Adaptive Immune Responses and Treatment in Sarcoidosis and Pulmonary Fibrosis

The road towards personalized medicine

Jelle R. Miedema

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Adaptive Immune Responses and Treatment in Sarcoidosis and Pulmonary Fibrosis

The road towards personalized medicine

Het verworven immuunsysteem en behandeling van sarcoïdose en longfibrose

De weg naar gepersonaliseerde behandeling

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CHAPTER 1

Introduction and outline of the thesis

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Jelle R. Miedema, Ylva Kaiser, Caroline Broos, Marlies Wijsenbeek, Johan Grunewald, Mirjam Kool

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INTRODUCTION

Interstitial lung diseases (ILDs) encompass a broad spectrum of ultra-rare to more common pulmonary disorders. They are classified together because most – but not all - affect the lung interstitium with a variable degree of inflammation and fibrosis (1). In this thesis, we focus on two relatively common groups of ILD, representing two sides of the inflammation-to-fibrosis spectrum in the lung interstitium. On the one hand we investigate sarcoidosis, an inflammatory disease with complex immunopathogenesis, overall response to immunosuppressive therapy and relatively good outcome (1). On the other hand, we study progressive pulmonary fibrosis, which is irreversible, unresponsive to immunosuppressive therapy and usually has a poor prognosis (1). In both sarcoidosis and pulmonary fibrosis, the pathogenesis is only partly understood and current therapeutic options are limited (1). Improved knowledge and understanding of the pathogenesis may result in the identification of novel drug targets. In clinical care, the decision to start specific treatment for these diseases is particularly challenging given the scarcity of clinical trial data and the heterogeneity of disease behavior. Major unmet needs for people with sarcoidosis and pulmonary fibrosis include new and personalized treatment, taking prognosis and estimated treatment response into account.

In this thesis, we focus on the adaptive immune responses and new personalized treatment in sarcoidosis and pulmonary fibrosis. In **part 1**, we aim to (**I**) identify new T cell biomarkers in sarcoidosis that may correlate with long-term prognosis or favorable response to specific treatment and (**II**) unravel the role of aberrantly activated T cells in sarcoidosis pathogenesis using a novel mouse model. In **part 2** of this thesis, we aim to (**III**) investigate the safety and efficacy of antifibrotic therapy in progressive pulmonary fibrosis due to asbestosis, using daily home monitoring of forced vital capacity (FVC).

This introduction chapter will first give an overview of current knowledge on clinical and immunological features of sarcoidosis, with a specific focus on the role of T cells in disease pathogenesis. An in-depth overview of the immunopathogenesis of sarcoidosis will be provided in chapter 2 of this thesis. In the second part of this introduction chapter, progressive pulmonary fibrosis is discussed, as well as unmet needs for patients with sarcoidosis and pulmonary fibrosis. Finally, the aims and outline of this thesis are described in more detail.

PART 1

Clinical presentation of sarcoidosis

Sarcoidosis is a complex systemic disease, heterogeneous in both clinical presentation and disease course. Compatible symptomatic presentation, histological identification of granulomas and exclusion of other diagnoses are the main criteria for evaluation of sarcoidosis patients. Although sarcoidosis can affect virtually any part of the body, pulmonary involvement is present >90% of cases. Approximately half of all patients with sarcoidosis show disease regression within 2 years, others will develop chronic disease (duration ≥ 3-5 years) (2), but in up to 20% of patients granuloma formation persists and progresses to pulmonary fibrosis (3, 4). This permanent organ damage may lead to disabling symptoms like progressive dyspnea and cough (5) and impaired quality of life. Other commonly affected extra-pulmonary organs are the liver (20-30%), eyes (10-30%), and skin (15%). Less prevalent but potentially catastrophic are cardiac (2-5%) or central nervous system involvement (5%) (2). Pulmonary fibrosis, cardiac or CNS involvement are the main reasons for an increased risk of mortality in sarcoidosis (6).

When sarcoidosis is suspected based on clinical and radiological presentation, confirmation of granulomatous tissue in a biopsy sample is required in most patients. Moreover, a thorough effort to exclude alternative causes of granulomas (e.g. infections such as aspergillosis, beryllium exposure, environmentally, malignancy- or drug-induced sarcoid-like reactions) is mandatory before the diagnosis of sarcoidosis can be made. Lymphocytosis marked by an elevated BALF CD4/CD8 ratio (> 3.5) can support the diagnosis (2, 7).

The incidence and clinical presentation of sarcoidosis vary greatly depending on ethnicity, geographic location, genetic background and gender. In parts of Europe, the specific acute phenotype termed Löfgren's syndrome (LS) is characterized by a usually favorable outcome, even without treatment (8, 9) (**Table 1**). In contrast, the non-acute form or non-LS sarcoidosis may lead to chronic inflammation and eventually pulmonary fibrosis in some cases. Constituting a much more heterogeneous patient group than LS, non-LS patients often present with a broader range of symptoms and vary greatly in clinical and radiographic resolution as well as well as long term outcome (10). Currently, we are not able to accurately predict which patients with sarcoidosis are at risk for developing progressive organ damage (3).

Sarcoidosis or sarcoidos*es*: Löfgren's syndrome as separate disease entity?

Although sarcoidosis was first described in 1877, LS was described in 1946 by Swedish pulmonologist Sven Löfgren. Similarly to non-LS sarcoidosis, the hallmark pathophysiological finding is non-caseating granulomas in the affected organs and an elevated BALF CD4/CD8 ratio (6, 7). In contrast to the often-insidious onset, slow disease progression and heterogeneous phenotype of non-LS sarcoidosis, LS patients typically experience an acute disease presentation (**Table 1**). Moreover, LS usually manifests with characteristic clinical symptoms of fever, erythema

nodosum and/or ankle arthritis, in addition to bilateral hilar lymphadenopathy on chest radiography, either with or without parenchymal infiltrates (11-13). Erythema nodosum is significantly more common in women, arthritis is more common in men (14, 15), suggesting hormonal and/or genetic influence on symptomatic appearance.

Gender differences are also observed in terms of incidence. While LS generally arises in young adults of both sexes between 25 and 40 years of age, a second peak of incidence is also observed in women around menopause, at the age of 45 to 60 (8, 15). Global incidence of LS highly varies, with LS patients constituting up to a third of all sarcoidosis patients in Scandinavia, the Netherlands and Spain (16-18), but radically less, down to one percent, in e.g. the UK, the US, and Asia (18-21).

The perhaps most striking distinction between LS and non-LS sarcoidosis lies in disease outcome. The distinct differences in presentation, disease course, genetic susceptibility, and immunology between LS and non-LS sarcoidosis suggest possible reconsideration of these conditions as separate disease entities (9, 22). Moreover, the gradual unraveling of not only clinical but also genetic and immunological differences between LS and non-LS sarcoidosis now calls for a more stringent separation of the two conditions in studies conducted worldwide. In clinical care, accurate prediction of the risk for developing progressive organ damage is mostly warranted in non-LS sarcoidosis.

Table 1: Clinical features of sarcoidosis

	Löfgren's syndrome	**Non-Löfgren sarcoidosis**
Onset	Acute	Insidious
Clinical presentation	Bilateral hilar lymphadenopathy Sometime parenchymal infiltrates Erythema nodosum and/or Bilateral ankle arthritis/arthralgia (often) Fever (often) (23)	Variable Often parenchymal infiltrates, ranging from lymph node involvement only to fibrotic changes >90% pulmonary involvement >50% involvement of skin, liver, peripheral lymph node or eye (19)
Diagnosis	Clinical presentation, usually does not require tissue confirmation (7) CD4/CD8 > 3.5 T-cell ratio BALF supportive (2)	Clinical presentation and tissue confirmation (granulomas) (7) CD4/CD8 > 3.5 T-cell ratio BALF supportive (2)
Prognosis	~ 85% recovery	50-70% recovery 30-50% chronic disease (> 3-5 years) 20% irreversible fibrosis (2)

Immunological features of sarcoidosis and granuloma formation

A key pathological feature of sarcoidosis is the presence of non-necrotizing granulomas in the affected organs. These aggregates of differentiated immune cells contain a core of alveolar macrophages, converted into epithelioid cells and multinucleated giant cells (MGCs) (24) and a shell comprised of T-cells and a few B cells (25). These granulomas are primarily found in lungs and lung-draining mediastinal lymph nodes (MLN), but can also be present in for example the eyes, skin, central nervous system and heart (26). The exact cause and triggers of sarcoidosis are still unknown, but the current concept is an exaggerated antigen-driven immune response inducing the formation of disease-typical granulomas (27). The increase of oligoclonal $CD4^+$ T helper (Th)-cells specifically in the lung in sarcoidosis implicates these cells as the primary drivers of inflammation (28). Historically, sarcoidosis has been considered a Th1-mediated disease, defined by a typical increase in interferon (IFN)-γ production by Th-cells in bronchoalveolar lavage fluid (BALF) (29), as well as the presence of Th1-skewing cytokines interleukin (IL)-12 and IL-18 in both granulomatous tissue and BALF (30, 31). Recently, the perception of sarcoidosis as a purely Th1-driven disease has shifted to a general understanding that a combination of Th1- and Th17-associated factors contribute to disease progression and outcome. Furthermore, the high prevalence of IFN-γ-producing Th17-cells in sarcoidosis BALF and the presence of all Th17-lineage cells in T-cell-priming MLN have contributed to our understanding of Th17-cell plasticity (**Fig. 1**) (32).

Different Th-cell subsets have been recognized based on their cytokine profile and master transcription factor (TF) expression: Th1-cells mainly produce IFN-γ and TNF-α and express the TF T-bet, Th2-cells produce IL-4, IL-5, and IL-13 and are GATA3 positive, and Th17-cells produce IL-17A and IL-22 and are controlled by RORγT (**Fig 1**) (33). Regulatory T-cells (Tregs) express the TF FoxP3 and secrete immune-regulatory cytokines IL-10 and transforming growth factor (TGF)-β and can also dampen inflammatory responses through cell-cell interaction. Differentiation of naive Th0-cells into different effector subsets, e.g. Th1-cells or Th17-cells requires T-cell receptor (TCR) triggering by a specific antigen-HLA complex, co-stimulation, and importantly signals from the cytokine milieu **(Fig. 1)**. Th17-cell differentiation occurs in the presence of IL-1β, IL-6, and/or TGFβ, whereas IL-12 and IFN-γ induce Th1-cell differentiation. IL-23 exerts regulatory effects on the maintenance of Th17-cells, but is also involved in conversion of Th17-cells to a more pathogenic phenotype (34-36). All these cytokines (IL-1β, IL-6, IL-12, IL-23, TNFα, and TGFβ) have been identified as produced by activated macrophages and DCs from sarcoidosis patients (37-41) (**Fig. 1**). IFN-γ stimulates macrophages and DCs, thereby inducing MGC formation and TNF-α production (42-44). Next, TNF-α propagates the migration of immune cells, contributing to granuloma formation (45)**.** Over the recent years, Th17-cells have been observed within granulomas of sarcoidosis patients with active disease, as well as in relapsing disease (41, 46-50). The primary Th17-cytokine, IL-17A, is an important mediator of granuloma formation and maturation (51), as it can induce fusion of DCs into MCG, shown e.g. in patients with

granulomatous Langerhans cell histiocytosis (52, 53). Next to this, IL-17A can even potentiate the IFN-γ-induced giant cell formation (52). Lastly, IL-17A can stimulate TNF-α production by macrophages (54). Together, the presence of these cytokines will promote the development of mature granulomas.

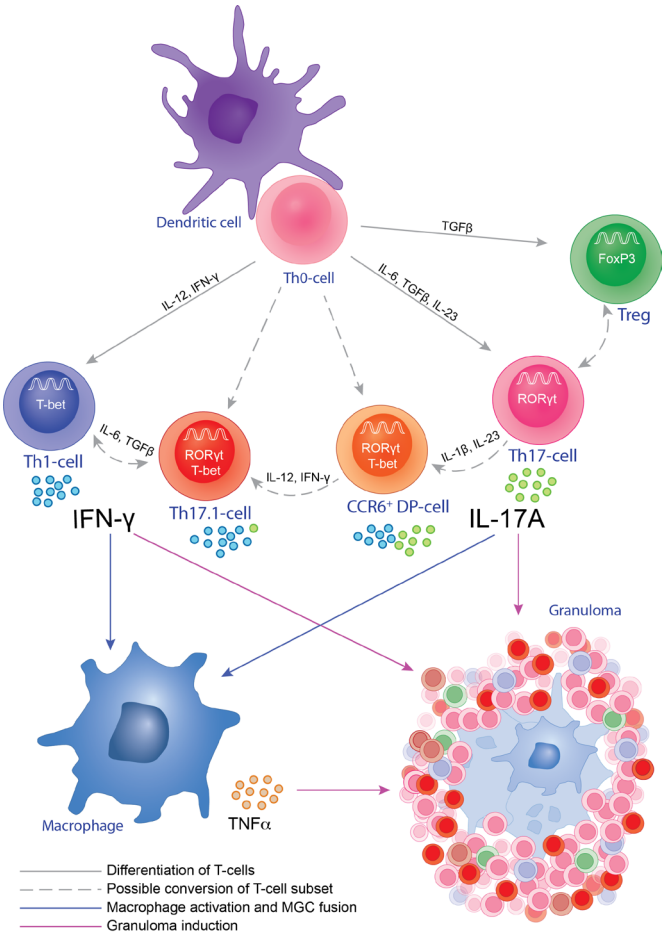

Figure 1: Th17-cell plasticity and influence on granuloma formation/maintenance
Polarization of naïve Th0-cells into Th1-cells, Th17-cells, and Tregs (solid grey line) and hypothesized conversion of Th1-cells, Th17-cells and Tregs (dotted grey lines). The discrimination of Th-subsets is based on chemokine receptor expression; Th1-cells (CCR6-CXCR3+CCR4-), Th17-cells (CCR6+CXCR3-CCR4+), CCR6+ DP Th-cells (CCR6+CXCR3+CCR4+), and Th17.1-cells (CCR6+CXCR3+CCR4-). Influence of effector cytokines IFN-γ, IL-17A, and TNFα on MCG induction (blue lines) and granuloma formation/maintenance (purple lines). Abbreviations: Th-cell: helper T-cell, Treg: regulatory T cell, IFN: interferon, IL: interleukin, TNF: tumor necrosis factor, TGF: transforming growth factor, DP: CCR6⁺DP Th cell

Postulated antigens involved in LS and non-LS sarcoidosis

The exact cause of sarcoidosis is currently still unknown. An exaggerated immune response to a specific antigen is most probable, highlighted by the Kveim reaction that was formerly used for diagnostic purposes (55). With this reaction, intradermal injection of lysates of sarcoidosis-affected lymph nodes and/or spleen tissue resulted in well-formed granulomas after 2-4 weeks, and importantly only in genetically susceptible individuals (56). In the recent decades, several disease-triggering antigens have been suggested **(Table 2)**, such as mycobacterial or proprionibacterial products (57), with some of their glycolipoproteins resisting degradation (2). Microbial DNA has been detected in sarcoid granulomas (58); however, live mycobacteria have never been found, in contrast to *Proprionibacterium acnes* (59, 60). Suspected antigens derived from *M. tuberculosis* are catalase peroxidase (mKatG), superoxide dismutase A (sodA), the 6 kDa early secretory antigenic target (ESAT-6), as well as heat-shock proteins (26, 58). Trigger factor protein RP35 from *Proprionibacterium acnes* can also induce proliferation of T-cells from sarcoidosis patients (61).

