti-CD83 (HB15e) and anti-CD40 (5C3), anti-CD11c PE-Cy5 (B-ly6) and anti-HLA-DR PE-Cy7 (L243), all from BD Biosciences, as well as anti-CD123-APC (AC145, Miltenyi), anti-CXCR3-PE (49801)(R&D) and anti-DC-LAMP-PE (104.G4) (Immunotech). 1% heat inactivated human serum was added to prevent non-specific antibody staining. Fixable Aqua Dead Cell Stain kit for 405 nm (Invitrogen, Molecular Probes) was used as a live/dead marker. Cells were measured on a LSRII Flowcytometer (BD Biosciences). pDCs and mDCs were recognized based on forward and side scatter characteristics and expression of different markers. A FITC-labeled lineage mix, containing antibodies against CD3, CD14, CD16, CD19 and CD56 was used. mDCs were alive, low autofluorescent, lineage mix negative, HLA-DR$^+$ cells, CD11c$^+$ cell, pDCs were CD11c$^-$ but CD123$^+$. Isolation of mDCs from BAL cells was performed using a FACS ARIA (BD Biosciences with Diva software and for analysis FlowJo software (Tree Star Inc.) was used, as previously described [27].

2.4 Immunohistochemistry of airway mucosa biopsies

Immunohistochemistry was performed in a half automatic stainer (Sequenza) using primary monoclonal antibodies against human CD11c (SHCL-3; BD Biosciences) and CD86 (2331(FUN-1); BD Biosciences), as previously described [28]. Control staining was performed with an irrelevant mAb of the same subclass. All biopsy sections were stained in one session to reduce inter-staining variation and analysed in a blinded fashion by two different researchers. Sections from lung mucosal biopsies fulfilled the following criteria: intact epithelium (32 out of 48 biopsies), a subepithelial mucosa of 100 µm depth (38 out of 48 biopsies) and a good overall morphological quality.

2.5 Functional testing of DCs in vitro

Isolated BAL mDCs were mixed with allogeneic naïve $CD4^+$ T cells isolated from a healthy donor as described previously [27] in a 1:20 ratio and cultured in RPMI 1640 medium at 37°C for 5 days. At day 5, a portion of the cells was harvested for flow cytometry measurements and to the remaining cells 3H-thymidine (0.5 µCi/well, Packard) was added and after 16 hours cell proliferation was determined by scintillation counting (Top count model B9912/VI, Packard Bioscience Benelux). In order to culture mo-DCs, monocytes were isolated from PBMCs using CD14 beads (Miltenyi), according to the manufacturer's instructions. Monocytes were then resuspended at 1 x 10^6 cells/ml in RPMI 1640 culture medium, supplemented with 10% FCS, 50 µg/ml gentamycin (Gibco), 1000U/ml GM-CSF (Immuno Tools) and 200U/ml IL-4 (R&D) and cultured at 37 °C for 7 days [29]. Mo-DCs were co-cultured with allogeneic naïve $CD4^+$ T cells for 5 days. T cell labeling with carboxyfluorescein succinimidyl ester (CFSE)-was performed using standard procedures.

Cytokine measurements in supernatants of DC-T cell co-cultures was performed using the human in a *25-plex* Luminex assay cytokine and chemokine kit (*Invitrogen*, Carlsbad, CA) and run on a Luminex 100 System (Luminex Corporation, Austin, TX), according to the manufacturer's protocol.

2.6 Statistical analysis

Results were compared using a two-tailed Mann Whitney U test. Differences were considered significant at $P \leq 0.05$.

3. RESULTS

3.1 BAL characteristics

In this study we investigated BAL, peripheral blood and mucosal biopsies from newly diagnosed sarcoidosis patients and healthy controls (see: Mat & Methods for patient information). The sarcoidosis patients displayed increased BAL cellularity (median: 327x10^3 total cells/ml), compared to healthy controls (median: 118x10^3 total cells/ml, $p = 0.06$), in agreement with previous reports [30-31]. This increased cellularity in BAL was paralleled by increased proportions of lymphocytes and increased CD4/CD8 ratios (median: 4.9; range 1.2-22). BAL lymphocytes in sarcoidosis are reported to be highly positive for the Th1-specific chemokine receptor CXCR3 [32]. Accordingly, the closely related CXCR3 ligands CXCL9, also known as monokine induced by IFNγ- (MIG) and CXCL10, also known as IFNγ-induced protein of 10 kDa (IP-10), are elevated in BAL fluid [33]. When we measured the levels of these chemokines in BAL fluid in the same patients tested for mDC function, we found that they were elevated: for CXCL9 the median was 533 pg/ml (range 65-1397) in patients and 43 pg/ml (range 21-59) p=0.0004) in healthy

controls and for CXCL10 these values were 360 pg/ml (range 34-957) in patients and 0 pg/ml (0-96; p<0.003) in controls.

3.2 Increased mDCs in BAL and blood in sarcoidosis

To investigate DCs in pulmonary sarcoidosis, we first analyzed the numbers of DC subsets in BAL and peripheral blood from patients (mDC (n= 16), pDC (n=8)) and healthy controls (n= 6). The proportions of HLA-DR$^+$CD11c$^+$ mDCs in BAL did not significantly differ between sarcoidosis patients and healthy controls (median: 0.26%, range: 0.07-1.4 and median: 0.17%, range: 0.03-0.43, respectively; Figure 1A), corroborating earlier studies [24]. However, the absolute numbers of mDCs were significantly elevated in patients (median 608 cells/ml, range: 217-3,965), when compared with healthy controls (median 123 cells/ml, range: 101-1,941, p= 0.05).

In the BAL from sarcoidosis patients the populations of interleukin-3 receptor (IL-3R)/CD123-expressing HLA-DR$^+$CD11c$^-$ pDCs were increased, both in proportions (median 0.06%, range 0.02-0.36, compared with healthy controls: median 0.03%, range 0.01-0.08, p= 0.03) and in absolute numbers (median: 201 cells/ml, range: 23-1740, compared with healthy controls: median 17 cells/ml, range 11-133, p = 0.02; Figure 1A).

In a concurrently drawn blood sample, we observed an increase in mDCs in sarcoidosis patients, both in proportions (median: 0.24%, range: 0.03-1.71, compared with healthy controls 0.11%, range 0.08-0.13, p = 0.01) and in numbers (median: 1,441/ml, range:

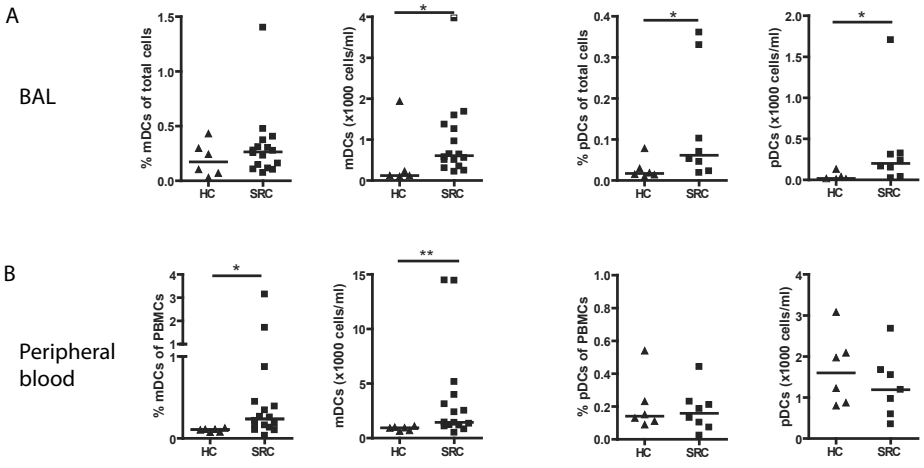

Figure 1. Increased mDCs in BAL and blood in sarcoidosis.
Proportions and numbers of mDCs and pDCs in BAL and peripheralblood from sarcoidosis patients and healthy controls. Using 9-color flowcytometry, the proportion of mDCs (low autofluorescent, lineage mix negative, HLA-DR$^+$, CD11c$^+$ cells) and pDCs (low autofluorescent, lineage mix negative, HLA-DR$^+$, CD11c$^-$, CD123$^+$ cells) was determined of total BAL cells (A) and PBMCs from peripheral blood (B). The number of mDCs and pDCs was calculated as a proportion of total counted BAL cells and PBMCs. Each symbol represents an individual patient or control; lines indicate medians. * p<0.05; ** p<0.01.

511-14,432, compared with healthy controls: median 933, range 638-1,102, $p<0.01$; Figure 1B). In contrast, no differences were observed in pDC numbers or proportions between patients and controls (Figure 1B). Peripheral blood from sarcoidosis patients and healthy controls also did not differ in the total numbers of PBMCs per ml (data not shown). Overall, we did not observe an association between numbers of mDCs or pDCs in BAL or blood and disease stage, as classified by Scadding [34] (data not shown).

In summary, in recently diagnosed sarcoidosis patients we found an increase in mDCs and pDCs in the BAL and mDCs in peripheral blood, irrespective of disease stage.

3.3 BAL mDCs manifest a more mature phenotype in sarcoidosis

Next, we set out to investigate whether the increase in DCs in BAL of sarcoidosis patients was associated with enhanced maturation. After antigen encounter, DCs upregulate various cell surface maturation markers, including CD80 (B7-1) and CD86 (B7-2), which work in tandem to prime T cells, CD40, which act as a regulator of DC cytokine production, and CD83, the function of which in T cell activation is less clear. As shown in Figure 2A, we observed increased expression of CD86, CD80 and CD40 on BAL mDCs in sarcoidosis patients (n=16), compared with healthy controls (n=6). Differences were significant for CD86 and CD40 ($p<0.05$; Figure 2B). Expression of CD83 on BAL mDCs from sarcoidosis patients was low (Figure 2A, 2B). Although the numbers of pDCs in BAL were very low, we were able to analyze typically 2-5x10^3 pDCs for expression of CD40, CD80, CD83 and CD86. We found a similar trend of increased expression of CD86 and reduced CD83, but differences did not reach significance (Figure 2B).

Finally, we noticed that expression of CD208/DC-LAMP, which is induced in later stages of DC maturation [35], was significantly increased on BAL mDCs but not pDCs of sarcoidosis patients, when compared with healthy controls ($p=0.05$; Figure 2C). In peripheral blood the expression of the CD80, CD86, CD40, CD83 and CD208 maturation markers on mDCs or pDCs was low and did not differ between sarcoidosis patients and healthy control subjects (data not shown). Taken together, these findings show that the increased presence of DCs in BAL and blood in sarcoidosis patients is accompanied by enhanced DC maturation in the BAL, but not in blood. We therefore conclude that pulmonary sarcoidosis is associated with an increase in mature DCs at the site of active disease.

3.4 BAL mDCs from sarcoidosis patients are immunocompetent

Next, we evaluated whether enhanced mDC maturation in the BAL from sarcoidosis patients was associated with either enhanced or diminished T cell activation, the latter of which has been suggested for blood-derived DCs in sarcoidosis [25]. We applied our previously developed flow cytometric strategy for the isolation of mDCs from BAL with high purity [27] to sort mDCs from BAL of 11 sarcoidosis patients and 6 healthy controls.

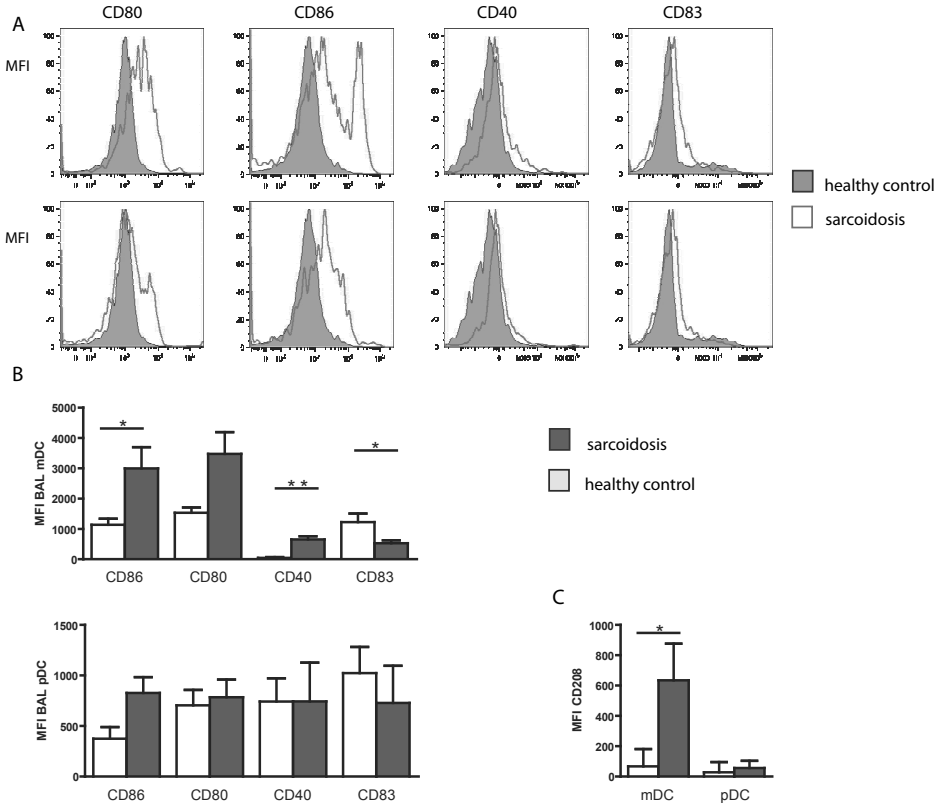

Figure 2. BAL mDCs manifest a more mature phenotype in sarcoidosis.
Expression of maturation markers on mDCs and pDCs in BAL from sarcoidosis patients and healthy controls. Using 9-color flowcytometry, mDCs (low autofluorescent, lineage mix negative, HLA-DR+, CD11c+ cells) and pDCs (low autofluorescent, lineage mix negative, HLA-DR+, CD11c-, CD123+ cells) in BAL from sarcoidosis patients and healthy controls were analyzed for expression of CD40, CD80, CD83, CD86 and CD208 (DC-LAMP). (A) Representative examples of the expression of the indicated markers is shown from two sarcoidosis patients and a healthy control. (B) Quantification of the expression levels (MFI, median fluorescence intensity) of the indicated activation markers on BAL mDCs (*upper panel*) of sarcoidosis patients (n=16) compared with healthy controls (n=6) and on BAL pDCs (*lower panel*) of sarcoidosis patients (n=8) compared with healthy controls (n=6). (C) Expression of DC-Lamp (CD208) on BAL mDCs (16 patients, 6 controls) and pDCs (8 patients, 6 controls). Bars represent mean ± SEM. * $p \leq 0.05$.

To test the capacity of these sorted mDCs to induce T cell proliferation and differentiation, we performed co-culture experiments with allogenic naïve $CD4^+$ T cells. Upon 5 days of co-culture, naïve $CD4^+$ T cells displayed clear proliferation, when compared with unstimulated $CD4^+$ T cells. No differences were found between co-cultures with BAL mDCs sorted from healthy controls or from sarcoidosis patients (as measured by 3H-thymidine incorporation; Figure 3A). In these co-culture experiments naïve $CD4^+$ T cells differentiated into $CD25^+CD127^{low}Foxp3^+$ regulatory T cells, $CD45RO^+$ memory T cells, $CXCR3^+$ Th1 or $CCR4^+$ Th2 cells, whereby we did not observe significant differences

between co-cultures with mDCs from sarcoidosis patients and with mDCs from healthy controls (Figure 3B). Likewise, we found similar levels of cytokines, including TNFα, IFNγ, IL-4, IL-5, IL-13 and IL-17 (Figure 3C).

Taken together, these findings indicate that mDCs from the BAL from sarcoidosis patients were functional and did not display diminished immunoreactivity, when compared with mDCs from healthy controls.

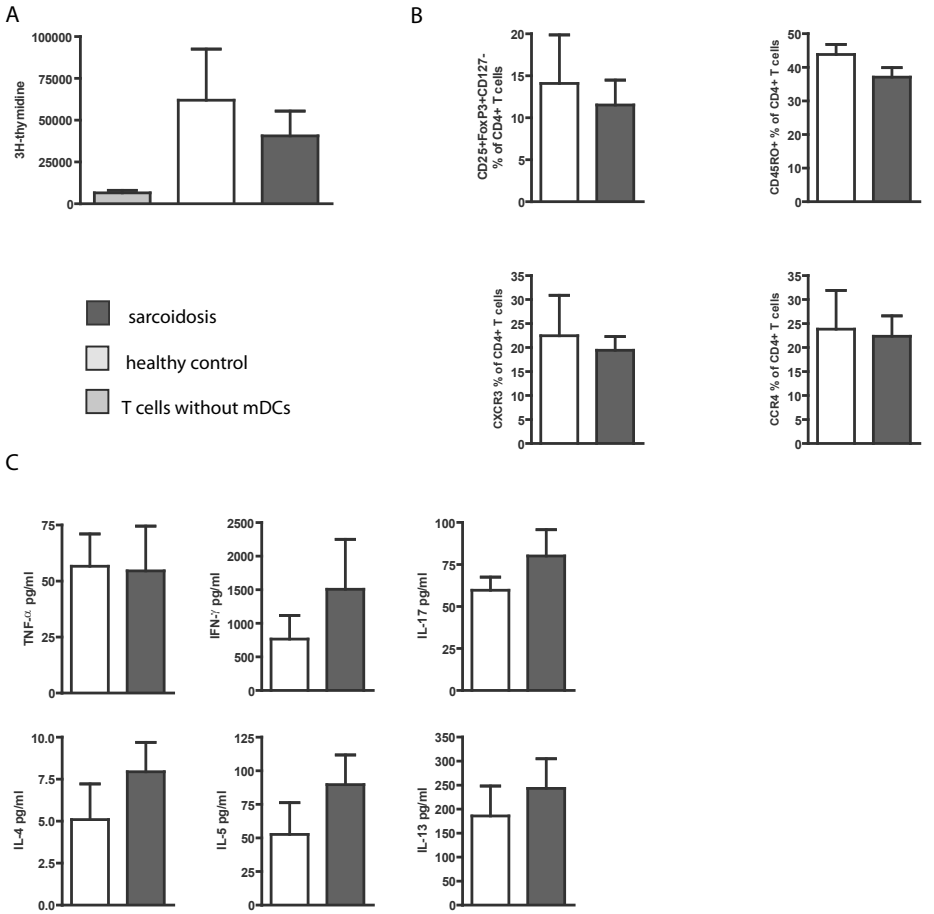

Figure 3. BAL mDCs from sarcoidosis patients are immunocompetent in co-cultures with T cells. Induction of T cell proliferation and differentiation by mDCs isolated from BAL from sarcoidosis patients. mDCs (defined as low autofluorescent, lineage mix negative, HLA-DR$^+$, CD11c$^+$ cells), isolated from BAL from sarcoidosis patients and healthy controls, were tested in a MLR with naïve allogeneic CD4$^+$ T cells. (A) After 5 days of co-culture, T cell proliferation was measured using 3H-thymidine incorporation. (B) T cell differentiation was assessed using markers for Tregs (CD25+ Foxp3$^+$ CD127 ^{low}, memory T cells (CD45RO$^+$), Th1 (CXCR3$^+$) and Th2 (CCR4$^+$) cells. (C) Cytokines were measured in the supernatant of the co-culture using a bead-based cytokine kit. Bars represent mean ± SEM.

3.5 Monocyte-derived DCs from sarcoidosis patients induce increased TNFα expression

To investigate whether intrinsic DC defects in sarcoidosis might lead to aberrant T cell responses independent of the lung microenvironment, we cultured monocyte-derived DCs (mo-DCs) from 10 sarcoidosis patients and 10 healthy controls. Expression of CD80, CD86, CD40 and CD83 was comparable in the two groups (Figure 4A). When mo-DC function was tested in co-cultures with allogeneic naïve $CD4^+$ T cells, mo-DCs from sarcoidosis patients and healthy controls did not manifest differences in their capacity to induce T cell proliferation (Figure 4B), T cell activation as assessed by membrane expression of the IL-2R/CD25 (Figure 4C), or regulatory T cell differentiation (Figure 4D). When cytokine production was measured by Luminex bead assays, we observed increased

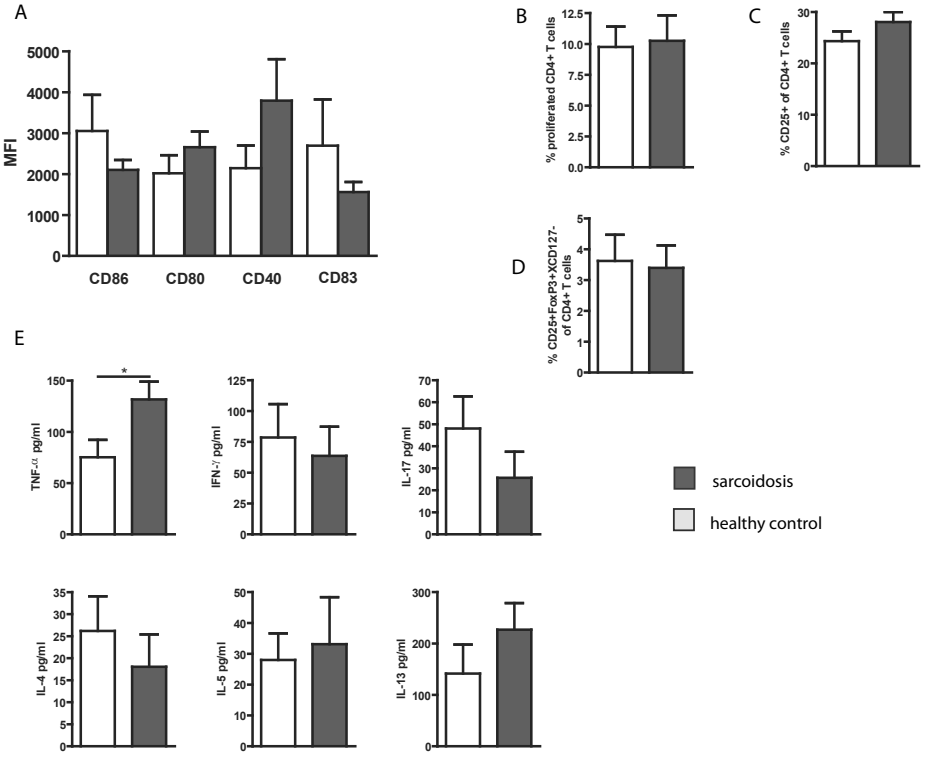

Figure 4. Monocyte-derived DCs from sarcoidosis patients induce increased TNFα expression
Activation and function of mo-DCs from sarcoidosis patients and healthy controls. (A) After culturing mo-DCs from monocytes, mean expression of DC maturation markers CD40, CD80, CD83 and CD86 was assessed by flowcytometry. (B) Mo-DC induced T cell proliferation was tested in a MLR after 5 days of co-culture with CFSE-labeled naïve $CD4^+$ T cells, using flowcytometry. (C,D) Mo-DC induced T-cell differentiation was measured by expression of CD25 (C) and Treg markers ($CD25^+Foxp3^+CD127^{low}$) (D). (E) Cytokine production in DC-T cell co-cultures. Indicated cytokines were measured in the culture supernatants by Luminex. Bars represent mean ± SEM; * $p \leq 0.05$.

TNFα expression in co-cultures of mo-DCs from sarcoidosis patients, when compared with co-cultures of mo-DC from healthy controls (Figure 4E). Other cytokines tested, including IFNγ, IL-17, IL-4, IL-5 and IL-13 were not different between the two groups of cultures.

Taken together, these data show that DCs from sarcoidosis patients examined outside of the disease microenvironment were not intrinsically more mature, but do show an increased propensity to induce the production of TNFα, a cytokine pivotal in sarcoidosis pathogenesis.

