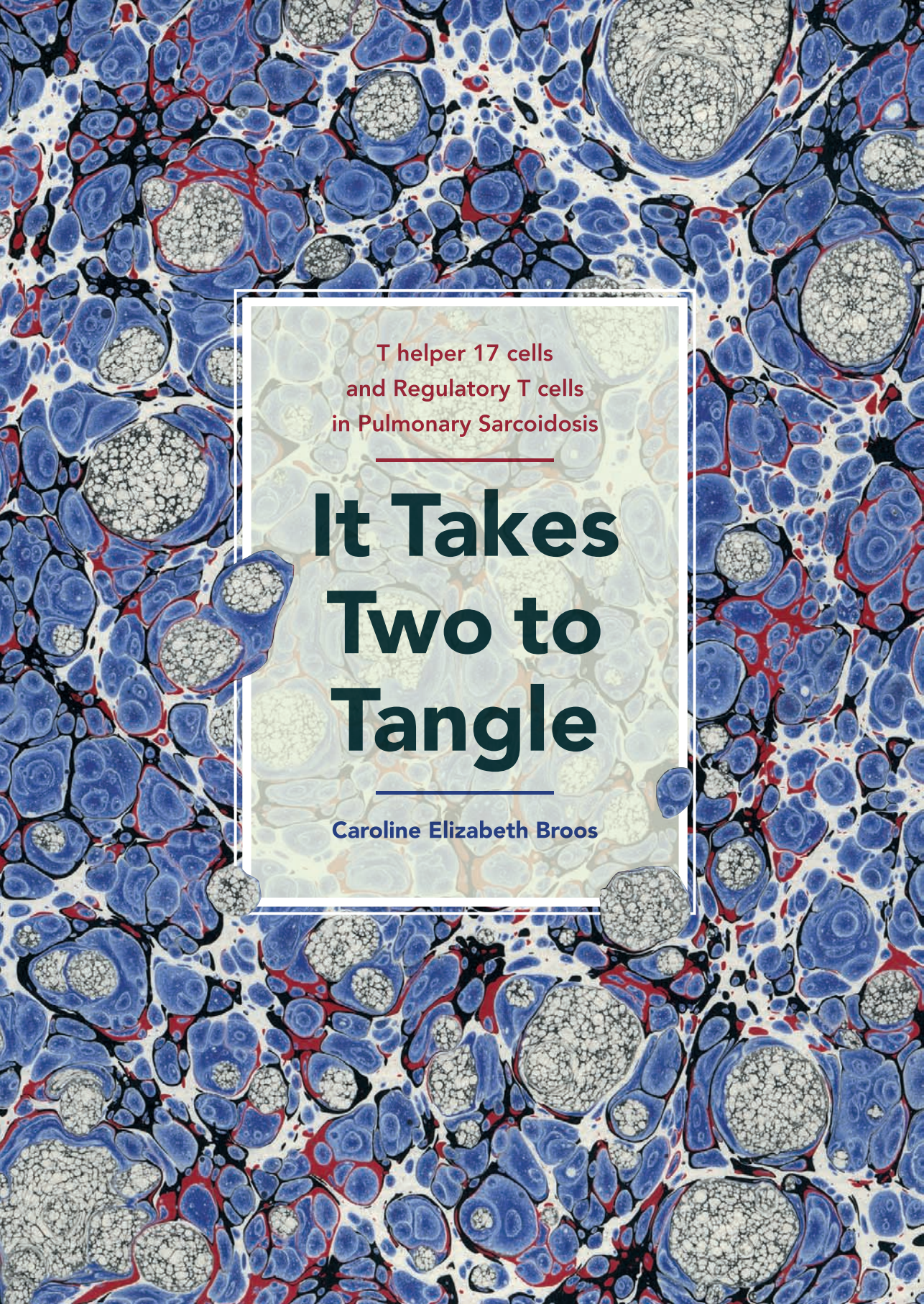

T helper 17 cells
and Regulatory T cells
in Pulmonary Sarcoidosis

It Takes Two to Tangle

Caroline Elizabeth Broos

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PROLOGUE

General outline of the thesis

‘When you talk, you are only repeating what you already know; but if you listen, you may learn something new’

Dalai Lama

PROLOGUE

Sarcoidosis is an intriguingly complex immunological disorder. It is characterized by the formation of non-necrotizing granulomas that are most commonly found in the mediastinal lymph nodes (MLN) and lungs of patients (1). Sarcoid granulomas also often affect the eyes and the skin, and in rare cases the heart, liver, central nervous system or bones (1).

A granuloma is a conglomerate of immune cells, and is formed in response to chronic antigenic stimulation (2). Granuloma formation likely occurs in order to optimize interactions between antigen presenting cells (APC) and adaptive immune cells, such as T cells. Typically, a fibrotic rim encapsulates the granuloma, aiding in preventing dissemination of the (infectious) antigen. A granuloma can arise in response to infectious agents such as mycobacteria and fungi, but also inorganic agents, such as silica and beryllium, can induce granuloma formation (2). Although several antigens have been proposed to be part of sarcoidosis etiology, not one antigen has yet been identified that can explain all cases of the disease (3, 4). Therefore, sarcoidosis is considered a multisystem granulomatous disorder of unknown cause (1).

The disease affects people all over the world, but the incidence/prevalence rates and natural course of the disease vary greatly (1, 5, 6), probably as a consequence of diverse local environmental triggers interacting with the genetic background of the affected population (7, 8). For example, the incidence rate of sarcoidosis is approximately three times higher in Afro-Americans than Caucasians (1, 6). Furthermore, the incidence in Japan is lower, however in this population the heart is more often affected, leading to higher mortality (1).

Intriguingly, granulomas tend to resolve spontaneously in the majority of patients, but in approximately 30% of the patients, granulomas persist, which can lead to chronic, sometimes progressive disease with need for treatment (1, 9). Some features at disease presentation have been identified that can help gain perspective on disease prognosis (10). For example, Afro-American women with pulmonary sarcoidosis have a higher chance of developing chronic disease (11). Clinical presentation with the Löfgren triad, that is lung bihilar lymphadenopathy on the lungs alongside uveitis and erythema nodosum or ankle arthritis, is typically associated with a good prognosis and occurs more often in Caucasians (12). Interestingly, Löfgren patients that have a specific human leucocyte antigen (HLA) genotype, i.e. HLA-DR*03, are most likely to undergo resolution (12).

Although a lot of progress has been made in Löfgren patients (which is likely a distinct disease entity than sarcoidosis (13)), it still remains largely unknown what environmental, genetic and/or immunological factors determine the disease course of non-Löfgren sarcoidosis patients. Thus, no variables have yet been identified that are routinely used in clinical practice to help predict disease course of sarcoidosis patients.

Gaps in knowledge on sarcoidosis disease etiology and determinants of disease course have thus far impeded rational decisions on 'Who, When and How' to treat. The mainstay of sarcoidosis therapy remains immunosuppressive, using relatively high doses of systemic prednisone. However, although often effective, corticosteroid treatment can be accompanied by severe side effects and knowledge on optimal dosing strategies balancing effects and side effects, is lacking (14). Detailed insight in the immunological response occurring in pulmonary sarcoidosis patients can help find determinants of disease etiology and disease course, which can yield new therapeutic targets. Therefore, the first aim of this thesis is to further unravel the immune-related pathogenesis of pulmonary sarcoidosis (**Part 1**). The second aim of this thesis is to gain insight into current prednisone treatment, in order to optimize treatment strategies to reduce side-effects and increase quality of life of pulmonary sarcoidosis patients (**Part 2**).

General outline of the thesis

Part 1: Unraveling the pathogenesis of pulmonary sarcoidosis

Several clinical features and lines of evidence in sarcoidosis support a key role for $CD4^+$ T cells in sarcoidosis, making them attractive candidates to help unravel the pathogenesis of this disease. First, the hallmark of sarcoidosis is the non-necrotizing granuloma that consists of a core of epithelioid- and multinucleated-giant cells, that is encircled by specifically $CD4^+$ T cells (1). Second, a cardinal feature of sarcoidosis clinical presentation is $CD4^+$ T-cell alveolitis (15). Third, lung $CD4^+$ T cells in sarcoidosis were found to be of limited clonal origin, suggesting that they are responsive to the putative antigen (16). Fourth, genes that have repeatedly been associated with sarcoidosis susceptibility and phenotype encode HLA class II antigens, which are well known to play a key role in $CD4^+$ T-cell activation (17). And lastly, other genes that have been associated with sarcoidosis code for proteins involved in T-cell activation, differentiation, proliferation and survival, including *butyrophilin-like* protein (BTNL)-2, NOTCH 4 and ANXA11 (17). Therefore, in-depth characterization of $CD4^+$ T cells in sarcoidosis will help shed light on the etiology of the disease, since these cells are very likely to respond to the putative antigen presented by HLA class II antigens on APCs.

In **Chapter 1** current knowledge on phenotypical and functional properties of sarcoidosis APCs and $CD4^+$ T cells is reviewed. A model is proposed for granuloma formation in sarcoidosis combining careful clinical observations with current literature that describes immunological findings in sarcoidosis blood, lungs and lymph nodes.

Sarcoidosis is characterized by an exaggerated T helper (Th)1/Th17 response upon exposure to one or several antigens in genetically susceptible individuals (see Chapter 1). However, mechanisms leading to this exaggerated immune response remain largely obscure. Key players in a disease are often identified by mouse models that lack or overexpress a specific protein or cell type of interest. Development of a universally accepted

mouse model for sarcoidosis has proven difficult, hampering research efforts employing this strategy. Interestingly, in **Chapter 2** we describe how emerging clinical observations in human cancer treatment shed new light on key players in sarcoidosis pathogenesis. Since treatment with anti-cytotoxic T-lymphocyte antigen 4 (CTLA4) agents in human cancers can induce sarcoidosis or sarcoid-like disease, we questioned whether CTLA4 expression is affected in pulmonary sarcoidosis patients.

Results described in Chapter 2 suggest a key role for specifically regulatory T cells (Tregs) and Th17 cells in pulmonary sarcoidosis. Although both Tregs and Th17 cells have been investigated in sarcoidosis, findings remain conflicting or inconclusive, respectively.

Failure of immune regulation by Tregs has long been suggested to contribute to ongoing inflammatory responses in pulmonary sarcoidosis (18). However, factors contributing to this phenomenon remain largely unknown and conflicting data is published regarding their proportions in various affected organs (see Chapter 1). In **Chapter 3** we aimed to get grip on the role of Tregs in sarcoidosis and systematically investigate the role of Tregs in pulmonary sarcoidosis by analyzing proportions, phenotype, survival and apoptotic susceptibility.

While Th17 cells were previously found to be increased in patient lungs, sarcoidosis was largely thought to be a Th1 mediated disease, because the total proportions of interleukin (IL)-17-producing cells remained very low. However, Chapter 2 demonstrates a key role for Th17 cells in pulmonary sarcoidosis and concomitantly it became clear in the T-cell field that stringent classification of T cells based on cytokine production may not be correct. Certain T-cell subsets, such as Th17 cells, are found to be more prone to plasticity and can adopt cytokine production capabilities of other T-cell subsets (19). We previously identified increased proportions of IL-17A/interferon (IFN)-γ-double-producing memory Th cells in a small sarcoidosis patient cohort (20) and therefore hypothesized that the role of Th17 cells in pulmonary sarcoidosis may be greater than anticipated. T helper 17 cells perhaps play an important role in the disease pathogenesis by mimicking Th1 cytokine (IFN-γ) production. Indeed, so called Th17.1 cells are known for this ability in other disease states (21). In **Chapter 4** we aimed to identify the origin of IFN-γ-producing cells in pulmonary sarcoidosis.

Primary Th cell activation and subset differentiation occurs in lung draining MLN. Above mentioned IFN-γ-producing Th17.1 cells can derive from Th17 cells under influence of a specific cytokine milieu (22, 23). Therefore, we hypothesized that in pulmonary sarcoidosis Th17 cells are primarily induced in the MLN and that the granulomatous environment in the lungs induces plasticity towards Th17.1 cells. In **Chapter 5** we challenged this hypothesis and aimed to gain more insight in the induction of Th17.1 cells in sarcoidosis by looking into proportions and phenotypic characteristics of $CCR6^+$ Th17-lineage cells in sarcoidosis and control MLN, and sarcoidosis lungs.

Finally, in **Chapter 6** recent insights in sarcoidosis T-cell immunology are reviewed. Based on new findings in Tregs, Th17.1 cells and checkpoint inhibitors a comprehensive model is proposed that integrates all observations that highlight a key role for both Tregs and Th17-lineage cells in the pathogenesis of pulmonary sarcoidosis.

Part 2: Optimization of prednisone treatment in pulmonary sarcoidosis

Prednisone is the main stay of treatment in pulmonary sarcoidosis because it quickly relieves symptoms and controls inflammation (1, 4, 9, 24). A meta-analyses of randomized controlled trials however concluded that limited evidence was found that (early) prednisone therapy in pulmonary sarcoidosis alters disease progression in the long term (24). Thus, although prednisone remains the first choice regimen for treatment of pulmonary sarcoidosis (1, 24-26), therapy is only indicated in certain cases, such as during development of a dangerous health situation or when there is a significant decrease in the patient's quality of life (27). Unfortunately, the optimal dose and duration of prednisone treatment remains uncertain, thus current guidelines include a broad range (1, 25), which could result in low dose-treated patients versus high dose-treated patients, depending on the physician's practice, which might lead to unmet therapeutic goals or unnecessary side effects.

Identifying the optimal treatment strategy that preserves organ function and relieves symptoms, but simultaneously minimizes side-effects, may increase quality of life of newly-treated pulmonary sarcoidosis patients. Therefore, in order to gain insight in what dosing strategy has the best balance between effect and side-effects, in **Chapter 7** we aimed to describe forced vital capacity (FVC) (effect) and weight (side-effect) changes during different prednisone doses used in daily practice for treatment naïve pulmonary sarcoidosis patients.

Available evidence and results presented in Chapter 7 suggest that early dose tapering is very likely to reduce side-effects in prednisone therapy for pulmonary sarcoidosis, while retaining effect. It has previously been suggested that prednisone effect on FVC occurs quickly within one month (28, 29). However, a prospective study monitoring individual lung function changes on a daily basis in newly-treated sarcoidosis patients to identify effect optimum, and also including clinical symptoms, is lacking. Therefore, in **Chapter 8** results are presented of a prospective, observational study, evaluating early FVC and symptom changes during prednisone treatment and tapering in newly-treated sarcoidosis patients, using daily home spirometry.

Finally, findings of Part 1 and Part 2 of the thesis are integrated into a general discussion (**Epilogue**) of the thesis, highlighting results of this thesis that could be of specific interest for clinical practice, providing an overview of outstanding questions and suggestions for future research.

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PART 1

Unraveling the pathogenesis of pulmonary sarcoidosis

CHAPTER 1

Granuloma Formation in Pulmonary Sarcoidosis

'Make it simple, but significant'
Don Draper

Front Immunol. 2013 Dec 10;4:437

Caroline E. Broos, Menno van Nimwegen, Henk C. Hoogsteden, Rudi W. Hendriks, Mirjam Kool, and Bernt van den Blink.

ABSTRACT

Sarcoidosis is a granulomatous disorder of unknown cause, affecting multiple organs, but mainly the lungs. The exact order of immunological events remains obscure. Reviewing current literature, combined with careful clinical observations, we propose a model for granuloma formation in pulmonary sarcoidosis. A tight collaboration between macrophages, dendritic cells and lymphocyte subsets, initiates the first steps towards granuloma formation, orchestrated by cytokines and chemokines. In a substantial part of pulmonary sarcoidosis patients, granuloma formation becomes an on-going process, leading to debilitating disease and sometimes death. The immunological response, determining granuloma sustainment is not well understood. An impaired immunosuppressive function of regulatory T cells has been suggested to contribute to the exaggerated response. Interestingly, therapeutical agents commonly used in sarcoidosis, such as glucocorticosteroids and anti-TNF agents, interfere with granuloma integrity and restore the immune homeostasis in autoimmune disorders. Increasing insight into their mechanisms of action may contribute to the search for new therapeutical targets in pulmonary sarcoidosis.

INTRODUCTION

Sarcoidosis is a granulomatous disorder of unknown cause, affecting multiple organs, but mainly the lungs. In 10-30% of the cases, sarcoidosis becomes chronic and progressive leading to debilitating disease and sometimes death (1). Its etiology is intriguing, since a part of its definition (i.e. *unknown cause)* makes it uniquely different from granulomatous disorders arising from exposure to a known chronically persisting antigen, such as tuberculosis, visceral leishmaniasis and chronic beryllium disease (2, 3). Nevertheless, several observations support an antigen-induced disease etiology. First, epidemiological research identified environmental and occupational risk factors, such as exposure to musty odors and insecticides (4). Second, infectious agents, including *Propionibacterium acnes (P.acnes)* and *Mycobacterium tuberculosis (Mtb),* have been implicated, since genomes of these species are detected within sarcoid granulomas (5). A role for mycobacterial peptides is further supported by the presence of T lymphocytes that are highly responsive towards 6-kDa early secreted antigenic protein (ESAT-6) or catalase peroxidase (KatG) in the bronchoalveolar lavage fluid (BALF) of sarcoidosis patients (6-8). Third, a limited clonality of $CD4^+$ T cells, expressing the AV2S3 T cell receptor, was demonstrated within the lungs of HLA-DRB1*03 positive sarcoidosis patients, which is consistent with an antigenic response (9-12). Finally, evidence for an antigen-induced disease lies within the granulomatous reaction that is virtually indistinguishable from sarcoid granulomas and occurs in individuals with sarcoidosis upon subcutaneous injection of homogenates from allogeneic sarcoid spleen or lymph nodes (LN), i.e., the Kveim-Siltzbach test (13, 14).

Genetic risk factors in sarcoidosis

People all over the world suffer from sarcoidosis (15). Familial clustering (16), increased concordance in monozygotic twins (17) and variations in susceptibility and disease presentation among different ethnic groups (18), suggest the importance of genetic, next to environmental risk factors in the etiology.

Genome-wide association studies (GWAS) identified polymorphisms within genes coding for proteins involved in T-cell activation, differentiation, proliferation and survival, including NOTCH 4 and ANXA11 (19, 20). Additionally, GWAS and case-control studies identified important genetic risk factors within the antigen presentation locus at 6p21.3, which contains genes encoding proteins involved in both antigen presentation and T-cell regulation, including human leucocyte antigen (HLA) and *butyrophilin-like* protein (BTNL)-2, respectively (20-23).

Specific HLA class II antigens are associated with certain sarcoidosis disease phenotypes. For example, the HLA-DRB1*03 and DQB1*0201 alleles have been associated with an acute disease onset, Löfgren syndrome and resolving disease, whereas in contrast HLA-DRB1*15 and DQB1*0601 are associated with chronic sarcoidosis (24-27). It is

conceivable that both resolving and persistent sarcoidosis arise due to a unique combination of a specific genetic background and exposure to one or several environmental triggers (28). This unique combination might lead to persistent stimulation of the immune system, contributing to granuloma formation and sustainment.

In this article we review the current knowledge on the role of the immune activation in pulmonary sarcoidosis and propose a hypothesis on the origin of granuloma formation. Secondly, we aim to discuss granuloma integrity, highlighting areas for research into new therapeutical targets.

GRANULOMA FORMATION

A well-developed sarcoid granuloma consists of a tightly formed conglomerate of epithelioid- and multinucleated giant cells (MGCs) encircled by lymphocytes, especially $CD4^+$ T helper (Th) cells, but also rare $CD8^+$ T cells and B cells (1). Both granuloma formation and integrity depend on the availability and supply of these different cells (29). The chronological order of immunological events and the exact role of these cells during the sarcoid granulomatous response remain obscure, due to the lack of an animal model for sarcoidosis. Nevertheless, careful clinical observations and in-depth research on functional properties of different cells involved provide essential information to unravel the cellular and molecular mechanisms of granuloma formation.

Clinical signs

Cardinal features of pulmonary sarcoidosis are mediastinal lymphadenopathy, parenchymal and airway granulomas, giving rise to upper lobe nodules in a perilymphatic or bronchovascular distribution and signs of a $CD4^+$ T-cell alveolitis. An interstitial pneumonitis, found on open lung biopsy, is classically thought to represent a very early stage of granuloma formation (30). Spontaneous remission and reactivation of sarcoidosis makes it difficult to ascertain the exact sequence of these cardinal features, however several findings strongly suggest a certain order in the majority of patients, which may add to the hypothesis on granuloma formation as described below.

Although it is well known that patients do not go through all disease stages as described by Scadding (from I to IV) sequentially, arguably pulmonary sarcoidosis starts in the draining LN. As stage I (bihilar lymphadenopathy) is most often asymptomatic, it is conceivable that it precedes pulmonary involvement, seen in stage II and III. Additionally, progression of stage I to II disease is well-known, while development of stage I after stage III is uncommon. Finally, a recent trial found an increased diagnostic sensitivity of LN-derived fine needle aspirates, compared with transbronchial lung biopsies (31). These data suggest that the first granulomas are formed within the mediastinal LN, only later followed by granuloma formation within the lungs.

Consequently, LN-specific immune reactions are important in early sarcoid granuloma formation, such as antigen presentation by dendritic cells (DCs). DCs are the only cells capable to pick up antigens and migrate to the LN where they present antigens to naïve T cells. Hereby they initiate highly specific clonal T cell differentiation and proliferation (32). Alternatively, LN-resident DCs may encounter antigenic particles, which we propose are submicroscopic and may therefore have passively migrated through the afferent lymph. The activated and differentiated Th cells migrate towards the site of inflammation, orchestrated by chemokines.

Macrophages contribute to early recognition of the putative sarcoid antigen in the lungs, thereby attracting mononuclear cells, including monocytes and LN-activated lymphocytes. The ensuing influx of cells leads to an interstitial pneumonitis, characterized by a mixed mononuclear cell infiltrate in the alveolar wall and $CD4^+$ T-cell alveolitis (30).

At the site of antigen encounter, antigen-presenting cells (APCs) induce persistent stimulation of the immune response, mediated by HLA-related proteins, leading to continuous recruitment and local expansion of lymphocytes and eventually granuloma formation. The central localization of macrophages within the final epithelioid aggregate supports an important role in antigen presentation at the site of granuloma formation. Alternatively, DCs may play a critical role in antigen presentation within the granuloma. Their capacity for antigen sampling within the lymph fluid makes them likely candidates to contribute to the induction of the perilymphatic localized granulomas (33, 34).

In the following paragraphs we describe the current knowledge on the role of macrophages, DCs and lymphocytes in sarcoid granuloma formation in more detail, also summarized in Figure 1.

Macrophages

Upon activation, macrophages release nuclear factor (NF)-κB-dependent pro-inflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor-α (TNF-α) (35). In sarcoidosis, BALF cells and monocytes highly express toll-like receptor (TLR)-2 (36, 37) and produce increased amounts of TNF-α, IL-1β and IL-6 compared with controls, when stimulated with TLR-2 ligands, including ESAT-6 and KatG (7, 36-38). A role for TLR-2 in immune activation and granuloma formation in sarcoidosis is further supported by genetic and mouse studies (38, 39). Lately, continuous TLR-2 ligation by macrophage-derived serum amyloid A has been suggested to contribute to persistent stimulation of the immune response in sarcoidosis (37).

Intrinsically, unstimulated sarcoid-derived alveolar macrophages (AMs) produce increased amounts of IL-1 and TNF-α (40-43) and are highly activated (44, 45). The amounts of spontaneously produced TNF-α by BALF cells *in vitro* correlate with the presence of aggregates of AMs in the tissue (46, 47). Only AMs from patients with active and progres-

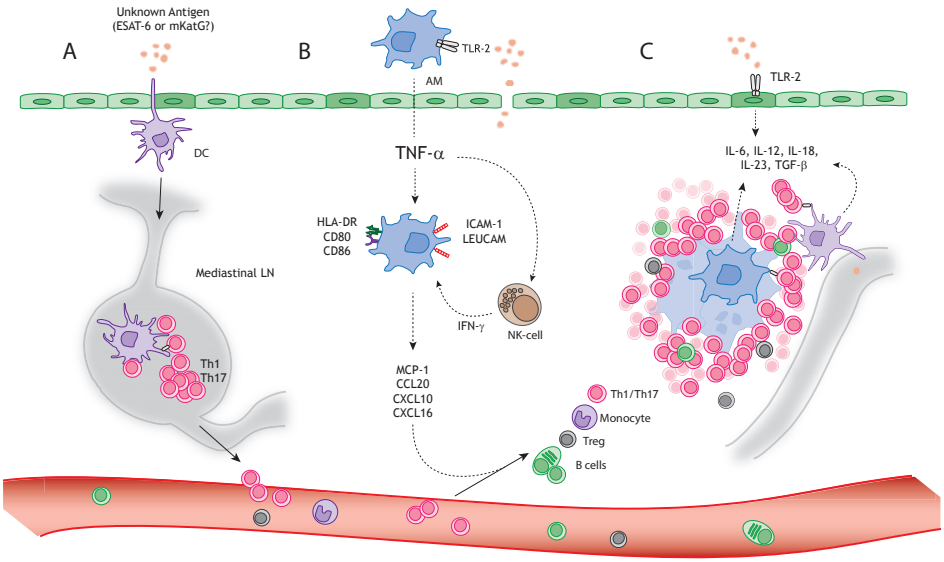

Figure 1. A schematic model for granuloma formation in pulmonary sarcoidosis. An unknown airborne-antigen activates (A) interstitial dendritic cells (DCs), (B) alveolar macrophages (AMs), and (C) alveolar epithelial cells type II (AEC-II) (*dark green)*, simultaneously. This process is initiated by toll-like receptor-2 (TLR-2) ligands, possibly *mycobacterium tuberculosis*-derived ESAT-6 or mKatG. (A) The interstitial DCs pick up the putative antigen and migrate towards the mediastinal lymph nodes (LN), where they initiate differentiation and clonal expansion of T helper (Th)1 and 17 cells. (B) Simultaneously, AMs produce tumor necrosis factor-α (TNF-α), which initiates upregulation of activation (HLA-DR and CD80/86) and adhesion (ICAM-1 and LeuCAM) molecules. Macrophages produce chemokine ligands (MCP-1, CCL20, CXCL10 and CXCL16) under stimulation of both TNF-α and natural killer (NK) cell-derived interferon-γ (IFN-γ), thereby attracting Th1/17 cells, monocytes, regulatory T cells (Tregs) and B cells. (C) The lung environment is characterized by the presence of Th1 and Th17 favoring cytokines, such as IL-6, IL-12, IL-18, IL-23 and TGF-β, produced by macrophages, perilymphatic DCs and AEC-II. Persistent stimulation, mediated by antigen presenting cells (APCs), leads to continuous cellular recruitment to the site of inflammation, which leads to granuloma formation. Tregs infiltrating the granuloma fail to diminish the exaggerated immune response, thereby contributing to granuloma persistence and integrity.

sive disease produce increased amounts of TNF-α (48-51). These data highlight the role of TNF-α in granuloma formation and integrity, also supported by mouse studies (52-54).

Important mechanisms of action of TNF-α include macrophage activation, promotion of cellular migration towards the site of inflammation and leucocyte adhesion (52, 55, 56). In a mycobacterial-driven mouse model, TNF-α is responsible for the early production of chemokines that attract mononuclear cells to the site of inflammation, such as RANTES, MIP-1α, MIP-1β, MIP-2 and MCP-1 (55), of which increased amounts are found in sarcoidosis BALF (57-59). In active sarcoidosis, AMs produce high amounts of CCL20, when stimulated by TNF-α and IL-1β (60). CCL20 is a chemokine with high affinity for chemokine receptor CCR6, therefore attracting DCs, B cells and specific T cell subsets

towards the lungs (60, 61). Similarly, AM-derived CXCL10 and CXCL16 contribute to $CXCR3^+$ and $CXCR6^+$ $CD4^+$ Th cell recruitment (62, 63).

In a mycobacterial-driven granuloma model, efficient cellular recruitment, mediated by AM-derived CXCL10 and CXCL16, depends on interferon-γ (IFN-γ) (53). During the early innate response natural killer (NK) cells are important producers of IFN-γ, when stimulated by TNF-α, IL-1 and IL-12. In sarcoidosis, the size of a distinct NK cell subpopulation ($CD56^{bright}CD94^{high}KIR^{low}$) is increased in the BALF compared with controls (64). Furthermore, higher proportions of NK cells were found to correlate with a poor outcome (65).

Once recruited, TNF-α is needed for leucocyte adhesion, since an abrogation of tightly formed granulomas in TNF-α-deficient mice is observed following mycobacterial infection (55). In sarcoidosis, TNF-α induced the expression of intracellular adhesion molecule-1 (ICAM-1) on AMs, leading to cellular aggregation (66). Additionally, leucocyte adhesion molecule (LeuCAM) expression, such as CD11a/b/c and CD18 (67), is increased in sarcoid AMs compared with controls.

Following adhesion, epithelioid histiocytes and monocyte-derived DCs can fuse to multinucleated giant cells (MGCs) when stimulated by local cytokines, such as TNF-α, GM-CSF, IL-17A, CCL20 and IFN-γ (68, 69). Patient-derived macrophages and monocytes show an enhanced potential to form MGCs *in vitro*, compared with healthy controls and other granulomatous diseases (70).

Importantly, sarcoid-derived AMs have an increased accessory function on autologous blood- and lung-derived T lymphocytes, when compared with controls (71-73). Macrophages are not capable to migrate to the LN to induce naive T cell activation, making them weak APCs. Nonetheless, in sarcoidosis, macrophages might contribute to local antigen presentation, enhancing proliferation of chemokine-recruited memory Th cells.

In summary, macrophages are important for the initial accumulation, aggregation and fusion of the cellular building blocks needed for granuloma formation. This process is mediated by the strong immune modulatory capacities of TNF-α and assisted by NK cells, which produce IFN-γ.

Dendritic cells

Only a few studies investigated the role of DCs in sarcoid granuloma formation (47, 74). Our group has shown that granuloma formation surrounding intravenously injected antigen-loaded beads trapped in the lung vasculature is dependent on DC-initiated Th cell proliferation within the mediastinal LN (75). In sarcoidosis, an accumulation of mature ($Fascin^+HLA\text{-}DR^+DC\text{-}LAMP^+$) DCs is found surrounding LN granulomas, adjacent to $CD3^+$ lymphocytes, suggesting DC-T cell interaction at this site (76). Mature ($CD11c^+CD86^+$) DCs are found surrounding granulomas in sarcoid-derived mucosal biopsies (77), further supporting a role for DCs in airway and parenchymal granuloma formation.

An impaired accessory function of *ex vivo* blood-derived myeloid DCs (mDCs) has been suggested to contribute to granuloma formation, as clearance of the putative antigen may be ineffective and the immune system turns to granuloma formation as a default immunological response (78). In contrast, our group isolated BALF mDCs of sarcoidosis patients and found them to be immunocompetent, initiating proliferation of allogeneic, naïve T lymphocytes comparable with mDCs from healthy controls (77). Similarly, *in vitro* cultured monocyte-derived DCs (moDCs) showed a comparable accessory capacity as controls, although they are intrinsically prone to produce TNF-α (77, 79). Hence, it is most likely that DCs are involved in granuloma formation, instead of displaying diminished antigen presenting capacities.

Differentiation of T lymphocytes depends on the local cytokines surrounding the initiating APC (80). Although it is very likely that LN-specific interactions, mediated by DCs, are responsible for the initial T cell polarization towards a Th1 and Th17 phenotype as observed in sarcoidosis, direct evidence is still lacking.

Lymphocytes

Sarcoidosis is characterized as a Th1- (81) and more recently a Th17-mediated disease (61, 82), based on the accumulation of IFN-γ, IL-2 and IL-17-producing Th cells in the lungs of patients with active sarcoidosis (44, 61, 82-84).

Th1 differentiation depends on IL-12 and IL-18, which are increased in BALF of sarcoidosis patients (85, 86). Alveolar epithelial cells type II (AEC-II) may contribute to this Th1-favoring environment, since patient-derived AEC-II produce IL-18 upon TLR-2 stimulation (87, 88). Additionally, AEC-II may contribute to $CXCR3^+$ Th1 cell recruitment by production of CXCL10 (89) .

Th17 differentiation is driven by IL-6 and TGF-β, both produced by sarcoid-derived BALF cells (90, 91), whereas survival and proliferation of this subset is IL-23-dependent (92-94). Increased expression of the IL-23-receptor and IL-17, both expressed by Th17 cells, is found in blood-, lung- and LN-derived lymphocytes of active sarcoidosis patients, and not in inactive disease (61, 82). Recently, ESAT-6-specific Th17 cells in the BALF of sarcoidosis patients were found (95). Additionally, IL-17A is essential for granuloma formation in the lung during mycobacterial infection (96) or in chronic granulomatous disease (97).

We recently found that the proportions of circulating IL-17A/IFN-γ and IL-17A/IL-4 double-producing cells are significantly increased in the peripheral blood of patients and are present in substantial numbers in BALF (82). Findings in several autoimmune diseases have indicated the pathogenic potential of $CD4^+$ Th cells producing both IL-17 and IFN-γ (98, 99). Processes underlying Th17 cell induction in sarcoidosis remain obscure, but the presence of these cells can suggest a role for autoimmune responses in sarcoidosis. B lymphocytes and plasma cells are found surrounding sarcoid granulomas

(100). Additionally, active sarcoidosis patients have increased serum levels of B-cell-activating factor (BAFF) (101). Since B cell maturation and function depends on BAFF, its aberrant expression can initiate defective selection of autoreactive B cells, leading to autoantibody production (101, 102). In sarcoidosis, approximately 30-60% of the patients exhibit antinuclear antibody (ANA) positivity (101, 103).

A SNP in the IL-23 receptor gene has been associated with chronic sarcoidosis (104), which may contribute to Th17 cell development in sarcoidosis. Since IL-23 is a heterodimer of the p19 subunit and the p40 subunit of IL-12 (105) the Th1- and Th17-promoting cytokines share a common therapeutical target. Ustekinumab, a neutralizing antibody against the IL-12/IL-23 p40, was shown to be successful in the Th1/Th17-mediated diseases psoriasis and Crohn's disease (CD) (106, 107), but not in chronic pulmonary or skin sarcoidosis (108, 109).

GRANULOMA INTEGRITY

In the majority of the sarcoidosis patients, granulomas spontaneous resolve within several years, without need for therapy. However, a substantial proportion of the patients develop chronic progressive disease, whereby granulomas persist and form fibrotic lesions, leading to debilitating disease and sometimes death (1, 110). The immunological response, determining granuloma sustainment is not well understood.

Regulatory T cells

Regulatory T cells (Tregs) play an important role in diminishing Th cell specific responses and are pivotal for maintenance of self-tolerance and immune homeostasis (111). An impaired immunosuppressive function of sarcoid-derived Tregs has been suggested to contribute to the on-going, exaggerated immune response, since sarcoid blood-derived ($CD4^+CD25^{high}$) Tregs fail to inhibit granuloma growth in an *in vitro* granuloma culture model (112, 113). Subsequently, an impaired immunosuppressive function of both blood- and BALF-derived sarcoidosis Tregs has repetitively been described on autologous and allogeneic healthy Th cell proliferation (114-116). These studies also show that sarcoid-derived Tregs fail to inhibit production of TNF-α, IFN-γ and IL-2, contributing to granuloma formation, rather than diminishing the immune response (112, 113, 116). It remains unknown what mechanism(s) underlies this impaired function.

Active and persisting sarcoidosis was recently associated with a global $CD4^+$ T cell subset dysfunction (116). Notably, both Th anergy and Treg malfunctioning were restored in patients with disease resolution (116). These results highlight the complex interplay between pro-inflammatory and anti-inflammatory responses needed for granuloma integrity. This fine balance may explain contradictory results with regard to reported Treg numbers in the BALF (112-119) (Table 1). Low BALF Tregs (i.e. less immunosup-

Table 1. An overview of studies reporting regulatory T cell (Treg) proportions and functional properties in pulmonary sarcoidosis

	Methods			Proportions			Function			
Study	**Population**	**Treg definition**	**Technique**	**Blood**	**BALF**	**LN**	**Blood**	**BALF**	**LN**	**Remarks**
Miyara et al, 2006[112]	Active disease	$CD4^+CD25^+$ (% *of* $CD4^+$)	FC/IHC	↑	↑*	↑	↓^	=*	=	^ Blood-derived Tregs reduce autologous T cell proliferation similarly as controls, but do not inhibit the release of TNF-α and INF-γ.
Idali et al, 2008[117]	Active disease	$CD4^+FoxP3^+$ (% *of* $CD4^+$)	FC/PCR	↓	↓					BALF Treg proportions are significantly higher than blood Treg proportions in both healthy controls and patients.
Taflin et al, 2009[113]	Active disease	$CD4^+CD45RA^-FoxP3^{++}$ (% *of* $CD4^+$)	FC/IHC	↑		↑*	↓			$FoxP3^+$ Tregs in the sarcoid LN are highly proliferative ($Ki67^+$).
Prasse et al, 2010[115]	Pre-treatment patients	$CD4^+CD25^+CD127^-$-(% *of* $CD4^+$)	FC		↓^			↓**,#		^ BALF Treg proportions are decreased in patients who develop active chronic disease, defined after 1 year follow-up. # Vasoactive intestinal peptide (VIP) inhalation increased the number of BALF Tregs and the immunosuppressive function.
Rappl et al, 2011[114]	Unknown	$CD25^+CD7^-$ % of ($CD4^+CD45RO^+FoxP3^+CD127^-$)	FC		↑		↓			Increased proportions of $CD4^+FoxP3^+CD127^-$ Tregs are $CD7^-$, compared with healthy controls.
Wiken et al, 2012[118]	Active disease$	$FoxP3^+$(% *of* $CD4^+CD45RO^+CD27^+$)	FC		↓***					BALF Tregs proportions are siginificantly decreased in HLA-DRB1*0301 positive patients, which are mostly (82%) Lofgren patients.
Darlington et al, 2012[119]	Active disease	$CD4^+FoxP3^+$ (% *of* $CD4^+$)	FC			=*				% FoxP3 expressing $CD4^+$ T cells is inversely correlated with % T cells with AV2S3 > 10% in BALF.
Oswald-Richter et al, 2013[116]	Active disease	$CD4^+CD45RO^+$ $CD25^{high}$ (% *of* $CD4^+$)	FC	↑			↓			Treg malfunctioning restored during disease resolution

All results are compared with healthy controls, unless specified otherwise. Flowcytometry (FC), Immunohistochemistry (IHC), Polymerase chain reaction (PCR), broncho-alveolar lavage fluid (BALF), lymph node (LN).

$ HLA-DRB1*0301 positive sarcoidosis patients were analyses vs HLA-DRB1*0301 negative sarcoidosis patients

* Compared with diseased controls

** Compared with post-treatment

*** Compared with HLA-DRB1*0301 negative patients

pression) in patients have been associated with a favorable prognosis in a Scandinavian population (118). In contrast, a German study reported decreased BALF Treg numbers in sarcoidosis patients who develop chronic (active) disease, when compared with controls and patients who develop spontaneous resolution (115). Similarly, CD1d-restricted natural-killer T (NKT) cells with immunoregulatory function are greatly reduced in the peripheral blood of all sarcoidosis patients, except Löfgren patients (120).

Taken together, these studies imply different roles for immune regulatory cells in sarcoidosis, either contributing to or preventing an on-going, exaggerated immune response. Arguably, whereas in the early sarcoid response there may be no need for Tregs to inhibit an effective immune response, during persistent stimulation immune regulatory cells should function as a natural brake on the exaggerated response to prevent immunopathology and autoimmunity.

Interfering with granuloma integrity

Effective treatment agents used for sarcoidosis interfere with granuloma integrity and would ideally prevent fibrogenesis. Glucocorticosteroids (GCs), the main stay of sarcoidosis therapy, partially exert their beneficial effect by repression of NF-κB-related cytokine gene transcription and induction of lymphocyte apoptosis (121, 122). Using a mouse model, Tregs are found to be less sensitive to GC-induced apoptosis compared with Th cells, favoring an anti-inflammatory milieu (123, 124). Similarly, anti-TNF agents induce monocyte and lymphocyte apoptosis (125-127), while improving Treg numbers (123). Interestingly, infliximab, which blocks membrane-bound TNF-α, is uniquely associated with a high risk of reactivation of latent *Mtb* infection, whereas etanercept, solely blocking secreted TNF-α, is not (29). This phenomenon implies a critical role of membrane-bound TNF-α signaling in granuloma integrity (29), which is further supported by mouse studies (128).

Whether GCs and anti-TNF agents interfere with the delicate Th/Treg balance in pulmonary sarcoidosis, remains to be elucidated. Research into this field will shed more light on the role of Tregs in sarcoid pathology and whether Treg induction holds a promising new therapeutical strategy. Finally, an interplay between anergic Th cells, IL-10, alternatively activated macrophages (M2), CCL18 and lung fibroblasts has recently been suggested to contribute to fibrotic remodeling of the lung in chronic sarcoidosis (129). These insights yield new therapeutical targets to prevent irreversible organ damage in chronic pulmonary sarcoidosis patients.

CONCLUSION

Sarcoidosis is an intriguingly complex granulomatous disorder, characterized by an exaggerated Th1/17 immune response, initiated by APCs, and maintained due to malfunc-

tioning of Tregs. Refining insight into immunological events that determine granuloma fate may help identify new therapeutical targets and patients who will benefit such therapy in the future.

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CHAPTER 2

Decreased Cytotoxic T-lymphocyte Antigen 4 Expression on Regulatory T Cells and Th17 Cells in Sarcoidosis: Double Trouble?

'Challenge the obvious'
Filosofie van Nauta*Dutilh*

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To the editor:

Cytotoxic T-lymphocyte antigen 4 (CTLA4) is an important suppressor of T-cell-mediated immune responses, and has become a major target in tumor therapy (1). Intriguingly, various case reports on initiation or exacerbation of sarcoidosis during anti-CTLA4 treatment of metastatic melanoma have recently emerged. This phenomenon is considered biotherapy–induced sarcoidosis, as in all reported cases, granuloma lesions occurred and progressed during biotherapy employment and disappeared after drug discontinuation (2). In patients with metastatic melanoma, CTLA4 blockade increased circulating proportions and absolute numbers of IL-17-producing $CD4^+$ T cells, whereas it only marginally changed T helper(Th)1 or Th2 cell percentages (3). Although the effect on regulatory T cell (Treg) proportions remains a matter of debate, CTLA4 blockade was found to impair Treg-mediated suppression (1). Moreover, in both mice and humans, CTLA4 deletion, blockade or dysfunction is strongly associated with the development of autoimmune lesions (1), in which Tregs and Th17 cells are known to play a pivotal role. Because sarcoidosis is characterized by an abnormal Th1/Th17 cell response and impaired Treg function (4), it is conceivable that CTLA4 blockade exacerbates underlying or pre-existent disease.

These clinical observations provide a rationale to examine CTLA4 expression on $CD4^+$ T cells in sarcoidosis in lung-draining mediastinal lymph nodes (MLN), which are an important site of primary and memory T-cell activation by dendritic cells. We hypothesize that altered CTLA4 expression contributes to ongoing Th1/Th17 cell responses in sarcoidosis. Using flow cytometry, we determined CTLA4 expression on $CD4^+$ T cell subsets (for gating strategy, *see* Figure E1 in the online supplement*)* in MLN from patient with sarcoidosis (n=18) and controls (n=24) (for methods, *see* Materials and Methods in the online supplement and Table E1).

Activated T cells from sarcoidosis MLN showed decreased CTLA4 expression compared with control MLN (Figure 1A). CTLA4 expression was also decreased on nonactivated memory T cells, but normal on naive T cells (Figure E2). Evaluating Th cell subsets showed a significant decrease in CTLA4 expression specifically on sarcoidosis Th17 cells (P=0.004) but not on Th1 or Th2 cells (Figures 1B and 1C). Furthermore, proportions of Th17 cells were significantly increased in sarcoidosis MLN (Figure 1D), consistent with our previous finding of enhanced Th17 cell proportions in peripheral blood and lungs of patients (5).

Notably not only Th17 cells but also activated Tregs showed decreased CTLA4 expression in sarcoidosis MLN compared with controls (P=0.016) (Figure 1B and 1C). Treg proportions were not increased (Figure 1E). Intriguingly, as CTLA4 protein expression is critical for Treg function (6) and Th17 cells are uniquely sensitive towards CTLA4 co-inhibition (7), impaired CTLA4 expression on these specific subsets contributes to Th17 cell differentiation and activation and to ongoing Th(17) cell proliferation.

Although there is evidence that sarcoidosis MLN parallel the lungs in terms of $CD4^+$ T cell (8) and Th17 cell (this manuscript) influx, we also determined CTLA4 expression on T cells in broncho-alveolar lavage fluid. Importantly, similar to in sarcoidosis MLN, sarcoidosis broncho-alveolar lavage fluid memory Th cells showed significantly decreased CTLA4 expression compared with disease controls ($P<0.0001$) (Figure E3).

CTLA4 expression depends on the strength of T cell stimulation, mediated by the T-cell receptor and CD28 expression (9). Decreased CTLA4 expression on $CD4^+$ T cells in sarcoidosis can therefore be a consequence of ongoing inflammation, leading to loss of CD28 expression, as described in chronic beryllium disease (10). However, we only found a slight, non-significant decrease of CD28 expression on sarcoidosis Th17 cells compared with controls (Figure 1F). Beryllium-responsive $CD4^+$ T cells in the lung-upregulated programmed death (PD)-1 reflecting T-cell exhaustion in response to chronic antigenic (beryllium) exposure (10). We did not find increased PD-1 expression on sarcoidosis Th17 cells (Figure 1G). Taken together, our findings do not support T-cell differentiation status or exhaustion as an explanation for low CTLA4 levels, but do suggest a specific defect in CTLA4 expression instead.

It is conceivable that genetic changes could underlie impaired CTLA4 protein expression in sarcoidosis. Genetic variations in the *CTLA4* gene are associated with various autoimmune disorders (1), and *CTLA4* gene mutations have been identified in families with common variable immunodeficiency, including patients with granulomatous lung disease (6). These mutations led to impaired CTLA4 up-regulation in activated Tregs, indicating that two functional CTLA4 alleles are necessary to drive high protein levels (6). We found up-regulation of CTLA4 on sarcoidosis activated Tregs compared with Th1, Th2, or Th17 cells (Figure 1C). Paired analyses showed that this was significant ($P=0.001$). Furthermore, *CTLA4* gene polymorphisms in sarcoidosis were not reported to be associated with disease susceptibility but merely with the extent of disease (11). Taken together, it is unlikely that genetic mutations within the *CTLA* gene impair CTLA4 protein expression in sarcoidosis.

The mechanisms that regulate CTLA4 expression on individual T-cell subsets remain largely unclear to date. The cause of impaired CTLA4 expression in sarcoidosis Tregs and Th17 cells may be T-cell non-intrinsic and depend on the local inflammatory microenvironment. Characterizing cytokine profiles in sarcoidosis MLN might help unravel further causes of diminished CTLA4 expression.

In summary, we are the first to demonstrate a significant decrease in CTLA4 expression specifically on Tregs and Th17 cells at an important site of T-cell activation in sarcoidosis. Because Th17 cell, and not Treg, proportions were increased, impaired CTLA4 expression in sarcoidosis could influence the delicate Th cell/Treg balance (4) and contribute to ongoing inflammation. Importantly, our data are in agreement with clinical observations

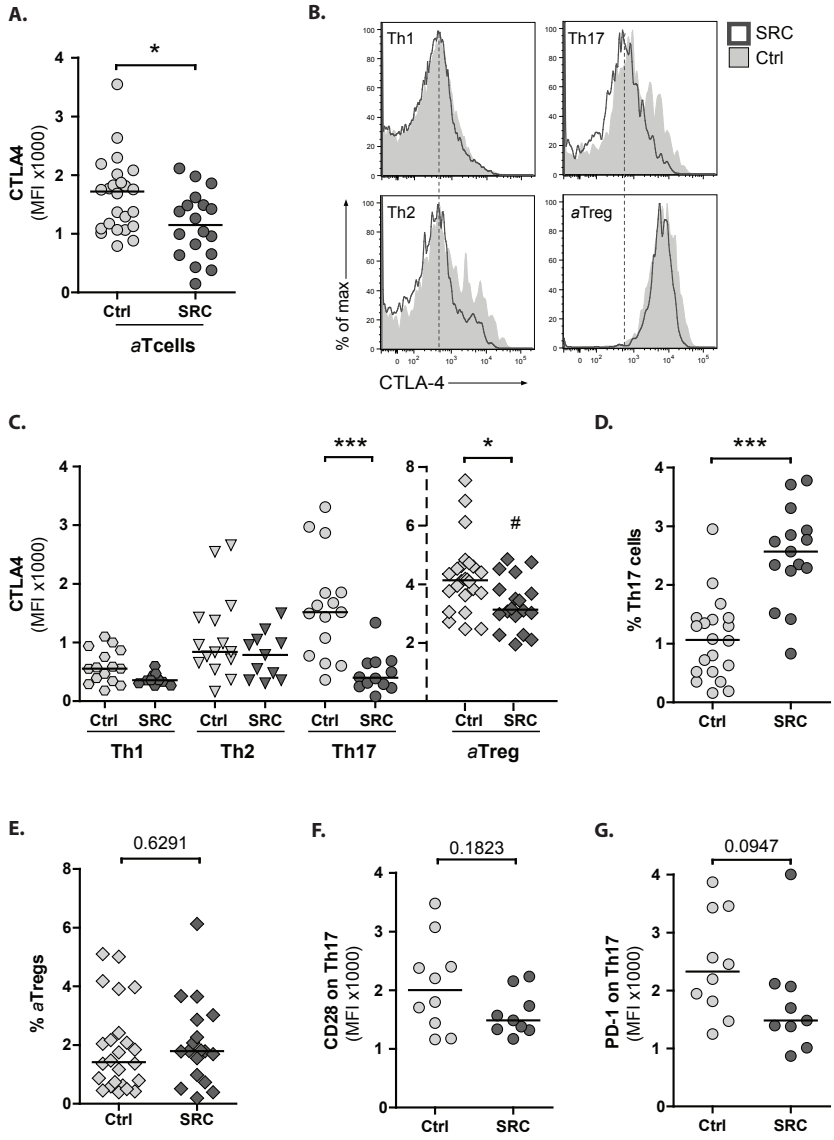

Figure 1. Decreased cytotoxic T-lymphocyte 4 antigen (CTLA4) expression on regulatory T cells (Tregs) and Th17 cells in sarcoidosis. CTLA4 expression was determined on $CD4^+$ T cells in control and sarcoidosis mediastinal lymph nodes. (*A*) Mean fluorescence intensity (MFI) of CTLA4 on activated ($CD45RA^-FoxP3^{int}$) T cells. (*B*) Flow cytometry analysis of CTLA4 expression on Th1, Th2 and Th17 cells and activated ($CD45RA^-FoxP3^{high}$) Tregs of a control patient and a patient with sarcoidosis. (*C*) MFI of CTLA4 on Th1, Th2, and Th17 cells and activated Tregs. (*D*) Th17 proportions of total memory T cells. (*E*) Activated Treg proportions of total $CD4^+$ T cells. (*F*) MFI of CD28 on Th17 cells. (*G*) MFI of programmed death (PD)-1 on Th17 cells. Statistics: *Horizontal lines* indicate the median, and significance was determined using a Mann-Whitney *U* test, $^*P < 0.05$ $^{***}P < 0.001$. Significance between medians of paired samples was determined using a Wilcoxon signed rank test ($^{\#}P$=0.001: sarcoidosis activated Treg vs. sarcoidosis Th1,Th2, or Th17 cells). *a*Tcells = activated T cells, *a*Treg = activated Treg, Ctrl = control, SRC = sarcoidosis.

that anti-CTLA4 treatment can exclusively increase Th17 cells (3) and induce sarcoidosis (2).

In conclusion, our data and clinical observations argue for a central role of CTLA4 in sarcoidosis pathology. Decreased CTLA4 protein expression on Tregs and Th17 cells in sarcoidosis MLN can cause double trouble: on the one hand contributing to Th17 priming and activation (7), and on the other, impairing Treg-mediated suppression (1). The treatment-naive sarcoidosis population studied displayed limited inter-patient variability regarding CTLA4 expression values, precluding an analysis of their correlation with disease prognosis. However, prospective studies can help provide a rationale to evaluate CTLA4 Ig (e.g. abatacept) efficacy in sarcoidosis (for a proposed model concerning effects of various CTLA4 expressions on Tregs and Th17 cells and a rationale for the effect of CTLA4 Ig treatment in sarcoidosis *see* Figure E4).

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SUPPLEMENTARY MATERIAL

METHODS

Study design and subjects

Patients with pulmonary sarcoidosis were included at time of diagnosis. The diagnosis of sarcoidosis was made conform the guidelines of the ATS/ERS/WASOG (1).

Exclusion criteria were use of immunomodulatory medication 3 months prior to study inclusion; respiratory tract infection 4 weeks prior to study inclusion; concomitant pulmonary disease (including chronic obstructive pulmonary disorder and asthma), autoimmune diseases, malignancies, human immunodeficiency virus seropositivity, pregnancy, and allergies.