Next to these bacterial components, several lines of evidence point to a self-antigen. Molecular modelling of an LS-specific HLA-TCR complex (HLA-DRB1*03-TCR Vα2.3/Vβ22) showed an ideal fit of a peptide derived from cytoskeletal protein vimentin into the peptide-binding cleft (62), implicating vimentin as a candidate autoantigen. Identical vimentin-derived peptides have on separate occasions been eluted from HLA molecules of BALF macrophages from sarcoidosis patients (63, 64), and also been observed to induce T-cell IFN-γ responses in LS patients with active disease (65). Furthermore, T-cell responses to vimentin were recently documented in a study on the still-elusive Kveim reagent (66). Furthermore, other candidate autoantigens specific for LS and non-LS sarcoidosis respectively, have been proposed based on detailed studies of antibody profiles (67).

Additional antigens are also associated with sarcoidosis (68). For instance, 26 rescue workers during the 2001 World Trade Center attack developed sarcoid-like granulomatous pulmonary disease (69); this excess of 'sarcoidosis' in WTC-exposed workers was also confirmed by another study (70). Although it can be questioned whether the granulomatous reaction is truly sarcoidosis, it shows an association between environmental dust exposure and new onset of granulomas highly comparable to sarcoidosis.

Unfortunately, due to the heterogeneity of stipulated sarcoidosis antigens, it is still unknown whether these antigens are a direct cause of disease or whether they are the result of a bystander immunological reaction and rather involved in maintaining or propagating disease.

Table 2: Speculated antigens involved in the onset of LS and/or non-LS sarcoidosis

Possible antigen source	Suggested antigen	Reference
Mycobacterium tuberculosis	ESAT-6 mKatG SodA	(57, 58, 71-74)
Proprionibacterium acnes	Trigger factor protein (RP35)	(57, 58, 61, 71-74)
Proprionibacterium granulosum	Yet unidentified	(61)
Auto-antigens	Vimentin Tubulin α-actinin-4	(62, 65, 66)
Anorganic / environmental	Yet unidentified	(69, 70)

Role of autoimmunity in LS and non-LS sarcoidosis

In LS, the combination of a strong HLA association, presence of potentially autoreactive T-cell responses with vimentin as a proposed auto-antigen (Table 2) and the occurrence of relapsing disease in a subset of patients, suggests that at least this particular form of sarcoidosis might have an autoimmune component. It is an intriguing observation that approximately 5% of LS patients (all being DR3-positive) experience disease relapse years after clinical recovery (8, 75), as this is reminiscent of disease "flares" observed in autoimmune diseases such as systematic lupus erythematosus (SLE) and multiple sclerosis (MS). Such recurrence raises the question of whether LS might constitute a discrete form of sarcoidosis with an autoimmune origin. It is plausible that different antigens are involved in induction of LS and non-LS, possibly under influence of external environmental or microbial triggers. In addition, genetic and epigenetic factors together with the unique microenvironment of the lung may contribute to shaping the inflammatory reaction that results in either LS or non-LS sarcoidosis. However, overall similarities in disease presentation, such as $CD4^+$ T cell expansion and granuloma formation, indicate that it would be worthwhile to explore whether proposed antigenic candidates such as vimentin are causative of disease also in non-LS sarcoidosis.

Genetic basis for favorable or detrimental T-cell responses and disease outcome

Several genes within the antigen presentation and T-cell activation/survival pathways are associated with disease susceptibility and outcome. Prognosis is especially favorable in patients positive for the *HLA-DRB1*03* (DR3) allele **(Table 3)**. In a study on 301 LS patients, an unusually strong association between DR3 and clinical recovery was found. Close to every DR3-positive LS patient (185 out of 187) experienced clinical resolution within two years. In sharp contrast, only around 50% of DR3-negative LS patients recovered within the same time span (8). In contrast to LS, non-LS sarcoidosis patients are more prone to develop chronic disease, especially if positive for *HLA-DRB1*15* (DR15) and *-DRB1*14* (DR14) (16) (Table 4). Moreover, presence of DR15 and *HLA-DRB1*04* (DR4) significantly increases the risk of developing extra-pulmonary

symptoms, with ocular involvement being particularly prominent in DR4-positive patients (76, 77). Second to HLA variants, single-nucleotide polymorphisms (SNPs) in genes regulating T-cell activation, such as *BTNL2*, a costimulatory molecule expressed on DCs, appear to be key predisposing factors for sarcoidosis (78) (Table 4). BTNL2 is important in sequestering T-cell activation; it stimulates *de novo* FoxP3 expression and the development of suppressive Tregs (79). Interestingly, different *BTNL2* SNPs have been found to influence development of either LS or non-LS sarcoidosis (78, 80, 81). Moreover, *BTNL2* SNPs have been associated with disease susceptibility in other Th17-driven diseases such as CD and RA (82, 83). A balance between T-cell activation and regulation is important in maintenance of the exaggerated immune response in sarcoidosis. Prolonged survival of effector T-cells or reduced survival of protective Tregs (84) might be a consequence of several SNPs found in survival-associated genes like *NOTCH4*, *ANXA11,* and *XAF1* (85-89)**.** The identification of an independent SNP in the *IL23R* (IL-23 receptor) gene further indicates a prominent role for the IL-23/Th17 signaling pathway in the genetic etiology of sarcoidosis, again showing overlap with CD (9, 90, 91). Strikingly, one *IL23R* SNP was associated with poorer prognosis and development of chronic sarcoidosis (91).

Table 3: Genes and SNPs associated with antigen-presentation and T-cell activation in sarcoidosis patients.

Gene	Function	Predisposition for	Reference
HLA-DRB1*03	Antigen presentation	LS	(8)
MHC2TA rs3087456	Antigen presentation	LS	(16)
HLA-DRB1*12	Antigen presentation	Non-LS	(16, 18)
HLA-DRB1*14	Antigen presentation	Non-LS (chronic)	(16)
HLA-DRB1*15	Antigen presentation	Non-LS (chronic)	(16)
SNPs			
CCR2 haplotype 2	Migration of APCs	LS	(80)
BTNL2 rs3117099T	Control of T-cell activation	LS	(80)
BTNL2 rs2076530A	Control of T-cell activation	Non-LS (chronic?)	(78, 92)
NOTCH4	Regulation of cell survival		(85)
ANXA11 rs230C	Regulation of cell survival		(86, 88, 89)
XAF1	Regulation of cell survival		(87)
IL23R rs12069782 rs11209026A	Control of T-cell differentiation	Non-LS chronic	(90, 91)
TNF	APC-derived cytokine T-cell derived cytokine	LS	(93-96)

The role of T cells in sarcoidosis

Recent years have seen a shift from the perception of sarcoidosis as a purely Th1-cell driven disease to a general understanding that a combination of Th1- and Th17-associated factors contribute to disease progression and outcome. Previous studies show a typical increase of

IFN-γ production by $CD4^+$ T cells in BALF (29), as well as Th1-skewing cytokines IL-12 and IL-18 in both granulomatous tissue and BALF (30, 31). Moreover, Th17-cells infiltrate the vicinity and core of granulomas in active disease, as well as in sarcoidosis relapse (46, 47). Functional prediction and protein network analyses also identified a prominent role for IL-23/Th17-signalling pathway in the genetic etiology of sarcoidosis (90)**.** It has been demonstrated that especially Th17-cells are highly plastic and by stimulation of IL-12 (97) and IL-23 (98) can differentiate into IFN-γ/IL-17A double-producing cells termed Th1/Th17-cells (**Table 4**). In sarcoidosis patients, these Th1/Th17-cells appear central in driving pulmonary inflammation, as increased percentages are found in BALF and peripheral blood (46-49, 99). Interestingly, in BALF of sarcoidosis patients, the proportion of Th1/Th17-cells increased with Scadding stage (49). This would indicate that the hybrid Th1/Th17-cells are pathogenic, which is also described for other chronic (autoimmune) inflammatory disorders e.g. rheumatoid arthritis and Crohns disease (97, 100). In line with high plasticity within the Th17-lineage, these Th1/Th17-cells can further develop into IFN-γ-single-producing Th-cells under the influence of Th1-skewing cytokines like IFN-γ and IL-12 (97, 101). These IFN-γ-producing Th17-cells can be discriminated based on their chemokine receptor expression CCR6 (Th17-lineage) as well as CXCR3 (Th1 lineage), and are referred to as non-classical Th1-cells or Th17.1-cells (**Table 4**), containing both IFN-γ/IL-17A-double-producing Th1/Th17 cells and IFN-γ single-producers. In three independent patient cohorts, the majority of Th-cells in sarcoidosis BALF co-express CXCR3 and CCR6, suggesting the lung microenvironment to promote a Th17.1-cell phenotype that is augmented in sarcoidosis (22, 99).

The initiation of Th-cell polarization is most often initiated by DCs in the draining lymph nodes (**Fig. 1**). In the lung-draining MLN of sarcoidosis patients, the proportion of all $CCR6^+$ Th17-lineage cells, including Th17-cells and Th17.1-cells, was increased (102). Strikingly, in contrast to the BALF, the MLN contained increased proportions of $CCR6^+CXCR3^+CCR4^+$ Th-cells or $CCR6^+$ double-positive (DP) Th-cells (**Table 4**), which are believed to be an intermediate stage between Th17-cells and Th17.1-cells (103-105) (**Fig. 1**). Especially $CCR6^+$ DP Th-cells and Th17-cells were highly proliferative, both in BALF and MLN, whereas Th17.1-cells were not. This supports a model in which Th17-cells are primed within sarcoidosis MLN and show plasticity towards pathogenic Th17.1-cells at chronically inflamed sites such as sarcoidosis lungs, but also granulomatous parts of the MLN. In contrast to their precursors, Th17.1-cells do not proliferate, suggesting this subset to constitute a set of end-stage effector cells (**Fig. 3**). In CD, human Th17.1, and not Th17-cells, have a pathogenic signature and express high levels of the multi-drug resistance type 1 membrane transporter (MDR1) (100). $MDR1^+$ Th17.1-cells are refractory to glucocorticoids that are used to treat clinical (auto)immune disease and sarcoidosis (100).

Interestingly, significantly higher BALF Th17.1-cell proportions were found at time of diagnosis in sarcoidosis patients developing chronic disease compared to patients with disease resolution within two years (103). Additionally, a sarcoidosis population that included progressive

Table 4: Terminology of Th17 lineage cells used, based on IL-17A and IFN-γ production, transcription factor and/or chemokine receptor (CCR6, CXCR3 and CCR4) expression

	Identification based on	IL-17A and/or IFN-γ production
Th17-cells	- IL-17A single-producers - Transcription factor RORγT - $CCR6^{+}CCR4^{+}CXCR3^{neg}$ Th-cells	- IL-17A single-producers
Th1-cells	- Transcription factor T-bet - $CXCR3^{+}CCR6^{neg}CCR4^{neg}$ Th-cells	- IFN-γ single-producers
Th1/Th17-cells	- IL-17A and IFN-γ double-producers	- IL-17A/IFN-γ double-producers
Th17.1-cells	- Transcription factors T-bet and RORγT - $CCR6^{+}CXCR3^{+}CCR4^{neg}$ Th-cells	- Mainly IFN-γ single-producers - Some IL-17A/IFN-γ double-producers
$CCR6^{+}$ DP-cells	- $CCR6^{+}CXCR3^{+}CCR4^{+}$ Th-cells	- Mainly IL-17A single-producers* - Some IL-17A/IFN-γ double-producers*

*: Speculated production

patients who are on first-, second- and/or third-line therapy also showed increased BALF Th17.1-cells proportions (99). These findings, together with the dominant IFN-γ signature, strongly argue for a pathogenic role for Th17.1-cells in the development and progression of non-LS pulmonary sarcoidosis and suggest that tissue resident Th17.1 cell proportions can be evaluated as a diagnostic and/or prognostic marker in clinical practice, and possibly in the future serve as a potential therapeutic target (103).

Little is known about underlying mechanisms leading to the exaggerated Th-cell response in sarcoidosis. Both regulatory mechanisms within T-cells (e.g. by checkpoint molecules) and the balance between pro-inflammatory Th-cells and Tregs are critical to maintaining tolerance and prevent inappropriate immune activation. Tregs are known to have an important role in mediating tolerance to (auto)antigens (106). Not surprisingly, they are involved in sarcoidosis pathogenesis, where decreased immunosuppressive capacity of Tregs has been consistently reported (106, 107).