3.6 Increased numbers of mature CD86$^+$ DCs surrounding granulomas

Finally, we determined the numbers, location and activation status of DCs in the airways in mucosal airway biopsies from 26 sarcoidosis patients and 20 healthy controls. Cells positive for CD11c showed a characteristic DC morphology and were found in the subepithelial layer of the airways in healthy subjects, as reported previously [36] (Figure 5A). Ten out of 26 mucosal biopsies from sarcoidosis patients contained clear granulo-

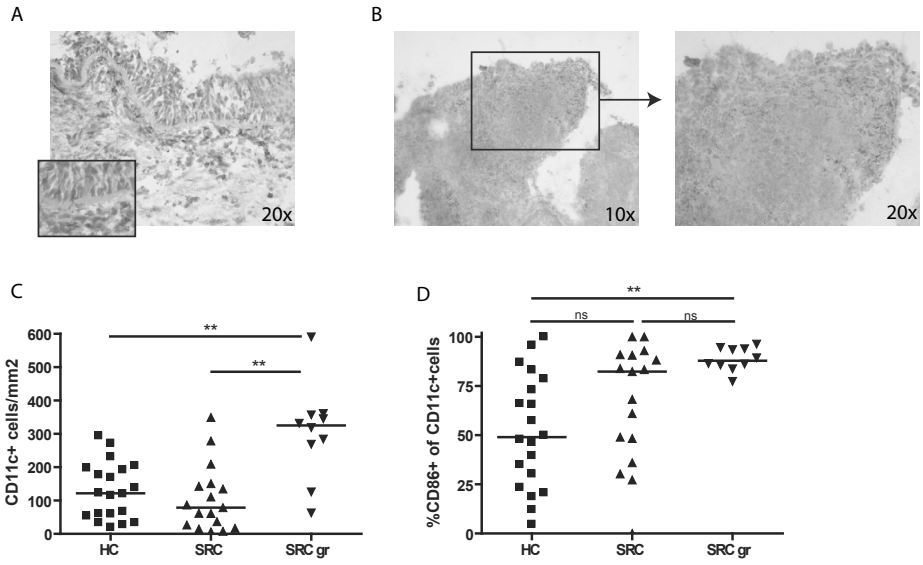

Figure 5. Increased numbers of mature CD86$^+$ DCs surrounding granulomas in sarcoidosis
Location, number and activation of DCs in mucosal airway biopsies. (A) CD11c staining on a frozen section from a mucosal airway biopsy from a healthy control, showing DCs in the subepithelial layer. Characteristic dendritic cell morphology was observed with dendrites crossing the basement membrane (*insert*). (B) In mucosal biopsies from sarcoidosis patients DCs were observed surrounding the granuloma. (C) Quantification of the numbers of CD11c$^+$ cells in lamina propria in biopsies from healthy controls (HC), sarcoidosis patients without (SRC) and with granuloma (SRC gr), bars indicate median values, ** $p<0.0015$. (D) Proportions of CD11c$^+$ cells co-expressing CD86 in biopsies from healthy controls (HC), sarcoidosis patients without (SRC) and with granuloma (SRC gr). Each symbol represents an individual patient or control. Lines indicate median value, ** $p<0.0015$ (D).

mas, and in those CD11c$^+$ DCs were seen predominantly surrounding the granulomas, confirming earlier studies [21] (Figure 5B). We also observed weak CD11c expression in the center of granulomas, perhaps reflecting CD11c expression on myeloid derived epithelioid histiocytes or interdigitating dendrites. Granuloma-containing biopsies displayed significantly increased CD11c$^+$ cell numbers, when compared with either healthy controls (p=0.0011) or non-granulomatous biopsies (p=0.0014) (Figure 5C).

To assess the maturation state of the mucosal DCs, double stainings were performed for CD11c and CD86. In granuloma-containing biopsies, the proportions of CD11c$^+$ cells co-expressing CD86 was significantly increased (median 88%, range 77-96%), when compared with healthy controls (median 50%, range 4-95) (p=0.0012), but not significantly different from values in non-granulomatous biopsies from patients (median 82 %, range 0-100%). The proportion of CD86$^+$ DCs tended to be higher in non-granulomatous biopsies, compared with healthy controls, but differences did not reach significance, probably related to the variation of CD86$^+$ expression in healthy individuals.

Taken together, these results show that granuloma formation was associated with an increase in number and maturation status of DCs, providing evidence for the involvement of DCs in granuloma formation or maintenance in sarcoidosis.

4. DISCUSSION

DCs are pivotal antigen presenting cells and the prime suspects for initiating granuloma formation and T cell alveolitis characteristic for pulmonary sarcoidosis. Paradoxically, local DCs have been suggested to be phenotypically and functionally immature [25]. In contrast, we provide in this report several lines of evidence for the involvement of mature, functional DCs in pulmonary sarcoidosis. First, pulmonary sarcoidosis patients have increased numbers of mDCs in BAL, granuloma containing mucosal biopsies and blood. Second, mDCs in BAL and granuloma-containing biopsies, but not in blood, show increased expression of maturation markers. Third, mDCs from BAL are very well capable of inducing T cell proliferation and differentiation and show no signs of anergy. Finally, mo-DCs from sarcoidosis patients induce more TNFα in co-cultures with allogeneic CD4$^+$ T cells, compared to mo-DCs from healthy controls. Taken together these results indicate that pulmonary sarcoidosis is associated with increased numbers of mature, functionally competent DCs that intrinsically induce increased levels of a central mediator in sarcoidosis, TNFα.

Previous studies and ours report different results in maturation status and numbers of mDCs in blood or BAL from sarcoidosis patients [21, 23-24]. These differences may reflect variations in obtaining (e.g. volume of total BAL fluid), isolating (enriched cell populations vs. flow cytometric sorting), calculating (number per ml vs. proportions) and mark-

ers used for defining DC subsets. To the best of our knowledge we report for the first time functional tests on mDCs from the site of disease in pulmonary sarcoidosis. Despite enhanced expression of maturation markers, mDCs isolated from BAL from sarcoidosis patients did not induce enhanced T cell proliferation or a skewed T cell differentiation in a MLR with allogeneic naïve CD4+ T cells, compared to controls. Several explanations are possible: First, mature DCs from BAL from sarcoidosis patients may have already interacted with T cells, and are beyond their functional maximum upon isolation from BAL. This is supported by studies showing that after initial exposure to a stimulus, DCs produce IL-12, important for inducing T cell proliferation, for a limited period of 10-18 hours [37]. Indeed, we found low IL-12 levels at day 5 in the MLR supernatant. Second, it is perhaps the sheer number of DCs and not the maturation status that determines the *in vivo* outcome of T cell proliferation in sarcoidosis. Although the number of DCs in BAL is in general very low, DCs in the BAL are thought to reflect only a small percentage of pulmonary DCs [38]. We found increased numbers of mDCs per ml of BAL in sarcoidosis patients, perhaps indicating increased numbers of interstitial DCs that may travel to the draining lymph node for antigen presentation. Future investigations in the lymph node compartment may shed light on this issue. Third, perhaps an intrinsic T cell factor is (additionally) required to induce the exaggerated T cell response observed in sarcoidosis. Interestingly, a single nucleotide polymorphism (SNP) in the IL-23 receptor was recently associated with sarcoidosis [39]. IL-23 is a cytokine that is essential for the induction of IL-17 producing $CD4^+$ T cells (Th17 cells) that were recently associated with granuloma formation in sarcoidosis [39]. Finally, influx of $CD4^+$ T cells into the bronchoalveolar space may primarily be determined by chemotactic factors. Our data on enhanced levels of MIG and IP10 in BAL, well-known chemotactic factors for Th1 cells, support this notion and confirm earlier reports [33, 40].

TNFα is a pivotal mediator of granuloma formation and maintenance, and is thought to play an important role in sarcoidosis pathogenesis [41]. Indeed, enhanced TNFα secretion by BAL macro-phages is observed in sarcoidosis [3]. In addition, TNFα is also expressed by Th1 and Th17 cells and both T helper cell subsets are likely involved in sarcoidosis pathogenesis [39]. Polymorphisms in the *TNFα* locus were associated with sarcoidosis phenotype and prognosis and have been linked to altered TNFα expression [42-43]. Importantly, TNFα is an essential target for treatment [44] and we found that mo-DCs from patients with sarcoidosis, not influenced by the micro-environment of the lung, induced increased TNFα release upon interaction with naïve $CD4^+$ T cells when compared to controls. Our results indicate that DCs are intrinsically different in sarcoidosis patients. Nevertheless, we found that mo-DCs from sarcoidosis patients were equally capable of inducing proliferation and differentiation in allogeneic naïve T cells, compared to healthy controls. In co-cultures with PBMCs, mo-DCs were previously reported to demonstrate a reduced capacity to induce T cell proliferation (26). However,

it is conceivable that other cells in the PBMC fraction, e.g. regulatory T cells (Tregs), influenced T cell proliferation. Indeed, we found increase numbers of Tregs in the PBMC fraction from sarcoidosis patients' blood (unpublished observations).

Granulomas are thought to arise upon failure of the immune system to clear invading pathogens. Earlier reports on diminished DC maturation and immune reactivity led to the notion that DC anergy may contribute to granuloma formation in sarcoidosis, e.g. due to diminished antigen presenting capabilities or ineffective induction of a T helper cell response [25]. However, we found that both mDCs in BAL and in granuloma-containing airway biopsies were increased in number and had enhanced expression of maturation markers. Furthermore, mDCs isolated from BAL displayed normal immune reactivity compared to healthy controls. Intriguingly, also in non-granulomatous mucosal airway biopsies from sarcoidosis patients there was a tendency towards increased numbers and maturation of mDCs, compared to healthy controls, although this was not statistically significant. It is tempting to speculate that these mucosal DCs are activated upon acquiring antigen from the airway lumen and subsequently present the antigen in a draining lymph node or are involved in mucosal granuloma formation. Taken together our results strongly support the notion that mDCs are involved in granuloma formation and maintenance in sarcoidosis, rather than the alternative that DCs are defective in supporting the adaptive immune system in antigen clearance.

In conclusion, we provide evidence for the involvement of DCs in antigen presentation and granuloma formation at the site of disease in pulmonary sarcoidosis patients. Intrinsic genetic alterations in key APCs may underlie the exaggerated immune response to a hard to discern antigen that is characteristic of sarcoidosis. Immunological measurements and functional examination of DC and T cell subsets from large groups of carefully genotyped patients should prove very interesting.

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Increased IL-17A expression in granulomas and in circulating memory T cells in sarcoidosis

Bregje ten Berge[1#], Marthe S. Paats[1#], Ingrid M. Bergen[1], Bernt van den Blink[1], Henk C. Hoogsteden[1], Bart N. Lambrecht[1,2], Rudi W. Hendriks[1*] and Alex KleinJan[1*]

[1] Department of Pulmonary Medicine, Erasmus MC, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

[2] Department of Respiratory Diseases, Laboratory of Mucosal Immunology, MRBI, University Hospital Gent, De Pintelaan 185, B9000 Ghent, Belgium

[#] These authors contributed equally to this work;

[*] These authors shared the supervision of this study.

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ABSTRACT

Objective: Sarcoidosis is a systemic inflammatory disorder characterized by granulomas. Although the etiology is unknown, sarcoidosis is thought to be mediated by T-helper (Th)1 lymphocytes. Recently, IL-17A has been implicated in granuloma formation in various diseases, including tuberculosis. Therefore, we hypothesized that Th17 cells play role in sarcoidosis, paralleling recent findings in autoimmune diseases such as rheumatoid arthritis.

Methods: T cells were investigated by intracellular flow cytometry and immunohistochemistry, in blood, bronchoalveolar lavages (BAL) and bronchial mucosal biopsies from a cohort of newly diagnosed sarcoidosis patients and healthy controls.

Results: Circulating memory $CD4^+$ T cell populations of sarcoidosis patients contained significantly increased proportions of IL-$17A^+$ cells, when compared with healthy controls. Interestingly, proportions of IL-17A/IFNγ and IL-17A/IL-4 double-producing cells were significantly increased in blood of sarcoidosis patients and were present in substantial numbers in BAL. In granuloma-containing, but not in non-granulomatous sarcoidosis biopsies, we found significantly increased numbers of IL-$17A^+$ T cells, located in and around granulomas throughout the lamina propria. IL-22^+ T cells were increased in the subepithelial layer.

Conclusions: Enhanced IL-17A expression in granulomas and the presence of IL-$17A^+$, IL-$17A^+$IFNγ^+ and IL-$17A^+$IL-4^+memory T helper cells in the circulation and BAL indicate Th17 cell involvement in granuloma induction or maintenance in sarcoidosis. Therefore, neutralization of IL-17A activity may be a novel strategy to treat sarcoidosis.

1. INTRODUCTION

Sarcoidosis is a systemic inflammatory disease characterized by the presence of non-caseating granulomas in various organs with pulmonary involvement in over 90% of patients [1]. These granulomas are compact, organized collections of macrophages and epithelioid cells, surrounded by and infiltrated with $CD45RO^+$ memory T lymphocytes. Besides granulomas, pulmonary alveolitis and peripheral blood lymphopenia are typically present in sarcoidosis [1]. The pathological processes that result in granulomatous inflammation are largely unknown. Nevertheless, the findings of $CD4^+$ T cell accumulation, oligoclonal $TCR\alpha\beta^+$ expansions and production of IFNγ and T helper (Th)1-promoting cytokines, including interleukin (IL)-12, chemokines and chemokine receptors at sites of inflammation provide evidence for a pathological antigen-driven Th1 response [2-3].

Recently, the proinflammatory cytokine IL-17A has been implicated in the pathogenesis of various granulomatous diseases, in particular in the formation of a mycobacterial infection-induced granuloma in the lung [4] Although IL-17A can be produced by various cell types, it is the main cytokine produced by the novel subset of Th17 cells, which is distinct from the Th1 and Th2 subset. Th17 cells were shown to be crucially involved in many autoimmune diseases, including rheumatoid arthritis (RA), inflammatory bowel disease, multiple sclerosis, autoimmune uveitis and in allergic lung disease [5-8]. Th17 cells have the capacity to confer protection against extracellular bacterial and fungal pathogens such as Klebsiella pneumonia, Citrobacter rodentium and Candida albicans, although accumulating evidence demonstrates that Th17 cells also provide protective effects during infection with more traditional intracellular pathogens [4, 9]. Interestingly, there are many reports of sarcoidosis coexisting with or mimicking rheumatic diseases such as systemic lupus erythematosus, rheumatoid arthritis, and ankylosing spondylitis [10]. In this context, the observation that IL-17A is highly expressed in synovium of RA patients and that the cellular source is mainly $CD4^+$ cells is particularly important [11-14]. Differentiation and maintenance of Th17 cells in human is critically dependent on IL-1β, IL-6, TGF-β and IL-23 [15]. The finding in sarcoidosis of increased IL-12p40 (which is not only part of the Th1-inducing cytokine IL-12 but also of the Th17-inducing cytokine IL-23), together with increased IL-1β mRNA expression in lymph nodes [16-18], may therefore also point to a role of Th17 cells.

Human Th17 cells comprise heterogeneous subsets. Next to IL-17A, these cells produce various other proinflammatory cytokines, including IL-17F, IL-22 and in some conditions IFNγ. IL-22 is a cytokine involved in mucosal immunity against extracellular pathogens and can also be produced independently of IL-17A, as was recently found in the context of psoriasis [19-21]. Also IL-17A/IFNγ double producing cells have been described [22-

24], which could possibly be more pathogenic, since e.g. these cells preferentially cross the blood-brain barrier in patients with multiple sclerosis.

Recently, sarcoidosis was suggested as a Th1/Th17 multisystem disorder [25], based on the presence of IL-17 positive CD4 T-cells in sarcoid lung tissue and their ability to respond to the chemotactic stimulus CCL20. Moreover IL-17A was expressed by macrophages infiltrating sarcoid tissue. However, the involvement of IL-22 or the recently identified pathogenic populations of IL-17A/IFNγ and IL-17A/IL-4 double-producing Th cells [26-27] in sarcoidosis pathogenesis remains unknown.

Therefore, in this report we studied the presence of double-producing Th cells. Moreover we investigated whether there would be granuloma-dependent differences in the presence of IL-22 and IL-17A positive cells in granuloma-containing compared with non-granulomatous lung mucosa biopsies obtained from sarcoidosis patients. Because of the heterogeneous cytokine profile of the Th17 subset, we analyzed peripheral blood, BAL cells and lung mucosal biopsies of newly diagnosed immunosuppressive drugs free stage 1 or stage 2 sarcoidosis patients. Flow cytometric (FACS) analyses were performed for the expression of the main Th17 cytokines IL-17A, IL-17F and IL-22, along with IFNγ, TNFα and IL-4 and immunohistochemistry for IL-17A and IL-22.

2. MATERIALS AND METHODS

See the online supplement for more details regarding bronchoscopy procedure, peripheral blood mononuclear cell (PBMC) processing, flow cytometric analysis and immunohistochemical staining of lung mucosa biopsies.

2.1 Patients and healthy control subjects

After informed consent (according to the Declaration of Helsinki) 33 patients and 33 healthy volunteers underwent fibre-optic bronchoscopy. The protocol was approved by the Medical Ethical Committee of the Erasmus MC Rotterdam. The patient group consisted of 15 males and 18 females with newly diagnosed sarcoidosis (mean age: 37.3 y). The diagnosis of sarcoidosis was made conform the guidelines of the ATS/ERS/WASOG statement on sarcoidosis [1]. All patients were newly diagnosed with stage 1 (19 patients) or stage 2 (14 patients) sarcoidosis. None of the patients were on corticosteroid or immunosuppressive drugs at the time of diagnosis and sampling. Healthy volunteers had a male/female ratio of 13/20 and the mean age was 24.2 y. Bronchoalveolar lavage (BAL) was performed with a flexible fibre-optic bronchoscope (Olympus) according to standard procedures (see Online Supplement).

2.2 Immunohistochemical analysis of lung mucosa biopsies and cytospins

Immunohistochemical stainings are detailed in the Online Supplement. All biopsy sections were stained in one session to reduce inter-staining-variation and analysed in a blinded fashion by two different researchers. Sections from lung mucosal biopsies fulfilled the following criteria: intact epithelium (32 out of 48 biopsies), a subepithelial mucosa of 100µm depth (38 out of 48 biopsies) and a good overall morphological quality. Cell numbers were expressed as cells per mm length basal membrane. The entire lamina propria region was analysed in all 48 biopsies and cell numbers were expressed as cells per square mm.

2.3 Statistical analysis

For statistical evaluations the Kruskal-Wallis 1-way ANOVA and the Mann-Whitney U-test were performed. A p-value <0.05 indicated significant differences. Associations between cells were assessed with Pearson rank correlations.

3. RESULTS

3.1 Increased Th17 profile in circulating memory $CD4^+$ T cells from sarcoidosis patients

To investigate the involvement of Th17 cells in the pathogenesis of sarcoidosis, we analyzed peripheral blood samples of nine recently diagnosed sarcoidosis patients and ten healthy controls by flow cytometry. Surface stainings for CD3, CD4, CD8 and CD45RO revealed a specific $CD4^+$ T cell lymphopenia in the sarcoidosis patients (Supplementary Figure S1A). The proportions of $CD3^+$ T cells in the mononuclear cell fractions were significantly reduced ($p=0.02$) in sarcoidosis patients (median: 45%; range: 8-56%), when compared with healthy controls (median: 58%; range: 35-74%; Supplementary Figure S1A). Likewise, the proportions of $CD4^+$ T cells of the total $CD3^+$ populations were significantly reduced ($p=0.001$) in sarcoidosis patients (median: 33%; range 11-61%; and healthy controls: median: 63%; range 51-90%) (Supplementary Figure S1B). The proportions of $CD8^+$ T cells in the mononuclear cell fractions were not different between sarcoidosis patients and healthy controls (Supplementary Figure S1A).

We did not detect significant differences in the fractions of antigen-experienced memory $CD45RO^+$ $CD4^+$ T cells between sarcoidosis patients (median: 51%; range: 31-92%) and healthy controls (median: 41%; range: 14-64%) (Figure 1A). We used intracellular flow cytometry to determine the expression profiles for IL-17A, IL-17F, IL-22, IFNγ, IL-4 and TNFα in total mononuclear cell fractions upon 4 hours of stimulation with PMA and ionomycin (See Supplementary Figure S1C for gating strategy). Intracellular expression of these cytokines was almost completely confined to the $CD45RO^+$ memory

T cell fractions (data not shown). Importantly, the proportions of IL-17A$^+$ cells within the CD45RO$^+$CD4$^+$ memory T cell population were significantly higher (p=0.009) in sarcoidosis patients (median: 3.7%; range: 3.0 - 6.6%) than in healthy controls (median: 2.4%; range: 1.4-5.8%; Figure 1B). We did not detect differences between patients and healthy controls for the other two Th17 cytokines tested, IL-17F and IL-22 (Figure 1B). The frequencies of IFNγ-expressing cells tended to be higher in sarcoidosis patients (median: 30% of CD45RO$^+$CD4$^+$T cells, range: 10-38%), when compared with healthy controls (median: 19%; range: 16-34%), but this difference did not reach significance (Figure 1B). Also, the proportions of IL-4$^+$ cells were significantly higher (p=0.05) in sarcoidosis patients (median: 1.8% of CD45RO$^+$CD4$^+$T cells, range: 0.7-3.8%; healthy controls: median: 1.2%, range: 0.8-2.3%; Figure 1B). No differences were observed for TNFα between sarcoidosis patients and healthy controls (Figure 1B). Because IL-17A/IL22 and IL-17A/IFNγ double-producing cells have been described, whereby particularly IL-17A$^+$IFNγ$^+$ cells might be

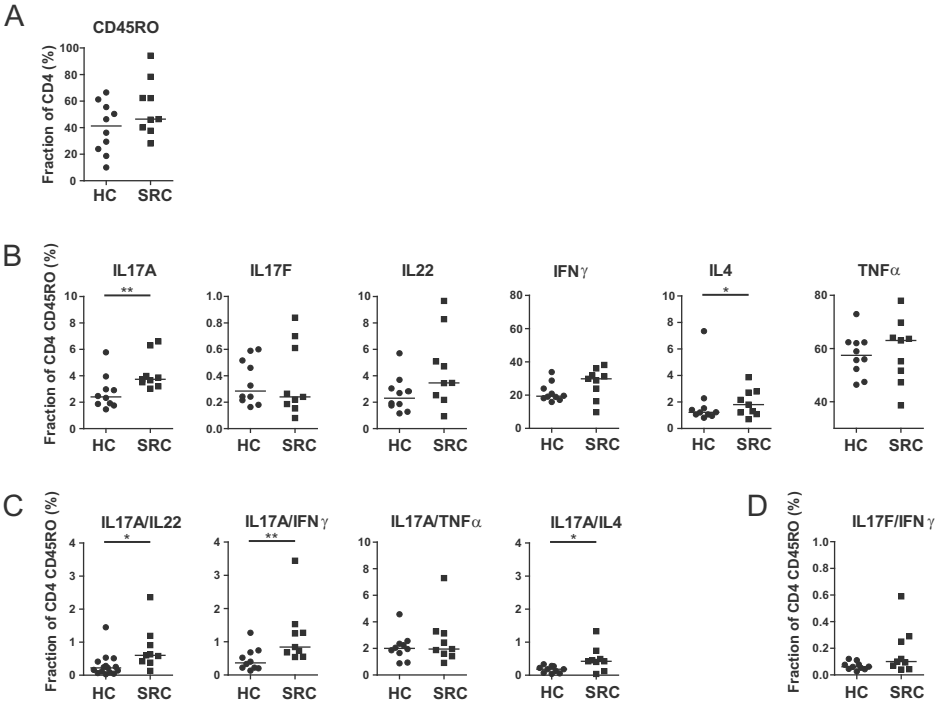

Figure 1. Cytokine profiles of circulating memory T helper cells in sarcoidosis patients and healthy controls.

(A) Proportions of antigen-experienced memory CD45RO$^+$ cells within the populations of CD4$^+$ T cells in PBMC fractions. (B) Proportions of CD45RO$^+$CD4$^+$CD3$^+$ cells expressing the indicated cytokines, as determined by intracellular flow cytometry. (C, D) Proportions of CD45RO$^+$CD4$^+$CD3$^+$ cells that were double positive for the indicated cytokines, as determined by intracellular flow cytometry. Symbols represent individual healthy controls (HC, l) and sarcoidosis patients (SRC, n) and lines indicate median values. * p<0.05; ** p < 0.01.

more pathogenic (21-22), we next analyzed coexpression of Th17 cytokines. We observed significantly higher proportions of $CD45RO^+CD4^+$ T cells expressing IL-17A, together with IL-22, IFNγ or IL-4 in sarcoidosis patients than in healthy controls (Figure 1C). No differences were observed for the frequencies of IL17A/TNFα or IL-17A/IL-17F (Figure 1C and data not shown) or IL17F/IFNγ double producers (Figure 1D).