For this study, 18 sarcoidosis patients donated lymph nodes aspirates from lung draining lymph nodes. Control mediastinal lymph nodes were collected from 24 lung transplantation donors without signs of pulmonary inflammation (routinely assessed by a chest X-ray and bronchoscopy). The cause of death of these donors was a cerebrovascular incident (n=16), cardial arrest (n=3), trauma capitis (n=3) or was not specified (n=2). Due to limitations in the number of cells isolated per lymph node aspirate per patient, we were not able to determine all subset analysis in all patients.

Furthermore, 27 additional sarcoidosis patients, 8 patient with community-acquired pneumonia (CAP) and 4 patients with COPD donated BALF for this study.

The Medical Ethical Committee of the Erasmus University Medical Centre Rotterdam approved this study. Written informed consent was obtained from every participant before study inclusion. Further subject characteristics are shown in Supplementary Table E1 (MLN analyses) and Supplementary Table E2 (BALF analyses).

Study materials

Eosophageal or endobronchial ultrasound guided fine-needle aspiration (EUS- or EBUS-FNA) from draining mediastinal lymph nodes was performed with a 22G (or 19G) needle. Mediastinal lymph node aspirates were filtered through a 100 µm cell strainer (BD Biosciences) and centrifuged. The cells were 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, and stored at -150 °C.

Control mediastinal lymph nodes were collected from lung transplantation donors. These lymph nodes are routinely removed from the donor lungs and discarded before implantation in the acceptor. Control mediastinal lymph nodes were processed as lymph node aspirates.

Bronchoscopy with BAL was performed as previously described (2) and BALF cells were processed as previously described (2).

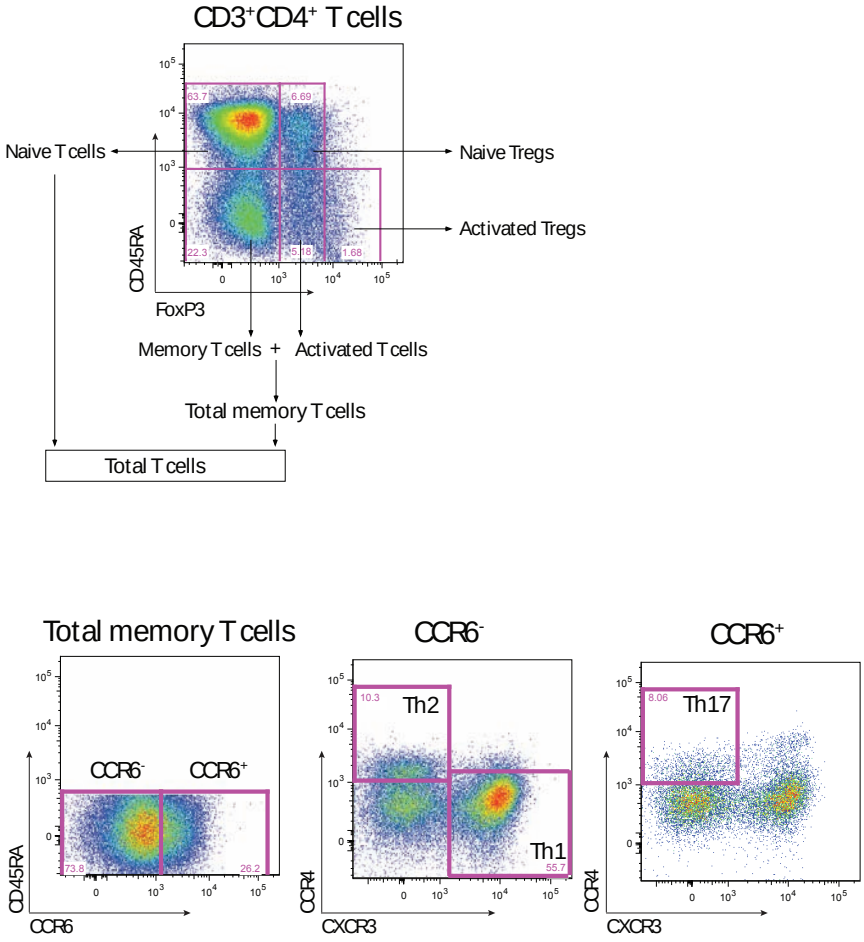
Flow cytometry staining

Mediastinal lymph node and BALF cells were stained for extra- and intracellular markers using the following antibodies: CD3-APC-eFluor780 (SK7), CD4-AF700 (OKT4), FoxP3-APC (PCH101), CTLA4-PerCp-eFluor710 (14D3) (eBiosciences) and CCR6-APC (11A9), CXCR3-BV711 (1C6/CXCR3), CTLA4-BV421 (BNI3), CD28-BV605 (CD28.2), PD-1-BV711 (EH12) (BD biosciences) and CCR4-FITC (205410) (R&D) and CXCR3-BV421 (G025H7) (Biolegend) and CD45RA-PE-Texas Red (MEM-56) (Invitrogen). Fixable Aqua Dead Cell Stain kit for 405 nm (Invitrogen, Molecular Probes) was used as live-dead marker. At least 100.000 cells per sample were measured on a Flow cytometer LSRII (BD Biosciences), and the mean fluorescence intensity of CTLA4, CD28 and PD-1 was standardized to average expression in healthy control peripheral blood cells.

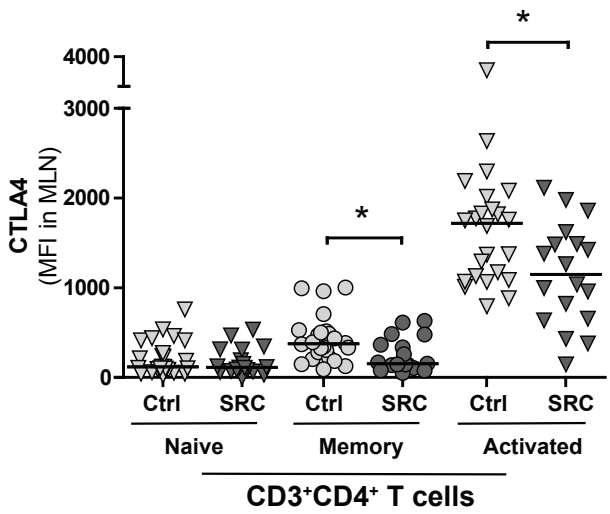
Statistical analyses

Comparisons were performed using a Mann-Whitney U test or Wilcoxon signed rank test. *P*-values were two sided, and analyses were performed using IBM SPSS Statistics 21. $P < 0.05$ was considered statistically significant.

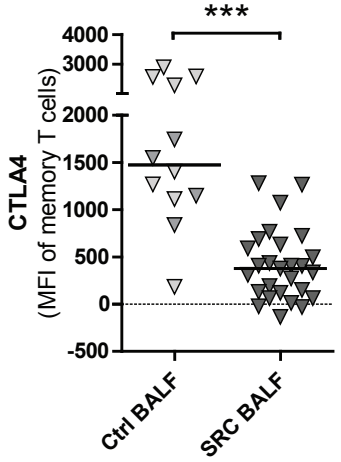
SUPPLEMENTARY FIGURES



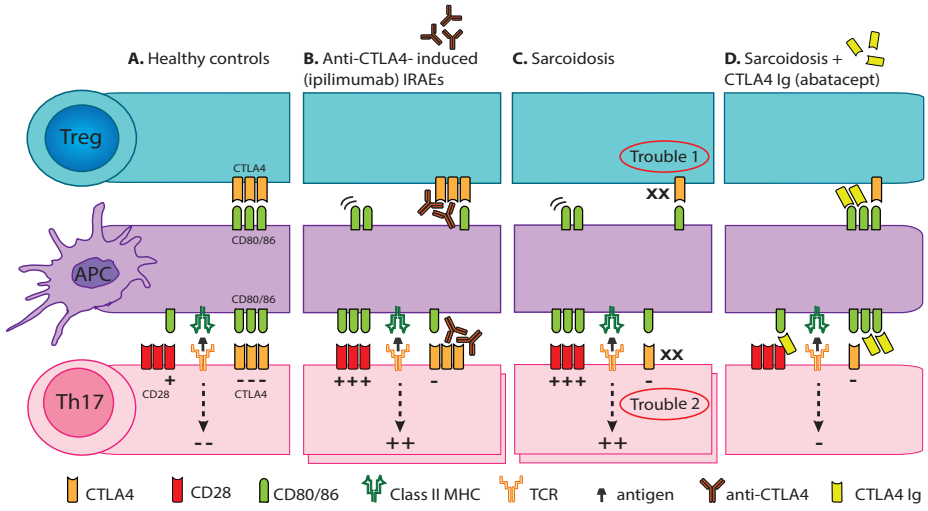
Supplementary Figure E1. Gating strategies to determine $CD3^+CD4^+$ T cell subsets. (*A*) Five $CD4^+$ T cell populations were characterized within mediastinal lymph nodes according to CD45RA and intracellular FoxP3 expression (3), i.e. $CD45RA^+FoxP3^-$ naïve T cells, $CD45RA^-FoxP3^-$ memory T cells, $CD45RA^-FoxP3^{int}$ activated (non-suppressive/-regulatory) T cells, $CD45RA^+FoxP3^{int}$ naïve regulatory T cells and $CD45RA^-FoxP3^{high}$ activated Tregs. The total memory T cell pool consists of memory T cells plus activated T cells. The total (non-suppressive/-regulatory) T cell pool consists of naïve T cells plus total memory T cells. (*B*) Within several mediastinal lymph nodes T helper subsets were characterized within the total memory T cells, using chemokine receptor expression, i.e. $CCR6^-CXCR3^+$ Th1, $CCR6^-CCR4^+$ Th2 and $CCR6^+CCR4^+$ Th17 cells (4). *Abbreviations:* Treg = regulatory T cells.



Supplementary Figure E2. CTLA4 expression was determined on $CD4^+$ T cells in control and sarcoidosis mediastinal lymph nodes. Mean fluorescence intensity of CTLA4 on naïve, memory and activated T cells is shown. *Statistics:* Horizontal lines indicate the median and significance was determined using a Mann-Whitney U test, * $P < 0.05$. *Abbreviations:* CTLA4 = cytotoxic T lymphocyte antigen 4, MFI = mean fluorescence intensity, Ctrl = control, SRC = sarcoidosis.



Supplementary Figure E3. CTLA4 expression was determined on $CD4^+$ memory T cells in sarcoidosis (n=27) and disease control (i.e. community-acquired pneumonia (n=8: lighter grey) and chronic obstructive pulmonary disease (n=4: darker grey) patients) BALF. Mean fluorescence intensity of CTLA4 on memory $CD4^+$ T cells in MLN and BALF is shown. Sarcoidosis BALF-derived memory $CD4^+$ T cells showed a significant decrease of CTLA4 expression compared with disease controls ($P < 0.0001$). *Statistics:* Horizontal lines indicate the median and significance was determined using a Mann-Whitney U test, *** $P < 0.0001$. *Abbreviations:* CTLA4 = cytotoxic T lymphocyte antigen 4, MFI = mean fluorescence intensity, BALF = bronchoalveolar lavage fluid, Ctrl = control, SRC = sarcoidosis.



Supplementary Figure E4. A proposed model for the effect of CTLA4 expression by Tregs and Th17 cells in healthy control (*A*), anti-CTLA4-treated (*B*) and sarcoidosis (*C*) mediastinal lymph nodes, and a rationale for the effect of CTLA4 Ig treatment in sarcoidosis (*D*). (*A*) Healthy Tregs highly express CTLA4 and indirectly inhibit T cell activation via CD80/86 engagement on APCs. This leads to restricted CD80/86 ligand availability on APCs, inducing tolerance (5). Healthy control Th17 cells highly express CTLA4 which competes with CD28-ligand binding, since CTLA4 interacts with CD80/86 with higher affinity and avidity than does CD28 (5). Therefore, healthy Th17 cells are sensitive towards CTLA4 co-inhibition, leading to suppressed Th17 differentiation, activation or proliferation (6). (*B*) Anti-CTLA4 treatment (e.g., ipilimumab) leads to enhanced CD80/86 ligand availability on APCs and decreased competition with CD28-ligand binding on Th17 cells. This can increase Th17 cell differentiation, activation and therefore enhance their proportions (7), contributing to IRAEs, including biotherapy-induced sarcoidosis (8). (*C*) Sarcoidosis Tregs show decreased CTLA4 protein expression (Figure 1B/C) leading to enhanced CD80/86 ligand availability on APCs (Trouble 1). Additionally, sarcoidosis Th17 cells show decreased CTLA4 protein expression (Figure 1B/C), leading to impaired competition with CD28-ligand binding on Th17 cells and therefore enhanced Th17 differentiation, activation and increased Th17 proportions (Figure 1D) (9) (Trouble 2). (*D*) Using a CTLA4-Ig fusion protein (e.g., abatacept) in sarcoidosis will bind to CD80/86 with high affinity and therefore restrict ligand CD80/86 availability on APCs (5). Additionally, it will compete with CD28-ligand binding on Th17 cells, leading to suppressed Th17 cell responses in sarcoidosis. *Abbreviations:* Treg = regulatory T cell, APC = antigen presenting cell, Th = T helper, CTLA4 = cytotoxic T lymphocyte antigen 4, TCR = T cell receptor, IRAEs = immune-related adverse events.

Supplementary Table E1. Study subject characteristics MLN samples

Subject characteristics	**Controls (n=24)**	**Sarcoidosis (n=18)**
Median age (min-max)	47 (15-70)	45 (24-75)
Sex (Male/Female)	6/18	12/6
Scadding Stage (n (% of total))		
Stage 0		3 (16,7)
Stage I		7 (38,9)
Stage II		5 (27,8)
Stage III		1 (5,6)
Unknown		2 (11,1)[†]
Diagnosis was assessed by (n (% of total))		
E(B)US-FNA		16 (88,9)
Mediastinoscopy		2 (11,1)
Extrathoracic involvement (n (% of total))		
No/Yes		5/13 (27,8/72,2)
Skin		5 (27,8)
Eyes		7 (38,9)
Articular [$]		8 (44,4)
Central nervous system		1 (5,6)
Other (nasopharynx, parotis, thyroid)		3 (16,7)
Residual radiological activity at 6 months following diagnosis (n (% of total))		
No		2 (11,1)
Yes		11 (61,1)
No chest X-ray and/or CT thorax		4 (22,2)
Lost to follow up		1 (5,6)
Need for treatment within 6 months following diagnosis (n (% of total))		
No		11 (61,1)
Yes		6 (33,3)
Lost to follow up		1 (5,6)

[†] One patient had Stage I and the other patient had Stage II sarcoidosis, determined by CT scan. [$] Self-reported articular involvement.

Supplementary Table E2. Study subject characteristics BALF samples

Subject characteristics	Controls (n=12)	Sarcoidosis (n=27)
Median age (min-max)	54 (18-80)	42 (24-73)
Sex (Male/Female)	7/5	15/12
Scadding Stage (n (% of total))		
Stage 0		2 (7,4)
Stage I		11 (40,7)
Stage II		13 (48,1)
Stage III		0 (0,0)
Unknown		1 (3,7)[†]
Diagnosis was assessed by (n (% of total))		
E(B)US-FNA		4 (14,8)
TBB-EBB		15 (55,6)
BAL CD4/CD8 > 3,5		8 (29,6)
Extrathoracic involvement (n (% of total))		
No/Yes		12/15 (44,4/55,6)
Skin		1 (3,7)
Eyes		8 (29,6)
Articular [$]		8 (29,6)
Central nervous system		0 (0,0)
Other (submandibular LN and parotis)		1 (3,7)
Residual radiological activity at 6 months following diagnosis (n (% of total))		
No		0 (0,0)
Yes		18 (66,7)
No chest X-ray and/or CT thorax		4 (14,8)[‡]
Lost to follow up		5 (18,5)
Need for treatment within 6 months following diagnosis (n (% of total))		
No		16 (59,3)
Yes		6 (22,2)
Lost to follow up		5 (18,5)

[†] This patient had Stage I sarcoidosis, determined by CT scan.

[$] Self-reported articular involvement.

[‡] Two patients had spontaneous resolution as determined with radiology at 2 years follow up and disease course of 2 patients is yet to be determined at 2 years follow up.

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CHAPTER 3

Impaired Survival of Regulatory T Cells in Pulmonary Sarcoidosis

'Try before you die'
Televisieprogramma van BNN

Respir Res. 2015 Sep 16;16:108

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ABSTRACT

Background: Impaired regulatory T cell (Treg) function is thought to contribute to ongoing inflammatory responses in sarcoidosis, but underlying mechanisms remain unclear. Moreover, it is not known if increased apoptotic susceptibility of Tregs may contribute to an impaired immunosuppressive function in sarcoidosis. Therefore, the aim of this study is to analyze proportions, phenotype, survival, and apoptotic susceptibility of Tregs in sarcoidosis.

Methods: Patients with pulmonary sarcoidosis (*n*=58) were included at time of diagnosis. Tregs were analyzed in broncho-alveolar lavage fluid and peripheral blood of patients and healthy controls (HC).

Results: In sarcoidosis patients no evidence was found for a relative deficit of Tregs, neither locally nor systemically. Rather, increased proportions of circulating Tregs were observed, most prominently in patients developing chronic disease. Sarcoidosis circulating Tregs displayed adequate expression of FoxP3, CD25 and CTLA4. Remarkably, in sarcoidosis enhanced CD95 expression on circulating activated $CD45RO^{+}$ Tregs was observed compared with HC, and proportions of these cells were significantly increased. Specifically sarcoidosis Tregs - but not Th cells - showed impaired survival compared with HC. Finally, CD95L-mediated apoptosis was enhanced in sarcoidosis Tregs.

Conclusion: In untreated patients with active pulmonary sarcoidosis, Tregs show impaired survival and enhanced apoptotic susceptibility towards CD95L. Increased apoptosis likely contributes to the insufficient immunosuppressive function of sarcoidosis Tregs. Further research into this field will help determine whether improvement of Treg survival holds a promising new therapeutic approach for chronic sarcoidosis patients.

INTRODUCTION

Sarcoidosis is a multisystem granulomatous disorder of unknown cause, often affecting the lungs (1). The disease is characterized by an exaggerated T helper (Th)1/Th17 response upon exposure to one or several antigens in genetically susceptible individuals (2, 3). Clinical presentation and disease prognosis vary greatly. Although the majority of the patients undergo spontaneous resolution, a substantial proportion develops chronic, progressive disease with need for therapy (1). Factors that determine granuloma fate remain to be elucidated (1, 3).

Failure of immune regulatory mechanisms to limit duration of inflammation has been suggested to contribute to persisting granulomatous responses in sarcoidosis (4), and may explain the need for immunosuppressive drugs. Effective immunosuppressive agents for (chronic) sarcoidosis include corticosteroids and anti-TNF agents (5). Interestingly, these drugs can induce Th cell apoptosis, while sparing or even inducing regulatory T cell (Treg) proportions and function (6-10), thereby favoring an anti-inflammatory milieu.

Tregs are an indispensable subset of T cells with strong immunosuppressive capacities on a wide range of immune cells, including Th cells, B cells, and antigen presenting cells (11). They have a fine sensitivity for immune dynamics, mediated by interleukin (IL)-2 signaling, and can quickly adjust their numbers and function during immune challenge (12). Upon activation, Th cells produce IL-2, which contributes to Treg proliferation and survival (12). Appropriate Treg function is required to terminate the immune response after antigen eradication, thus preventing (redundant) continuing inflammation (12). Importantly, defective Treg function contributes to induction, sustainment or progression of various autoimmune diseases, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), but also granulomatous disorders such as antineutrophil cytoplasmic antibody-associated vasculitis (AAV) and hypersensitivity pneumonitis (HP) (13-15).

The role of Tregs in the pathogenesis of sarcoidosis remains controversial. An impaired immunosuppressive function of sarcoidosis peripheral blood (PB) Tregs has been reported consistently (4, 16-18). Furthermore, broncho-alveolar lavage fluid (BALF)-derived Treg suppressive efficacy was found to increase during short-term inhaled vasoactive intestinal peptide (VIP)-treatment in pulmonary sarcoidosis, which was also associated with amelioration of clinical symptoms (i.e. dyspnea and cough) (19). Decreased inhibition of Th cell proliferation and cytokine production by Tregs therefore likely contributes to the ongoing and exaggerated immune responses seen in sarcoidosis. Since contradictory results have been reported about Treg proportions with respect to Th cells in both BALF and PB of sarcoidosis patients (*See for review:* (3)), it remains to be determined what mechanism(s) underlies this impaired immunosuppressive function (3).

Pro- and anti-apoptotic pathways play an important role in Treg homeostasis (12). Intriguingly, in sarcoidosis granulomas and PB increased proportions of activated (i.e. $CD45RO^{+}FoxP3^{high}$) proliferating Tregs are found (4). The significance of this finding for sarcoidosis pathogenesis remains unclear, since $CD45RO^{+}FoxP3^{high}$ Tregs are described to be both more suppressive and more sensitive towards apoptosis in healthy individuals (20). Although it has been suggested that increased apoptotic susceptibility of Tregs may contribute to an impaired immunosuppressive function in sarcoidosis (4), it remains unknown if survival of patient-derived Tregs is affected.

Therefore, the aim of this study is to analyze proportions, phenotype, survival, and apoptotic susceptibility of sarcoidosis Tregs. Our results demonstrate that in patients with active pulmonary sarcoidosis, Tregs show impaired survival and enhanced apoptotic susceptibility towards CD95L.

METHODS

Study design and subjects

Patients with pulmonary sarcoidosis were included at time of diagnosis. The diagnosis of sarcoidosis was made conform the guidelines of the ATS/ERS/WASOG (1).

Exclusion criteria were use of immunomodulatory medication 3 months prior to study inclusion; respiratory tract infection 4 weeks prior to study inclusion; concomitant pulmonary disease (including chronic obstructive pulmonary disorder and asthma), autoimmune diseases, malignancies, human immunodeficiency virus seropositivity, pregnancy, and allergies.

For this study, in total 58 newly diagnosed sarcoidosis patients donated PB and/or BALF. Due to limitations in the number of cells isolated per patient, we were not able to perform all experiments on all patients. Furthermore, 47 healthy controls donated PB and 5 healthy controls underwent bronchoscopy with BAL for this study.

The Medical Ethical Committee of the Erasmus MC Rotterdam approved this study. Written informed consent was obtained from every participant before study inclusion. Further subject characteristics are shown in Additional file: Table E1.

Study materials

Bronchoscopy with BAL and mucosal biopsy was performed as previously described (21). BALF cells, mucosal biopsies and PB were processed as previously described (21).

Flow cytometry staining

BALF cells and PBMCs were stained for extra- and intracellular markers using the following antibodies: CD3-APC-eFluor780 (SK7), CD4-AF700 (OKT4), CD45RO-FITC (UCHl-1), CD95-APC (DX2), FoxP3-APC (PCH101) (eBiosciences) and CD25-PE (M-A251), CD25-PE-

Cy7 (M-A251), CD127-V450 (hIL7R-M21) anti-CTLA-4-BV421 (BNI3) (BD biosciences) and CD45RA-PE-Texas Red (MEM-56) (Invitrogen). Fixable Aqua Dead Cell Stain kit for 405 nm (Invitrogen, Molecular Probes) was used as live-dead marker. Cells were measured on a Flow cytometer LSRII (BD Biosciences).

Suppression assays

Th cells and Tregs were sorted from PBMCs using a human $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (MACS, Miltenyi Biotec). Th cells were labeled with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) according to manufacturer's protocol. CFSE-labeled Th cells were stimulated with Anti-Biotin MACSiBead™ Particles (MACS, Miltenyi Biotec) and co-cultured with unlabeled Tregs. Th cell division was quantified based on serial halving of CFSE intensity, using algorithms provided by FlowJo software (Treestar).

Cytokine measurements in supernatants of co-cultures were performed using the Bio-Plex Pro Human Cytokine 17-plex Panel (Biorad), run on a Luminex 100 System (Luminex Corporation), according to manufacturer's protocol.

Survival and apoptosis assays

$CD25^{low\text{-}int}CD127^{high}$Th cells and $CD25^{int\text{-}high}CD127^{low}$Tregs were isolated from PBMCs using the BD FACSAria Cell Sorter (BD Biosciences) and cultured with 20 ng/ml recombinant human IL-2 (hIL-2; R&D systems). Survival was determined with DAPI Nucleic Acid Stain (Life Technologies)-negative cells. To measure Treg survival and apoptosis in co-culture with Th cells, $CD4^+$ T cells were isolated from PBMCs using the human $CD4^+$ T cell isolation kit (MACS, Miltenyi Biotec), cultured with 20 ng/ml hIL-2 and examined using the FITC Annexin V Apoptosis Detection Kit I (BD biosciences). To assess Treg apoptotic susceptibility, $CD4^+$ T cells were cultured either with 20 ng/ml hIL-2 or with hIL-2 and 500 ng/ml soluble CD95L (recombinant human soluble FasL, Enzo Life Sciences).

Statistical analyses

Comparisons were performed using a Mann-Whitney U test or Wilcoxon matched pairs test. Correlations were analyzed using Spearman's rank-order correlation test. *P*-values were two sided, and analyses were performed using IBM SPSS Statistics 21. $P < 0.05$ was considered statistically significant.

RESULTS

Increased proportions of circulating Tregs in patients developing chronic sarcoidosis

Conflicting results have been reported with regard to Treg proportions in lungs and PB of sarcoidosis patients (3). Since a relative deficit of Tregs has been suggested to contribute

to a persisting granulomatous response in chronic sarcoidosis (19, 22), we first quantified $CD25^{int\text{-}high}FoxP3^{high}$ Tregs in BALF and PB of active sarcoidosis patients and healthy controls by flow cytometry. No evidence was found for a relative deficit of Tregs in BALF of untreated patients with active sarcoidosis compared with healthy control BALF (Additional file: Figure E1), suggesting an intact migration of Tregs towards site of inflammation. Rather, in PB of these patients increased proportions of $CD25^{int\text{-}high}FoxP3^{high}$ Tregs were found (Figure 1A and 1B). Interestingly, we determined the disease course of a subgroup of our study cohort with 2 years clinical follow-up, and found that in patients developing chronic sarcoidosis, but not in patients undergoing spontaneous resolution, significantly increased Treg proportions were detected at time of diagnosis, compared with healthy controls (Figure 1C).

In summary, in untreated patients with active sarcoidosis, no evidence was found for a relative deficit of Tregs, neither systemically nor at the site of inflammation. In contrast, significantly increased proportions of circulating Tregs were observed, most prominently in patients developing chronic disease. These data show that Treg homeostasis is affected in active sarcoidosis, which could contribute to disease course.

Circulating Tregs of sarcoidosis patients express adequate levels of FoxP3, CD25 and CTLA4

Since no numeral deficit of Tregs was found in patients at time of diagnosis, we questioned whether malfunctioning of circulating sarcoidosis Tregs is associated with an altered suppressive phenotype. Therefore, we analyzed circulating $CD25^{int\text{-}high}FoxP3^{high}$ Tregs of active sarcoidosis patients and healthy controls for expression of forkhead box P3 (FoxP3), CD25 and cytotoxic T lymphocyte antigen 4 (CTLA4) (three proteins pivotal for adequate Treg function (11)) by flow cytometry.

We confirmed that PB-derived $CD25^{+}$ Treg suppressive capacity on autologous Th proliferation and cytokine production was significantly less in sarcoidosis compared with healthy controls (Additional file: Figure E2). Circulating $CD25^{int\text{-}high}FoxP3^{high}$ Tregs of patients showed a trend towards increased expression levels of FoxP3 (Figure 2A). Furthermore, CD25 and CTLA4 expression (downstream molecules of FoxP3) were significantly increased on PB $CD25^{int\text{-}high}FoxP3^{high}$ Tregs of sarcoidosis patients compared with healthy controls (Figure 2B and 2C).

These data suggest that the impaired suppressive capacity of sarcoidosis PB-derived Tregs is not mediated by decreased expression of FoxP3 and its downstream molecules. Rather, a significant increase in CD25 and CTLA4 expression was found on circulating Tregs from sarcoidosis patients compared with healthy controls.

Activated CD45RO$^+$ Tregs from sarcoidosis patients highly express CD95

In healthy individuals, increased CD25 and CTLA4 expression on circulating Tregs has been associated with an activated, apoptotic-prone Treg population (20). Therefore, increased CD25 and CTLA4 expression on sarcoidosis PB-derived Tregs could point towards increased apoptosis, counteracting their functionality. In order to analyze their apoptotic phenotype, CD45RO and CD95 (FAS; death receptor) expressing Th cells and Tregs were examined in PB of healthy controls and patients (Figure 3A).

Within the PB Treg, but not Th cell population, proportions of CD45RO and CD95 expressing cells were significantly increased in sarcoidosis patients compared with healthy controls (Figure 3B). The majority of CD45RO$^+$ Tregs were CD95$^+$ in both patients and controls (Figure 3A) and their proportions strongly correlated ($P < 0.0001$, R = 0.87) (Figure 3C). Importantly, surface expression of CD95 was significantly increased on sar-

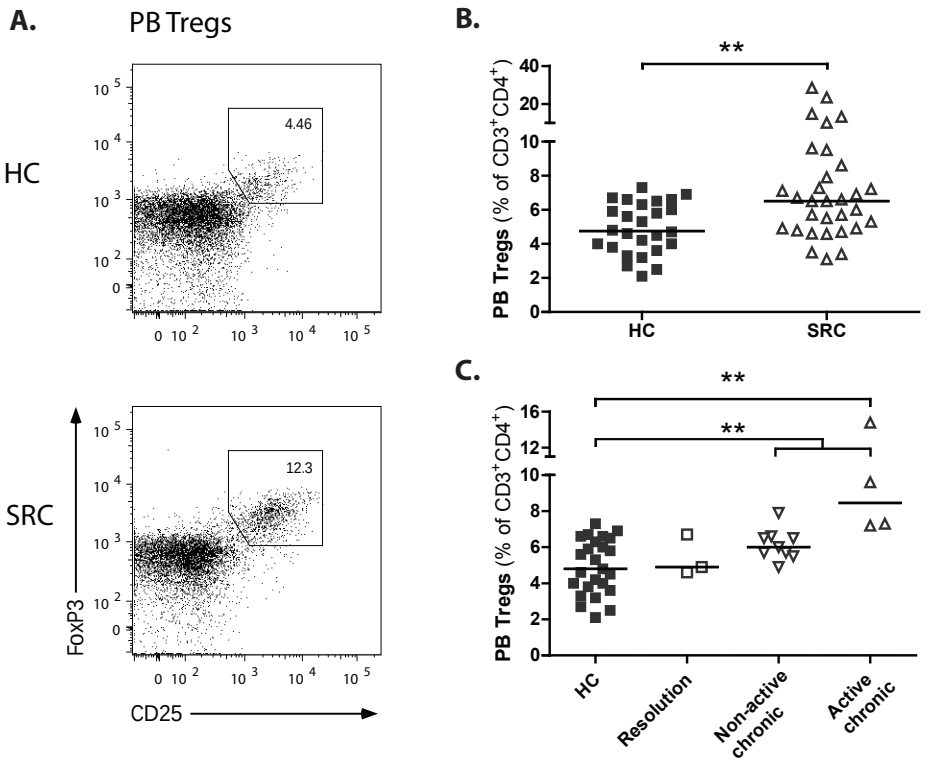

Figure 1. Increased proportions of circulating Tregs in patients developing chronic sarcoidosis. Treg proportions were determined in PB of HCs and SRC patients. (*A*) Representative flow cytometry analysis of an HC and SRC patient to determine Tregs in PB. (*B*) PB Treg proportions. (*C*) Subgroup analyses of PB Treg proportions in patients undergoing disease resolution, or developing (non-) active chronic disease. *Statistics:* Horizontal lines indicate the median and significance was determined using a Mann-Whitney U test, ** $P < 0.01$. *Abbreviations:* PB = peripheral blood, HC = healthy control, SRC = sarcoidosis.

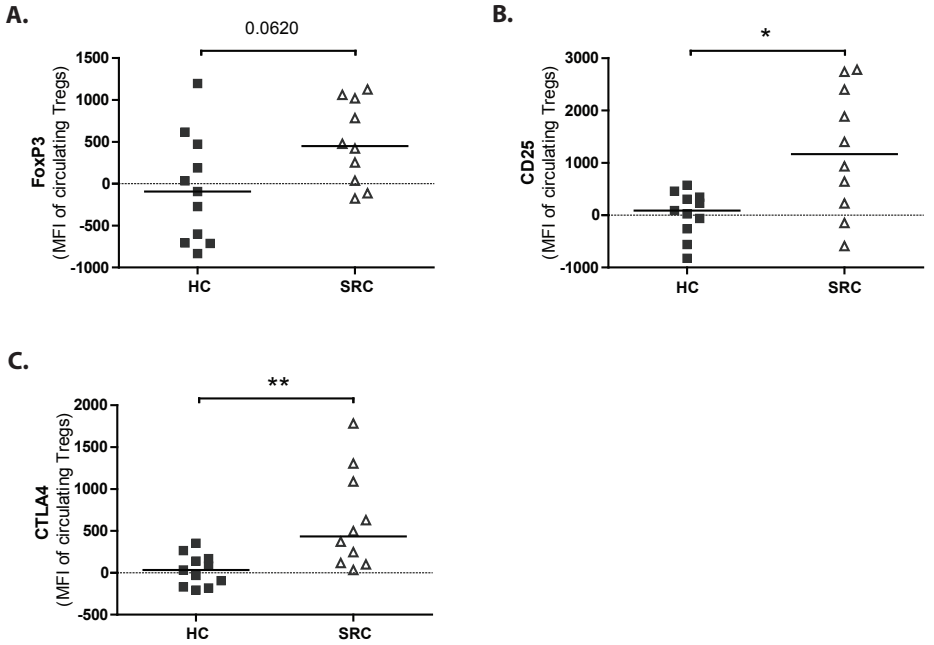

Figure 2. Adequate expression of FoxP3, CD25 and CTLA4 on sarcoidosis circulating Tregs. FoxP3, CD25 and CTLA4 expression was determined on circulating $CD25^{int\text{-}high}FoxP3^{high}$ Tregs of HC and SRC patients by flow cytometry. (*A-C*) Mean fluorescence intensity of FoxP3 (*A*), CD25 (*B*) and CTLA4 (*C*). Mean fluorescence intensity was standardized to average expression in healthy control peripheral blood cells. *Statistics:* Horizontal lines indicate the median and significance was determined using a Mann-Whitney U test, * $P < 0.05$ ** $P < 0.01$. *Abbreviations:* FoxP3 = forkhead box P3 , CTLA4 = cytotoxic T lymphocyte antigen 4, HC = healthy control, SRC = sarcoidosis.

coidosis $CD45RO^+$ Tregs compared with healthy control $CD45RO^+$ Tregs, whereas CD95 expression on $CD45RA^+$ Tregs was low and not different between healthy controls and patients (Figure 3D).

Altogether, increased proportions of circulating, activated $CD45RO^+$ Tregs were detected in sarcoidosis. Importantly, these activated $CD45RO^+$ Tregs highly express CD95 in patients compared with controls, suggesting altered apoptotic susceptibility.

Impaired survival of sarcoidosis Tregs

We questioned whether sarcoidosis-derived circulating Tregs would display altered survival. To investigate survival of sarcoidosis Th cells and Tregs, we isolated $CD25^{low\text{-}int}CD127^{high}$ Th cells and $CD25^{int\text{-}high}CD127^{low}$ Tregs from PB of patients and healthy controls. Proportions of $CD25^{int\text{-}high}FoxP3^{high}$ Tregs, as measured in the PB of our study subjects (Figure 1), strongly correlated with proportions $CD25^{int\text{-}high}CD127^{low}$ Tregs (data not shown). These data underlined previously published data that $CD4^+CD25^{int\text{-}high}CD127^{low}$

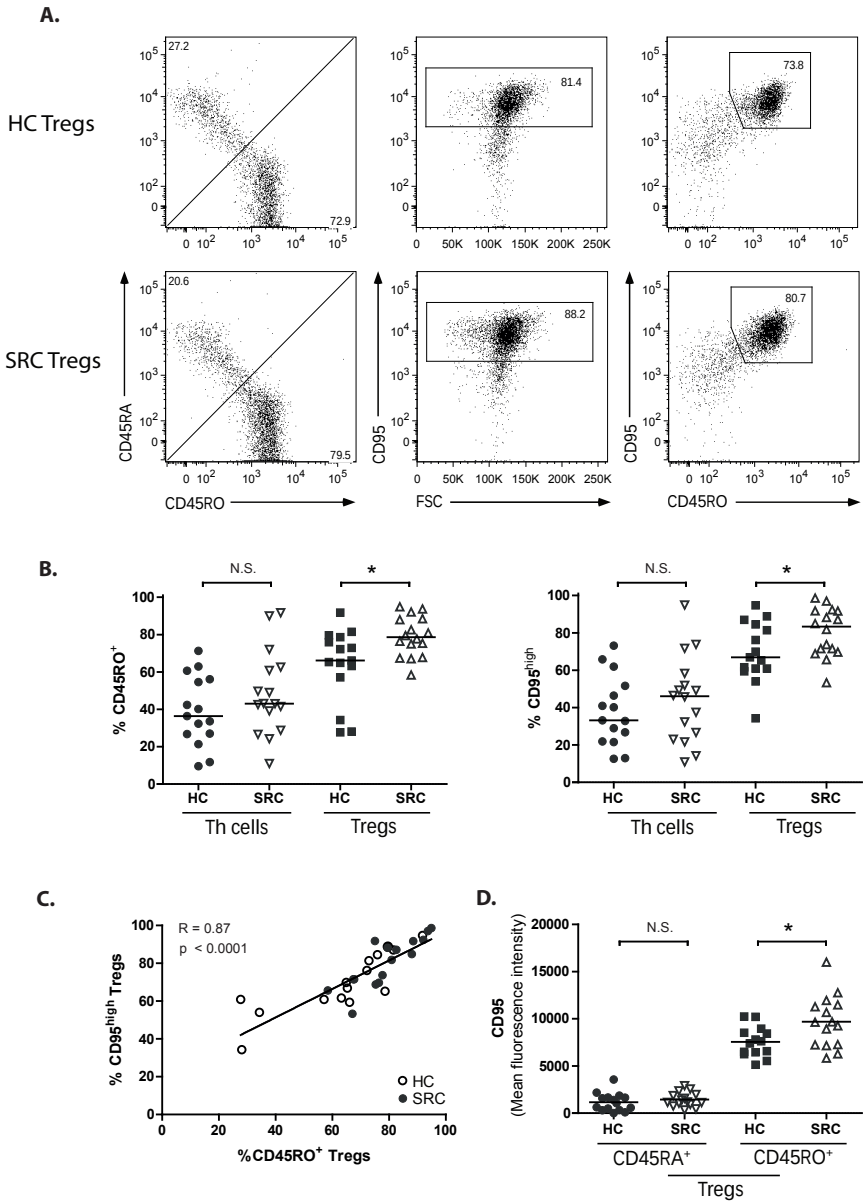

Figure 3. Activated CD45RO$^+$ Tregs from sarcoidosis patients highly express CD95. The proportions of CD45RO and CD95 expressing Th cells and Tregs were determined in PB of HCs and SRC patients. (*A*) Representative flow cytometry analysis of an HC and SRC patient to determine the proportions CD45RO$^+$, CD95^{high} and double positive Tregs (gated on CD3$^+$CD4$^+$CD25$^{int\text{-}high}$CD127^{low}). (*B*) Proportions of CD45RO$^+$ and CD95^{high} Th cells and Tregs. (*C*) Correlation between proportions CD45RO$^+$ Tregs and CD95^{high} Tregs in PB. Open dots represent HC Tregs and closed dots represent SRC patients. (*D*) Mean fluorescence intensity of CD95 on CD45RA$^+$ and CD45RO$^+$ Tregs. *Statistics:* Horizontal lines indicate the median and significance was determined using a Mann-Whitney U test, * $P < 0.05$. Correlation was analyzed using Spearman's rank-order correlation test. Regression line with R and *P*-value are shown in the plot. *Abbreviations:* Th = T helper, CD95 = Fas; death receptor, PB = peripheral blood, HC = healthy control, SRC = sarcoidosis.

(sortable) can be used as surrogate marker for $CD4^{+}CD25^{int\text{-}high}FoxP3^{high}$ Treg isolation and functional studies (23).

Isolated cells were cultured with IL-2 and spontaneous survival was measured at 72 hours. Both healthy- and sarcoidosis-derived Tregs showed decreased survival compared with isolated Th cells (Additional file: Figure E3), confirming that Tregs are an apoptotic-prone population. Importantly, patient-derived Tregs showed significantly decreased survival compared with healthy control Tregs (Figure 4A). This impaired survival was Treg-specific, since sarcoidosis-derived Th cells showed comparable survival to their healthy counterparts (Additional file: Figure E3). The survival defect of sarcoidosis Tregs was not restored when co-cultured with autologous Th cells (Figure 4B and 4C (*upper plot*)). Moreover, increased proportions of apoptotic Tregs (annexin V^{+} and low FSC values) were observed in sarcoidosis (Figure 4B and 4C (*lower plot*)).

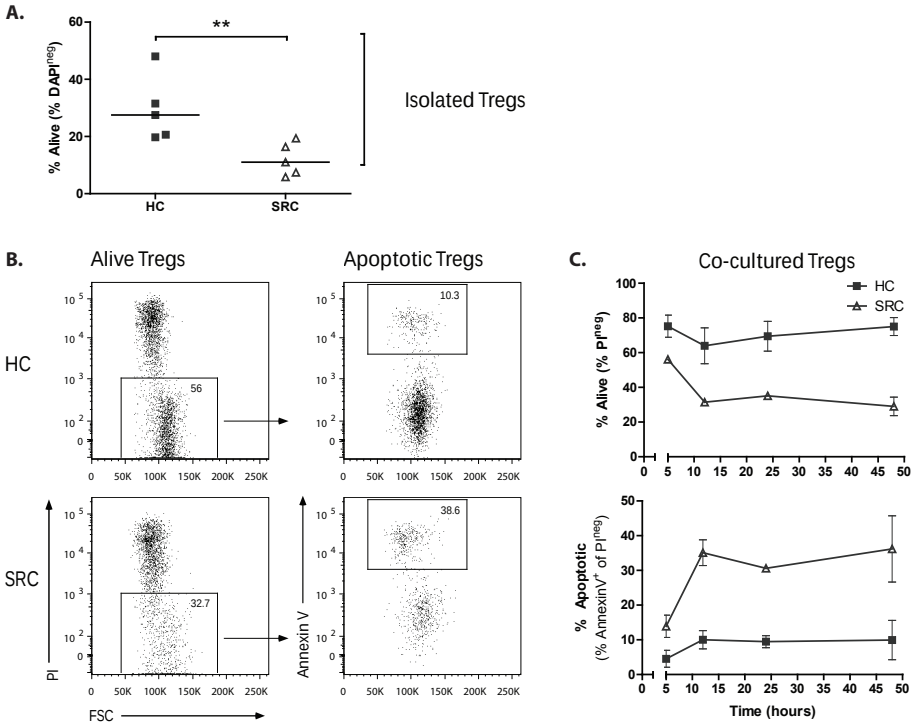

Figure 4. Impaired survival of sarcoidosis Tregs. Isolated Tregs (purity >97%) were cultured with recombinant human IL-2. (*A*) Percentage alive Tregs at 72 hours of culture. Horizontal line indicates the median. Significance was determined using a Mann-Whitney U test, ** $P < 0.01$. (*B*) Representative flow cytometry analysis of an HC and SRC patient after 12 hours co-culture with autologous Th cells to determine Treg survival and apoptosis. (*C*) Survival (above) and apoptosis (below) graph of Tregs cultured for 48 hours with autologous Th cells. Dots indicate mean +/- SEM of 3 HCs and 3 SRC patients. One representative experiment is shown of 3 independent experiments. *Abbreviations:* HC = healthy control, SRC = sarcoidosis.

These data provide evidence for an impaired survival of sarcoidosis Tregs, associated with increased apoptotic susceptibility.

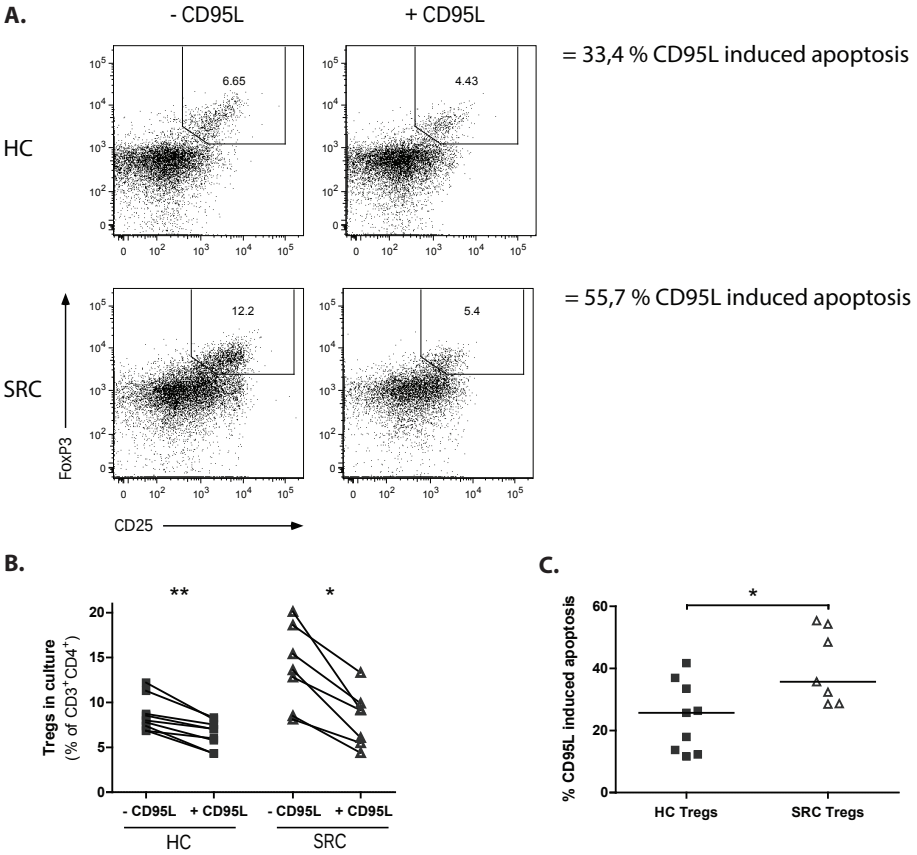

Figure 5. Increased sensitivity of sarcoidosis Tregs towards CD95L-mediated apoptosis. Freshly isolated Tregs were analyzed for apoptotic susceptibility towards soluble CD95L. (*A*) Representative flow cytometry analysis of an HC and SRC patient after 20 hours of culture with IL-2 only or with IL-2 and soluble CD95L. Numbers indicate percentage of $CD25^+FoxP3^+$ Tregs in culture. Induced apoptosis by CD95L was calculated, using the following formula: ((% $CD25^+FoxP3^+$ Tregs cultured with IL-2 - % $CD25^+FoxP3^+$ Tregs cultured with IL-2 and CD95L)/(% $CD25^+FoxP3^+$ Tregs cultured with IL-2))*100%. (*B*) Percentage Tregs in culture of 9 HCs and 7 SRC patients after 20 hours. Paired data is shown of the Treg cultures without or with soluble CD95L. (*C*) Percentage CD95L-induced apoptosis in HC and SRC Tregs. *Statistics:* Horizontal lines indicate the median. Significance was determined using a Mann-Whitney U test (*C*) or Wilcoxon matched pairs test (*B*), * $P < 0.05$ ** $P < 0.01$. *Abbreviations:* CD95L = CD95 ligand, HC = healthy control, SRC = sarcoidosis.

Increased sensitivity of sarcoidosis Tregs towards CD95L-mediated apoptosis

Since increased CD95 expression was observed on sarcoidosis $CD45RO^+$ Tregs (Figure 3) alongside impaired survival and increased apoptosis (Figure 4), we investigated apoptotic susceptibility of freshly isolated $CD4^+$ T cells towards soluble CD95L. Although both

healthy control and sarcoidosis Tregs were sensitive towards CD95L-mediated apoptosis (Figure 5A and 5B), CD95L-induced apoptosis was significantly enhanced in sarcoidosis Tregs compared with healthy controls (Figure 5C).

These data provide evidence for increased susceptibility of sarcoidosis Tregs towards CD95L-mediated apoptosis.

DISCUSSION

In this study, for the first time, we provide evidence for an increased apoptotic susceptibility and impaired survival of sarcoidosis Tregs. Untreated patients with active pulmonary sarcoidosis showed enhanced CD95 expression levels on circulating activated $CD45RO^+$ Tregs at time of diagnosis. Additionally, proportions of $CD95^+$ and $CD45RO^+$ circulating Treg were significantly increased. Furthermore, sarcoidosis Tregs were specifically more susceptible towards CD95L-induced apoptosis compared with healthy controls. Increased apoptosis likely contributes to the insufficient immunosuppressive function of sarcoidosis Tregs.

Failure of Treg-mediated immunosuppression is widely reported in autoimmune diseases (13) as well as granulomatous disorders, including AAV and HP (14, 15). In sarcoidosis, impaired immunosuppression by Tregs has been found both in active and in chronic disease (4, 16-18). It has been suggested to contribute to granuloma persistence (4) and was reported to recover during disease resolution (18). Decreased immunosuppression by Tregs on Th cells may either result from deregulated Tregs (decreased proportions and/or malfunctioning), resistance of Th cells towards suppression or a combination of both (13). We confirmed an impaired immunosuppressive function of PB-derived Tregs on Th cell proliferation and cytokine production. Although our assays cannot exclude that sarcoidosis Th cells contribute to this phenomenon, a previous study showed that sarcoidosis Th cells were responsive towards the suppressive capacity of healthy Tregs (18). Furthermore, we did not find evidence for a numeral deficit of Tregs, neither systemically nor in the lungs of untreated patients with active pulmonary sarcoidosis at time of diagnosis. Rather, we and others (4, 18) found increased proportions of circulating Tregs in active sarcoidosis patients.

Importantly, we are the first to report that increased proportions of circulating Tregs at time of diagnosis are mainly attributable to patients who develop chronic disease and not patients who undergo spontaneous resolution. Interestingly, *Prasse et al.* previously found significantly decreased Treg proportions in lungs of patients who develop chronic active disease compared with controls, but not in patients who undergo spontaneous resolution (19). Taken together, these data suggest that enhanced circulating Treg proportions in sarcoidosis reflect impaired migration towards the site of inflammation. Alternatively, during homeostatic conditions Tregs rapidly adjust their numbers in

response to IL-2 variations, directly reflecting Th cell activity. Therefore, in agreement with published findings (12), it is also conceivable that at time of diagnosis in sarcoidosis patients circulating Treg proportions expand as a result of local Treg failure, specifically in patients who will develop chronic sarcoidosis. Therefore, the numbers of circulating Tregs at the time of diagnosis potentially serve as a new biomarker indicating need for immunosuppressive drugs that restore immune homeostasis. To further address this issue prospective studies are warranted.

To the best of our knowledge, it thus far remained unclear what mechanism(s) underlies Treg dysfunction in PB of active sarcoidosis patients. We did not find differences in FoxP3 expression or diminished expression of its downstream molecules CD25 or CTLA4 in circulating Tregs of sarcoidosis patients. Interestingly, we found that Tregs in lymph nodes from sarcoidosis patients have reduced CTLA4 expression, compared with controls (24), indicating that CTLA4 expression levels are differentially regulated between compartments. Thus, despite adequate Treg proportions and expression of FoxP3-downstream effector molecules, sarcoidosis-derived PB Tregs fail to suppress autologous Th cell responses.

Pro- and anti-apoptotic pathways govern Treg homeostasis (12). In this study a Treg-specific survival defect was found in sarcoidosis patients, whereby CD95L-mediated apoptosis was increased. Although factors determining T cell sensitivity towards CD95L-mediated apoptosis remain to be fully elucidated, T cell activation status and CD95 expression appear critical (25). Indeed, our data argue that in sarcoidosis circulating activated $CD45RO^+$ Tregs highly expressing CD95 compared with control $CD45RO^+$ Tregs, can contribute to impaired Treg survival.

Our finding that in sarcoidosis Tregs are hypersensitive towards CD95L-mediated apoptosis parallels earlier findings in SLE (26) and adds to our previously described similarities between sarcoidosis and systemic autoimmune disorders, which include the involvement of pathogenic IFN-γ-producing Th17 cells (27, 28). In SLE increased sensitivity of Tregs towards CD95L-mediated cell death was proposed to exacerbate the extent of tissue damage during flares (26). Increased Treg apoptosis in sarcoidosis might hamper restoration of the immune balance and contribute to the development of (chronic) sarcoidosis. Interestingly, CD95 signaling has previously been suggested to contribute to chronic sarcoidosis pathology, since an activating CD95 promotor polymorphism (-670A) has been associated with disease severity in Afro-American patients (29). This -670A variant in the CD95 gene promotor has also been associated with SLE and was shown to influence CD95 gene expression, whereby the A (instead of the G) allele induces increased CD95 transcription (30). It is tempting to speculate that increased transcription of CD95 in sarcoidosis, mainly affecting activated $CD45RO^+$ Tregs due to their physiological CD95 expression (25), contributes to the development of chronic sarcoidosis. Importantly, increased Treg sensitivity towards CD95L-mediated

apoptosis does not seem to be a general consequence of chronic inflammation, since it was excluded in multiple sclerosis and granulomatosis with polyangiitis (31, 32).