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is an important suppressor of T-cell-mediated immune responses. It is highly expressed on Tregs, but also on Th-cells and thus acts both cell-extrinsically and -intrinsically to control Th-cell responses. Notably, of all Th-cell subsets, Th17-cells display the highest degree of CTLA-4 expression (107). Through a higher affinity for CD80/86 on APCs, CTLA-4 is able to compete out co-stimulatory molecule CD28, thereby leading to suppression of T-cell activation (28). Recent years have seen an increasing collection of reports describing the induction or exacerbation of sarcoid-like granulomatous disease in patients treated with anti-CTLA-4 (108-111) or anti-programmed cell death protein-1 (PD-1) antibodies (112-115) for malignancy. A link between CTLA-4 inhibition and control of Th17-cell activation was suggested by observations in a patient with an anti-CTLA-4 treatment-associated sarcoid-like reaction, where increased numbers and proportions of Th17-cells were found during therapy compared to baseline (109). PD-1 also appears crucial in control of Th17-cell (plasticity), as

melanoma patients with anti-PD-1-induced sarcoid-like disease presented with elevated peripheral mononuclear Th17.1-cells before the first dose of treatment, compared to patients that did not develop sarcoidosis and healthy controls (115), further strengthening the relationship between pathogenic Th17(.1)-cells and sarcoidosis.

As CTLA-4 and PD-1 are responsible for restriction of T cell responses as well as induction and maintenance of immune tolerance, these findings further address the notion of an autoimmune or auto-inflammatory mechanism in sarcoidosis. Indeed, genetic variations within the *CTLA4* gene are associated with autoimmune diseases (116), but have as yet not been linked to sarcoidosis. Gene pathway analysis of whole blood mRNA expression revealed that the TCR pathway was, as expected, enriched in sarcoidosis compared to controls, but also that this included down-regulated *CTLA4* mRNA expression (117)**.** Broos *et al* previously described reduced protein levels of CTLA-4 in non-LS patients compared to healthy individuals, especially on Th17-cells and Tregs, which normally have the highest expression (107) (**Fig. 2**). Also, the highly proliferative CCR6^{+} DP Th-cells in sarcoidosis MLN show a reduced CTLA-4 expression compared to control MLN (103) (**Fig. 3**). *CTLA4* gene mutations have also been identified in families with common variable immunodeficiency (CVID), including patients with granulomatous lung disease (118). These gene mutations lead to one dysfunctional allele and consequently to

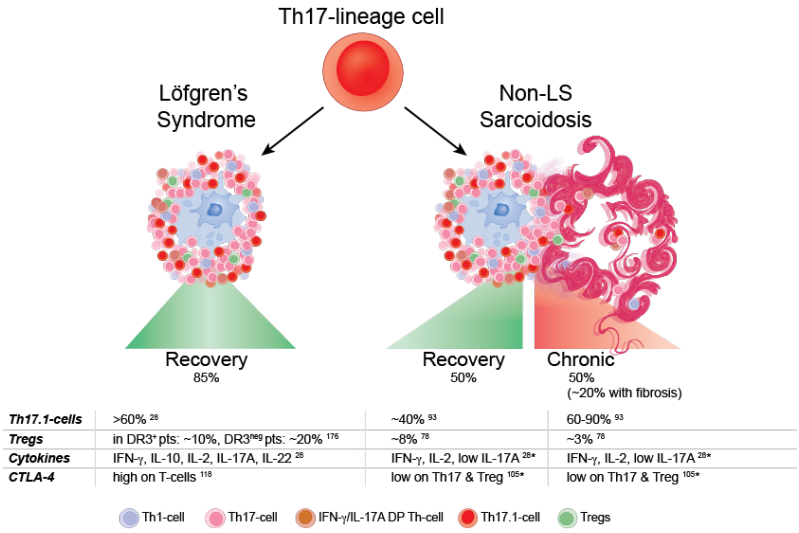

Figure 2: Comparison of Th-cell activity in LS versus non-LS sarcoidosis
Schematic overview of Th-cell-related factors between LS patients and non-LS sarcoidosis patients either undergoing recovery or developing chronic disease. Proportions of Th17.1-cells and Tregs are listed as % of total CD4^{+} T cells in BALF. Cytokine and CTLA-4 expression in BALF are shown. *No discrimination is made between recovery and chronic non-LS sarcoidosis. Abbreviations: IFN: interferon, IL: interleukin, Treg: regulatory T cell, Th-cell: helper T-cell, CTLA-4: cytotoxic T-lymphocyte-associated antigen 4

lower CTLA-4 expression on Tregs. This decrease of CTLA-4 on both Th17-cells and Tregs can cause “double trouble”, as reduced CTLA-4 on Tregs will lead to a reduced regulation of T-cell activation (116) and low CTLA-4 on Th17-cells and CCR6⁺ DP Th-cells will increase their proliferation and activation state (119).

Taken together, sarcoid granuloma formation is accompanied by the accumulation of activated Th cells in the inflamed organs with signs of aberrant activation as shown by decreased CTLA-4 expression on Th17-cells. Additionally, less efficient control of activated Th cells by tissue resident CTLA-4low Tregs in sarcoidosis possibly propagates a chronic inflammatory state. The substantial differences of Th cell proportions between the peripheral blood and inflamed organs in sarcoidosis argue for the circulation to be considered as an immunological compartment with distinctive T cell characteristics. It is currently unknown if circulating T cells from the peripheral blood can be used as clinical or therapeutic biomarkers. Because peripheral blood can be easily sampled during the disease course in contrast to the invasive procedures to obtain granulomatous tissue, studies investigating the biomarker potential of circulating T cells in sarcoidosis are highly warranted in sarcoidosis.

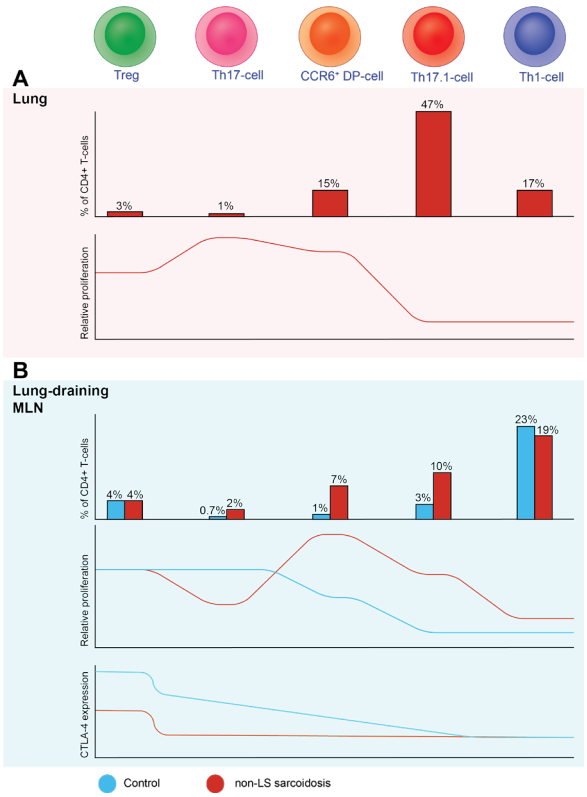

Figure 3: Proportions, proliferation, and CTLA-4 expression on Th-cell subsets
A) Proportion and proliferation of Th-cell subsets in the BALF of sarcoidosis patients. B) Proportions, proliferation and CTLA-4 expression on Th-cell subsets in the lung-draining MLN of control and sarcoidosis patients is shown. Figure is based on data from (102). Abbreviations: Treg: regulatory T cell, Th-cell: helper T-cell, CTLA-4: cytotoxic T-lymphocyte-associated antigen 4

PART 2

Progressive pulmonary fibrosis: personalized antifibrotic treatment

Most fibrotic ILDs can be categorized as idiopathic, autoimmune related and exposure related and several different causes or triggers are known (1). The archetypal and most common fibrotic interstitial lung disease (ILD) is idiopathic pulmonary fibrosis (IPF), characterized by progressive clinical worsening and a very poor prognosis (120). The progressive pulmonary scarring in IPF is thought to include aberrant repair responses after repetitive epithelial injury in genetically susceptible and/or ageing patients (120). Currently, two registered drugs are available for the treatment of IPF: nintedanib and pirfenidone (121). These pharmacological interventions will slow down IPF disease progression as measured by lung function decline, but do not stop or reverse the fibrotic process, nor increase quality of life in patients (121). Non-IPF pulmonary fibrosis is usually characterized by an initial inflammatory phase that slowly progresses to a combination of interstitial inflammation and pulmonary fibrosis (1). In a subset of sarcoidosis and pulmonary fibrosis patients, ongoing inflammation and pulmonary tissue damage can eventually lead to downstream fibrotic tissue remodeling and extracellular matrix deposition leading to self-perpetuating fibrosis as seen in IPF, irrespective of the initial causing factor (120, 122). This disease behavior, defined as progressive pulmonary fibrosis (PPF), manifests in 13-40% patients with autoimmune- related and exposure related ILD and in ~15% of patients with sarcoidosis, (120, 122). The antifibrotic drug nintedanib showed good efficacy in progressive pulmonary fibrosis other than IPF (123). For Pirfenidone, the other fibrotic drug, there are also data suggesting a beneficial effect in PPF, but trial design and power precluded firm conclusions (124, 125). Increased understanding of key mechanisms in fibrosis pathogenesis poses the question whether the therapeutic efficacy of nintedanib and pirfenidone demonstrated in IPF can be replicated in specific other ILDs with a progressive fibrotic phenotype. Importantly, early identification of progressive pulmonary fibrosis or favorable response to antifibrotic treatment may allow for improvement of personalized medicine, as this will allow early and more selective treatment in a heterogeneous patient population. Inclusion of a large ILD patient group with PPF in clinical trials to investigate treatment efficacy is not easy and very expensive. Recent studies showed that daily home monitoring of forced vital capacity (FVC) in pulmonary fibrosis provided a sensitive prediction of disease behavior and correlated well with hospital-based measurements of pulmonary function (126, 127). Home monitoring of FVC has the potential to identify progression of pulmonary fibrosis in an early stage, demonstrate treatment efficacy and therefore improve personalized antifibrotic treatment strategies in clinical care (128).

AIM AND SCOPE OF THIS THESIS

More research is needed in the field of sarcoidosis and pulmonary fibrosis to improve current treatment and identify new drug targets. In this thesis, we focus on the adaptive immune responses and new personalized treatment in sarcoidosis and pulmonary fibrosis.

PART 1

T cell phenotype: from disease pathogenesis to personalized medicine in sarcoidosis

Knowledge on how to accurately subgroup T-cells remains incomplete, but with more advanced technology, enabling comprehensive and simultaneous detection of multiple cell surface markers, further phenotypic and functional categorization of T cells may reveal valuable biomarkers to predict disease course and gain insights in disease mechanisms and response to therapy in sarcoidosis and hopefully, new drug targets. As previous studies conducted on patient material offer a mere snapshot of sarcoidosis progression, the ideal approach would involve several points of sampling, preferably from the circulation throughout the disease course, in order to more accurately capture the presence and contribution of different T cells at various time points. It is plausible that Th-cells undergo a switch during the disease course, gradually transitioning from pro- to anti-inflammatory during the course of the disease and subsequently altering their cytokine profile and proliferative capacity. Understanding the phenotypical and functional changes of circulating T cells in sarcoidosis may help to identify new – easy to sample - biomarkers that may correlate with long-term prognosis or favorable response to specific treatment.

Second, a mouse model to unravel the immunopathogenesis of sarcoidosis is essential for the development of new drug targets. As outlined in the introduction, intrinsic loss of CTLA-4 in Th17 or Tregs may induce spontaneous pathogenic T-cell phenotypes and – antigen triggered - granuloma formation in sarcoidosis. Therefore, we aimed to investigate the immunopathogenesis of sarcoidosis using a novel mouse model with heterozygous expression of CTLA-4 as well as complete absence of CTLA-4 in interleukin (IL) 17 producing T cells or regulatory T cells.

PART 2

Treatment of progressive pulmonary fibrosis

In the second part of this thesis, we wanted to investigate if antifibrotic treatment of IPF with Pirfenidone can be applied to other progressive fibrotic lung diseases. To this end, we focused on patients with progressive lung fibrosis due to asbestosis, a rare occupational ILD resulting from inhalation of asbestosis fibers. Besides the fact that it is a rare disease, another challenge in this group of patients is that not all patients have disease progression, hence a treatment effect of slowing down disease decline might be hard to show. We hypothesized that daily home monitoring of forced vital capacity (FVC) to investigate disease behavior before and after the start of antifibrotic treatment will allow for the demonstration of treatment efficacy even in a study with a small sample size, and might be used in the near future for tailored start and personalized antifibrotic treatment in clinical care.

GENERAL OUTLINE OF THE THESIS

PART 1

Although the exact pathogenesis of sarcoidosis is still unknown, convincing evidence has been obtained that $CD4^+$ T cell immunology is central in sarcoid formation. **Chapter 2** gives a general introduction in sarcoidosis immunopathogenesis and summarizes current knowledge on the contribution of T cells in sarcoidosis pathogenesis.

In **chapter 3**, we aimed to identify characteristics of circulating T cells that would distinguish patients from healthy controls and would correlate with long-term prognosis. In this study, we performed an in-depth phenotypic characterization of peripheral blood (PB) T cells in treatment-naïve sarcoidosis patients and healthy controls, with a specific focus on surface expression of activation makers. The observed T cell phenotypes were analyzed in relation to disease outcome two years after diagnosis to investigate if T cell phenotype could be a reliable biomarker for prognosis. Additionally, we investigated if circulating naïve and memory T cells are functionally different in patients with sarcoidosis compared to controls by measuring surface expression of activation makers and cytokine profile upon stimulation.