Taken together, these findings show that in recently diagnosed sarcoidosis patients the peripheral blood memory T helper cell compartment contained increased proportions of $IL\text{-}17^+$ T cells, indicating enhanced Th17 differentiation. Moreover, the increased frequencies of IL-17A/ IL-22 and particularly IL-17A/IFNγ double producing T cells would indicate an active state of the disease. The increased frequency of $IL\text{-}4^+$ single and $IL\text{-}17A^+IL\text{-}4^+$ double positive memory T helper cells point to a possible involvement of IL-4 in sarcoidosis, as previously suggested by Hauber et al. [28].

3.2 Increased IL-17A, but not IL-22, in BAL memory CD4+ T cells from sarcoidosis patients

The majority of $CD4^+$ T cells in the alveolar space of sarcoidosis patients were $CD45RO^+$ memory T cells, as determined by analysis of BAL cells (median: 94%; range 91%-96%; Figure 2A). Stimulation of BAL cells by PMA/ionomycin, similar to PBMC, indicated the abundant presence of cells expressing IL-17A (median: 25%; range: 11-33%), IFNγ (median 54%; range: 33-70%), and TNFα (median: 60%; range: 33-72%) within the $CD45RO^+CD3^+CD4^+$memory helper T cell population. $IL\text{-}17F^+$, $IL\text{-}22^+$ or $IL\text{-}4^+$ T helper cells were present a very low frequencies (Figure 2B). Importantly, large proportions of $IL\text{-}17A^+$ memory T helper cells in BAL were also $IFN\gamma^+$. The frequency of IL-17A/IFNγ double producing memory T helper cells was therefore remarkably higher in BAL (~15%) than in peripheral blood (~1%) of sarcoidosis patients (Figure 2C).

IL-17A/IL-4 double-producing cells were present at low but detectable proportions, representing a novel subpopulation (Figure 2C). By contrast, $IL\text{-}17^+IL\text{-}22^+$ cells were almost completely absent and proportions of IL-17A/TNFα or IL-17A/IL-17F double producers were present in the same ranges in BAL and peripheral blood from sarcoidosis patients (Figure 2C and data not shown). IL17F/IFNγ double producers were virtually absent in BAL of sarcoidosis patients (Figure 2D). The proportions of $IL\text{-}17A^+$, $IL\text{-}17A^+IFN\gamma^+$ and $IL\text{-}17A^+IL\text{-}4^+$ memory T helper cells in BAL varied between patients and did not show a positive correlation with the T cell alveolitis in BAL (data not shown).

In summary, the observation of high proportions in BAL of $IL\text{-}17A^+$ memory T helper cells, and particularly of IL-17A/IFNγ double-producing cells, which are thought to be more pathogenic [22], clearly point to the involvement of Th17 cells in sarcoid inflammation.

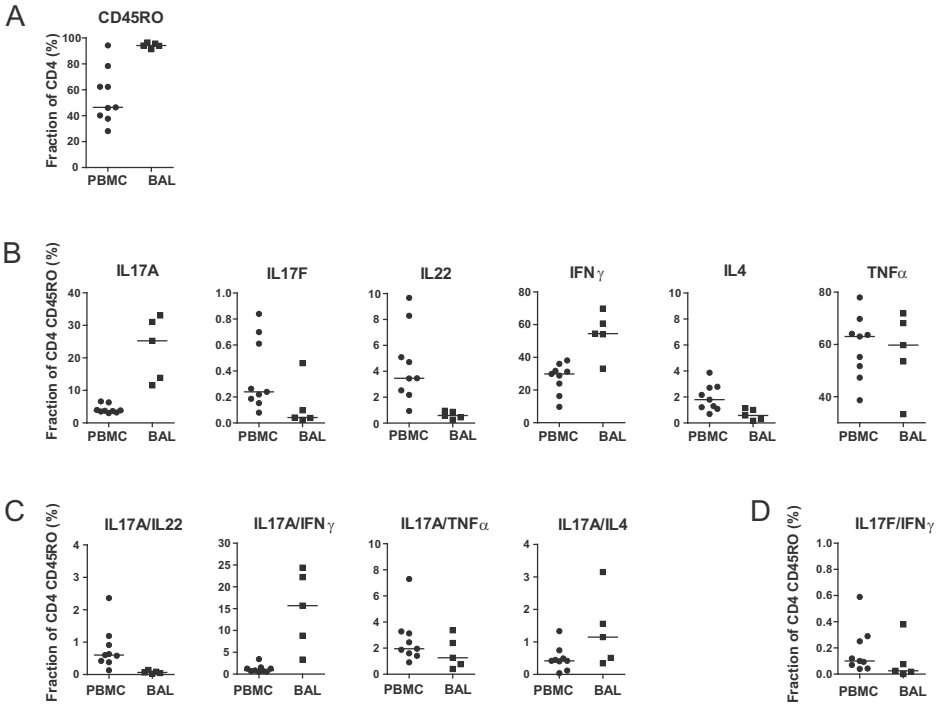

Figure 2. Cytokine profiles of circulating and BAL memory T helper cells in sarcoidosis patients. (A) Proportions of antigen-experienced memory CD45RO$^+$ cells within the populations of CD4$^+$ T cells in PBMC and BAL. (B) Proportions of CD45RO$^+$CD4$^+$CD3$^+$ cells expressing the indicated cytokines, as determined by intracellular flow cytometry. (C, D) Proportions of CD45RO$^+$CD4$^+$CD3$^+$ cells that were double positive for the indicated cytokines, as determined by intracellular flow cytometry. Symbols represent PBMC (l) and BAL (n) from individual patients and lines indicate median values.

3.3 Increased IFNγ and TNF-α, but not IL-17A in CD4$^-$ T cells in sarcoidosis

Little is known about CD8$^+$ and γδ T cells (the CD4$^-$CD3$^+$ T-cell population) in sarcoidosis, although the general observation is that these cells show a similar or a less pronounced cytokine pattern as CD4$^+$ T cells do [29-30]. Here, we observed that a substantial fraction of circulating CD8$^+$ and γδ T cells produced IFNγ, whereby the proportions were significantly higher ($p<0.01$) in sarcoidosis patients compared with healthy controls (Supplementary Figure S2). Frequencies of IL-17A, IL-17F, IL-4 or TNFα expressing CD8$^+$ and γδ cells T cells were not different between sarcoidosis patients and healthy controls. When we measured IL-22 expression in circulating CD3$^+$CD4$^-$ cells, we found fewer IL-22$^+$ cells in sarcoidosis patients when compared with healthy controls (Supplementary Figure S2). BAL CD8$^+$ and γδ T cell populations of sarcoidosis patients contained significant proportions of IL-22$^+$, IFNγ$^+$ and TNFα$^+$ cells and limited proportions of cells expressing IL-17A, IL-17F or IL-4.

Taken together, these findings show that IL-17A is not a prominent cytokine produced by $CD8^+$ and γδ T cells in sarcoidosis. Nevertheless, a substantial fraction of $CD8^+$ and γδ T cells in the BAL produced IL-22.

3.4 Increased IL-17A$^+$ cells in sarcoidosis lung biopsies containing granulomas

The presence of Th17 cytokines in memory T helper cells in the BAL, representing the alveolar space of the lungs, prompted us to analyze T cell cytokine expression in lung mucosal tissue of sarcoidosis patients.

We investigated the presence of IL-17^+ cells in lung mucosal biopsies from 27 sarcoidosis patients and 22 healthy controls (Figure 3). From the 27 biopsies from sarcoidosis patients ten contained clear granulomas (Figure 3C) and 17 were non-granulomatous, showing diffuse cellular infiltrates only (Figure 3B). IL-17A-expressing cells were particularly found in sarcoidosis lung biopsies containing granulomas. Low magnifications of immunohistochemical stainings revealed that IL-17^+ cells were specifically present in areas of inflammatory cells surrounding granulomas, as well as within the granulomas (Figure 3C). In addition, we observed diffuse IL-17A staining within the granuloma areas (Figure 3C). IL-17^+ cells were not detected in the epithelium. Quantification of IL-17-expressing cells in biopsies from healthy controls and non-granulomatous and granuloma-containing biopsies from sarcoidosis patients showed that in all three groups the subepithelial area contained very few IL-17^+ cells, although a trend of higher numbers was observed in granuloma-containing biopsies ($p=0.059$, Kruskal Wallis test; Figure 3D). Importantly, in the entire lamina propria, the numbers of IL-17^+ cells were significantly increased in granuloma-containing biopsies (median: 44 cells/mm^2, range: 0-505 cells/mm^2), when compared with biopsies from healthy controls or non-granulomatous biopsies (median values: 0 cells/mm^2 and ranges of 0-107 and 0-314 cells/mm^2, respectively; $p=0.001$, Kruskal Wallis test; Figure 3D). By immunohistochemical double stainings for and CD3, the IL-17^+ cells were characterized as T cells (Figure 3E).

In summary, we observed increased numbers of IL-17A$^+$ T cells in sarcoidosis in association with granulomas. Together with the presence of diffuse IL-17A staining in the granulomas, these findings suggest a role for IL-17A in granuloma formation or maintenance.

3.5 Increased subepithelial IL-22$^+$ T cells in sarcoidosis lung biopsies containing granulomas

Finally, we investigated the presence of IL-22^+ cells in the epithelium, subepithelium and lamina propria (Figure 4). The epithelium could be evaluated in 32 biopsies. IL-22^+ cells were detected in nine of them, but no significant differences were found between sarcoidosis patients and healthy controls. Importantly, granuloma-containing biopsies showed significantly more IL-22^+ cells in the subepithelial area (median: 15 cells/mm

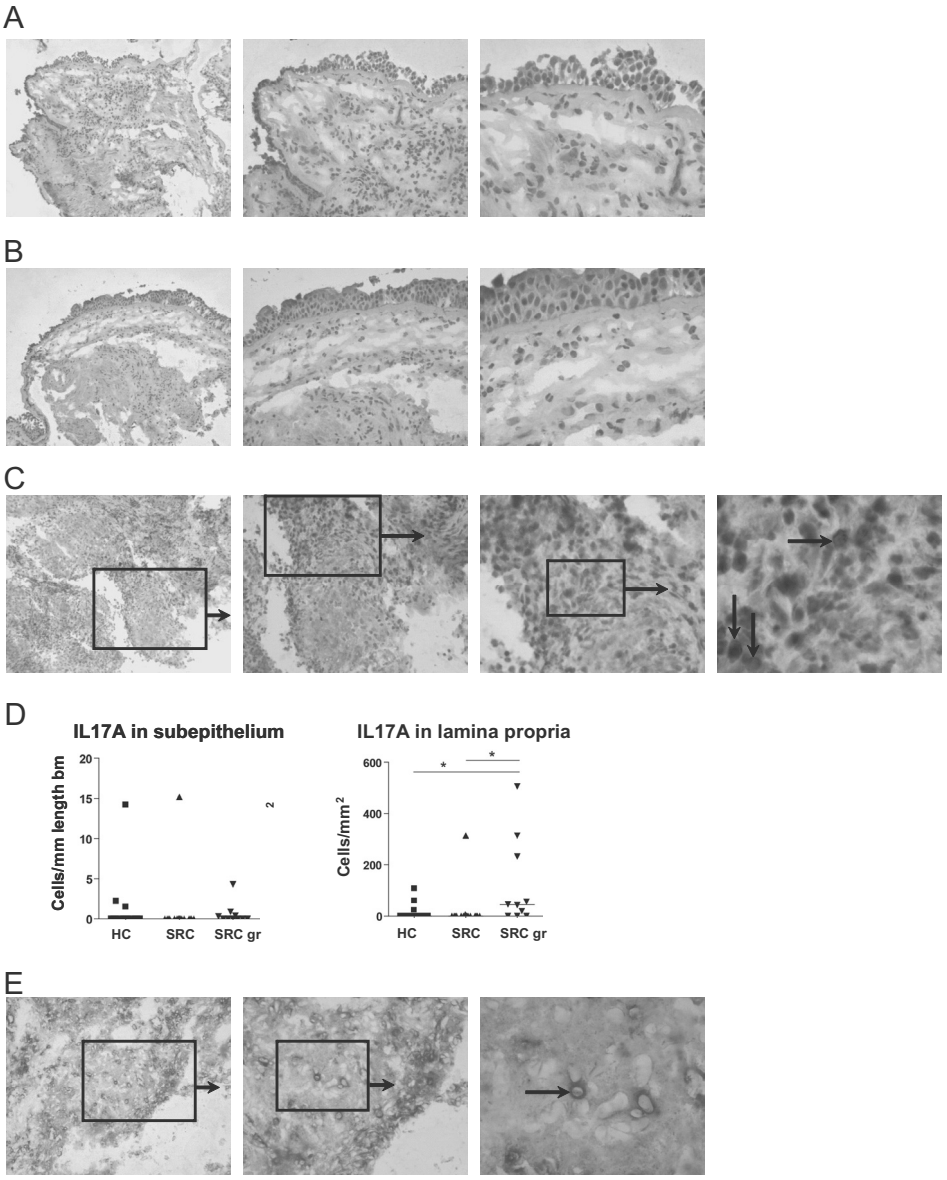

Figure 3. Increased numbers of IL-17A$^+$ cells in sarcoidosis lung biopsies containing granulomas. (A,B,C) Hematoxylin nucleus staining and IL-17A staining of lung mucosal frozen sections from a healthy control biopsy (A), a non-granulomatous sarcoidosis biopsy (B) and a granuloma-containing sarcoidosis biopsy (C) at 100x, 200x and 400x magnification. IL-17A$^+$ cells as well as diffuse IL-17A staining are observed in red. (C) The high magnification photograph (1000x, *far right*) illustrates cytoplasmic staining with anti-IL-17A antibodies (arrows). (D) Quantifications of the numbers of IL-17$^+$ cells in the subepithelium (*left*) and entire lamina propria area (*right*) in the indicated groups. Symbols represent biopsies from individual healthy controls (HC, n), sarcoidosis patients without (SRC, ▲) and sarcoidosis patients with granuloma (SRC gr, ▼); lines indicate median values. * $p<0.05$. (E) Co-localization of an IL-17$^+$ (in blue) and CD3$^+$ (in red) T cell in a lung mucosal biopsy.

basal membrane, range: 5-27 cells/mm; Figure 4B and 4C) than non-granulomatous biopsies (5 cells/mm, range: 0-13 cells/mm; p=0.004, Mann-Whitney U test) or healthy control biopsies (7 cells/mm, range: 0-14 cells/mm; Figure 4A and 4C). When we quantified the numbers of IL-22$^+$ cells in the total lamina propria areas, we did not detect significant differences between the three groups of biopsies (Figure 4D). Analyses of alveolar biopsies demonstrated the incidental presence of IL-22$^+$ cells in healthy controls as well as in sarcoidosis patients, without significant differences between these groups. Immunohistochemical double stainings showed that IL-22$^+$ cells were mainly CD3$^+$ T cells, as illustrated in Figure 4E. In those biopsies where IL-17$^+$ cells were detected in the lamina propria, we found an association with the presence of IL-22$^+$ cells in the subepithelial area (r=0.185; p=0.05, Pearson test, see Supplementary Figure S3).

In summary, our findings show that sarcoidosis patients have a significant increase in IL-22-producing cells in the subepithelial area in the lung. Thus, their main localisation is different from IL-17-producing cells, which are mainly localized around and within granulomas.

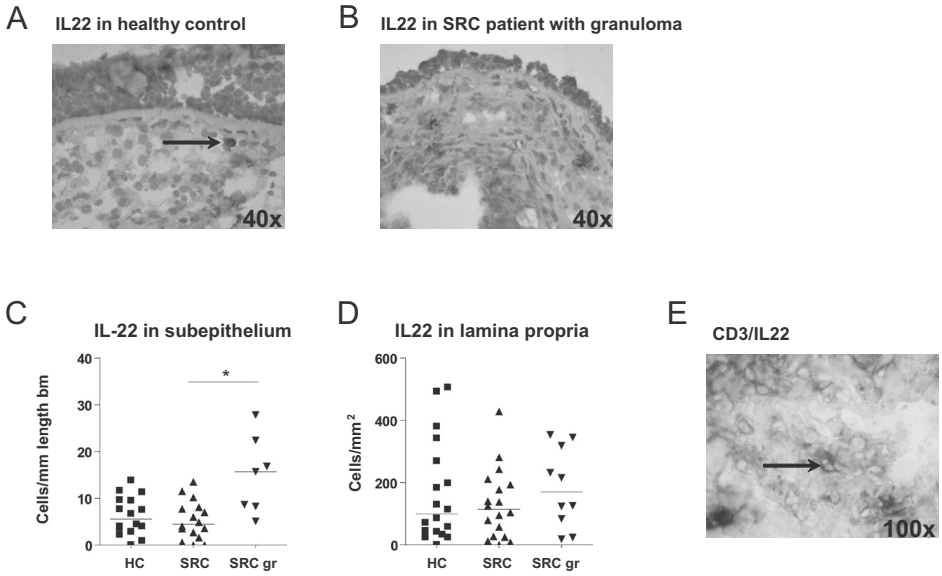

Figure 4. Increased numbers of IL-22$^+$ cells in sarcoidosis lung biopsies containing granulomas. HE and IL-22 staining (in red) of submucosal frozen sections from a healthy control (A) and a granuloma-containing sarcoidosis biopsy (B). Quantifications of the numbers of IL-22$^+$ cells in the subepithelium (C) and in the entire lamina propria area (D) in the indicated groups. Symbols represent biopsies from individual healthy controls (HC, n), sarcoidosis patients without (SRC, ▲) and sarcoidosis patients with granuloma (SRC gr, ▼); lines indicate median values. * p<0.05. (E) Co-localization of an IL-22$^+$ (in blue) and CD3$^+$ (in red) T cell in a lung mucosal biopsy.

4. DISCUSSION

The pathological mechanisms that control granulomatous inflammation in sarcoidosis are only poorly understood, but it is clear that cytokines play an important role in granuloma formation. To date, sarcoidosis pathogenesis has mainly been related to increased Th1 cytokines. In this report, we provide several lines of evidence for the involvement of proinflammatory Th17-lineage cytokines. First, we found increased proportions of circulating IL-17A$^+$ memory T helper cells. Second, IL-17A$^+$ cells, and in particular IL-17A/IFNγ and IL-17A/IL-4 double-producing cells, which are normally very rare, were also present in BAL samples of sarcoidosis patients. Third, we observed an increase in IL-17A-expressing T cells in the lamina propria of the lung in sarcoidosis patients, specifically in granuloma-containing biopsies, where IL-17A$^+$ cells were present around and inside granulomas. Fourth, we identified an increase in IL-22$^+$ cells, in particular in subepithelial regions in granuloma-containing biopsies. To the best of our knowledge, we show for the first time differential distribution of IL-17A$^+$ and IL-22$^+$ T cells in local granulomas, BAL and the circulation in sarcoidosis. Our data particularly point at a possible role for IL-17A/IFNγ and IL-17A/IL-4 double-producing CD4$^+$ T cells, while we did not find evidence for increased IL-17A production by other T cell subsets, such as γδ or CD8$^+$ T cells.

Until now sarcoidosis was considered a Th1-mediated multi-system disease and there is convincing evidence reported for the role of Th1 cells in the pathogenesis of sarcoidosis over the last few years [1]. Thus, Th17 cells are clearly not the only effector cells capable of inducing or regulating granuloma pathogenesis. This would parallel findings in autoimmune uveitis and in experimental autoimmune encephalomyelitis, in which both Th1 and Th17 cells can drive tissue damage [6, 31]. Cooperation of IL-17A and IFNγ is particularly of interest, since we observed high frequencies of IL-17A$^+$IFNγ$^+$ memory T helper cells in blood and BAL of sarcoidosis patients. Furthermore, it has recently been reported that IL-17A/IFNγ double producing CD4$^+$ T cells can become single IFNγ$^+$ cells or single IL-17A producing cells [22]. IL-17A$^+$IFNγ$^+$ cells are thought to be more pathogenic and have also been identified in Crohn's disease [32] and in coronary atherosclerosis [33].

Also our identification of IL-17A/IL-4 double-producing cells in sarcoidosis is of interest. Very few of these cells are present in the circulating memory T cell populations in healthy individuals, but their proportions were reported to be significantly increased in patients with chronic asthma [34]. In this regard, it is very well possible that these cells contribute to IL-4-induced pro-fibrotic features, such as fibroblast growth and collagen production, that are often observed in later stages of sarcoidosis [35]. In contrast to the IL-17A$^+$IFNγ$^+$ and IL-17A$^+$IL-4$^+$ memory T helper cells present in the circulation as well as BAL of sarcoidosis patients, we detected only few IL-17A$^+$IL-22$^+$ cells. On the contrary, our findings of (i) high proportions of IL-17A$^+$ and very low proportions of IL-22$^+$ T helper

cells in BAL and (ii) different locations of IL-17A$^+$ and IL-22$^+$ T helper cells in mucosal biopsies of sarcoidosis patients support the hypothesis that IL-22 can be produced in a IL-17 independent fashion by Th22 cells [19, 21].IL-17A has previously been implicated in various conditions characterized by granuloma formation. In a *Mycobacterium bovis* infection model, IL-17A expression was detected early after pulmonary infection and IL-17A-deficient mice showed impaired granuloma formation [36]. In humans living in regions with high prevalence of *Mycobacterium tuberculosis* infection, peripheral blood contains high frequencies of IL-17A$^+$ and IL-22$^+$ memory T helper cells, which may have protective properties against tuberculosis [37]. In mouse models and in humans with active pulmonary tuberculosis both IL-17A- and IL-22-producing CD4$^+$ T cells and IL-17A$^+$ γδ T cells were shown to contribute to the anti-mycobacterial immune response in human [7, 38]. Lung injury in a mouse model for chronic granulomatous disease with lethal aspergillosis was shown to involve unrestrained γδ T cell reactivity and dominant production of IL-17A [39]. In Langerhans cell histiocytosis, which is accompanied by aggressive chronic granuloma formation, yet another cell population, dendritic cells, was shown to synthesize IL-17A [6]. An IL-17A-dependent pathway for dendritic cell fusion was identified, which was potentiated by IFNγ and led to giant cell formation. In this context, interesting parallels between Langerhans cell histiocytosis and sarcoidosis further include the presence of multinucleated giant cells [2, 6, 40].

The etiology of sarcoidosis is still unknown. Epidemiological and histopathological data have been suggestive for occupational airborne antigens or infectious antigens underlying this disease, but until now attempts to link sarcoidosis to a causative pathogen are difficult and remain controversial. It is therefore presently unclear which mechanisms would initiate a Th17 response in sarcoidosis. It is conceivable that the involvement of Th17 cells in sarcoidosis points to an autoimmune process that is comparable to various other IL-17A-driven autoimmunity disorders, such as autoimmune uveitis, rheumatoid arthritis, inflammatory bowel disease or psoriasis. Future studies are required to determine putative genetic components that enhance IL-17A synthesis, e.g. IL-23R polymorphisms, which have also been associated with autoimmunity. As we observed diffuse IL-17A staining, it is very well possible that Th17 effector cells within granulomas are required to achieve local IL-17A concentrations that can activate various myeloid cell populations. Such a model would parallel the proposed role of local Th17 cells in the bone marrow, which may regulate myeloid development [41]. An alternative explanation for the observed diffuse IL-17A staining in granuloma may be that granuloma cells are a source for IL-17A, analogous to Langerhans cells in Langerhans cell histiocytosis [40].

In summary, we provide evidence for the involvement of the Th17 lineage in sarcoidosis: IL-17A-expressing T cells were present in and around the granuloma and IL-22-expressing T cells were found in the subepithelial lamina propria in mucosal biopsies of

sarcoidosis patients. This was accompanied by the presence of IL-17A$^+$, IL-17A$^+$IFNγ$^+$ and IL-17A$^+$IL-4$^+$ memory T helper cells in BAL and by a significant increase in the proportions of these cells in the circulation. Therefore, IL-17A and IL-22 represent targets that may have clinical value in the treatment of sarcoidosis. In this context, it is promising that clinical trials of the fully human antibody, AIN457, in RA, psoriasis and noninfectious uveitis, show that targeting IL-17A interrupts inflammation and reduces disease activity [42].