Previously it has been suggested that decreased apoptosis of antigen-specific T cells might contribute to granuloma persistence in sarcoidosis (22). However, sarcoidosis-derived BALF and PB T cells highly express CD95 (33, 34) and show signs of apoptosis (35). Lymphocytes infiltrating and surrounding sarcoid granulomas express CD95L (35), and increased amounts of soluble CD95L are found in BALF and serum of patients (36). Most interesting, patients with chronic sarcoidosis and need for therapy show an increased expression of CD95 on BALF and PB T cells (including CD45RO on PB T cells) at time of diagnosis compared with patients undergoing spontaneous resolution of disease (34). These data imply that increased apoptosis of T cells contributes to ongoing inflammation. However, the role of CD95L-mediated apoptosis in the homeostasis of pro- and anti-inflammatory T cell subsets (i.e. Th cells versus Tregs, respectively) in sarcoidosis thus far remained unclear (35). In this study, for the first time, evidence is provided for a Treg specific survival defect, which can lead to an imbalance between pro-inflammatory Th cells and properly functioning anti-inflammatory Tregs, resulting in on going inflammation.

The anti-TNF agent infliximab is known to induce Treg functionality in RA (7). Induction of Treg survival may very well contribute to its therapeutic effect as observed in patients with chronic sarcoidosis (5). Investigation of Treg proportions present in or around the granulomas, their functional capacities, and apoptosis susceptibility during the natural course of disease and in response to therapy should further unravel the role of Tregs in the development of chronic sarcoidosis. Research into this field will help determine whether improvement of Treg survival, e.g. by other immunosuppressive drugs, such as rapamycin (37, 38), holds a promising new therapeutic approach for chronic sarcoidosis patients.

CONCLUSION

In conclusion, this study is the first to demonstrate a role for deregulated Treg survival, mediated by CD95-signaling, in untreated patients with active pulmonary sarcoidosis. Increased apoptosis likely contributes to the insufficient immunosuppressive function of sarcoidosis Tregs. Further research into this field will help determine whether improvement of Treg survival holds a promising new therapeutic approach for (chronic) sarcoidosis patients.

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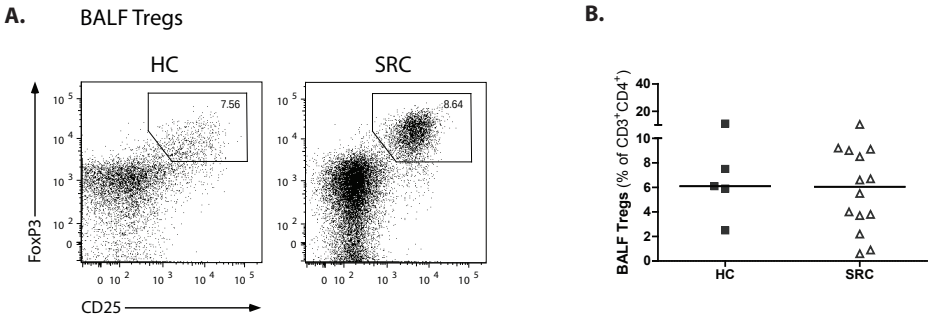
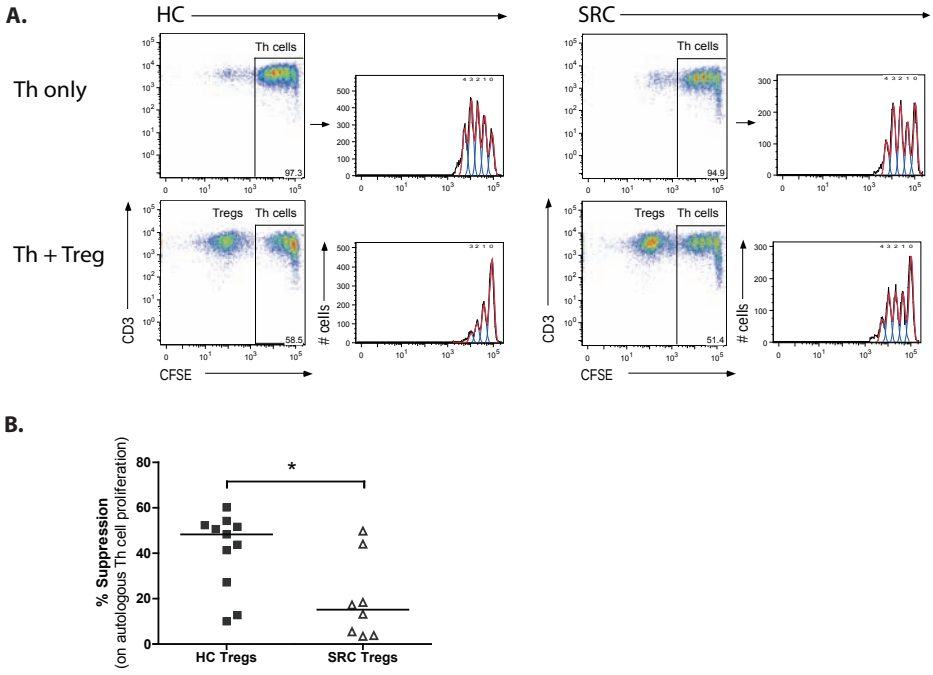
SUPPLEMENTARY MATERIAL

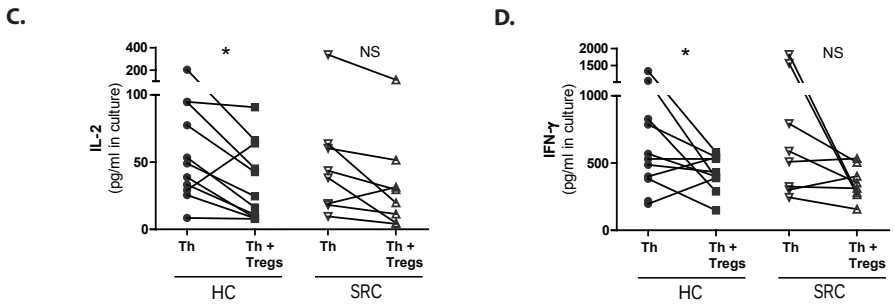
SUPPLEMENTARY FIGURES



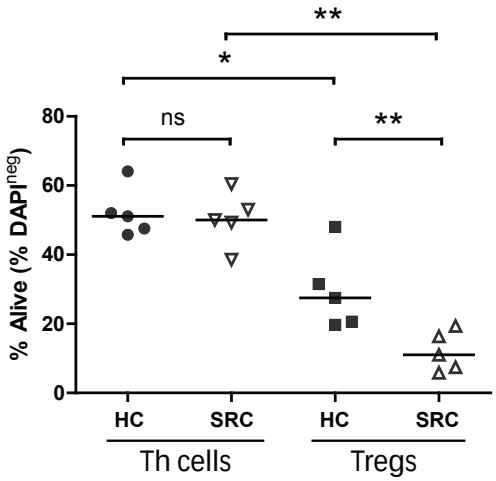
Supplementary Figure E1. Treg proportions were determined in BALF of HCs and SRC patients. (*A*) Representative flow cytometric analysis of an HC and SRC patient to determine Treg proportions in BALF. (*B*) BALF Treg proportions. *Statistics:* Horizontal lines indicate the median and significance was determined using a Mann-Whitney U test. *Abbreviations:* BALF = broncho-alveolar lavage fluid, HC = healthy control, SRC = sarcoidosis.



Supplementary Figure E2. (See legend on next page)



Supplementary Figure E2. Freshly isolated Th cells were CFSE-labeled, TCR-stimulated and cultured without or with autologous Tregs. (*A*) Flow cytometry analysis of one HC and SRC patient after 4 days of culture. (*B*) Percentage suppression by Tregs on autologous Th cell proliferation. Percentage suppression was calculated as follows: ((%divided Th cells only - %divided Th cells co-cultured with Tregs)/(%divided Th cells only))*100%. Horizontal lines indicate the median and significance was determined using a Mann-Whitney U test, * $P < 0.05$. (*C-D*) Amount of IL-2 (*C*) or IFN-γ (*D*) measured in culture supernatant of suppression assays at day 4. Paired data is shown of Th cell cultures without or with autologous Tregs. Significance was determined using a Wilcoxon matched pairs test, * $P < 0.05$. *Abbreviations:* Th = T helper, CFSE = carboxyfluorescein succinimidyl ester, TCR = T cell receptor, HC = healthy control, SRC = sarcoidosis, NS = not significant.



Supplementary Figure E3. Isolated Th cells and Tregs were cultured with recombinant human IL-2. Percentage alive Th cells and Tregs at 72 hours of culture is shown. Horizontal line indicates the median. Significance was determined using a Mann-Whitney U test, * $P < 0.05$ ** $P < 0.01$. *Abbreviations:* Th = T helper, HC = healthy control, SRC = sarcoidosis, NS = not significant.

Supplementary Table E1. Study subject characteristics

Subject characteristics	Healthy controls (n=47)	Sarcoidosis (n=58)
Age (y)*	36 (19-59)	44 (17-81)
Sex (Male/Female/Unknown[†])	23/22/2	31/27/0
Ethnicity (n (%))		
White	34 (72,3)	35 (60,3)
Black	2 (4,3)	12 (20,7)
Asian	2 (4,3)	6 (10,3)
Hispanic	0 (0)	1 (1,7)
Unknown[†]	9 (19,1)	4 (6,9)
Scadding Stage (n (%))		
Stage 0		5 (8,6)
Stage I		25 (43,1)
Stage II		21 (36,2)
Stage III		3 (5,2)
Stage IV		0 (0,0)
Unknown		4 (6,9)[‡]
CD4/8 ratio BALF (n=46)*		4,3 (0,5 – (>)10)
BALF lymphocytes (n=46)*		25,0 (0 - 84)
Diagnosis was assessed by (n (%))		
Trans- and/or endobronchial biopsy		18 (31,0)
E(B)US-FNA		17 (29,3)
BALF alveolitis + CD4/CD8 ratio > 3.5		22 (38,0)
Mediastinoscopy		1 (1,7)
Disease course[§] (n (%))		
Resolution		5 (8,6)
Non-active chronic		15 (25,9)
Active chronic		8 (13,8)
Lost to follow up		12 (20,7)
Not yet determined		18 (31,0)

Study subject characteristic. * Median (minimum-maximum). [†] Anonymous blood donors. [‡] Two of these patients had Stage I and two had Stage II sarcoidosis, determined by CT scan. [§] Disease course of a subgroup of patients (n=28) was determined 2 years after study inclusion. Resolution of disease was defined by the absence of abnormalities on the chest X-ray and clinical symptoms (n=5). Patients with residual abnormalities on chest X-ray, but without need for treatment were designated as non-active chronic (n=15); and patients with need for treatment (n=8) as active chronic (Prasse et al., Am J Respir Crit Care Med. 2010; 182: 540-548).

CHAPTER 4

IFN-γ-Producing T-Helper 17.1 Cells Are Increased in Sarcoidosis and Are More Prevalent than T-Helper Type 1 Cells

'The difficulty lies not so much in developing new ideas as in escaping from old ones'
John Maynard Keynes

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*Authors contributed equally to this study.

ABSTRACT

Rationale: Pulmonary sarcoidosis is classically defined by T-helper (Th) cell type 1 inflammation (e.g., interferon (IFN)-γ production by $CD4^+$ effector T cells). Recently, interleukin (IL)-17A-secreting cells have been found in lung lavage, invoking Th17 immunity in sarcoidosis. Studies also identified IL-17A-secreting cells that expressed IFN-γ, but their abundance as a percentage of total $CD4^+$ cells was either low or undetermined.

Objective: Based on evidence that Th17 cells can be polarized to Th17.1 cells to produce only IFN-γ, our goal was to determine whether Th17.1 cells are a prominent source of IFN-γ in sarcoidosis.

Methods: We developed a single-cell approach to define and isolate major Th-cell subsets using combinations of chemokine receptors and fluorescence-activated cell sorting. We subsequently confirmed the accuracy of subset enrichment by measuring cytokine production.

Measurements and Main Results: Discrimination between Th17 and Th17.1 cells revealed very high percentages of Th17.1 cells in lung lavage in sarcoidosis compared with controls in two separate cohorts. No differences in Th17 or Th1 lavage cells were found compared with controls. Lung lavage Th17.1-cell percentages were also higher than Th1-cell percentages and approximately 60% of Th17.1-enriched cells produced only IFN-γ.

Conclusions: Combined use of surface markers and functional assays to study $CD4^+$ T cells in sarcoidosis revealed a marked expansion of Th17.1 cells that only produce IFN-γ. These results suggest that Th17.1 cells could be misclassified as Th1 cells and may be the predominant producer of IFN-γ in pulmonary sarcoidosis, challenging the Th1 paradigm of pathogenesis.

INTRODUCTION

Sarcoidosis is a granulomatous disease in which the inflammation is thought to be driven in part by activated T-helper (Th) type 1 effector T cells (1-4). The idea that Th1 cells play a key role in sarcoidosis is largely based on experimental observations of increased numbers of $CD4^+$ effector T cells, which produce interferon-γ (IFN-γ), in sarcoidosis bronchoalveolar lavage (BAL) fluid (3, 5-7). After the recent discovery of a new class of Th cells, Th17 cells, several investigators have used a variety of flow cytometry gating strategies to identify interleukin-17A (IL-17A)-producing cells in BAL fluid from patients with sarcoidosis (8-13). Several of these studies also identified IL-17A-secreting cells that expressed IFN-γ, but their abundance as a percentage of total $CD4^+$ cells was either low or undetermined (9, 11, 12).

Recent studies in Crohn's disease identified a subset of IFN-γ-producing Th17 cells, called Th17.1 cells. This subset expressed a specific pattern of chemokine receptors and exhibited functional attributes indicating that they were proinflammatory and resistant to corticosteroids (14). Therefore, identification of these cells in sarcoidosis would have important implications for treatment approaches.

Th17.1 cells are thought to be derived from classically polarized Th17 cells. Many studies show that Th17 cells are plastic and can be polarized to differentiate into a Th1-like phenotype in which they produce significant IFN-γ (15-19). *In vitro* experiments have shown that cytokines prevalent in sarcoidosis, IFN-γ and IL-12, promote this transformation (18). The nomenclature for this "Th1-polarized Th17 subset" is not uniform, and these cells have been referred to Th17/Th1 (20, 21), Th1/17 (22), and Th17.1 cells to capture their transformed state (14). We refer to this Th17 subset as Th17.1 to be consistent with prior studies that used chemokine receptor expression as part of their definition for these cells (14, 23). Because the majority of Th17.1 cells produce only IFN-γ, we hypothesized that Th17.1 cells have largely been misclassified as Th1 cells because measurement of cytokine production has been the usual method for defining Th1 and Th17 cells. For example, production of IL-17A has been used to define "Th17" cells (8-13), and therefore the proportions of Th17 cells that produced only IFN-γ would be completely missed.

To address whether Th17.1 cells could be a predominant source of IFN-γ in pulmonary sarcoidosis, we used definitions for Th cells based on the latest immunology (14), which consisted of a combination of three chemokine receptors, CCR4, CCR6 and CXCR3. We first applied single-cell sorting techniques using chemokine receptor expression to isolate cells from paired blood and lung samples from sarcoidosis and controls. We then confirmed appropriate cytokine secretion in the sorted and enriched populations of Th cell subsets. These techniques allowed for a high degree of cell separation in which to study Th subsets (and subsets within subsets) and make new observations in sarcoidosis, such as finding that IFN-γ-producing Th17.1 cells are the predominant effector cell in sarcoidosis BAL in two separate cohorts.

METHODS

Subjects

Participants in the U.S. cohort underwent written informed consent and the study was approved by the University of California, San Francisco Committee on Human Research. Sarcoidosis diagnosis was based on consistent clinical features, absence of alternative diagnoses, and biopsy of the lung or mediastinal lymph nodes showing non-caseating granulomas according to accepted criteria (24). Exclusion criteria included a smoking history, cancer, chronic infections, autoimmune diseases, other pulmonary diseases, or organ transplant. Subjects underwent chest X-ray, high-resolution chest computed tomography (CT) scan, BAL and blood collection. Noncontrast axial images (1.25 mm) were obtained supine during full inspiration for a 10-second breath hold. Imaging protocol was defined by the National Institute of Health (NIH) study (NCT01831739). Organ involvement was determined as described previously (25). Healthy control data were obtained from a concurrent study (NCT01484691) to measure the same immunological parameters.

The validation cohort, referred to as the Erasmus MC cohort, consisted of newly diagnosed pulmonary sarcoidosis European patients using the same diagnostic and exclusionary criteria (24). In addition, patients could not be taking immunomodulatory medication in the three months before enrollment, however a smoking history was accepted. The control group consisted of individuals who underwent bronchoscopy for community-acquired pneumonia or chronic obstructive pulmonary disease. The Medical Ethical Committee of the Erasmus MC (Rotterdam, the Netherlands) approved this study.

BAL and peripheral blood mononuclear cells

The bronchoscopy protocol with BAL was developed by the NIH study, Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis (NCT01831739). Cells were resuspended in 0.1% bovine serum albumin + 2mM ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS) and immediately processed for flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated as described previously (26).

Flow cytometry and sorting

For surface staining, BAL cells and PBMCs were incubated with fluorescent antibodies (CD3 [BD Horizon, San Jose, CA], CCR4 [BD Pharmingen, San Jose, CA], CD127, CD4, CD25 and CCR6 [eBioscience, San Diego, CA], and CD45RO, CD45RA and CXCR3 [Biolegend, San Diego, CA]), and CD1d-tetramer-PBS57 [NIH Tetramer Facility, Atlanta, GA] for 30 minutes at 4°C using methods recommended by the manufacturers. Viability was measured using Propidium Iodide (BD Bioscience, San Jose, CA). We used the ARIA II fluorescence activated cell sorter (FACS) to isolate CCR4- and CCR6-positive and –negative cells (BD Biosciences). FACS sorting was performed using a number of gating strategies: we gated

on live cells, followed by lymphocytes, singlets, non-invariant natural killer T cells, $CD4^+$ T cells, nonregulatory T cells, and effector cells (Figures 1A & 1B). $CCR6^+$, $CCR4^+CCR6^-$, and $CCR4^-CCR6^-$ effector cells were sorted into separate chambers to analyze cytokine function. Figures 1A & 1B show the three-gate sorting strategy. After discovery of the high prevalence of IFN-γ-producing $CCR6^+$ cells, a fourth gate was added to sort effector T cells into four chambers based on chemokine expression: $CCR4^+CCR6^+$, $CCR4^+CCR6^-$, $CCR4^-CCR6^+$, and $CCR4^-CCR6^-$. CXCR3 was used in the FACS-sorting panel and functional panel to allow more stringent gating of Th17, Th17.1, Th1, and Th2 populations as defined in Table 1 and Figure E1 in the online supplement, and as previously described (14). Samples from the European cohort were analyzed similarly (*see* the online supplement).

Table 1. T effector cell subset phenotypes

	$CCR4^-/CXCR3^+$	$CCR4^+/CXCR3^-$
$CCR6^-$	Th1	Th2
$CCR6^+$	Th17.1	Th17

Cell stimulation and cytokine analysis

Freshly isolated cells were stimulated at 37°C for 4 hours in 10 nM Phorbol 12-myristate 13-acetate, 1 μM ionomycin, and 5μg/mL brefeldin A. Antibody staining against CXCR3 (eBioscience), and live/dead Amcyan stain (Invitrogen, Grand Island, NY) was performed for 30 minutes at 4°C. Cells were fixed in 2% paraformaldehyde and permeabilized (BD Biosciences) for intracellular cytokine staining of IL-17A, IL-4, and IFN-γ (eBioscience). Additional details are provided in the online supplement.

Statistical analysis

FlowJo 9 (FlowJo, Ashland, OR), JMP 10 (SAS Institute, Cary, NC), and GraphPad Prism 6 (GraphPad Software, San Diego, CA) software were used. Normality was measured using Shapiro-Wilk test. Two group comparisons were made using an unpaired Student's *t* test or rank sum test as appropriate. Chi-square analysis was used to assess sex, race, and ethnicity differences, and Student's *t* test for age. A *P*-value less than 0.05 was considered significant.

RESULTS

Patient characteristics

A total of 35 patients with sarcoidosis and 18 healthy controls were enrolled in the U.S. cohort. Patients with sarcoidosis had pulmonary disease (Table 2) and met criteria for chronic disease (lack of disease resolution by 2 yr after diagnosis (27)). There were no significant differences in sex between groups, however the control population was

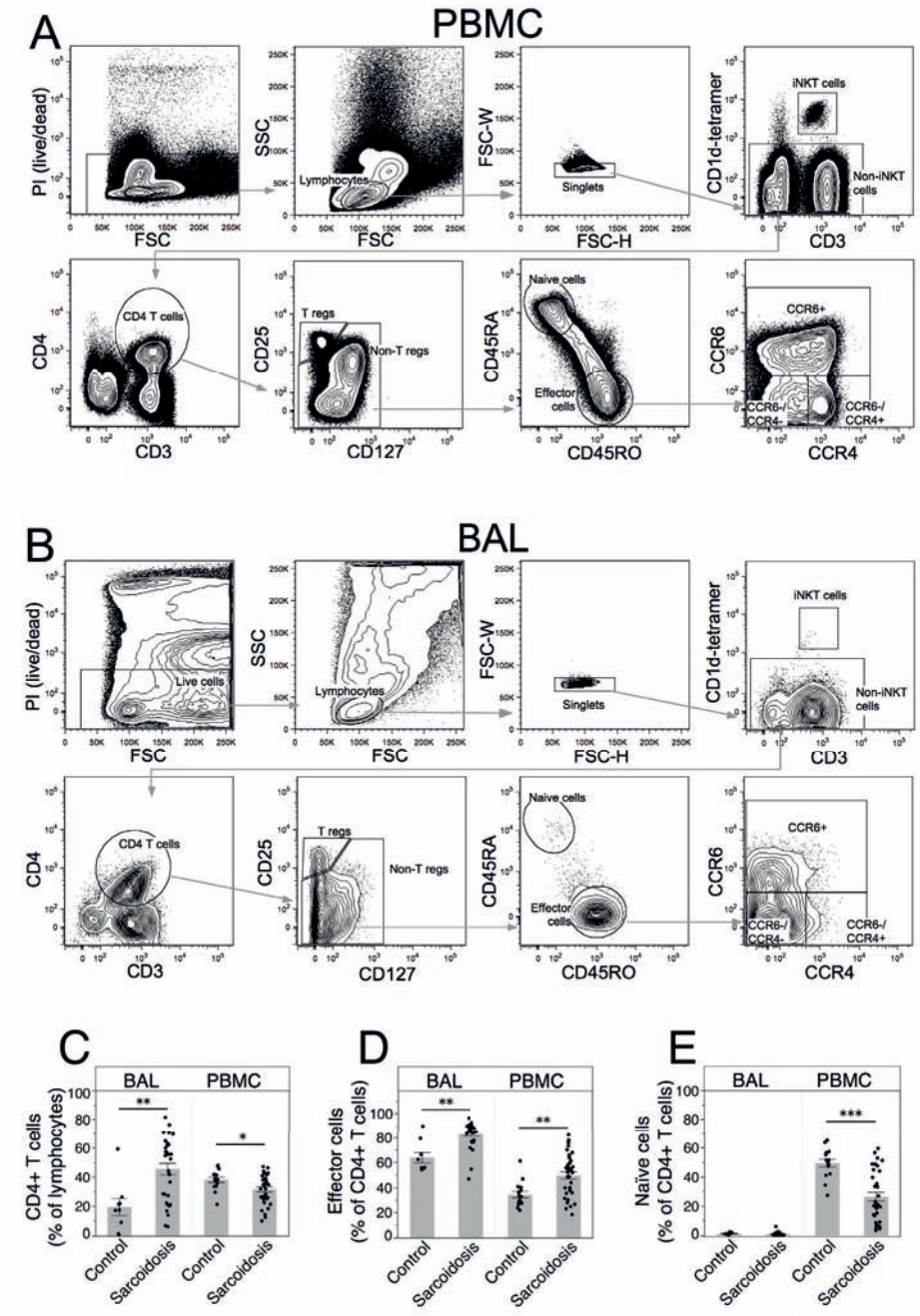

Figure 1. Flow cytometry sorting strategy and comparative analysis of $CD4^+$ T cell subsets. Peripheral blood mononuclear cells (PBMCs) and bronchoalveolar lavage (BAL) cells were stained and analyzed by an LSRII Fortessa cytometer (BD Biosciences). (*A* and *B*) Representative sample is shown for blood (*A*) and BAL cells (*B*) from a sarcoidosis patient. Lymphocytes were analyzed using the following series of sub-gates: live

cells, lymphocytes, singlets, non-invariant natural killer (iNKT), CD4 T cells ($CD4^+CD3^+$), nonregulatory T cells ($CD4^+CD127^+CD25^-$ T cells), memory effector cells ($CD45RA^-/CD45RO^+$ $CD4^+$T cells), and three sub-populations of memory effector cells using CCR4 and CCR6. We excluded iNKT cells (CD1d tetramer-PBS57), regulatory T cells ($CD4^+CD3^+CD127^-CD25^+$) and naïve T cells ($CD4^+CD3^+CD45RA^+CD45RO^-$) from the $CD4^+$ population. (*C*) $CD4^+$ T cells as a percentage of lymphocytes are significantly increased in the lungs of patients with sarcoidosis (**$P = 0.0019$), and decreased in the blood (*$P = 0.012$). (*D*) Effector cells as a percentage of $CD4^+$ T cells are significantly increased in both the lungs (**$P = 0.0015$) and the blood (**$P = 0.0004$) of patients with sarcoidosis. (*E*) There are essentially no naïve cells in the lungs and a significant decrease of naïve cells as a percentage of $CD4^+$ T cells in the blood of sarcoidosis patients (***$P < 0.0001$). Data are expressed as mean (histogram bars) ± SEM. *Overlaid dots* represent individual patient values. BAL: $n = 32$ subjects with sarcoidosis and 9 control subjects; PBMC: $n = 35$ subjects with sarcoidosis and 18 control subjects. FSC = forward scatter; PI = propidium iodide; SSC = side scatter; T regs = T regulatory cells.

younger, on average. Both groups consisted of more white than African American subjects or those of other races. Six patient with sarcoidosis were on immunosuppression for progressive pulmonary disease at enrollment. Three patients were taking 20 mg or less of prednisone daily; two patients were taking 15 mg of methotrexate weekly, and one of these was also taking infliximab. One patient was taking 50 mg of azathioprine daily. We found no significant differences between T cell profiles of these 6 patients compared with those not taking immunosuppression. Fewer subjects were Stage I compared with Stages II/III and IV. Fibrosis was confirmed in Stage IV patients by CT scanning. There was a trend in decreasing lung function with increasing stage (Table 3). Average BAL fluid return was 114 ml (60-169 ml) with 77% (69-81%) alveolar macrophages, 22% (17-30%) lymphocytes, and 1% (0-3%) neutrophils.

Table 2. Demographics and clinical characteristics

	Control	**Sarcoidosis**	***P* value**
Number	18	35	
Female*	9 (50%)	16 (46%)	0.78
Age (median, range)	30 (25-54)	53 (32-69)	<0.01
Immunosuppression	-	6 (17%)	-
Stage, I/II-III/IV	-	5/17/13	-
Lung/heart/LN/eye/skin		35/2/4/1/2	
Race			
African American*	3 (17%)	3 (9%)	0.39
Caucasian*	11 (61%)	31 (89%)	0.02
Other*	4 (22%)	1 (3%)	0.03
Ethnicity			
Hispanic*	1 (6%)	2 (6%)	0.98

* Percentage of total in parentheses unless otherwise stated

A second validation cohort from Europe, the Erasmus MC cohort, consisted of 30 subjects with sarcoidosis and 12 disease control subjects, eight of whom underwent bronchoscopy for community-acquired pneumonia and four patients with chronic obstructive pulmonary disease. The control group showed a trend towards older age (Table 4). There were no statistically significant differences in sex or age between groups. The majority of the patients were white. One patient had Lofgren syndrome. Patients with sarcoidosis were newly diagnosed and not on immunosuppressive treatment. The lymphocyte percentages were consistent with alveolitis (Table 4).

$CD4^+$ T-cell analysis and enrichment of T helper subsets using chemokine receptor expression

Sarcoidosis is characterized by abnormal increase of IFN-γ-producing BAL $CD4^+$ T cells (2, 3, 28). Our goal was to use chemokine receptor expression to define and distinguish among Th17 subsets to understand their full capacity to produce IFN-γ and how numerous they were (14, 23). We used single-cell analysis and FACS-sorting to isolate these Th subset populations (Figures 1A & 1B). We use the terminology "enriched" subsets to acknowledge that these populations cannot be 100% pure, but we show, using cytokine measurements, that the purities are very high.

As expected, we found that $CD4^+$ T cells (as a % of lymphocytes) were markedly increased in BAL from patients with sarcoidosis compared with healthy control subjects (Figure 1C). In contrast, we found that $CD4^+$ T cells were decreased in the blood in sarcoidosis compared with control, consistent with the idea of $CD4^+$ T cells homing to the lungs from the blood (2). Decreased circulating $CD4^+$ T-cell numbers have been associated with more severe forms of sarcoidosis, including decreased pulmonary func-

Table 3. Clinical characteristics by radiographic stage of disease

Total N = 35	**Stage I (N=5)**	**Stage II/III (N=17)**	**Stage IV (N=13)**
Age (median, range)	53 (37-71)	53 (34-68)	58 (34-71)
Female, n	3 (60%)	8 (47%)	5 (38%)
Race, n			
Caucasian	5	14	12
African American	0	3	0
Other	0	0	1
Current immunosuppression use	0	3	3
Forced Expiratory Volume, 1 sec (FEV1) †	101% ± 24%	98% ± 14%	91% ±18%
Forced Vital Capacity (FVC) †	102% ± 21%	100% ± 10%	97% ± 14%
Diffusing Capacity (DLCO) †	80% ± 22%	80% ± 11%	75% ± 9%

†Data are presented as average values expressed as percent predicted
± Standard Deviation

Table 4. Demographics and clinical characteristics for the Erasmus MC cohort

	Control	Sarcoidosis	*P* value
Number	12	30	
Female*	5 (42%)	12 (40%)	0.92
Age (median, range)*	57 (18-80)	43 (24-70)	0.07
Immunosuppression†	-	0	
Stage, I/II-III/IV/CT	-	11/16/0/3§	-
Lung/heart/LN/eye/skin		30/0/0/9/2	
Smoking, never/former/current/unknown	-	16/8/4/2	-
Race*			
African	-	2 (7%)	-
Caucasian	-	19 (63%)	-
Asian	-	4 (13%)	-
Unknown	-	5 (17%)	-
Ethnicity*			
Hispanic	-	1 (3%)	-
Lymphocyte % BALF (median, range), (average, SD)	-	27% (3-68%), 32% (±21%)	-
CD4/CD8 ratio BALF (median, range), (average, SD)	-	4.4 (0.5-14.34), 5.2 (±3.4)	-

*Percentage of total in parentheses unless otherwise stated

†Patients were newly diagnosed and not on immunosuppressive treatment

§Three subjects found to have mediastinal and hilar lymph node involvement by CT scan

tion (29). In support of this idea, we also found that patient with sarcoidosis with fibrosis on chest CT scan had significantly lower numbers of $CD4^+$ T cells in the blood compared to those without fibrosis (Figure E2). As anticipated, there were essentially no naive cells ($CD3^+CD4^+CD1d^-CD25^{lo}CD45RA^+CD45RO^-$) in the BAL fluid in any participant (Figure 1E); in the blood, naive cells as a percentage of $CD4^+$ T cells were significantly decreased in sarcoidosis compared with control, as has been recently observed in a separate cohort (30). Effector memory cells ($CD3^+CD4^+CD1d^-CD25^{lo}CD45RA^-CD45RO^+$) as a percentage of $CD4^+$ T cells were significantly increased in sarcoidosis BAL (average, ~83%) compared with controls (Figure 1D). This expansion of effector memory cells is consistent with the idea of antigen-driven stimulation, as has been suggested by prior findings of oligoclonal expansion of T cells expressing specific αβ T-cell receptor genes in the lung and blood in sarcoidosis (31, 32).

Within the population of effector memory cells, we found that, on average approximately 50% of the $CD4^+$ T effector cells in the lung expressed CCR6 (Figure 2). Almost one-third of patient with sarcoidosis had over approximately 60% $CCR6^+$ effector cells in the BAL. Although CCR6 is expressed on other cell types, it is a marker for Th17 cells, and this finding supports the hypothesis that large numbers of Th17 cells (or their subsets) are present in sarcoidosis BAL (33). In contrast, we did not find an increase in the per-

centage of CCR4$^+$ effector cells in the lungs of patients with sarcoidosis compared with control subjects ($P = 0.25$).

Functional analysis of Th-cell CCR4/CCR6 subset enrichments

We next assessed the accuracy of the Th subset enrichment strategy by stimulating the FACS-sorted CCR4 and CCR6 subsets and measuring production of IL-4, IL-17A, and IFN-γ. The CCR4$^-$CCR6$^-$ population produced IFN-γ and virtually no IL-4 or IL-17A, consistent with Th1 characteristics (Figure 3). The CCR4$^+$CCR6$^-$ effector cells produced IL-4 with little to no production of IL-17A or IFN-γ, supporting Th2 characteristics. Finally, the CCR6$^+$ effector cells produced IL-17A as expected, but in addition, a significant percent-

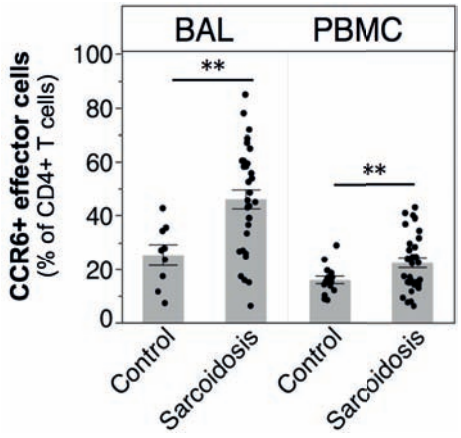

Figure 2. Significant increases in CCR6$^+$ effector cells, consistent with the Th17 lineage, in sarcoidosis. Bronchoalveolar lavage (BAL) cells and peripheral blood mononuclear cells (PBMCs) were stained and analyzed by flow cytometry as described in Methods. As a percentage of CD4$^+$ T cells, CCR6$^+$ effector cells are significantly increased in both the lungs (**$P = 0.0006$) and the blood (**$P = 0.0051$) of patients with sarcoidosis compared with control subjects. Data are shown as mean (histogram bars) ± SEM. *Overlaid dots* represent individual patient values. BAL: $n = 32$ subjects with sarcoidosis and 9 control subjects; PBMC: $n = 35$ patients with sarcoidosis and 18 control subjects.

age also produced IFN-γ, and a very small population showed polyfunctional capacity to produce both IL-17A and IFN-γ. These data demonstrate the presence of at least three functionally diverse Th17 cells in sarcoidosis: a large percentage of CCR6$^+$ cells that only produced IFN-γ, a smaller percentage of cells that produce only IL-17A, and a very small percentage of cells able to produce both IFN-γ and IL-17A.

Discrimination of Th cell subsets using CXCR3, CCR6, and CCR4

Given the large sub-population of IFN-γ-producing CCR6$^+$ cells we analyzed the expression of CXCR3, a chemokine receptor known to be expressed on Th1 and Th17.1 cells (14, 34). Adding CXCR3 to our analysis allowed for more stringent gating strategy to determine the percentages of Th1, Th17, and Th17.1 subsets (Table 1 and Figure E1) as well as to measure cytokine production. Functional analysis of both BAL and PBMC confirmed IFN-γ production with no significant IL-17A production by the sorted Th1-enriched (CCR6$^-$CCR4$^-$CXCR3$^+$) population (Figure 4). In comparison, we found marked production of IFN-γ by Th17.1-enriched cells (CCR6$^+$CCR4$^-$CXCR3$^+$), but only a small percentage of

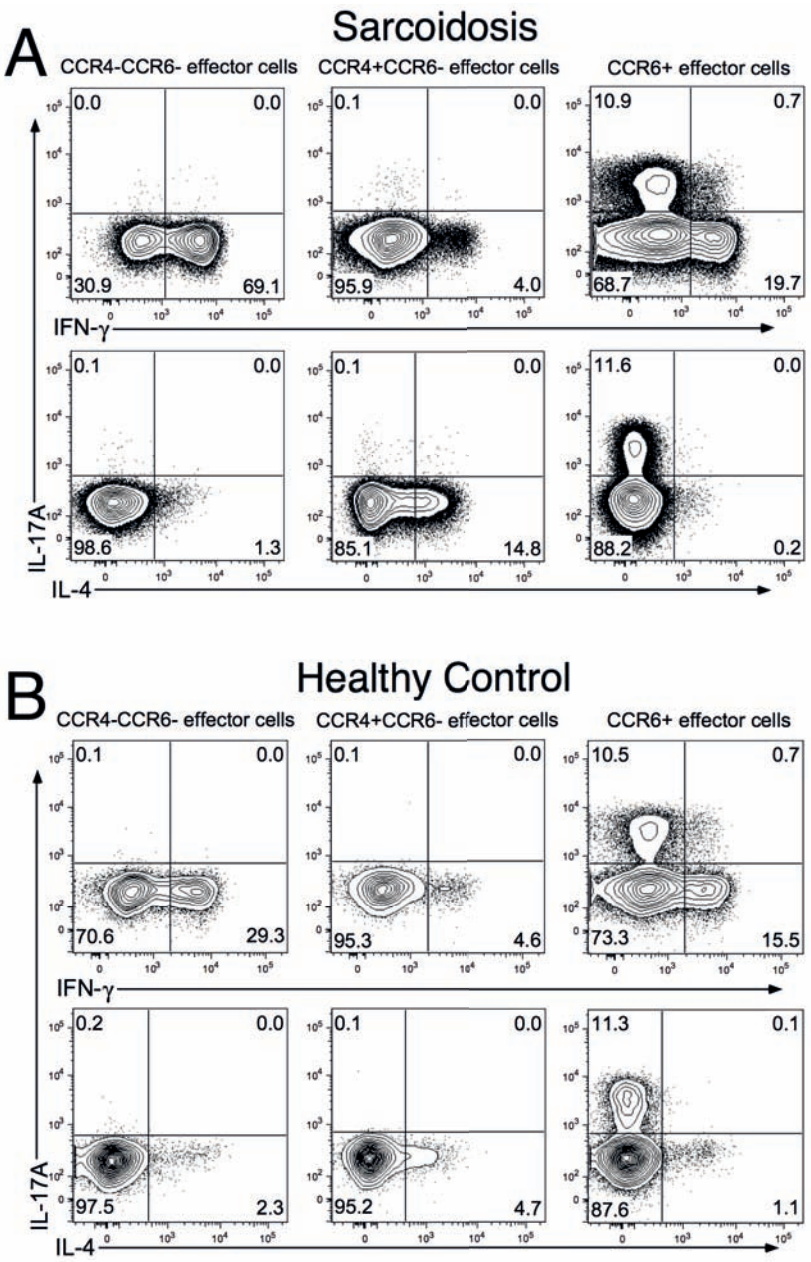

Figure 3. Isolation of discrete T-helper (Th)-cell populations from peripheral blood mononuclear cells (PBMCs) using FACS-sorting confirmed accurate separation and revealed that $CCR6^+$ cells demonstrated different patterns of cytokine secretion. FACS-sorted cells were stimulated and stained with fluorescent antibodies, as described in Methods. Shown is a representative sarcoidosis (*A*) and healthy control (*B*) sample of FACS-sorted PBMC effector cell subsets demonstrating predicted patterns of cytokine secretion for Th1 ($CCR6^-CCR4^-$) and Th2 ($CCR4^+CCR6^-$) cells. FACS-sorted $CCR6^+$ effector cells show increased frequencies of cells expressing IFN-γ or IL-17 and a small frequency of cells expressing both cytokines. FACS = fluorescence-activated cell sorter.

Th17.1 cells were capable of IL-17A expression (Figure 4, row "Th17.1-enriched"). In contrast to Th17.1 cells, the Th17-enriched cells ($CCR6^+CCR4^+CXCR3^-$) showed essentially no production of IFN-γ and an increased capacity to produce IL-17A compared with Th17.1 (Figure 4, row "Th17-enriched"). Figure E3 compiles the functional data for PBMC samples from all subjects with sarcoidosis and demonstrates the striking differences in cytokine production between the Th17 subsets. Thus, these data show that we have identified three discernible populations of Th cells, namely, Th1, Th17, and Th17.1 in human sarcoidosis, and, specifically, that a large population of Th17.1 cells produce only IFN-γ.

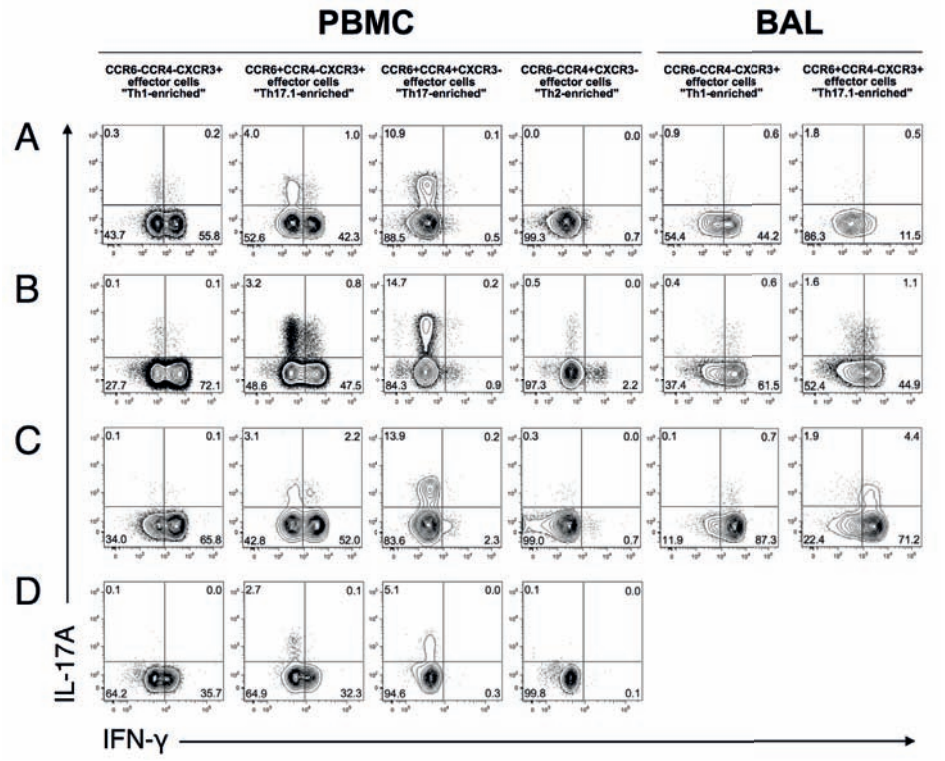

Figure 4. T-helper (Th) 17.1 cells showed co-production of IFN-γ and IL-17A to a small extent but significant production of IFN-γ, which was not found for Th17 cells. Shown are representative *dot plot images* displaying IFN-γ versus IL-17A production from FACS-sorted effector cell subsets from subjects with sarcoidosis (*A-C*) and a healthy control subject (*D*). After FACS-sorting by surface markers, the memory effector cell subsets were stimulated, fixed, permeabilized and stained intracellularly for cytokines, and analyzed by flow cytometry. These methods revealed marked differences in IL-17 and IFN-γ cytokine production between Th17- and Th17.1-enriched cells. In the broncho-alveolar lavage (BAL) samples, too few events fell within the $CCR4^+$ gate to quantify $CCR6^+CCR4^+CXCR3^-$ "Th17-enriched" cells and $CCR6^-CCR4^+CXCR3^-$ "Th2-enriched". Functional stimulation assays were not available from healthy control BAL samples due to a paucity of available cells. FACS = fluorescence-activated cell sorter.

We next compiled the functional data from the sarcoidosis cohort to compare cytokine production between BAL and PBMC and Th1 and Th17.1 cells. We found that a remarkably high percentage (average, ~60%) of Th17.1-enriched cells from BAL produced IFN-γ (Figure 5A). Importantly, the frequency of the Th17.1 cells producing IFN-γ was just as high as that measured from Th1-enriched cells. The percentage of IFN-γ-producing Th1 and Th17.1 cells were also similar in the PBMC compartment (Figure 5A). Figure 5B shows that the frequency of Th17.1 cells producing IL-17 was modest (~5% of total Th17.1 sorted cells in both BAL and PBMC) as predicted from the individual FACS plots shown in Figure 4. Finally, the frequency of Th17.1 cells capable of co-production of IL-17A and IFN-γ was even lower (~2% or lower; Figure 5C). Taken together, these data show a much greater capacity for Th17.1 cells to produce IFN-γ alone compared to IL-17A or both cytokines together, supporting the hypothesis that Th17.1 cells expressing only IFN-γ could have been misclassified as Th1 cells.

Th17.1, but not Th1 or Th17, cells are increased in sarcoidosis

Sarcoidosis is characterized as a Th1-mediated disease. However, we found no significant differences in the percentage of Th1 cells in both BAL and blood in patients with sarcoidosis compared with control subjects (Figure 6A). Furthermore, the size of the Th1 population in BAL was low in both groups (~20% of $CD4^+$ T cells in BAL) (Figure 6A). Consistent with our hypothesis, we found, on average, that approximately 30% of the $CD4^+$ T cells in sarcoidosis BAL were Th17.1 cells, which was significantly increased compared with control subjects and not found for Th17 cells, respectively (Figure 6B & 6C). Th17.1 cells accounted for up to 60% of all the BAL $CD4^+$ T cells in some subjects. In sarcoidosis

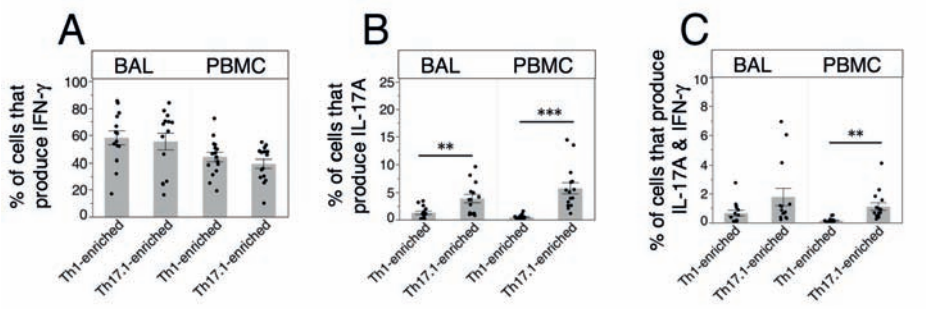

Figure 5. T-helper (Th) 17.1-enriched cells were equipotent in their ability to produce IFN-γ compared with Th1-enriched cells. Bronchoalveolar lavage (BAL) and peripheral blood mononuclear cells (PBMCs) were FACS-sorted and subsequently stimulated and stained for intracellular cytokines, as described in Methods. (*A-C*) Functional data collected from flow cytometry from patients with sarcoidosis were compiled and displayed. (*A*) Percentage of cells producing IFN-γ only. (*B*) Percentage of cells producing IL-17A only (BAL, $^{**}P = 0.0056$; PBMC, $^{***}P < 0.0001$). (*C*) Percentage of polyfunctional cells producing both IL-17A and IFN-γ (PBMC $^{**}P = 0.0024$). Data are expressed as mean (histogram bars) ± SEM. *Overlaid dots* represent individual patient values. Sarcoidosis BAL: $n = 14$, and PBMC: $n = 15$. FACE = fluorescence-activated cell sorter.

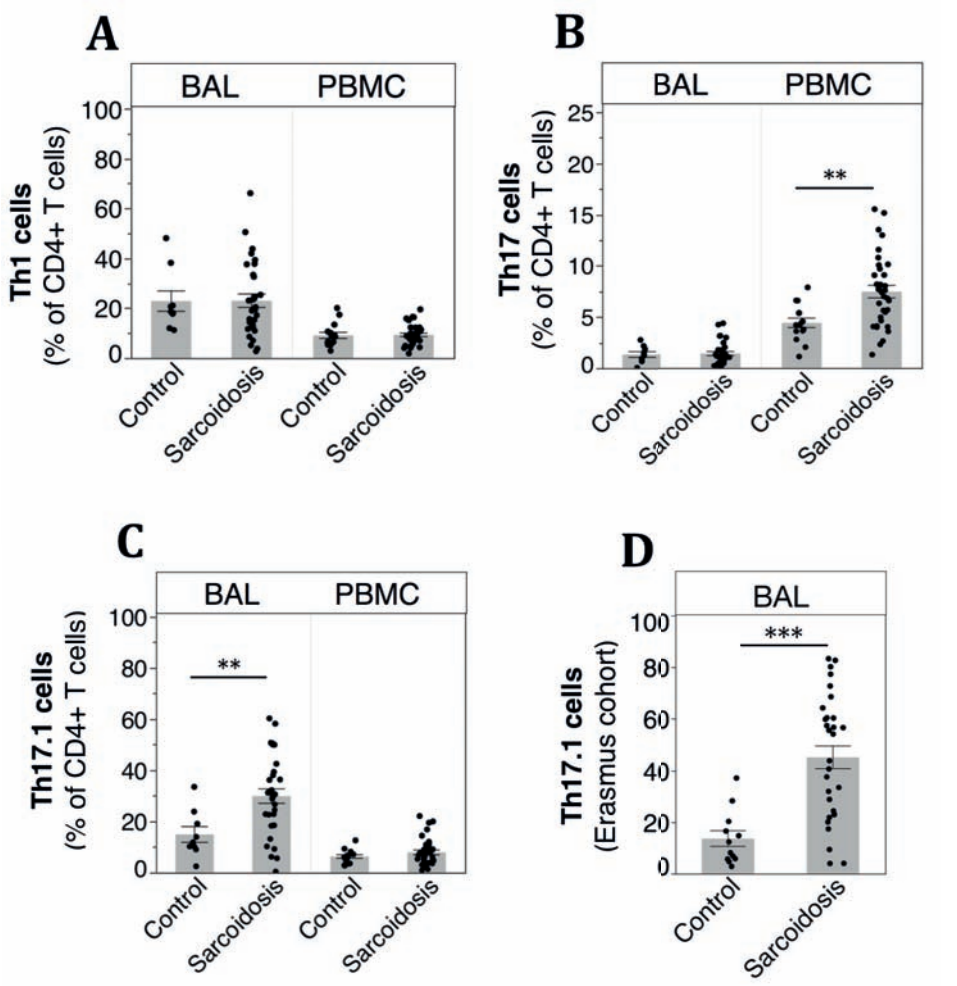

Figure 6. T-helper (Th) 17.1 cells from sarcoidosis bronchoalveolar lavage (BAL) fluid were significantly increased in two separate sarcoidosis cohorts and present in higher percentages compared with Th1 cells. BAL cells and peripheral blood mononuclear cells (PBMCs) were stained, FACS-sorted, and acquisition data was analyzed to include all three chemokine receptors to define Th1 and Th17.1 cells, as described in Methods, Table 1 and Figure E1. (*A*) There were no significant differences in the percentages of Th1 cells ($CCR6^{-}CCR4^{-}CXCR3^{+}$ effector cells) in the blood or lungs of patients with sarcoidosis compared with healthy control subjects. (*B*) The percentages of Th17 cells (defined $CCR6^{+}CCR4^{+}CXCR3^{-}$ effector cells) are significantly increased in the blood of patients with sarcoidosis (**$P = 0.0002$), but not in the lungs, compared with healthy control subjects. (*C*) The percentages of Th17.1 cells (defined $CCR6^{+}CCR4^{-}CXCR3^{+}$) were significantly increased in the lungs of patients with sarcoidosis (**$P = 0.0016$), but not in the blood. (*D*) The percentages of Th17.1 cells were markedly increased in the BAL of patients with sarcoidosis from the Erasmus MC cohort compared with similarly aged disease control subjects (***$P < 0.0001$). Data are expressed as mean (histogram bars) ± SEM. *Overlaid dots* represent individual patient values. U.S. cohort: BAL: $n = 32$ subjects with sarcoidosis and 9 control subjects; PBMC: $n = 35$ subjects with sarcoidosis and 18 control subjects. Erasmus MC cohort: BAL: N=30 subjects with sarcoidosis and 12 control subjects. FACS = fluorescence-activated cell sorter.

blood, we found the opposite pattern: Th17 cells were increased, but Th17.1 cells were not. Since the U.S. control group was younger than the patients with sarcoidosis (Table 2), we also studied a second cohort of European control subjects and newly diagnosed patients with sarcoidosis that were better matched for age (Table 4). Figure 6D displays the marked expansion of Th17.1 cells in patients with sarcoidosis compared with the disease control subjects, which validates the U.S. cohort findings.