Sarcoidosis disease course is variable and unpredictable. The decision to initiate first line glucocorticoid treatment for sarcoidosis is based on an individual assessment of dangerous or progressive organ involvement and quality of life, but the individual response to prednisone is highly variable and may have significant toxicity and side effects. In **chapter 4**, we hypothesized that the circulating $CD4^+$ T cell phenotype of patients with sarcoidosis might correlate with lung function response to prednisone treatment. We evaluated baseline frequencies and phenotypes of circulating T cell populations in newly diagnosed patients with pulmonary sarcoidosis before standardized prednisone treatment started and correlated these T cell parameters with lung function response at 3 and 12 months.

In **chapter 5**, we review categories of drugs that have been associated with the development of a systemic granulomatous disease that is clinically and histopathologically indistinguishable from sarcoidosis. Unraveling the mechanisms involved may greatly improve our understanding of immunopathogenesis and the link between aberrant T cell activation and sarcoidosis.
Abatacept, a recombinant CTLA-4-immunoglobulin (Ig) fusion protein, inhibits T cell activation by binding to CD80/CD86 on antigen presenting cells and thereby depleting the ligand for CD28, which serves as a co-stimulatory receptor on T cells. A prospective open-labelled single arm phase II trial (ABASARC) was conducted in Germany to assess the safety and efficacy of abatacept treatment in chronic, steroid-refractory sarcoidosis. In **chapter 6**, we investigated the effect of CTLA-4-Ig on the phenotype of circulating T cells in patients with chronic sarcoidosis participating in the ABASARC trial and aimed to identify patients who benefit most from this potential new treatment. The observed baseline T cell phenotypes and activation marker expression were analyzed in relation to one-year pulmonary function response and compared with paired samples at 52 weeks.

Currently, great effort has been put into the development of various murine sarcoidosis models, but there is still a lack of *in vivo* models that recapitulate all aspects of human disease (129). In particular, the development of an animal model to understand the role of T cells in sarcoidosis pathophysiology and investigate new therapeutic targets is warranted. In **chapter 7**, we analyzed mice with CTLA4 haplo-insufficiency (expression from only one allele) as well as complete absence of CTLA-4 specifically on IL17-producing T cells or Tregs. To this end, we made use of the Cre-loxP system, which is a widely used powerful technology for mammalian gene targeting. Hereby a single-Cre recombinase recognized two directly repeated LoxP sequences, and subsequently excises the DNA that is flanked by the LoxP sites, i.e. floxed DNA. $CTLA4^{fl/fl}$ mice were crossed with Il17a-cre transgenic (targeting IL-17A-producing cells) and FoxP3-cre transgenic mice (targeting Treg cells). Mice that were haplo-insufficient or completely deficient for CTLA in IL-17-expressing T cells, haplo-insufficient for CTLA4 in Tregs were studied, whereby wild-type littermates served as controls. These mice were evaluated for signs of autoimmunity, spontaneous T cell activation and granulomatous responses. Additionally, we aimed to describe the effects of exposure to trehalose 6,6'-dimycolate (cord factor) of mycobacterium tuberculosis in our mice, which is an existing murine model to induce granulomas (130).

There is an unmet need for new treatment options in sarcoidosis. In **chapter 8**, we critically discuss the mechanisms of action of potential therapeutic targets based on disease pathogenesis and summarize ongoing clinical trials in sarcoidosis, including treatment strategies in progressive fibrotic sarcoidosis.

PART 2

Pirfenidone slows down disease progression in IPF. However, the safety and effectiveness of pirfenidone in asbestosis remain unclear. In **chapter 9**, we aimed to investigate the safety, tolerability and efficacy of pirfenidone in asbestosis patients with a progressive phenotype in a multicenter prospective study at three sites in the Netherlands performed by the Dutch Association of Pulmonologists (NVALT). We use daily home monitoring of forced vital capacity (FVC) before and after the start of antifibrotic treatment, to obtain a more granular overview of lung function change over time and measure treatment efficacy.

Finally, I integrated the findings of this thesis in a general discussion in **chapter 10**. The main results are evaluated in the context of current and new scientific literature and their contribution to our understanding of disease pathogenesis and improvement of personalized treatment is discussed. Furthermore, outstanding research questions are summarized and additional scientific studies to address them are suggested.

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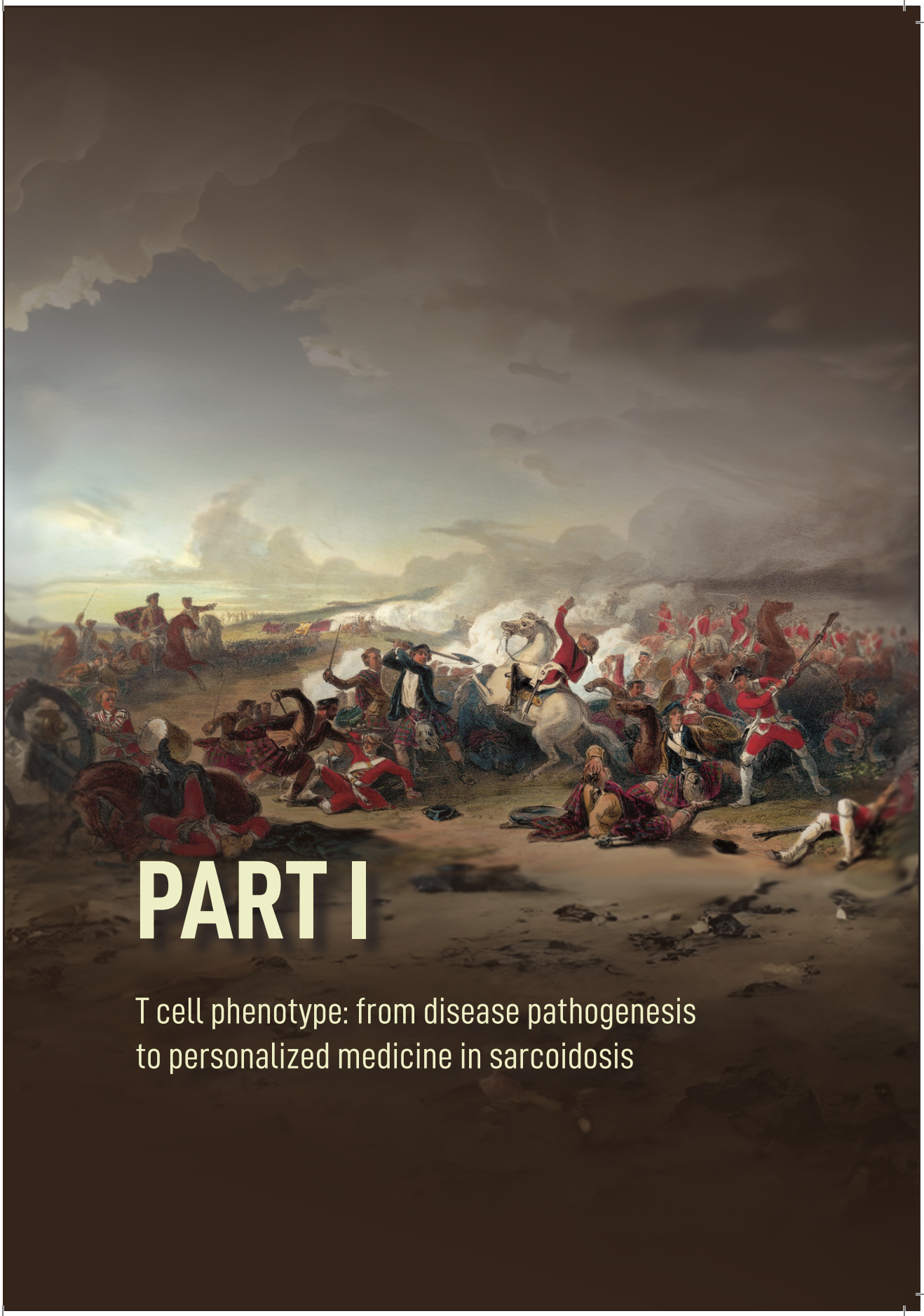

PART I

T cell phenotype: from disease pathogenesis to personalized medicine in sarcoidosis

CHAPTER 2

The immunopathogenesis of sarcoidosis

Jelle R. Miedema, Francesco Cinetto, Anna Smed-Sörensen, Paolo Spagnolo

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INTRODUCTION

The pathogenesis of sarcoidosis is often described as an "immune paradox" because of the apparent inconsistency between peripheral anergy (i.e. reduced delayed-type hypersensitivity to tuberculin and common antigens) (1) and the exaggerated inflammatory response in affected organs (2). Several mechanisms are believed to contribute to this immune paradox **(Table 1)**, including a disequilibrium between effector T and regulatory T lymphocytes (Tregs), notably cluster of differentiation (CD)$4^+CD25^{bright}FoxP3^+$ cells (2), the compartmentalization of activated T cells at disease sites with consequent peripheral blood lymphopenia (3) and the immunosuppressive effect of $CD8^+$ T cells that accumulate in peripheral tissues in patients with active disease (4, 5). The hypergammaglobulinemia observed in sarcoidosis suggests that B cells may also be involved in the granulomatous inflammatory response (6).

Noncaseating epithelioid cell granulomas are the histological hallmark of sarcoidosis (**Figure 1**). Macrophages differentiate into epithelial cells, which then fuse to form multinucleated giant cells. $CD4^+$ T helper (Th) cells are scattered throughout the granuloma whereas $CD8^+$ T cells, Tregs, fibroblasts, and B cells surround the periphery of the granuloma (7). Tregs have been found at increased levels in bronchoalveolar lavage fluid (BALF) and peripheral blood of patients with active sarcoidosis (8), but results are conflicting as decreased Treg proportions in BALF associated with worse prognosis and need for prednisone treatment after 1-year follow up (9). Notably, Tregs exert a profound antiproliferative activity, and inhibit the production of interleukin (IL)-2 but not of interferon-gamma (IFN-γ) or tumor necrosis factor-alpha (TNF-α), which are key determinants of granuloma formation and maintenance (2). Granulomas are not unique to sarcoidosis as they are found in several other diseases, including infection and malignancy (10). Indeed, granulomas represent a pathologic response to an insoluble or poorly degradable antigenic substance, forming a physical barrier to isolate the causative agent and protect adjacent tissues from injury. The development and progression of sarcoid granulomas involve both innate and adaptive immune responses (11).

Sarcoid granulomas result from a complex interplay between immune cells and their mediators. However, the first event is the phagocytosis and degradation by antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs), of yet unidentified foreign material, which is expressed on the surface of APCs as small peptide fragments complexed in the groove of class II Major Histocompatibility Complex (MHC) molecules (12). $CD4^+$ T cells recognize and bind the MHC/oligopeptide complex through T-cell receptors (TCRs) on their surface, thus forming a "trimolecular complex" (MHC/peptide/TCR), which provides the activation signal for the antigen-specific T cells along with several co-stimulatory molecules (13). T cell differentiation into effector subsets requires further important signals from the local cytokine milieu, including IL-1β, IL-6, IL-12, IL-23 and transforming growth factor beta (TGF-β), produced by activated DCs and macrophages (14). Activated T cells are now ready to orchestrate the immune response that

culminates with granuloma formation by producing IFN-γ, IL-2 and IL-17A (14, 15). Increased expression of the chemokine receptor CXCR3 and transcription factor T-bet contributes to the highly polarized Th1-type cytokine response typical of sarcoidosis (16). Th17 cells, one of the major pathogenic $CD4^+$ Th cell populations (17), also contribute to granuloma formation (14). It is possible that Th17 cells, that express the Th17 chemokine receptor CCR6, are primed in mediastinal lymph nodes of sarcoidosis patients and further differentiate at affected sites into CCR6 and CXCR3 co-expressing Th17.1 cells, that are able to produce IFN-γ. In the BALF of sarcoidosis patients, Th17.1 cells are increased and represent the predominant proportion of total memory $CD4^+$ T cells (18). Additionally, Th17.1 cells are increased in mediastinal lymph nodes of patients with sarcoidosis (compared to healthy controls) and increased Th17.1 proportions in BALF correlate with the development of chronic disease (18).

In sarcoidosis, the increased expression of Th1 cytokines and chemokines that is observed at the time of diagnosis, if untreated, can perpetuate granulomatous inflammation for many years (19). However, whether irreversible fibrosis results from chronic inflammation secondary to a long-lasting Th1 cytokine response or a switch to fibrosis promoting Th2 cytokine response remains unclear. Overall, sarcoidosis is a highly dynamic process involving multiple immune cell subsets, and the regulation of the Th1/Th17/Th2 balance is likely to be critical in determining disease burden and clinical behavior.

Table 1: Mechanisms believed to contribute to the sarcoidosis immune paradox*

Increased percentage of Tregs in peripheral blood of sarcoidosis patients
Increased levels of activated $CD4^+$ and $CD8^+$ T cells in BAL fluid and at sites of granulomatous inflammation with peripheral lymphopenia
Increased expression of Th1 chemokines and chemokine receptors (CXCR3, CCR5, IL-12R and IL-18R) on $CD4^+$ T cells in BAL fluid compared to peripheral blood.
Elevated $CD4^+$:$CD8^+$ ratio at sites of localized granulomatous inflammation
Imbalance of Tregs and Th17.1 cells between peripheral blood and BAL fluid
Impaired circulating blood dendritic cell function
Differentially expressed inflammatory markers (angiotensin-converting enzyme, chitotriosidase, soluble IL-2 receptor, IL-12, IL-18, neopterin, monocyte chemoattractant
protein-1 and TNF receptors) peripherally and at sites of granulomatous inflammation
Higher percentage of T lymphocytes expressing IL-2, IFN-γ and TNF-α in BAL fluid compared to peripheral blood lymphocytes

*The term "immune paradox" refers to the coexistence of intense localised granulomatous inflammation and peripheral anergy (poor response to common antigens *in vitro* and *in vivo*).