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Impaired survival of regulatory T cells in pulmonary sarcoidosis patients

Ten Berge B.[1], KleinJan A.[1], Muskens F.[1], D. Korporaal[1], Hoogsteden H.C.[1], Lambrecht B.N.[1,2], Hendriks R.W.[1]*, Van den Blink B.[1]*

(1) Department of Pulmonary Medicine, Erasmus MC, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

(2) Department of Respiratory Diseases, Laboratory of Mucosal Immunology, MRBI, University Hospital Gent, De Pintelaan 185, B9000 Ghent, Belgium

** These authors shared the supervision of this study.*

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ABSTRACT

Sarcoidosis is a systemic inflammatory disorder characterized by the presence of granulomas in the lungs and other organs and is associated with a T helper (Th) –1 and Th17-driven inflammation. Conflicting data have been reported on the number and the function of regulatory T cells (Tregs) in sarcoidosis patients. It is therefore unknown whether a lack of Tregs or an impaired function of these cells, which are essential to control inflammation, can contribute to sarcoidosis pathogenesis. In this report, we studied 43 newly diagnosed sarcoidosis patients and 33 healthy controls. Using flow cytometry, we found increased numbers of Tregs in the bronchoalveolar lavage (BAL) and in peripheral blood. In mucosal biopsies, Tregs were found in and outside the granulomas by immunohistochemistry, but not all granulomas contained Tregs. Using flow cytometric cell sorting, $CD4^+CD25^+CD127^{low/-}$ Tregs were purified from peripheral blood and functionally tested *in vitro*. Isolated Tregs from sarcoidosis patients showed a significantly reduced suppressive capacity on effector T cells, when compared with healthy controls. This was associated with significantly reduced survival of sarcoidosis Tregs *in vitro*, while survival of $CD4^+$ and $CD8^+$ T cells was apparently normal. Our findings in sarcoidosis of increased numbers of Tregs in BAL and peripheral blood but not in pulmonary granulomas, together with reduced survival *in vitro* indicate that impaired Treg function contributes to sarcoidosis pathogenesis.

1. INTRODUCTION

Sarcoidosis is a multisystem disorder, characterized by the presence of non-caseating granulomas affecting various organs, but in over 90% of patients the respiratory tract is involved [1]. The clinical presentation is diverse, depending on the organs involved. The course of sarcoidosis is in most cases benign but severe complaints can be present according to involvement of extrapulmonary sites. Moreover, the course of sarcoidosis can become chronic leading to pulmonary fibrosis [2]. The granulomatous inflammation in sarcoidosis occurs in the absence of clearly defined immunological targets. Epidemiological and histopathological data have been suggestive for occupational airborne antigens [3-5], or infectious antigens underlying this disease [5-10]. Attempts to link sarcoidosis to a causative pathogen are difficult until now and remain controversial. Besides an immunological target, several studies show evidence for a genetic contribution to the pathogenesis of sarcoidosis [11]. Associations were found in genes coding for the human leucocyte antigen (HLA) region, the apoptosis-inducing protein Fas/CD95 [12] and annexin A11, of which the function is unknown but may be related to apoptosis or cell-cycle regulation [13]. Several specific alleles associate with disease risk and phenotype [14, 15].

Immunologically, sarcoidosis is characterized by a peripheral blood T cell lymphopenia and a $CD4^+$ T cell alveolitis in the lung, resulting in an increased bronchoalveolar lavage (BAL) cell CD4/CD8 ratio. T cells in the BAL have a Th1 phenotype, are highly activated, expressing CD25, CD69 and CD26 [16] and produce spontaneously Th1 cytokines IL-2, IFNγ and TNFα. Recently sarcoidosis was suggested as a Th1/Th17 multisystem disorder [17], based on the presence of $IL\text{-}17^+CD4^+$ T cells in sarcoid lung tissue and their ability to respond to the chemotactic stimulus CCL20. Moreover, IL-17 was expressed by macrophages infiltrating sarcoid tissue.

Regulatory T cells (Tregs) comprise an important cell population in our immune system capable of suppressing inflammation. At least two different types of Tregs can be recognized and both have a $CD4^+CD25^+$ surface phenotype and express the key transcription factor forkhead box P3 (FoxP3). Whereas naturally occurring Tregs are programmed in the thymus, adaptive Tregs are generated from naive $CD4^+$ T cells in the periphery by unique differentiation signals including stimulation by immature DCs, retinoic acid or TGFβ [18]. Tregs are capable of inhibiting imummunopathologic inflammation driven by $CD4^+$ and $CD8^+$ T cells in various ways. Tregs can suppress the cytologic function of effector T (Teff) cells by IL-2 depletion and can modulate their cytokine composition by secretion of TGFβ and IL-10. Another mechanism involves direct cytotoxic killing of T cells by perforin, granzyme or through the induction of Fas-mediated apoptosis [19].

Conflicting results have been reported concerning the number of Tregs in sarcoidosis patients. Idali et al. [20] reported decreased expression of FoxP3 mRNA in BAL $CD4^+$ T

cells from BAL, together with lower numbers of FoxP3^{+}CD4^{+} T cells in BAL and peripheral blood of sarcoidosis patients. These findings are in stark contrast with published results showing an increase in CD4^{+}CD25^{high} Tregs in BAL and blood of sarcoidosis patients, and an increase in CD45RA negative and positive CD4^{+}FoxP3^{+} T cells in peripheral blood [21, 22]. Furthermore Tregs were found to be present and proliferating in and around lymph node and kidney granulomas in sarcoidosis [21]. Functional testing of CD4^{+}CD25^{high} blood Tregs in sarcoidosis revealed a decreased inhibition of the TNFα and IFNγ production by Teff cells [22]. In a granuloma model using peripheral blood mononuclear cells (PBMCs) to create granulomas around Bacille Calmette Guerin (BCG) coated beads, depletion of CD25^{+} cells in sarcoidosis did not affect granuloma grow rates, suggesting a defect in Treg function in sarcoidosis patients[21].

Decreased suppressive function, either due to reduced Treg numbers or diminished suppressive capabilities, perhaps bearing an intrinsic functional defect as suggested in patients with hypersensitivity pneumonitis [23], might explain the excessive inflammatory response in sarcoidosis with CD4^{+} T cell alveolitis and granuloma formation. In this study, we therefore measured Treg numbers in peripheral blood and in BAL of newly diagnosed sarcoidosis patients and healthy control subjects. We defined the localization and number of Tregs in the airways of patients and controls and performed functional tests on isolated peripheral blood Tregs *in vitro*. Our findings indicate an intrinsic survival defect of Tregs in sarcoidosis.

2. MATERIALS AND METHODS

2.1 Patients and healthy control subjects

After written informed consent, 43 sarcoidosis patients and 33 healthy volunteers were included in our study and underwent fibre-optic bronchoscopy. The protocol was approved by the Medical Ethical Committee of the Erasmus MC Rotterdam. The patient group consisted of 20 males and 23 females with newly diagnosed sarcoidosis (mean age: 38.3 y). The diagnosis of sarcoidosis was established conform the guidelines of the ATS/ERS/WASOG statement on sarcoidosis [1] on the basis of compatible clinical and radiographic features: histological evidence of non-caseating granulomata on mucosal biopsy or an increased CD4/CD8 ratio in the BAL fluid and the exclusion of other granulomatous diseases. All patients were diagnosed, mainly within a few months after first symptoms, with radiographic stage 1 (23 patients) or stage 2 (20 patients) sarcoidosis. None of the patients were on corticosteroid or immunosuppressive drugs at the time of diagnosis and sampling. Healthy volunteers, with no history of pulmonary disease, had a male/female ratio of 12/21 and the mean age was 24.5 y.

2.2 Collecting and storage of BAL, peripheral blood mononuclear cells and mucosal biopsies

BAL was performed with a flexible fibre-optic bronchoscope (Olympus) placed in the right middle lobe in wedge position. Four aliquots of 50 ml saline were instilled and subsequently gently aspirated. BAL fluid was collected in siliconized bottles to prevent cell adherence and kept at 4 ºC. BAL fluid was filtered through a 100 µm cell strainer (BD Biosciences) and centrifuged for 7 min at 450g at 4ºC. The supernatant was removed and frozen at –80ºC. The BAL fluid cells were counted and directly used for flow cytometry.

Peripheral blood was diluted 1:1 with RPMI 1640, (Gibco) and centrifuged over a Ficoll-Paque (GE Healthcare) density gradient for 20 min, 1200g, at room temperature (RT). The mononuclear cells at the interface were collected and washed in RPMI 1640 (Gibco). Cells were counted and used directly to assess Treg numbers, or subsequently frozen and stored.

Mucosal biopsies were taken at the right middle lobe, processed in PBS and Tissue Tek, O.C.T. compound, (Sakura Finetek Europe) and afterwards directly frozen in Tissue Tek, O.C.T. compound and stored at -80°C.

2.3 Flow cytometric analysis of Tregs in BAL and peripheral blood

Peripheral blood mononuclear cells (PBMCs) and BAL cells were stained extracellularly with the following antibodies: anti-CD3 APC-Cy7 (SK7) (BD biosciences), anti-CD4 PE-CY5 (RPA-T4) and anti-CD25 PE (BC96) (eBioscience). Heat-inactivated human serum (1%) was added to prevent non-specific antibody staining. Subsequent intracellular staining with anti-FoxP3 APC or rat-IgG2a APC isotype control (eBioscience) was performed according to the manufacturers' protocol.

For apoptosis marker analysis, PBMCs were extracellularly stained with the following antibodies: anti-CD3 and anti-CD4 (described above), anti-CD127-PE (hIL7R-M21) (BD Biosciences), anti-CD25 PE-Cy7 (M-A251) (BD Biosciences) and anti-CD95 (FAS, clone DX2) (eBioscience), followed by Annexin V binding (Annexin V FITC apoptosis detection kit; BD Pharmingen).

Cells were measured on a LSRII Flowcytometer (BD Biosciences) and collected data were analysed by FlowJo software (Tree Star Inc).

2.4 Immunohistochemical staining of FoxP3 on lung mucosa biopsies

Immunohistochemical single stainings were performed in a half-automatic stainer (Sequenza), as previously described [24]. Briefly, aceton-fixed slides were blocked in diluted 10% normal goat serum (CLB, Amsterdam, the Netherlands) and were incubated with primary monoclonal Abs against human FoxP3 (PCH101, eBioscience) for 1 hour, rinsed with PBS and incubated for 30 min with diluted long chain biotin-conjugated goat-anti-mouse (Biogenix). After a second wash slides were incubated with alkaline

phosphatase-conjugated streptavidin for 30 min, washed and incubated with New Fuchsin substrate. Finally, the sections were counterstained with Gills triple strength haematoxylin and mounted in glycerin gelatin.

2.5 Isolation of Tregs from peripheral blood

Upon thawing in RPMI 1640 supplemented with 20% FCS, PBMCs were stained with the following antibodies: anti-CD3 APC-Cy7 (SK7) (BD biosciences), anti-CD4 PE-CY5 (RPA-T4)(eBiosciences), anti-CD127PE (hIL7R-M21) (BD biosciences) and anti-CD25 PE-Cy7 (M-A251) (BD biosciences). 4′,6-diamidino-2-phenylindole (DAPI, Molecular probes) was used as a dead-live marker in the violet channel. Tregs were sorted as live $CD3^+CD4^+CD25^+CD127^{low}$ cells [25], using a FACS ARIA (BD Biosciences) with Diva software and for analysis FlowJo software (Tree Star Inc.) was used. The overall purity of the sorted Tregs, measured by flow cytometry was ~97%, both from healthy controls and from sarcoidosis patients.

2.6 Mixed leukocyte reaction and Treg culture

PBMCs were isolated as described above. Monocytes were isolated from a single batch of peripheral blood buffy coat, using $CD14^+$ microbeads (Miltenyi Biotec), according to the manufacturers' protocol. Monocytes were resuspended in culture medium (RPMI 1640, 10% FCS and gentamycin (Gibco (50 ug/ml final concentration)) to 1 x 10^6 cells/ml. GM-CSF (1000 U/ml; Immuno Tools) and IL-4 (200 U/ml; R&D) were added and cells were cultured at 37°C for 6 days. At day 6, to mature the monocyte-derived DCs (mo-DCs) 10 µg/ml LPS was added. At day 7, naive $CD4^+$ T cells were isolated from another batch of peripheral blood buffy coat, using microbeads (130-091-894, Miltenyi Biotec). For fluorescent cell labeling with carboxyfluorescein succinimidyl ester (CFSE), T cell fractions were washed twice with serum free medium and labeled in a final concentration of 5 µM CFSE (Molecular Probes) in serum-free medium at 37°C for 10 min. Adding excess ice-cold culture medium (containing 10% FCS) stopped the reaction.

The mixed leukocyte reaction (MLR) consisted of day 7 LPS-stimulated mo-DCs with CFSE labeled CD4 naive T cells in a 1:20 ratio. Sorted Tregs were labeled with PKH fluorescent cell linker dye (Sigma-Aldrich), according to the manufacturers' protocol. After labeling, Tregs were added to the MLR in a 1:5, 1:10 and 1:20 ratio. At day 5, cells were analyzed by flow cytometry using anti-CD4 PE-Cy5 (RPA-T4) (eBiosciences) and 4′,6-diamidino-2-phenylindole (DAPI) as a live-dead marker.

To determine survival of Tregs, sorted Tregs were cultured for 5 days with or without 2ng/ml recombinant human IL-2 (rhIL-2, Peprotech). At day 3 and day 5 Tregs were analysed with flow cytometry, as described above.

2.7 Statistics

Results were compared using a two-tailed Mann Whitney U test. Correlations were analyzed using the Spearman's rank correlation test. Differences were considered significant at $p< 0.05$.

3. RESULTS

3.1 Increased numbers of Tregs in BAL and peripheral blood from sarcoidosis patients

To analyze the involvement of Tregs in the pathogenesis of sarcoidosis, we first determined the numbers of Tregs in peripheral blood and BAL of sarcoidosis patients and healthy controls. In flow cytometric analyses we gated for $CD25^+CD3^+CD4^+$ T cells expressing intracellular FoxP3, as illustrated in Figure 1A. In peripheral blood the proportions of $CD3^+CD4^+$ T cells that were $CD25^+FoxP3^+$ were significantly increased in sarcoidosis patients (median: 6.8%; range 4.6-8.9), when compared with healthy controls (median: 2.7%; range: 1.6-6.6, p=0.03). The absolute numbers of Tregs appeared also higher in sarcoidosis patients, but this difference was not significant (median: 13 x 10^3 cells/ml; range: 5.3 x 10^3-23 x 10^3 in patients and 4,7 x 10^3 cells/ml (2.9 x 10^3-15 x 10^3) in healthy controls)(Figure 1B).

Moreover, also in the BAL of sarcoidosis patients an increase in Tregs was observed, when compared to healthy controls. The increase was evident when measured as proportions of $CD3^+CD4^+$ T cells (median: 8.6%; range: 6.8-9.1 in patients, and median: 5.3%; range: 3.3 - 8.0 (p= 0.01) in controls (Figure 1C). In agreement with published findings [26], sarcoidosis patients manifested an increase of $CD4^+$ T cells in the BAL, leading to an increased CD4/CD8 ratio. Therefore, an even more prominent increase of the absolute numbers of Tregs in BAL was observed. Median values were 3.4 x 10^3 cells/ml (range: 1.1 x 10^3 – 5.4 x 10^3) in patients, compared with 92 cells/ml (range: 12 – 140; $p<0.01$) in healthy controls (Figure 1C). Finally, we observed that in the BAL of sarcoidosis patients the number of Tregs positively correlated with the number of $CD3^+$ T lymphocytes ($r^2= 0.8$; p=0.01) (Figure 1D).

In summary, these findings show that in sarcoidosis patients the numbers of Tregs were increased in BAL and peripheral blood. Therefore, a reduced Treg population cannot explain the seemingly exaggerated immune response in sarcoidosis.

3.2 Localization of Tregs in mucosal lung biopsies in sarcoidoisis patients

After investigating airway lumen, we set out to determine the amount and localization of Tregs in the airways by analysis of cryosections of mucosal airway biopsies. We used FoxP3 as a marker for Tregs and counterstained with heamatoxillin (Figure 2A). As from the 26 biopsies of sarcoidosis patients analyzed, 10 contained clear granulomas, we divided these biopsies into two separate groups: sarcoidosis with granuloma (SRC

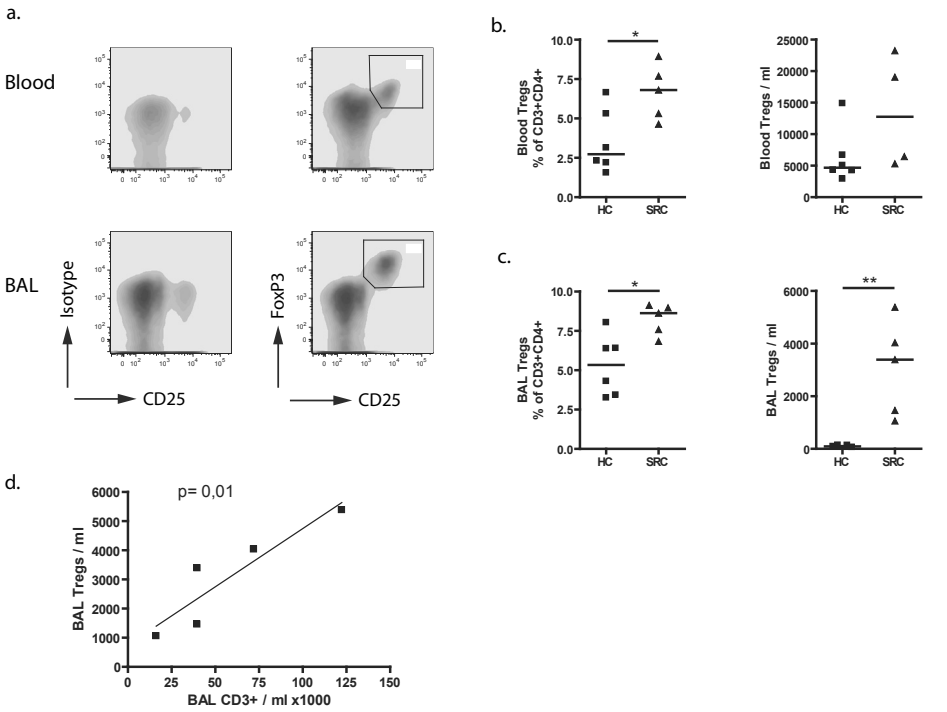

Figure 1. Increased numbers of Tregs in BAL and peripheral blood from sarcoidosis patients. (A) Blood and bronchoalveolar lavage (BAL) Tregs were identified by flow cytometry. $CD3^+CD4^+$ T cells were gated and analysed for the expressing both CD25 and FoxP3. The indicated gate contains $CD25^+FoxP3^+$ Tregs. (B) Frequencies in blood of $CD25^+FoxP3^+$ Tregs as fraction of $CD3^+CD4^+$ T cells (*left panel*) or as the number of $CD25^+FoxP3^+$ $CD3^+CD4^+$ T cells per ml (*right panel*). Each symbol represents and individual healthy control (HC) or sarcoidis patient (SRC). *, p=0.03. (C) Frequencies in BAL of $CD25^+FoxP3^+$ Tregs as fraction of $CD3^+CD4^+$ T cells (*left panel*) or as the number of $CD25^+FoxP3^+CD3^+CD4^+$ T cells per ml (*right panel)*. Each symbol represents an individual healthy control (HC) or sarcoidis patient (SRC). *, p<0.01. (D) Correlation between the number of $CD3^+$ T-lymphocytes and Tregs in BAL (r^2=0.8; p=0.01).

gr, n=10) and sarcoidosis without granuloma (SRC, n=16). In these analyses, we also included mucosal airway biopsies from 20 healthy controls. We found no differences in $FoxP3^+$ cell numbers per mm^2 between the three groups (Figure 2B). Thorough analysis of the localization of $FoxP3^+$ cells in granuloma-containing biopsies revealed that not all granulomas contained $FoxP3^+$ cells: in 6 out of 10 granuloma-containing biopsies no $FoxP3^+$ cells were detected. This was not correlated to disease course or disease stage, $CD3^+$ T cells alveolitis or CD4/CD8 ratio. In granulomas were $Foxp3^+$ cells were detected, they were localized both within the granulomas and in the peripheral infiltrate.

These immunohistochemical analyses show that many granulomas did not contain Tregs, and when present they were in or outside granulomas. From these data we conclude that it is not likely that the numbers of Tregs have an effect on granuloma formation or maintenance. Rather, it is conceivable that Treg function is impaired.

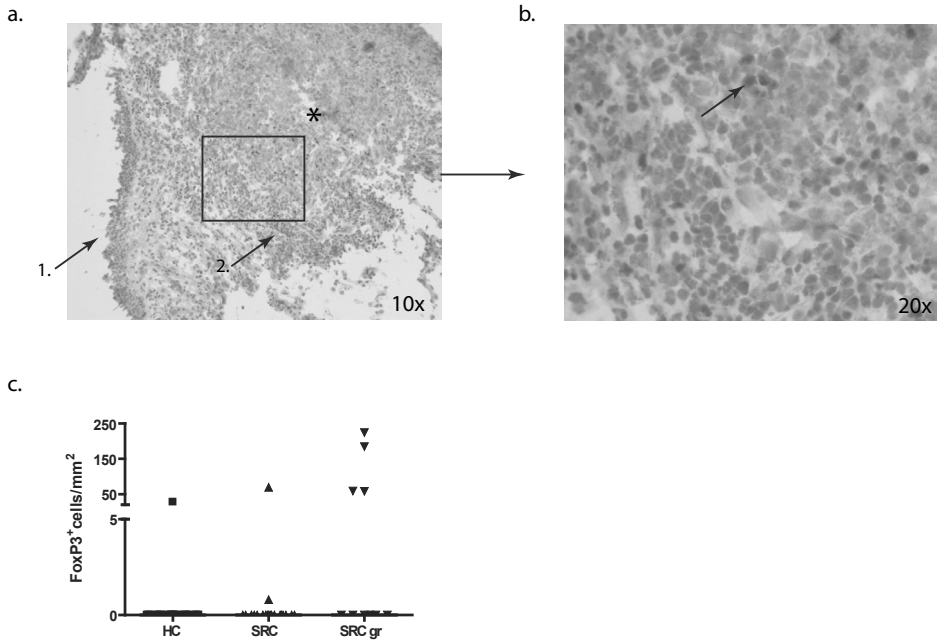

Figure 2. Localization of Tregs in mucosal lung biopsies in sarcoidoisis patients.
(A) Pulmonary mucosal biopsy from a sarcoidosis patient with a submucosal granuloma (1= mucosal layer; 2= lymphocyte rim around granuloma; * = centre of granuloma), stained with FoxP3 (*red*) and counterstained with haematoxylin (original magnification 10x). (B) Enlargement of the same mucosal biopsy showing in more detail the FoxP3 positive Tregs (*red*)(original magnification 20x). (C) Quantification of FoxP3$^+$ cells in healthy controls (HC, n=20), non-granuloma-containing sarcoidosis (SRC, n=16) and granuloma- containing sarcoidosis (SRCgr, n=10).