DISCUSSION

Sarcoidosis is a classic Th1-mediated disease (35-37). Given the evidence for plasticity of Th17 cells towards a Th1 phenotype (14, 17-20, 22, 23, 34, 38-40), we speculated that the intense IFN-γ inflammation in the lungs of patients with sarcoidosis could transform Th17 cells into cells with characteristics of Th1 cells. These cells have been referred to as Th17.1, and large subpopulations of them have been shown to produce only IFN-γ. Using methods that are highly accurate in discriminating subsets of Th cells (14), we identified two separate Th17 subsets in sarcoidosis, namely, Th17 and Th17.1. These subsets express different chemokine receptors and demonstrate different cytokine effector functions. Importantly, Th17.1 cells have shown pathogenic characteristics, such as up-regulation of proinflammatory cytokines and corticosteroid resistance (14), demonstrating the importance of identifying this Th subset in sarcoidosis. We found that Th17.1 cells were markedly expanded in sarcoidosis BAL, and were present in even higher frequencies than classical Th1 cells. Furthermore, sarcoidosis Th17.1-enriched cells were equipotent in their ability to produce IFN-γ compared with Th1 cells. We also analyzed Th17.1 cells from a separate European cohort, which validated the original observations. Taken together, these findings stimulate questions regarding the established view that Th1 cells are the major contributor in sarcoidal inflammation. This study also demonstrates the importance of detailed characterization of human T cells, because Th17.1 cells, which are skewed to produce only IFN-γ, have likely been misclassified as Th1 cells due to their cytokine profiles. Ultimately, functional studies of $CD4^+$ T cells in sarcoidosis, without regard to more detailed distinction of the type of Th subset, may be misleading.

Direct comparison of the frequencies of Th17 and Th17.1 effector cell subsets between the blood and BAL compartment identified significant elevations of Th17-enriched cells in the blood, but not the BAL, of subjects with sarcoidosis compared with control subjects. Conversely, Th17.1-enriched cells were significantly increased in the BAL, but not in the blood, when compared with healthy control subjects. This observation could relate to plasticity of Th17-cell subsets *in vivo* if they are transiting from blood to the inflamed lung (36, 41, 42). Interesting prior research supports our results by finding elevated expression of CCR6 on $CD4^+$ T cells in sarcoidosis, but this study did not use

immunophenotyping assays that would allow for identification of the full population of Th17.1 cells or identify the marked differences in cytokine production of Th17.1 cells compared with Th17 cells (43). Other studies focused on $CD4^+$ cells that secreted IL-17 or IFN-γ without incorporation of multiple phenotypic markers and FACS-sorting, resulting in the possible misclassification of Th17.1 cells as Th1 cells (9, 11, 12). By highlighting the heterogeneity of IFN-γ-producing cells in this chronic human disease, our results suggest the more general conclusion that lymphocytes *in vivo*, especially in chronic diseases, may be more heterogeneous than those observed in controlled experimental models.

Studies such as that by Ramesh and colleagues (14) provide insight into the complicated functional role of Th17-cell subsets. The authors asked why patients with Crohn's disease receiving Secukinumab, a fully humanized anti-IL-17A monoclonal antibody, developed exacerbations of their disease (44), even though IL-17A is thought to be an important contributor of gut inflammation. Using human blood and gut tissue, they showed that Th17.1 cells (which the authors defined as $CCR6^+CXCR3^{hi}CCR4^{lo}CCR10^-CD161^+MDR1^+$) expressed a pro-inflammatory transcriptional signature that mimicked disease-inducing Th17 subsets in mice and also predicted increased responsiveness to IL-23, which is thought to be a regulator of pathogenic Th17 function (14). In contrast, a study examining T cells expressing $CCR6^+CXCR3^+CCR4^-$ (i.e., Th17.1 cells) found that their circulating numbers were increased in patients with latent tuberculosis infection compared with control subjects (45). The authors performed RNA sequencing of these cells and found lineage-specific signatures of both Th1 and Th17 cells, which would be expected, but also differential expression of genes associated with susceptibility to tuberculosis, and enhanced T-cell activation and cell survival (45). Thus, the functional complexity of Th17 subsets appears to relate to the local inflammatory signals and, although considered pathogenic in one inflammatory disease, they may be necessary for eradication of the infection in another. Further studies are needed to understand the role of Th17-cell subsets, their cellular products, and the regulators of Th17 pathology (e.g. IL-23) in sarcoidosis to make sense of results from existing studies (46) and facilitate the development of more effective therapies.

Our study has some potential limitations. We found that Th17.1-cell numbers were markedly increased in sarcoidosis BAL as compared with that from two control groups, whereas Th1 cell numbers were not. However, each control group has a potential limitation. Our first control group was comprised of healthy control subjects, but they were somewhat younger, on average, than those in our sarcoidosis group. Our second control group was comprised of control subjects with disease rather than healthy control subjects. Nonetheless, we did not find any expansion of Th17.1 cells in BAL in either of these control groups and we believe the consistency of our findings across these two control groups is supportive of our general conclusions.

In summary, we isolated $CD4^+$ T effector cells by their chemokine receptors to understand their distribution and functional attributes in the BAL and blood of sarcoidosis to uncover significantly elevated frequencies of BAL Th17.1 cells. It is likely that these cells have been misclassified as Th1 cells in sarcoidosis. These new findings raise questions about prior assumptions of disease pathogenesis and may provide new directions for clinical studies and treatment targets. We also need to learn more about their presence in granulomatous tissues and what role(s) they play in disease progression.

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SUPPLEMENTARY MATERIAL

METHODS

Subjects

Participants underwent written informed consent and the study was approved by the UCSF Committee on Human Research. Sarcoidosis diagnosis was based on consistent clinical features, absence of alternative diagnoses and biopsy of the lung or mediastinal lymph nodes showing non-caseating granulomas according to accepted criteria (1). Patients were excluded for the following conditions: a history of smoking, cancer, chronic infections (hepatitis, HIV, TB, etc.), autoimmune diseases (Crohn's, Lupus, etc.), other pulmonary diseases or organ transplant. After enrollment, patients underwent a chest X-ray, high-resolution chest computed tomography (CT) scan, BAL and blood collection. Fibrosis was determined on the basis of lung scarring by chest CT scan analysis. Healthy control subject data were obtained from a concurrent study (study number NCT01484691) to evaluate the same immunological parameters.

The validation cohort referred to as "Erasmus MC cohort" consisted of 30 newly diagnosed European patients in which the pulmonary sarcoidosis diagnosis was based on the same criteria as above (1). Exclusion criteria included conditions described above in addition to use of immunomodulatory medication three months prior to study enrollment, however a smoking history was accepted. The control group consisted of 12 age-matched individuals, eight of whom underwent bronchoscopy for community-acquired pneumonia and four patients with COPD. The Medical Ethical Committee of the Erasmus MC Rotterdam approved this study.

Bronchoalveolar lavage

Subjects underwent bronchoscopy with BAL following the procedures set forth by the protocol committee of the NIH-funded study, Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis (ClinicalTrials.gov Identifier: NCT01831739). Cells were resuspended in FACS buffer (0.1% BSA + 2mM EDTA in PBS), counted and immediately processed for flow cytometry.

Peripheral blood mononuclear cell (PBMC) isolation

PBMCs were isolated from peripheral blood (collected in EDTA tubes) by Ficoll density centrifugation as previously described (2) and processed immediately for flow cytometry.

Flow cytometry and sorting

For surface staining, BAL cells and PBMCs were incubated with fluorescent antibodies (CD3 [BD Horizon], CCR4 [BD Pharmingen], CD127, CD4, CD25 and CCR6 [eBioscience], CD45RO, CD45RA and CXCR3 [Biolegend]), and CD1d-tetramer-PBS57 [NIH Tetramer Facility] for 30 minutes at 4°C in the dark using methods recommended by the manufacturers. After incubation, cells were washed, and viability was measured using Propidium Iodide (BD Bioscience). To FACS-sort CCR4 and CCR6 positive and negative expressing cells, we gated on live cells, followed by lymphocytes, singlets, non-invariant natural killer T (iNKT) cells, CD4 T cells, non-regulatory T cells, and effector cells (Figure 1A & B). $CCR6^+$, $CCR4^+CCR6^-$, and $CCR4^-CCR6^-$ effector cells were sorted into separate chambers and used in phenotypic and functional studies. Figure 1A & 1B show our three-gate sorting strategy. After discovery of the high prevalence of IFN-γ-producing $CCR6^+$ cells, a fourth gate was added to sort effector T cells into four chambers based on chemokine expression: $CCR4^+CCR6^+$, $CCR4^+CCR6^-$, $CCR4^-CCR6^+$, and $CCR4^-CCR6^-$. CXCR3 was used in the FACS-sorting panel and functional panel to allow for a more stringent gating such that Th1-enriched cells were defined by $CCR6^-CCR4^-CXCR3^+$, Th2-enriched defined by $CCR6^-CCR4^+CXCR3^-$, Th17-enriched defined by $CCR6^+CCR4^+CXCR3^-$, and Th17.1-enriched defined by $CCR6^+CCR4^-CXCR3^+$ (Table 1) (3). Titration of each reagent and "Fluorescence Minus One" controls (4) were used for optimization and placement of gates. One million events were recorded for analysis during FACS acquisition.

Cell stimulation & cytokine analysis

After overnight rest in RPMI 1640 + 10% FBS (Hyclone), 1% pen/strep, 1% glutamine at 37°C, the FACS-sorted effector cells subsets were stimulated at 37°C for 4 hours in 10 nM PMA and 1 μM ionomycin in the presence of 5μg/mL brefeldin A. Cells were stained for surface markers, CCR6, CCR4, and CXCR3 (eBioscience), and live/dead Amcyan stain (Invitrogen) for 30 minutes at 4°C in the dark and washed. Cells were fixed in 2% paraformaldehyde for 20 minutes on ice and permeabilized (BD Biosciences) for 10 minutes at RT prior to intracellular cytokine staining for IL-17A, IL-4, and IFN-γ (eBioscience). Cells were washed and analyzed immediately. Data analysis included determining the percentage of each effector subset making one or all of the above cytokines. As was done for the phenotypic analysis of each subset, we again used 3 chemokine receptors to define Th1-, Th2-, Th17-, and Th17.1-enriched groups (Table 1) and report their respective production of cytokines.

Methods for validation Erasmus MC cohort: flow cytometry staining and Th17.1 analyses

Bronchoscopy with BAL and processing of fluid and cells performed as previously described (5). BAL fluid cells were stained for intra- and extracellular markers using the

following antibodies: CD3-APC-eFluor780 (SK7), CD4-AF700 (OKT4), FoxP3-PE (236A/E7), CTLA4-PerCp-eFluor710 (14D3) (eBiosciences) and CCR6-APC (11A9), CXCR3-BV711 (1C6/CXCR3) (BD biosciences) and CCR4-FITC (205410) (R&D) and CXCR3-BV421 (G025H7) (Biolegend) and CD45RA-PE-Texas Red (MEM-56) (Invitrogen). Fixable Aqua Dead Cell Stain kit for 405 nm (Invitrogen, Molecular Probes) was used as live-dead marker. At least 100,000 cells per sample were measured on a Flow cytometer LSRII (BD Biosciences). In sarcoidosis and disease control BAL fluid we identified subsets of $CD45RA^{-}FoxP3^{low/intermediate}$ total memory T helper cells (6) on the basis of chemokine-receptor expression (3, 7-9), as we have previously shown in mediastinal lymph nodes (10). We determined $CCR6^{-}$ T helper cell subsets ($CCR4^{-}CXCR3^{+}$ Th1 cells and $CCR4^{+}CXCR3^{-}$ Th2 cells) and $CCR6^{+}$ T helper cell subsets ($CCR4^{+}CXCR3^{-}$ Th17 cells, and $CCR4^{-}CXCR3^{+}$ Th17.1 cells).

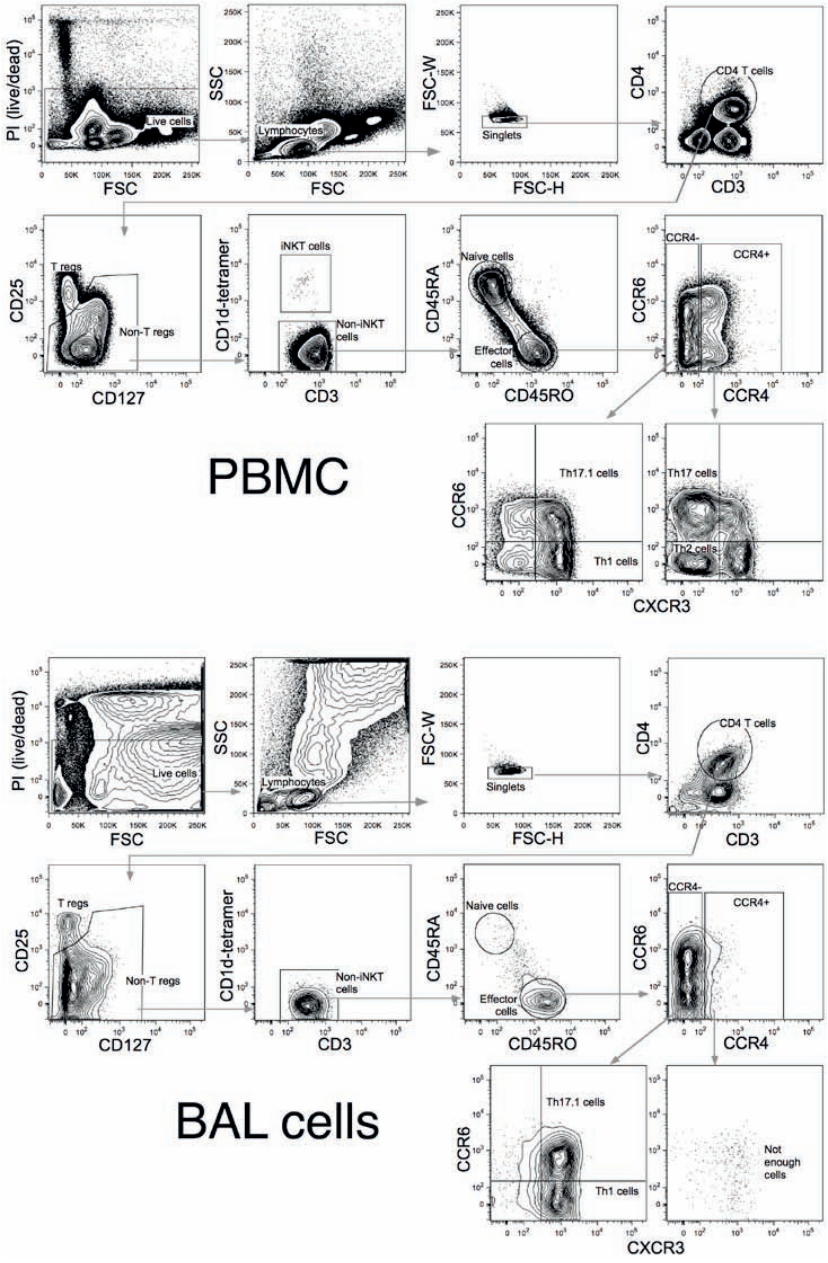
CT scanning

Patients underwent computed tomography (CT) performed on a General Electric 64 multidetector CT scanner. 1.25-mm noncontrast axial images were obtained while supine during full inspiration for a 10 s breath hold. Radiation settings were adjusted to body mass index. Imaging protocol was defined by the NIH-funded study, Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis (ClinicalTrials.gov Identifier: NCT01831739).

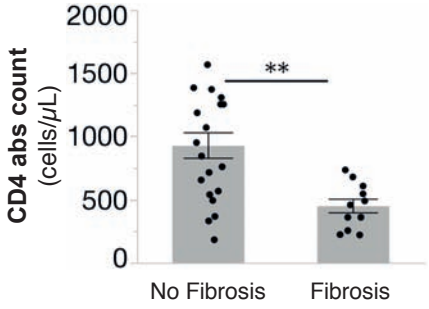
Statistical analysis

Data were analyzed using FlowJo 9, JMP 10 and GraphPad Prism 6 software. Normality was measured using Shapiro-Wilk test. Differences between two groups were analyzed using an unpaired student t-test with Welch's correction or Rank sum test as appropriate. Chi-square analysis was used for gender, race and ethnicity, and a student t-test was used for the age. For all statistical tests, a P-value < 0.05 was considered significant.

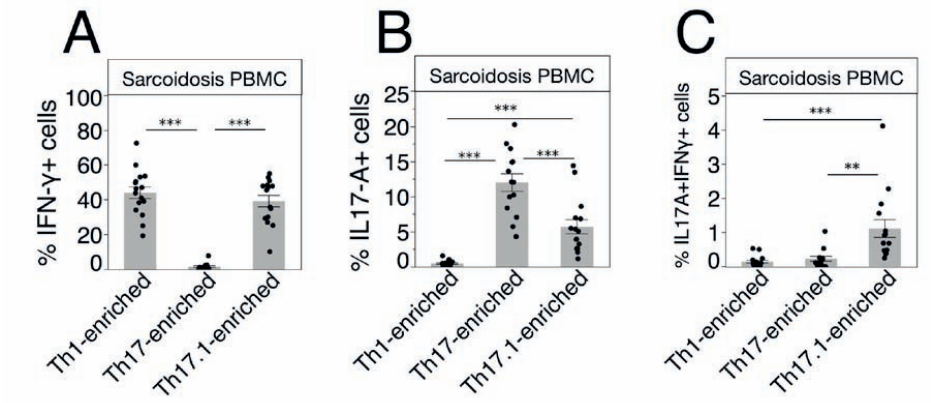
SUPPLEMENTARY FIGURES



Supplementary Figure E1. Flow cytometry gating strategy using all three chemokine receptors to determine percentages of T helper subsets. CXCR3 was used in the FACS-sorting and functional panels to allow more stringent gating of Th17, Th17.1, Th1, and Th2 populations as defined in Table 1.



Supplementary Figure E2. Absolute $CD4^+$ T cell count in the blood of sarcoidosis patients, with and without fibrosis. Absolute CD4 counts were obtained on sarcoidosis participants on the same day their radiology images were performed. On average, the counts were found to be significantly higher in the patients without evidence of fibrosis compared to the fibrotic patients. $^{**}P = 0.0002$.



Supplementary Figure E3. Compilation of the functional data for PBMC samples from all sarcoidosis subjects demonstrates the marked differences in cytokine production between Th17 subsets. PBMC cells were FACS-sorted and subsequently stimulated and stained for intracellular cytokines as described in Methods. (A-C) Functional data from sarcoidosis patients were compiled and displayed. (A) Percentage of cells producing IFN-γ only. (B) Percentage of cells producing IL-17A only. (C) Percentage of polyfunctional cells producing both IL-17A and IFN-γ. These data show three discernible populations of Th cells, namely, Th1, Th17, and Th17.1 in human sarcoidosis and specifically that a large percentage of Th17.1 cells produce only IFN-γ. Data are expressed as mean histogram bars ± SEM. Overlaid dots represent individual patient values. Sarcoidosis PBMC: $n = 15$.

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CHAPTER 5

Increased CCR6^{+} Th17-Lineage Subset Polarization in Sarcoidosis Mediastinal Lymph Nodes

'Life is like riding a bicycle. To keep your balance, you must keep moving'
Albert Einstein

Submitted for publication

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ABSTRACT

Background: We reported that T helper 17.1 (Th17.1) cells are responsible for the exaggerated IFN-γ production in pulmonary sarcoidosis, but it is currently unknown where these cells are induced.

Methods: Using flow cytometry, proportions, proliferation, and activation status of $CCR6^-$ Th subsets ($CCR4^-CXCR3^+$Th1 and $CCR4^+CXCR3^-$Th2) and $CCR6^+$ Th17-lineage subsets ($CCR4^+CXCR3^-$Th17, $CCR4^+CXCR3^+$ double-positive (DP) and $CCR4^-CXCR3^+$Th17.1) were compared between mediastinal lymph nodes (MLN) from treatment-naive sarcoidosis patients and healthy controls, and within broncho-alveolar lavage fluid (BALF) from patients.

Results: Higher proportions of $CCR6^+$ Th17-lineage subsets were detected in sarcoidosis MLN than in control MLN. Sarcoidosis BALF contained predominantly Th17.1 cells, whereby proportions were significantly higher in patients developing chronic disease than in patients undergoing resolution. Sarcoidosis $CCR6^+$ DP cells exhibited a high fraction of proliferating Ki-67^+ cells and displayed lower CTLA4 expression in sarcoidosis MLN compared with controls. Th17.1 cells in sarcoidosis MLN exhibited significantly reduced CD28 expression.

Conclusion: Our data are the first to support a model in which sarcoidosis $CCR6^+$ Th17-lineage cells are polarized in the MLN, which show plasticity towards Th17.1 cells in the lung microenvironment, where their presence correlates with development of chronic disease. This study provides a new perspective on sarcoidosis pathogenesis and therapeutic targets.

INTRODUCTION

Sarcoidosis is a multisystem granulomatous disorder of unknown cause, predominantly affecting the lungs (1). Sarcoidosis was initially characterized as a $CD4^+$ T helper (Th)1-mediated immunological response upon exposure to a likely poorly degradable antigenic stimulus, leading to granuloma formation (1). Immunological characterizations included non-caseating granulomas with enhanced local expression of Th1-cytokines/chemokines, such as interleukin (IL)-2, interferon (IFN)-γ, IL-12, IL-18 and IFN-γ-induced protein (IP)-10 (C-X-C motif chemokine ligand 10 (CXCL10)) (2-6), and clonal expansion of Th cells reflecting a chronic antigenic nature of stimulation (7).

Over the last years, IL-17-producing cells were detected in sarcoidosis patients during active and progressive pulmonary disease (8-10). Both lung and circulating Th cells from patients showed increased IL-17 and IL-23 receptor (IL-23R) expression (8-10). We and others found significantly increased proportions of IL-17A/IFN-γ-double-producing memory Th cells within sarcoidosis peripheral blood (PB) and broncho-alveolar lavage fluid (BALF) (9-11). Interleukin-17A/IFN-γ-double-producers are described to be pathogenic in human chronic inflammatory disorders, including Crohn's disease (CD) (12, 13) and juvenile idiopathic arthritis (JIA)(14). Proportions of $IL\text{-}17A^+/IFN\text{-}\gamma^+$ Th cells in sarcoidosis BALF increased with Scadding stage (11), suggesting a role in disease prognosis.

Evidence has accumulated that $IL\text{-}17A^+/IFN\text{-}\gamma^+$ Th cells (Th17/Th1) express both Th17-associated markers, including C-C chemokine receptor type 6 (CCR6), CD161, IL-23R and RAR-related orphan receptor (ROR)γt, and Th1-associated markers, such as C-X-C motif chemokine receptor 3 (CXCR3), IL-12Rβ2 and TBX21 (T-bet)(12-21). These $IL\text{-}17A^+/IFN\text{-}\gamma^+$ Th cells originally derive from IL-17A-single-positive Th cells (Th17) and can develop into IFN-γ-single-positive cells (12-21). Hence, there are two types of IFN-γ-producing Th cells, i.e. $CCR6^-$ Th1 cells and $CCR6^+$ Th17-derived cells. The latter are referred to as Th17.1 cells, based on CCR6 and CXCR3 chemokine-receptor expression (13, 15, 20, 21) (see Figure E1 for subset clarification).

Insight in the role of Th17-Th17.1 cell plasticity in sarcoidosis lungs is highly relevant since human Th17.1, and not Th17 cells, were specifically found to have a pathogenic signature and to highly express the multi-drug resistance type 1 membrane transporter (MDR1), enhancing steroid resistance (13). Importantly, we recently reported that Th17.1 cells rather than Th1 cells are responsible for the exaggerated IFN-γ production in pulmonary sarcoidosis (22). Since Th17.1 cells are primarily thought to derive from Th17 cells (12-21), Th17 cell plasticity could take place in pulmonary sarcoidosis.

Lung-draining mediastinal lymph nodes (MLN) have become an attractive clinical site for sarcoidosis diagnosis, since detection of granulomas is more accurate in MLN than in lung (23). Clinical findings in pulmonary sarcoidosis suggest that the first granulomas are formed within MLN (24), and enhanced $CD4^+$ T-cell and Th17-lineage cell presence in sarcoidosis MLN (25) reflects the pulmonary immunological response. Classifying Th

subsets in MLN will increase insight in the primary Th cell response that occurs in pulmonary sarcoidosis. Therefore, in the current study we investigated the distribution and proliferative capacities of $CCR6^+$ Th17-lineage subsets in MLN and BALF to gain insight into Th17/Th17.1 cell induction in pulmonary sarcoidosis.

METHODS

Study design and subjects

Patients with pulmonary sarcoidosis were included at time of diagnosis. The diagnosis of sarcoidosis was made conform to the guidelines of the ATS/ERS/WASOG (1).

Exclusion criteria were use of immunomodulatory medication 3 months prior to study inclusion; respiratory tract infection 4 weeks prior to study inclusion; concomitant pulmonary disease (including chronic obstructive pulmonary disorder and asthma), autoimmune diseases, malignancies, human immunodeficiency virus seropositivity, pregnancy, and allergies.

For this study, in total 55 sarcoidosis patients donated BALF, MLN-derived fine-needle aspiration (FNA) or PB. Disease course of a subgroup of patients was determined 2 years after study inclusion. Resolution of disease was defined by the absence of abnormalities on chest X-ray and clinical symptoms. Patients with residual abnormalities on chest X-ray, but without need for treatment were designated as non-progressive chronic; and patients with need for treatment as progressive chronic.

Control MLN were collected from 22 lung transplantation donors without signs of pulmonary inflammation (routinely assessed by a chest X-ray and bronchoscopy).

The Medical Ethical Committee of the Erasmus University Medical Centre Rotterdam approved this study. Written informed consent was obtained from every participant before study inclusion. Further subject characteristics are shown in Supplementary Table E1.

Study materials

Bronchoscopy with BAL was performed as previously described(9). BALF cells and PB were processed as previously described (9).

Esophageal or endobronchial ultrasound guided (EUS- or EBUS)-FNA from draining MLN were performed with a 22G (or 19G) needle. MLN aspirates were filtered through a 100 µm cell strainer (BD Biosciences) and centrifuged. Cells were stored at -150 °C.

Control MLN were collected from lung transplantation donors. Control MLN were processed as lymph node aspirates.

Flow cytometry

BALF, MLN and PB mononuclear cells were stained for intra- and extracellular markers using the antibodies shown in Supplementary Table E2. Fixable Aqua Dead Cell Stain kit for 405 nm (Invitrogen, Molecular Probes) was used as live-dead marker. At least 100.000 cells per sample were measured on a Flow cytometer LSRII (BD Biosciences), and the mean fluorescence intensity of cytotoxic T-lymphocyte antigen 4 (CTLA4), programmed cell death protein 1 (PD-1), CD28, CD25 and CD278 (inducible T-cell costimulator (ICOS)) was standardized to average expression in healthy control PB cells.

Statistical analyses

Comparisons were performed using a Mann-Whitney U test or Wilcoxon signed rank test. *P*-values were two-sided, and analyses were performed using IBM SPSS Statistics 21. $P<0.05$ was considered statistically significant.

RESULTS

Predominant presence of Th17.1 cells in sarcoidosis BALF

We recently found an increased presence of Th17.1 cells in sarcoidosis BALF compared with controls (22). Because Th17.1 cells are primarily thought to be progeny of Th17 cells(12-19), we aimed to quantify all $CCR6^+$ Th subpopulations in sarcoidosis BALF. T helper subsets of $CD45RA^-FoxP3^{low/int}$ total memory Th cells (Figure E2) were identified on the basis of chemokine-receptor expression (Figure E1) in BALF of 36 sarcoidosis patients. We quantified two $CCR6^-$ Th cell subsets ($CCR4^-CXCR3^+$ Th1 cells and $CCR4^+CXCR3^-$ Th2 cells) and three $CCR6^+$ Th cell subsets ($CCR4^+CXCR3^-$ Th17 cells, $CCR4^+CXCR3^+$ double-positive (DP) Th cells and $CCR4^-CXCR3^+$ Th17.1 cells), as depicted for a representative sarcoidosis BALF in Figure 1A. Within $CCR6^+$ Th cells, $CCR4^+CXCR3^+$ DP cells likely reflect an intermediate stage between Th17 and Th17.1 cells(20, 21, 26), expressing both RORγt/IL-17A and T-bet/IFN-γ in patients with rheumatoid arthritis (RA) (26).

In sarcoidosis Th17.1 cell proportions were higher than either Th1, Th2, Th17 or DP cells (Figure 1B) and thus represented the most predominant Th population in BALF (Figure 1C). Interestingly, the proportions of $CCR6^+$ DP cells were higher than Th17 cells ($P < 0.0001$).

Together, these data show a predominance of Th17.1 cells and the presence of $CCR6^+$ DP cells in sarcoidosis BALF.

Specific increase of all $CCR6^+$ Th17-derived cell subpopulations in sarcoidosis MLN

Next, we quantified and classified total memory Th cells according to chemokine-receptor expression (Figure E1), both in sarcoidosis MLN (n=17) and in control MLN

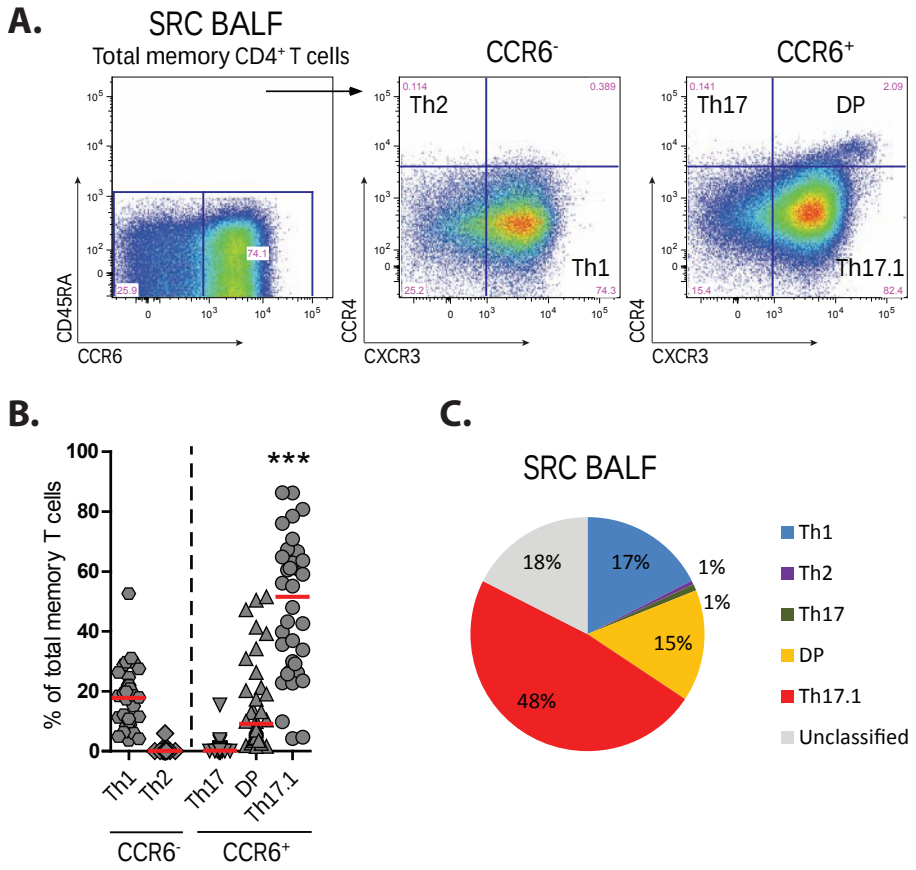

Figure 1. Predominant presence of Th17.1 cells in sarcoidosis BALF. $CCR6^-$ T-cell subsets ($CCR4^-CXCR3^+$ Th1 cells and $CCR4^+CXCR3^-$ Th2 cells) and $CCR6^+$ T-cell subsets ($CCR4^+CXCR3^-$ Th17 cells, $CCR4^+CXCR3^+$ DP Th cells and $CCR4^-CXCR3^+$ Th17.1 cells) were determined in $CD45RA^-FoxP3^{low/int}$ total memory $CD4^+$ T cells (see Figure E2 for gating strategy) from sarcoidosis BALF (n=36). (*A*) Representative flow cytometry analysis of BALF from one sarcoidosis patient. (*B*) Proportions of Th1, Th2, Th17, $CCR6^+$ DP and Th17.1 cells of total memory $CD4^+$ T cells. Horizontal lines indicate median values. Significance was determined using a Wilcoxon signed rank test. *** $P \leq 0.001$ (represents sarcoidosis Th17.1 cells versus either sarcoidosis Th1,Th2 or Th17 or DP cells). (*C*) A pie-chart showing mean percentages of Th1, Th2, Th17, $CCR6^+$ DP, Th17.1 and unclassified cells (which include $CCR6^-CCR4^-CXCR3^-$ cells, $CCR6^-CCR4^+CXCR3^+$ cells and $CCR6^+CCR4^-CXCR3^-$ cells) of total memory $CD4^+$ T cells. *Abbreviations:* CCR = C-C chemokine receptor, CXCR = CXC chemokine receptor, BALF = broncho-alveolar lavage fluid, DP = double-positive, Th = T helper, SRC = sarcoidosis.

(n=22). A representative gating strategy is shown for one control and one sarcoidosis MLN in Figure 2A. Similar to sarcoidosis BALF (22), sarcoidosis MLN contained higher proportions of $CCR6^+$ Th cells than control MLN (Figure 2B). We found that all Th17-derived subsets, e.g. Th17 (25), $CCR6^+$ DP and Th17.1 cells were significantly increased in sarcoidosis compared with control MLN (Figure 2C/**D**). In sarcoidosis MLN, Th1 cell proportions were significantly decreased compared with controls, although Th1 cells

remained (like in controls) the most prominent memory T-cell population in sarcoidosis MLN (Figure 2C**/D**).

Taken together, these data show that sarcoidosis MLN contain increased proportions of Th17.1 cells compared with controls, paralleling the lungs (22). However, in contrast to sarcoidosis BALF, sarcoidosis MLN additionally contain increased proportions of Th17.1 precursor cells, i.e. Th17 and CCR6$^+$ DP cells. These data indicate enhanced Th cell differentiation towards CCR6$^+$ Th17-lineage cells in sarcoidosis MLN.

CCR6$^+$ DP cells are highly proliferative in sarcoidosis

The specific increase in all CCR6$^+$ Th cells in sarcoidosis MLN could be caused by enhanced proliferation, which we assessed using the proliferation marker Ki-67.

Proportions of proliferative CD4$^+$ memory T cells were significantly increased in sarcoidosis MLN compared with controls (Figure 3A). Interestingly, specifically CCR6$^+$ DP cells and Th17.1 cells exhibited high fractions of proliferating Ki-67$^+$ cells compared with controls (Figure 3B**/C**). This was particularly striking for the Th17.1 subpopulation, which in controls contained only very few Ki-67$^+$ cells. However, CCR6$^+$ DP cells contained the highest fraction of proliferating cells (~15%, which was significantly higher than any of the other T-cell subsets in sarcoidosis MLN (Figure 3C). In sarcoidosis BALF not the predominant Th17.1 cells (22), but rather the suggested precursors (12-21, 26) Th17 and CCR6$^+$ DP subsets were highly proliferative (Figure 3D).

In summary, *ex-vivo* phenotyping identified CCR6$^+$ DP cells as a highly proliferative Th subset in both MLN and BALF of sarcoidosis patients

Both Th17 cells and CCR6$^+$ DP cells show decreased CTLA4 expression

Since proliferative capacity depends on co-inhibitory CTLA4 and PD-1, and co-stimulatory CD28 signals, we determined their expression on Th17-derived subpopulations. Strikingly, both Th17 cells (25) and CCR6$^+$ DP cells showed decreased CTLA4 expression in sarcoidosis MLN compared with controls (Figure 4A). PD-1 expression was lower on all Th17-lineage cells in sarcoidosis, however only significantly in Th17 cells compared with controls (Figure 4B). In sarcoidosis MLN, all CCR6$^+$ Th cells subsets showed lower CD28 expression than Th1 cells (Figure 4C). Compared with controls, sarcoidosis Th17.1 cells showed significantly lower CD28 expression (Figure 4C). No changes were observed in ICOS or CD25/IL-2R expression (Figure E3).

Thus, sarcoidosis CCR6$^+$ DP cells, which are highly proliferative in sarcoidosis, also show significantly decreased CTLA4 expression compared with controls (25).

Higher Th17.1 and lower Th17 cell proportions in BALF than MLN in sarcoidosis

Findings above suggest Th17 plasticity towards Th17.1 cells in pulmonary sarcoidosis, since we observed increased proportions of all CCR6$^+$ Th cells in MLN and only Th17.1

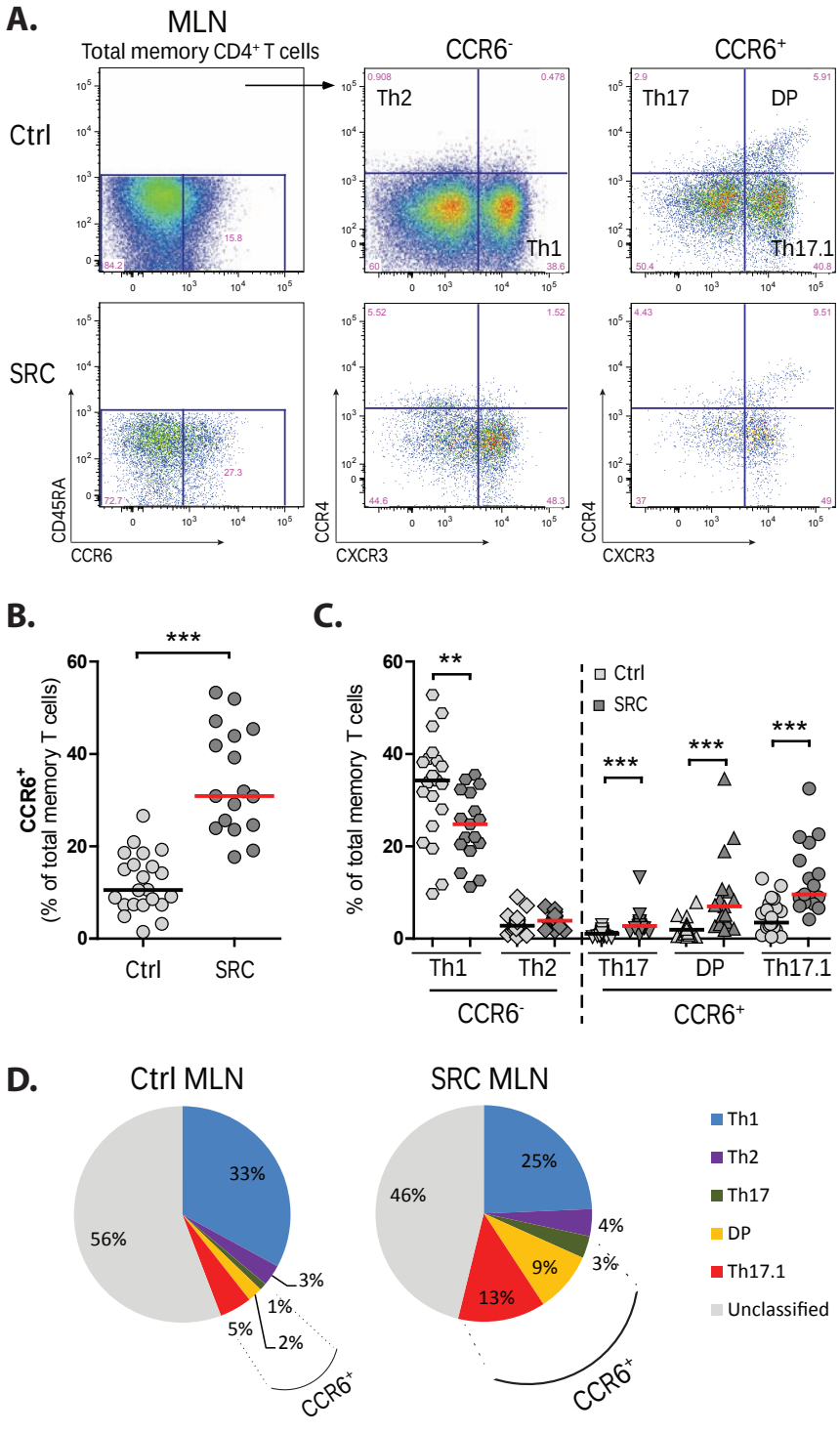
A.
MLN
Total memory CD4+ T cells
CCR6-
CCR6+
Ctrl
SRC
Th2
Th1
Th17
DP
Th17.1
CD45RA
CCR6
CCR4
CXCR3
B.
CCR6+ (% of total memory T cells)
Ctrl
SRC
C.
% of total memory T cells
Ctrl
SRC
Th1
Th2
Th17
DP
Th17.1
CCR6-
CCR6+
D.
Ctrl MLN
SRC MLN
33%
56%
3%
1%
5%
2%
CCR6+
25%
46%
4%
3%
9%
13%
CCR6+
Th1
Th2
Th17
DP
Th17.1
Unclassified

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Figure 2. Specific increase of all CCR6$^+$ Th cell populations in sarcoidosis MLN. CCR6$^-$ T-cell subsets (CCR4$^-$CXCR3$^+$ Th1 cells and CCR4$^+$CXCR3$^-$ Th2 cells) and CCR6$^+$ T-cell subsets (CCR4$^+$CXCR3$^-$ Th17 cells, CCR4$^+$CXCR3$^+$ DP Th cells and CCR4$^-$CXCR3$^+$ Th17.1 cells) were determined in CD45RA$^-$FoxP3$^{low/int}$ total memory CD4$^+$ T cells (see Figure E2 for gating strategy) from control (n=22) and sarcoidosis MLN (n=17). (*A*) Representative flow cytometry analysis of MLN from one control and one sarcoidosis patient. (*B*) Proportions of CCR6$^+$ total memory T cells. (*C*) Proportions of Th1, Th2, Th17, CCR6$^+$ DP and Th17.1 cells of total memory CD4$^+$ T cells. (*D*) Pie-chart showing mean percentages of Th1, Th2, Th17, CCR6$^+$ DP, Th17.1 and unclassified cells (which include CCR6$^-$CCR4$^-$CXCR3$^-$ cells, CCR6$^-$CCR4$^+$CXCR3$^+$ cells and CCR6$^+$CCR4$^-$CXCR3$^-$ cells) of total memory CD4$^+$ T cells. *Statistics:* Horizontal lines indicate the median values; significance was determined using a Mann-Whitney U test, ** P <0.01 *** P < 0.001. *Abbreviations:* CCR = C-C chemokine receptor, CXCR = CXC chemokine receptor, MLN = mediastinal lymph nodes, DP = double-positive, Th = T helper, Ctrl = control, SRC = sarcoidosis.

cells in BALF, and CCR6$^+$ DP cells, which likely reflect an intermediate Th17 and Th17.1 cell population (20, 21, 26), are highly proliferative in sarcoidosis-affected organs (Figure 3B-D).

To gain further insight into the *in-vivo* behavior of Th17-derived CCR6$^+$ populations, we compared their proportions within sarcoidosis PB, MLN, and BALF. Remarkably, in sarcoidosis patients, the proportions of memory Th cells that were CCR6$^+$ increased from ~19% in PB, to ~31% in MLN and to ~75% in BALF (Figure 5A). Whereas Th17 cell proportions were significantly lower in BALF than in MLN and PB (Figure 5B), Th17.1 cells were significantly higher (Figure 5D). Proportions of CCR6$^+$ DP cells were similar in MLN and BALF, but were significantly higher in BALF than PB (Figure 5C). Finally, CCR6$^-$ Th1 cell proportions were lower in sarcoidosis BALF compared with MLN (Figure 5E).

In conclusion, whereas Th17.1 cell proportions were significantly higher in sarcoidosis BALF than MLN, Th17 and Th1 cell proportions were significantly lower. CCR6$^+$ DP cells were also higher in sarcoidosis lungs than PB. Taken together, these data suggest that Th17.1 cells arise from Th17/CCR6$^+$ DP cells, indicating a role for Th17 cell plasticity in sarcoidosis, specifically in the lungs.

Higher BALF Th17.1 cell proportions in patients developing chronic disease than in patients undergoing resolution

As Th17.1 cells are described to be pathogenic, we questioned whether proportions of Th17.1 cells in BALF would associate with disease prognosis. Therefore, we determined the disease course of a subgroup (n=25) of our study cohort at ~2 years clinical follow-up. Interestingly, in patients developing chronic sarcoidosis, and specifically progressive chronic sarcoidosis with need for treatment, significantly higher BALF Th17.1 proportions were detected at time of diagnosis, compared with patients undergoing resolution (Figure 6A). In contrast, Th1 proportions were lower in patients developing progressive chronic disease than patients undergoing disease resolution (Figure 6B), resulting in an

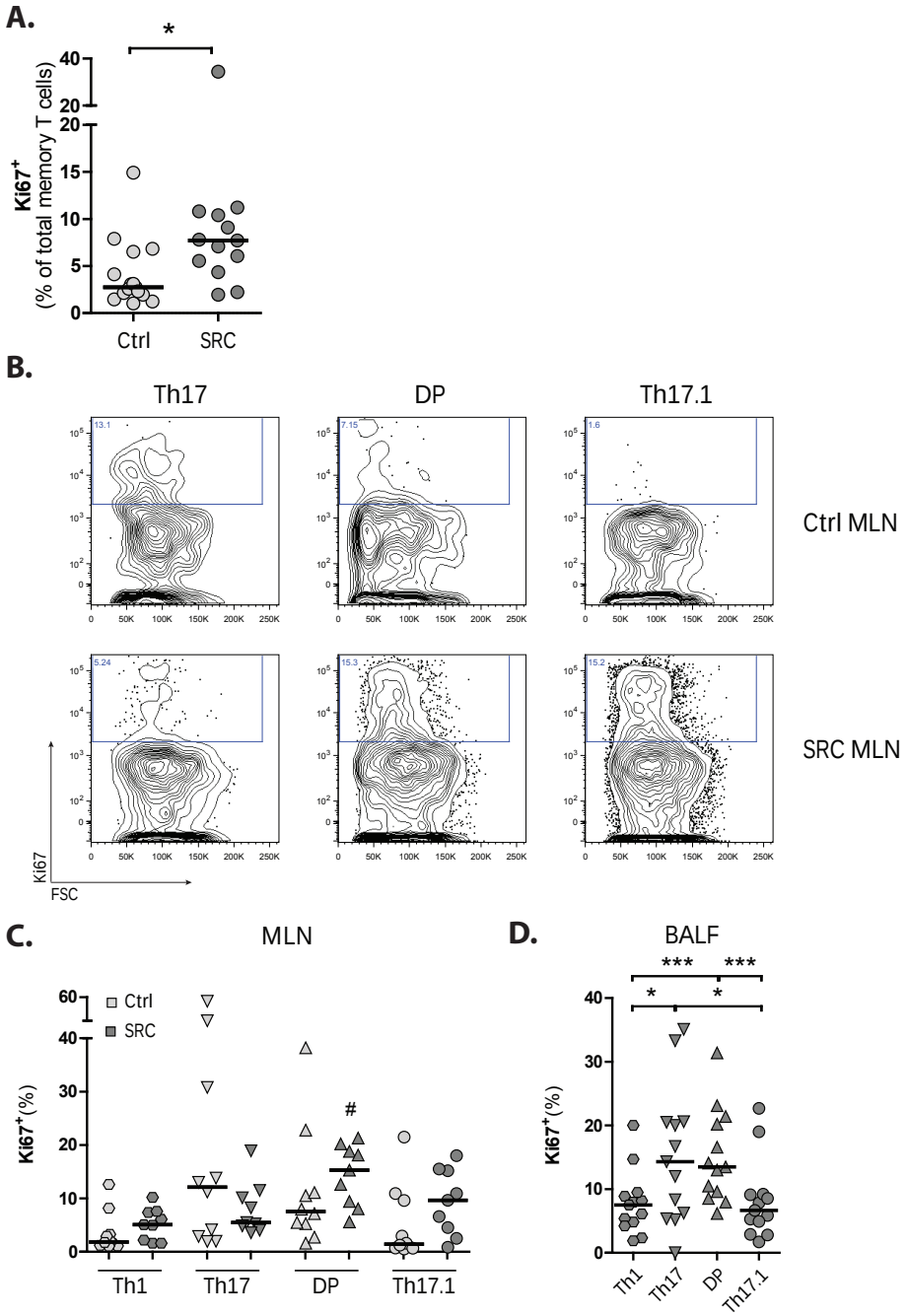

Figure 3. CCR6$^+$ DP cells are highly proliferative in sarcoidosis. Expression of Ki67 was determined *ex-vivo* in Th1, Th17, CCR6$^+$ DP and Th17.1 cells in control and sarcoidosis MLN, and sarcoidosis BALF. (*A*) Proportions Ki67$^+$ total memory T cells (data are from 15 controls and 13 patients). (*B*) Representative flow cytometry analysis of Th17, CCR6$^+$ DP and Th17.1 cells in MLN from one control and one sarcoidosis patient. (*C*) Pro-

portions of Ki67$^+$ cells in control (n=10) and sarcoidosis MLN (n=9). Horizontal lines indicate median values. Significance was determined using a Mann-Whitney U test. Significance between median values of paired samples was determined using a Wilcoxon signed rank test (# $P < 0.05$: sarcoidosis DP cells versus either sarcoidosis Th1,Th17 or Th17.1). (*D*) Proportions of Ki67$^+$ cells in sarcoidosis BALF (n=13). Horizontal lines indicate median values. Significance was determined using a Wilcoxon signed rank test, * $P < 0.05$ *** $P < 0.001$. *Abbreviations:* DP = double-positive, Th = T helper, Ctrl = control, SRC = sarcoidosis.

increased Th17.1/Th1 ratio within BALF of patients developing chronic disease (Figure 6C). Although non-significant, Th17 proportions also showed a trend to be lower in patients developing chronic disease than in patients undergoing resolution (Figure E4A), and patients developing progressive chronic disease showed a higher Th17.1/Th17 ratio within BALF (Figure E4B).

In conclusion, these data suggest that development of chronic disease is associated with Th17.1 cells, but not with Th1 or Th17 cells in sarcoidosis BALF, highlighting a pathogenic role for Th17.1 rather than Th1 or Th17 cells in the development of (chronic) pulmonary sarcoidosis.

DISCUSSION

Our study supports a model in which sarcoidosis Th17-lineage cells are induced in MLN and manifest plasticity towards Th17.1 cells in the (lung) granulomatous microenvironment, where they are particularly critical for development of chronic disease.

Since it was recognized that Th17 cells display considerable plasticity and can produce IFN-γ, it has become increasingly clear that Th17 cells, rather than Th1 cells, play a key role in the development of autoimmune diseases (21, 27). Interferon-γ-producing Th17 cells, or so called Th17.1 cells, are described to be pathogenic in several autoimmune diseases and chronic inflammatory disorders, including CD (12, 13) and arthritis (14). In a previous study we clarified that specifically CCR6$^+$ Th17.1 cells rather than classical CCR6$^-$ Th1 cells are the key source for IFN-γ production in sarcoidosis BALF (22). Here, we found that in sarcoidosis MLN not only Th17.1 cells, but all CCR6$^+$ Th cell subsets, including Th17 and CCR6$^+$ DP cells were significantly enhanced compared with control MLN. Since Th17 and CCR6$^+$ DP cells can be precursor cells of Th17.1 cells, these data are consistent with the hypothesis that initial Th17-lineage subset differentiation occurs within sarcoidosis MLN (24).

Although Th17.1 cells were abundantly present within sarcoidosis affected-organs, we identified CCR6$^+$ DP cells as the subset with the highest fraction of proliferating cells. Furthermore, sarcoidosis CCR6$^+$ DP cells showed decreased co-inhibitory CTLA4 expression compared with controls, likely contributing to increased proliferative capacity. Previously, we have also observed a reduced CTLA4 expression in sarcoidosis MLN Th17 cells (25). Aberrant expression of co-inhibitory receptors, such as CTLA4 (25) and PD-1

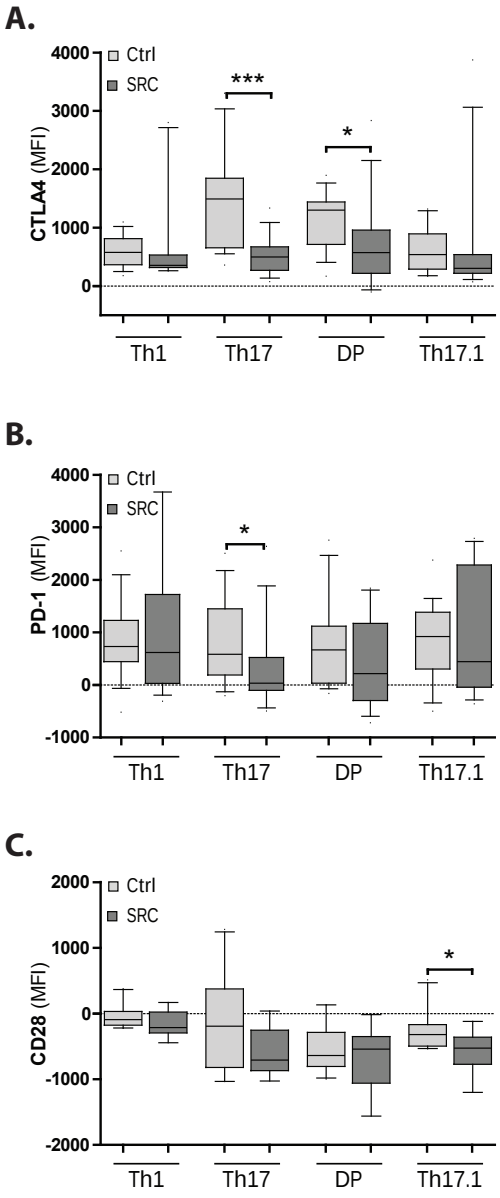

Figure 4. CCR6$^+$ DP cells show decreased CTLA4 expression. Expression of CTLA4, PD-1 and CD28 was determined *ex-vivo* in Th1, Th17, CCR6$^+$ DP and Th17.1 cells in control and sarcoidosis MLN. (*A*) Mean fluorescence intensity of CTLA4 (data are from 17 controls and 13 patients). (*B*) Mean fluorescence intensity of PD-1 (data are from 17 controls and 13 patients). (*C*) Mean fluorescence intensity of CD28 (data are from 10 controls and 9 patients). *Statistics:* Data are presented as boxplots and whiskers that show the 10-90 percentile of the data. Significance was determined using a Mann-Whitney U test, * $P < 0.05$ *** $P < 0.001$. Mean fluorescence intensity was standardized to average expression in healthy control PB cells. *Abbreviations:* CTLA4 = cytotoxic T lymphocyte antigen 4, PD-1 = programmed cell death protein 1, MFI = mean fluorescence intensity, DP = double-positive, Th = T helper, Ctrl = control, SRC = sarcoidosis.