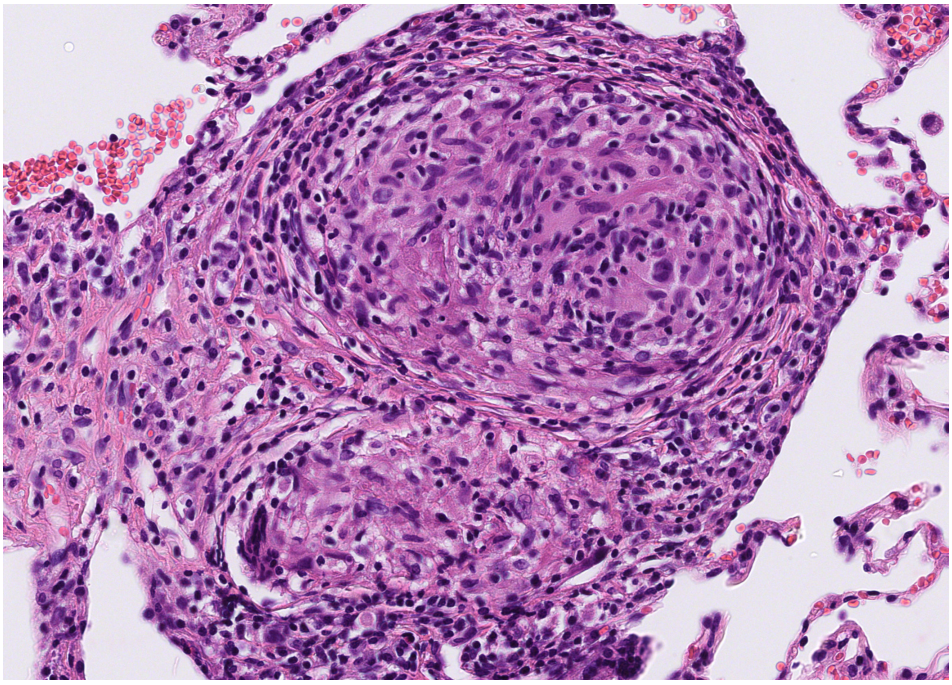

Figure 1: Structure of a typical non-caseating sarcoid granuloma, demonstrating a core of epithelioid histiocytes and multinucleated giant cells and a rim of T lymphocytes (hematoxylin-eosin staining, magnification x200).

Building granulomas in the susceptible host: the role of the innate immune system

In the past few years, the key regulatory role of the innate immune system in the pathogenesis of sarcoidosis via its cellular and humoral arms has been increasingly disclosed. Surprisingly, animal models such as severe combined immunodeficiency (SCID) mice and zebrafish embryos (lacking T cells in the first days of life) demonstrated that aggregates of epithelioid macrophages with pathological hallmarks of granulomas can still be generated after mycobacterial infection in the complete absence of adaptive immunity (20, 21). Moreover, sarcoid-like granulomatous disease may occur in humans with adaptive immunity defects, including common variable immune deficiency (CVID) (22) or recombination activating gene (RAG) deficiency (23).

Macrophages in particular do not only form the core of the granuloma, but seem to be crucial in its initiation (**Figure 2**)(24, 25). Both tissue resident and monocyte-derived macrophages are known to be involved in tissue homeostasis and repair. Due to their broad pathogen recognition receptor (PRR) repertoire including toll-like receptors (TLR) and their phagocytic activity, they exert a surveillance function acting as interface towards environmental warning cues (**Figure 2**) (26). Upregulated and disordered toll-like receptor response and related genetic polymorphisms

have been implicated in the pathogenesis and clinical behavior of sarcoidosis (27-30). TLR9 expression was found increased in alveolar macrophages and giant cells which associated with $CD4^+$ T cells in BALF, and its activation induced CXCL10 release in patients compared to controls (31). A dysregulated TLR2 response was found to correlate with increased TNF-α and Il-6 production in BALF cells (27). Additionally, TLR3 polymorphism L412F has been associated with chronic disease (30).

Detection of danger signals derived from tissue injury, dead or dying cells, infectious agents or foreign substances can activate macrophages, and subsequent cell aggregation and interaction with T cells can ultimately lead to granuloma formation. In case of persistent microenvironmental triggers, macrophages can differentiate into epithelioid and subsequently multinucleated giant cells, while recruiting other immune cells such as T cells and monocytes, but also B lymphocytes, granulocytes, DCs and fibroblasts (32). Furthermore, monocytes/monocyte-derived cells from BALF of sarcoidosis patients were found to produce high amount of TNF-α even without stimulation, thus contributing to local and systemic inflammation. Of note, high frequencies of these TNF-α-producing cells in BALF have been associated with progressive disease (33).

In combination with TLR engagement, cytokines like IFN-γ and TNF-α are considered inflammatory signatures of sarcoidosis. These cytokines are known to induce an initial classic (M1) macrophage polarization with nuclear factor kappa B (NF-kB) mediated activation of several interferon-regulatory factors (26). However, an alternative M2 polarization of macrophages and multinucleated giant cells has been shown in granulomas of sarcoidosis patients with lung, lymph node, skin, and neuromuscular involvement (34-36). Of note, these alternatively activated M2 macrophages were shown to be relatively insensitive to conversion to M1 state in the presence of Th1 cytokines. Moreover, they were able to induce myofibrosis in neuromuscular sarcoidosis (36). Well described *in vitro* and *in vivo* granuloma models support the presence of M2 macrophage polarization during initial granuloma formation (24, 25). In particular, an M2-like macrophage phenotype was demonstrated within granulomas in lung, liver, heart and lymph nodes in a murine sarcoidosis model (24, 37). It is important to note that, starting from the concept of a 'dual nature' of macrophages being either classically activated (M1) or alternatively activated (M2), it has been hypothesized that macrophages might transit from an initial M1-driven phase (promoting granuloma formation) to a more M2 polarized phase during disease progression, possibly promoting fibrosis (34). All these findings suggest that the M2 macrophage signature might represent a potential marker of chronic disease. However, the M1/M2 polarization is likely a simplified model. Our current understanding of macrophage physiology is less "dogmatic" and considered more complex and plastic, with a broad spectrum of responses that these cells are required to provide *in vivo* (38, 39). During chronic stimulation, TLR2-mediated signaling is able to promote both M1 and M2 immune responses, depending on the microenvironmental conditions and local nutrient supply (40, 41). For instance, arginine availability has been proposed as a determinant of macrophage activation fate (41). Also, peroxisome

proliferator-activated receptor gamma (PPAR-γ), which belongs to the nuclear hormone receptor family, is known to promote M2 activation. A significant reduction of PPAR-γ was found in alveolar macrophages from pulmonary sarcoidosis patients (42), thus favoring a Th1 immune response (43). In addition to PPAR-γ, intracellular signals induced by hypoxia, amino acids, lack of nutrients and growth factor deprivation, as well as exposure to chemicals and toxins may all lead to an

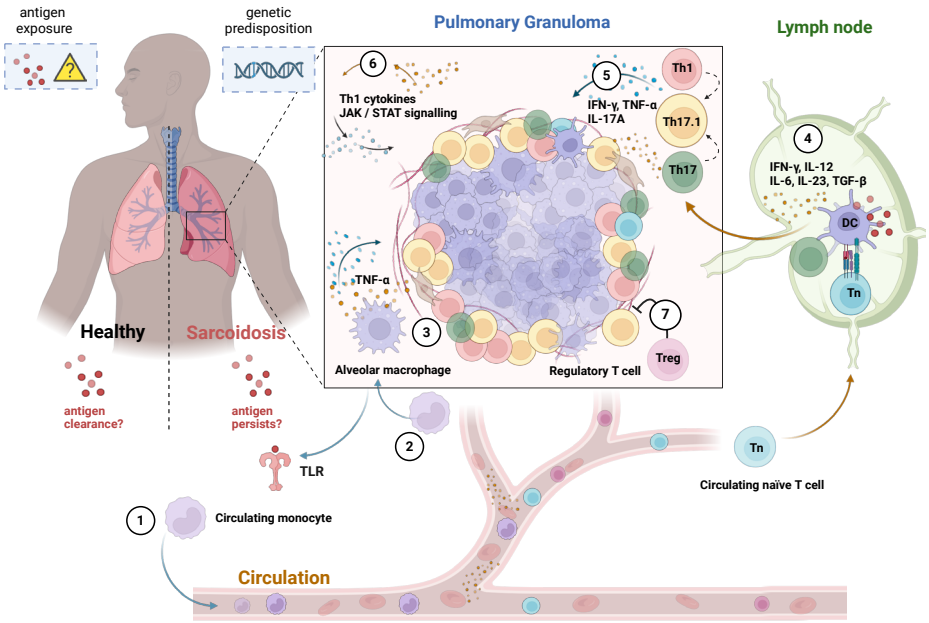

Figure 2: Proposed immunopathogenesis of sarcoidosis: innate and adaptive responses
Exposure to unidentified antigens triggers an exaggerated immune response in genetically predisposed individuals. Persistent antigen is sensed by pattern recognition receptors including Toll-like receptors (TLR) on circulating monocytes (**1 and 2 in figure**) and tissue resident macrophages (**3**). Circulating monocytes are attracted to the affected tissue and differentiate into monocyte derived alveolar macrophages (**3**), where they further contribute to the local inflamed microenvironment, through antigen presentation and secretion of pro-inflammatory mediators and chemokines that attract T cells to the inflammatory site (**3**). Upon contact with antigen, dendritic cells (DC) migrate towards draining lymph nodes to prime T cell responses by presenting the antigen as MHC/oligopeptide complex in combination with co-stimulatory signals and pro-inflammatory cytokines that polarize naïve T cells (Tn) into different effector subsets, e.g. T helper (Th) 1 or Th17-cells, which are then recruited to the lung (**4**). In the inflamed tissue, interferon gamma (IFN-γ)-producing Th17 cells, called Th17.1-cells, represent the predominant fraction of total memory $CD4^+$ cells (**5**). In the lung, levels of the Th1 promoting cytokines interleukin (IL)-12 and IL-18 are increased and T cells produce Th1 cytokines including IL-2, tumor necrosis factor alpha (TNF-α) and IFN-γ, further amplifying the inflammatory cascade (**6**). Downstream signaling of several key cytokines in the local granulomatous inflammation involve the Janus Kinase (JAK) pathway, that subsequently phosphorylates signal transducers and activators of transcription (STATs) (**6**). Additionally, the immunosuppressive function of regulatory T cells (Tregs) is compromised, which contributes to ongoing inflammation (**7**). Figure created with BioRender.com.

upregulation of autophagy (44). It is likely that these signals and their influence on metabolic pathways in macrophages are involved in sarcoid granuloma initiation and disease progression. Another feature of activated metabolism in sarcoid macrophages is the involvement of the pentose phosphate pathway (45).

In line with the idea that the metabolic signature is a key determinant of innate immune involvement in sarcoidosis, the mechanistic target of rapamycin (mTOR) pathway was found to be involved in granuloma formation in a murine model (24, 37). To this end, conditional knockout of its inhibitor tuberous sclerosis complex (TSC) 2 in myeloid cells in a murine model led to spontaneous and self-maintaining noncaseating granulomatous aggregates in the lung, heart, liver, and lymph nodes (24, 37). The mTOR complex 1 (mTORC1) acts as an autophagy inhibitor by regulating catabolic and anabolic cell processes and mTORC1 activation has been identified through gene set enrichment analysis in clinical progressive versus self-limiting sarcoidosis in humans (24, 46). Bone marrow transplantation from TSC2-deleted mice recapitulated the granulomas in wild type recipient mice. Importantly, treatment with the mTOR inhibitor everolimus led to complete resolution of granulomatous inflammation (24). Sustained activation of mTORC1 in the murine model resulted in apoptosis inhibition, M2-like macrophage differentiation and CDK4 expression with consequent cell metabolic re-programming and increased transcription of E2F targets and glycolysis-related genes (24). Interestingly, whereas mTORC1 is a negative regulator of autophagy, aberrant autophagy and hampered clearance of antigens were proposed to promote granuloma formation (44).

One of the key regulators of glycolysis is the oxygen-sensitive transcription factor hypoxia inducible Factor-1α (HIF-1α). mTORC1 can activate HIF-1α irrespective of decreased oxygen concentration, thus inducing the transcription of hypoxia-associated genes (47, 48). These genes are commonly responsible for tissue adaptation to decreased O_2 availability and promote glycolysis, cell differentiation, proliferation, and angiogenesis. The HIF-1α signaling pathway was found activated in alveolar macrophages from patients with sarcoidosis cultured under normoxic conditions (49). The enrichment of this pathway was mechanistically linked to IL-1β, IL-6 and IL-17 production, because HIF-1α inhibition in peripheral blood mononuclear cells (PBMCs) from patients reduced the production of these cytokines (49). Notably, BALF exosomes from sarcoidosis patients, compared to healthy controls, were also shown to induce a dose-dependent elevation of intracellular IL-1β in monocytes, as well as IL-6 and TNF release (50).

Innate humoral immunity, such as the complement cascade, serum amyloid A (SAA) and pentraxine-3 (PTX3), has also been implicated in the pathogenesis of sarcoidosis (51-53). A marked increase of C4d and C5a levels was found in BALF of sarcoidosis patients, in association with locally produced ficolin-3. Pattern recognition molecules of the lectin system may indeed trigger complement activation, leading to a sustained lung inflammation (52). Lectin proteins may also act as opsonins, attracting alveolar macrophages and other phagocytes. SAA is an innate receptor ligand, whose localization to macrophages and giant cells within sarcoid

granulomas was found to correlate with $CD3^+$ lymphocytes (54). SAA can drive pro-inflammatory differentiation of macrophages in the lung and may be secreted at tissue level by different cell types, including macrophages. Its aggregation within granulomas, together with the putative sequestered antigen, has been associated with promotion and maintenance of chronic granulomatous inflammation in sarcoidosis (54). Acting through TLR2, this protein has been shown to activate NF-kB, regulating Th1-mediated granulomatous inflammation in the lungs of sarcoidosis patients (54). More recently, its role in Th17 differentiation has also been highlighted, since SAA 1 and 2 proteins have been shown to drive a distinct Th17 pro-inflammatory differentiation program independent on TGF-β microenvironmental levels (55). SAA can also activate the NLRP3 inflammasome pathway, which has been shown to be hyper-activated in sarcoidosis (56).