3.3 Peripheral blood Tregs of sarcoidosis patients do not suppress allogeneic T cell proliferation

To compare the function of Tregs of sarcoidosis patients and healthy controls, we investigated the capacity of the Tregs to suppress a mixed leukocyte reaction (MLR) *in vitro*. To this end, we isolated Tregs from peripheral blood by cell sorting, using the IL-7R/CD127 and the IL-2R/CD25 as surface markers. CD127 inversely correlates with FoxP3 expression and the immunosuppressive function of Tregs [27, 28]. We sorted CD25$^+$CD127$^{low/-}$ CD3$^+$CD4$^+$ T cells [25] and tested their immunosuppressive function on allogeneic T cell proliferation, using CD25$^-$CD127$^+$ CD3$^+$CD4$^+$ effector T cells (Teffs) as a control. In an allogeneic MLR of monocyte derived DCs (mo-DCs) and CD4$^+$ naive T cells in a 1:20 ratio, we added Tregs or Teffs *in vitro*, in a 1:1 ratio with the naïve CD4$^+$T cells. As shown in Figure 3A, we found that T cell proliferation in the MLR was further increased by addition of Teffs and decreased by addition of Tregs, when measured by ^{3}H-thymidine incorporation. In the presence of Tregs, T cell proliferation was decreased to the same level as in a culture of CD4$^+$ naive T cell culture without added DCs (Figure 3A). Thus, sorting of

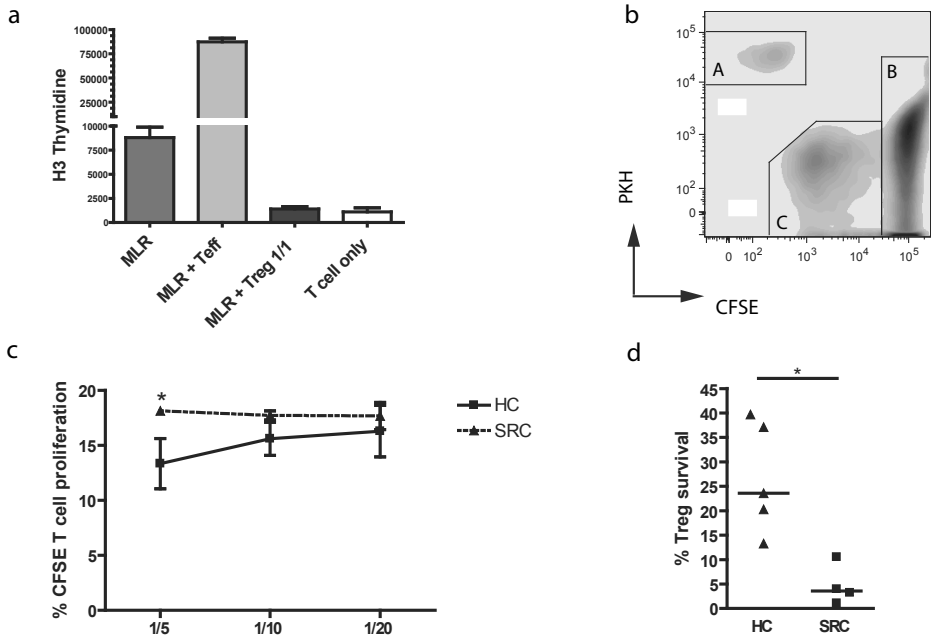

Figure 3. Peripheral blood Tregs of sarcoidosis patients do not suppress T cell proliferation
(A) In vitro co-culture of mo-DC and isolated peripheral blood naive T cells in a 1:20 ratio (MLR), in the presence of sorted $CD25^{-}CD127^{+}$ $CD3^{+}CD4^{+}$Teff (1:1, MLR+Teff) or sorted $CD25^{+}CD127^{low/-}$ $CD3^{+}CD4^{+}$Tregs (1:1, MLR+Treg). As a control we measured proliferation rates of $CD4^{+}$ naive T cells only, in the absence of mo-DC. Total T cell proliferation was measured with ^{3}H thymidine incorporation at day 5. (B) Flow cytometric analysis of proliferation of $CD4^{+}$ naive T cells, stained with CFSE, in the presence of Tregs, stained with PKH, and stimulated with mo-DCs, measured at day 5. Gated populations are Tregs (A), resting $CD4^{+}$ T cells (B) and $CD4^{+}$ T cells that have proliferated (C) in vitro. (C) Proportions of proliferating T cells at day 5, as measured according to the method in panel (B), when Tregs of sarcoidosis patients (SRC) or healthy control subjects (HC) were added in the indicated ratios. T cell proliferation is significantly lower when the HC Tregs were added compared to the SRC Treg in a 1: 5 ratio. * p=0.02. (D) Survival, as measured by the proportions of DAPI-negative cells (in %), at day 5 of the MLR culture. Treg survival is significantly decreased in SRC Tregs (median: 3.6%; range: 1.1-10.5) compared to HC Tregs (median: 23.6% ; range: 13.3-39.7). * p=0.02.

$CD3^{+}CD4^{+}CD25^{+}CD127^{low/-}$ Tregs yields a T cell population with a good suppressive effect on allogeneic T cell proliferation. However, measurements of ^{3}H-thymidine incorporation cannot discriminate between proliferation of naive T cells only, Tregs only, or both. Therefore, we stained the Tregs with PKH dye and the $CD4^{+}$ naïve T cells with CFSE before incubation. At day 5 we analysed the MLR cultures by flow cytometry and quantified the proliferation of the CFSE- labeled $CD4^{+}$ T cells separately (Figure 3B).

To functionally test Tregs in sarcoidosis patients we isolated peripheral blood Tregs of four sarcoidosis patients and five healthy controls in two independent experiments. Again, mo-DCs and naive $CD4^{+}$ Tcells were cultured in a 1:20 ratio. Isolated Tregs of sarcoidosis patients and healthy controls were added in a 1:5, 1:10 or 1:20 ratio to the MLR.

After 5 days, we measured the proliferation of the CFSE-labeled $CD4^+$T cells. In contrast to Tregs of healthy donors, Tregs from sarcoidosis patients did not have a suppressive function on T cell proliferation in the MLR cultures. The difference in T cell proliferation was significant when Tregs were added in a 1:5 ratio (p=0.02; Figure 3C). Interestingly, at day 5 of the MLR cultures, only ~3.6% (range 1.1 - 10.5%) of the Tregs of sarcoidosis patients were still alive, compared to 24% (13 – 40%) of Tregs of healthy controls (Figure 3D).

Taken together, these findings show that Tregs isolated from peripheral blood of sarcoidosis patients do not suppress allogeneic T cell proliferation. This lack of suppressive function of the sarcoidosis Tregs was associated with impaired survival of these cells in MLR cultures.

3.4 Tregs of sarcoidosis patients manifest reduced survival in vitro

Based on the impaired survival of Tregs in the MLR cultures, we decided to investigate if Tregs in sarcoidosis patients have an intrinsic survival defect resulting in impaired suppressive function. This was a likely possibility, since (i) in sarcoidosis the apoptotic pathway protein Fas is overexpressed and (ii) Fas promotor genetic variants are linked to sarcoidosis disease risk [12], and (iii) apoptosis is in general a mechanism for Treg homeostasis [19].

First, we analysed Fas and Annexin V expression on $CD25^+CD127^{low/-}$ $CD3^+CD4^+$ Tregs and $CD25^-CD127^+$ $CD3^+CD4^+$ T cells from five patients and five healthy controls, but did not find significant differences (data not shown). The mean fluorescent expression (MFI) of IL-2R/CD25 on Tregs also did not differ between patients and healthy controls.

Next, we isolated $CD25^+CD127^{low/-}$ $CD3^+CD4^+$ Tregs from peripheral blood (six patients and five healthy controls) and cultured them *in vitro* for 5 days. Both on day 3 and on day 5 we found decreased survival of Tregs from sarcoidosis patients, when compared with Tregs from healthy controls (41% ± 6 versus 65% ± 3, respectively, at day 3 ($p<0.05$) and 33% ± 5 versus 52% ± 5, respectively, at day 5 (p=0.01), as shown in Figure 4A.

In an independent experiment we determined Treg survival in cultures with or without IL-2. We found that on day 3 the numbers of residual viable Tregs from sarcoidosis patients was about ~50% of the values found for Tregs from healthy donors, irrespective of the presence of added IL-2 in the culture (Figure 4B). Because addition of IL-2 did not restore Treg survival, we conclude that impaired Treg survival in sarcoidosis cannot be explained by reduced IL-2 production by Tregs. The impaired survival Tregs from sarcoidosis patients is not a general feature seen in all T cells from these patients, because $CD25^-CD127^+$ $CD3^+CD4^+$ T cell fractions from healthy controls and sarcoidosis patients manifested similar survival both in cultures with and without additional IL-2 (Figure 4B).

In summary, these findings show that circulating Tregs of sarcoidosis patients had a significantly reduced cellular survival *in vitro,* which was not due to defective IL-2 production by the Tregs. This impaired survival was specific for Tregs and was not observed for $CD4^+$ T cells.

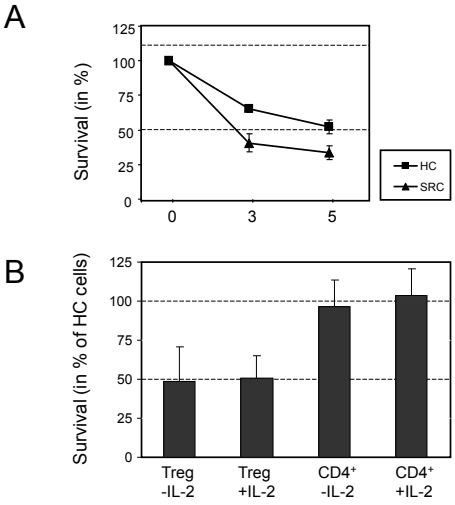

Figure 4. Tregs of sarcoidosis patients manifest reduced survival in vitro
(A) Survival (as % DAPI-negative cells) of cultured peripheral blood-derived $CD25^{+}CD127^{low/-}$ $CD3^{+}CD4^{+}$ Tregs from healthy controls (HC) or sarcodosis patients (SRC) at the indicated days of culture. (B) Survival of Tregs and $CD4^{+}$ T cells, cultured in the absence (-) or presence (+) of IL-2 for 3 days. Mean survival of purified Treg and $CD4^{+}$ T cell fractions from healthy controls (n=6) were set to 100%. Data shown are relative survival values (mean ± SEM) of purified Treg and $CD4^{+}$ T cell fractions from peripheral blood of five sarcoidosis patients.

4. DISCUSSION

In the present study we show that in sarcoidosis patients Tregs are increased in BAL, both in proportions of total $CD4^{+}$ T cells and in absolute numbers. In peripheral blood significant differences were only found for the proportions of Tregs of total $CD4^{+}$ T cells. Nevertheless, isolated Tregs from peripheral blood of sarcoidosis patients showed a significantly reduced suppressive capacity on effector T cells, when compared with healthy controls. Together with the observed reduced survival of Tregs in vitro, these findings indicate that impaired Treg function contributes to sarcoidosis pathogenesis.

Our results are consistent with the previously reported increase in $CD4^{+}CD25^{high}$ Tregs in the BAL and blood of sarcoidosis patients [22], and the increase in both $CD45RA^{+}$ naïve and $CD45RA^{-}$ memory $FoxP3^{+}$ Tregs [21]. In contrast to these reports, Idali et al showed a decrease in the percentages of BAL and blood $FoxP3^{+}$ Tregs [20], but about half of the included patients were diagnosed as Löfgren's syndrome. Löfgren's syndrome is generally regarded as an acute subtype of sarcoidosis with typical clinical aspects and a good prognosis. To our knowledge no data are reported on the numbers of Tregs in Löfgren's syndrome, but it cannot be excluded that in patients with Löfgren's syndrome Treg cell numbers are reduced, which could then explain the finding of reduced Treg numbers

in this study. Interestingly, Prasse et al. [29] very recently showed that the numbers of Tregs in the BAL of sarcoidosis patients were reduced in patients with a chronic course of sarcoidosis, and were significantly higher in patients with spontaneous remission, but not increased compared to healthy controls [29]. This finding therefore clearly indicates that during sarcoidosis disease course the numbers of Tregs are subject to change.

The increase of Tregs in BAL is positively correlated with an increased level of BAL $CD3^+$ T lymphocytes. Therefore, in our patients increased Treg numbers are not associated with a decreased inflammatory response in sarcoidosis, suggesting a possible functional defect in Tregs in sarcoidosis. We observed that in contrast to isolated Tregs from healthy controls, Tregs from sarcoidosis patients did not show suppressive activity on T cell proliferation in MLR cultures. This lack of suppression was associated with impaired survival *in vitro*. Altered suppressive activity of regulatory Tcells in sarcoidosis has previously been reported by Miyara *et al.* [22]. The authors purified Tregs from the peripheral blood as $CD25^{high}CD4^+$ T cell fractions. These cells exhibited good antiproliferative activity, but did not completely inhibit TNFα and INFγ secretion by autologous $CD4^+$ T cells. The observed good anti-proliferative activity does not match with our results, as we demonstrated decreased anti-proliferative activity. However, activated T cells also express CD25, so when Treg fractions are isolated only on the basis of CD25 expression, activated T cells are included. Although it is not expected that activated T cells have anti-proliferative capacities, $CD25^{high}CD4^+$ T cell fractions cannot be directly compared with $CD25^+CD127^-$ T cell fractions. Nowadays FoxP3 is regarded as a reliable maker for Tregs in humans [30], although recent studies provided evidence for transient FoxP3 expression in activated human non-regulatory T cells [31]. Although there is a major overlap between $CD25^{bright}$ and FoxP3 expressing T cells, all $Foxp3^+$ cells are $CD25^+$ but not all are $CD25^{bright}$, and not all $CD25^{bright}$ cells are $FoxP3^+$ [32]. In our study we used CD127 (IL-7R) as a marker for isolating regulatory T cells, as CD127 is negatively correlated to FoxP3 expression on Tregs [27]. CD127 is an extracellular marker on T cells and is suitable, as previously described [25], for discriminating Tregs as $CD3^+CD4^+CD25^+$ and $CD127^{-/low}$ with cell sorting. However, recent study suggests that there is no complete overlap between $CD3^+CD4^+CD25^+CD127^{-/low}$ Tregs and $CD3^+CD4^+CD25^+Foxp3^+$ Tregs and therefore $FoxP3^+$ and $CD127^{-/low}$ characterize different Treg populations in the human blood [33]. In our study we measured the level of Tregs in the blood of sarcoidosis patients using both gating strategies and found that the proportions of $CD127^{low/-}$ Tregs were slightly higher than those of $FoxP3^+$ Tregs, but for both strategies the levels of Tregs as proportions of $CD4^+$ T cells were significantly increased in sarcoidosis patients.

Different study results may also partly originate from differences in patient population characteristics. Whereas Miyara *et al* [21] included patients with inactive disease, we only included newly diagnosed patients with active disease. Interestingly, it has recently been suggested that Treg function may be different between active and inactive sarcoidosis,

on the basis of the analysis of a BCG-coated bead model for *in vitro* granuloma formation [21]. *In vitro* PBMCs formed granulomatous infiltrates around the coated beads. Using $CD25^+$ T cell depletion as a means to deplete Treg, this study showed that Tregs in active sarcoidosis patients did not influence the *in vitro* growth rate of granuloma, whereas healthy control Tregs and Tregs of an inactive sarcoidosis patient inhibited the growth rate [21].

In airway biopsies of sarcoidosis patients we did not find Tregs in all the granulomas and when they were present, they were localized in the inside as well as in the outer peripheral layer of the granulomas. We did not find any correlations of granulomas with or without Tregs with BAL $CD3^+$ lymphocyte number, CD4/CD8 ratio, or clinical disease stage in sarcoidosis. The fact that we and others do not find any correlation of Tregs with the amount of granulomas or clinical parameters, may imply that Tregs do not play a major role in granuloma formation or maintenance. Nevertheless, it has been reported that more Tregs in lymph nodes or renal granulomas of sarcoidosis patients expressed the proliferation marker Ki67, when compared with healthy controls or patients with nongranulomatous interstitial nephritis [21]. However, it might also be argued that the numbers and location are only marginally important, since the function of Tregs is considerably impaired in sarcoidosis. While our studies were ongoing, it was very recently shown that those Treg cells present in affected lung lesions were mainly derived from activated natural Treg cells and exhibited reduced repressor capacities despite high IL-10 and TGFβ levels[34]. This was associated with shortened telomeres, indicating an extensive cell division history. However, these Tregs did express IL-4, which induces fibroblast expansion and mast cell activation. The authors [34] also found that the repressive capacity of blood Treg cells was not impaired compared to age-matched healthy donors. This is in contrast to our findings, since we noticed reduced repressor function and reduced survival of sarcoidosis Tregs. Tregs use apoptosis as a mechanism for homeostasis. Under physiological conditions fast proliferating Tregs exists, which are more sensitive for FasL apoptosis, next to slow proliferating Tregs. The suppressive function of innate Tregs is independent of proliferation. This duality in proliferation and sensitivity to apoptosis may prevent accumulation of an excess of Tregs under baseline conditions [19].

We did not find enhanced expression of Fas or reduced IL-2R expression on the cell surface of Tregs from sarcoidosis patients. Interestingly, we previously observed that in sarcoidosis patients DCs may have an intrinsic propensity to produce higher levels of TNFα (Ten Berge et al., this thesis, chapter 4) and in the lungs of sarcoidosis patients levels of TNFα were found the be increased [35]. As TNFα can induce a reduced suppressive function of Tregs [36], elevated TNFα may well contribute to reduced Treg function in sarcoidosis. Nevertheless, further studies are required to identify the molecular mechanisms responsible for the reduced survival or increased apoptosis of Tregs in sarcoidosis.

For example, micro array comparisons of the expression profiles of sorted Treg fraction from sarcoidosis patients and healthy controls should reveal reduced expression of survival genes or increased expression of apoptosis-associated genes.

In summary, we report here that in newly diagnosed sarcoidosis patients the numbers of Tregs in blood and BAL were increased. However, Tregs from sarcoidosis patients were not capable of inhibiting T cell proliferation and showed reduced survival *in vitro*. Decreased Treg survival was independent of IL-2 and contrasted with normal survival of $CD4^+$ T cells. It is therefore conceivable that Tregs contribute to the pathogenesis of sarcoidosis because they are not capable to dampen the excessive T cell proliferation in the lung, resulting in a $CD4^+$ T cell alveolitis.

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General discussion and concluding remarks

Pulmonary sarcoidosis is characterized by the presence of a T cell alveolitis in the lungs of patients. These T cells in the lung have a Th1 profile, secreting high levels of IFNγ, IL-2 and TNFα [1]. Furthermore, an oligoclonal expansion of T cells in both lungs and peripheral blood can be seen in sarcoidosis patients [2, 3]. These findings are characteristic for a classic, antigen-driven immune response. However, in sarcoidosis the provoking antigen remains undefined.

Antigens entering the alveolar space will be recognized by dendritic cells (DCs) in the alveolar space and the bronchial epithelium. DCs take up the antigens and mature afterwards expressing their co-stimulatory molecules. They travel to the regional lymph nodes attracted by CCL19. In the lymph nodes DCs present the antigens to naive T cells and secrete pro-inflammatory cytokines. In sarcoidosis most activated T cells become Th1 cells [4], but also regulatory T cells and Th17 cells are found in the lungs of sarcoidosis patients, as shown in this thesis. Activated T cells travel back from the lymph nodes to the inflammatory sites of the lung.

1. DCS IN SARCOIDOSIS

By presenting an unknown antigen DCs could be responsible for initiating the immune response characteristic for sarcoidosis. But paradoxically, it has been suggested that DCs at the site of disease are phenotypically and functionally immature. In chapter 4 of this thesis, we provide several lines of evidence for the involvement of mature, functional DCs in pulmonary sarcoidosis. First, we found that in patients with pulmonary sarcoidosis, myeloid DCs are increased in number in BAL, granuloma containing mucosal biopsies and blood. Second, myeloid DCs in BAL and granuloma-containing biopsies, but not in blood, show increased expression of various maturation markers, including CD40, CD80 and CD86. Third, mDCs from BAL are very well capable of inducing T cell proliferation and differentiation and show no signs of anergy. Furthermore, we did not observe functional differences between DCs that were differentiated *in vitro* from monocytes present in peripheral blood from sarcoidosis patients or healthy controls in co-cultures with T lymphocytes. We only found that monocyte-derived DCS from sarcoidosis patients induce more TNFα, a cytokine pivotal in sarcoidosis pathogenesis and treatment. Taken together, these results indicate that in sarcoidosis DCs are not anergic, but more mature and functionally intact at the site of disease and associated with granuloma formation.

Previous studies reported different results in maturation status and number or proportions of mDCs and pDCs in blood or BAL from sarcoidosis patients [5-7]. We found increased proportions and numbers of mDCs in peripheral blood from sarcoidosis patients, when compared with healthy controls. In contrast, Mathew *et al*. reported that numbers of mDCs did not differ between patients and healthy controls [6]. However,

these results are not directly comparable, because Mathew et al. analyzed whole blood samples after lysis of red blood cells and calculated the number of DCs as a proportion of total leucocytes, thus including granulocytes, from a concurrent drawn blood sample, while we calculated mDCs as the proportion of mDCs within the peripheral blood mononuclear cell (PBMC) fraction. Ota *et al.* reported decreased numbers of mDCs and pDCs in blood of sarcoidosis patients, calculated by using proportions of a DC-enriched cell population [5]. Similar numbers and proportions of mDCs and pDCs in the BAL of sarcoidosis patients and controls were reported [7]. Indeed, we found similar proportions of mDCs in the BAL of sarcoidosis patients and healthy controls as well. However, paralleling an increased cellularity in our patient group, a typical phenomenon in sarcoidosis BAL [4], we found a significant increase in numbers of mDCs/ml in the BAL. In contrast to Lommatzsch *et al.*, we found increased numbers and proportions of pDCs in the BAL [7]. We performed BAL with 200 ml saline, compared to 100 ml by Lommatzsch *et al*., which perhaps increased our yield of pDCs. Next to methodological variations in the quantification of DCs, variations in defining DC subsets might as well be responsible for differences in reported cell numbers. The mDCs in the studies mentioned above were defined in a similar manner as in our experiments (lineage mix-, HLA-DR+, CD11c+); however, pDCs were not. We defined pDCs as lineage mix-, HLA-DR+, CD11c-, CD123+ cells [8]. Ota *et al.* defined pDCs as lineage mix -, HLA-DR+, CD11c-, cells without using CD123, while Lommatzsch et al. defined pDCs as lineage mix-, HLA-DR+, CD123+ cells. It is conceivable that these populations used by Ota et al. contained other cells as well [9] because HLA-DR+, lineage mix-CD11c- cells are not exclusively CD123+. This fraction therefore also contain other cells, e.g. in blood at least also CD34+ hematopoietic stem cells, but in BAL this population is not well defined yet [10].

The next step was to isolate DCs from the lungs in sarcoidosis patients to analyze their function. Over the years it has proven to be difficult to analyze purified DCs from the lung and to establish methods that do not have any effect on the phenotype or the function of DCs. Multiple studies on the function of human lung DCs used lung tissue, mostly resection tissue after surgery [8,11]. This approach has several limitations. First of all, the need for resection material limits the number of different diseases that can be studied: most pulmonary diseases do not need invasive surgery for diagnosis or treatment. Secondly, to obtain DCs from lung tissue a digestion step is required. This digestion step is likely to induce phenotypical or functional changes in the cells, with unknown effects on the outcome of subsequent functional tests. BAL is a minimally invasive and low-risk method to obtain cells from the lung. The cells collected in the BAL fluid are suggested to be representative for the interstitial and alveolar compartment of the lung [12]. But, the number of DCs in the BAL is very low [7], which makes isolation and purification very difficult.

Another problem in the last few years was to identify the proper DC markers to define a pure population of DCs. Several studies using flow cytometric sorting were only able to test a DC-enriched fraction instead of pure DCs. Such a DC-enriched fraction is not suitable to test the exact functional role of DCs in different diseases [13-15]. With the use of coated beads to isolate DCs cells from resection tissue or BAL more pure populations were isolated [11, 16], but the beads coated to the DCs are likely to induce phenotypical or functional changes to the DCs.