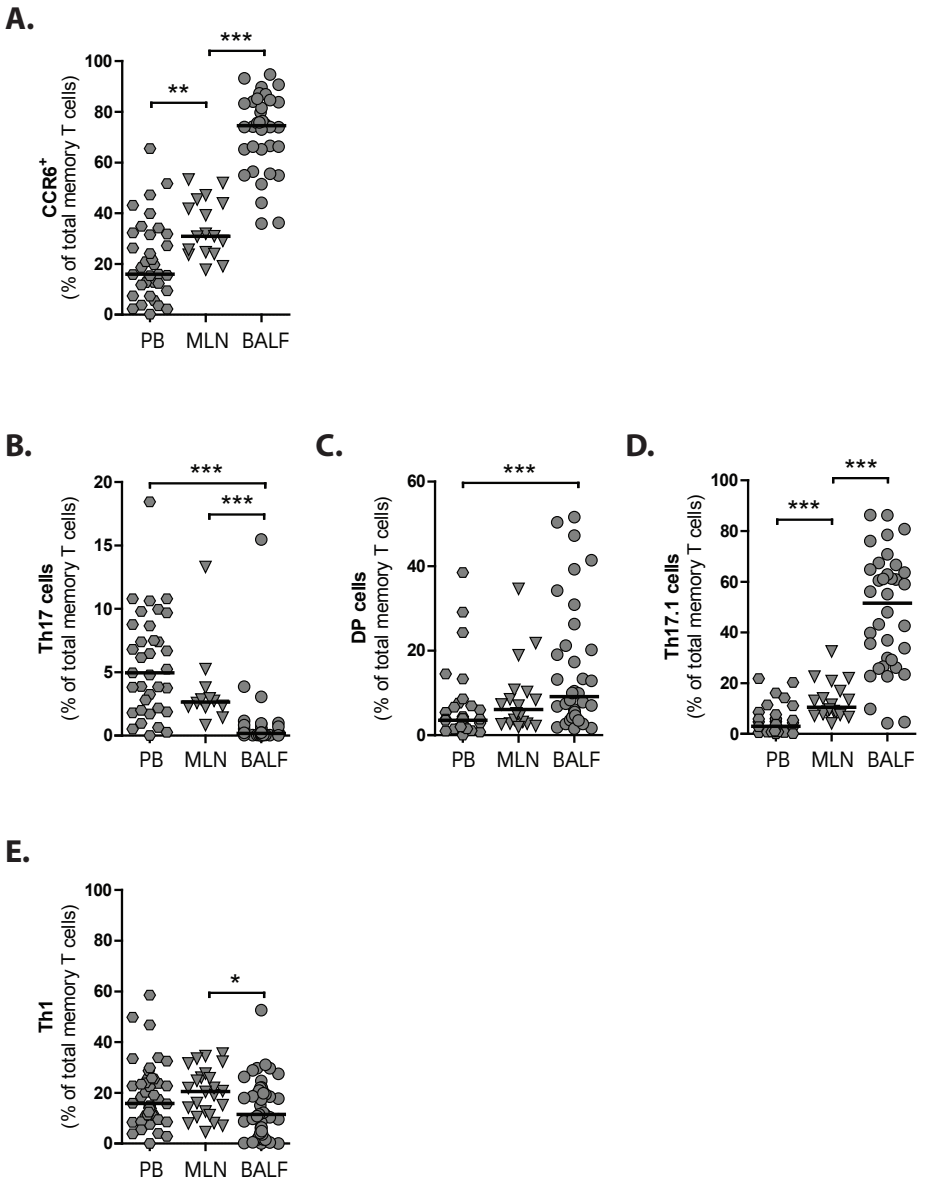

Figure 5. Increase of Th17.1 cell and decrease of Th17 cell proportions within sarcoidosis BALF compared with MLN. Proportions Th1, Th17, CCR6$^+$ DP and Th17.1 cells were determined in total memory CD4$^+$ T cells from sarcoidosis PB, MLN and BALF. (*A*) Proportions of CCR6$^+$ total memory T cells. (*B-D*) Proportions of Th17, CCR6$^+$ DP and Th17.1 cells of total memory CD4$^+$ T cells. (*E*) Proportions of Th1 cells of total memory CD4$^+$ T cells. *Statistics:* Horizontal lines indicate median values and significance was determined using a Mann-Whitney U test, * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. Data are from 35 (PB), 17 (MLN) and 36 (BALF) patients. *Abbreviations:* CCR = C-C chemokine receptor, PB = peripheral blood, MLN = mediastinal lymph nodes, BALF = broncho-alveolar lavage fluid, DP = double-positive, Th = T helper.

(Figure 4B) on specific T- cell subsets in sarcoidosis is very likely to be part of disease pathogenesis, since clinical treatment with anti-CTLA4/anti-PD-1 has been associated with development of sarcoid-like granulomas in MLN of cancer patients (25). Th17.1 cells are thought to be highly differentiated cells, which would be confirmed by their low CD28 expression. These findings are in agreement with Th17 plasticity towards a differentiated Th17.1 phenotype, where $CCR6^+$ DP cells represent an intermediate stage (20, 21, 26).

Comparing different involved immunological compartments in sarcoidosis provided further support for Th17 cell plasticity towards Th17.1 cells, as specifically Th17.1 cells were augmented in sarcoidosis lungs compared with MLN and PB (19). In joints of patients with JIA, Th17.1 cell fractions were also found increased compared with PB and shared clonal ancestry with Th17 cells (14).

Taken together, our data support a model in which Th17 cells are primed within sarcoidosis MLN and show plasticity towards pathogenic Th17.1 cells at chronically inflamed sites (14, 19) such as sarcoidosis lungs, but also granulomatous parts of the MLN.

High levels of cytokines, such as IL-12 or IL-23, which can induce a Th17 shift towards Th17.1 cells are found in sarcoidosis lungs (14, 17, 19, 28). Increased protein expression of IL-12 (p70 and p40) is found consistently within sarcoidosis lungs (4, 6, 29). Although IL-23p19 expression in sarcoid lungs remains uncertain, high expression of IL-12p40 compared with IL-12p70 may very well indicate elevated levels of IL-23 (29), since IL-12p40 is also a subunit of IL-23 (21). Notably, IL-23 transcription was enhanced in sarcoid skin lesion compared with controls (30), and serum amyloid A (SAA), an antigen that was speculated to contribute to development of chronic (pulmonary) sarcoidosis (31), has been described to increase IL-23 production by dendritic cells (32). Strikingly, BALF IL-12p40 protein levels are highest in patients developing chronic disease (33). Nevertheless, our data do not exclude the possibility of additional selective Th17.1 cell migration towards sarcoidosis lungs (19), attracted by high local co-expression of IP-10/CXCL10 (5) and CCL20 (34). To provide further evidence for enhanced Th17-Th17.1 plasticity in sarcoidosis, future studies could focus on T-cell receptor repertoire distribution within sarcoidosis $CCR6^+$ Th cell populations in different immunological compartments and should aim to identify indispensable contributors to sarcoidosis Th17 plasticity.

Interestingly, we found significantly higher Th17.1 cell proportions at time of diagnosis in lungs of patients who develop chronic disease compared to patients who underwent resolution. Together with our previous finding of increased BALF Th17.1 cells proportions in a population that included progressive patients who are on first-, second- and/or third-line therapy (22), these data strongly argue for a pathogenic role for Th17.1 cells in the development/progression of pulmonary sarcoidosis. Remarkably, two independent single-nucleotide polymorphisms (SNPs) within the *IL23R* gene locus were found to predispose for (chronic) sarcoidosis (35, 36) and IL-23R expression is

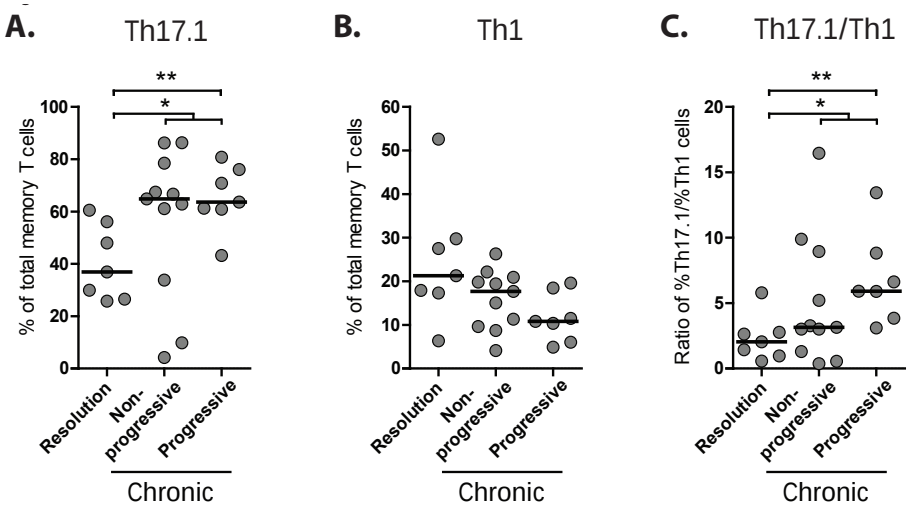

Figure 6. Higher BALF Th17.1 cell proportions in patients developing chronic disease than patients undergoing resolution. Disease course of a subgroup of patients (n=25) was determined 2 years after study inclusion and flow cytometric analyses. Resolution of disease (n=7) was defined by the absence of abnormalities on the chest X-ray and clinical symptoms. Patients with residual abnormalities on chest X-ray, but without need for treatment were designated as non-progressive chronic (n=11); and patients with need for treatment as progressive chronic (n=7). (*A-B*) Proportions Th17.1 and Th1 cells of total memory $CD4^+$ T cells at time of diagnosis. (*C*) Ratio of the proportions of Th17.1 versus Th1 cells. *Statistics:* Horizontal lines indicate median values and significance was determined using a Mann-Whitney U test, * $P < 0.05$ ** $P < 0.01$. *Abbreviations:* Th = T helper.

essential for conversion of Th17 cells towards pathogenic Th17.1 cells during chronic inflammation in mice (17, 37). The most recent genome-wide association study (GWAS) identified one SNP within the putative promoter region of the *IL23R* gene, which might influence IL-23R expression (36). The functional prediction and protein network analyses of the identified candidate susceptibility genes provided a prominent role for IL-23/Th17-signaling pathway in the genetic etiology of the sarcoidosis (36). Together with our data, this implies that genetic variations within the *IL23R* gene or other genes involved in the IL-23/Th17-signaling pathway may predispose for (chronic) sarcoidosis, by supporting the conversion of Th17 to Th17.1 cells or by stabilizing Th17.1 cells. Therefore, specific targeting of IL-23R signaling, e.g. by Tildrakizumab/Guselkumab (anti-IL-23p19) which was recently described to be successful in psoriasis (38), might be a promising new therapeutic approach in (chronic) sarcoidosis.

However, additional prospective studies investigating $CCR6^+$ Th17-lineage function, such as cytokine production, in sarcoidosis patients with a distinct disease prognosis are needed to address the functional complexity of Th17-lineage cells. This is highlighted by recently reported findings in Löfgren's disease (39), a subtype of acute sarcoidosis

whereby patients are unlikely to develop chronic disease. In these patients lung T-bet^{+}RORγT^{+} CD4^{+} T cells, co-expressing CCR6 and CXCR3, were associated with a favorable disease outcome (39). Löfgren BALF however contained higher levels of favorable Th17 cytokines such as IL-17A, IL-10 and IL-2, but lower levels of IFN-γ compared with non-Löfgren patients, likely contributing to the favorable prognosis in Löfgren patients (39).

In conclusion, this study is the first to show evidence that sarcoidosis Th17 cells are induced in MLN and supports a model in which they manifest plasticity towards Th17.1 cells in the lung granulomatous microenvironment, where they are particularly critical for development of chronic disease. Our findings provide a new perspective on sarcoidosis pathogenesis and the potential of new therapeutic targets.

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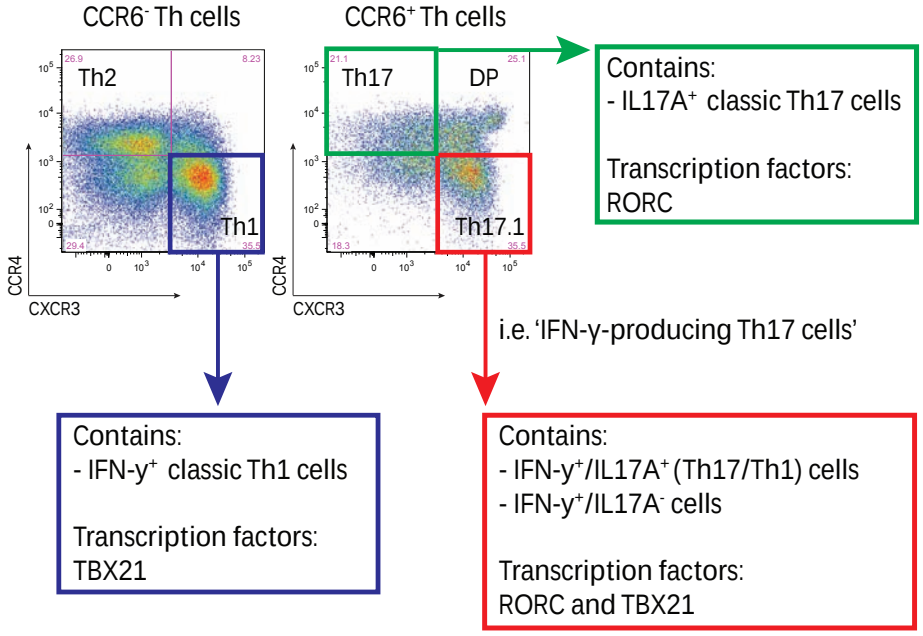
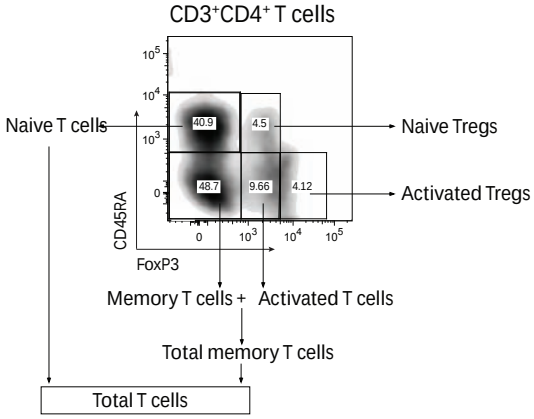
SUPPLEMENTARY MATERIAL

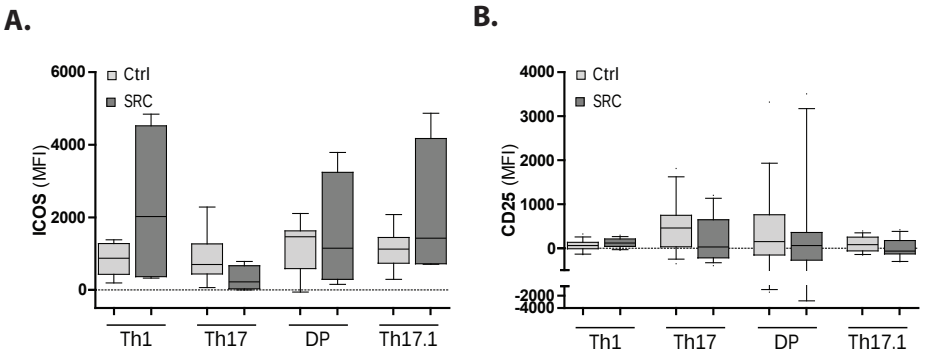
SUPPLEMENTARY FIGURES



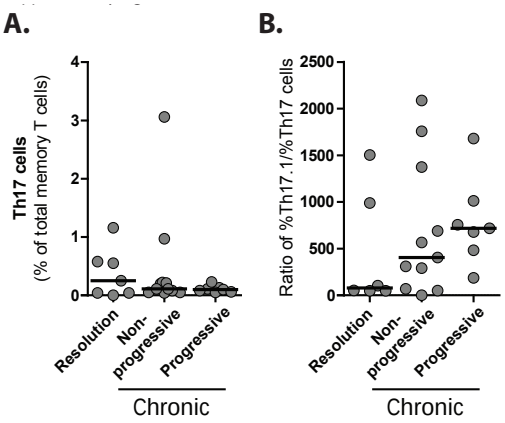
Supplementary Figure E1. Th cell subset classification according chemokine-receptor expression. Th cell subsets can be classified according to chemokine-receptor expression (1-5). $CCR6^-$ memory T cells subsets, including $CCR4^-CXCR3^+$ Th1 cells and $CCR4^+CXCR3^-$ Th2 cells; and $CCR6^+$ T cell subsets, which include $CCR4^+CXCR3^-$ Th17 cells, $CCR4^+CXCR3^+$ DP Th cells and $CCR4^-CXCR3^+$ Th17.1 cells. Th17.1 cells (i.e. IFN-γ-producing Th17 cells) contain both $IL\text{-}17A^+/IFN\text{-}\gamma^+$ (Th17/Th1) cells and IFN-γ-single-positive cells. $CCR6^+CCR4^+CXCR3^+$ DP cells are thought to reflect an intermediate Th17 and Th17.1 cell population (3, 4, 6). *Abbreviations:* CCR = C-C chemokine receptor, CXCR = CXC chemokine receptor, DP = double-positive, Th = T helper.



Supplementary Figure E2. Gating strategy to determine $CD3^+CD4^+$ T cell subsets. Five $CD3^+CD4^+$ T cell populations were characterized according to CD45RA and intracellular FoxP3 expression (7), i.e. $CD45RA^+FoxP3^-$ naïve T cells, $CD45RA^-FoxP3^-$ memory T cells, $CD45RA^-FoxP3^{int}$ activated (non-suppressive/-regulatory) T cells, $CD45RA^+FoxP3^{int}$ naïve regulatory T cells and $CD45RA^-FoxP3^{high}$ activated Tregs. The total memory T cell pool consists of memory T cells plus activated T cells. The total (non-suppressive/-regulatory) T cell pool consists of naïve T cells plus total memory T cells. *Abbreviations:* Treg = regulatory T cells.



Supplementary Figure E3. Expression of ICOS and CD25 in Th1, Th17, $CCR6^+$ DP and Th17.1 cells in control and sarcoidosis mediastinal lymph nodes. (*A*) Mean fluorescence intensity of ICOS (data are from 7 controls and 4 patients) (*B*) Mean fluorescence intensity of CD25 (data are from 15 controls and 13 patients). *Statistics:* Data are presented as boxplots and whiskers show the 10-90 percentile of the data. Mean fluorescence intensity was standardized to average expression in healthy control PB cells. *Abbreviations:* ICOS = Inducible T-cell costimulator, MFI = mean fluorescence intensity, DP = double-positive, Th = T helper, Ctrl = control, SRC = sarcoidosis.



Supplementary Figure E4. Quantification of Th subset ratios in various patient groups. Disease course of a subgroup (n=25) of patients was determined 2 years after study inclusion. Resolution of disease (n=7) was defined by the absence of abnormalities on the chest X-ray and clinical symptoms. Patients with residual abnormalities on chest X-ray, but without need for treatment were designated as non-progressive chronic (n=11); and patients with need for treatment as progressive chronic (n=7) (8). (*A*) Proportions Th17 cells of total memory $CD4^+$ T cells at time of diagnosis. (*B*) Ratio of proportions Th17.1 versus Th17 cells. *Statistics:* Data are presented as boxplots and whiskers that show the 10-90 percentile of the data. Significance was determined using a Mann-Whitney U test. *Abbreviations:* BALF = broncho-alveolar lavage fluid, Th = T helper, SRC = sarcoidosis.

Supplementary Table E1. Study subject characteristics

Subject characteristics	MLN Ctrl (n=22)	Sarcoidosis (n=55)
Median age (min-max)	47 (15-70)	43 (24-75)
Sex (Male/Female)	6/16	36/19
Scadding Stage (n (% of total))		
Stage 0		5 (9)[†]
Stage I		20 (36)
Stage II		23 (42)
Stage III		4 (7)
Unknown		3 (6)[††]
Diagnosis was assessed by (n (% of total))		
TBB-EBB		17 (31)
BALF CD4/CD8 > 3,5		8 (15)
E(B)US-FNA		26 (47)
Other (e.g. mediastinoscopy, other biopsy)		4 (7)
Extrathoracic involvement (n (% of total))		
No/Yes		21/34
Skin		8 (15)
Eyes		16 (29)
Articular[$]		22 (40)
Central nervous system		3 (6)
Other		6 (11)

[†] Three patients had Stage I and two patients had Stage II sarcoidosis, determined by CT scan. [††] Two patients had Stage I and one patient had Stage II sarcoidosis, determined by CT scan. [$] Self-reported articular involvement. *Abbreviations:* TBB: transbronchial biopsy, EBB: endobronchial biopsy, BALF: broncho-alveolar lavage fluid, E(B)US-FNA: Esophageal or endobronchial ultrasound guided fine-needle aspiration.

Supplementary Table E2. Overview antibodies

Company	Antigen	Fluor chrome	Clone
eBiosciences	CD3	APC-eFluor780	SK7
	CD4	AF700	OKT4
	FoxP3	PE	236A/E7
	CTLA4	PerCp-eFluor710	14D3
BD biosciences	CCR6	APC	11A9
	CXCR3	BV711	1C6/CXCR3
	Ki67	Pe-Cy7	B56
	CD28	BV605	CD28.2
	PD-1	BV786	EH12.1
	PD-1	BV711	EH12.1
	CD25	PE-Cy7	M-A251
	CD25	BV650	M-A251
	CD278 (ICOS)	BV650	DX29
R&D	CCR4	FITC	205410
Biolegend	CXCR3	BV421	G025H7
Invitrogen	CD45RA	PE-Texas Red	MEM-56

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CHAPTER 6

T-cell Immunology in Sarcoidosis: Disruption of a Delicate Balance between Helper and Regulatory T cells

'The true sign of intelligence is not knowledge but imagination'
Albert Einstein

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Caroline E. Broos, Rudi W. Hendriks, and Mirjam Kool.

ABSTRACT

Purpose of review

Although the etiology of sarcoidosis is not yet completely understood, immunological changes within the T-cell compartment are characteristic for an exaggerated antigen-driven immune response. In this review, we describe the most recent findings on T-cell subset responses and regulation in sarcoidosis. We discuss how future immunological research can advance the field to unravel pathobiological mechanisms of this intriguingly complex disease.

Recent findings

Research into the field of T-cell plasticity has recently challenged the long-held T helper type (Th1) paradigm in sarcoidosis and striking parallels with autoimmune disorders and common variable immunodeficiency were recognized. For instance, it was demonstrated that Th17.1 cells rather than Th1 cells are responsible for the exaggerated interferon (IFN)-γ production in pulmonary sarcoidosis. Furthermore, impaired regulatory T-cell (Treg) function and alterations within the expression of co-inhibitory receptors that control T-cell responses, such as PD-1, CTLA4 and BTNL2, raise new questions regarding T-cell regulation in pulmonary sarcoidosis.

Summary

It becomes increasingly clear that Th17(.1) cells and Tregs are key players in sarcoidosis T-cell immunology. New findings on plasticity and co-inhibitory receptor expression by these subsets help build a more comprehensive model for T-cell regulation in sarcoidosis and will finally shed light on the potential of new treatment modalities.

INTRODUCTION

Sarcoidosis is a complex systemic inflammatory disease of unknown etiology characterized by the presence of noncaseating granulomas in various organs, with pulmonary involvement in more than 90% of patients (1). In pulmonary sarcoidosis, granulomas are most commonly found in the alveolar septa, near lymphatic vessels and in lung-draining lymph nodes. Granulomas consist of a core of histiocytes, that is differentiated tissue macrophages and multinucleated giant cells, surrounded by lymphocytes, especially $CD4^+$ T helper (Th)-cells (2). In 30-50% of sarcoidosis cases, progression towards active chronic disease is seen with need for therapy (3).

Sarcoidosis etiology is incompletely understood, but immunological changes within the T-cell compartment are characteristic for an exaggerated antigen-driven immune response (4). Immunological changes include enhanced local expression of Th1/Th17 cytokines and chemokines, dysfunctional regulatory T-cell (Treg) responses, and oligoclonal expansion of Th cells consistent with chronic antigenic stimulation. Candidate T-cell targets may be pathogen-derived (e.g. mycobacterial 6-kDa early secreted antigenic target (ESAT-6) or catalase-peroxidase (KatG)) or auto-antigens (e.g. vimentin and zinc finger protein 688 (ZNF688)) (5, 6). Alternatively, pulmonary infections may initiate local pathogen-specific T-cell responses cross-reacting with sarcoidosis-associated autoantigens, referred to as molecular mimicry (7). Although it was traditionally believed that memory T cells can only be stimulated in an antigen-dependent manner, recently it became clear that they can also be activated by key cytokines without the need for T-cell receptor (TCR) stimulation (8, 9).

In this review, we describe recent findings on T-cell subset responses and regulation in sarcoidosis. It is well accepted that regulatory mechanisms within T cells and the balance between T-cell subsets is critical to maintain tolerance and prevent inappropriate activation. Moreover, emerging evidence for T-cell plasticity raise new questions within sarcoidosis etiology. We end with speculations on how future immunological research can advance the field to unravel pathobiological mechanisms of this intriguingly complex disease.

T-cell response in sarcoidosis

In sarcoid granulomas $CD4^+$ T-cells are abundantly present and play a key pathogenic role (2). Naive Th cells are primed by TCR stimulation through peptide/HLA-DR complex from an antigen-presenting cell (APC), whereby proliferation occurs before lineage specification begins. Concomitant with their activation, naive T cells undergo differentiation under the influence of cytokines, resulting in the induction of lineage-specific transcription factors, and epigenetic modifications at the cytokine loci, which then result in subset-specific cytokine production and lineage commitment. Consequently, different Th subsets have been identified based on their cytokine secretion: Th1 cells

mainly produce interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), Th2 cells interleukin-4 (IL-4), IL-5, and IL-13, and Th17 cells IL-17A and IL-22 (10). Furthermore, Tregs dampen inflammatory responses through cell-cell interaction and secretion of IL-10 and transforming growth factor beta (TFG-β). However, it has become increasingly clear that Th cells do not always commit to one lineage, but maintain flexibility to change subset type or to increase the range of cytokines that they produce (11). Additionally, differentiated Th cells can adopt mixed phenotypes, whereby stable hybrid cells co-express lineage-specific transcription factors and co-produce cytokines characteristic for two opposing differentiation programs (12-14). Discoveries in the field of T-cell plasticity have recently challenged long-held paradigms on T-cell responses in sarcoidosis. A schematic model including novel findings on T-cell responses in sarcoidosis is shown in Figure 1 and discussed in text below.

T helper cells

Sarcoidosis was thought to be a Th1-cell-driven disease, because of high expression of IFN-γ in Th cells and the presence of Th1-skewing cytokines IL-12 and IL-18 in granulomatous organs (15-17). Subsequently, next to IFN-γ-producing cells, the presence of IL-17-producing cells was recognized in sarcoidosis patients during active and progressive pulmonary disease (18-23). Although their fractions remained lower than IFN-y-producing cells, both lung and circulating Th cells from patients showed significantly increased IL-17 and IL-23 receptor (IL-23R) expression compared with controls (18-20). However, it became clear that stringent classification of Th lineages based on cytokine production is unsuitable, as cells were found that produce cytokines of both the Th1 and Th17 lineage. Significantly increased proportions of IL-17A$^+$/IFN-γ$^+$ double-producing memory Th cells were observed within sarcoidosis peripheral blood and broncho-alveolar lavage fluid (BALF) (18-21). In sarcoidosis BALF, the proportions of IL-17A$^+$/IFN-γ$^+$ Th cells also increased with Scadding stage (21). Intriguingly, IL-17A$^+$/IFN-γ$^+$ double-producers are described to be pathogenic in various human chronic auto-immune inflammatory disorders, such as Crohn's disease and rheumatoid arthritis (RA) (24, 25). In these diseases, considerable progress has been made concerning the origin of IL-17A$^+$/IFN-γ$^+$ double-producing Th cells (26). It is generally believed that they originate from IL-17-single-positive Th cells (Th17) which are subsequently exposed to Th1-skewing cytokines, such as IFN-γ and IL-12 (25, 27). They express the chemokine receptor 6 (CCR6), CD161, IL-23R and RAR-related orphan receptor (ROR)γt, as well as IL-12Rβ2 and T-box transcription factor (TBX21)(T-bet). Furthermore, IL-17A$^+$/IFN-γ$^+$ Th cells can develop into IFN-γ-single-positive Th cells. Thus, two types of IFN-γ-producing Th cells exist, i.e. CCR6$^-$ Th1 cells and CCR6$^+$ Th17-derived cells. The latter are referred to as Th17.1 cells based on CCR6 expression (Th17-lineage) in combination with CXC-motif

chemokine receptor 3 (CXCR3) (Th1-lineage) (24-26, 28, 29). Accordingly, expression of chemokine receptors can be used to define the origin of IFN-γ-producing cells.

In collaboration with the group of dr. Koth, we have recently demonstrated that Th17.1 cells rather than Th1 cells are responsible for the exaggerated IFN-γ production in pulmonary sarcoidosis (Figure 1), challenging the Th1 paradigm (30). In two independent sarcoidosis patient cohorts, including newly-diagnosed patients versus progressive patients, lung Th17.1-cell proportions were strikingly increased compared with controls (30). These findings raise new questions in the field, such as the role of these cells in disease development and treatment. For instance, both IFN-γ and IL-17 are essential in granuloma formation and multinucleated giant cell fusion (31-33) (Figure 1). Human Th17.1, and not Th17 cells, have a pathogenic signature and highly express the multi-drug resistance type 1 membrane transporter (MDR1) (24). $MDR1^+$ Th17.1 cells are refractory to glucocorticoids that are used to treat clinical (auto)-immune disease and sarcoidosis (24). Although the pathogenic signature of human Th17.1 cells is characterized by high expression of *IL23R*, *CSF2* (granulocyte-macrophage colony-stimulating factor (GM-CSF)), *CCL20*, and *TBX21* (T-bet) (24, 34), classical Th17 cells display a non-pathogenic Th17-signature that includes *IL10, MAF,* and *AHR* transcription (24)(Figure 1). Interestingly, while IL-17 can influence the expression of proinflammatory cytokines such as TNF-α (35) and is able to mature granuloma formation (31), it was also found that HLA-$DR3.01^+$ Löfgren patients have an increase in IL-17 in BALF compared with non-Löfgren sarcoidosis patients (22), suggesting that classical Th17 cells in sarcoidosis can exhibit a non-pathogenic phenotype. In Crohn's disease, the non-pathogenic role for Th17 cells was recognized, as clinical trials with blocking IL-17A or IL-17RA showed exacerbation of symptoms in some cases (36, 37). Lastly, IL-23R expression is essential for conversion of Th17 cells towards pathogenic Th17.1 cells during chronic inflammation (38), and functional prediction and protein network analyses identified a prominent role for IL-23/Th17-signaling pathway in the genetic etiology of sarcoidosis (39, 40).

Although involvement of IFN-γ-producing Th17 cells point to a striking parallel between sarcoidosis and RA/Crohn's disease, it cannot be taken as evidence for an autoimmune etiology of sarcoidosis, as pathogens such as *Candida albicans* can also induce IFN-γ-producing Th17-cells (41). However, one of the outstanding questions that remain in sarcoidosis is why specific T cells are driven into the Th17 lineage. In this context, it is interesting that IFN-γ can promote B-cell-activating factor (BAFF) production by monocytes (42) and strikingly BAFF is needed for APC-driven Th17-cell differentiation *in vitro* in RA (43). This has relevance for sarcoidosis, since elevated serum BAFF levels are observed (42, 44). It is currently unclear whether increased BAFF levels in sarcoidosis affect B-cell selection or activity. However, peri-granuloma localization of B cells and plasma cells (45) may indicate the involvement of antigen-specific B cells.

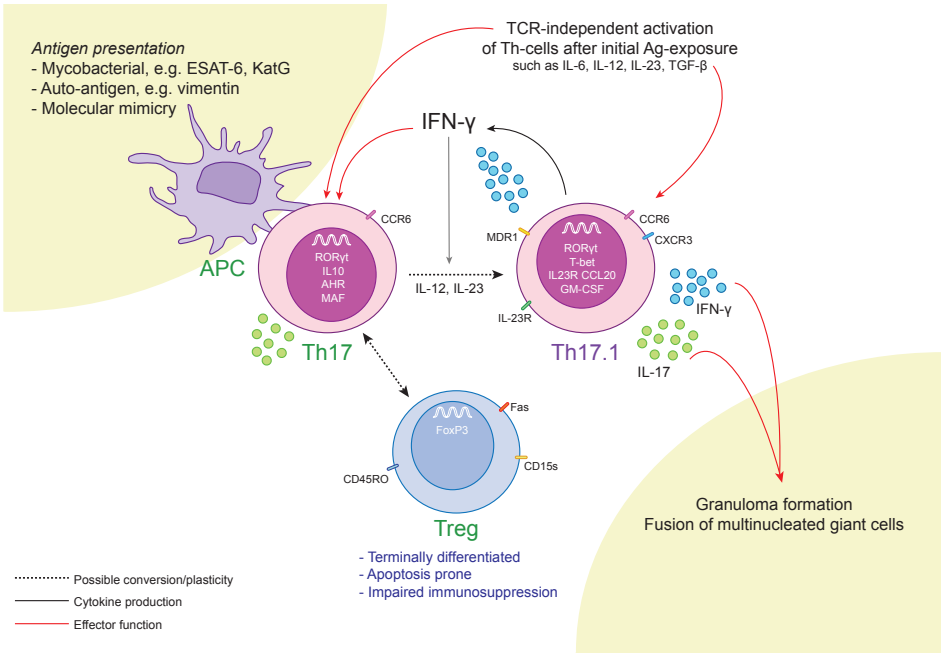

Figure 1. T-cell activation in pulmonary sarcoidosis. T-cell activation is initiated by antigenic peptide presentation in HLA-DR on APCs recognized by their responsive TCR on T cells. Possible antigens in sarcoidosis are thought to be of mycobacterial origin, auto-antigens or even molecular mimicry of pathogens. Memory Th cells might also be activated through a TCR-independent manner, for example, by key cytokines. IL-17-producing Th cells have been recognized in the pathobiological response in sarcoidosis and come in different flavors, being either benign (Th17 cells, expressing also IL-10, AHR and MAF) or pathogenic (Th17.1 cells, expressing next to Th17-lineage markers such as RORγt and CCR6, markers from the Th1-lineage, such as T-bet, CXCR3). Th17.1 cells are also capable of producing a sarcoidosis key cytokine, IFN-γ, either solely or together with IL-17. IFN-γ, next to IL-12 and IL-23, also promotes the conversion of Th17 cells into Th17.1 cells. IFN-γ and IL-17 both promote granuloma formation and fusion of multinucleated giant cells. Tregs in sarcoidosis have a terminally differentiated phenotype shown by CD45RO and CD15s expression, are apoptosis-prone probably because of increased Fas expression, and consequently have impaired immunosuppressive function. *Abbreviations:* APC = antigen presenting cell, CCR6 = chemokine receptor 6, CXCR3 = C-X-C chemokine receptor 3, FoxP3 = forkhead box P3, GM-CSF = granulocyte-macrophage colony-stimulating factor, HLA = human leukocyte antigen, IFN-γ = interferon gamma, IL = interleukin, MDR1 = multi drug resistant receptor 1, RORγt = RAR-related orphan receptor, T-bet = T-box transcription factor TBX21, TCR = T-cell receptor, TGF-β = transforming growth factor beta, Th = T helper, Treg = regulatory T-cell.

Regulatory T cells

Tregs are a subpopulation of T cells that control the immune system, maintain tolerance to self-antigens, and abrogate autoimmune disease. A role for Tregs in the pathogenesis of sarcoidosis has long been suggested (46, 47), although there is inconsistency in the reported findings. Depending on the affected-organ and time of evaluation (e.g. at time of diagnosis or during progressive/chronic disease), either an increase or decrease in their proportions in BALF or peripheral blood have been shown (2, 48, 49). However, decreased immunosuppressive function of Tregs from sarcoidosis patients has

consistently been observed (47, 49-53). On the basis of CD45RA and forkhead box P3 (FoxP3) expression Tregs can be classified into naïve ($CD45RA^{+}FoxP3^{int}$) and activated ($CD45RA^{-}FoxP3^{high}$), both having the capacity to reduce T-cell responses (54). In sarcoidosis patients, especially activated Tregs are increased in peripheral blood (54) and express CD15s (sialyl Lewis x), a ligand for the selectins CD62E and CD62P (55). CD15s expression is highly specific for activated, terminally differentiated, and the most suppressive $FoxP3^{high}$ Tregs (55). Particularly in sarcoidosis patients, these $CD15s^{+}FoxP3^{+}$ Tregs were reported to be increased, however whether their immunosuppressive capacity was altered was not investigated (55). As $CD15s^{+}$ Tregs are terminally differentiated, it is plausible that they are 'exhausted' in sarcoidosis, as suggested before (50). Interestingly, Tregs from sarcoidosis patients were recently found to express more CD45RO and Fas, both pointing towards an activated and apoptotic-prone phenotype (49). Since sarcoidosis Tregs indeed displayed impaired survival compared with control Tregs, this may contribute to their insufficient immunosuppressive capacities (49). Loss of T-cell regulation in sarcoidosis can also be caused by a reduced number and exhaustion of invariant natural killer T cells, an additional regulatory lymphocyte (56-58).

Early developmental programs of induced Tregs and Th17-cells show that these cell types are intimately linked. This is reflected by the lineage-specific transcription factors FoxP3 and RORγt, which are both downstream of TGFβ signaling (59). Differentiation of Tregs is not static and Tregs can differentiate into Th17 cells (60). Even IL-17-secreting Tregs are observed and they exert suppressive capacities *in vitro* but lose this capacity upon stimulation with the pro-inflammatory cytokines IL-1β and IL-6 (61). As both loss of Treg function and induction of Th17 cells are observed in sarcoidosis, and since pathogenic conversion of $FoxP3^{+}$ T cells into Th17 cells has been found in autoimmune arthritis (62), it would be worthwhile to investigate the plasticity of Tregs towards Th17 cells and the presence of IL-17-producing Tregs in sarcoidosis.

T-cell regulation in sarcoidosis

Little is known about underlying mechanisms leading to the above described exaggerated Th-cell response and Treg impairment in sarcoidosis (Figure 1). Both regulatory mechanisms within T cells (e.g. by checkpoint inhibitors) and the balance between proinflammatory and anti-inflammatory T cells are critical to maintain tolerance and prevent inappropriate activation.

Co-stimulatory and co-inhibitory receptors on T cells play a pivotal immune-regulatory role, as they determine the functional outcome of TCR signaling and consequently T-cell proliferation and cytokine production. Alterations in the expression of co-inhibitory receptors that keep T-cell responses in check, such as cytotoxic T-lymphocyte antigen 4 (CTLA4), butyrophilin-like 2 (BTNL2) and programmed death-1 (PD-1), have recently been described in the context of sarcoidosis.

CTLA4 is an important suppressor of T-cell–mediated immune responses by competing with CD28 for the shared ligands CD80 and CD86 expressed on APCs. As CTLA4 has a higher affinity for CD80/86 relative to CD28, CTLA4 restrains CD28 signaling (63). Therefore, while CD28 engagement promotes T-cell proliferation and differentiation, CTLA4 engagement attenuates T-cell responses. A possible role of CTLA4 in sarcoidosis pathogenesis linked to the Th17 subset was first suggested by the striking phenomenon of 'biotherapy-induced sarcoidosis'. In several case reports, cancer patients receiving CTLA4 checkpoint inhibition (e.g. ipilimumab treatment) developed granulomas (64, 65). Interestingly, in patients receiving CTLA4 blockade the numbers and proportions of circulating IL-17–producing Th cells increased (66). Although it remains unclear whether Treg proportions are affected, CTLA4 blockade is known to impair Treg-mediated suppression (63). As in sarcoidosis abnormal Th17-cell and Treg responses are observed (2), we analyzed CTLA4 expression and intriguingly found that specifically Th17 cells and Tregs in sarcoidosis mediastinal lymph nodes (MLN) displayed decreased CTLA4 expression (64). Solely Th17-cell proportions in sarcoidosis MLN were increased, whereas Treg proportions remained unaffected (64). Decreased CTLA4 expression is likely to impair Treg immuno-regulatory function and specifically induce proinflammatory Th17 differentiation and proliferation, and thus to cause 'double trouble' (Figure 2) (64). Hereby, it is conceivable that reduced CTLA4 expression aggravates underlying or pre-existent disease, or alternatively that it is driving the disease.

BTNL2 negatively regulates T-cell activation independently of CD28 and CTLA4. It promotes de-novo FoxP3 expression during T-cell activation and the development of suppressive Tregs (67). A splicing variant in the *BTNL2* gene has been associated with sarcoidosis (68) and a recent meta-analysis showed that *BTNL2* rs2076530 polymorphism even contributes to the risk of sarcoidosis (69). Strikingly, *CTLA4* and *BTNL2* gene mutations have also been identified in families with common variable immunodeficiency (CVID), including patients with granulomatous lung disease that shows some striking resemblance with sarcoidosis (70, 71). Additionally, coding and loss-of-function variants in *BTNL2* have been associated with susceptibility to other immune related disorders such as RA and Crohn's disease (72, 73), in which Th17 cells and Th17.1 cells are also important drivers of disease. Whether BTNL2 expression, like CTLA4 (74), is important in Th17 lineage commitment needs further investigation. However, in the view of the closely developmental programs of Tregs and Th17 cells and the role of BTNL2 in driving Tregs (67), its importance in Th17-cell development is plausible.

Thus, hypothetically, due to decreased CTLA4/BTNL2 expression, decreased suppression of T(h17)-cell function occurs, leading to on-going inflammation (64). However, during active disease, increased proportions of PD-1-expressing cells have been found within Th cells in sarcoidosis peripheral blood and BALF compared with healthy controls (75). Sarcoidosis IL-17-producing Th cells highly expressed PD-1 (75); however, it remains

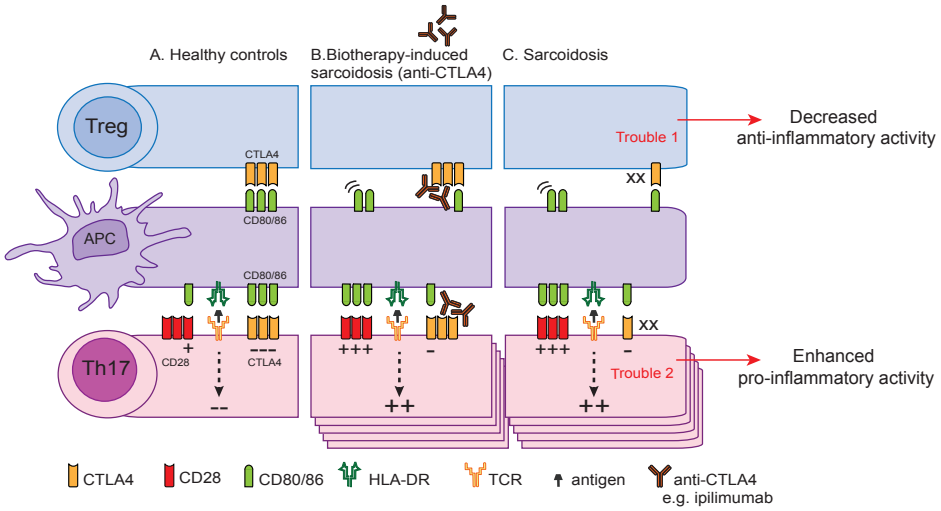

Figure 2. Effect of reduced CTLA4 expression on Tregs and Th17 cells in sarcoidosis mediastinal lymph nodes. (*A*) In healthy controls Tregs highly express CTLA4 and indirectly inhibit T-cell activation via CD80/86 engagement on APCs, leading to restricted CD80/86 ligand availability on APCs, inducing tolerance (63). Also Th17 cells highly express CTLA4, which competes with CD28-ligand binding. Therefore, Th17 cells are sensitive towards CTLA4 co-inhibition, leading to suppressed Th17 differentiation, activation and/or proliferation in healthy individuals (74). (*B*) Biotherapy-induced sarcoidosis by anti-CTLA4 treatment (e.g. ipilimumab) leads to enhanced CD80/86 ligand availability on APCs and decreased competition with CD28-ligand binding on Th17-cells. This can increase Th17-cell differentiation and activation. (*C*) In sarcoidosis patients, Tregs show decreased CTLA4 protein expression leading to enhanced CD80/86 ligand availability on APCs (Trouble 1) and thus a reduced anti-inflammatory response. Additionally, sarcoidosis Th17 cells show decreased CTLA4 protein expression, leading to impaired competition with CD28-ligand binding on Th17 cells and therefore enhanced Th17-cell differentiation and activation (Trouble 2). *Abbreviations:* APC = antigen presenting cell, CTLA4 = cytotoxic T lymphocyte antigen 4, HLA = human leukocyte antigen, TCR = T-cell receptor, Th = T helper, Treg = regulatory T-cell. Figure adapted from Broos *et al.* (64). Reprinted with permission of the American Thoracic Society.

unknown whether PD-1 expression is increased compared with BALF control IL-17$^+$ T cells. Paradoxically to these findings, recently sarcoid-like granulomas were observed in MLN and skin in a patient receiving anti-PD1 treatment (65). Strikingly, in a small sarcoidosis patients cohort a trend towards decreased PD-1 expression was observed on Th17 cells in MLN (64). Co-inhibitory receptor expression is likely differentially regulated in sarcoidosis-affected organs, since expression of co-inhibitory receptors depends on the strength of T-cell stimulation, mediated by TCR and CD28 expression (76). These data highlight the need for further studies to clarify co-inhibitory receptor expression of distinct Th subsets in different sarcoidosis-affected organs. Also, clarification of conflicting results regarding the co-stimulatory receptor CD28 in sarcoidosis (51, 64, 77, 78) is warranted in order to devise a comprehensive model explaining impaired immune regulation in sarcoidosis. Whereas an impaired T-cell function in response to TCR stimu-

lation was previously found in active sarcoidosis (53), but spontaneous IL-2 and IFN-γ production is highly increased compared with controls; Th cells in sarcoidosis may have become less dependent on CD28 signaling as is observed in chronic beryllium disease, resulting in dysregulated PD-1 and CTLA4 pathways (79, 80).

Finally, co-inhibitory and co-stimulatory receptors differentially affect function and proportions of distinct T-cell subsets, which is critical to maintain tolerance and prevent inappropriate activation. Interestingly, it was recently found that Treg/Th17 cell ratios are higher in peripheral blood of sarcoidosis patients who remained stable after corticosteroid withdrawal compared with relapsing patients (81). Furthermore, during relapse treatment with corticosteroids and methotrexate the proportions of Tregs increased, Th17 cells decreased, and Th1 cells and Th2 cells remained stable (81). Since Treg/Th17 ratios also inversely correlated with serum angiotensin converting enzyme levels, and positively correlated with lung function parameters, these data, together with above-described recent findings in sarcoid T-cell immunology, highlight that Th17 and Treg cells are key players in sarcoidosis pathogenesis (Figure 1 and Figure 2).

CONCLUSION

These recent immunological findings in pulmonary sarcoidosis highlight the need for an in-depth characterization of T-cell subsets. Experimental findings in other chronic inflammatory diseases, such as RA and Crohn's disease, can help determine the most relevant markers to identify T-cell subsets. Once T-cell subsets can be accurately characterized and isolated, T-cell function and regulation in sarcoidosis warrant careful reviewing, since current results sometimes remain contradictory, such as the role of checkpoint inhibitors that we have highlighted above.

*Future directions: how can we get 'from the core to a cure'?***:** Because sarcoidosis is characterized by an immune paradox, that is, a hyperactive immune system at site of inflammation and anergy in peripheral blood (47), it is important to clarify Th-cell function and regulation in different sarcoid-affected organs in order to build a comprehensive model of the in-vivo behavior and dynamics of different Th-cell subsets in sarcoidosis. This will also help on-going endeavors to identify candidate antigens in sarcoidosis. If key players of (chronic) sarcoidosis are well defined, sophisticated studies that have already recently taken place in Löfgren patients (5), can help analyze the structure of the TCR and use prediction models to identify new candidate antigens. For example, in-depth characterization of TCR specificity in HLA-DR3.01^{+} Löfgren patients has led to identification of new peptides, such as vimentin, as potentially involved antigens (5). While the search for the Holy Grail, that is, the antigen, remains essential, it is obvious that T-cell plasticity warrants further attention and research, because determinants of T-cell plasticity may turn out to be promising new therapeutical targets in (chronic) sarcoidosis.

Note added in proof: During the publication processes of this review, a new paper of specific interest was published by Kaiser *et al.* (82), showing that also in BALF of Löfgren patients T-bet^{+}RORγt^{+} CD4^{+} T cells, co-expressing CCR6 and CXCR3, were significantly increased. Interestingly, cytokine production was more heterogeneous, with higher IL-17A, IL-10 and IL-2, but lower IFN-γ, which may contribute to a favorable prognosis in Löfgren patients.

Key points

- Th17(.1) cells and Tregs are key players in sarcoidosis T-cell immunology
- Th17.1 cells, rather than Th1 cells, are the main source of IFN-γ in sarcoidosis-affected organs.
- T-cell plasticity likely occurs in granulomatous tissues of sarcoidosis patients
- Sarcoidosis likely advances due to an imbalance between co-inhibitory and co-stimulatory molecules on Th17 cells and Tregs.
- Clarification of Th-cell characteristics, function and regulation in different sarcoid-affected organs can help build a comprehensive model of the in-vivo behavior and dynamics of different Th-cell subsets in sarcoidosis.

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PART 2

Optimization of prednisone treatment in pulmonary sarcoidosis

CHAPTER 7

Little Association between Cumulative Prednisone Dose and FVC Change in Newly-Treated Pulmonary Sarcoidosis

'Conformity is the jailer of freedom and the enemy of growth'
John F. Kennedy

Submitted for publication

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ABSTRACT

Background: Prednisone is used as first-line therapy for pulmonary sarcoidosis. What dosing strategy has the best balance between effect and side-effects is largely unknown. Therefore, we analyzed change in forced vital capacity (FVC) and weight during different prednisone doses used in daily practice for treatment naïve pulmonary sarcoidosis patients.

Methods: Multilevel models were used to describe FVC and weight change over time. Correlations were calculated using linear regression models.

Results: Fifty-four patients were included. FVC changed over time ($P < 0.001$), with an average increase of 9.6% predicted (95% CI: 7.2 to 12.1) at 12 months. Weight changed significantly over time ($P < 0.001$), with an average increase of 4.3 kg (95% CI: 3.0 to 5.6) at 12 months. There was little correlation between prednisone dose and FVC change at 3, 12 and 24 months, while weight increase correlated significantly with cumulative prednisone dose at 24 months. In patients treated with a high cumulative prednisone dose, baseline FVC was on average lower ($P = 0.001$) compared to low dose treated patients, while no significant differences were observed in need for second/third-line therapy or number of exacerbations. A strategy leading to a low cumulative dose at 12 months was defined by rapid dose tapering to 10 mg/day within 3.5 months.

Conclusions: These results suggest that prednisone therapy aimed at improving or preserving FVC in newly treated pulmonary sarcoidosis can often be reduced in dose, and highlight the need for prospective trials carefully monitoring treatment efficacy of a lower dose treatment regimen, characterized by early dose tapering.