PTX3, a pattern recognition molecule with a fundamental role in innate immunity, has recently been suggested as another potential key link between innate humoral immunity, macrophages, and granulomatous inflammation (53). It was demonstrated that PTX3 deficiency enhances inflammation and granuloma formation in the lung of knock-out mice. Interestingly, PTX3 can hinder complement-mediated inflammation during granuloma formation and regulate the metabolic activity of macrophages, preventing their mTORC1-dependent proliferation and aggregation and ultimately influencing clinical features of sarcoidosis (53). Of note, SAA influences PTX3 secretion and the interplay between these two proteins might coordinate the functional activity of macrophages, also in response to putative triggers, potentially leading to granuloma formation.

In summary, the innate immune system, in particular, alveolar and monocyte-derived macrophages are critically involved in granulomatous inflammation. An mTORC1-dependent HIF-1α mediated transcription program seems to be a cornerstone of the pro-inflammatory granulomatous microenvironment, characterized by IL-1β and IL-6 enrichment. Additionally, SAA and PTX3 appear to be key links between innate and adaptive immunity, involved in the induction and maintenance of sarcoid granulomatous inflammation (44).

Ongoing granulomatous inflammation: the role of dendritic cells and T cells

Dendritic cells and antigen presentation

Dendritic cells (DCs) are a heterogeneous population of innate immune cells found in blood and throughout tissues. As innate immune cells, DCs contribute to inflammation through cytokine and chemokine secretion in a similar way as described earlier for macrophages. In addition, DCs are antigen-presenting cells with the unique capacity to activate naïve T cells, and are thus described to bridge innate and adaptive immune responses (57). While the precise role of DCs in sarcoidosis remains to be elucidated, it is likely that DCs are important in disease pathogenesis by contributing to inflammation as well as by initiating and maintaining T cell activation.

DCs are often divided into three subsets, $CD141^+$ conventional dendritic cell 1 (cDC1), $CD1c^+$ conventional dendritic cell 2 (cDC2) and CD11c-, $CD123^+$ plasmacytoid dendritic cells (pDC) (58-60). In addition, more heterogeneity among the cDC2s than previously appreciated has recently been reported (61-64). The different DC subsets share many features, but also specialize in certain functions. pDCs secrete high levels of type 1 interferons, particularly during antiviral responses, and can induce $CD4^+$ T cell activation or self-tolerance, depending on the immunological context (65). cDCs are highly efficient antigen presenting cells and potent inducers of T-cell activation and differentiation (57), with cDC1 (now sometimes termed DC1) being specialized in cross-presentation of antigens on MHC-I to $CD8^+$ T cells (59) and cDC2s presenting antigens on MHC-II to $CD4^+$ T cells (58). In the lung, DCs reside in close proximity to the epithelium and within the interstitium (66) and are also recruited during inflammation to take up antigens (67). Upon antigen uptake, cDCs upregulate activation markers and migrate towards draining lymph nodes to prime T cell responses by presenting the antigen on MHC in combination with co-stimulatory signals and pro-inflammatory cytokines (68, 69). The differentiation of $CD4^+$ T cells into different effector subsets, e.g. Th1 or Th17-cells, requires specific signals from the local cytokine milieu. IL-12 derived from DCs is critical for Th1 polarization and subsequent IFN-γ production (70), while IL-6, IL-23 and TGF-β are essential for Th17 differentiation (71) (**Figure 2A**). Maturation of cDCs is complex and can be assessed by the expression of human leukocyte antigen (HLA)-DR and co-stimulatory molecules such as CD80 and CD86 (72, 73). During inflammation, classical $CD14^+$ monocytes are recruited from the circulation and differentiate into monocyte-derived DCs (moDCs) at the site of inflammation (74). Monocyte-derived DCs control the effector T-cell response through antigen presentation and the secretion of pro-inflammatory chemokines that subsequently attract T cells to the inflammatory site (75).

Conflicting results on DCs in sarcoidosis have been reported and only a few studies addressed their functionality, probably due to their complex biology, low proportions in BALF and different strategies used for their evaluation (1, 66, 76, 77). Advances in definition, classification and identification have increased our understanding of the integral role of DCs in disease pathogenesis (72, 78, 79). In sarcoidosis, the frequency or number of circulating DCs were either found reduced (80, 81), increased (82), or equal (1) compared to controls. Measuring surface expression of co-stimulatory molecules on circulating cDC and pDCs showed no difference with healthy subjects (82). Early functional analysis of moDCs from peripheral blood of patients with sarcoidosis showed a decreased ability to stimulate T cells compared to healthy and diseased controls that correlated with pulmonary disease severity (1).

However, the biology and role of DCs in disease pathogenesis can only be understood properly if their heterogeneity and tissue distribution in the inflamed organs are taken into consideration. In sarcoidosis tissue, mature DCs with high CD86 expression were observed in the rim of the granuloma in close proximity to T-cells, which suggests their interaction and contribution to ongoing T-cell activation (80, 82). In BALF, enhanced proportions of moDCs and

pDCs were found in patients, while the amount of pDCs was similar to controls in another study (82, 83). An altered maturation status of pulmonary moDCs in sarcoidosis was suggested by increased CD80 and decreased CD86 expression compared to controls (83). More mature DCs with high HLA-DR and CD80 expression in BALF compared to blood were found in sarcoidosis (76). Importantly, this indicates a more pronounced DC activation in the inflamed tissue compared to the circulation, although this finding was not compared to healthy controls. Co-culture experiments with isolated moDCs from the lungs of controls and sarcoidosis patients with allogenic naïve T-cells demonstrated similar T cell proliferation and differentiation, which indicates an intact pulmonary DC function (77). In these co-culture experiments, moDCs from patients induced significantly increased TNF-α release, which suggests they are intrinsically altered. Co-culture experiments with allogenic T cells from healthy donors were performed to elucidate the T cell activating potential of DC subsets, monocytes and monocyte-derived cells from peripheral blood and lung of sarcoidosis patients (84). In this study, cDC2 from the circulation and BALF of sarcoidosis patients most potently induced T cell proliferation. Importantly, $CD4^+$ T cells with IFN-γ production were primarily induced, with some T cells also producing IL-17 (84). This typical and pathogenic T cell polarization profile is found in the lungs and lymph nodes of patients with sarcoidosis (discussed in detail below).

Taken together, these data suggest that DCs are major contributors of T cell activation and polarization in the lymph nodes and inflamed tissue of patients with sarcoidosis.

Localization, phenotype, and function: the role of $CD4^+$ T cells

Convincing evidence exists that $CD4^+$ T cells are central to sarcoidosis pathogenesis (85). During active disease, blood lymphopenia can be observed that correlates with severity and non-resolving disease (86, 87). In the periphery, naïve T cells circulate between the blood and secondary lymphoid organs, where they differentiate into effector and memory T cells upon antigen recognition and polarization by the local cytokine milieu (**Figure 3A**) (88). Evidence of antigen-independent $CD4^+$ naïve T cell activation and decreased apoptosis was found in the peripheral blood of patients using single-cell RNA-sequencing (89). Additionally, increased proportions of activated naïve T cells in the circulation correlated with worse prognosis (90). Although naïve T cells have - by definition - not encountered antigens yet, their pre-activated state probably contributes to the exaggerated T cell immune response typical of sarcoidosis (90).

In contrast to their naïve precursors, circulating $CD4^+$ T cells were found to have an exhausted phenotype, reduced proliferative capacity and increased expression of co-inhibitory receptor programmed death-1 (PD-1) (91, 92). Single-cell RNA-sequencing demonstrated upregulated anergy associated genes (89). Importantly, cytokine expression on $CD4^+$ Th cells derived from the periphery was significantly decreased upon T cell receptor stimulation (91). However, a substantial difference in proportions and function of T helper (Th) cells was demonstrated between the circulation and chronically inflamed sites such as the mediastinal lymph nodes

(MLNs) or lung (18, 93-95). An expanded population of highly activated $CD4^+$ T cells was found in the lung of sarcoidosis patients, suggesting that the disease is mostly restricted to the involved organs (95-97). The characteristic combination of peripheral anergy, highlighted by the lack of hypersensitivity skin reaction to several antigens, and local immune activation in sarcoidosis has been described as the immunological paradox (2). It is therefore crucial to acknowledge this compartmentalization of T cells when interpreting results from published studies.

In BALF from sarcoidosis patients, the levels of the Th1 promoting cytokines IL-12 and IL-18 are increased and the vast majority of lymphocytes express the Th1 cytokines IL-2, TNF-α and IFN-γ (93, 94, 98). Accordingly, downstream expression of Th1 chemokines CXCL9, CXCL10, CXCL11, CCL2, RANTES/CCL5 and their respective receptors (CXCR3, CCR5) was found in the inflamed lung (99-104). The typical increase of IFN-γ production by $CD4^+$ T cells in BALF was initially ascribed to Th1-cells (93). However, increased proportions of IFN-γ-producing Th17 cells, called Th17.1-cells, were found to be the main source of IFN-γ in sarcoidosis BALF, and high proportions at diagnosis correlated with the development of chronic disease (18, 95).

T helper 17.1 cells can be defined based on concomitant chemokine receptor expression of the Th1 (CXCR3) and Th17 lineage (CCR6). They represent the most predominant fraction of total memory $CD4^+$ cells in the inflamed lung, while proportions are intermediate in MLNs and low in peripheral blood of patients (**Figure 3B**) (18). The vast majority of Th17.1 cells in sarcoidosis BALF produce IFN-γ and only a small proportion produce both IL-17 and IFN-γ (95). It is hypothesized that Th17 cells, after priming within sarcoidosis MLNs, show plasticity towards pathogenic end-stage Th17.1 effector cells in the lung microenvironment (14, 95). Importantly, Th17 lineage cells show signs of aberrant activation in sarcoidosis, highlighted by decreased expression of Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) in the MLNs (105). Because CTLA-4 is a crucial suppressor of T cell-mediated immune responses, reduced expression on Th17 cells will likely contribute to their activation.

Increased $CD4^+$ T cell proportions simultaneously expressing transcriptional regulators T-bet (Th1 lineage) and RORγT (Th17 lineage) were found most pronounced in the lung of patients with Löfgren's syndrome, who generally recover spontaneously from the disease (106). Importantly, this characterization includes IL-17/IFN-γ double producing (DP) Th cells, which are believed to be a highly proliferative intermediate stage between Th17 and Th17.1 cells (**Figure 3A**) (14). The correlation with favorable prognosis in this study may be explained by IL-17/IFN-γ DP cells, a broader range of Th17 cytokines and lower levels of IFN-γ in the BALF of patients with Löfgren's syndrome (106, 107).

In contrast to Th1 cells, Th17.1 cells are associated with resistance to the immunosuppressive effect of glucocorticoids, as a proportion of them express the multidrug resistance protein-1 (108). Many cytokines involved in the aberrant immune response and T cell activation observed in sarcoidosis signal via the Janus Kinase (JAK) pathway (109, 110). Cytokines bind to their respective receptors, thereby activating JAK complexes that promote phosphorylation of signal

transducers and activators of transcription (STATs) (109). Interfering with the JAK pathway will target key cytokines involved in sarcoidosis, such as IFN-γ, IL-2, IL-6, IL-12, IL-23 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (**Figure 2**) (110, 111).

In contrast to the proinflammatory effects of $CD4^+$ effector T cells, Tregs exhibit strong immunosuppressive capacities on various immune cells (112). The inhibitory function of Tregs is accomplished by the expression of regulatory co-receptors such as CTLA-4 and the production of the anti-inflammatory cytokines IL-10, TFG-β and IL-35 (113). In recent years, efforts have been made to elucidate the role of Tregs in sarcoidosis pathogenesis. Although conflicting results were reported regarding the proportion of circulating Tregs, most studies found increased levels in sarcoidosis compared to healthy controls (90, 114-116). Tregs were most pronounced in the circulation of active or fibrotic sarcoidosis patients (114, 117). Baseline increased Treg proportions with an aberrant phenotype were found in peripheral blood of patients who developed chronic sarcoidosis within the following two years (90). Circulating Tregs demonstrated an aberrant phenotype with increased expression of multiple surface markers, particularly in patients with worse long-term prognosis (90). With regard to Treg function, an impaired immunosuppressive capacity has been consistently reported in studies based on peripheral blood in sarcoidosis (2, 116). Interestingly, restoration of Treg function was associated with disease resolution (116). Possible molecular mechanisms of Treg dysfunction were studied with RNA sequencing analysis, which demonstrated alterations in several pathways including TLR and chemokine signaling (115). Additionally, circulating Tregs in sarcoidosis show increased expression of CD95, the receptor for apoptosis inducer CD95-ligand, making them more susceptible to apoptosis (114). This increase in surface expression of CD95 was specifically found in patients with poor outcome (90).

Global analysis of microRNA expression showed major differences between Tregs isolated from peripheral blood and BALF in sarcoidosis, highlighting the importance of the local microenvironment (115). Conflicting results have been reported about the proportions of Tregs in the lungs of sarcoidosis patients, which were found both increased (118) and comparable (117) to those in peripheral blood. A study that measured Tregs in BALF of patients with sarcoidosis assessed disease activity at 1-year follow up as either chronic-active, non-active or resolution. Hereby, proportions of Tregs were found significantly decreased in the chronic-active disease group compared to controls and other outcome groups. Conversely, patients with resolving disease had unchanged baseline Treg proportions in the lung (9).

Compared to their circulating counterparts, BALF derived Tregs showed reduced proliferation and increased signs of apoptosis, suggesting that their function is even more compromised in affected tissues (**Figure 3**) (118). Additionally, Tregs isolated from MLN showed decreased CTLA-4 expression, which probably impairs their suppressive function (105). In contrast, the expression of inducible co-stimulator (ICOS) was found to be increased on Tregs in patients with Löfgren's syndrome, suggesting enhanced activation and Treg mediated suppression in patients with acute sarcoidosis (119).