In our method the cells are sorted by flow cytometry, which involves only labeling with monoclonal antibodies. Such monoclonal antibodies are much smaller than beads, which makes it less likely that they interfere with the function of the sorted cells. During the cell sorting procedure antibodies to CD11c and HLA (human leukocyte antigen)-DR are bound to the DCs. CD11c, or Integrin alpha X (complement component 3 receptor 4 subunit) protein ITGAX, is a membrane molecule of which the exact cellular role is unclear. In the field of tumor biology, targeted delivery of tumor antigens to activated DCs via CD11c has been shown to induce robust antigen-specific immune responses *in vivo* [17]. Targeting DCs with antigens via CD11c resulted in efficient antigen processing, presentation on MHC classes I and II products, and robust $CD4^+$ and $CD8^+$ T-cell immunity [17]. So, therefore, CD11c seems to play a role in T cell activation by DCs. HLA-DR, present on dendritic cells is up regulated after activation of the dendritic cells. The function of the HLA-DR molecule is to present the antigens to the TCR on the cell surface of T cells [18]. HLA-DR is also up regulated on activated B cells, and it has been shown that antibodies reactive with MHC class II antigens may inhibit human B cell activation [19]. In addition to the classical function in antigen presentation in DC-T cell interaction, cell surface MHC class II molecules can function as receptors to mediate reverse signal transduction after ligation with agonist antibodies, T cell antigen receptors or CD4 molecules. Engagement of cell surface MHC class II may regulate cell adhesion, cytokine production and the expression of costimulatory molecules on DCs [20]. We cannot formally exclude the possibility that binding of the antibodies to CD11c or HLA-DR had some effects on DCs, but we did not find any evidence for activation of DCs on the basis of cell surface markers tested. Also, in our hands, the FACS antibodies we used against CD11c and HLA-DR did not block the T cell activating function of DCs, when we tested the initiation of T cell proliferation, activation and cytokine production of the isolated dendritic cells.

After sorting, the purity of the sorted fractions were high and the morphology showed nicely all typical characteristics known to be distinctive for mDCs and pDCs. The mDCs isolated with our method were capable of contacting with naïve T cells as recorded by confocal microscopy. When incubated with allogenic CD4+ naïve T cells, these mDCs demonstrated potent stimulatory capacity of T cells. Unfortunately, we were not able to sort enough pDCs from BAL for functional analysis.

In sarcoidosis the number of mDCs in the BAL is increased and using our method we were able to isolate pure mDCs for functional analysis. We found that mDCs of sarcoidosis patients were very well capable of activating naive T cells *in vitro* and they do not show signs of decreased immunoreactivity. But despite enhanced expression of maturation markers, mDCs isolated with a high degree of purity from BAL from sarcoidosis patients did not induce enhanced T cell proliferation. Several explanations are possible: Firstly, mature DCs from BAL from sarcoidosis patients may have already interacted with T cells and are beyond their functional maximum upon isolation from BAL. This is supported by studies showing that after initial exposure to a stimulus, DCs produce IL-12, important for inducing T cell proliferation for a limited period of 10-18 hours [21]. Indeed, we found low IL-12 levels at day 5 in the supernatant of the T cell co-cultures. Furthermore, in the field of cancer research it has been reported that phenotypically mature DCs release diminished IL-12 [22, 23]. Thirdly, it is perhaps the sheer number of DCs and not the maturation status per cell that determines the *in vivo* outcome of T cell proliferation. Although the number of DCs in BAL is in general very low, DCs in the BAL are thought to reflect only a small percentage of pulmonary DCs [24]. We found increased numbers of DCs per ml of BAL in sarcoidosis patients, perhaps indicating increased numbers of interstitial DCs that may travel to the draining lymph node for antigen presentation. Future investigations in the lymph node compartment may shed light on this issue. Finally, influx of CD4+ T cells into the bronchoalveolar space may be determined primarily by chemotactic factors. Our data on enhanced levels of MIG and IP10 in BAL, well known chemotactic factors for Th1 cells, support this notion and confirm earlier reports [25, 26].

We found that monocyte-derived DCs (mo-DCs) from patients with sarcoidosis, which were not influenced by the micro-environment of the lung, induced increased TNFα release upon interaction with CD4+ T cells, when compared to mo-DC from healthy controls. TNFα is an important Th1 cytokine involved in the pathogenesis of sarcoidosis and granuloma formation and maintenance and currently an essential target for treatment [27]. Our results indicate that DCs are intrinsically different in sarcoidosis patients. Interestingly, a polymorphism in the gene encoding TNFα was linked to altered TNFα expression and an altered disease course in sarcoidosis [28, 29]. It is therefore conceivable that the increased TNFα release is directly connected with the polymorphisms in the TNFα locus, although many other genetic or environmental factors may explain our findings.

2. TH17 CELLS IN SARCOIDOSIS

In sarcoidosis it is clear that cytokines play an important role in granuloma formation, but to date mainly Th1 cytokines have been described in the pathogenesis of sarcoidosis

[4, 30]. When BAL CD4+ and CD8+ T cells were stimulated in vitro, Wahlstrom et al. [31] showed at the single cell level that these cells secreted high levels the cytokines interferon gamma (INF-γ), interleukin-2 (IL-2) and TNFα. In the BAL fluid of sarcoidosis patients and at sites of inflammation also increased levels of Th1 cytokines are seen [32, 33]. Furthermore, expression of Th1 markers on pulmonary T cells in sarcoidosis is found [34].

Recently another subset of T helper cells, the Th17 cells, has been described and was found to be increased in peripheral blood of sarcoidosis patients [35]. We provide further evidence for the role of Th17-lineage cytokines in sarcoidosis. We found increased proportions of circulating IL-17A$^+$ memory T helper cells and IL-17A-IFNγ double producing cells, and in BAL samples of sarcoidosis patients we detected high levels of IL-17A$^+$ cells, and in particular IL-17A/IFNγ and IL-17A/IL-4 double-producing cells, which are normally very rare. Th1 and Th17 cells can work cooperative to induce tissue damage as described in autoimmune uveitis and experimental autoimmune encephalitis [36, 37]. The recently identified double-producing Th17/IFNγ CD4$^+$ T cells in Crohn's disease [38], in coronary atherosclerosis [39] and in MS patients [40] are thought to be more pathogenic, because these cells e.g. preferentially cross the blood brain barrier in MS. IL-17A/IL-4 double producing cells like we found in blood and BAL of sarcoidosis patients, were also observed in patients with chronic asthma [41]. A novel Th2/Th17 subset was reported, co expressing the transcription factors GATA3 and RORγt and coproducing T_H17 and T_H2 cytokines [42]. Classical T_H2 memory/effector cells have the potential to produce IL-17 after stimulation with proinflammatory cytokines IL-1β, IL-6, and IL-21. The number of IL-17/T_H2 cells was found to be significantly increased in blood of patients with atopic asthma. [42]In a mouse model of allergic lung diseases, IL-17–producing CD4$^+$ T_H2 cells were induced in the inflamed lung and persisted as the dominant IL-17–producing T cell population during the chronic stage of asthma. Compared with classical T_H17 and T_H2 cells, antigen-specific IL-17–producing T_H2 cells induced a profound influx of heterogeneous inflammatory leukocytes and exacerbated asthma. These findings highlight the plasticity of T_H2 memory cells and suggest that IL-17–producing T_H2 cells may represent the key pathogenic T_H2 cells promoting the exacerbation of allergic asthma [42]. In sarcoidosis IL-4, a Th2 cytokine, is thought to have pro-fibrotic features, like induction of fibroblast growth and collagen production, which have been implicated in later stages of sarcoidosis [43].

In granulomatous disease, the exact role of IL-17 is still unknown but particularly in mycobacterium infections several published results are pointing towards IL-17 involvement in the pathogenesis of this other granulomatous disease. In a Mycobacterium bovis infection model, IL-17A expression was detected early after pulmonary infection and IL-17-deficient mice showed impaired granuloma formation [44]. In humans living in regions with high prevalence of Mycobacterium tuberculosis infection, peripheral blood contains high frequencies of IL-17+ and IL-22+ memory T helper cells, which may

have protective properties against tuberculosis [45]. In mouse models and in humans with active pulmonary tuberculosis both IL-17- and IL-22-producing CD4+ T cells and IL-17+ γδ T cells were shown to contribute to the anti-mycobacterial immune response in human [46]. Also other diseases which are accompanied by granuloma formation show involvement of Th17 cells [47, 48]. It is presently unclear which mechanisms would initiate a Th17 response in sarcoidosis. One possibility would be persistence of pathogenic antigens, since T cell responses to Mycobacterium tuberculosis catalase-peroxidase have been identified in sarcoidosis [49]. Alternatively, cross-reactivity between microbial and auto-antigens may play a role. It is conceivable that the involvement of Th17 cells in sarcoidosis points to an autoimmune process that is comparable to various other IL-17A-driven autoimmunity disorders, such as autoimmune uveitis, rheumatoid arthritis, inflammatory bowel disease or psoriasis.[50-53]

We found that IL-22 cells are not increased in the blood or BAL of sarcoidosis patients compared to controls. These results do not match earlier reported results were a decreased level of IL-22 in the BAL and serum of patients were measured [54]. In contrast we see an increase of IL-22 cell in the subepithelial area of bronchial biopsies. These different results and the different location of Th17 cells and IL-22 cells in the bronchial biopsies support the hypothesis that IL-22 can produced independently from Il-17 by Th22 cells [55, 56].

IL-17 induces the production of many other pro-inflammatory cytokines (such as IL-6, G-CSF, GM-CSF, IL-1β, TGF-β, TNFα), chemokines (including IL-8, GRO-α, and MCP-1), and prostaglandins (e.g., PGE_2) from many cell types (fibroblasts, endothelial cells, epithelial cells, keratinocytes, and macrophages) [57, 58]. The release of cytokines causes many features, such as airway remodeling, a characteristic of IL-17 responses [59] which can be important in later stages of sarcoidosis where fibrosis is found in pulmonary tissue [1]. The increased expression of chemokines attracts other cells including neutrophils but not eosinophils [60]. IL-17A can also induce enhanced secretion of CCL20, a chemoattractant for dendritic cells, resulting in an increased recruitment of inflammatory dendritic cells to the lungs [61]. And in Langerhans cell histiocytosis an IL-17A-dependent pathway for DC fusion, which was highly potentiated by IFN-γ and led to giant cells, was described. Giant cells are also present in sarcoid granulomas [48]. As earlier reported, TNF-α, which can be induced by IL-17 is an important cytokine in the pathogenesis of sarcoidosis and a main target for treatment at this time [27].

IL-22 can contribute to immune disease through the stimulation of inflammatory responses, like in psoriasis or RA, but can also have an protective role in inflammation as in hepatitis or in IBD [62]. IL-22 also promotes hepatocyte survival in the liver [63]. Furthermore, IL-22 is expressed at barrier surfaces and can play a critical role in the maintenance of normal barrier homeostasis [62]. Mouse studies have identified a critical role for signaling by IL-22 through its receptor (IL-22R) in the promotion of antimicrobial

immunity, inflammation and tissue repair at barrier surfaces [64]. In more than 90% of sarcoidosis patients the lungs are affected and it is suspected that a sarcoidosis antigen is entering the body via the epithelial barrier in the airways. In the bronchial airway biopsies we analyzed, an increased subepithelial expression of IL-22+ cells was seen. It is very well possible that IL-22 plays a role in the pathogenesis of the inflammation seen in sarcoidosis. Recently, IL-22 was found to have a protective effect against pulmonary fibrosis [65]. It is therefore important to identify the precise role of IL-22 in fibrosis and in sarcoidosis, and therefore IL-22 is a crucial target for further research in sarcoidosis. In some contexts, the pro-inflammatory versus tissue-protective functions of IL-22 are regulated by the often co-expressed cytokine IL-17A [66].

In the alveolar space of sarcoidosis patients, mainly Th1 cells are present, resulting in increased levels of IFNγ, IL-2 and TNFα. These cytokines contribute to the inflammation and formation of granulomas as seen in sarcoidosis [67]. The Th2 cytokines IL-4, IL-5 and IL-13 are known to be important in inducing pulmonary fibrosis in e.g. systemic sclerosis of the lungs [68]. In sarcoidosis not all T cells in the BAL fluid are of the Th1 subset and there are a few IL-4+ Th2 cells present. Only a minor fraction of sarcoidosis patients develop a chronic course of the disease involving pulmonary fibrosis at a later stage. It is very well possible that also in sarcoidosis the Th2 cells, and not the Th1 cells, play a role in the initiation of fibrosis. A shift from Th1 to Th2 inflammation may evolve during the course of sarcoidosis, resulting in fibrosis at later stages. Although this scenario may seem likely, detailed studies on this subject are lacking to date. We propose that there might be a role for the IL-17/IL-4 double producing T cells in this context, since we found significantly elevated proportions of these cells in sarcoidosis patients and IL-17 has also profibrotic features [69]. In our study we only included stage I and stage II patients, because we aimed to identify cytokines that are involved in diseases initiation. Obviously, it would be interesting to analyze stage III and IV sarcoidosis patients to investigate if there are increased proportions of IL-4-expressing or IL-17/IL-4 double-producing T cells in BAL or peripheral blood. Furthermore, follow-up of patients is expected to be informative, as we see considerable variability in cytokine expression levels between patients. Such analyses may e.g. reveal whether patients with high levels of IL-17/IL-4 double producing T cells have an increased risk of developing pulmonary fibrosis, or if patients with increased numbers of IL-17/IFNγ expressing cells develop a more severe form of sarcoidosis.

3. TREGS IN SARCOIDOSIS

Tregs that are present in the in the alveolar space in sarcoidosis patients should have a suppressive effect on the Th1 alveolitis. We found increased numbers of Tregs in the al-

veolar space, in line with previous reported data. But Idali et al showed a decrease in the percentages of BAL and blood FoxP3$^+$ Tregs [70]. In this study a substantial part of the included patients were diagnosed as Löfgren's syndrome, an acute subtype of sarcoidosis with typical clinical aspects and a good prognosis. To the best of our knowledge no data on the numbers of Tregs in Löfgren's syndrome have been reported but it is conceivable that in patients with Löfgren's syndrome Treg cell numbers are reduced, which could explain the finding of reduced Treg numbers in the patient group analyzed in this study. Interestingly, Prasse et al. [71] very recently showed that the numbers of Tregs in the BAL were reduced in patients with a chronic course of sarcoidosis, and were significantly higher in patients with spontaneous remission, but not increased compared to healthy controls [71]. This finding therefore indicates that during sarcoidosis disease course the numbers of Tregs are subject to change, which could explain the differences in reported Treg numbers in sarcoidosis.

Despite the increased numbers of Tregs present in the alveolar space ([72]; chapter 6) the Th1 alveolitis remains. These findings suggest a diminished suppressive function of the Tregs, possibly reflecting an intrinsic functional defect in Tregs in sarcoidosis. Tregs are capable of inhibiting immunopathologic inflammation by CD4$^+$ and CD8$^+$ T cells in different ways. Tregs can suppress the function of effector T (Teff) cells by IL-2 depletion and can modulate their cytokine composition by secretion of TGFβ and IL-10. In this context, it has previously been known that IL-2 depletion also reduces the synthesis of IFNγ by human T lymphocytes [73]. Furthermore, Tregs are capable of direct cytotoxic killing of T cells by perforin/granzyme or Fas ligand (FasL) [74]. Due to low cell numbers in the BAL, we were not able to isolate Tregs from BAL for detailed *in vitro* investigations, but we did examine the function of peripheral blood Tregs *in vitro*. An altered suppressive activity of regulatory T cells in sarcoidosis has previously been reported by Miyara et al. [72], who showed *in vitro* that Tregs from sarcoidosis patients exhibited good anti-proliferative activity, but did not completely inhibit TNFα and IFNγ secretion of autologous CD4$^+$ T cells. We found in our study that Tregs of sarcoidosis patients did not have an antiproliferative effect on activated CD4$^+$ T cells *in vitro*. This is in contrast to the results published by Miyara et al. [72]. As previously discussed in chapter 6, over the last few years different markers have been described to identify Tregs and not all markers comprise the same cell populations. The Treg populations tested were slightly different in the two studies, as were the patient populations according to disease stage, either of which may explain the discordant study results. In this context, it is important to note that it has been found that FoxP3$^+$ Tregs suppress early stages of granulomas formation but have little impact on established sarcoidosis lesions [75].

In our study we show that Tregs of sarcoidosis patients manifest a decreased suppressive capacity *in vitro*. This could very well be explained by impaired survival of Tregs, as at day 5 of our MLR cultures almost all sarcoidosis Tregs had died. Tregs use apoptosis as

mechanism for homeostasis [74]. Interestingly, in sarcoidosis different studies show that polymorphisms in apoptosis-related proteins Fas and annexin 11 are associated with an increased disease risk [76, 77]. Tregs from sarcoidosis patients did not show enhanced expression of Fas or reduced IL-2R.

Tregs exist in two different types, naïve Tregs (CD45RA+) and memory or activated Tregs (CD45RA-). In sarcoidosis the increase in Tregs is mostly due to an increase in memory Tregs [75]. Miyara *et al.* [78] found even a decrease in resting naïve Tregs. Recent published results show that after activation naïve Tregs turn into memory Tregs and start to proliferate. These memory Tregs are highly activated and die very quickly after isolation *in vitro* [78, 79]. Furthermore, another very recent study, using the markers CD7 on Tregs, shows that $CD7^-$ Tregs (activated Tregs) are increased in sarcoidosis and exhibit shorter telomeres, indicating more rounds of cell division. In this study it was also shown that *in vitro* activated $CD7^-$ Tregs from BAL - but not from peripheral blood - manifested decreased suppressive activity in sarcoidosis patients, when compared with healthy controls [80]. These conflicting results even further increased the need to subdivide Tregs into different functional subtypes. According to published results based on telomere length analysis, most of the Tregs present in sarcoidosis granulomas lesions are memory Tregs, in contrast to Tregs isolated from healthy controls. It is very well possible that the highly activated sarcoidosis Tregs which have undergone more rounds of amplification die more quickly, resulting in a diminished suppressive function. In addition, Treg function in the lung can also be impaired by mechanisms that are not intrinsic to the Tregs, e.g. the micro-environmental levels of TNFα in the lungs of sarcoidosis patients [81]. TNFα can induce a reduced suppressive function of Tregs [82]. In addition, it has been reported that in sarcoidosis lymphocytes in the BAL are less sensitive to apoptosis, because of enhanced expression of the anti-apoptotic protein Bcl-2 [83]. Since apoptosis of proliferating T cells induced by Fas-FasL interaction is one the most important mechanisms used by Tregs as a means to dampen T cell proliferation [74], it is conceivable that Tregs are less functionally effective in sarcoidosis.

Future experiments, including expression profiling of sorted Treg populations of sarcoidosis patients and healthy controls, should reveal if there are intrinsic differences in the expression levels of genes involved in survival or apoptotic pathways in Tregs. In this context, it is important that it now has become technically possible to perform RNA expression profiling studies on very limited numbers of cells. As a result, it should be feasible to perform genome-wide quantitative analyses of transcripts of purified Treg (sub) populations, thereby comparing healthy controls and sarcoidosis patients, both in peripheral blood and in BAL. Finally, it would be interesting to investigate if sarcoidosis is accompanied by intrinsic defects in development, proliferation, maintenance or survival of Tregs, e.g. by analysis of in vitro differentiated Tregs from naïve T cells using TGFβ.

4. LUNG MUCOSAL BIOPSIES

To learn more about the localisation of the different cell types in the lungs of sarcoidosis patients we collected bronchial mucosal biopsies and stained these biopsies with different markers to identify DCs, Tregs, and IL-17 and IL-22+ T cells. Earlier reports showed that sarcoidosis is associated with mature DCs in lymph node granulomas expressing the lysosome-associated membrane glycoprotein DC-LAMP, which is induced upon DC maturation. DC-LAMP+ DCs were typically located in the lymphocyte layer of granulomas and adjacent to CD3+ T cells, suggesting functional DC-T cell interaction [5]. In addition, we found that mDCs in granuloma-containing airway biopsies were increased in number and maturation (in particular CD86 expression), when compared with healthy controls. Intriguingly, also in non-granulomatous biopsies from sarcoidosis patients there was a tendency for increased numbers and maturation of mDCs, compared with healthy controls, although this was not statistically significant. It has been demonstrated that pulmonary granulomas in sarcoidosis are distributed along the lymphatic routes [84]. It is very well conceivable that DCs are activated by antigen and involved in granuloma formation, while travelling in the lymphatic routes to the draining lymph node. We found IL-17-producing T cells around and in the granulomas, but not in non-granulomatous lung tissue from sarcoidosis patients or in healthy controls. This is consistent with earlier reports, in which Th17 cells were also identified around the sarcoidosis granulomas in the lung [35]. We found very few Tregs in and around the granulomas and we did not find any correlation of granulomas with or without Tregs with BAL CD3+ lymphocytes, CD4/CD8 ratio, or disease stage course in sarcoidosis. Perhaps Tregs are present in all the granulomas, but express lower levels of FoxP3 and were not stained by immunohistochemistry. Because we and others do not find any correlation of Tregs with the amount of granulomas or clinical parameters [75] and because Tregs do not seem to affect the grow rate of in vitro granulomas [75], it is not very likely that Tregs play a major role in granuloma formation. Very recently, Tregs in sarcoidosis were found to secrete IL-4 in blood and BAL fluid [80]. IL-4 can sustain granuloma formation through fibroblast amplification and the activation of mast cells. Chronic sarcoidosis is complicated by the formation of pulmonary fibrosis and it would be very interesting to see if Tregs associated with granulomas in lung tissue do also secrete IL-4 and if these cells will induce the formation of fibrosis. Also IL-17-producing cells have pro-fibrotic features as discussed above, and because we found IL-17/IL-4+ T cells in pulmonary sarcoidosis, these cells can also be important in fibrosis formation. Later on we will discuss the recently found plasticity of the T helper cells lineages, but it can very well be possible that the Treg/IL-4 and IL-17/IL-4 T cells will be T cells that are undergoing a transformation into Th2 cells in a later stage of sarcoidosis.

Increased numbers of IL-22 producing T cells are found in the submucosal layer of sarcoidosis lung biopsies. Il-22 is thought to play role in anti-microbial host defence [85] but, as discussed above, the exact role of these cells is sarcoidosis is still unclear and needs further research.

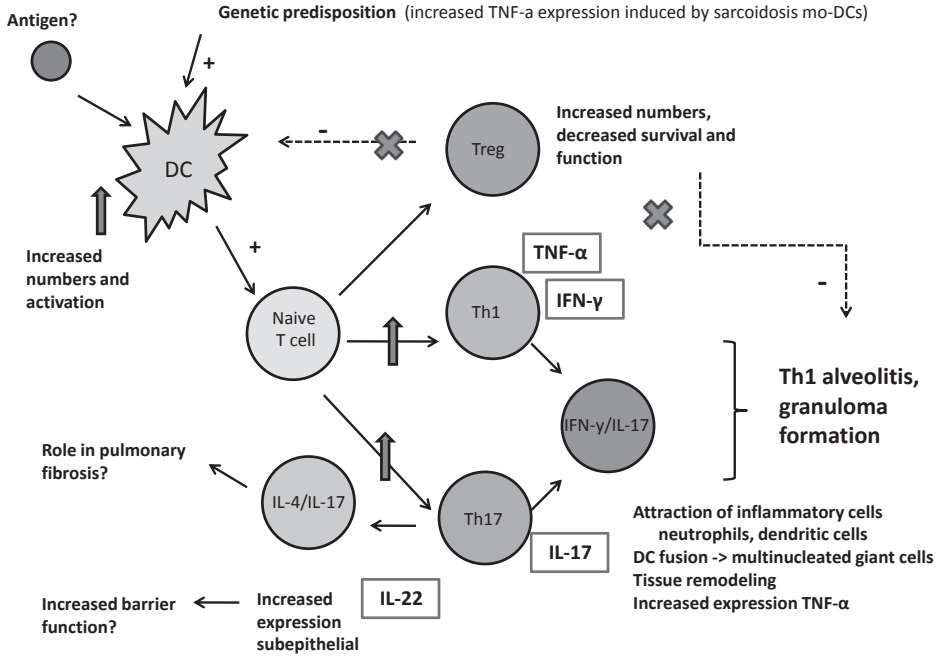
5. MICRO-ENVIRONMENTAL INFLUENCE AND DC- T CELL INTERACTIONS

In sarcoidosis raised plasma levels of the active form of vitamin D, 1,25 dihydroxyvitamin D3, are found, resulting in hypercalcaemia and hypercalciuria leading to kidney failure. In sarcoidosis the source of active vitamin D is not only the kidneys, but active vitamin D is also produced locally. This takes place particularly at sites of inflammation, the granulomas, by 1 alpha-hydroxylase produced by activated macrophages in the granuloma [86, 87]. The vitamin D receptor is present on many immune cells, such as macrophages, DCs, T and B cells, mainly after activation [88]. In the presence of active vitamin D3 tolerogenic DC are initiated with a less mature phenotype and an altered expression of cytokines. The overall effect is a switch from the Th1/Th17 response to the Th2/Treg profile [89-91]. Interestingly, 1,25 dihydroxyvitamin D3 was shown to directly modulate human Th17 polarization, accompanied by suppression of IL-17A, IL-17F, TNFα, and IL-22 production by memory T cells, sorted by FACS from patients with early rheumatoid arthritis (RA), another disease in which Th1/Th17 cells play an important role [92]. The presence of 1,25(OH)(2)D(3) reduced interleukin-17A (IL-17A) and interferon-gamma levels and increased IL-4 levels in stimulated PBMCs from treatment-naive patients with early RA [92]. T cells activated in the presence of active vitamin D and IL-2 show a T-regulatory phenotype with upregulation of CTLA- 4 and FoxP3 and less expression of IFN-y, IL-17 and IL-21, and these T cells are able to suppress the proliferation of normally responsive T cells [93]. The presence of active vitamin D locally in the granulomas of sarcoidosis patients can alter the function of the local immune cells. Perhaps after formation of the granulomas the local vitamin D levels influence immune cells to a more tolerogenic state, in which they are incapable to clear the antigens resulting in the persistence of granulomas. Because vitamin D-activated tolerogenic DC initiate a more Th2-like profile, this mechanism can contribute to the initiation of pulmonary fibrosis [43], because IL-4 is important in pulmonary fibrosis, as discussed above .