INTRODUCTION

Sarcoidosis is a multisystem, granulomatous disease affecting the lungs in 90% of the cases (1). Approximately 30-50% of the patients develop progressive and debilitating disease with need for therapy (1, 2). The recommended first-line therapy for pulmonary sarcoidosis is prednisone (1, 3-5). Although prednisone treatment in pulmonary sarcoidosis is reported to induce short-term benefits on clinical symptoms and inflammation, it remains unclear whether the therapy modifies long-term progression of the disease (4). Therapy should therefore primarily be aimed at symptom relief, inflammation control to prevent (further) organ damage and improving patient's quality of life while avoiding unnecessary side effects (6-9).

A meta-analysis of corticosteroids for pulmonary sarcoidosis concluded that evidence for the best corticosteroid treatment strategy is lacking (4). The suggested initial prednisone dose varies between 20-40 milligrams (mg) (1) or 0,5 mg per kilogram (kg) (5) per day for 1-3 months. Subsequently, prednisone dose should be tapered to a maintenance dose of 5-10 mg/day, which is commonly continued for 6-12 months before discontinuation (1, 5). As these guidelines include a broad range in recommended prednisone doses, variation in treatment regimen in clinical practice is suspected, varying from low- to high-dose treated patients. Prolonged high dose corticosteroid therapy is associated with numerous side effects, including weight gain, diabetes and osteoporosis (7, 10).

What dosing strategy has the best balance between effect and side-effects is largely unknown. Therefore, in this study we aimed to evaluate treatment effect on forced vital capacity (FVC) (effect) and weight (side-effect) of different prednisone doses used in daily practice.

METHODS

Study design

This study is a multicenter retrospective study, performed in one academic sarcoidosis referral center (Erasmus MC) and three regional training hospitals (Franciscus Gasthuis, Ikazia hospital and Amphia hospital) in the Netherlands. Medical records were reviewed for demographic and diagnostic data, organ involvement, radiographic Scadding stage, prednisone dose, weight, pulmonary function parameters and exacerbations. An exacerbation was determined as an increase in daily prednisone dose from 5-10 mg/day maintenance dose to ≥ 20 mg/day. Data that was available up to 5 years following therapy initiation were collected per patient. Individual prednisone regimens were analyzed. Weight was collected from pulmonary function records.

Patients

Treatment naïve sarcoidosis patients, in whom prednisone therapy was started for a pulmonary indication between January 2000 and December 2013, were included in this study. Patients were identified by screening medical records of patients that were in hospital-specific databases that track sarcoidosis diagnosed patients over time. Patients were included when they met standard criteria for diagnosis of the disease (1), were treatment naive and had a treatment indication for pulmonary sarcoidosis as determined by the treating physician. Patients were excluded when they were: 1. primarily treated for a non-pulmonary indication; 2. treated solely with methotrexate for a pulmonary indication; and 3. when there were less than two hospital visits documented.

Ethical requirements

Formal consultation with the Medical Ethical Committee of the Erasmus MC learnt that, under the Dutch act for medical research involving human subjects (Wet Medisch Onderzoek), approval of this study by the Medical Ethical Committee was not required. The local institution review board of all participating centers approved with registration number MEC-2014-089.

Statistical analysis

The comparison of means of continuous variables were tested with the student *t* test, the categorical variables were tested with the X^2 or the Fisher exact test. Absolute changes in FVC and weight were used as outcome in multilevel models. Log time appeared to be an adequate transformation to enter as fixed factor in the model, while patient functioned as a random intercept. For different points in time we analyzed the correlation between cumulative prednisone dose and the absolute change in outcome between start of treatment and the specific time point. Correlations were calculated using linear regression models.

FVC is shown as mean percent (%) predicted (± standard deviation (SD)) or as mean absolute change of % predicted (including a 95% confidence interval (CI)) compared to baseline. Weight is shown as mean kg (± SD) or as mean absolute change (including 95% CI) in kg compared to baseline. Prednisone dose is shown as mean daily dose in milligrams (mg) or as cumulative dose in mg.

For pulmonary function tests the European Community for Steel and Coal 1993 prediction equations were used in all hospitals.

Statistical analyses were performed using SPSS (version 21.0.0.1) and R software (version 3.2.2). Figures were created with R software. A *P*-value < 0.05 was considered as statistically significant.

RESULTS

Patients

A total of 54 treatment naïve sarcoidosis patients that were initiated on prednisone therapy for a pulmonary indication were identified for this study; two third from regional hospitals and one third from an academic referral center for sarcoidosis. Mean % predicted FVC at start of prednisone treatment was 83.4 ± 20.4, and mean % predicted diffusing capacity of the lung for carbon monoxide (DLCO) (corrected for hemoglobin levels) was 69.9 ± 25.0 (Table 1). Average weight was 79.2 ± 19.2 and average body mass index (BMI) was 26.6 ± 5.9 kg/m^2; 48.1% of the patients were men and 71.1% of the patients had Scadding stage II sarcoidosis. Additional baseline characteristics are shown in Table 1.

FVC change upon prednisone treatment

Mean initial prednisone dose was 32.6 ± 8.7 mg (Table 1). On average, prednisone was tapered to 10 mg/day at approximately 6 months (Figure 1 and Figure E2). Mean FVC (% predicted) change over time was calculated using a multilevel model that incorporated regression lines of all 54 individual patients (Figure E1). FVC changed significantly over time ($P < 0.001$), with an average increase of 7.4 % predicted (95% CI: 5.5 to 9.3) at 3 months and 9.6 % predicted (95% CI: 7.2 to 12.1) at 12 months (Figure 1). At 24 months, an average increase of 10.8 (95% CI: 8.0 to 13.5) was observed (Figure 1), which was largely preserved in patients with data available at 3 and 5 years following therapy initiation (Figure E1A). Interestingly, the major increase in FVC occurred within 1-3 months of treatment (Figure 1).

FVC change and prednisone dose

The association between change in FVC and prednisone dose used, was determined. Although all correlations were weakly positive, no significant correlation was found

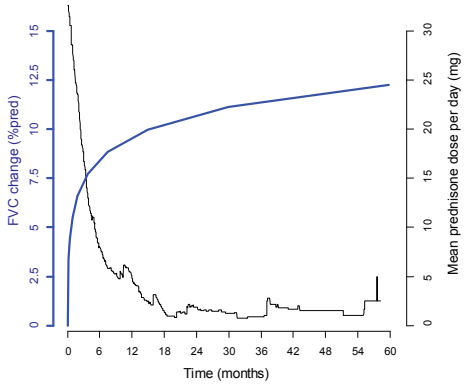

Figure 1. FVC change after initiation of prednisone therapy. FVC change over time during prednisone treatment. Percent predicted FVC change compared to baseline (absolute change; blue line) and the mean prednisone dose given per day in milligrams of patients with available data is shown. *Statistics:* Data is calculated using a multilevel model with FVC over time as outcome. *Abbreviations:* FVC = forced vital capacity, mg = milligrams.

Table 1. Baseline characteristics of study cohort

Characteristics	Patients n=54
Age	44 ± 13
Gender	
Male	26 (48,1)
Female	28 (51,9)
Treated in	
Academic center	18 (33,3)
Training hospital	36 (66,7)
Race/Ethnicity	
Unknown	17
White	23 (62,2)
Black	7 (18,9)
Hispanic	7 (18,9)
BMI in kg/m2 (n=52)	26,6 (± 5,9)
Weight in kg (n=52)	79,2 (± 19,2)
Smoking	
Unknown	8
Never	21 (45,7)
Current	7 (15,2)
Former	18 (39,1)
Scadding stage	
Unknown	9
I	8 (17,8)
II	32 (71,1)
III	5 (11,1)
Extra pulmonary organ involvement*	
No/Yes	26/ 28
Skin	7 (13)
Eyes	14 (25,9)
Joints	15 (27,8)
Other (e.g. liver, heart, neural)	9 (16,7)
Prednisone dose at start	32,6 ± 8,7
>10 ≤20 mg	6 (11,2)
>20 ≤30 mg	32 (59,3)
>30 ≤40 mg	12 (22,2)
>40 ≤50 mg	2 (3,7)
>50 ≤60 mg	2 (3,7)
Pulmonary function tests	
% predicted FVC (n=42)	83,4 ± 20,4
% predicted DLCO (n=34)	69,9± 25,0

Categorical data is presented as No. (% of total patients with available data) and continuous data as mean ± SD of patients with available data (No. of patients with available data is presented behind the continuous variable). *Abbreviations:* CNS: central nervous system, DLCOc: diffusing capacity of lung for carbon monoxide (corrected for hemoglobin levels), FVC: forced vital capacity, kg: kilograms, mg: milligrams. *As assessed and described by the treating physician.

between FVC change and cumulative prednisone dose used in the short-term (3 (Figure 2A), 6 (not shown) and 9 months (not shown)) and long-term (12 (Figure 2B) and 24 months (Figure 2C)). At 12 months, the correlation became almost zero after correction for start FVC (correlation: -0.07, $P = 0.76$). Similarly, no significant correlation was found between the average daily prednisone dose used in the first 3 months and FVC change (Figure E3).

Weight change upon prednisone treatment

To gain insight in side effects occurring during prednisone treatment, we captured weight change over time using a multilevel model (Figure E4). Weight changed significantly over time ($P < 0.001$), with an average increase of 3.3 kg (95% CI: 2.3 to 4.3) at 3 months and 4.3 kg (95% CI: 3.0 to 5.6) at 12 months (Figure 3). After tapering of the prednisone dose to a mean of ≤ 5 mg/day, weight remained increased compared with

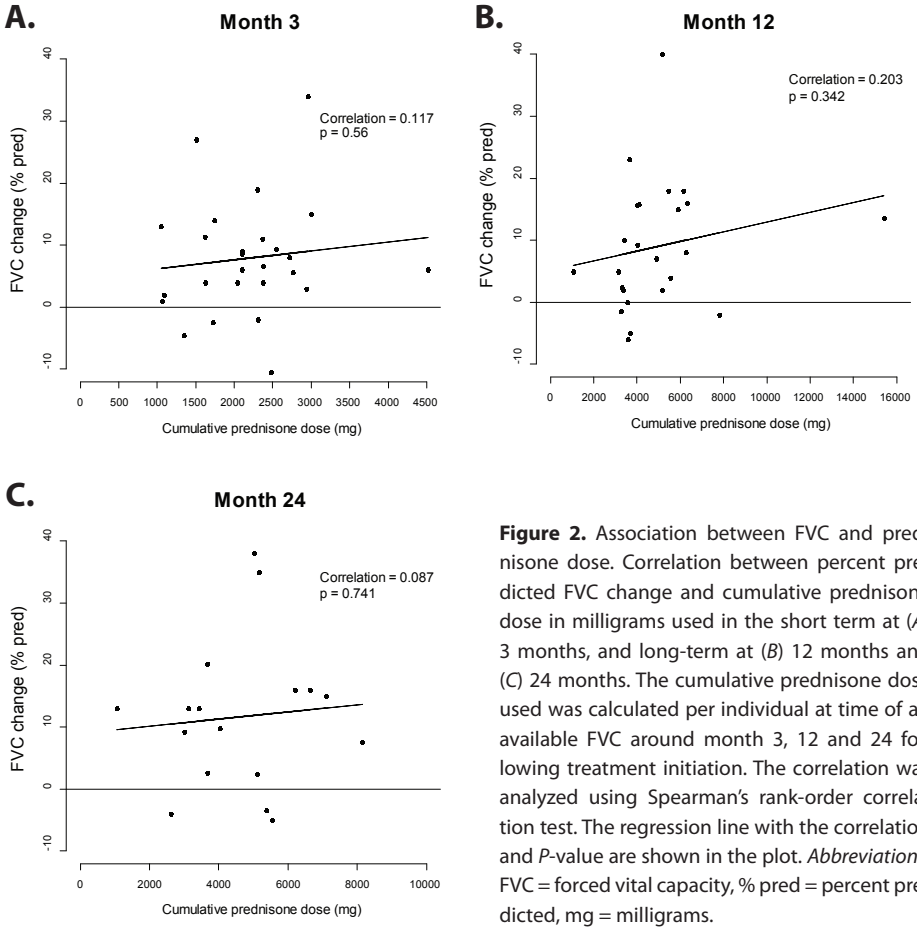

Figure 2. Association between FVC and prednisone dose. Correlation between percent predicted FVC change and cumulative prednisone dose in milligrams used in the short term at (*A*) 3 months, and long-term at (*B*) 12 months and (*C*) 24 months. The cumulative prednisone dose used was calculated per individual at time of an available FVC around month 3, 12 and 24 following treatment initiation. The correlation was analyzed using Spearman's rank-order correlation test. The regression line with the correlation and *P*-value are shown in the plot. *Abbreviations:* FVC = forced vital capacity, % pred = percent predicted, mg = milligrams.

baseline with an average of 4.8 kg (95% CI: 3.3 to 6.3) at 24 months, which persisted up to 5 years in patients with data available (Figure 3 and Figure E2). Similar to FVC change (Figure 1), the largest proportion of weight gain occurred during the first 1-3 months of treatment (Figure 3).

Weight change and prednisone dose

We aimed to determine whether weight change depends on total prednisone dose used. No significant correlation was found between weight change and cumulative prednisone dose used in the short-term at 3 (Figure 4A), 6 (not shown) and 9 months (not shown). Also, no significant correlation was found between daily prednisone dose used and weight change in the first 3 months (Figure E5). However, an association between cumulative prednisone dose and weight change became apparent and significant in the long-term at 12 (Figure 4B) and 24 months (Figure 4C), respectively.

Prednisone regimen analysis

In order to gain insight in clinical practices that are associated with reduced prednisone use, we aimed to determine the average daily prednisone dose regimen administered to patients that received a relatively low versus a higher cumulative dose at 12 months. Therefore, patients with available data on prednisone therapy at 12 months (n=39) (Figure E2) were divided into a low dose and a high dose group. The median cumulative dose given at 12 months was 4000 mg (range 1050 to 13090 mg) thus patients who had received less than 4000 mg prednisone were assigned to the low dose group (n=20), and patients who had received 4000 mg prednisone or more were assigned to the high dose group (n=19).

The average start dose of prednisone was lower in the low dose treated group than the high dose treated group, but this did not reach statistical significance (30.3 ± 9.1 mg versus 36.1 ± 9.8 mg; $P = 0.06$) (Table 2). The treatment strategy leading to a lower

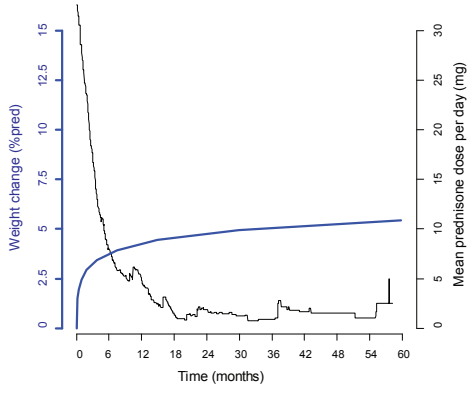

Figure 3. Weight change after initiation of prednisone therapy. Weight change in kilograms over time during prednisone treatment compared to baseline (absolute change; blue line) and the mean prednisone dose given per day in milligrams of patients with available data. The blue line is calculated using a multilevel model with weight over time as outcome. *Abbreviations:* kg = kilograms, mg = milligrams.

cumulative dose prednisone at 12 months seemed mainly characterized by earlier dose tapering, i.e. less than 10 mg/day at 3.5 months, whereas the high cumulative dose group was characterized by later dose tapering i.e. less than 10 mg/day at 6.9 months (Figure 5).

Low versus high dose prednisone

We examined baseline and therapy-response characteristics in order to determine factors associated with a treatment strategy leading to a high or low cumulative dose.

No significant differences were observed between the high and low dose groups in baseline characteristics such as race/ethnicity (P = 0.76), Scadding stage (P = 1.00), weight (P = 0.83) or BMI (P = 0.87) (Table 2). Furthermore, no statistical significant difference was observed in the presence of extra pulmonary organ involvement (P = 0.15), but the low dose treated group tended to have more skin involvement (including 2 patients with erythema nodosum) than the high dose treated group (20% versus 0%, P = 0.11),

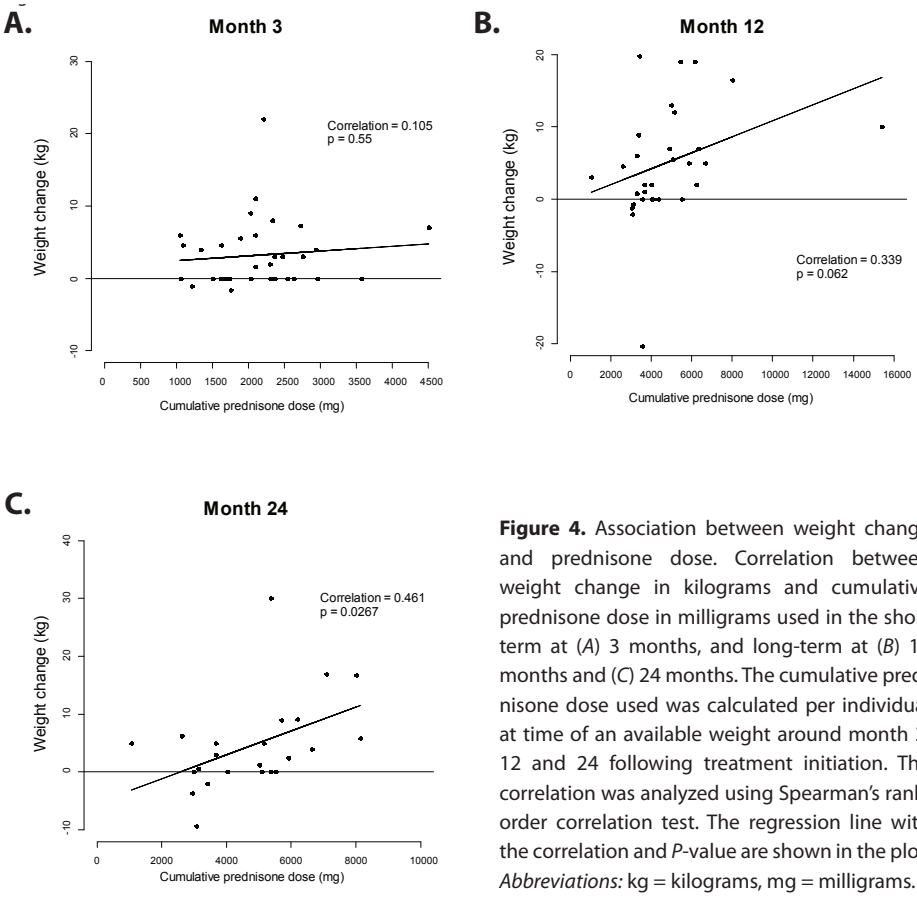

Figure 4. Association between weight change and prednisone dose. Correlation between weight change in kilograms and cumulative prednisone dose in milligrams used in the short term at (*A*) 3 months, and long-term at (*B*) 12 months and (*C*) 24 months. The cumulative prednisone dose used was calculated per individual at time of an available weight around month 3, 12 and 24 following treatment initiation. The correlation was analyzed using Spearman's rank-order correlation test. The regression line with the correlation and *P*-value are shown in the plot. *Abbreviations:* kg = kilograms, mg = milligrams.

Table 2. Baseline characteristics of low versus high dose treated patients

	Low dose N=20	**High dose N=19**	***P*-value**
Age	46 ± 14,0 (20)	43 ± 12,5 (19)	0.54
Gender			
Male	9 (45)	9 (47,4)	0.88[c]
Female	11 (55)	10 (52,6)	
Treated in			
Academic center	3 (15)	6 (31,6)	0.27[f]
Regional hospital	17 (85)	13 (68,4)	
Race/Ethnicity			
Unknown	8	5	
White	8 (66,7)	7 (50,0)	0.76[f]
Black	2 (16,7)	4 (28,6)	
Hispanic	2 (16,7)	3 (21,4)	
BMI in kg/m^2	27,5 ± 5,1 (18)	27,9 ± 6,8 (19)	0.87
Weight in kg	82,1 ±17,6 (18)	83,6 ± 23,1 (19)	0.83
Smoking			
Unknown	4	1	
Never	7 (43,8)	9 (50,0)	0.34[f]
Current	4 (25,0)	1 (5,6)	
Former	5 (31,2)	8 (44,4)	
Scadding stage			
Unknown	1	3	
I	4 (21,1)	3 (18,8)	1.00[f]
II	13 (68,4)	11 (68,8)	
III	2 (10,5)	2 (12,5)	
Extra pulmonary organ involvement*	12 (60)	7 (36,8)	0.15[c]
Skin	4[$] (20)	0 (0)	0.11[f]
Eye	4 (20)	5 (26,3)	0.72[f]
Joint	6 (30)	7 (36,8)	0.65[c]
Other	1 (5)	4[$$] (21,1)	0.18[f]
Multiple organs	2 (10)	7 (36,8)	0.07[f]
Prednisone dose use at start	30,3 ± 9,1	36,1 ± 9,8	0.06
>10 ≤20 mg	5 (25)	0 (0)	0.08[f]
>20 ≤30 mg	9 (45)	12 (63,2)	
>30 ≤40 mg	5 (25)	4 (21,1)	
>40 ≤50 mg	1 (5)	1 (5,3)	
>50 ≤60 mg	0	2 (10,5)	
PFT, % predicted			
FVC	96,5 ±20,8 (16)	70,7 ±15,7 (14)	0.001
DLCOc	76,9 ± 20,6 (13)	64,7 ± 18,2 (13)	0.12

Categorical data is presented as No. (%) and continuous data as mean ± SD (No. of patients with available data and were included in the analyses). *Statistics:* We tested the continuous outcomes for residuals and they had a normal distribution. Significance between continuous data was analyzed with an unpaired student t-test. Categorical data was analyzed with a X^2 (=[c]) or the Fisher (=[f]) exact test. *P* values are given for significant differences between the two groups. *Abbreviations:* NS = not significant; PFT: Pulmonary function tests. DLCOc: diffusing capacity of lung for carbon monoxide (corrected for hemoglobin levels). FVC: forced vital capacity, kg: kilograms, mg: milligrams. [$] Two patients had erythema nodosum. [$$] One patient had heart involvement, one had parotids involved and two patients had peripheral neurological involvement. *As assessed and described by the treating physician.

while high dose treated patients seemed to have more involvement of other organs, such as cardiac and neurological involvement (5% versus 21.1%, *P* = 0.18). Also, a trend was observed that high dose treated patients had more often multiple organs affected (10% versus 36.8%, *P* = 0.07) (Table 2). Interestingly, the baseline percent predicted FVC was significantly lower in the high dose treated group (70.7 ± 15.7) than the low dose treated group (96.5 ± 20.8) (*P* = 0.001) (Table 2). When we looked into therapy-response characteristics, no significant differences were however observed between the two groups in the number of patients that experienced an exacerbation during tapering (*P* = 0.66) or patients that received additional second or third line drugs (*P* = 0.34) (Table 3).

DISCUSSION

It currently remains unknown what dosing strategy for first-line prednisone treatment in pulmonary sarcoidosis has the best balance between effect and side effect. In this study, we aimed to determine treatment outcome of different prednisone regiments used in current clinical practice for newly treated pulmonary sarcoidosis. No strong correlation was found between prednisone dose used and FVC change at different time points, while weight increased significantly more in patient receiving a higher cumulative prednisone dose in the long-term. These data suggest that a treatment strategy leading to a lower cumulative dose prednisone in the long term has the potential to be equally effective in treating pulmonary sarcoidosis patients as a higher dose regimen, while reducing side-effects.

In this study, we gained more insight in prednisone therapy-induced effects. Serial FVC is currently the best end-point to monitor pulmonary sarcoidosis patients' response to therapy (11). We observed an increase of approximately 10% predicted FVC after prednisone treatment initiation. Strikingly, the major part of the FVC change seems to occur within the first 1-3 months of therapy, as has been described in exacerbation patients (12). The FVC increase observed after prednisone initiation is likely a clinical significant therapy effect (11), although we cannot formally discount the notion that this improvement is in part due to the natural history of the disease. Importantly, no clear

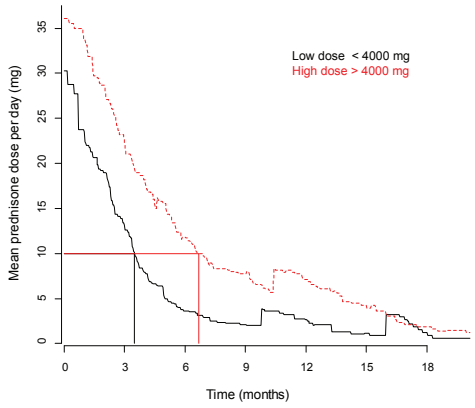

Figure 5. Prednisone regimen analysis. The mean prednisone dose given per day in milligrams (mg) to patient who had received a low (< 4000 mg; n=20) or high (≥ 4000 mg; n=19) cumulative dose prednisone at 12 months. Patients who received a low or high cumulative prednisone dose at 12 months were treated with more than 10 mg/day up to 3.5 or up to 6.9 months, respectively.

correlation was found between average daily or cumulative prednisone dose given and FVC change.

Weight gain is a well-known side effect of prednisone therapy (10). In this study, we found a correlation between weight increase and cumulative dose used, specifically in the long term. A treatment strategy leading to a lower cumulative dose at 12 months was characterized by early dose tapering, i.e. to less than 10 mg/day at 3.5 months, while the strategy leading to a higher dose was characterized by prolonged use of more than 10 mg/day up to 6.9 months. Prolonged prednisone therapy for pulmonary sarcoidosis at a dose of more than 10 mg a day was reported to induce significant more side-effects than a lower dose (13), which is now further supported by the results of this study. These data highlight that early dose tapering can be essential in reducing cumulative prednisone dose and hazardous side-effect such as weight gain.

Taken together, our data suggest that a lower prednisone dose increases and/or maintains FVC similar to a higher dose, thus meeting an important therapy objective, i.e. preserving organ function, while avoiding side effects (6).

Table 3. Response to therapy of low versus high dose treated patients

	Low dose N=20	**High dose N=19**	***P*-value**
Exacerbation			
No	18 (90)	16 (84,2)	0.66
Yes	2 (10)	3 (15,8)	
Additional 2nd or 3th line drugs			
No	19 (95)	16 (84,2)	0.34
Yes	1 (5)	3 (15,8)	

Data are presented as No. (%) or mean ± SD. Categorical data was analyses with the Fisher exact test. *P* values are given for significant differences between the two groups. *Abbreviations:* NS = not significant; FVC = forced vital capacity, kg: kilograms.

There are a number of limitations to our study. Our study included a small number (n=7) of black patients, thus it remains to be determined whether these patients, who more often suffers from a severe chronic form of sarcoidosis, responds identically to first-line prednisone therapy. While a number of characteristics such as radiographic Scadding stage and BMI were similar, baseline FVC was significantly lower in patients treated with a higher cumulative prednisone dose. Also these patients tended to have features associated with severe disease, such as multiple organ involvement more often. We did not observe a clear correlation between cumulative prednisone dose and FVC change; when correcting for FVC at baseline the weak non-significant association between cumulative prednisone dose and FVC change at 12 months ($r = 0.203$) was no longer found ($r = -0.07$). Furthermore, high dose treated patients did not more often experience an exacerbation during tapering or receive additional second or third line drugs than low dose treated patients. Nevertheless, the retrospective design of our study does not allow for firm conclusion on whether these patients may have benefitted from a lower dose. Other factors may have been taken into account by the treating physicians when determining a treatment strategy, such as change in symptoms, DLCO, radiological features or extra-thoracic organ involvement (11, 14, 15).

In conclusion, we did not find a clear association between prednisone dose and FVC change in newly treated pulmonary sarcoidosis patients, while weight gain positively correlated with cumulative prednisone dose used in the long term. These results support that prednisone therapy that is mainly aimed at maintaining and/or improving FVC in pulmonary sarcoidosis may be dose-reduced in current clinical practice by early dose tapering and highlight the need for prospective clinical trials on treatment strategies that carefully balance effects-side-effects.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY FIGURES

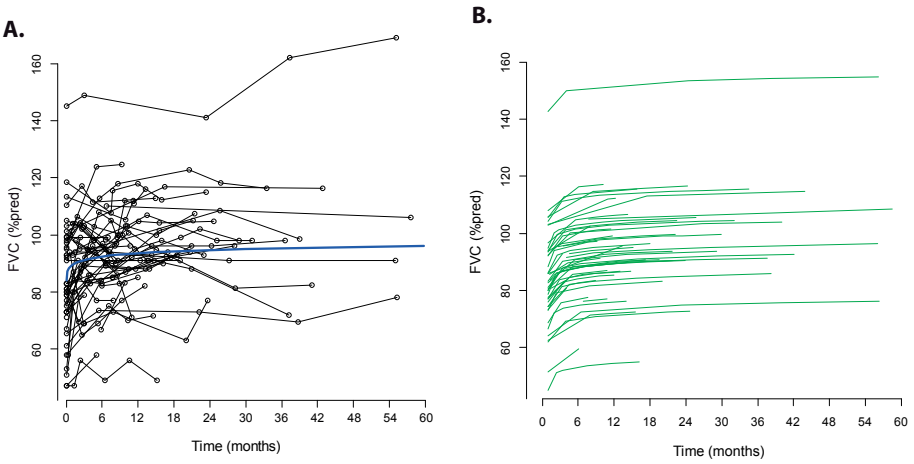

Figure E1. Mean FVC change over time was calculated using a multilevel model that incorporated regression lines of all 54 individual patients. (*A*) All individual FVC observations per patient, including the calculated multilevel regression fitted values (blue) for the median patient. (*B*) Individual regression lines of percent predicted FVC change over time. *Statistics:* Data is calculated using a multilevel model with FVC over time as outcome. *Abbreviations:* FVC = forced vital capacity, % pred = percent predicted.

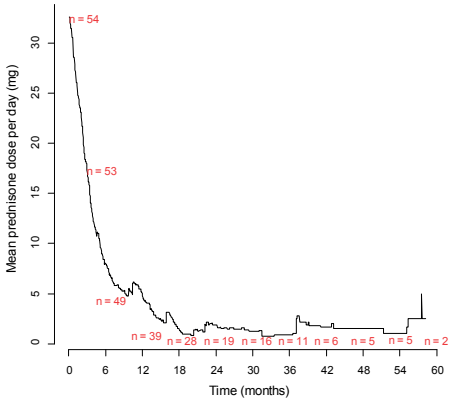

Figure E2. The mean prednisone dose given per day in milligrams of patients with available data. The total number of patients with available data (in red text) is shown at baseline, month 3, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, respectively. *Abbreviations:* mg = milligrams.

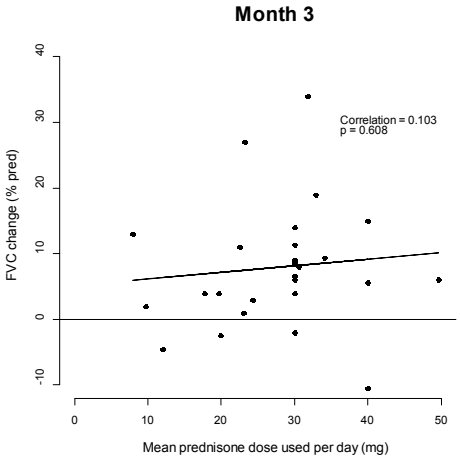

Figure E3. Correlation between percent predicted FVC change and mean prednisone dose used per day in milligrams over 3 months. The correlation was analysed using Spearman's rank-order correlation test. The regression line with the correlation and *P*-value are shown in the plot. *Abbreviations:* FVC = forced vital capacity.

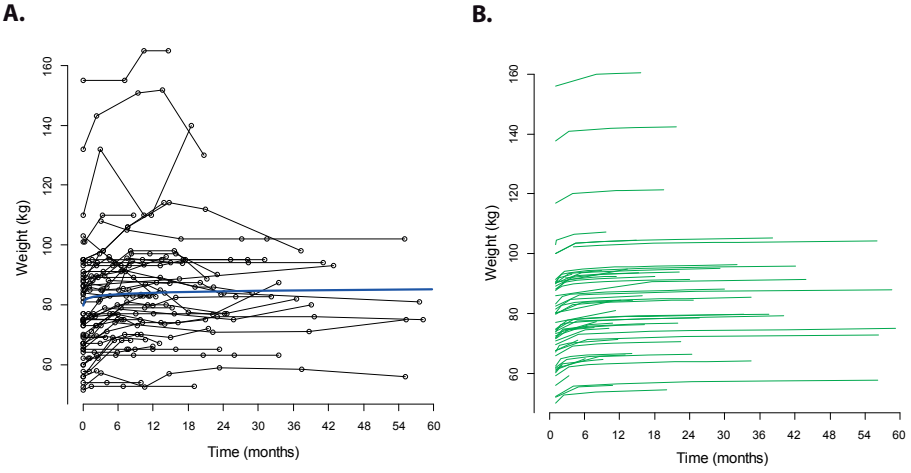

Figure E4. Mean weight change over time was calculated using a multilevel model that incorporated regression lines of all 54 individual patients. (*A*) All individual weight observations per patient, including the calculated multilevel regression fitted values (blue) for the median patient. (*B*) Individual regression lines of weight change over time. *Statistics:* Data is calculated using a multilevel model with weight over time as outcome. *Abbreviations:* kg = kilograms.

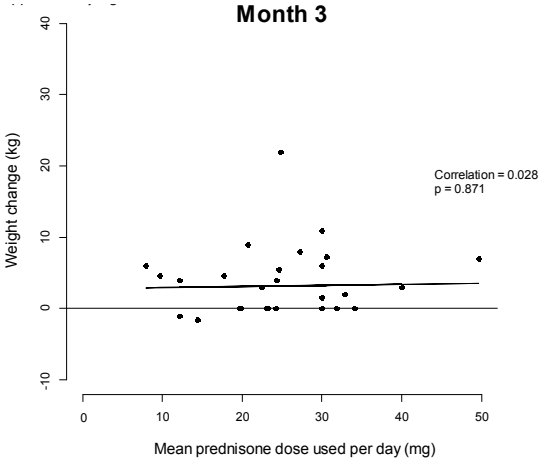

Figure E5. Correlation between weight change in kilograms and mean prednisone dose used per day in milligrams over 3 months. The correlation was analysed using Spearman's rank-order correlation test. The regression line with the correlation and *P*-value are shown in the plot. *Abbreviations:* kg = kilograms, mg = milligrams.

CHAPTER 8

Daily Home Spirometry to Detect Early Steroid Treatment Effects in Newly-Treated Pulmonary Sarcoidosis

'Instruction does much, but encouragement does everything'
Johann Wolfgang von Goethe

Manuscript in preparation

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ABSTRACT

Background: Optimization of first-line prednisone therapy for sarcoidosis is urgently needed, since side effects can be severe and long-term benefits are debated. Prospective data on the early response towards prednisone treatment is lacking. We initiated a prospective study to evaluate the early lung function response to prednisone treatment and tapering, using daily home spirometry.

Methods: Treatment naïve sarcoidosis patients in whom prednisone therapy was about to be initiated for a pulmonary indication were eligible. Prednisone treatment was standardized for the first three months (mo) of treatment. Forced vital capacity (FVC) was monitored daily at home using a hand-held spirometer and in hospital at baseline, 1-mo and 3-mo. Symptom scores were filled out weekly. FVC is shown as mean percent (%) predicted (± SD).

Results: Eighteen patients performed daily home spirometry. In-hospital measurements showed a significant change in FVC of 12.3 (±9.8) at month 1 ($P < 0.001$) and a smaller change between month 1 and 3 (3.8 (±6.9), $P = 0.045$). A similar pattern was observed in ACE and sIL-2R, and symptom scores (MRC, SGRQ and FAS). Interestingly, home-monitoring revealed that home spirometry of FVC correlated with in hospital measurements (Pearson correlation: 0.97 ($P < 0.001$)) and that already at 17 days (95% confidence interval (CI): 16 to 18) after prednisone initiation 90% of the total FVC increase was reached, whereas a plateau in FVC improvement was already reached at 23 days (95% CI: 20 to 28). MRC and FAS decreased significantly within 2 weeks following treatment initiation.

Conclusion: These results show that in newly-treated sarcoidosis patients most of the prednisone effect on FVC, fatigue and dyspnea symptoms is reached within approximately 2-3 weeks. Timely and precise measurement of pulmonary function and symptoms using daily home spirometry can help individualize first-line prednisone therapy in pulmonary sarcoidosis patients, aiming at early dose tapering and side-effect reduction.

INTRODUCTION

Sarcoidosis is characterized by granuloma formation upon an unknown cause, often affecting mediastinal lymph nodes and lungs (1). The immune response observed in sarcoidosis-affected organs is suggestive for an exaggerated immune response upon chronic antigenic stimulation (2). Although granulomas resolve spontaneously in the majority of the cases, in 30-50% of the sarcoidosis patients lung granulomas remain chronically present and may cause burdensome symptoms, such as dyspnea, chest pain and cough, that warrant therapy (3).

Since the etiology of the disease remains unknown, sarcoidosis treatment is not curative, but characterized by immune suppression. Prednisone is the main stay of sarcoidosis treatment, as it is known to induce symptom relieve, improve the chest X-ray and increase forced vital capacity (FVC) in the short-term (4-6). However, since the long term benefit of prednisone therapy is debated (e.g. slowing down progression or development of fibrosis), the treatment regimen should mainly be aimed at symptom control, inflammation control to prevent (further) organ damage, and improving patient's quality of life (3, 4, 6, 7).

The optimal treatment time and dose remain unknown (1, 6, 8), thus clinical guidelines include a broad range, leading to variability of doses used in clinical practice (*Broos et al., submitted*). Prolonged high dose prednisone therapy, in particular at higher doses, is associated with burdensome side-effects (3, 9)(*Broos et al., submitted*). These data highlight that prednisone treatment optimization is warranted, and that early dose tapering has the potential to reduce side-effects.

Gaining insight in the early treatment response can help determine when tapering could be initiated. Two retrospective studies in either exacerbation or newly-treated sarcoidosis patients found that the major change in FVC upon treatment occurred within 1 month, and remained stable up to 3-12 months during tapering (10)(*Broos et al., submitted*). An older, small, prospective study monitored (mostly newly-treated) patients regularly with in-hospital lung function equipment during the first 2 months of treatment and found a steep increase in FVC already within the first 10 to 21 days, and remaining stable up to 2 months (11).

However, a prospective study monitoring patient-administered lung function on a daily basis also including clinical symptoms is lacking, and can help gain further insight in the early treatment response towards prednisone in sarcoidosis. Therefore, we have initiated a prospective, observational study to evaluate early FVC changes during prednisone treatment and tapering in newly-treated sarcoidosis patients, using daily home spirometry.

METHODS

Study design

This study was a multicenter, prospective and observational study in order to determine early treatment responses in newly-treated pulmonary sarcoidosis patients (Trial registration number in the Dutch National Trial Register (http://www.trialregister.nl/trialreg): NTR4328 (Acronym: STEPS study). Six hospitals in the Netherlands participated, of which 1 academic referral center for sarcoidosis (Erasmus MC (Rotterdam)) and 5 training-hospitals (Franciscus Gasthuis (Rotterdam), Ikazia hospital (Rotterdam), Amphia hospital (Breda), Haaglanden Medical Center (The Hague) and Zuyderland Medical Center Heerlen (Heerlen)).

Study subjects

Treatment naïve sarcoidosis patients in whom prednisone therapy was about to be initiated for a pulmonary indication were eligible for this study. Disease diagnosis was established according to criteria recognized by the joint statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) (1). At screening for eligibility patients had to further meet the following criteria: an FVC of ≤ 80% predicted and/or FVC had declined ≥ 10% predicted (absolute decline) within the previous 12 months. Potential subjects who met any of the following criteria were excluded from participation: previous immunosuppressive treatment for sarcoidosis (e.g. prednisone, methotrexate, anti-TNF); use of systemic immunosuppressive therapy within the previous 3 months for another disease than sarcoidosis; contra-indications for steroids; co-morbidities of other conditions that could influence pulmonary function, such as chronic obstructive pulmonary disorder (COPD), active asthma (diagnosis established according to GINA guidelines), pulmonary malignancies, fibrotic disease, pregnancy or morbid obesity (body mass index (BMI) ≥ 35 kg/m^2).

Treatment policies

Study patients were treated according to current guidelines (1, 8). Prednisone regimen was standardized for the first three months according to a consensus that was reached between all participating pulmonary physicians. Accordingly, patients were intended to be treated with the following prednisone regimen: 4 weeks 40 milligrams (mg)/day, 2 weeks 30 mg/day, 2 weeks 20 mg/day, 2 weeks 15 mg/day, 2 weeks 10 mg/day. At any time the treating physician was allowed to deviate from this consensus regimen, if the clinical situation so demanded.

In-hospital monitoring

Routine in-hospital clinical monitoring with lung function and laboratory tests was performed at baseline, 1 and 3 months following treatment initiation. Besides these, patients were asked to fill out symptom- and quality of life-related questionnaires, including the St. George Respiratory Questionnaire (SGRQ), Short Form Health Survey (SF-36), King's Sarcoidosis Questionnaire (KSQ), the Medical Research Council Dyspnea (MRC) scale and Fatigue Assessment Scale (FAS). Also, patients were weighted at each visit. European Respiratory Society standard prediction equations for pulmonary function were used in all hospitals.

Home monitoring

During the first 3 months of treatment daily home monitoring of the lung function was performed by the patient on a calibrated hand-held spirometer that is available and used in regular care (Micro Diary, Carefusion). Participants were asked to undertake 3 maneuvers each day at approximately the same time in order to enhance compliance. An alarm clock would remind the patient that same day if a maneuvers was late and therefore would possibly be forgotten. Next to lung function data, daily prednisone dose was automatically recorded on the home spirometer. Additionally, patients were asked to fill out a MRC and FAS score at the end of each treatment week.

Home spirometer training

Participants were trained how to use the home spirometer for approximately 45 minutes at start of the study. A refreshment training was performed at the routine follow-up hospital visit after 4 weeks. Trainers were either respiratory physiologists or research nurses who were officially certified to perform lung function tests on hospital lung function equipment. A detailed training protocol was used by all trainers in participating hospitals to ensure and level training quality. A patient was declared to be adequately trained when at least 3 FVC maneuvers on the home spirometer were reproducible, of which the top 2 best were less than 150 ml apart. Also, the trainer had to ensure that the record of the FVC on the home spirometer did not deviate more than 10% of the FVC measured on in-hospital lung function equipment. After one week patients were contacted and results of the first week were reviewed with the patient. When 3 FVC records on the home spirometer of the previous week were lower than 80% of the baseline FVC measurement on the home spirometer, while the patient did not experience an increase in disease symptoms, a refreshment training was considered with the participant.

Statistical analysis

Export records of daily FVC measurements on the hand-held spirometer were used for analyses. The written home diary was used to read out weekly MRC and FAS scores. The

comparison of means of continuous variables measured at different time points during in-hospital monitoring at baseline, month 1 and month 3 were tested with the paired student *t* test. Daily FVC and weekly MRC/FAS scores measured during home monitoring were used as outcome in multilevel models with patient as random factor. For analyses of the FVC a multilevel model was used that incorporated the change of the curvature at an estimated time point at which the maximum improve in lung function was reached. This model assumes that after a certain number of days a maximum level is reached (*tau*) and the curvature of the trajectory between start and maximum has some freedom (*gamma*)(see Supplementary Figure E1). For MRC/FAS scores we modelled linear splines with a knot at 4 weeks, using multilevel models. Statistical analyses and figures were performed/created using SPSS (version 21.0.0.1) and R software (version 3.2.2), using the package lme4. A *P*-value <0.05 was considered as statistically significant.

Ethical requirements

The Medical Ethical Committee of the Erasmus MC approved this study, under the Dutch act for medical research involving human subjects (Wet Medisch Onderzoek). The local institution review board of all participating centers approved with registration number MEC-2013-244.

RESULTS

Patients

Thirty-eight sarcoidosis patients with a pulmonary treatment indication for first-line prednisone therapy were screened for eligibility for this study. Eighteen patients were excluded due to various reasons (Figure 1), including a FVC>80% (5); contra-indication for prednisone (e.g. diabetes mellitus) (4); active asthma (1); morbid obesity (1); incapable or practical contra-indications to perform daily spirometry, including a multi-trauma, a recent cerebral event and a planned aortic valve replacement during study procedures (3); not treatment naïve (1); incapability to give informed consent due to language (1); and finally one patient had already started with prednisone (1) and one patient did not want to participate (1). In total 20 sarcoidosis patients were enrolled in this study. However, 1 patient chose to withdraw from the study at day one enrolment, and from 1 patient the FVC at start of study was >10% higher than the screening FVC, suggesting a major spontaneous improvement before start of treatment. This leaves 18 patients that were home monitored and analysed for this study (Figure 1).

The study group consisted of 11 males and 7 females. Mean age was 44 (± 11) years. The majority of the patients (78%) was diagnosed with Scadding stage II sarcoidosis. In half of the patients, sarcoidosis also affected extra thoracic organs. Patient demographics are shown in Table 1.

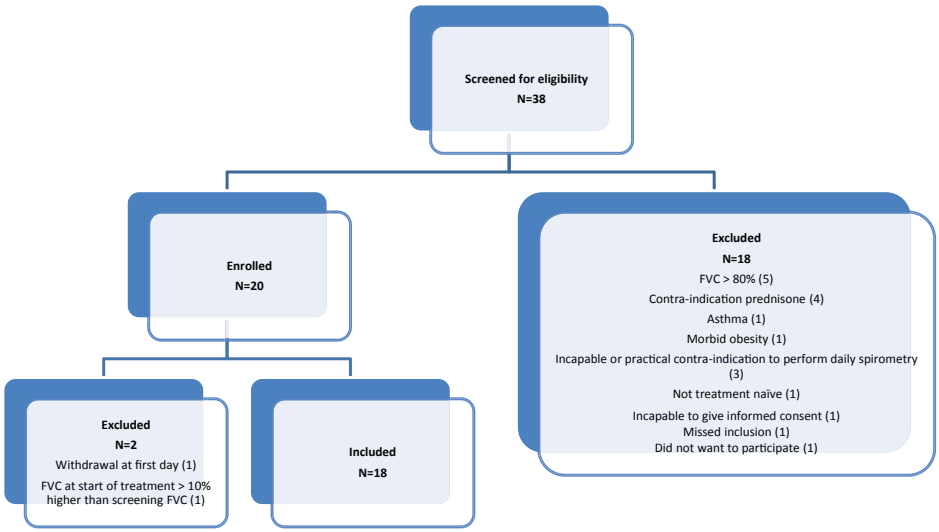

Figure 1. Flowchart of patient that were eligible for this study and that were enrolled, included or excluded. *Abbreviations:* FVC = forced vital capacity.

Prednisone treatment

Thirteen patients were treated according to the agreed standard prednisone regiment. In 2 patients the treating physician felt that prednisone could not be tapered to 10 mg/day within 3 months due to an unsatisfactory clinical response at 4-8 weeks after treatment initiation, thus prednisone was kept on a higher daily doses (≥ 20 mg/day). One of these received additional methotrexate therapy at month 2. Another patient experienced retro sternal pains and nervousness after initiation of prednisone, and the treating physician decided on earlier tapering to 10 mg/day. One patient by accident took 40 mg/day for an additional two weeks instead of tapering. Finally, one patient chose to stop prednisone treatment and also home-spirometry after 2 months, without report of any adverse event or other reason.

In-hospital monitoring

In-hospital clinical parameters, including lung function tests, laboratory tests, quality of life and weight, were monitored at baseline and month 1 and 3 after prednisone treatment initiation.

Lung function

In-hospital clinical monitoring showed a significant FVC increase upon treatment from 68.7 (±14.9) to 80.9 (±14.8) percent predicted at month 1 (mean change 12.3 (±9.8), $P < 0.001$) (Figure 2a**)**. Interestingly, a smaller FVC improvement was observed between

Table 1. Study subject characteristics

Patient baseline characteristics	Total study cohort (n=18)
Age (Mean years (SD))	44 (11)
Sex (Male/Female)	11/7
Race (n (%))	
White	9 (50)
Black	6 (33)
Asian	3 (17)
Weight (mean kg (SD))	80,1 (17,6)
BMI (mean kg/m^2 (SD))	26,7 (6,1)
Smoking	
Never	12 (67)
Former	4 (22)
Current	2 (11)
Scadding Stage at start treatment (n (%))	
Stage II	14 (78)
Stage III	4 (22)
Extrathoracic involvement (Yes/No)	9/9
Extrathoracic involvement (n (%))	
Skin	3 (17)
Eyes	2 (11)
Articular$^{\$}$	6 (33)
Central nervous system	0 (0)
Other (e.g. liver, spleen, skeletal)	2 (11)
PFT (mean % predicted (SD))	
FVC	69,4 (14,2)
DLCO	61,0 (22,6)(n=15)

Abbreviations: SD: standard deviation, kg: kilogram, PFT: pulmonary function tests. $ Self-reported articular involvement.

month 1 (80.9 (±14.8)) and month 3 (84.7 (±14.4)) (mean change 3.8 (± 6.9), $P = 0.045$) (n=16) (Figure 2a).

Furthermore, in a subgroup of patients diffusing capacity of lung for carbon monoxide (DLCO) (corrected for hemoglobin level) was measured, and a significant increase in percent predicted DLCO was observed within 1 month from 59.4 (±22.5) to 71.6 (±20.6) (mean change 12.2 (±8.1), $P < 0.001$). However, DLCO decreased somewhat again between month 1 and 3 to 66.7 (±16.2) (mean change -5.0 (±8.5), $P = 0.047$) (n=14) (Figure 2b).

These data show that most of the effect on FVC is reached within 1 month of prednisone treatment in newly-treated sarcoidosis patients.

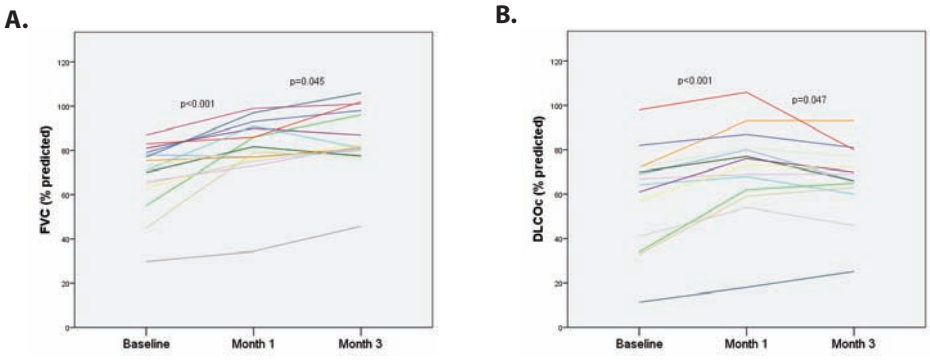

Figure 2. In-hospital clinical monitoring of percent (%) predicted FVC (*A*) and DLCO (corrected for hemoglobin levels) (*B*) values at baseline, month 1 and 3 during prednisone treatment of patients with data available at all three time points. *Statistics:* Lines represent paired results from one patient (*A*: n=16 and *B*: n=14). Significance was determined using a two-tailed paired T-test. *Abbreviations:* FVC = forced vital capacity, DLCOc = diffusing capacity of lung for carbon monoxide (corrected for hemoglobin levels).

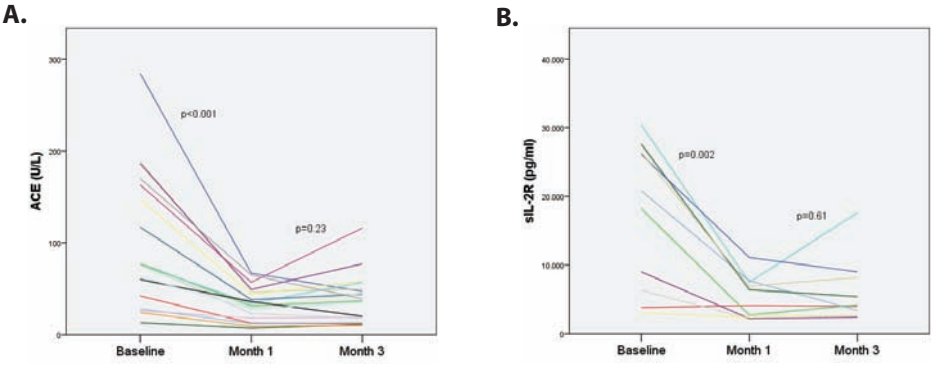

Figure 3. In-hospital clinical monitoring of ACE (*A*) and sIL-2R (*B*) at start (baseline) and month 1 and 3 during prednisone treatment of patients with data available at all three time points. *Statistics:* Absolute values are shown. Lines represent paired results from one patient (*A*: 18 and *B*: 10). Significance was determined using a two-tailed paired T-test. *Abbreviations:* ACE = angiotensin-converting-enzyme, U/L = units per liter, sIL-2R = soluble interleukin-2 receptor, pg/ml = pictograms per milliliter.

Serum markers

Proposed serum markers of disease activity in sarcoidosis include angiotensin-converting-enzyme (ACE) and soluble interleukin-2 receptor (sIL-2R)(12, 13).

Since several variables could have influenced ACE levels between patients assessed in our study cohort (such as ACE genotypes (14) and distinct methods used within participating centers to determine ACE levels), merely level changes were analyzed. Interestingly, ACE levels decreased significantly upon treatment between baseline and

month 1 ($P < 0.001$), however no significant change was observed between month 1 and 3 ($P = 0.23$) (n=18) (Figure 3a).