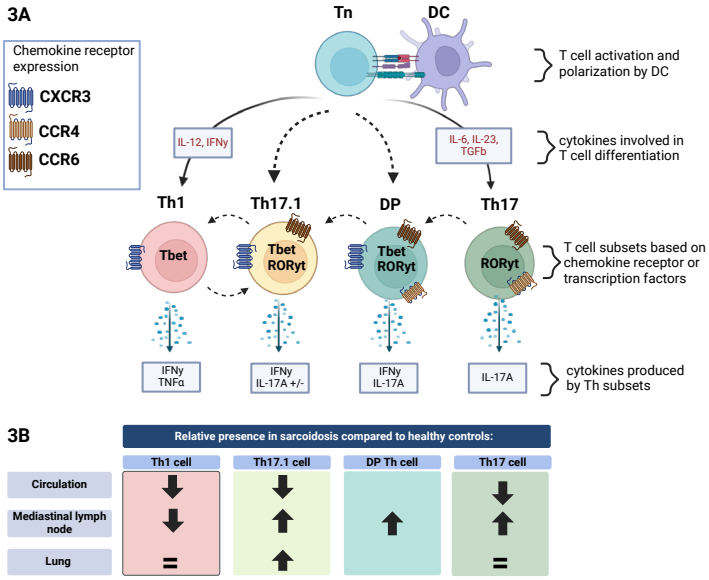

Figure 3: T cells, chemokines and transcription factors involved in sarcoidosis pathogenesis and their proportions in the blood, mediastinal lymph nodes and bronchoalveolar lavage.
3A. Dendritic cell (DC) priming and differentiation of naïve T cells (Tn) into different effector subsets, e.g. T helper (Th) 1, Th17.1, IFN-γ and interleukin (IL)-17 double producing (DP) cells or Th17-cells. T effector subsets can be defined based on chemokine receptor expression of the Th1 lineage (CXCR3) and Th17 lineage (CCR6), the expression of transcriptional regulators of the Th1 lineage (T-bet) and Th17 lineage (RORγT) and their cytokine expression profile. Interleukin-12 derived from DCs favors Th1 polarization and subsequent interferon gamma (IFN-γ) production. Transforming growth factor beta (TGF-β), IL-6 and IL-23 are involved in Th17 differentiation. **3B.** Table demonstrating differences in the relative presence of Th1, Th17.1, DP and Th17 subsets in the circulation, mediastinal lymph nodes and lungs of patients with sarcoidosis. Figure created with BioRender.com.

Many aspects of sarcoidosis pathogenesis are still being unraveled and challenges remain on the road towards personalized treatment. T cells hold promise as potential biomarkers that may associate with disease phenotypes, long-term prognosis, and response to specific treatment. Increased understanding of the role of T cell subsets in progressive sarcoidosis may lead to novel therapies targeting pathogenic T cell phenotypes.

Other immune cells in sarcoidosis: bystanders or contributors?

A role for the interplay between T and B cells in the pathogenesis of sarcoidosis has been suggested, supported by the hypergammaglobulinemia often found in active disease and reports on the successful use of anti-CD20 monoclonal antibodies in refractory sarcoidosis (120). No impairment in antigen-specific antibody response has been shown in sarcoidosis patients, but an altered homeostasis of peripheral blood B cells has been reported, with a decrease in memory

B cell subsets and a predominance of "naïve" and activated B cells (121-123). Moreover, at least two subsets ($CD24^{+++}$ $CD38^{+++}$ and $CD5^{+}$ $CD27^{neg}$) of IL-10 producing regulatory B cells were found highly increased in sarcoidosis patients. An increase in serum B cell activating factor (BAFF) concentration has also been shown in sarcoidosis (124, 125). Furthermore, granuloma surrounding B cells and IgA-producing plasma cells have been reported, together with an increase of $CD27^{neg}$ IgA^{+} B cells in peripheral blood (122). High frequency of somatic hypermutations and increased usage of down- stream IgG subclasses suggest an underlying prolonged or repetitive antigen response. Interestingly, anti-TNF-α treatment was able to reverse this B cell signature in peripheral blood (122). More recently, moving from the finding of an in-situ expansion of $Vα2.3^{+}Vβ22^{+}$ T cells recognizing vimentin peptides in HLA-DRB1*03^{+} sarcoidosis patients, Kinloch et al. (126) were able to demonstrate a selective humoral immune response to the vimentin C-terminus in the lung of these patients. More cellular and inflamed lungs, indeed, presented higher quantities of vimentin, together with $CD4^{+}$ and $CD20^{+}$ cells resembling tertiary lymphoid neo-genesis. In line with previous findings, anti-vimentin C-terminus antibodies (AVA) were found more frequently in BALF of HLA-DRB1*03^{+} patients with BALF titers correlating with the percentage of BALF $CD4^{+}$ $Vα2.3^{+}Vβ22^{+}$ T cells (126). Furthermore, an expansion of memory Th subsets and an imbalance of circulating T follicular helper (Tfh) cell subsets have been suggested to be involved in the development of sarcoidosis (123, 127). Of note, the expansion of Tfh2- and Tfh17-like cells strongly support B-cell activity, particularly in isotype switching and antibody production, compared to the more "regulatory" function of Tfh1 cells (128). This expansion has been implicated in the pathogenesis of different autoimmune diseases (129, 130). A specific population of Tfh-like cells was found in BALF of sarcoidosis patients, characterized by high expression of CD40L and IL-21, providing potent help for B cells to differentiate into antibody-producing cells (131). Consistently, despite being in low numbers, BALF B cells mainly presented a $CD27^{+}$ antigen-experienced phenotype, while a large number of B cells and plasmablasts were detected in sarcoidosis-affected lung tissue on immunohistochemistry staining (131). Although these findings require further investigations, the data support the importance of B and T cells cross-talk and the idea of an in situ adaptive immune response to a defined antigen in the pathogenesis of sarcoidosis.

A recent study on granuloma biopsies from patients with cutaneous sarcoidosis using single-cell sequencing and spatial transcriptomics, clearly identified macrophages, T cells and fibroblasts as the main cell types in the inflammatory lesions (132). Moreover, fibroblasts in granuloma microenvironment were found to have a distinct transcriptional cell state. The study identified two types of granuloma-associated fibroblasts: immune-interacting fibroblasts, involved in the attraction, activation, and retention of immune cells, and tissue-remodeling fibroblasts that contribute to the structure of granulomas (132). This study results underline the contribution of fibroblasts to sarcoid granulomas and suggests therapeutic targeting of granuloma associated fibroblasts as a promising future strategy.

The frequency and role of NK cells has also been investigated in peripheral blood and BALF of sarcoidosis patients. An expansion of a specific and more immature $CD56^{bright}CD16^{neg}$ subset of NK cells has been shown in BALF, producing higher levels of TNFα and IFN-γ compared with BALF from healthy controls, and possibly contributing to the Th1 alveolitis and the perpetation of granulomatous inflammation. NK cells from sarcoidosis BALF were characterized by a high expression of the marker of terminal differentiation CD57 and of NKG2C, the activating counterpart of NKG2A (133, 134). An increased BALF percentage of NK cells in sarcoidosis has been associated with a worse prognosis and higher probability of steroid treatment, as well as higher frequency of Scadding stage IV and extrapulmonary disease, thus suggesting a possible link with fibrogenesis and systemic disease biology (135, 136). Moreover, the more mature $CD56^{dim/neg}$ $CD16^{+}$ BALF NK cells from sarcoidosis patients were characterized by a high expression of activated NK cell markers such as NKp44, CD69 and CD25 compared to autologous peripheral blood. Interestingly, an increase in PD-1 expression by mature NK cells in the BALF of patients was also detected, but the significance of this finding in terms of disease progression is currently unknown (134). Overall, these findings support a role of NK cells in the development of a more severe and chronic sarcoidosis phenotype.

Finally, although the role of neutrophils in the pathogenesis of sarcoidosis is still under investigation, they have been proposed as a potential prognostic marker in a small exploratory study (137). Additionally, higher BALF neutrophil counts were seen in Scadding stage 2 and 3 compared with stages 0 and 1, and patients with high BALF neutrophil counts were more prone to relapse after corticosteroid tapering. A pro-fibrotic role of neutrophils has been hypothesized, due to their capability of elastase and metalloproteases secretion, as demonstrated in other fibrotic lung diseases (138, 139).

Lessons learned from drug-induced sarcoid-like reactions

Multiple categories of drugs have been associated with the development of a systemic granulomatous disease that is clinically and histopathologically indistinguishable from sarcoidosis (140). These drug-induced sarcoid-like reactions (DISRs) occur after initiation of the offending drug and usually resolve following its discontinuation (141). Moreover, a DISR may recur after rechallenge with the culprit drug (142). The majority of DISRs have a relatively indolent clinical course and discontinuation of the pharmacological trigger or treatment of the granulomatous inflammation are not always necessary (142). Because of the striking similarities between sarcoidosis and DISR, it is not known whether DISR represents “true” sarcoidosis or a separate granulomatous syndrome. Either way, it is likely that DISR immunopathogenesis closely resembles sarcoidosis and unraveling the mechanisms involved in DISR may greatly improve our understanding of sarcoidosis.

An analysis from the World Health Organization pharmacovigilance database reported a wide spectrum of drugs associated with DISR, but most common categories include TNF-α

antagonists, interferons and immune checkpoint inhibitors (140). Several possible immunological mechanisms for DISR have been hypothesized (142).

TNF-α antagonists are used as treatment in several immune-mediated diseases, including sarcoidosis. Most of the reported DISRs occur after prolonged anti-TNF-α treatment (143). Although TNF-α has important proinflammatory effects, it also inhibits IFN-α production and pDC maturation (144). Increased proportions of circulating Th17 cells were found in rheumatoid arthritis patients during anti-TNF-α treatment (145). Additionally, increased Th17 cytokines and decreased Tregs were associated with exacerbation of skin inflammation during anti-TNFα treatment in a murine psoriasis model (146). It has been hypothesized that anti-TNF-α treatment may disrupt a delicate immune balance in a subset of patients, leading to increased DC maturation, autoantigen presentation and cytokine imbalance (147).

Interferons are used for the treatment of a range of diseases including multiple sclerosis, hepatitis C and several types of cancer (148). The majority of DISRs associated with this class of drugs occurs within the first 6 months of treatment (142). Proposed mechanisms by which interferons may exacerbate inflammation and induce a DISR include overexpression of MHC II class antigens on APCs and Th1 polarization with increased IFN-γ production (148). In a murine model of experimental autoimmune encephalomyelitis (EAE) that used transferred myelin specific Th1 or Th17 cells, administration of recombinant IFN-β ameliorated symptoms in the Th1 induced EAE but exacerbated the Th17 induced EAE (149). It can be hypothesized that susceptible patients with unknown antigen exposure and Th17 polarization may develop DISR during interferon therapy due to its exacerbating effects on Th17 induced disease.

Treatment with immune checkpoint inhibitors is increasingly used to treat several types of cancer. This expanding category of drugs includes antibodies targeting PD-1, the PD-1 ligand and CTLA-4 (141, 142). Anti-tumor T cell responses are enhanced through blockage of co-inhibitory signals, but the immune activation may also lead to immune-related adverse events including DISR (142). A systematic review found the interval between treatment and occurrence of DISR to be 8.4 months for CTLA-4 and 6 months for PD-1 checkpoint inhibitors (150). Proposed mechanisms that cause DISR during immune checkpoint inhibition include enhanced Th cell activation, exposure to neo-(tumor)antigens and altered Th1/Th17 cell polarization (142). A small study compared pre-treatment blood samples of two patients with melanoma treated with anti-PD-1 who developed DISR to 13 melanoma patients who did not (151). Immunophenotypic analysis demonstrated increased proportions of Th17.1 cells in 5 of 15 patients before treatment, including both patients that subsequently developed DISR. Interestingly, a case study on pulmonary sarcoidosis-like inflammation after CAR T-cell therapy for refractory multiple myeloma also identified a Th17.1 driven mechanism (152). The authors speculate that a cytokine release syndrome that occurred after CAR T-cell infusion may have triggered the expansion of preexisting pulmonary Th17.1 cells, leading to a DISR.

In addition to more common drug categories, DISRs have also been described during treatments that directly interfere with lymphocytes such as anti-CD20 (140), anti-CD25 (153) or

anti-CD52 (154). Other reports describe DISRs during therapies that target T cell polarizing or effector cytokines such as anti-IL-6 (155), anti-IL12/23 (156) and anti-IL-17A (157).

A different drug category associated with DISR is combined antiretroviral therapy (cART) for treatment of human immunodeficiency virus (HIV) infection (141, 142). Before the availability of cART, limited cases of coexistent HIV infection and sarcoidosis were reported (158). This coexistence seems paradoxical as untreated HIV infection is characterized by progressive loss of $CD4^+$ Th cells (159). More recent studies report incident cases of sarcoidosis in people living with HIV and CD4 counts of more than 200 cells/microliter (160). After initiation of cART, an early increase in $CD4^+$ memory Th cells is generally observed, while naïve $CD4^+$ T cells expand later (161). A study that analyzed the effect of cART on sarcoidosis showed that patients had received prolonged cART (29±16 months) before the diagnosis of sarcoidosis was made (162). These findings suggest that cART related restoration of functional peripheral $CD4^+$ T cells, specifically naïve T cells, leads to a regained ability to form sarcoid granulomas during HIV infection (159, 162).

Many aspects of DISR immunopathogenesis remain to be unraveled and the underlying mechanisms can only be speculated upon. Most of the associated classes of drugs are able to alter a delicate immune balance in susceptible patients. In addition to other proposed mechanisms, expansion of pre-existing Th17 subsets or altered Th17 polarization during treatment with the pharmacological trigger are likely to contribute to DISR immunopathogenesis.