We were not able to completely phenotype or functional test the DCs around the granulomas, but it should be informative to investigate if DCs locally around the granulomas show functional differences or initiate more Th2 cytokines.

6. T HELPER CELL LINEAGE PLASTICITY AND THE IMPLICATIONS FOR SARCOIDOSIS

Besides the function assessed from individual cell types, the various cells types involved in sarcoidosis obviously have effects on each other (Figure 1). Together with the plasticity of the different T helper lineages in relation to disease progression, this makes it even more difficult to elucidate the exact role of individual cells types in the pathogenesis of sarcoidosis. As described in this thesis, DCs are crucial antigen presenting cells that are capable of initiating an immune response against invading pathogens. Hereby, they will activate different T cell populations, including Th1, Th17 cells and Tregs. DCs are able to activate naturally occurring Tregs and to induce Tregs from CD4+CD25-Foxp3- T cells. The activation of the various T cell subtypes depends on the antigens presented by DCs and micro-environmental factors at the time of presentation (as discussed in chapter 2). In their turn Tregs are able to influence the function and phenotype of DCs. Interaction of Tregs with DCs *in vitro* can result in down regulation of co-stimulatory molecules on the DC membrane and in upregulation of the inhibitory molecule B7-H3 [94, 95]. Taken together, this may result in an impaired T cell stimulatory function of DCs. Furthermore, a decreased production of DC-associated inflammatory molecules was seen, together



Figuur 1. Schematic overview of the main findings of DCs and T cells in pulmonary sarcoidosis and their role in the pathogenesis of sarcoidosis.

with an enhanced expression of IL-10. Treg-educated DCs were poor stimulators of T cell proliferation [96]. Chronically activated DCs, as in autoimmune diseases, can escape surveillance by Tregs and can generate activated T cells that are refractory to suppression by Tregs [96]. Moreover, prolonged exposure to TNFα, which is an important cytokine in the pathogenesis of sarcoidosis [27], can inhibit Treg function and their ability to inhibit DCs [82].

Very recently Treg were also found to decrease the inflammatory function of Th17 cells [97]. Perhaps more so than Th1 and Th2 cells, which appear to be committed cell populations due to the action of transcription factors T-bet and Gata3, respectively, the Th17 and Treg cell lineages display more plasticity. Naturally occurring Tregs develop during thymic selection, independently of TGFβ. In contrast, adaptive regulatory T cells and the Th17 lineage both need TGFβ for induction of the key transcription factor, FoxP3 and RORγt, respectively. The development of Th17 cells requires next to TGFβ also IL-6 [98-100], produced by DC, macrophages or monocytes activated by microbial products [101] or IL-21 produced by IL-6-stimulated T cells to establish an autocrine loop [102-104]. So in the absence of pro-inflammatory signals from the innate immune system, priming of naïve CD4+ T cells by antigen in a TGFβ environment promotes the development of Tregs, whereas activation in an environment where both TGFβ and IL-6 are available promotes Th17 development. TGFβ suppresses Th1 and Th2 development [105]. Naïve T cells stimulated with TGFβ alone were found to up regulate both Foxp3 and RORγt, although they failed to express IL-17 and progressively extinguished RORγt, as they further develop into Tregs. Likewise, Th17 differentiation induced by both IL-6 and TGFβ was shown to be accompanied by the transient expression of Foxp3 and RORγt, in which Foxp3 is subsequently extinguished [106]. Furthermore, Tregs show late plasticity as IL-17 and IFNγ expression by Tregs is reported [107-109], and mature Foxp3-expressing Tregs were found to differentiate into Th17 cells under the influence of IL-6 [108]. As mentioned above, Tregs were also found to produce IL-4 [80], and we identified IL-17/IL-4 double producing cells in BAL and peripheral blood, indicating that in sarcoidosis there is an evident overlap of Tregs and Th17 cells with the Th2 lineage.

TGFβ is present in the BAL of sarcoidosis patients but not in increased levels, compared with healthy controls [110]. In sarcoidosis patients with altered lung function an increase in TGFβ was reported [111]. TGFβ was also present in the histiocytes in granulomas of sarcoidosis patients [112]. Studies on *TGF*β gene polymorphisms in sarcoidosis patients showed that different genetic variants were associated with the risk of developing lung-fibrosis [113] and particular other variants were associated with protection to relapsing sarcoidosis [114]. Therefore, in sarcoidosis it is very well possible that higher levels of TGFβ can induce differentiation of Tregs or Th17cells, whereas lower levels may result in increased Th1 or Th2 differentiation. Thus TGFβ expression may have a major effect on the course of sarcoidosis. In sarcoidosis patients, concentrations of IL-6 were increased

in the BAL [110], and a previous study on granuloma formation *in vitro* implicated IL-6 as an important cytokine in granuloma formation [115]. More recently, IL-17 has been described as an important cytokine in granuloma formation in tuberculosis, chronic granulomatous disease and Langerhans cell histiocytosis [46, 48, 116]. As a result, it could be possible that in sarcoidosis the TGFβ present in developing granulomas, together with IL-6, favors the induction of Th17 cells. This would suppress the induction of Tregs, resulting in low numbers of Tregs in granulomas, in agreement with our findings. IL-17 favors granuloma formation and the presence of Th17 cells could therefore be crucial in orchestrating the persistence of granulomas in sarcoidosis. However, in BAL fluid from sarcoidosis patients an increased level of IL-6 was found [110], together with increased levels of Tregs [72], which cannot be explained by this mechanism. It is clear that further research is necessary to clarify the role of Tregs during early and later phases of inflammation and in the formation and persistence of granulomas.

Th17 cells do also show overlap in expression of cytokines with Th1 cells. As mentioned before, cells expressing both IL-17 and IFNγ, as we identified them in the blood and BAL of sarcoidosis patients, have been found previously in autoimmune diseases, such as multiple sclerosis and Crohn's disease [40, 117-119]. It has been shown that naïve T cells stimulated under Th17-polarizing conditions may still be pluripotent. Their re-stimulation with TGFβ induced subsets that co-expressed IFNγ and IL17 [118], whereas re-stimulation with IL-12 led to a Th1-phenotype expressing IFNγ [120]. Similar plasticity is reported for IL-4 which re-polarized Th17 to a Th2 phenotype [121]. As described before, because the double producers are possibly more pathogenic, this cell population is an interesting and important target for further research in sarcoidosis.

7. POSSIBLE TARGETS FOR TREATMENT AND FURTHER RESEARCH

At this moment the treatment of choice for sarcoidosis patients is systemic corticosteroids. But sustained treatment even with modest doses of systemic steroids may result in disabling side effects like increased infection risk, gain of weight, nervousness, restlessness, sleep problems, and various, less common, side effects. In refractory cases of sarcoidosis methotrexate, azathioprine or cyclophosphamide are used, all immune modulating drugs with several serious side effects, including increased risk of infections due to reduced immune responses and increased risk to develop lymphoreticular malignancies. More recently, TNF inhibitors have been investigated for the treatment of sarcoidosis, especially for chronic forms of sarcoidosis after failure of initial therapy [27, 122, 123]. In several studies a modest improvement was found, but patients can experience serious side effects, including infections (tuberculosis, histoplasmosis and other invasive fungal infections), congestive heart failure, neurologic events, malignan-

cies and autoimmunity. The overall results are unfortunately still disappointing [124]. The fact that a TNF-blocker does not completely resolve sarcoidosis is complementary to our results showing that sarcoidosis is not exclusively a Th1 disease: DCs, Treg and Th17 do also play a role in the pathogenesis. Blocking only Th1 cytokines does not resolve the disease and inhibiting the function of DCs and Th17 cells or increasing the function of Tregs can be possible targets for treatment in the near future. Several studies have been performed in autoimmune disease to induce tolerogenic DC as a means to diminish the inflammatory response. Possible candidates described are vitamin D3 and Estriol [91, 125]. In sarcoidosis, these substrates can have theoretically an ambivalent effect, leading on the one side possibly to less Th1 inflammation but on the other side perhaps also to a diminished clearance of putative antigens, resulting in a more chronic course of sarcoidosis. For Th17 cells ustekinumab, an human monoclonal antibody against IL-12 (Th1) and IL-23 (Th17) is currently tested in the treatment of chronic sarcoidosis [124]. In addition, an anti-IL-17A antibody is currently evaluated in rheumatoid arthritis and is likely to be a specific future treatment for sarcoidosis as well [126]. Very recently, suppression of Th17 differentiation and autoimmunity by a synthetic ROR ligand has been described [127], which will be a very interesting compound to test in sarcoidosis patients. Moreover, it is expected that combined therapy to two or more identified targets will be important in the future. Although in our studies we have been able to identify aberrant function of DC and various T cell subsets, it will be necessary to evaluate these aberrant parameters, such as DC activation, cytokine production by DC and T cells, Treg survival, etc. in more patients. Particularly, because sarcoidosis is a heterogeneous disease with different stages and a fluctuating disease course, varying from spontaneous remission to chronic disease with severe pulmonary fibrosis. We also found considerable diversity between different patients. Probably, variation in genetic predisposition in the patients contributes to this phenomenon[76,128] It would be informative to investigate if values for immunologic parameters correlate with disease stage or severity and whether biological markers or special features in cytokine profiles may have predictive value for the course of sarcoidosis in individual patients or their response to therapy.

To further elucidate the DC-T cell interaction in inflammatory human pulmonary disease, nowadays endoscopic ultrasound (EUS) or endobronchial ultrasound (EBUS) can be used as a minimally invasive technique to obtain material from mediastinal lymph nodes [129] Hopefully in the future this will help to better understand the immunopathogenesis of sarcoidosis and other pulmonary inflammatory diseases.

It would be very helpful to develop a representative granuloma mouse model that resembles the granulomatous inflammation seen in sarcoidosis. In the past, attempts have been made to establish such a model and very recently a few promising results have been published using mostly infectious agents like propioni bacterium acnes or mycobacterium superoxide dismutase A peptide together with beads, which show a Th1

inflammatory response and granulomas in the lungs.[130-133] At this moment in our lab a granulomatous mouse model using beryllium is tested. This model is may be promising as chronic beryllium disease resembles the pathogenesis of sarcoidosis. Importantly, it was found that many months after beryllium exposure mice showed evidence for increased DC activation, since membrane MHC class II and CD40 expression on DCs was enhanced. Moreover, proportions of T cells that showed a Th17 profile, including IL-17 and IFNγ co-production, was significantly increased, when compared with untreated control mice (M.v.N., A.K., unpublished results).

In summary, the results presented in this thesis show that in sarcoidosis not only Th1 cells play a role, but also DCs, Th17 cells and Tregs contribute to the pathogenesis (Figure 1). Thus, the pathogenesis of sarcoidosis is very complex and more research is required to elucidate the exact disease cause and to develop more specific personalized treatment strategies.

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Summary

Sarcoidosis is a multisystemic disease commonly affecting young and middle-aged adults. There is a genetic predisposition in females and in Afro-Americans. The etiology of sarcoidosis remains unknown to date, but it is conceivable that sarcoidosis is the result of immune responses to different environmental stimuli together with various genetic factors.

Various organs can be involved in the pathology causing diversity of symptoms, but in more than 90% of patients the lungs are affected. According to the chest X-rays, sarcoidosis has been classified in four different stages. Nowadays the diagnosis of sarcoidosis is confirmed on the basis of compatible clinical and radiological findings, together with histological evidence of non-caseating granuloma in one or more organs. An increased number of lymphocytes in bronchoalveolar lavage (BAL), together with an increased ratio of CD4/CD8 cells (>3.5) can also point to the diagnosis of sarcoidosis when biopsies cannot be obtained.

The pulmonary lesions in sarcoidosis can resolve spontaneously but can also result in pulmonary fibrosis, with pulmonary hypertension as a major complication. Two thirds of patients with sarcoidosis will have a spontaneous remission within a decade after diagnosis with few or no consequences. In most of the patients remission occurs within 3 years. One third of the patients have relenting disease leading to significant organ impairment. Recurrence after one or more years is uncommon but may develop at any age in any organ. Less than 5% of patients die from sarcoidosis and death is usually the result of pulmonary fibrosis with respiratory failure or cardiac or neurologic involvement.

By histology, sarcoidosis is characterized by the presence of non-caseating granulomas in involved organs. Clinically, sarcoidosis is characterized by peripheral blood T cell lymphopenia, a cutaneous anergy to tuberculin and other skin tests, and a T cell alveolitis in the lung with an increased CD4/CD8 ratio. Lung T cells in sarcoidosis patients are highly activated and secrete high levels of the Th1 cytokines interferon gamma (INFγ), interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNFα). To become activated, T cells need to engage the proper antigen peptide in a major histocompatibility complex (MHC-complex) together with correct co-stimulatory molecules presented by antigen presenting cells (APCs).

Dendritic cells (DCs) are professional antigen presenting cells. They are present at sides with high antigen exposure like the lungs. In most tissues, DCs are present in a so-called immature state, unable to stimulate T cells but extremely well equipped to capture antigens. After an antigenic stimulus in the lung, DCs and monocytes are rapidly recruited to the lung, most likely by the expression of CCL5 on the DCs. When the DC has captured an antigen, it will provide a signal to mature. The skills to capture antigens rapidly decline and the MHC class II complexes and expression of costimulatory markers are up regulated on the cell membrane. After activation DCs express CCR7 and CXCR4 and travel to the lymphoid tissue such as the spleen and lymph nodes expressing CCL19,

where they will complete their maturation. DCs present their antigen to the T helper cells inducing a strong T cell response. DCs do not only activate T cells, they can also tolerate T cells to self-antigens, to prevent autoimmune reactions. Moreover DCs can influence the polarization of naïve T cells into Th1, Th2, Th17 or T regulatory direction depending on the type of microorganism that is recognized and the tissue factors present on the site of activation. In the human lung three distinct DC populations can be recognized, myeloid dendritic cells (mDC) type 1, mDC type 2 and plasmacytoid dencritic cells (pDCs), with different expression of receptors, different T cell stimulative capacities and production of different cytokines after stimulation.

As DCs are the most important APC in the lung, we hypothesized that DCs could play a role in the pathogenesis in sarcoidosis. In addition, it is known that Th1 cells play an important role in sarcoidosis, but less is known about the other T helper cells and their function in sarcoidosis. In this thesis we intended to clarify the role of DCs, Th17 cells and Tregs in the pathogenesis of sarcoidosis. A short overview of the clinical presentation and the immunopathology of sarcoidosis, as well as the role of DCs and T cells in the lung is given in **Chapter 1**.

In sarcoidosis DCs are present in the lymphocyte layer surrounding the granulomas making contact with the lymphocytes present. There is no consensus about the number of DCs in the peripheral blood, but the T cell activating function of mDCs in the blood seems to be decreased compared to controls despite the normal maturation status of the DCs. In BAL $CD1a^+$ mDCs are increased and they express a more mature phenotype. The past few years many studies on DCs in sarcoidosis have been performed, but detailed information about the function and role of DCs in sarcoidosis still lacking. Our current knowledge of DCs in sarcoidosis, as well as in other interstitial lung diseases is discussed in **Chapter 2**.

To gain more insight into the initiation of the pulmonary immune response in sarcoidosis we developed a method to isolate DCs from the bronchoalveolar lavage fluid, which is described in **Chapter 3.** BAL is a minimally invasive procedure that instills and subsequently recovers lavage fluid and its components from the alveolar space. It yields an *in situ* specimen from affected lung tissue. The number of DCs in BAL fluid is low. In the past few years many DC markers have been described but isolation of a pure DC population have been proved to be very difficult and time consuming. With the use of nine color flowcytometry using a lineage mix for exclusion the of non-DCs, and the DC markers HLA-DR, CD11c (mDC) and CD123 (pDC), we isolated pure DCs. The isolated pDCs and mDCs were assessed by microscopy and they show the specific characteristics as described for mDCs and pDCs. To analyse series of specimens at the same time, we froze the BAL cells upon sorting. We prove that freezing of BAL mDCs does not induce phenotypic changes or an altered T cell proliferation capacity of the mDCs. With confocal microscopy we evaluated T cell-mDC contact dynamics. The numbers of pDCs in

BAL samples are very low and unfortunately we were not able to sort enough pDCs for functional testing. The sorted mDCs were tested in a mixed leukocyte reaction (MLR). Isolated BAL mDCs were able to induce T cell proliferation, differentiation and cytokine production by activated T cells.

To elucidate the number, phenotype and function of DCs in the pathogenesis of sarcoidose, we collected blood, BAL and bronchial biopsies of sarcoidosis patients and healthy controls and analyzed mDCs en pDCs in **chapter 4**. In recently diagnosed sarcoidosis patients we found more mDCs and pDCs in the BAL and mDCs in the peripheral blood. We observed a clearly increased expression of the co-stimulatory molecules CD40 and CD86 (B7-2) on BAL mDCs in sarcoidosis patients, when compared with controls. This shows that the increase in number of DCs in the BAL and blood in sarcoidosis patients is accompanied by an increase in DC maturation in the BAL, but not in blood, and pulmonary sarcoidosis is associated with more mature DCs at the site of active disease. In addition, we isolated mDCs from the BAL of sarcoidosis patients and healthy controls and we tested mDCs *in vitro*, as described in chapter 3. BAL mDCs of sarcoidosis patients induced proper T cell activation and differentiation, but no differences were seen compared with healthy control BAL mDCs. These results indicate that BAL mDCs from sarcoidosis patients are functional and do not display diminished immunoreactivity. When we analyzed cultured monocyte-derived DCs (mo-DCs) of sarcoidosis patients and healthy controls, we found that mo-DCs from patients with sarcoidosis examined outside of the disease micro-environment were not intrinsically more mature, but did show the propensity to induce more TNFα, a cytokine pivotal in sarcoidosis pathogenesis. In bronchial granulomas-containing biopsies of sarcoidosis patients increased numbers of $CD11c^+$ cells were found and these CD11c positive cells showed increased expression of CD86. In summary, these results show that granuloma formation is associated with an increase in number and maturation of DCs. We concluded that DCs are involved in granuloma formation in sarcoidosis.

Recently, the pro-inflammatory cytokine IL-17A has been implicated in the pathogenesis of various granulomatous diseases, in particular in the formation of mycobacterial infection-induced granulomas in the lung. Although IL-17A can be produced by several cell types, it is mainly produced by the novel subset of Th17 cells, which has a distinct function compared to the Th1 and Th2 subsets. Th17 cells were shown to be crucially involved in many autoimmune diseases as well as in the host defense against intracellular pathogens. Human Th17 cells contain heterogeneous subsets. Next to IL-17A, these cells produce various other pro-inflammatory cytokines, including IL-17F, IL-22 and in some conditions IFNγ. IL-22 is a cytokine involved in mucosal immunity against extracellular pathogens, and can also be produced independently of IL17A. In addition IL17A/IFN-γ

double producing cells have been described in autoimmune diseases and these cells could be possibly more pathogenic.

In **Chapter 5** we found that more circulating CD45RO$^+$ memory T cells in sarcoidosis expressed IL-17A and IL-4 compared to controls. IL-22$^+$ T cells were not increased in sarcoidosis patients. In addition double-producing T cells expressing IL-17 together with IFNγ or IL-4 were also increased in sarcoidosis. In BAL of sarcoidosis patients we found substantial numbers of IL-17A$^+$ memory T helper cells, and particularly of IL-17A/IFN-γ$^+$ double-producing cells. In bronchial biopsies of sarcoidosis patients we observed increased numbers of IL-17A$^+$ T cells in association with granulomas and an increased number of IL-22 positive cells in the subepithelial layer of the long. In summary these results are pointing towards IL-17 involvement besides the known Th1 inflammation in sarcoidosis.

Cells in our immune system capable of suppressing the Th1/Th17 inflammation present in sarcoidosis are the regulatory T cells (Tregs). Tregs are subdivided as naïve and adaptive Tregs and have a CD4$^+$CD25$^+$ and Foxp3$^+$ phenotype. Tregs suppress the cytological function of effector T cells by IL-2 depletion, by modulating the T cell cytokine secretion using TGFβ and IL-10 and by direct cytotoxic killing using perforin/granzyme or Fas ligand. A decreased number of Tregs or a diminished function of the Tregs can be responsible for the T cell alveolitis present in sarcoidosis and conflicting results have been reported concerning T regs in sarcoidosis. We collected blood, BAL and bronchial biopsies of sarcoidosis patients and we analyzed numbers, localisation and function of Tregs in sarcoidosis in **Chapter 6.**

In blood of sarcoidosis patients we found increased percentages of Tregs, when compared with healthy controls. Also in the BAL, increased numbers and percentage of Tregs were present, which were positively correlated with the number of CD3$^+$ T cells in the BAL. So, there is not a lack of Tregs responsible for the T cell alveolitis in sarcoidosis. By immunohistochemistry, we found that Tregs were present in most of the granulomas, but not in all. Tregs were localized in the outside lymphocyte layer, as well as in the middle of the granuloma. We found no differences in Treg numbers in sarcoidosis patients and healthy controls. We subsequently isolated Tregs of peripheral blood and tested their function *in vitro*. Tregs of sarcoidosis patients appeared to have a reduced suppressive effect on T cell proliferation compared to healthy control Tregs. The lack of suppressive function of the sarcoidosis Tregs may likely be caused by impaired survival of the sarcoidosis Tregs *in vitro*. The impaired suppressive function and survival was independent of the presence of IL-2 in vitro. By comparison, CD4$^+$ T cells of sarcoidosis patients did not show impaired survival compared to healthy controls. Therefore, we conclude that in sarcoidosis not a lack of Tregs, but a decreased suppressive function probably caused by an impaired survival of the Tregs, contributes to the T cell alveolitis seen in sarcoidosis.

As discussed in **Chapter 7**, taken together the results presented in this thesis show that in sarcoidosis not only Th1 cells play a role but also the dendritic cells, Th17 cells and the Tregs have their own essential contribution to the pathogenesis in sarcoidosis. The pathogenesis of sarcoidosis is very complex and additional research, both in patients and in model systems, is required in the future to further elucidate the cause of this disease and to develop more specific treatment strategies.

Nederlandse samenvatting

SARCOÏDOSE

Sarcoïdose is een ziekte die verschillende organen van het lichaam kan aantasten en waarbij bepaalde ontstekingen, zogenaamde granulomen, gevonden kunnen worden in onder andere de huid, spieren, gewrichten, zenuwweefsel en ogen maar ook in andere organen. In meer dan negentig procent van de gevallen zijn de longen bij het ziekteproces betrokken. De klachten van de patiënt zijn afhankelijk van de organen die zijn aangedaan.