Also sIL-2R levels decreased significantly upon treatment between baseline (17160 (±10685)) and month 1 (5320 (±3052)) ($P = 0.002$), but not between month 1 (5320 (±3052)) and 3 (5940 (±4701) ($P = 0.61$)(n=10) (Figure 3b).

These data show that serum inflammatory markers decrease significantly during sarcoidosis prednisone treatment within 1 month, remaining stable over 3 months.

Adverse events and weight

In total 18 different adverse events were reported. Table 2 shows adverse events that were reported in more than 1 patient (n=9). One patient was hospitalized during the study for an elective surgical operation (parathyroidectomy). No further SAE's occurred.

In order to further objectify an important side-effect of prednisone treatment in pulmonary sarcoidosis, weight was assessed during hospital visits. Average weight at start of treatment was 80.1 (± 17.6) kg and BMI was 26.7 (± 6.1) kg/m^2 (Table 1). Paired analyses showed that weight increased significantly between baseline and month 1 (mean change 2.3 (± 2.6) kg ($P = 0.002$)) and month 3 (mean change 5.4 (± 4.2) kg ($P < 0.001$)) (n=18), respectively (Figure 4).

These data show that weight increased significantly during prednisone treatment and tapering.

Symptoms and quality of life

Change in symptoms and/or quality of life during prednisone treatment is shown in Table 3**.** Importantly, MRC score significantly decreased at month 1 compared with baseline ($P = 0.001$), indicating less dyspnea. FAS score was also significantly improved at month 1 compared with baseline ($P = 0.001$), indicating less fatigue (15). Similar to

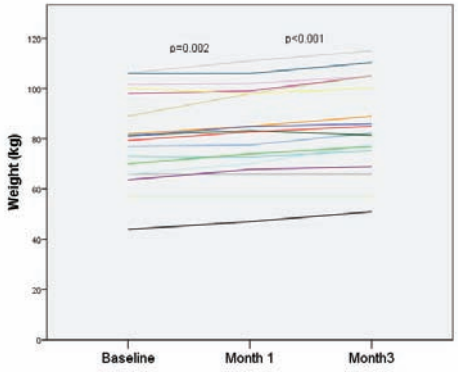

Figure 4. In-hospital clinical monitoring of weight at baseline, month 1 and 3 during prednisone treatment. *Statistics:* Absolute kilograms are shown. Lines represent paired results from one patient (n=18). Significance was determined using a two-tailed paired T-test. *Abbreviations:* kg = kilograms.

Table 2. Reported adverse events

Adverse event reported in > 1 patient	Total patients (%)
Weight increase/Cushingoid features	3 (15,8)
Upper airway infection†	3 (15,8)
Insomnia	3 (15,8)
Chest pain	2 (10,5)
Muscle cramps	2 (10,5)
Gastrointestinal disturbance	2 (10,5)
Neuropsychiatric	2 (10,5)
Other bacterial/viral infection††	2 (10,5)
Acne	2 (10,5)

† All three patients received antibiotics for the upper airway infection. †† One patient had a bacterial testicular infection and one patient had herpes zoster.

Table 3. Symptoms and quality of life assessed during prednisone treatment

Symptom and/or quality of life-related questionnaire	Baseline Mean (SD)(No.)	Month 1 Mean (SD)(No.)	Month 3 ¥ Mean (SD)(No.)
MRC	2,56(1,03)(16)	1,44(0,89)(16)*	1,50(0,89)(16)*
FAS	28,0(10,9)(17)	21,2(6,8)(17)*	21,1(7,6)(17)*
SGRQ			
Symptoms	45,4(21,7)(16)	31,5(21,8)(16)*	27,5(25,9)(16)*
Activity	56,8(25,4)(16)	41,2(25,9)(16)*	39,1(27,7)(16)*
Impact	29,8(23,6)(16)	19,4(15,7)(16)	19,0(14,8)(16)*
Total	41,2(22,1)(16)	28,0(17,6)(16)*	26,6(18,9)(16)*
SF-36			
PCS	38,3(7,2)(13)	39,3(7,0)(13)	36,2(6,6)(13)
MCS	33,8(6,6)(13)	36,8(7,2)(13)	39,5(8,2)(13)*
KSQ			
GHS	59,8(15,1)(9)	70,5(19,0)(9)*	63,6(14,4)(9)
Lung	57,1(14,3)(8)	66,5(23,3)(8)	67,3(21,8)(8)*

Data is shown as mean (± SD) (No. of patients with available paired data over the 3 time points). * $P < 0.05$ compared with baseline, using a paired sample T-test. ¥ None of the values is statistically significant at month 3 compared with values at month 1. *Abbreviations:* SD: standard deviation, No. = number, MRC: medical research council dyspnoea score, FAS: Fatigue Assessment Scale, SGRQ: St. George Respiratory Questionnaire, SF-36: Short Form Health Survey, PCS: Physical Component Score, MCS: Mental Component Score, KSQ: King's Sarcoidosis Questionnaire, GHS: General Health Status.

FVC and serum markers (Figure 2 and 3), MRC and FAS score remained stable between month 1 and 3 (Table 3).

SGRQ sub- and total scores showed a similar pattern of improvement as MRC and FAS scores (Table 3). Total SF-36 score did not show much change, although the mental component summary (MCS) score was statistically significant improved at month 3 com-

pared with baseline ($P = 0.04$). A subgroup filled out the KSQ score (16) during the study after it was validated in the Netherlands (17), and this sub analyses showed that general health status (GHS) improved significantly within one month of treatment ($P = 0.009$). This however did not remain stable over 3 months, whereas lung specific symptoms recorded in this questionnaire were still significantly better at 3 months compared with baseline ($P = 0.03$) (Table 3).

These data show that symptoms such as dyspnea (MRC/SGRQ) and fatigue (FAS) decreased significantly during prednisone treatment within 1 month.

Home monitoring

In the home setting, FVC and dyspnea (MRC) and fatigue (FAS) scores were monitored by the patient at a daily and weekly basis, respectively.

Home spirometry

In order to determine when the maximum improve in lung function occurs, individual FVC changes over time were recorded on a daily basis after prednisone initiation, using a calibrated home-spirometer. Figure 5a shows the best daily FVC measurements on the home spirometer per individual patient over time in liters. Data was recalculated to percent predicted FVC, using the predicted values on the individual in-hospital baseline FVC record, and in-hospital FVC measurements at baseline, 1 month and 3 months are shown next to the home spirometry data in red dots (Figure 5b). Although FVC measures tended to be lower on the home spirometer than on in-hospital lung function equipment (~197 ml), there was a strong correlation between those two parameters (Pearson correlation: 0.97 ($P < 0.001$)). Furthermore, individual analyses of the home spirometer-derived results showed that the mean standard deviation of the 3 repeated FVC measurements within an individual patient on one day was 100 ml. These data imply that the home spirometer can be used as a reliable tool to measure FVC changes in pulmonary sarcoidosis patients on a daily basis.

Mean FVC change over time was calculated using a multilevel model that incorporated regression lines of all 18 individual patients (Figure 6)**.** Mean percent predicted FVC at start of treatment was 65.9 (95% CI: 60.9 to 73.4). A maximal mean increase of 10.0 (95% CI: 9.6 to 10.5) % predicted FVC was observed after treatment initiation. A plateau of FVC increase was observed at 23 days (95% CI: 20 to 28), and 90% of the total FVC increase was reached at day 17 (95% CI: 16 to 18).

Weekly symptom scores

In order to get insight whether the early change in lung function correlated with patient-relevant outcomes (i.e. symptom relieve) short symptom scores, such as MRC and FAS were assessed weekly in the home-setting. Weekly symptom scores showed a

decreasing trend during the first month (see Supplementary Figure E2). Multilevel analyses of MRC scores showed that symptoms decreased significantly with 0.3 (95% CI: 0.2 to 0.4) MRC points per week ($P < 0.001$), stabilizing after 1 month. Similarly, FAS scores decreased significantly with 2.1 (95% CI: 1.7 to 2.5) FAS points per week ($P < 0.001$), stabilizing after 1 month.

Together these data show that MRC and FAS scores were also already significantly decreased within 2 weeks of prednisone treatment in sarcoidosis patients.

DISCUSSION

This is the first study showing prospectively by daily home spirometry that most of the change in FVC and symptoms occurs within 2-3 weeks after prednisone therapy initiation in a cohort of newly-treated sarcoidosis patients.

As long term disease modifying benefits of prednisone are debated (4-6), carefully balancing potential beneficial effects on symptoms and quality of life with potential side effects in first-line treatment of pulmonary sarcoidosis is warranted (3, 4, 6, 7). Current guidelines differ in recommendation on the dose and duration of treatment (1, 8), but daily home spirometry could facilitate a personalized approach to treatment for each patient aiming at achieving the maximum effect of lung function and symptom improvement with the lowest possible dose of prednisone, in order to minimize side effects. This study shows that daily home spirometry of the FVC is an effective tool to monitor sarcoidosis response to therapy (18). Moreover, multi-level analyses of daily home monitored FVC showed that most (90%) of the prednisone effect on FVC already occurred around 17 days, reaching its maximum improvement around 23 days. A significant decrease in dyspnea (MRC) and fatigue (FAS) (15) scores was also observed within 2 weeks of treatment. These data argue that FVC changes observed at approximately 2-3 weeks can help physicians evaluate response to therapy in newly-treated sarcoidosis patients. Consequently, physicians might earlier decide on dose tapering and/or the need for initiation of second-line steroid-sparing therapies than is now advised on (1, 8).

Our results are in line with other studies that have suggested that the major increase in FVC during prednisone treatment in sarcoidosis occurs within 1 month (10, 11)(*Broos et al., submitted*). These studies were however either performed retrospectively in exacerbation patients that have previously shown to be responsive to prednisone (10) and/or evaluated changes on in-hospital lung function equipment at determined time points, (possibly) missing early and individual daily changes in FVC (10, 11). This study is the first to perform daily home spirometry and include home monitoring of weekly symptom scores, evaluating early effects on dyspnea (MRC) and fatigue (FAS) (15) next to FVC, strengthening the accuracy of clinical evaluation in pulmonary sarcoidosis (13). Furthermore, results showed that inflammatory markers that have been suggested to

reflect disease activity in sarcoidosis, such as ACE and sIL-2R (12, 13), also decreased significantly within 1 month, remaining stable up to 3 months. Similarly, quality of life (SGRQ) scores improved clinically significantly within 1 month (19, 20). In a sub cohort DLCO increased within one month during prednisone therapy, but it did not remain fully stable during tapering. However, although DLCO measurements are likely to reflect earlier interstitial changes in sarcoidosis (21), reproducibility of DLCO measurements

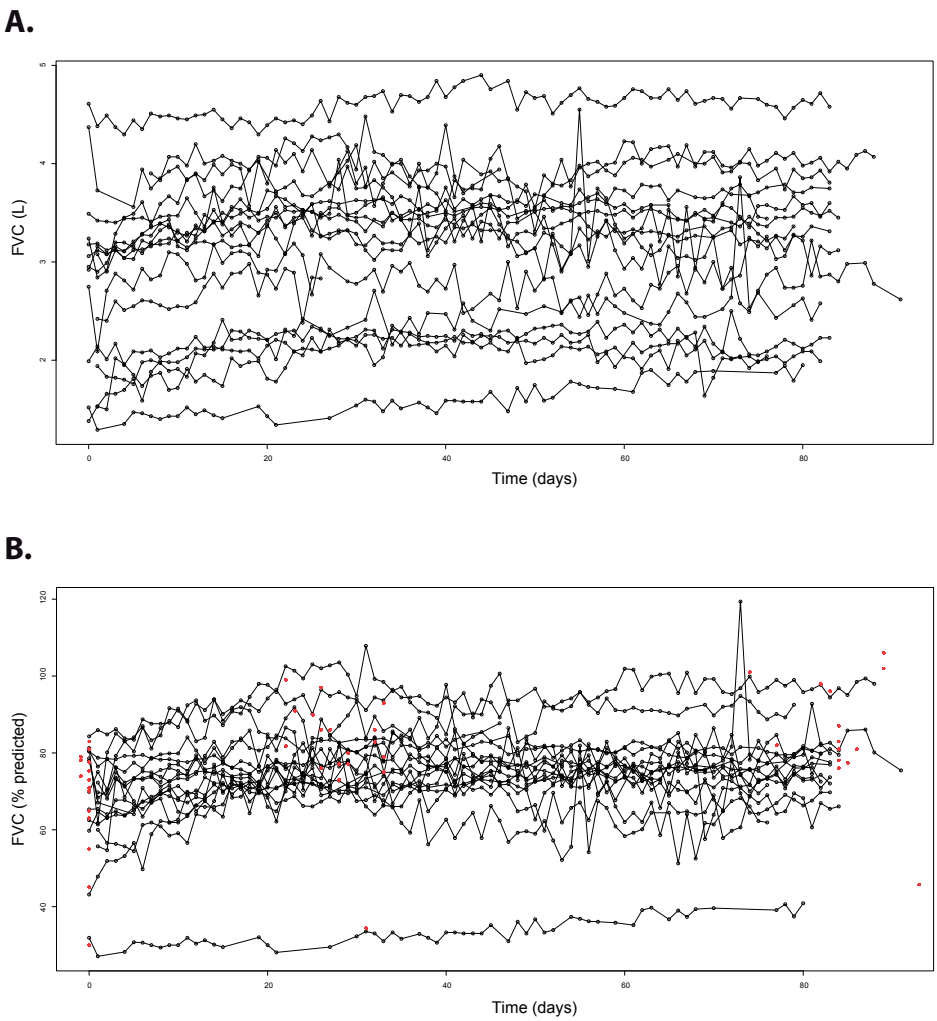

Figure 5. Daily best FVC measurements recorded on the home spirometer (Micro Diary, Care Fusion) per individual patient (n=18) over time (days) in liters (*A*) or as percentage of predicted (*B*). *Calculations:* Data was recalculated to percent predicted FVC, using the predicted values on the individual in-hospital baseline FVC record, and in-hospital FVC measurements at baseline, 1 month and 3 months were added to the home spirometry data in red dots (*B*). *Abbreviations:* FVC = forced vital capacity, L = liters.

is lower than FVC measurements, thus only a major change (>15%) in DLCO has been suggested to reliably exclude confounding by measurement variation (13).

Early dose tapering to a maintenance dose is very likely to reduce important side-effects in pulmonary sarcoidosis. It has been found that a daily dose of lower than 10 mg prednisone is less likely to induce side effects and is regarded as a successful tapering (3, 9, 22). Furthermore, our retrospective study in newly-treated sarcoidosis patients showed that specifically a high cumulative dose prednisone received in the long term (at 12 and 24 months) significantly correlated with weight increase. Remarkably, the treatment regimen leading to a high cumulative dose in the long term was characterized by a prolonged use of more than 10 mg prednisone per day compared with the low dose regimen (*Broos et al., submitted)*.

Interestingly, questionnaires that were more focused on general health status, such as SF-36 and KSQ-GHS showed either no or less pronounced changes during prednisone treatment in our study group than scores purely including physical symptoms such as MRC and FAS. This could have been caused by concurrent side-effects that are likely to occur during prednisone treatment, counterbalancing the positive (physical) outcomes of prednisone treatment. Indeed, in our study a number of adverse events were reported that are associated with prednisone treatment, and our data also showed that sarcoidosis patients underwent significant weight gain within the first 3 months of

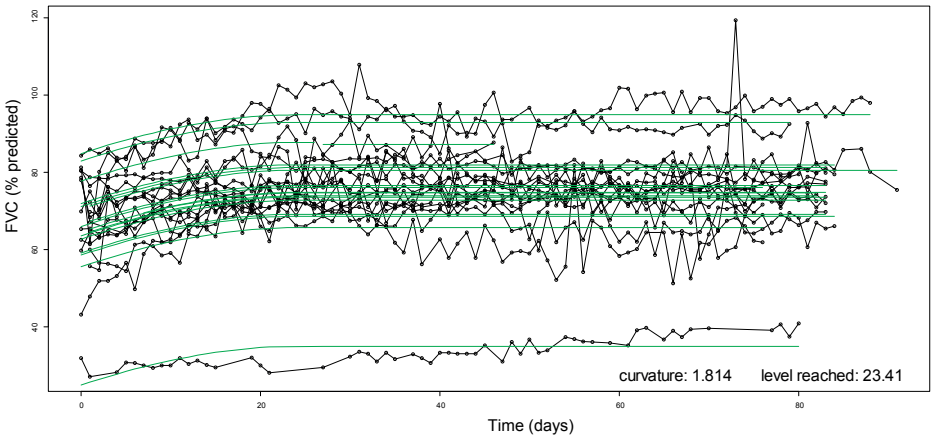

Figure 6. Daily best FVC measurements recorded on the home spirometer (Micro Diary, Care Fusion) per individual patient over time (days) in as percentage of predicted, including regression lines of all 18 individual patients. *Calculations:* Data was recalculated to percent predicted FVC, using the predicted values on the individual in-hospital baseline FVC record. *Statistics:* A multilevel model was used that incorporated the change of the curvature at an estimated time point when the maximum improve in lung function was reached. This model assumes that after a certain number of days a maximum level is reached (*tau*) and the curvature of the trajectory between start and maximum has some freedom (*gamma*)(see Supplementary Figure E1). *Abbreviations:* FVC = forced vital capacity.

treatment (~ 5 kg). These data confirm that it remains relevant to continuously evaluate benefit-risk ratio of prednisone treatment in consultation with the individual patient (3, 6, 7). Although prednisone seems highly effective in the short term on FVC, symptoms and inflammatory markers, side-effects remain a prominent problem during therapy and can therefore influence quality of life of sarcoidosis patients.

A multi-level model provided evidence that most of the FVC effect in the majority of the newly-treated sarcoidosis patients seems to occur within 2-3 weeks, remaining stable up to 3 months. However, the individual home monitoring data also highlight that patients can respond uniquely to prednisone and suggest the need for tailored medicine in sarcoidosis treatment (6, 7). For example, in a few cases FVC kept increasing or partly decreased again after 2-3 week or during tapering. Also, in 2 patients, prednisone therapy could not be tapered below 20 mg because of an unsatisfactory response and one of these patients received additional methotrexate during this study. Sarcoidosis is well known for great heterogeneity in clinical course, thus long-term follow-up of this study cohort may reveal differences in disease course and ultimate treatment outcome. Long-term follow-up of an expanded cohort should reveal whether early FVC changes can predict treatment outcome, and whether home spirometry can guide physicians in early initiation of second- and or third-line therapy to spare prednisone. In idiopathic pulmonary fibrosis it has been shown that change in disease course could well be detected with home spirometry and was predictive for long term outcomes (23).

Taken together, results of this study validate the use of home spirometry in first-line treatment of sarcoidosis patients and suggest that daily home monitoring of FVC changes in clinical practice has the potential to personalize sarcoidosis treatment, aiming at early dose tapering and side-effect reduction. Importantly, quality of the home spirometry maneuvers, and therefore the reliability of home-monitored FVC measurements can be influenced by training quality and patient-related factors, thus critical review of individual results always remain warranted before the physician includes such results in clinical treatment decisions. Up till now, not one clinical test has been found to be sufficiently accurate in assessing disease severity in pulmonary sarcoidosis (13). Future studies are needed to evaluate whether careful home monitoring of prednisone treatment (including FVC and symptom scores) and personalized dose titration (24, 25), will allow for a non-inferior treatment effect compared to current clinical practice, while increasing pulmonary sarcoidosis patients' quality of life (6, 7).

CONCLUSION

This study validates the use of home spirometry in first-line treatment of pulmonary sarcoidosis and shows that the major improvement in lung function occurs within 2-3 weeks. Early (home) monitoring of lung function and symptoms has the potential to

individualize prednisone therapy, aiming at early dose tapering, side-effect reduction and patient-related quality of life.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY FIGURES

$$\widehat{\mathrm{FVC}} = \alpha_i + \beta.t'$$

$$t' = \begin{cases} 1 - \left(\frac{\tau - t}{\tau}\right)^{\gamma} & \text{if } t < \tau \\ 1 & \text{otherwise} \end{cases}$$

where:

$\widehat{\mathrm{FVC}}$	fitted value of FVC	estimated FVC without random error
α_i	random intercept	estimated FVC at start for individual i
β	effect of the intervention	difference between start and final level
t'	transformation of time t	shape of the change between start and end of the followup
τ	time at wich the level is reached	non linear parameter in the regression model
t	time in days	
γ	curvature of the change before τ	$\gamma = 1$ means straight line from 0 to τ $\gamma = 2$ means parabola from 0 to τ

Figure E1. Mathematical model that was used to predict FVC change over time. A multilevel model was used that incorporated the change of the curvature at an estimated time point when the maximum improve in lung function was reached. This model assumes that after a certain number of days a maximum level is reached (*tau*) and the curvature of the trajectory between start and maximum has some freedom (*gamma*).

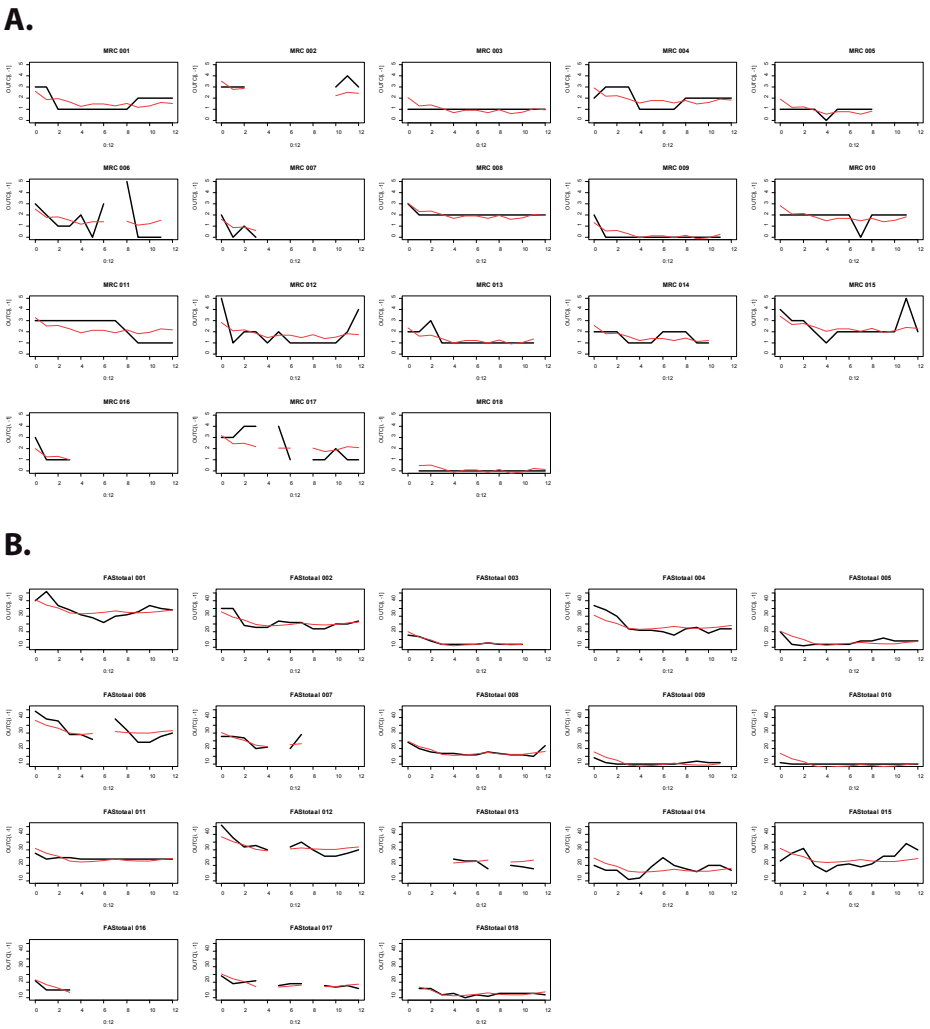

Figure E2. Weekly MRC (*A*) and FAS (*B*) scores filled out in a home diary per individual patient over time (weeks), including regression lines of all 18 individual patients. *Statistics:* A multilevel model was used that modelled linear splines with a knot at 4 weeks. *Abbreviations:* MRC = Medical Research Council Dyspnea score, FAS = Fatigue Assessment Scale.

EPILOGUE

General discussion of the thesis

**'Live out of your imagination,
not your history'**
Stephen R. Covey

EPILOGUE

Important goals of sarcoidosis research are to identify predictors of disease course, biomarkers of disease activity and targets for effective (new) therapies. Insight in these factors can help guide sarcoidosis specialists on 'Who, When and How' to treat, helping them to fine-tune treatment strategies, thereby increasing quality of life of pulmonary sarcoidosis patients.

The first aim of this thesis was to (further) unravel the immune-related pathogenesis of sarcoidosis, so as to discover contributors to disease etiology and disease course (**Part 1**). The second aim was to gain insight into the current mainstay of sarcoidosis treatment, that is prednisone therapy, in order to optimize treatment strategies to reduce side-effects (**Part 2**). In this epilogue, results of both parts of the thesis are put into the perspective of implications for clinical practice, ending with a summary of outstanding questions and concluding remarks.

GENERAL DISCUSSION OF THE THESIS

Implications for practice: Who to treat?

Sarcoidosis affects all people over the world with different races/ethnicities, but the disease course varies greatly per individual patient (1). Some patients will undergo spontaneous resolution without any treatment, and others develop chronic, sometimes progressive disease with need for treatment. Consequently, an intriguing question in sarcoidosis research is what factors determine the difference between these patients. This can help identify biomarkers that predict disease course and therefore help physicians decide which patient to treat and which patient not. Results of this thesis that are valuable in this sense are discussed below.

Predictors of disease course: A role for Treg and Th 17-lineage cell-associated factors?

Results of this thesis and current evidence suggest that immunological factors related to regulatory T cells (Tregs) and T helper (Th)17-lineage cells are attractive candidates to study as biomarker predicting disease course in pulmonary sarcoidosis.

The distribution within and/or functionality (e.g. cytokine production) of Th17-lineage cells might be predictors of disease course. Sarcoidosis lung proportions of Th17.1 cells are significantly higher than Th17 cells in newly-diagnosed and progressive patients (this thesis). Within the limited number of patients analyzed in our study, we found that patients that develop chronic sarcoidosis have significantly higher proportions of Th17.1 cells in the lungs at time of diagnosis than patients who undergo resolution within 2 years (Chapter 5). Also, the ratio Th17.1/Th1 cells and (although it did not reach statistical significance) Th17.1/Th17 cells was increased at time of diagnosis in patients that develop chronic disease. Although these data merely show an association, and do

not proof causality, Th17.1 cells have previously been found to be pathogenic in other autoimmune disorders, by upregulation of pro-inflammatory cytokines and corticosteroid resistance (2), making it conceivable that Th17.1 cells do contribute to development of progressive disease.

Recently, results of Grunewald and colleagues also suggested a role for Th17.1 cells in sarcoidosis disease prognosis. They however identified increased proportions of T-bet$^+$RORγT$^+$ CD4$^+$ T cells, also co-expressing CCR6 and CXCR3 like Th17.1 cells (Chapter 4 (3) and 5), in lungs of Löfgren patients; a subtype of acute sarcoidosis that is associated with a favorable disease outcome (4). In contrast to our results, observed in non-Löfgren patients (Chapter 5), a positive correlation was found between lung T-bet$^+$RORγT$^+$ CD4$^+$ T-cell proportions and resolving disease in Löfgren patients. However, interestingly, although Löfgren lung CD4$^+$ T cells consisted of ~65% T-bet$^+$RORγT$^+$ CD4$^+$ T cells, IFN-γ production by Löfgren BALF cells was lower than by non-Löfgren BALF cells in vitro (4). Additionally, benign Th17-lineage cytokine (IL-17A and IL-10) production was higher. These data suggest that maybe not so much Th17.1 cell proportions, but mostly the distribution of cytokine production by these cells is a contributor to sarcoidosis disease course. It is however important to mention that Löfgren's syndrome may constitute a separate disease from sarcoidosis, as suggested by a distinct clinical presentation, a separate genetic background (5), and in most patients, an antigen-specific immune response. Therefore, careful research of the predictive value of Th17.1 cells is needed in both disease entities, as the immune pathology may be different.

Failure of immunosuppressive Tregs has also been suggested to contribute to ongoing inflammation in sarcoidosis (Chapter 1 (6)). Decreased Treg proportions have been detected in broncho-alveolar lavage fluid (BALF) of patients that develop chronic active disease (7), suggesting that Tregs can contribute to disease course. In this thesis we did not find evidence for reduced Treg numbers at site of disease activity (Chapter 2 (8) and 3 (9)). In contrast, circulating Treg proportions were significantly enhanced at time of diagnosis in patients developing (active) chronic disease (Chapter 3 (9)). Together these data could suggest that enhanced circulating Treg proportions reflect impaired Treg migration towards the site of inflammation and/or failure of local Tregs to control Th cell inflammation, leading to ongoing IL-2 production and homeostatic Treg expansion (Chapter 3 (9)). Therefore, circulating Treg proportions might serve as a biomarker predicting chronic disease in sarcoidosis.

However, Treg proportions have also been described to be significantly lower in BALF of Löfgren patients than non-Löfgren sarcoidosis patients (10)(Chapter 1), and like in Th17-lineage cells, that would suggest an opposite role of these cells as contributor to sarcoidosis disease course. Although Löfgren syndrome is often accompanied by a favorable prognosis, the disease is also characterized by an acute onset of pronounced inflammation, reflected in noticeable fever (11). Therefore, the excessive presence of

Th17.1 cells and decreased Treg proportions might be more an indicator of disease activity (see below) alongside other important determinants of disease course, such as the ability to adequately get rid of the putative antigen. Alternatively, as mentioned above, although insights in immunological responses observed in Löfgren patients might help shed light on sarcoidosis pathogenesis, Löfgren disease very likely evolves different than non-Löfgren disease (5), thus contradictory results between these patients may exist.

It is important to notice that studies in this thesis and reported in current literature were not primarily aimed at evaluating Th17-lineage cell or Treg proportions/function as potential predictor of disease course in pulmonary sarcoidosis, and were thus not powered to make a firm conclusion on 'Who' to treat. From these data, it is however evident that Th17-lineage cells and Tregs are likely involved in immunological responses that contribute to ongoing inflammation in pulmonary sarcoidosis. Prospective studies should aim to clarify whether Th17-lineage cell (Chapter 5) and/or Treg (Chapter 3 (9)) proportions and/or function of these cells (4) (e.g. cytokine production) can predict disease outcome, serving as a prognostic biomarker that could indicate the need for treatment. Therefore, ideally, different sarcoidosis-affected organs should be studied paired-wise at time of diagnosis in distinct, well-defined sarcoidosis disease entities, including Löfgren patients and non-Löfgren patients. Furthermore, serial data during active, progressive, resolved and treated disease can further shed light on the role of these cells and related factors as determinant of sarcoidosis disease course.

Implications for practice: When to treat?

Once determined who needs (or will need) treatment, it remains to be decided when to start therapy. Biomarkers that reflect disease activity can help physicians decide on the need to initiate treatment, but also help evaluate response to therapy. Several serological biomarkers have been suggested to reflect disease activity in pulmonary sarcoidosis, including angiotensin-converting-enzyme (ACE) and soluble interleukin-2 receptor (sIL-2R) (12, 13). These markers are however not specific for sarcoidosis activity, and serial testing has not proven an additional predictive value to pulmonary function testing and radiology (13). The benefit of a reliable peripheral blood biomarker for disease activity is evident, as it could assist in monitoring and detecting (subclinical) disease activity and/or evaluate response to therapy.

Biomarkers of disease activity: A role for the Treg/Th17 ratio?

Results of this thesis and current literature suggest a potential role for Treg/Th17 ratio as candidate biomarker of disease activity in pulmonary sarcoidosis. Chapter 3 (9) shows that circulating Tregs are significantly increased in active pulmonary sarcoidosis patients at time of diagnosis. Also, enhanced circulating Th17 cells proportions were detected during active disease and at time of progression (3, 14-16) (Chapter 4 (3)). Although

both subsets increase in peripheral blood during active/progressive disease, the Treg/Th17 ratio was found decreased (17-19), suggesting that induction of inflammatory cells outbalances anti-inflammatory cells. Importantly, candidate biomarkers should correlate with clinical parameters and response to treatment. Indeed, circulating Treg proportions are lower in patients at time of relapse than at time of remission and Th17 cells vice versa (17). This study also published that the Treg/Th17 ratio associates with clinical parameters, such as pulmonary function and serum ACE and remission after treatment (17).

Furthermore, data presented in this thesis suggest that BALF Th17.1 cells correlate with disease activity (Chapter 4 (3) and 5). However, to confirm this assumption, ideally, sarcoidosis Th17.1 BALF proportions should also be measured during inactive disease and/or at time of resolution. This is however not very feasible, since patients should then undergo a second broncho-alveolar lavage at time of disease inactivity. Furthermore, as mentioned above, the advantage of a peripheral blood biomarker is evident above performing serial invasive techniques, such as a broncho-alveolar lavage, to detect disease activity. Interestingly, the epigenome is very valuable in in this sense, since it provides information on the developmental history, present identity and future potential of cells. Since lung Th17.1 cells likely derive from circulating Th17 cells that are initiated in the lung mediastinal lymph nodes (MLN) (Chapter 5) it is an attractive idea to examine the epigenomic signature of sarcoidosis circulating T cells. Hypothetically, the epigenetic profile of circulating Th17 cells reflects the local aberrant Th17.1 cell inflammatory response in pulmonary sarcoidosis and can therefore potentially serve as a biomarker. Future studies should reveal whether epigenetic profiling of T cells can help optimize and personalize pulmonary sarcoidosis treatment in the future.

In conclusion, these data highlight that there is a role for the Treg/Th17 ratio as disease activity marker in pulmonary sarcoidosis, however these studies should be extended, evaluating Treg/Th17 ratios at time of diagnosis, at time of resolution or progression and during treatment, correlating findings with clinical parameters. Insight could help further evaluate the role of the circulating Treg/Th17 ratio as candidate biomarker of disease activity in pulmonary sarcoidosis. Furthermore, although still in its infancy, an attractive line of investigation to identify new biomarkers for pulmonary sarcoidosis would be to examine the epigenome of circulating T cells in pulmonary sarcoidosis.

Implications for practice: How to treat?

The ideal therapy for pulmonary sarcoidosis would alter the natural course of the disease. This means: inducing resolution in patients that suffer (or will suffer) from chronic sarcoidosis. The cornerstone of current pulmonary sarcoidosis therapy, i.e. prednisone, does suppress symptoms and inflammation in pulmonary sarcoidosis, but does not seem to alter disease course in the long-term (20). Therefore, therapeutic targets that do

change disease course are warranted. Additionally, current prednisone therapy induces significant side-effects, thus treatment strategies reducing these side-effects could increase quality of life of patients. Results of this thesis that are valuable in this sense are discussed below.

Potential new therapy (1): A role for CTLA4-Ig?

The heterogeneity of sarcoidosis suggests that determinants of disease etiology (Why does someone develop the disease in the first place?) in sarcoidosis are (at least partly) distinct from factors determining disease course (Why does the disease not resolve in some patients?). New therapeutic targets that have the potential to change disease course may be identified from looking into factors determining disease etiology or disease progression.

Importantly, findings in Chapter 2 (8) of this thesis argue for a key role for cytotoxic T lymphocyte antigen (CTLA)4 and specific $CD4^+$ T-cell subsets (Tregs and Th17 cells) in sarcoidosis disease etiology. The cause of impaired CTLA4 expression in specifically Tregs and Th17 cells in sarcoidosis MLN remains unclear. Based on findings presented in Chapter 2 (8), it is not very likely that T-cell exhaustion underlies impaired CTLA4 expression in sarcoidosis. This idea is indeed further strengthened by results in Chapter 5 providing evidence for a significant decrease in programmed cell death protein (PD)-1 expression on specifically sarcoidosis Th17 cells, rather than an increase as would be expected during exhaustion. Currently, also more and more case reports emerge regarding sarcoidosis development or exacerbation during anti-PD1 therapy in cancer patients (21-24), making it more likely that global deregulation of co-inhibitory receptor expression might be a determinant of disease etiology, rather than aberrant expression of CTLA4 alone.

Interestingly, butyrophilin-like 2 (BTNL2) also negatively regulates T-cell activation, independently of CTLA4 and PD-1, and a recent meta-analyses showed that a BTNL2 polymorphism contributes to disease susceptibility in sarcoidosis (Chapter 6 (25)). Furthermore, CTLA4 and BTNL2 gene mutations have also been identified in families with common variable immunodeficiency (CVID), including patients with granulomatous lung disease (26, 27). However, although it seems conceivable that genetic changes can (partly) drive the aberrant expression of checkpoint inhibitors that contribute to granuloma formation in sarcoidosis, results in Chapter 2 (8) and 3 (9) suggest that the genetic contribution is likely very low. Mutations in CTLA4 in patients with CVID led to impaired upregulation of this protein on activated Tregs (27), yet, we found that CTLA4 on activated Tregs is significantly upregulated compared with other Th subsets in sarcoidosis MLN (Chapter 2 (8)) and circulating Tregs even showed a significant increase in CTLA4 expression compared with their control counterpart (Chapter 3 (9)). Finally, in a screening experiment we did not find evidence for the presence of CTLA4 mutations

in 38 sarcoidosis patients (data not shown). Together, these data rather suggest that CTLA4 expression is differentially regulated in sarcoidosis-affected organs, for example by epigenetic mechanisms and/or due to the local inflammatory environment.

For instance, expression of co-inhibitory receptors depends on the strength of T-cell receptor (TCR) signaling (28), which can vary between affected immunological compartments. Findings in our studies do not provide evidence for decreased CD28 expression on Th17 cells in pulmonary sarcoidosis MLN (Chapter 2 (8) and 5). However, an impaired response of sarcoidosis peripheral blood (PB)- and BALF-derived T cells towards anti-CD3 and anti-CD28 has been reported in active sarcoidosis (29). Thus, theoretically, distinct sarcoidosis T-cell subsets could have become less dependent on CD28 signaling, possibly due to chronic antigenic stimulation, resulting in deregulated PD-1 and CTLA4 pathways, which is remarkably similar to findings in chronic beryllium disease (30); an occupational lung disease that is also characterized by granuloma formation on the lungs that can be indistinguishable from sarcoidosis.

Nonetheless, consistent with previous literature and our results in Chapter 2 it is at least very likely that decreased CTLA4 (and possibly also PD-1) expression on specifically Tregs and Th17 cells in sarcoidosis MLN can cause double trouble in sarcoidosis, i.e. by increasing Th17 cell responses (thereby affecting the delicate Treg/Th17 balance (17-19), since Treg proportions remain unaffected (Chapter 2 (8))) and impairing Treg function (6) (see Figure 2 in Chapter 6 (25)). Therefore, future research should aim to clarify the role of co-inhibitory and co-stimulatory receptors on distinct T-cell subsets in sarcoidosis-affected organs, helping to gain further insight in this likely important determinant of sarcoidosis disease etiology and further providing a rationale to evaluate the role of CTLA4-Ig (e.g. Abatacept) in the treatment of (chronic) pulmonary sarcoidosis.

Potential new therapy (2): A role for anti-IL23p19?

This thesis is the first to identify a role for failure of immune regulation within a Th cell in sarcoidosis, which is decreased CTLA4 expression on specifically Th17 cells (Chapter 2 (8)). Interestingly, as CTLA4 expression displayed limited interpatient variability in Th17 cells of newly-diagnosed and treatment-naïve sarcoidosis patients (Chapter 2 (8)), it is more likely that reduced CLTA4 expression is a contributor to disease etiology rather than to disease course.However, as previously highlighted, in this thesis also a significant association was found between lung Th17.1 cells and disease prognosis (Chapter 5). It is thought that Th17.1 cells can derive from Th17 cells (2, 31, 32), and data in Chapter 5 indeed argue for a role of Th17-Th17.1 cell plasticity in sarcoidosis lungs. Therefore, it is tempting to speculate that decreased CTLA4 expression on Th17 cells is a trigger that contributes to Th17 differentiation in sarcoidosis MLN (8, 14, 15)(Chapter 5), and that a distinct factor contributes to ongoing inflammation and possibly development of fibrosis in the future. Although research of this thesis did not look into factors contributing

to development of fibrosis in pulmonary sarcoidosis, aberrancies in Tregs and Th17 cells and related factors have been described to contribute to pulmonary fibrosis (33-38), and $CCR6^+$-lineage cells have been found to affect synovial fibroblasts in rheumatoid arthritis (31), thus further investigation of these cells in patients with Scadding stage IV seems an attractive line of investigation. Nevertheless, based on findings in this thesis it is very likely that factors contributing to Th17-Th17.1 plasticity in sarcoidosis lungs are likely to include target candidates to inhibit ongoing chronic inflammation.

For example, IL-23R signaling was found to be essential for conversion of Th17 cells towards Th17.1 cells in mice (39, 40) and two independent polymorphisms within the IL23R gene were associated with (chronic) sarcoidosis (41, 42). A monoclonal antibody that binds to the shared p40 subunit of IL-12 and IL-23 (Ustekinumab) has been evaluated in chronic pulmonary sarcoidosis with the rationale to inhibit both Th1 and Th17 cell induction (43). However, no obvious effect on forced vital capacity (FVC) change and secondary endpoints were detected in this trial (43), although it was argued that the trial design of this study may have contributed to a failure to detect a potential effect of Ustekinumab (44). Data of this thesis (Chapter 4 (3) and 5) show that not Th1 cells, but specifically Th17-lineage cells are affected in pulmonary sarcoidosis. Since Th17.1 cells are likely most pathogenic in sarcoidosis (Chapter 4 (3)), an antibody directed against solely IL-23R-signaling, i.e. anti-IL-23p19 (Tildrakizumab/Guselkumab), which was recently described to be successful in psoriasis (45), may be a more attractive alternative for evaluation in pulmonary sarcoidosis.

Nevertheless, future research should first aim to clarify function (e.g. T-cell receptor specificity and cytokine production) of Th17-lineage cells in pulmonary sarcoidosis, since the role of these distinct T-cell subsets in pulmonary sarcoidosis (and Lofgren's disease) remain relatively under-investigated and inconclusive (as previously discussed). Identifying for example T-cell receptor specificity of these cells can help evaluate whether they are of oligoclonal or polyclonal origin and therefore targeted at the putative antigen or randomly attracted, respectively. Finally, *in-vitro* studies should aim to evaluate the role of IL-23R-signaling and other factors in Th17-Th17.1 cell plasticity in chronic pulmonary sarcoidosis.

Optimizing current prednisone treatment: A role for early dose tapering?

Prednisone, the current mainstay of pulmonary sarcoidosis therapy, is immunosuppressive but not curative. It restores the Treg/Th17 ratio in pulmonary sarcoidosis, while leaving Th1 and Th2 cells unaffected (17-19), confirming an important role for a disruption of a delicate balance between these cells in active pulmonary sarcoidosis (Chapter 6) (25). As it is debated whether prolonged therapy affects the course of the disease or prevents complications such as developing fibrosis in the future (20), it is now

thought that prednisone therapy in pulmonary sarcoidosis needs to be given as long as the disease is active and clinical symptoms demand suppression.

The optimal prednisone treatment strategy in newly-treated patients that controls symptoms and inflammation, while minimizing serious side-effects, remains unknown. Findings described in this thesis (Chapter 7 and 8) suggest that current treatment strategies can be adjusted in order to optimize prednisone therapy, i.e. by early initiation of dose tapering, guided by daily home spirometry.

Results described in Chapter 7 indicate that there is no clear correlation between prednisone dose given for newly-treated pulmonary sarcoidosis and FVC change, while cumulative prednisone did correlate with weight increase in the long term. A lower cumulative prednisone dose in the long term (12 months) was mainly characterized by early dose tapering, i.e. to below 10 milligrams (mg)/day within 3.5 months, and to a lesser extent by the initial starting dose. Results of a prospective, observational study that carefully home-monitored early treatment responses in newly-treated pulmonary sarcoidosis patients (Chapter 8) further showed that most (>90%) of the change in FVC and dyspnea symptoms already occurs within 2-3 weeks, remaining stable up to 3 months during dose tapering.

Together these data suggest that prednisone therapy in newly-treated pulmonary patients, that is predominantly focused on preserving lung function (i.e. FVC) and controlling pulmonary symptoms, will likely benefit from earlier clinical evaluation, which could lead to earlier and personalized prednisone dose tapering, preventing side effects while retaining effect.

In our retrospective study in Chapter 7 we did not find evidence that FVC response to a lower prednisone dose treatment strategy is influenced by characteristics of severe disease at start of treatment. It is tempting to speculate that patients receiving a high cumulative dose, often exhibiting more severely impaired lung function at the start of treatment, were tapered off of prednisone more slowly in pursuit of a 'normal' lung function. Indeed, prednisone therapy with more than 10 mg/day in pulmonary sarcoidosis patients might be warranted in certain cases. For example, a very low FVC at start of treatment can prolong time to recovery of the lung function to a level that relieves symptoms (Chapter 8). Ultimately, the treatment goal (e.g. prevention of organ damage, improvement versus 'normalization' of lung function, improvement of symptoms and/or quality of life) and with that the treatment strategy will remain a topic of careful, individual assessment and discussion between patient and caregiver. Our data on short term effects and long term side effects may help in making a balanced assessment. Long-term follow up of our prospective trial cohort (Chapter 8) should reveal whether early changes in FVC can predict therapy outcome in the long-term, such as the need for second- and third-line treatments (46).

Importantly, data in Chapter 8 of this thesis validate the (daily) use of a hand-held spirometer to careful home monitor patients' individual early lung function response to therapy in pulmonary sarcoidosis. However, ideally, in the future, an approach that integrates multiple home monitored features, such as pulmonary function, but also patient-reported outcomes, would further increase accuracy of clinical evaluation (13) and will help further personalize treatment of pulmonary sarcoidosis patients, both of new onset as well as patients suffering from chronic sarcoidosis. In order to reach such a goal, a study comparing effectivity and steroid-sparing potential of an internet-based home monitoring system that adjusts prednisone treatment daily and/or weekly (47) with current clinical practice of prednisone treatment for pulmonary sarcoidosis patients in a randomized fashion could be conceived.

Outstanding questions for future research

This thesis provided new questions for future research in pulmonary sarcoidosis, that is to say:

- Can a Treg and/or Th17-lineage cell-associated factor serve as a new biomarker in pulmonary sarcoidosis?
- Can the epigenomic signature of circulating T cells function as new potential biomarker in pulmonary sarcoidosis?
- What is the TCR specificity of Th17.1 cells in pulmonary sarcoidosis?
- What factors contribute to Th17-Th17.1 plasticity in pulmonary sarcoidosis?
- What is the role of $CCR6^+$ Th17-lineage cells in fibrotic (Scadding stage IV) sarcoidosis?
- What molecular/cellular link between $CCR6^+$ Th17-lineage cells and Tregs contributes to granuloma formation in pulmonary sarcoidosis?
- Can CTLA4-Ig and/or anti-IL23p19 serve as a new therapy for pulmonary sarcoidosis?
- Can frequent home monitoring be employed to optimize and personalize treatment of pulmonary sarcoidosis patients?

Concluding remarks

Findings described in this thesis provide a new perspective on sarcoidosis pathogenesis, challenging long-held paradigms (Chapter 3 (3)) and providing novel (technical) approaches to further unravel this immunological complex disease (Chapter 6 (25)). The importance of carefully characterizing T-cell subsets (including proportions, phenotype and predisposition to plasticity) in distinct affected organs (lungs, mediastinal lymph nodes and peripheral blood) in pulmonary sarcoidosis is highlighted, and is likely part of the key to further understand this enigmatic disease.

In this sense it should be noticed that research presented in this thesis has focused on the role of T-cell subsets in pulmonary sarcoidosis, however the importance of an-

tigen presenting cells (macrophages, dendritic cells and B cells (48)) as being part of sarcoidosis etiology is acknowledged by the author (see Chapter 1 (6)). Ideally, the total immune profile of distinct organs in pulmonary sarcoidosis patients is characterized, and techniques analyzing 'big immunological data' in an unsupervised fashion should be applied (49) to determine other key role players or interactions between immune cells and derived factors that determine disease etiology/outcome.

Furthermore, because of the heterogeneity of disease presentation, it is conceivable that the immunological profile of patients is also personally determined and that similar to prednisone therapy (Chapter 7 and 8), treatment with other (new) immune modulating therapies should be personalized, depending on what aberrant immunological response is predominant. Detailed clinical characterization of patients is highly warranted during immunological profiling.

Data in this thesis also point towards a striking overlap between sarcoidosis and other auto-immune disorders, such as rheumatic arthritis, systemic lupus erythematosus and psoriasis. Developments in the autoimmunity field in both immunology and clinical therapies should be carefully followed and validated/applied in sarcoidosis research. Indeed, this thesis helped to provide a rationale to evaluate the potential of certain existing therapies that are used/evaluated in other auto immune diseases. Although the search for the ideal experimental model for sarcoidosis remains ongoing, current models mimicking granuloma formation can at least be considered for pre-clinical evaluation of potential efficacy of these drugs (50).

Finally, results of this thesis provided practical clinical suggestions that may be applied in current treatment policies. The main challenge for future research lies within integrating these basic immunological and practical findings into current clinical practice and therefore a tight collaboration between physicians and researchers remains most essential. Thus to conclude, to suffer from pulmonary sarcoidosis, *It Takes Two To Tangle*, to solve it, it will (at least) *Take Two To Tango!*

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Summary of the thesis (English)

SUMMARY OF THE THESIS

Pulmonary sarcoidosis is an intriguingly complex immunological disorder. It is characterized by granuloma formation in the lung and the lung-draining mediastinal lymph nodes (MLN), upon chronic antigenic stimulation of unknown origin. Sarcoid granulomas can also affect the eyes and the skin, and in rare cases the heart, liver, central nervous system or bones.

A granuloma is a conglomerate of immune cells and formation of a granuloma likely occurs in order to optimize interactions between antigen presenting cells and adaptive immune cells, such as T cells. Typically, a fibrotic rim encapsulates the granuloma, aiding in preventing dissemination of the (infectious) antigen. A granuloma can arise in response to infectious agents such as mycobacteria and fungi, but also inorganic agents, such as silica and beryllium, can induce granuloma formation. Although several antigens have been proposed to be part of sarcoidosis etiology, not one antigen has yet been identified that can explain all cases of the disease. Therefore, sarcoidosis is considered a multisystem granulomatous disorder of unknown cause.

The disease affects people all over the world, but the incidence/prevalence rates and natural course of the disease vary greatly. For example, the incidence rate of sarcoidosis is approximately three times higher in Afro-Americans than Caucasians. Also, granulomas tend to resolve spontaneously in the majority of patients, but in approximately 30% of the patients, granulomas persist, which can lead to chronic, sometimes progressive disease with need for treatment. Afro-American women with pulmonary sarcoidosis have a higher chance of developing chronic disease. In contrary, clinical presentation with the Löfgren triad, that is lung bihilar lymphadenopathy on the lungs alongside uveitis and erythema nodosum or ankle arthritis, is typically associated with a good prognosis and occurs more often in Caucasians.

Gaps in knowledge on sarcoidosis disease etiology and determinants of disease course have thus far impeded rational decisions on 'Who, When and How' to treat. The mainstay of sarcoidosis therapy remains immunosuppressive, using relatively high doses of systemic prednisone. However, although often effective, corticosteroid treatment can be accompanied by severe side effects and knowledge on optimal dosing strategies balancing effects and side effects, is lacking. Detailed insight in the immunological response occurring in pulmonary sarcoidosis patients can help find determinants of disease etiology and disease course, which can yield new therapeutic targets. Therefore, the first aim of this thesis is to further unravel the immune-related pathogenesis of pulmonary sarcoidosis (**Part 1**). The second aim of this thesis is to gain insight into current prednisone treatment, in order to optimize treatment strategies to reduce side-effects and increase quality of life of pulmonary sarcoidosis patients (**Part 2**).

Part 1: Unraveling the pathogenesis of pulmonary sarcoidosis

A well-developed sarcoid granuloma consists of a tightly formed conglomerate of epithelioid- and multinucleated-giant cells encircled by lymphocytes, especially $CD4^+$ T helper (Th) cells, but also rare $CD8^+$ T cells and B cells. In **Chapter 1** a model for granuloma formation in pulmonary sarcoidosis is proposed, combining careful clinical observations with existing evidence in literature. Available evidence suggests that an unknown antigen in pulmonary sarcoidosis activates interstitial dendritic cells (DCs) and alveolar macrophages (AMs). This process is possibly initiated by toll-like receptor-2 (TLR-2) ligands in pulmonary sarcoidosis, such as *Mycobacterium tuberculosis*-derived ESAT-6 or mKatG proteins. Following stimulation, DCs migrate towards MLN where they initiate differentiation and expansion of Th1 and Th17 cells. Simultaneously, TLR-2 stimulation of AMs initiates a cascade of cytokine and chemokine production in the lungs, including production of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), C-C motif chemokine ligand 2 (CCL2), CCL20 and C-X-C motif chemokine ligand 10 (CXCL10). This process leads to cellular aggregation, formation of multinucleated giant cells, and attraction of important building blocks of the sarcoid granuloma, namely Th1/Th17 cells, monocytes and regulatory T cells (Tregs).