Jaarlijks wordt bij ongeveer 3000 Nederlandse patiënten de diagnose sarcoïdose gesteld. De ziekte treedt meestal op bij jong volwassenen of na het veertigste levensjaar en komt meer voor bij vrouwen en negroïde mensen. Soms ziet men meerdere personen met sarcoïdose binnen een familie. Het vaker voorkomen van sarcoïdose bij eeneiige tweelingen duidt op een genetische component in het ontstaan van de ziekte. Van de patiënten met sarcoïdose geneest meer dan twee derde spontaan, maar bij een aantal van deze patiënten kan de ziekte wel terug komen. Bij ongeveer een derde van de patiënten geneest sarcoïdose niet spontaan en ontstaat er een chronische vorm die ernstige schade, fibrosering, aan de longen tot gevolg kan hebben. Een klein deel (<5%) van de patiënten overlijdt aan sarcoïdose, meestal ten gevolge van longfibrose, pulmonale hypertensie of aantasting van hart of zenuwstelsel.

De verdenking op de diagnose sarcoïdose ontstaat op basis van klachten, bevindingen bij lichamelijk onderzoek en de vaak karakteristieke beelden bij röntgen of nucleaire beeldvorming. Aan de hand van de afwijkingen die op röntgenfoto's van de longen te zien zijn wordt longsarcoïdose ingedeeld in vier verschillende stadia: bij stadium I is er sprake van lymfkliervergroting bij de longhilus en/of langs de luchtpijp, bij stadium II is er tevens sprake van fijnvlekkige afwijkingen van het longparenchym, bij stadium III is er geen lymfkliervergroting (meer) maar zijn er nog duidelijke parenchymafwijkingen en bij stadium IV is er sprake van onomkeerbare veranderingen van het longweefsel (fibrose). Om de diagnose te bevestigen wordt gestreefd naar het aantonen van granulomen in aangedane weefsels door middel van een biopt, terwijl andere oorzaken van granulomen, zoals bepaalde infecties, zo goed mogelijk worden uitgesloten. Soms lukt het niet goed om een weefseldiagnose te verkrijgen. In het geval van longbetrokkenheid kan het aantonen van een ophoping van zogenaamde CD4+ T lymfocyten in de alveolaire ruimte middels een longspoeling (bronchoalveolaire lavage, BAL) ook dienen om de diagnose sarcoïdose te stellen.

Hoewel de ziekte sarcoïdose al meer dan 100 jaar bekend is, is de oorzaak nog steeds onbekend. De granulomen die zo kenmerkend zijn voor sarcoïdose zijn normaal gesproken ophopingen van ontstekingscellen bedoeld ter afscherming van een moeilijk op te ruimen lichaamsvreemd voorwerp of bacterie. Bij onderzoek van door sarcoïdose aangedaan weefsel blijken de granulomen opgebouwd te zijn uit verschillende soorten

afweercellen: weefsel macrofagen (histiocyten) en meerkernige reuscellen omgeven door een rand van geactiveerde lymfocyten. Er is bijna nooit celverval in het centrum (necrose) van het granuloom, zoals wel wordt gezien bij granulomen in het kader van een infectieziekte. Een oorzakelijke boosdoener, zoals een virus of bacterie, wordt bij patiënten met sarcoïdose niet gevonden. Wel wordt de betrokkenheid vermoed van diverse van bacteriën afkomstige eiwitten bij het ontstaan van sarcoïdose. Waarschijnlijk ontstaat sarcoïdose door een combinatie van een genetische aanleg en blootstelling aan één of meerdere soorten binnendringende deeltjes. Zoals boven al genoemd is, is er ook in de luchtruimte van de long bij sarcoïdose vaak sprake van een enorme toevloed van afweercellen (CD4+ T lymfocyten), zonder dat er een ziekteverwekker wordt gevonden. Al met al lijkt er dus sprake van een ogenschijnlijk overmatige afweerreactie op een onzichtbare indringer.

Het in gang zetten van een dergelijke afweerreactie in de long is normaal gesproken de taak van dendritische cellen (DCs), cellen die gespecialiseerd zijn in het opnemen, verwerken en presenteren van lichaamsvreemde eiwitten (antigenen). Een antigeen is een molecuul dat in staat is een reactie van het afweersysteem op te wekken, waarbij antistoffen worden aangemaakt. Deze DCs zijn in staat om een efficiënte afweerreactie in gang te zetten die specifiek gericht is op een binnendringende lichaamsvreemd eiwit. Dit doen ze door af te reizen naar de lymfeklieren van de long en daar het schadelijke eiwit aan zogenaamde T helper cellen te presenteren. Deze T helper cellen raken op hun beurt geactiveerd, differentiëren zich voor een bepaalde taak en vertrekken naar de plaats des onheils en verzorgen ('helpen') daar de verdere afweer- en opruimreactie.

Het lijkt dan ook aannemelijk dat bij het ontstaan van sarcoïdose dendritische cellen en/of T helper cellen een cruciale rol spelen.

DENDRITISCHE CELLEN

DCs zijn professionele antigeen presenterende cellen in ons lichaam. Ze zijn aanwezig in het lichaam op plaatsen waar het lichaam blootstaat aan verschillende antigenen, zoals in de longen. In de meeste weefsels zijn de DCs aanwezig in een niet-geactiveerde staat, niet in staat om T cellen te stimuleren, maar juist geschikt om antigenen op te vangen. Nadat antigenen in de long terecht komen worden extra DCs en monocyten (voorlopers van DCs) aangetrokken naar de longen. Na contact met antigeen worden de DCs geactiveerd en gaan zij major histocompatibility complex (MHC) moleculen op hun celmembraan tot expressie brengen, eiwitten die gespecialiseerd zijn in het presenteren van antigeen. Ook co-stimulatoire moleculen zoals CD80, CD86, CD83 en CD40 komen op het celmembraan tot expressie. Na activatie door antigeen gaan de DCs onder invloed van bepaalde cytokinen via de lymfebanen naar de lymfeklieren om

T cellen te activeren. In het MHC complex wordt het antigeen gepresenteerd door de DC aan de T cel, de co-stimulatoire moleculen zijn ook nodig om de T cellen te activeren. De geactiveerde T cellen worden dan T helper cellen. T helper cellen worden ingedeeld in o.a. T helper 1 (Th1), T helper 2 (Th2) T helper 17 (Th17) of regulatoire T cellen (Tregs) . Welke T helper cel de T cel wordt na activatie, hangt af van het gepresenteerde antigeen en verschillende omgevingsfactoren tijdens de presentatie. In de humane long kunnen 3 verschillende typen DCs herkend worden: myeloide DCs type 1 (mDCs type1), myeloide DCs type 2 (mDCs type2) en plasmacytoide DCs (pDCs), elk met verschillende T cel stimulerende capaciteiten en productie van verschillende cytokinen.

Ondanks onderzoek de afgelopen jaren is er geen duidelijkheid over het aantal en de functie van DCs bij sarcoïdose. Er wordt gezien dat de T cellen, betrokken in de ontstekingsreactie, vooral Th1 cellen zijn. Maar is er geen duidelijkheid over de rol van andere T helper cellen. Ons doel was de rol te onderzoeken van DCs, Th17 cellen en Tregs in het ontstekings proces zoals gezien wordt bij sarcoïdose.

Als eerste hebben we gekeken naar de DCs bij sarcoïdose. We ontwikkelden een methode om zuivere DCs te isoleren uit de BAL (beschreven in **hoofdstuk 3)**. Een gedeelte van de longen wordt gespoeld met vloeistof en in de opgezogen vloeistof bevinden zich dan de cellen die aanwezig zijn in de longblaasjes. Het aantal DCs in de vloeistof is erg laag. Door het gebrek aan juiste markers en door het lage aantal DCs was het de afgelopen jaren moeilijk een zuivere DC populatie die afkomstig is van de longen te isoleren. Met behulp van de DCs markers CD11c, HLA-DR en CD123 isoleerden we een zuivere populatie mDC en pDCs uit de lavage. Deze cellen zagen er microscopisch uit als DCs, en de mDCs waren in staat contact te maken met T cellen en ze te stimuleren en activeren. Om de procedure makkelijker te maken werden de lavage cellen ingevroren tot aan de DC isolatie. We laten zien dat dit invriezen van deze cellen geen effect heeft op hun membraanmarkers of hun functie. Door het ontwikkelen van deze methode, waren we in staat functioneel onderzoek te doen naar long DCs in sarcoïdose.

De afgelopen jaren heeft onderzoek uitgewezen dat sarcoïdose waarschijnlijk een antigeen gemedieerde ziekte is. DCs zijn de cellen die dat antigeen opnemen en presenteren aan T cellen en zo een immuun reactie starten. In **hoofdstuk 4** vonden we bij sarcoïdose patiënten dat de aantallen mDCs en pDCs in de lavage samen met het aantal mDCs in het perifere bloed verhoogd waren. Toen we het fenotype van deze cellen verder onderzochten, vonden we dat er verhoogde expressie was van de co-stimulerende moleculen CD40 en CD86 op mDCs in de lavage van sarcoïdose patiënten. Dit liet zien dat de toename van de aantallen DCs gepaard ging met verhoogde activatie van de mDCs. En dat de sterk geactiveerde mDCs voornamelijk in de long aanwezig waren bij sarcoïdose. Om de functie van de sterk geactiveerde mDCs uit de lavage te testen, isoleerden we de mDCs uit de lavage van gezonde mensen en van patiënten met sarco-

idose. In vitro zagen we dat mDCs van sarcoïdose patiënten goed in staat zijn T cellen aan te zetten tot activatie en proliferatie en dat er geen sprake was van verminderde immunoreactiviteit. Wel zagen we dat er geen verschillen waren vergeleken met gezonde mDCs. Omdat we geen verhoogde T cel proliferatie zagen door mDCs geïsoleerd uit de BAL van sarcoïdose patiënten, is de het de vraag wat er wel ten grondslag ligt aan de toevloed van T cellen in de alveolaire ruimte van sarcoïdose patiënten. Wellicht komt er primair een verhoogd aantal T cellen uit het perifere bloed naar de long door de secretie van cytokinen (MIG and CXCL10) in de long, zoals we die inderdaad in de BAL vonden. Anderzijds kan ook de invloed van omgeving van de long op de plaatselijke mDCs het zicht benemen op wat er intrinsiek aan de hand is met de DCs. Om die reden hebben we ook gekeken naar DCs die we gekweekt hadden in vitro uit monocyten (mo-DCs); dat waren dus DCs die nog geen antigeen hadden gezien en niet blootgesteld waren aan een inflammatoir milieu in de long. Hierbij zagen we dat mo-DCs van sarcoïdose patiënten T cellen aanzetten tot meer productie van TNF-α, een belangrijk cytokine bij sarcoïdose en geassocieerd met genetische afwijkingen bij sarcoïdose. Dit suggereert dat mDC van sarcoïdose patiënten intrinsiek anders zijn dan die van gezonde controles. Van patiënten en gezonde controles hebben we ook in mucosale bronchiale biopten gekeken naar DCs, met behulp van een kleuring voor CD11c. Hierbij zagen we dat in de biopten die granulomen bevatten er meer DCs aanwezig waren en die DCs lieten ook meer expressie zien van de co-stimulerende marker CD86; dus waren ze meer geactiveerd. Samenvattend laten deze resultaten zien dat de vorming van granulomen in de long geassocieerd is met hogere aantallen DCs die ook meer geactiveerd zijn en dat mo-DCs T cellen stimuleren tot meer aanmaak van TNF-α. We concluderen dat DCs betrokken zijn bij het ontstaan van granulomen in de longen van sarcoïdose patiënten.

IL-17 PRODUCERENDE T CELLEN

Bekend is dat T cellen die in de BAL en in granulomen worden gevonden bij sarcoïdose patiënten een T helper 1 profiel hebben. Hierbij produceren de T cellen interferon gamma (IFN-γ), Interleukine-2 (IL-2) en tumor necrosis factor alpha (TNF-α). Recent is beschreven dat ook het pro-inflammatoire cytokine IL-17A een rol speelt bij verschillende aandoeningen die met granuloomvorming gepaard gaan en dan voornamelijk de vorming van granulomen bij tuberculose. Il-17A kan geproduceerd worden door verschillende cellen maar vooral door de T-helper-17 cel (Th17). Er is gevonden dat Th17 cellen belangrijk zijn bij verschillende auto-immuunziekten en bij de afweer tegen intracellulaire pathogenen. Th17 cellen kunnen naast IL-17A ook nog andere cytokinen produceren als IL-17F en IL-22. Soms is er ook productie van het Th1-cytokine IFN-γ door Th17 cellen. Deze dubbel producerende cellen zijn in auto-immuunziekten

zoals reumatoïde artritis zelfs als meer pathogeen beschreven. IL-22 is een cytokine die een rol speelt in de afweer tegen binnendringende pathogenen in het lichaam en kan ook onafhankelijk van IL-17A geproduceerd worden. In **hoofdstuk 5** hebben we gekeken naar de aanwezigheid en lokalisatie van Th17 cellen in sarcoïdose patiënten. We vinden dat meer geheugen (memory) T cellen in het bloed van sarcoïdose patiënten expressie van IL-17A en IL-4 laten zien. IL-4 en andere Th2 cytokinen lijken belangrijk te zijn bij de vorming van fibrose in de long. Ook zijn er meer dubbel producerende T cellen aanwezig die IL-17A produceren samen met IFN-γ, IL-22 en IL-4. In lavage van sarcoïdose patiënten vinden we hoge aantallen van IL-17A positieve memory cellen, en ook van dubbel producerende IL-17A/IFN-γ cellen. In mucosale, bronchiale biopten van sarcoïdose patiënten vinden we in en rondom de granulomen IL-17A+ T cellen en een verhoogd aantal IL-22+ cellen in de laag net onder de mucosa, in vergelijking met biopten van gezonde controles. Naar aanleiding van deze resultaten concluderen we dat naast de Th1 cellen ook Th17 cellen een rol spelen in de inflammatie bij sarcoïdose. Wat de exacte rol is, en of dit voornamelijk bij de vorming van granulomen, is zal moeten blijken uit verder onderzoek.

REGULATOIRE T CELLEN

Er bestaan cellen in ons immuunsysteem die in staat zouden moeten zijn de Th1 inflammatie bij sarcoïdose te kunnen onderdrukken. Dit zijn de regulerende (regulatory) T cellen (Tregs). Tregs worden onderverdeeld in naïeve Tregs, die ontstaan in de thymus en altijd aanwezig zijn in ons lichaam, en de adaptieve Tregs, die in het perifere bloed ontstaan uit CD4+ T cellen bij inflammatie. De Tregs laten expressie zien van CD4+, CD25 en FoxP3. Er zijn verschillende manieren waarop Tregs andere T cellen kunnen remmen, door het wegvangen van IL-2, door het direct kapot maken van de T cellen met behulp van perforin/granzyme of Fasligand, of door het beïnvloeden van de cytokines die geproduceerd worden door de T cellen. Tregs bereiken dit door middel van de excretie van TGF-β en IL-10. Een verminderd aantal of een verminderde suppressieve functie van Tregs zou kunnen bijdragen aan het verhoogd aantal T cellen in de long en de vorming van granulomen. Uiteenlopende resultaten zijn tot nu toe gepubliceerd over het aantal Tregs en hun functie in sarcoïdose. We verzamelden bloed, lavage en biopten van sarcoïdose patiënten en gezonde controles om te kijken naar het aantal Tregs en hun lokalisatie en functie in sarcoïdose in **hoofdstuk 6.** In bloed van sarcoïdose patiënten vonden we een verhoogd percentage Tregs. In de lavage vonden we zowel een verhoogd percentage als een verhoogd aantal Tregs in vergelijking met gezonde controles. Het aantal Tregs in de lavage was positief gecorreleerd met het aantal T cellen in de BAL. Dit liet zien dat er geen gebrek is aan Tregs in de longen om de inflammatie te onder-

drukken. In het longweefsel zagen we dat Tregs vaak aanwezig zijn in granulomen, maar niet altijd. Tregs waren gelokaliseerd zowel in het midden als rondom de granulomen. Er was geen verschil in Tregs aantal in het weefsel van de longen in vergelijking met gezonde controles. We isoleerden Tregs uit het perifere bloed en testten hun functie in vitro. Hierbij zagen we dat Tregs van sarcoïdose patiënten in vitro de proliferatie van T cellen minder remmen dan gezonde controles, waarschijnlijk omdat zij (in ieder geval in vitro) heel snel dood gaan. De Tregs laten geen verhoogde expressie zien van apoptose markers voor de functionele test en de overleving van Tregs in vitro is onafhankelijk van IL-2. CD4 en CD8 cellen van sarcoïdose patiënten laten deze verminderde in vitro overleving niet zien. Concluderend zien we geen gebrek aan Tregs bij sarcoïdose maar vooral een verkorte overlevingduur. De verminderde remming van de inflammatie hierdoor kan zeker bijdragen aan de pathologie bij sarcoïdose, maar ook hier is verder onderzoek nodig de aankomende jaren.

Concluderend laten de resultaten gepresenteerd in dit proefschrift zien dat in sarcoïdose niet alleen Th1 cellen een rol spelen maar ook DCs, Tregs en Th17 cellen hun bijdrage leveren aan de pathogenese in sarcoïdose. The pathogenese van sarcoïdose is zeer complex en meer patiëntenonderzoek is noodzakelijk om het exacte ziektemechanisme te weten te komen, om meer te kunnen zeggen over de prognose van een individuele patiënt en om daarna in de toekomst een meer gerichte therapie te kunnen ontwikkelen.

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Mijn paranimfen:

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Tijdens mijn promotie kon ik natuurlijk rekenen op jullie onvoorwaardelijke steun en ik ben dan ook erg blij dat jullie samen mij paranimfen willen zijn. Ik heb heel mijn leven tussen jullie in gestaan, en doe dat ook erg graag tijdens de verdediging van mijn proefschrift.

Mijn collega's van het lab

Alex KleinJan: beste Alex, in de afgelopen jaren op het lab heb ik veel van je geleerd. Je hebt me met raad en daad ter zijde gestaan, niet alleen op technisch gebied en ik kon met al mijn vragen altijd bij je terecht. Je begreep ook als geen ander hoe het is om kinderen thuis en werk te combineren. Ik heb onze samenwerking altijd als bijzonder prettig ervaren, mijn grote dank hiervoor en ik wens je heel veel succes met al je onderzoek in de toekomst.

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Joost Hegmans: beste Joost, toen ik op het lab kwam heb je me op sleeptouw genomen en met engelen geduld samen met Peter proteomics geleerd. Jammer genoeg was proteomics niet de oplossing voor mijn sarcoidose onderzoek maar ik heb de jaren op het

lab een erg fijne collega aan je gehad. Dank je wel en veel succes met je mesothelioma studie.

Marthe Paats: beste Marthe, Hoewel we ons samen regelmatig af hebben gevraagd of het echt ooit zou lukken om een boekje te maken, is het voor mij toch goed gekomen en you are next! Dank je wel voor de ontzettend leuke tijd samen met je op het lab. Heel veel succes en sterkte met het afronden, voor je het weet staan we samen in de kliniek.

Al mijn andere collega's op het lab: Anoek, Brigitte, Claudia, Corine, Danielle, Dana, Hamida, Hermelijn, Hoyan, Ingrid, Joao, Karoline, Laurens, Leonie, Marjolein, Monique, Menno, Marco, Mirjam, Nanda, Odelia, Peter, Pieter Fokko, Sanne, Tanja, Van, Wilma, bedankt voor de gezellige tijd.

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CURRICULUM VITAE

Bregje ten Berge werd geboren op 21 juli 1979 te Nijmegen. In juni 1997 behaalde zij haar VWO diploma aan het Twickel College te Hengelo. Aansluitend startte zij met de opleiding geneeskunde aan de Rijks Universiteit Centrum Antwerpen (RUCA) waar zij in juni 2000 haar kandidaat-arts diploma behaalde. Hierna vervolgde zij haar geneeskunde studie aan de Universitaire instelling Antwerpen (UIA). In maart 2003 ging zij voor een co-schap kindergeneeskunde naar St. George's Hospital te Londen. Na het voltooien van haar scriptie met de titel: "corticoiden en COPD" met begeleiding van prof. De Backer en dr. Van Ranst behaalde zij in juni 2004 haar arts examen. In sept 2004 begon zij als AG-NIO interne geneeskunde in het Amphia ziekenhuis te Breda (opleider dr. Van Guldener). In nov 2005 werd gestart met haar promotie onderzoek naar "dendritische cellen en T cellen in sarcoidose" in het Erasmus MC te Rotterdam onder supervisie van Prof.dr. R.W. Hendriks en Prof.dr. B.N. Lambrecht. Per 1 jan 2010 is zij begonnen met de opleiding Longziekten en Tuberculose (opleider Prof.dr. H.C. Hoogsteden) in het Erasmus MC. De vooropleiding interne geneeskunde wordt thans verricht in het Ikazia ziekenhuis te Rotterdam.

PHD PORTFOLIO

Bregje ten Berge
Department of Pulmonary Medicine
Research school: Molecular Medicine Post- graduate School
Phd period: november 2005 - december 2009
Promotor: Prof.dr. R.W. Hendriks
Prof.dr. B.N. Lambrecht

Courses

2006	Molecular Immunology course, Department of Immunology, Erasmus MC, Rotterdam
2007	International Conference on Harmonisation Good Clinical Practice (ICH GCP) training: "Wet- en regelgeving van klinisch onderzoek in de dagelijkse praktijk" Clinical trial services
2005-2009	Weekly internal and external presentations at the Department of pulmonary medicine, Rotterdam

(Inter)national scientific symposia

Attended:

2006	9th international conference on dendritic cells, Edinburgh, Scotland
2006	Frontiers of mucosal immunology, Rotterdam The Netherlands,
2008	Molecular medicine day, Erasmus MC, Rotterdam, The Netherlands
2008	New perspectives of severe astma, Erasmus MC, Rotterdam, The Netherlands
2009	Molecular medicine day, Erasmus MC, Rotterdam, The Netherlands
2009	"sarcoidosis: an update" Maastricht, The Netherlands

Poster presentations:

2008	"Pulmonary dendritic cells are highly activated and induce CD4+ T cell proliferation and differentiation in sarcoidosis". WASOG meeting and BAL conference 2008, Athens, Greece
2008	"Pulmonary dendritic cells are highly activated and induce CD4+ T cell proliferation and differentiation in sarcoidosis". NVVI, Dutch association of immunology, Noordwijkerhout, The Netherlands
2009	"Pulmonary dendritic cells are highly activated and induce CD4+ T cell proliferation and differentiation in sarcoidosis". NRS, first spring meeting, Arnhem, The Netherlands

2010 "Regulatory T cells are increased in sarcoidosis patients but have a reduced suppressive effect on allogeneic T cell proliferation caused by impaired survival in vitro." American Thoracic Society, New Orleans, USA

Oral presentations:

2008 "A novel method for isolating dendritic cells from brochoalveolar lavage fluid" WASOG meeting and BAL conference 2008, Athens, Greece

2010 "IL-17 and IL-22 expressing cells are involved in granuloma pathogenesis in pulmonary sarcoidosis". American Thoracic Society, New Orleans, USA

2011 "Increased local and circulating T helper 17 cells in pulmonary sarcoidosis". WASOG meeting and BAL conference 2011, Maastricht, The Netherlands

Student coaching and teaching:

2007 "Try-out" study courses: fifth and sixth-grade VWO students. Erasmus faculty of medicine, Rotterdam

2008 "Try-out" study courses: fifth and sixth-grade VWO students. Erasmus faculty of medicine, Rotterdam

2008 Supervising the thesis on "dendritic cells in sarcoidosis" of Dana Korporaal, student at the Rotterdam University of Applied Sciences, medical laboratory research. Department of pumonary medicine, Rotterdam

2008 Introduction to first-grade medical students on PhD-research, Erasmus faculty of medicine, Rotterdam

2009 Journal club courses: First grade medical students. Erasmus faculty of medicine, Rotterdam

2009 "Try-out" study courses: fifth and sixth-grade VWO students. Erasmus faculty of medicine, Rotterdam

2009 Introduction to first-grade medical students on PhD-research, Erasmus faculty of medicine, Rotterdam

2009 Supervising an introduction course on Tregs in sarcoidosis. Master of science-student Hoyan Kexin Wen. Department of pulmonary medicine, Rotterdam.