Granuloma formation in pulmonary sarcoidosis is widely considered as an exaggerated Th1/Th17 immune response upon exposure to an unknown antigen. However, mechanisms contributing to this exaggerated response in pulmonary sarcoidosis patients remain largely obscure. Key players in a disease are often identified by mouse models that lack or overexpress a specific protein or cell type of interest. Development of a universally accepted mouse model for sarcoidosis has proven difficult, hampering research efforts employing this strategy. Interestingly, in **Chapter 2** we describe how emerging clinical observations in human cancer treatment shed new light on key players in sarcoidosis pathogenesis and helped identify a key role for Tregs and Th17 cells in pulmonary sarcoidosis.

As presented in Chapter 2, more and more case reports describe occurrence of sarcoidosis or sarcoid-like disease during "re-boosting" of the immune system in cancer patients using anti-cytotoxic T-lymphocyte antigen 4 (CTLA4) agents. CTLA4 is an important suppressor of T-cell mediated immune responses, thus this phenomenon provided a rationale to look into CTLA4 expression in sarcoidosis-derived T cells. Intriguingly, we found that specifically Tregs and Th17 cells in sarcoidosis MLN showed aberrant expression of this important immune suppressive protein. A model is proposed whereby this phenomenon can cause double trouble in sarcoidosis, i.e. by increasing Th17 cell responses (thereby affecting the delicate Treg/Th17 balance, since Treg proportions remained unaffected) and impairing Treg function.

Failure of immune regulation by Tregs has long been suggested to contribute to ongoing inflammatory responses in pulmonary sarcoidosis. However, factors contributing

to this phenomenon remained largely unknown and conflicting data was published regarding their proportions in various affected organs. In **Chapter 3** we aimed to systematically investigate the role of Tregs in pulmonary sarcoidosis by analyzing their proportions, phenotype, survival and apoptotic susceptibility. We confirmed that circulating sarcoidosis-derived Tregs have an impaired immune suppressive function, and clarified that no evidence was found for a numeral deficit, neither locally nor systemically. Rather, circulating Treg proportions were significantly increased in sarcoidosis patients, specifically in patients developing chronic disease. Also, no evidence was found for decreased expression of important immunosuppressive proteins, such as FoxP3, CD25 or CTLA4 on circulating Tregs. However, importantly, circulating sarcoidosis Tregs were found to be highly apoptotic, likely contributing to an impaired function of these cells in pulmonary sarcoidosis.

Whereas Th17 cells were previously found to be increased in patient lungs, sarcoidosis was still largely thought to be a Th1 mediated disease, because the total proportions of interleukin (IL)-17-producing cells remained very low. However, our findings in Chapter 2 suggested a key role for Th17 cells in pulmonary sarcoidosis and concomitantly it became clear in the T-cell field that stringent classification of T cells based on cytokine production may not be correct. Certain T-cell subsets, such as Th17 cells, are found to be more prone to plasticity and can adopt cytokine production capabilities of other T-cell subsets. We previously identified increased proportions of IL-17A/IFN-γ-double-producing memory Th cells in a small sarcoidosis patient cohort and therefore hypothesized that the role of Th17 cells in pulmonary sarcoidosis may be greater than anticipated. Th17 cells perhaps play an important role in the disease pathogenesis by mimicking Th1 cytokine (IFN-γ) production. So called Th17.1 cells are known for this ability in other disease states. In **Chapter 4** we aimed to identify the origin of IFN-γ-producing cells in pulmonary sarcoidosis. And indeed, we found that not Th1 cells, but specifically Th17-derived IFN-γ-producing cells (Th17.1 cells) are increased in sarcoidosis and responsible for the exaggerated IFN-γ production in sarcoidosis lungs, challenging long-held paradigms in the sarcoidosis field.

Primary Th cell activation and subset differentiation occurs in lung draining MLN. Above mentioned IFN-γ-producing Th17.1 cells can derive from Th17 cells under influence of a specific cytokine milieu. Therefore, we hypothesized that in pulmonary sarcoidosis Th17 cells are primarily induced in the MLN and that the granulomatous environment in the lungs induces plasticity towards Th17.1 cells. In **Chapter 5** we challenged this hypothesis and aimed to gain more insight in the induction of Th17.1 cells in sarcoidosis by looking into proportions and phenotypic characteristics of $CCR6^+$ Th17-lineage cells in sarcoidosis and control MLN, and sarcoidosis lungs. The results of this study suggest a model whereby Th17 cells are induced in sarcoidosis lymph nodes, and show plasticity

towards pathogenic Th17.1 cells in sarcoidosis lungs, where they are particularly critical for development of chronic disease.

Finally, in **Chapter 6** recent insights in sarcoidosis T-cell immunology are reviewed. Based on new findings in Tregs, Th17.1 cells and checkpoint inhibitors we propose that it is very likely that a disruption of the delicate balance between helper and regulatory cells contributes to the exaggerated Th cell response in pulmonary sarcoidosis. Data presented in Part 1 of this thesis provide a new perspective on sarcoidosis pathogenesis and consequently on therapeutic targets.

Part 2: Optimization of prednisone treatment in pulmonary sarcoidosis

Prednisone therapy in pulmonary sarcoidosis is only indicated in certain cases, such as during development of a dangerous health situation or when there is a significant decrease in the patient's quality of life. Unfortunately, the optimal dose and duration of prednisone treatment remains uncertain, thus current guidelines include a broad range, which could result in low dose-treated patients versus high dose-treated patients, depending on the physician's practice, which might lead to unmet therapeutic goals or unnecessary side effects.

Identifying the optimal treatment strategy that preserves organ function and relieves symptoms, but simultaneously minimizes side-effects, may increase quality of life of newly-treated pulmonary sarcoidosis patients. Therefore, in order to gain insight in what dosing strategy has the best balance between effect and side-effects, in **Chapter 7** we aimed to describe forced vital capacity (FVC) (effect) and weight (side-effect) changes during different prednisone doses used in daily practice for treatment naïve pulmonary sarcoidosis patients. Using a retrospective, multicenter set-up, this study indeed showed clinical variation in prednisone treatment, but did not find evidence for a strong association between prednisone dose used and FVC change in newly treated sarcoidosis patients in the short (3-9 months) and long (12-24 months) term. However, weight gain positively correlated with cumulative prednisone dose used in the long term. The strategy leading to a lower dose at 12 months was characterized by early dose tapering, i.e. less than 10 mg/day at 3.5 months. Importantly, we did not find evidence that FVC response to a lower prednisone dose treatment strategy is affected by characteristics of more severe disease at start of treatment. These results suggest that prednisone therapy, aimed at maintaining/improving FVC in pulmonary sarcoidosis can often be reduced in clinical practice and highlight the need for prospective trials that carefully monitor treatment efficacy of a lower dose treatment regimen, characterized by early dose taping, in newly treated sarcoidosis patients.

Results presented in Chapter 7 and available evidence suggested that early dose tapering is likely to reduce side-effects in prednisone therapy for pulmonary sarcoidosis. It was previously suggested that prednisone effect on FVC occurs quickly within one

month. However, a prospective study monitoring individual lung function changes on a daily basis in newly-treated sarcoidosis patients to identify effect optimum, and also including clinical symptoms, is lacking. Therefore, in **Chapter 8** results are presented of a prospective, observational study, evaluating early FVC and symptom changes during prednisone treatment and tapering in newly-treated sarcoidosis patients, using daily home spirometry. Results of this study showed that most of the improvement in lung function and symptoms occur within 2-3 weeks during first-line prednisone treatment. Importantly, these results suggest that frequent (home) monitoring of lung function and symptoms has the potential to individualize prednisone therapy, aiming at early dose tapering, side-effect reduction and increasing patient-related quality of life.

Several results of this thesis may be of value for clinical practice and are highlighted in the **Epilogue.** From this thesis it is evident that Th17-lineage cells and Tregs are likely involved in immunological responses that contribute to ongoing inflammation in pulmonary sarcoidosis, thus prospective studies evaluating Treg and Th17-lineage cell-associated factors as predictor of disease course or disease activity are expected to have implications for clinical practice in the future. Intriguingly, results of this thesis provide a rationale to evaluate the potential of both novel and existing therapies in pulmonary sarcoidosis, such as CTLA4-Ig (e.g. abatacept) or anti-IL-23p19 (e.g. tildrakizumab/guselkumab). And finally, results of this thesis provided practical clinical suggestions that could be applied in current prednisone treatment policies. The main challenge for future research lies within integrating these basic immunological and practical findings into current clinical practice and therefore a tight collaboration between physicians and researchers remains most essential. Thus to conclude, to suffer from pulmonary sarcoidosis, *It Takes Two To Tangle*, to solve it, it will (at least) *Take Two To Tango!*

Summary of the thesis (Dutch)

SAMENVATTING VAN HET PROEFSCHRIFT

Pulmonale sarcoïdose is een intrigerend complexe immunologische aandoening. De ziekte wordt gekarakteriseerd door granuloom vorming in de longen en in de long-drainerende mediastinale lymfeklieren. De granulomen ontstaan door chronische antigeen-gemedieerde stimulatie van het immuunsysteem. De oorzaak hiervan is echter onbekend. Granulomen kunnen bij sarcoïdose ook worden aangetroffen in de ogen, de huid en in zeldzame gevallen ook in het hart, de lever, het centrale zenuwstelsel en in de botten.

Een granuloom is een opeenhoping van afweercellen en ontstaat zeer waarschijnlijk om de interactie tussen antigeen presenterende cellen (APC) en cellen van de verworven afweer, zoals T-cellen, te optimaliseren. Vaak wordt een granuloom omgeven door een fibrotische rand om verspreiding van een (infectieus) antigeen te voorkomen. Een antigeen is een molecuul dat in staat is een reactie van de afweer op te wekken. Een granuloom kan ontstaan als reactie op chronische stimulatie van de afweer door een infectieus agens, zoals een tuberculose bacterie of schimmel. Echter, ook anorganische stoffen, zoals siliciumdioxide en beryllium, kunnen granulomen induceren. Ondanks dat er enkele antigenen zijn gesuggereerd als oorzaak van sarcoïdose, is er niet één gevonden die alle gevallen van de ziekte kan verklaren. Daarom wordt sarcoïdose gedefinieerd als een granulomateuze, multisysteem ziekte van onbekende oorzaak.

Sarcoïdose komt voor bij mensen over de hele wereld, maar de klinische presentatie en het ziektebeloop variëren enorm tussen verschillende populaties. Zo komt de ziekte ongeveer drie keer vaker voor bij mensen van Afro-Amerikaanse afkomst dan bij mensen van Kaukasische afkomst. Bij de meerderheid van de mensen verdwijnen de granulomen spontaan, maar bij ongeveer 30% van de patiënten blijven de granulomen chronisch aanwezig. Dit kan leiden tot blijvende, soms progressieve klachten met noodzaak tot therapie. Het is niet goed te voorspellen wie chronisch ziek zal worden, maar het is bijvoorbeeld wel bekend dat Afro-Amerikaanse vrouwen hier een grotere kans op hebben. Daartegenover staat dat een klinische presentatie met het Löfgren syndroom bijna altijd gepaard gaat met een snel herstel. Het syndroom wordt gekenmerkt door een trias van problemen bij klinische presentatie, namelijk: bihilaire lymfadenopathie, uveitis en erythema nodosum en/of enkel artritis. Het Löfgren syndroom komt vaker voor bij mensen van het Kaukasische ras.

Aangezien we nog weinig weten over de ziekte etiologie en voorspellers van het ziektebeloop, blijft het nog onzeker wie, wanneer en hoe we exact moeten behandelen. De huidige behandeling voor pulmonale sarcoïdose is slechts immunosuppressief, niet curatief, waarbij in de eerste instantie aanzienlijke hoeveelheden prednison worden voorgeschreven. Ook al is prednison behandeling vaak effectief in sarcoïdose; het leidt helaas ook tot het ontstaan van vervelende bijwerkingen. Goede kennis over de opti-

male behandelstrategie, waarbij het therapie effect van prednison in balans is met de bijwerkingen, mist.

Inzicht in de immunologische respons die optreedt bij pulmonale sarcoïdose draagt bij aan het vinden van factoren die een aandeel hebben in het ontstaan van de ziekte én factoren die het beloop van de ziekte beïnvloeden. Dit kan nieuwe aangrijpingspunten onthullen voor effectievere therapieën. Om deze reden was het primaire doel van dit proefschrift om de immuun-gerelateerde pathogenese van pulmonale sarcoïdose verder te ontrafelen (**Deel 1**). Het tweede doel was om inzicht te krijgen in de huidige prednison behandeling, ter optimalisatie van hedendaagse behandelstrategieën om daarmee bijwerkingen te minimaliseren en de kwaliteit van leven van patiënten te verbeteren (**Deel 2**).

Deel 1: Het ontrafelen van de pathogenese van pulmonale sarcoïdose

Een granuloom in sarcoïdose is een opeenhoping van epithelioide histiocyten en meerkernige reuscellen, die worden omringd door lymfocyten, met name $CD4^+$ T-helper (Th) cellen, maar ook in mindere mate $CD8^+$ T cellen en B cellen. In **Hoofdstuk 1** presenteren wij een model voor granuloomvorming in pulmonale sarcoïdose door klinische observaties te combineren met bestaande literatuur over granuloomvorming. Bestaande informatie suggereert dat een onbekend antigeen de interstitiële dendritische cellen (DCs) en alveolaire macrofagen (AMs) activeert. Dit proces wordt waarschijnlijk gemedieerd door Toll-like receptor-2 (TLR-2) agonisten, zoals bijvoorbeeld de van *Mycobacterium tuberculosis*-afkomstige eiwitten ESAT-6 of mKatG. Na stimulatie migreren interstitiële DCs naar de mediastinale lymfeklieren, alwaar ze differentiatie en expansie van Th1 en Th17 cellen initiëren. Tegelijkertijd leidt TLR-2-gemedieerde stimulatie van AMs tot de initiatie van een cascade aan cytokine- en chemokine productie in de longen. Eerst wordt de productie van tumor necrosis factor-α (TNF-α) door AMs geïnitieerd, waarna de productie van interferon-γ (IFN-γ), C-C-motief chemokine ligand 2 (CCL2), CCL20 en C-X-C-motief chemokine ligand 10 (CXCL10) volgen. Dit proces leidt tot cellulaire aggregatie, formatie van meerkernige reuscellen en chemoattractie van belangrijke bouwstenen van het granuloom. Zo worden onder andere monocyten, pro-inflammatoire Th1/Th17 cellen en immunosuppressieve regulatoire T-cellen (Tregs) aangetrokken.

Het wordt gedacht dat granuloomvorming in pulmonale sarcoïdose ontstaat door een (chronische) overmatige Th1/Th17 immuunrespons na expositie aan een onbekend antigeen (Hoofdstuk 1). Echter, de mechanismen die leiden tot het ontstaan van deze overmatige respons blijven grotendeels obscuur. Vaak worden met behulp van een muismodel factoren ontdekt die sterk bijdragen aan het ontstaan van een ziekte, bijvoorbeeld doordat een specifiek eiwit of een celtype niet of juist overmatig kan worden aanmaakt. Ontwikkeling van een universeel geaccepteerd muismodel voor sarcoïdose is moeizaam gebleken, waardoor bovengenoemde aanpak wordt belemmerd. Echter,

in **Hoofdstuk 2** beschrijven wij hoe klinische observaties in kankerbehandeling nieuwe inzichten geven in de pathogenese van sarcoïdose en een belangrijke rol identificeert voor Tregs en Th17 cellen.

Zoals wordt gepresenteerd in Hoofdstuk 2 worden er meer en meer casussen beschreven over het ontstaan van sarcoïdose of sarcoïde-achtige reacties tijdens het *boosten* van het immuunsysteem in kankerpatiënten met anti-cytotoxic T-lymfocyte antigen 4 (CTLA4). CTLA4 is een sterke onderdrukker van de T-cel-gemedieerde immuunrespons, dus dit fenomeen leverde een rationale om naar CTLA4 expressie in sarcoïdose T-cellen te kijken. Intrigerend genoeg vonden wij dat specifiek Tregs en Th17 cellen in sarcoïdose mediastinale lymfeklieren een verminderde expressie hebben van dit belangrijke immunosuppressieve eiwit. In dit hoofdstuk wordt een model voorgesteld waarbij dit fenomeen kan leiden tot een dubbel probleem ('double trouble') in pulmonale sarcoïdose, namelijk door het versterken van de Th17 cel respons (daarbij wordt de delicate Treg/Th17 balans ontwricht, aangezien de Treg proporties onveranderd bleven) en het verminderen van de immunosuppressieve Treg functie.

Een verminderde suppressieve Treg functie is eerder beschreven in pulmonale sarcoïdose en het is ook gesuggereerd dat dit kan bijdragen aan het ontstaan van chronische inflammatie. Echter, factoren die bijdragen aan deze verminderde Treg functie blijven onbekend. Ook is er nog veel tegenstrijdige data over Treg aantallen in verschillende aangedane organen. In **Hoofdstuk 3** analyseren wij systematisch de rol van Tregs in pulmonale sarcoïdose, door te kijken naar proporties, het fenotype, de overleving en apoptotische gevoeligheid. We bevestigen dat circulerende Tregs in pulmonale sarcoïdose een verminderde immunosuppressieve werking hebben, maar vinden geen aanwijzing voor een tekort in aantallen, zowel in het bloed als lokaal in de longen. In tegendeel, het aantal circulerende Tregs was juist verhoogd op het moment van diagnose, en met name bij patiënten die binnen twee jaar chronische, progressieve ziekte ontwikkelen. Ook vonden wij geen aanwijzingen voor een verminderde expressie van immunosuppressieve eiwitten, zoals FoxP3, CD25 en CTLA4, in of op de circulerende Tregs. Echter, circulerende Tregs in pulmonale sarcoïdose vertoonden wel een verhoogde expressie van CD95, een verhoogde apoptotische gevoeligheid en verminderde overleving in vergelijking met gezonde controles. Dit laatste fenomeen draagt zeer waarschijnlijk bij aan een verminderde immunosuppressieve functie van circulerend Tregs in pulmonale sarcoïdose.

De aanwezigheid van Th17 cellen is eerder beschreven in pulmonale sarcoïdose, echter de ziekte wordt nog voornamelijk beschouwd als een Th1-gemedieerde ziekte aangezien de aantallen interleukine (IL)-17-producerende cellen erg laag ligt. Echter, onze bevindingen in Hoofdstuk 2 suggereerden toch een belangrijke rol voor Th17 cellen in pulmonale sarcoïdose. Tegelijkertijd werd het in het T-cel veld bekend dat classificatie van T-cellen puur op basis van cytokineproductie alleen niet correct blijkt.

Sommige T-cellen, zoals Th17 cellen, kunnen plasticiteit vertonen en kunnen cytokines produceren die oorspronkelijk door andere T-cel lijnen worden geproduceerd. Eerder hebben wij al de aanwezigheid van IL-17A/IFN-γ-dubbel-producerende cellen aangetoond in de longen van een klein sarcoïdose cohort en daarom stelden wij de hypothese dat de rol van Th17 cellen in pulmonale sarcoïdose mogelijk veel groter is dan eerder geanticipeerd. Mogelijk spelen Th17 cellen een grotere rol dan we denken door de productie van IFN-γ, waarmee ze Th1 cellen nabootsen. Zogenoemde Th17.1 cellen staan hier bekend om en zijn eerder aangetroffen in andere immuun-gemedieerde ziektes, zoals reumatoïde artritis. In **Hoofdstuk 4** hadden we daarom als doel om de oorsprong van IFN-γ-producerende cellen vast te stellen. Wij vonden inderdaad dat niet Th1 cellen, maar specifiek Th17-afkomstige IFN-γ-producerende (Th17.1) cellen verhoogd zijn in sarcoïdose en verantwoordelijk zijn voor de eerder geobserveerde verhoging van IFN-γ in sarcoïdose longen. Dit inzicht resulteert in een ware paradigma verandering in het sarcoïdose veld.

Primaire T-cel activatie en differentiatie gebeurt in long-drainerende mediastinale lymfeklieren, terwijl bovengenoemde IFN-γ-producerende Th17.1 cellen elders kunnen ontstaan uit Th17 cellen onder invloed van een specifiek cytokine milieu. Om deze reden hadden wij als hypothese dat in pulmonale sarcoïdose, Th17 cellen primair worden geïnduceerd in de mediastinale lymfeklieren en dat de granulomateuze omgeving in de longen plasticiteit van Th17 cellen induceert richting Th17.1 cellen. In **Hoofdstuk 5** onderzochten wij deze hypothese en stelden als doel om meer inzicht te krijgen in de inductie van Th17.1 cellen in sarcoïdose door naar de proporties en het fenotype te kijken van $CCR6^+$ Th17-afkomstige cellen in sarcoïdose en controle mediastinale lymfeklieren en in sarcoïdose longen. Resultaten van deze studie tonen aan dat Th17 cellen in pulmonale sarcoïdose worden geactiveerd in de mediastinale lymfeklieren en suggereren sterk dat de granulomateuze omgeving van de longen plasticiteit induceert naar pathogene Th17.1 cellen, alwaar deze cellen kunnen bijdragen aan het ontstaan van chronische ziekte.

Tot slot vatten wij in **Hoofdstuk 6** recente bevindingen over de immunologische T-cel respons in pulmonale sarcoïdose samen. Gebaseerd op nieuwe bevindingen in Tregs, Th17.1 cellen en co-stimulatoire en co-inhibitoire moleculen stellen wij een model voor waarbij een ontregelde balans tussen helper en regulatoire cellen zeer waarschijnlijk bijdraagt aan de overmatige immuunrespons in pulmonale sarcoïdose. Data gepresenteerd in dit eerste deel van het proefschrift bieden een nieuw perspectief op de pathogenese van pulmonale sarcoïdose en daardoor op aangrijpingspunten voor nieuwe therapieën.

Deel 2: Optimalisatie van prednison therapie in pulmonale sarcoïdose

Prednison is de eerste keus voor de behandeling van pulmonale sarcoïdose. Prednison therapie is alleen geïndiceerd in bepaalde gevallen, zoals wanneer er orgaanschade op-

treedt of wanneer er een significante daling is in de kwaliteit van leven van een patiënt. Helaas is de optimale dosis en duur van de behandeling niet bekend, dus huidige richtlijnen handhaven ruime adviezen waardoor patiënten op verschillende manieren kunnen worden behandeld, vaak afhankelijk van de voorkeur van de behandelend arts. Mogelijk leidt dit tot suboptimale resultaten of juist onnodige bijwerkingen.

Het vaststellen van de optimale behandelstrategie die orgaanfunctie behoudt en symptomen reduceert, maar tegelijkertijd de bijwerkingen minimaliseert, kan de kwaliteit van leven van sarcoïdose patiënten verhogen. Om meer inzicht te krijgen in de balans tussen effect en bijwerkingen van huidige behandelstrategieën hebben wij in **Hoofdstuk 7** als doel gesteld om de geforceerde expiratoire vitale capaciteits- (FVC) (effect) en gewichts- (bijwerking) veranderingen te beschrijven die plaatsvinden in de huidige praktijk na het initiëren van prednison therapie in nieuw behandelde pulmonale sarcoïdose patiënten. Middels een retrospectieve, multicenter opzet hebben wij ontdekt dat de huidige klinische praktijk van prednison behandeling inderdaad varieert, echter dat er geen sterke correlatie is tussen de prednison dosis die werd gebruikt en de FVC-verandering die optrad. Echter, de toename in gewicht correleerde significant met de cumulatieve prednison dosis die was gebruikt op de lange termijn (12-24 maanden). Behandelstrategieën die leidden tot een lage cumulatieve prednison dosis op de lange termijn werden met name gekarakteriseerd door het snel afbouwen van de prednison dosis, namelijk naar minder dan 10 mg/dag binnen 3,5 maand. We vonden geen aanwijzingen dat de FVC-respons op therapie werd beïnvloed door karakteristieken van ernstigere ziekte tijdens start van de behandeling. Deze resultaten suggereren dat prednison therapie in pulmonale sarcoïdose, gericht op het behouden en/of verbeteren van de FVC, kan worden gereduceerd in de huidige klinische praktijk. Deze retrospectieve studie verschaft ook een rationale om een prospectieve studie op te zetten waarbij nieuw behandelde sarcoïdose patiënten eerder worden afgebouwd van de prednison, gegeven dat zij nauwlettend worden gevolgd.

Resultaten van Hoofdstuk 7 en bestaande publicaties suggereren dat het snel afbouwen van prednison zeer waarschijnlijk leidt tot het verminderen van bijwerkingen in pulmonale sarcoïdose. Het is eerder gesuggereerd dat het effect van prednison op de FVC al optreedt binnen een maand. Echter, een prospectieve studie die individuele longfunctie veranderingen dagelijks vastlegt om het daadwerkelijke effect optimum vast te stellen mist nog. In **Hoofdstuk 8** worden resultaten gepresenteerd van een prospectieve, observationele studie die vroege veranderingen in FVC en symptomen vastlegt tijdens prednison behandeling voor pulmonale sarcoïdose, middels thuis spirometrie. Resultaten van deze studie tonen aan dat het grootste deel van het behandel effect op longfunctie en symptomen al optreedt binnen 2-3 weken. Resultaten van deze studie suggereren verder dat dagelijkse thuismonitoring van de longfunctie en symptomen de potentie heeft om prednison behandeling van sarcoïdose patiënten te individualiseren.

Frequente monitoring is dan gericht op het snel afbouwen van de prednison dat de potentie heeft om bijwerkingen te verminderen en daarmee de kwaliteit van leven van pulmonale sarcoïdose patiënten verbetert.

Verschillende resultaten van dit proefschrift zijn waardevol voor de klinische praktijk en deze worden besproken in de **Epiloog**. Door de studies beschreven in dit proefschrift is het duidelijk geworden dat $CCR6^+$ (van Th17-afkomstige) cellen en Tregs betrokken zijn bij de immunologische respons die leidt tot overmatige inflammatie in pulmonale sarcoïdose. Naar verwachting zullen prospectieve studies, die evalueren of Treg- en Th17(.1) cel-geassocieerde factoren kunnen fungeren als betrouwbare biomarker, invloed hebben op de toekomstige klinische praktijk. Verder verschaffen resultaten van dit proefschrift een rationale om de potentie van reeds bestaande (CTLA4-Ig, zoals abatacept) en nieuwe (anti-IL-23p19, zoals tildrakizumab/guselkumab) therapieën te evalueren in pulmonale sarcoïdose. Als laatste geeft dit proefschrift suggesties om de huidige prednison behandeling aan te passen. Een grote uitdaging blijft natuurlijk om deze basale en klinische onderzoeksgebieden te integreren in studies die toepasbaar blijken in de klinische praktijk. Een nauwe samenwerking tussen experimentele wetenschappers en artsen blijft daarvoor essentieel. Kortom, om sarcoïdose te krijgen, *It Takes Two to Tangle*, om sarcoïdose op te lossen, *It Takes (at least) Two to Tango!*

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Mijn promotieteam

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Mijn *extended* promotieteam

Naast mijn officiële promotieteam, heb ik nog een *extented* promotieteam gehad.

Menno, *dude*!, hoe had ik dit promotietraject overleefd zonder jou?! Als ik terugdenk aan de eerste keer dat ik jou ontmoette dan had ik dit denk ik niet voor ogen gehad. Marthe stelde me aan je voor tijdens de lunch toen ik een dagje kwam kijken op het lab. Je schoof naast me en zei: 'Zo… jij gaat dus aan het sarcoïdose project werken? Succes ermee…Weet je zeker dat je de onderzoekswereld in wilt?' Je had me toen al voorbereid op jouw scepticisme. Voor mij bleek dit heel fijn. Ik houd ervan om goed werk af te leveren, en jij ook: voor niets gaat de zon op. Jij stelde mij kritische vragen en dan kwamen we er samen uit. Ik geloof dat ik vrij snel jouw vertrouwen had gewonnen en sindsdien ben je tot in de late uurtjes en vrije dagen loyaal geweest aan het sarcoïdose project. Nu nog steeds… Dankzij jou had ik elke dag een team gevoel als we weer begonnen aan de grote compartiment experimenten of tot in den treure de Treg assays moesten inzetten. Ik weet zeker dat dit traject veel zwaarder was geweest zonder jouw steun en plezierige aanwezigheid. In de flowkast hebben we samen kunnen lachen en huilen en op werk niveau hebben we naar mijn mening soms aan één blik genoeg. Ik voel me enorm vereerd dat jij me wil bijstaan tijdens mijn verdediging.

Prof. Hoogsteden, ik realiseer me maar al te goed wat een ruimte u voor mij heeft gecreëerd om mijzelf te kunnen ontwikkelen tijdens dit promotietraject. Ik ben erg dankbaar voor alle mogelijkheden die ik heb gekregen om mijn werk te kunnen presenteren op nationale en internationale congressen. Tevens heeft u tot het einde nog vertrouwen naar mij laten blijken door me te ondersteunen bij mijn plan om een research fellowship in Amerika te doen. Onder andere de kans om met zoveel verschillende nationaliteiten te kunnen brainstormen en samenwerken heeft mij doen realiseren hoe bijzonder ik onderzoek doen vind. Erg veel dank hiervoor.

Marlies, zoals al eerder beschreven, heb ik onder andere aan jou te danken dat ik in dit fantastische promotietraject terecht ben gekomen! Ik heb enorm genoten van jouw enthousiasme voor onderzoek doen en jouw netwerkkwaliteiten op congressen. Het was altijd enorm ontspannen om 's avonds een biertje met jou en Bernt te kunnen drinken na een intense congresdag. Bedankt voor je eerlijkheid, nuchterheid en eeuwig handige tips over carrière keuzes.

Linda, mijn eerste en enige student! Wat hebben wij onwijs veel gelachen. We hebben ons samen door een retrospectieve studie geworsteld waarover we tot op de dag van vandaag nog steeds een beetje '*tangled*' zijn. Wat mij in ieder geval heel duidelijk is

geworden is dat jij een doorzetter bent, want over de jaren heen ben je je altijd blijven inzetten om dit verhaal echt op papier te krijgen. Het kwam erop neer dat we dan onder het genot van een biertje, koffietje of taartje ons weer lachend blind zaten te staren op ons stuk. Een mooie bijkomstigheid hiervan is dat we elkaar over de jaren heen zijn blijven zien en op de hoogte zijn gebleven van elkaars ontwikkelingen: voornamelijk onze prachtige reizen in het buitenland! Voor mij blijft het WASOG-congres in Turkije een van de momenten waarop we elkaar beter hebben leren kennen en waarop ik me realiseerde dat ik ook maar beter geen 2e student kon gaan begeleiden, want die zou toch niet voldoen aan de lat die jij had gelegd! Zet hem op als ANIOS-gynaecologie, en we borrelen snel weer over het retroSTEPS stuk en de 'T3 die geen string heeft'.

De promotiecommissie

Mijn dank gaat ook uit naar Dr. E. Lubberts, Prof. Dr. J.C. Grutters en Prof. Dr. B.N.M. Lambrecht voor het kritisch lezen van het manuscript en het zitting nemen in de promotiecommissie.

Onderzoek deelnemers

Resultaten uit dit proefschrift zijn tot stand gekomen uit verschillende multicenter studies waar veel verschillende mensen bij betrokken zijn geweest.

Allereerst grote dank aan alle sarcoïdose patiënten die deel hebben genomen aan de sarcoïdose studie, GRANULOMA-trial, SARTREG-studie en STEPS-studie. De voortgang van sarcoïdose onderzoek staat of valt bij deze belangeloze deelname. Ook veel dank aan mensen die bloed of BAL hebben gedoneerd voor onze studies als controle weefsel. Verder wil ik Bregje ten Berge, Marthe Paats, Bob von Barthold en hun promotieteams bedanken voor hun inzet bij het verzamelen van sarcoïdose en controle weefsel waardoor mijn onderzoekstraject snel op gang kon komen.

Het opzetten van multicenter studies vraagt om veel organisatie, wat ik niet in mijn eentje had kunnen uitvoeren of bedenken. Annemarie, met jou werk ik al sinds het begin van mijn promotietraject, en ik ben enorm onder de indruk van het bedrijf dat jij in de tussentijd hebt opgezet om research op onze afdeling soepel te laten verlopen. Ik heb veel met je gelachen en ben jou en de andere research dames erg dankbaar voor de ondersteuning die jullie nog steeds geven bij het includeren en vervolgen van sarcoïdose studie patiënten. Marjolein, jij hebt veel werk van mij overgenomen het afgelopen jaar om de SARTREG en STEPS-studie door te kunnen laten lopen. Bedankt voor je punctualiteit en inzet hiervoor. Monique en Linda, bedankt voor jullie vergaande hulp en inzet bij het opzetten van de STEPS-studie en het protocolleren van het thuis-monitoren met spirometers. Frans en Hadassa, bedankt voor het opvangen van onverwachte inclusies en vragen uit andere klinieken! En ook dank aan Karin Lammering voor het ondersteunen van de STEPS-studie op de longfunctie afdeling.

Mirjam van Manen, dank voor je gezelligheid op congressen en je hulp bij het verwerken van vragenlijsten. Femke, dank voor je ondersteuning bij de STEPS-studie database.

Specifiek ook dank aan Leon, Marlies, Jelle en Karin (en de daaronder vallende artsassistenten) voor het includeren van patiënten voor de sarcoïdose studies in het EMC; Rogier en Kim voor het verzamelen van donor lymfeklieren; Robin en Robert voor de extra lymfeklier puncties en Marleen voor het zijn van onafhankelijk arts van al onze studies! Ook gaat mijn dank uit naar de prikzusters die vaak extra bloed hebben afgenomen van studie patiënten en controles. Dank ook aan de polidames voor hulp bij het inplannen van STEPS-studie patiënten. Dank aan Wilma en Orisia voor jullie administratieve ondersteuning.

Een enorm grote bijdrage aan het tot stand komen van dit proefschrift is geleverd door meerdere inclusie teams door deel te nemen aan de SARTREG en STEPS-studie. SFG-team: Hans, Benvinda en Simone (hoofd-includeerders van de SARTREG-studie!); Ikazia-team: Roxane en Jolanda; Amphia-team: Marco, Merijn, Brigitte en Ilse; MCH-team: Marieke, Jantine en Jacky; Zuyderland-team: Remy en Carla: heel veel dank aan jullie en jullie afdelingen voor de enorme inzet en hulp bij inclusies! Ik heb onze samenwerking als enorm prettig ervaren en hoop in de toekomst dit nog voort te kunnen zetten.

Verder wil ik erg graag benadrukken hoeveel geluk ik denk te hebben gehad met het promoveren op ons lab. Lab Longziekten, ik heb zo ontzettend veel leuke tijden met jullie gehad. De feestjes op de NVVI zijn onvergetelijk. Bedankt dat jullie allemaal zo benaderbaar zijn geweest tijdens mijn promotietraject, want ik denk dat ik ieder van jullie weleens heb lastiggevallen met vragen over het FACS-apparaat, buffers, antibodies en ga zo maar door. Speciaal wil ik nog even kort benoemen: Jen, dank voor je enorme hulp bij het verzamelen en opslaan van sarcoïdose materiaal en het *stand-by* staan voor mijn sarcoïdose projecten/experimenten. Ingrid, dank voor je hulp bij het in elkaar knutselen van FACS-kleuringen en actief meedenken tijdens de I&R-meeting over methodes. Koen, dank voor je vrolijkheid op het lab en je hulp bij onder andere de Luminex assay. Alex, dank voor je hulp bij het opstarten toen ik nog maar net was begonnen op het sarcoïdose project. Marjolein, dank voor je hulp bij het klaarmaken van samples voor genetische analyses. Margaretha en Floris, dank voor het helpen bij het verwerken van sarcoïdose materialen wanneer we het even niet hadden zien aankomen. En *last, but not least: Roomies*, bedankt voor het vormen van een hele fijne uitlaatklep en voor alle mooie tijden op feestjes bij congressen en op onze PhD kamer. Ik zal deze fijne tijd met jullie echt missen.

Caspar Looman, dank voor je enorme hulp bij het analyseren van alle (retro)STEPS-data in de afgelopen 1,5 jaar. Ondanks dat je nu met pensioen bent blijf je erg betrokken bij deze artikelen en daar ben ik je enorm dankbaar voor! Sandra en Jan Piet, bedankt voor de fijne samenwerking op analyses van T-cel subsets. Dankzij jullie hulp bij de

chemokine kleuring hebben wij veel relevante data kunnen halen uit zeldzame weefsels zoals de lymfeklieren.

Wat mij ook veel vertrouwen en stimulans heeft gegeven is dat zowel de Sarcoidose Belangenveneniging Nederland (SBN) als de Amerikaanse Foundation for Sarcoidosis Research (FSR) hun steun voor ons onderzoek hebben laten blijken. Best SBN-bestuur, dank voor jullie erkenning. *Dear FSR, thank you so much for your encouragements on our research on sarcoidosis.*

Furthermore, I would like to thank Laura Koth for a very successful collaboration on inducing a paradigm shift in sarcoidosis T cell immunology. It was such a pleasure to work with you on our paper(s) and brainstorming on interpretation of the data we both found independently on two other sides of the world. I hope to be able to keep on working with you on sarcoidosis research in the future.

Also, I would like to thank prof. Kaminski and the Kaminski lab for helping me discover that doing research wherever over the world means: being surrounded by passionate and pleasant people. Thank you so much for hosting me in New Haven in the summer of 2016. Now I know for sure that I want to keep on doing research in the future…

Ook nog speciale dank aan Arie Rietveld, mijn huidige opleider van de vooropleiding interne geneeskunde. Ik ken je al van voor mijn promotietraject en ben je dankbaar dat je zo flexibel bent geweest met wanneer ik kon starten met mijn vooropleiding. Mede dankzij u kreeg ik daarom de kans om de reis van mijn leven te maken en onderzoek te doen in Amerika. Veel dank hiervoor en ik kijk uit naar mijn verdere tijd in het Franciscus Gasthuis.

Als laatste wil ik benadrukken hoezeer ik heb ervaren tijdens dit promotietraject hoeveel geluk ik heb met lieve vrienden, tennisgenootjes en (schoon) familie om me heen. Dank allemaal voor jullie constante interesse in mijn onderzoek de afgelopen jaren en dat ik altijd zo mezelf mag zijn bij jullie.

Als laatste…

Sanne, mede-promovendus, mede-longarts-in-opleiding, mede-Linque-oprichtingsbestuursgenoot, mede-roeiteam/coach, maar het meeste nog: mijn hele goede vriendin! Wie had ooit kunnen bedenken dat wij beide via totaal verschillende ingangen op exact hetzelfde basale onderzoekslab terecht zouden komen, nadat we elkaar al zo lang kenden uit onze studententijd? Wat een geluk om 4 jaar lang een goede vriendin zo dicht in de buurt te mogen hebben tijdens een promotietijd. Lekker zeuren over hoe zwaar het allemaal wel niet is en lekker genieten van feestjes en congressen die daaraan verbonden zijn. San, wat top dat we nu ook nog eens op exact dezelfde dag mogen promoveren. Ik hoop nog lang samen in deze vriendin-carrière modus door te kunnen gaan met jou, want ik heb gemerkt dat wij dit erg goed kunnen samen! Dank lieverd!

Pap en Mam en Bro. Ik ben jullie enorm dankbaar voor alle steun en liefde die jullie me constant geven. Mijn dipjes tijdens het promotietraject hebben jullie ook levendig meegemaakt en jullie hebben me telkens weer opgepept met lieve woorden van vertrouwen. Ik ben zo blij met jullie!

Mariëlle, we kennen elkaar al zo lang en toch is er de afgelopen 5 jaar weer iets veranderd. Onze band lijkt alleen maar weer sterker te zijn geworden en ondertussen voel je een beetje als mijn zus, misschien zelfs wel een beetje als mijn tweelingzus. Door de jaren heen ontdekken we telkens weer hoeveel we toch op elkaar lijken en hoe we op dezelfde manier door onze levensfases, zoals een promotie- of assistententijd, heen gaan. Onze sterke en zwakke punten verschillen zo weinig van elkaar, en juist daarom kan jij me zo goed steunen en advies geven. Lieve Jip, dank je wel voor je onvoorwaardelijke steun.

Laurens, waar moet ik beginnen? De tranen springen al in mijn ogen als ik dit stukje aan jou moet schrijven en wat ik daarom ook als langste heb uitgesteld. Het is bijna niet te geloven hoeveel wij al samen hebben meegemaakt de afgelopen 12 jaar en hoe we weer samen dansend door deze fase zijn gekomen. Niets lijkt tot nu toe onze band te kunnen verstoren. Dit komt door jou onaantastbare geduld, zorgzaamheid en authenticiteit. Je stond altijd voor me klaar als ik weer in een onpeilbare dip zat. Jouw kracht zit in je enorm kunnen inleven in een ander en dit doe je door oprecht te luisteren en betrokken te zijn. Ik ben zo dankbaar voor jouw liefde en dat ik jou heb gevonden in dit leven. Ik vind je de liefste van de hele wereld…

About the author

ABOUT THE AUTHOR

Caroline Broos was born on September 24th 1986 in Muscat, Oman. The first ten years of her life she spent in Oman, The Netherlands (The Hague) and Syria (Damascus) with her brother and parents living an expatriate life. After returning from Syria the family settled in Assen, the Netherlands, where she completed her secondary education in 2004 (Dr. Nassau College). The same year she started medical school at Maastricht University. After finishing her bachelor degree in 2007, she did voluntary work at the Medical University of Tamale in Ghana. During her master degree, she did additional elective clinical internships in Radiotherapy (Netherlands Cancer Institute, Amsterdam) and Pulmonology (Maastricht University Medical Center, Maastricht). She ended her studies by executing fundamental research at the department of Experimental Therapy at the Netherlands Cancer Institute in Amsterdam. In August 2011, she obtained her medical doctor degree from Maastricht University.

We are very pleased that during all her study work, Caroline continued to broaden and develop herself through other activities: she succeeded in winning medals in rowing both as a team member as well as a coach. She also continued to spent time with her various networks of friends and her family. She always remained sociable and focused on the human interest.

In 2011 Caroline started as resident internal medicine at the Franciscus Gasthuis in Rotterdam. In 2012 she decided to start her PhD research at the research laboratory of the Department of Pulmonary Medicine at the Erasmus MC in Rotterdam (supervisor: Prof.dr. R.W. Hendriks). In 2016 she obtained a grant from the Longfonds to perform a research fellowship at the Department of Internal Medicine, Section of Pulmonary, Critical Care and Sleep Medicine at Yale University in New Haven (Connecticut) (supervisor: Prof. N. Kaminski). In October 2016, she finished her PhD research work at the Erasmus MC and started her pulmonary medicine training with two years of internal medicine in the Franciscus Gasthuis (supervisor: drs. A.P. Rietveld). The final part of the training will be undertaken at the Department of Pulmonary Medicine in the Erasmus MC (Head of the department: Prof.dr. H.C. Hoogsteden and supervisor: dr. L.M. van den Toorn).

We are very proud of the result of the research that lies in front of you. It is the result of Caroline's hard work and dedication but also her ability to motivate the necessary support from the people around her. She is a real team player and she would be the first to say that she couldn't have done it without others.

Roland Broos and Rieteke Broos-Munnik,
parents of Caroline

PhD Portfolio

Summary of PhD training and teaching

Name PhD student: C.E. (Caroline) Broos	PhD period: 2012-2016
Erasmus MC Department: Pulmonary Medicine	Promotor: Prof.dr. R.W. Hendriks
Research School: Molecular Medicine	Supervisor: Dr.ing. M. Kool and Dr. B. van den Blink

1. PhD training	Year	Workload ECTS
General courses, seminars and workshops		
Interstitial lung disease (ILD) winter course, Davos	2012	1,5
CPO Mini-course	2012	0,3
Basic Immunology course	2012	3,0
Endnote workshop	2012	0,3
NVVI Lunteren symposium: 'APC's revisited'	2012	0,5
Basic introduction course on SPSS	2012	0,8
BD training on Basic Flow Cytometry	2012	0,15
Course on Molecular Diagnostics VII	2012	1,0
BROK course	2012	2,0
Workshop on Photoshop & Illustrator	2012	0,3
Workshop on Indesign CS5	2012	0,15
Interstitial lung disease (ILD) winter course, Davos (for assistance)	2013	1,0
Open Clinica Training	2013	0,3
NVVI Lunteren symposium: 'Mucosal Immunology; crossing borders'	2014	0,5
Basic Human Genetics course: Genetics for Dummies	2015	0,5
Introduction course to 'R'	2015	0,3
BROK-course (re-registration)	2016	0,15
(Inter)national scientific presentations and conferences		
16th Molecular Medicine day, Rotterdam (poster)	2012	0,3
Longdagen, Utrecht (poster)	2012	0,3
Chronic inflammatory disorders of the lung symposium, Freiburg (poster)	2012	1,0
Symposium Sarcoidose & IPF, Rotterdam (presentation)	2012	0,5
NRS Young Investigator Meeting, Amsterdam (poster)	2012	0,3
NVVI Winter School, Noordwijkerhout (poster)	2012	0,8
Longdagen, Utrecht (poster)	2013	0,5
ATS International Conference, Philadelphia (poster discussion)	2013	1,5
WASOG International Meeting, Paris (poster)	2013	0,5
NRS Young Investigator Meeting, Amsterdam (poster discussion)	2013	0,3
NVVI, Winter School, Noordwijkerhout (poster)	2013	0,5
18th Molecular Medicine day, Rotterdam (poster)	2014	0,3
Longdagen, Utrecht (poster)	2014	0,5
ATS International Conference, San Diego (poster discussion)	2014	1,5
13th International Meeting on Dendritic Cells, Tours (poster)	2014	1,0
WASOG-BAL International Meeting, Kusadasi (poster)	2014	1,5
NVVI Winter School, Kaatsheuvel (poster)	2014	0,5
ATS International Conference, Denver (poster discussion)	2015	1,5
ERS Annual Conference, Amsterdam (poster)	2015	1,0
ERS Annual Conference, London	2016	1,0
2. Teaching		
Winter course, Research Master Infection & Immunity, Erasmus University, Rotterdam. Lecture: "Unraveling the pathogenesis of sarcoidosis"	2014-2015	0,5

Supervising Master's theses L.H.C. Poell, Erasmus University, Rotterdam.	2014	3,0
Journal Club tutor for first year medical students, Erasmus University, Rotterdam.	2013-2014	1,0
Symposium sarcoidosis and IPF for medical doctors, Erasmus MC, Rotterdam. Lecture: "The T cell in sarcoidosis"	2012	0,3
3. Awards		
Abstract Scholarship Award, ATS, Philadelphia (sponsored by the Foundation of Sarcoidosis Research (FSR))	2013	
Abstract Scholarship Award, ATS, San Diego (sponsored by the FSR)	2014	
NRS Young Investigator Travel Grant for WASOG-BAL, Kasadasi	2014	
Abstract Scholarship Award, ATS, Denver (sponsored by the FSR)	2015	
Sarcoïdose Belangenvereniging Nederland (SBN) Persoonlijke Onderzoeksondersteuning 2015 for the 'STEPS study'	2015	
Research fellowship grant from the Dutch Lung Foundation (Longfonds)	2016	
TOTAL		32,85

About the cover

ABOUT THE COVER

Fundamenteel immunologisch onderzoek heeft mij enorm doen realiseren en waarderen hoe bijzonder ons immuunsysteem is. Immunologische cellen zijn zo goed op elkaar afgestemd dat het ons lichaam erg vaak lukt om aanvallen van buitenaf adequaat af te weren. Toch weten micro-organismen of ontregelde cellen soms te ontsnappen aan deze scherp afgestelde patrouille van cellen, waardoor we infecties of kanker krijgen. Anderzijds lijkt ons afweersysteem soms zo overmatige enthousiast dat we juist tegenovergestelde ziektes ontwikkelen, zoals allergieën of auto-immuunziektes. Onderzoek doen naar de pathogenese van sarcoïdose heeft mijn kennis over de immunologie op de proef gesteld. Er blijkt in de sarcoïdose wereld nog een levendige discussie gaande over de vraag of het immuunsysteem overmatig of juist middelmatig reageert. Deze vraagstelling, die soms opspeelt tijdens congressen, daagt je enorm uit om af en toe los te komen van je eigen theorieën en weer terug te gaan naar de basis…

Het granuloom is bij uitstek de basis van sarcoïdose en dit granuloom fascineert me. Zonder de aanwezigheid van granulomen, bestaat sarcoïdose niet. Oorspronkelijk wordt er gedacht dat granulomen ontstaan door chronische stimulatie van het immuunsysteem, en meestal is dat doordat het ons lichaam niet lukt om (schadelijke) stoffen of bacteriën op te ruimen. Denk hierbij aan wat er gebeurt bij tuberculose of berylliose. In sarcoïdose granulomen zijn tot op heden nog geen stofjes gevonden die alle casussen kunnen verklaren. Dit laatste suggereert dat het sarcoïdose immuunsysteem overmatige reageert en wat waarschijnlijk niet echt nodig is. Dit wordt bevestigd door het feit dat sarcoïdose patiënten niet ernstig ziek worden als zij behandeld worden met prednison. In tegendeel: prednison is de huidige eerste keus voor de behandeling van de ziekte. Echter, prednison en andere therapieën lossen het probleem op de lange termijn niet op… Dus wat is hier gaande? Is granuloomvorming in sarcoïdose een uiting van het immuunsysteem dat in de basis niet adequaat is en daarom overschakelt op een van oorsprong oud afweersysteem om (gevaarlijke) aanvallen op afstand te houden? Of is het een uiting van een combinatie van fouten in specifieke cellen, waardoor specifiek overmatige stimulatie van het immuunsysteem ontstaat, zoals sommige resultaten uit dit proefschrift doen vermoeden? Al met al zijn we nog stappen verwijderd van het echte antwoord. Wat ik in ieder geval heb geleerd van sarcoïdose onderzoek is dat je moet uitkijken met aannames doen die al langer bestaan. Om verder te komen moet je jezelf telkens blijven uitdagen om weer even vanuit een andere hoek te durven kijken. Daarbij is het heel belangrijk om goed te blijven kijken naar wat je ziet: zowel in de kliniek als naar de basis, het granuloom…

De voorkant is ontstaan doordat ik mijn schoonvader, Olphaert den Otter, beeldend kunstenaar, microscopie plaatjes liet zien van sarcoïdose granulomen. Het duurde niet lang voordat hij met zijn talent voor gedetailleerd observeren, een verband legde met gemarmerd papier. Papier marmeren is een kunstmethode die oorspronkelijk is

ontstaan in Azië en waarbij de vormen op papier ontstaan door verdunde olieverf op een substantie te leggen waar het niet mee mengt en waardoor het niet mengt met andere verfkleuren. Met behulp van verschillende tools kunnen lijnen en figuren worden getrokken. Als de vormen naar wens zijn wordt het papier op de kop op de substantie gehouden, et voilà! Gemarmerd papier werd vroeger ook vaak gebruikt om boeken te kaften. Toen Olphaert mij voorbeelden hiervan liet zien, was ik meteen verkocht. Er gaat een wereld van verschillende soorten granulomen voor je open! En laten we eerlijk zijn: deze wirwar ontstaat toch eigenlijk vooral door *Two* substanties die *Tanglen*…

Ik wil hiermee mijn dank uiten aan Olphaert die mij enorm heeft geholpen om tot een voorkant van mijn proefschrift te komen waar ik erg content mee ben. Tevens wil ik mijn dank uitspreken aan Jonna Cohen (boekrestaurator): zij heeft mij geholpen bij het vinden van het juiste gemarmerde papier. Ook gaat mijn dank uit naar Marijn de Jong (fotograaf): hij heeft mij geholpen om dit papier goed digitaal te krijgen. Het gemarmerde papier komt van Michel Duval (Paris) en de voorkant is ontworpen door grafisch ontwerper Erwin Timmerman van Optima Grafische Communicatie. Mijn grote dank!

Sarcoidosis is an intriguingly complex immunological disorder. It is characterized by the formation of non-necrotizing granulomas that are most commonly found in the mediastinal lymph nodes and lungs of patients. Gaps in knowledge on sarcoidosis disease etiology and determinants of disease course have thus far impeded rational decisions on 'Who, When and How' to treat. The mainstay of sarcoidosis therapy remains immunosuppressive, using relatively high doses of systemic prednisone. However, although often effective, corticosteroid treatment can be accompanied by severe side effects. Detailed insight in the immunological response occurring in pulmonary sarcoidosis patients can help find determinants of disease etiology and disease course, which can yield new therapeutic targets. Therefore, the first aim of this thesis is to further unravel the immune-related pathogenesis of pulmonary sarcoidosis. The second aim is to gain insight into current prednisone treatment, in order to optimize treatment strategies to reduce side effects and increase quality of life of pulmonary sarcoidosis patients.

Findings described in this thesis provide a new perspective on sarcoidosis pathogenesis, challenging long-held paradigms and exploring novel approaches to further unravel this immunological complex disease. Furthermore, a rationale is provided to evaluate the potential of certain existing therapies that are currently used in other autoimmune diseases for pulmonary sarcoidosis treatment. Finally, results of this thesis lay a strong foundation for future studies that can help individualize sarcoidosis treatment and thereby increase pulmonary sarcoidosis-related quality of care and patient-related quality of life.

Caroline Elizabeth Broos