Disease resolution versus non-resolving sarcoidosis and fibrosis

Although the majority of patients with sarcoidosis demonstrate disease resolution with or without treatment, about a third of them experience persistent or recurrent disease requiring anti-inflammatory therapy for progressive organ involvement or impaired quality of life (163, 164). Patients with long-standing sarcoidosis are at increased risk of developing pulmonary fibrosis, which is reported in up to 20% of cases (14). Importantly, an estimated 15% of patients with pulmonary fibrotic disease develop a progressive phenotype (165). As anticipated in the previous paragraphs, both the innate and adaptive immune system are associated with resolution or persistence of sarcoidosis, although the precise mechanisms involved in the evolution of the disease remain poorly understood.

Increased proportions of Th17.1-cells in the lung and impairment in Treg number and function have been associated with chronic sarcoidosis and fibrosis (**Figure 4**) (18, 95, 114, 116, 117). In particular, in a mouse model of *Propionibacterium acnes*–induced sarcoidosis-like inflammation, IL-17A has been shown to be critical for the development of TGF-β dependent pulmonary fibrosis following chronic granulomatous inflammation (166).

SAA has also been implicated in chronic granulomatous inflammation and pro-fibrotic skewing in sarcoidosis and increased SAA levels have been associated with fibrotic sarcoidosis (**Figure 4**) (54, 167). Notably, SAA deposition has been shown to influence the imbalance between Th17 and Treg cells via CCL20 expression (168).

Increased proportions of circulating $CD14^+CD16^+$ intermediate monocytes at the time of diagnosis have been associated with the development of chronic sarcoidosis at 2-year follow up (33). Alternative metabolism of arginine by macrophages may also play a pro-fibrotic role, as shown in other fibrotic lung diseases (169). mTORc1-dependent M2-like macrophage differentiation has been related to the development of a pro-fibrotic microenvironment (**Figure 4**). In a mouse model, chronic activation of mTORC1 signaling in $CD11c^+$ cells was sufficient to initiate granulomatous inflammation in the heart and associated with increased fibrosis. Accordingly, treatment with the mTOR inhibitor everolimus did not only resolve the granulomatous inflammation but also prevented cardiac fibrosis (37). HIF-1α activation in monocyte-derived macrophages from active sarcoidosis patients may sustain the pro-fibrotic process, along with its contribution to the pro-inflammatory microenvironment, (47). The role of HIF-1α in the regulation of wound healing and fibrosis has been investigated in a bleomycin-induced pulmonary fibrosis mouse model, where its activation led to production of TGF-β-Induced plasminogen activator inhibitor-1 (PAI-1) from activated alveolar macrophages (170, 171). In monocyte-derived macrophages from patients with highly active sarcoidosis, hypoxia induced HIF-1α transcriptional activity to a significantly higher extent compared to controls, leading to increase of vascular endothelial growth factor (VEGF) and TGF-β1 transcripts, as well as PAI-1 protein, without significant NF-kB activation (47). In particular, PAI-1 is known to play a key role in the development of pulmonary fibrosis by sustaining the fibrotic matrix deposition (**Figure 4**) (172). Moreover, conditioned media from hypoxic monocyte derived macrophages of active sarcoidosis patients was able to inhibit pulmonary fibroblasts migration, which is known to be highly dependent on PAI-1 (47). It is conceivable that PAI-1 secretion by epithelioid cells from sarcoid granuloma may prevent lung fibroblasts from leaving the granuloma surroundings, leading to inefficient alveolar repair following injury and ultimately favoring peri-granulomatous fibrosis (47).

Increased production of the chemokine C-C motif ligand 18 (CCL18) by alternatively polarized alveolar macrophages was also associated with pulmonary fibrosis. Fibroblast contact and exposure to native collagen can increase spontaneous CCL18 release by alveolar macrophages, thus up-regulating collagen production by lung fibroblasts in a positive feedback loop that promotes collagen deposition (173).

Interestingly, collagen deposition may increase extracellular matrix stiffness, which, in turn, might enhance myofibroblast trans-differentiation through the activation of fibroblastic mechanoeffector proteins that are able to sense pathophysiologic-range lung stiffness, as shown in other lung fibrosis contexts (174).

Alternatively activated macrophages (M2) have also been implicated in the progressive fibrotic remodeling observed in the advanced stages of pulmonary sarcoidosis, possibly through release of TGF-β and CCL18. Indeed, an increase in $CD163^+$ M2 macrophages has been observed in the lungs and lymph nodes of 10 patients with sarcoidosis, including two with pulmonary fibrosis (34), although this macrophage subset is not specific to fibrotic sarcoidosis (175).

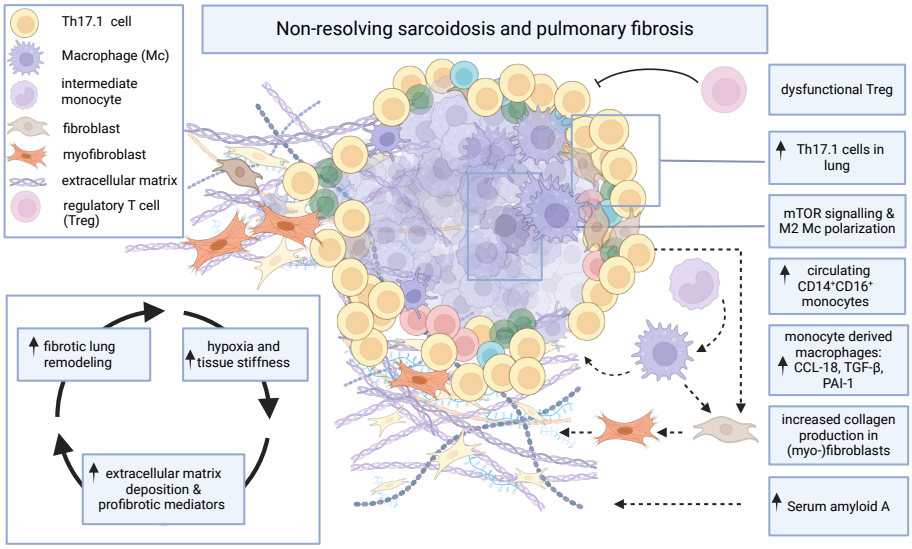

Figure 4. Immunological factors associated with chronic sarcoidosis and fibrosis
Created with BioRender.com

IFN-γ, the prototypical Th1 and Th17.1 cytokine and a key mediator of sarcoidosis pathogenesis, is a potent antifibrotic cytokine. Therefore, a switch from a Th1 to a Th2 phenotype has been suggested to occur in the transition from granulomatous inflammation to fibrotic disease (176). In keeping with this hypothesis, circulating levels of IL-5, an important mediator of fibrotic tissue remodeling, are significantly decreased in sarcoidosis patients with non-fibrotic disease (177). Overall, however, the evidence of Th1/Th2 switch in fibrotic sarcoidosis is limited. Moreover, while not profibrotic in itself, IFN-γ can indirectly promote fibrosis by inducing persistent inflammation and injury (178). Reduced or dysfunctional Tregs, may also contribute to fibrosis through the expansion of Th2 effector cells, which under normal conditions are suppressed by Tregs (114, 116, 117, 179).

Recently, fibrotic sarcoidosis has been studied by proteomics and transcriptomics. Mass spectrometry analysis of sarcoidosis BALF found proteins related to CD28 signaling in Th cells, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) 4 signaling and IL-12 production and signaling in macrophages to be differentially expressed between progressive and non-progressive sarcoidosis (180). A recent genome and transcriptome-wide association study of fibrotic sarcoidosis explored the role of telomere-related genes in sarcoidosis and identified *PARN* gene on chromosome 16 as associated with fibrotic (radiographic Scadding stage IV) vs. nonfibrotic sarcoidosis (Scadding stages I, II and III) sarcoidosis (181).

Targeting granulomatous inflammation in sarcoidosis: what does the future hold?

CTLA-4 immunoglobulin. Dysregulated CTLA-4 signaling is implicated in sarcoidosis pathogenesis, whereby decreased expression of CTLA-4 on Th17 and Tregs in the MLN of patients with sarcoidosis likely contributes to Th17 activation, while Treg function is impaired (105). Th cell activation requires a signal from the T cell receptor and a co-stimulatory signal, provided by CD28 on Th cells and CD80/CD86 on APCs (182). CTLA-4 limits this costimulatory signal, because it binds to CD80/CD86 on APCs with high affinity (182). Cancer treatment with antibodies that block CTLA-4 activates T cells and can induce a granulomatous disease indistinguishable from sarcoidosis (142). Conversely, abatacept, a recombinant CTLA-4-immunoglobulin fusion protein, inhibits T cell activation by binding to CD80/CD86 on APCs and thereby disrupting the costimulatory signal pathway (182-184). Restoring the dysregulated CTLA-4 pathway in chronic sarcoidosis with abatacept may offer an effective therapeutic strategy, which is currently investigated in a multi-center, single-arm phase II trial (183).

B cell blockers. While sarcoidosis is considered a T-cell mediated disease, the frequent occurrence of hypergammaglobulinemia and circulating immune complexes, the presence of peri-granuloma infiltration of B cells and increased T cell–independent $CD27^{-}IgA^{+}$ memory B cells in peripheral blood, suggest a role for humoral immunity in disease pathogenesis (122, 185). B cells may be involved through IFN-γ, which stimulates the B cell activating factor (BAFF), an antiapoptotic signal involved in B cell differentiation; notably, sarcoidosis patients with active disease display increased BAFF activity compared to patients with stable disease and healthy controls (186). Additionally, Th17 cells can also induce a proliferative response in B cells (187). These observations form the rationale for the use of B cell blocking agents in sarcoidosis (163). However, data on the efficacy of Rituximab in sarcoidosis, mostly in refractory pulmonary disease, either derives from small case series (188) or is inconclusive (189).

JAK-inhibitors. The JAK/STAT signaling pathway plays a key role in cell function and homeostasis (190) and its dysregulation has been implicated in the pathogenesis of various disease ranging from autoimmune disease to malignancies (191). Of note, several cytokines that are involved in macrophage activation and granuloma formation, including IFN-γ, IL-2, IL-4 and IL-23, signal via JAK/STAT (110). Messenger RNA transcripts associated with STAT signaling are upregulated in the lung parenchyma, thoracic lymph nodes and peripheral blood of patients with sarcoidosis compared to healthy controls (192), and genes differentially expressed in the lung of patients with pulmonary sarcoidosis are closely related to the JAK/STAT signaling pathway (193), thus providing the rationale for the use of JAK-inhibitors in sarcoidosis. Tofacitinib (194), ruxolitinib (195) and baricitinib (196) have been successfully used in refractory cutaneous or pulmonary sarcoidosis; in addition, in one study of patients with refractory cutaneous disease, tofacitinib was associated with a significant decrease in several Th1-mediated markers of inflammation (110).

Anti-IL-6. IL-6 is a pleiotropic pro-inflammatory cytokine produced primarily by innate immune cells which signals via JAK/STAT and MAPK pathways (197, 198). Additional functions of IL-6 include the ability to promote the differentiation of Th17 and Th17.1 effector T cells and to inhibit Tregs (199). In addition, IL-6 promotes the hepatic production of serum amyloid A (SAA) (200), which is abundant in the sarcoid granulomas and has been suggested as a key mediator of chronic granulomatous inflammation through stimulation of Toll-Like Receptor-2 (TLR2) (54). Elevated levels of IL-6 have been consistently found in the serum and BAL of patients with active pulmonary sarcoidosis (201, 202), and it is plausible that IL-6 blockade may mitigate sarcoidosis granulomatous inflammation. In a small case series, tocilizumab improved respiratory symptoms and measures of lung function in patients with persistent pulmonary disease despite chronic prednisone use (203). Conversely, in a phase II clinical trial of patients with refractory pulmonary sarcoidosis, sarilumab, an IL-6 receptor monoclonal antibody, was not superior to placebo in terms of flare-free survival, change in lung function, chest imaging, patient-reported outcomes and serological parameters (204).

Neuropilin modulators. Neuropilins (NRPs) are non-tyrosine kinase transmembrane glycoproteins expressed on the surface of several cell types, including T lymphocytes, macrophages and DCs (205). In sarcoidosis, NRP2 is enriched on circulating monocytes and is upregulated on the surface of activated immune cells involved in granuloma formation in the lung of patients with pulmonary disease (206). Efzofitimod, a first-in-class biological immunomodulator composed of a splice variant of histidyl-tRNA synthetase, binds selectively NRP2 (207), thus regulating the immune response (208) and reducing lung inflammation (209). In a phase I/II randomized, double-blind, placebo-controlled trial, efzofitimod (administered intravenously every 4 weeks for 24 weeks at the doses of 1, 3 and 5 mg/kg) was safe and well tolerated in patients with pulmonary sarcoidosis (210); in addition, all efzofitimod treatment groups showed a lower corticosteroid use at week 24 compared with the placebo group, with the largest difference observed in the 5 mg/kg group. Furthermore, compared with placebo, the highest doses of efzofitimod led to improvements in lung function (i.e., percentage of predicted forced vital capacity [FVC] and diffusing capacity of the lung for carbon monoxide [DLCO]), which were maintained throughout the study.

mTOR inhibitors. The serine/threonine protein kinase mechanistic target of rapamycin (mTOR) is involved in the regulation of the function and metabolism of innate and adaptive immune cells (24). Linke and co-workers showed that activation of the metabolic checkpoint kinase mTORC1 in macrophages by deletion of the gene encoding tuberous sclerosis 2 (Tsc2) induced macrophage proliferation, resulting in excessive granuloma formation *in vivo* whereas inhibition of mTORC1 completely resolved granulomas in myeloid TSC2-deficient mice (24). Moreover, in patients with sarcoidosis, mTORC1 activation and macrophage proliferation

correlated with disease progression. A single-centre randomised study in patients with glucocorticoid-refractory cutaneous sarcoidosis showed long lasting clinical and histological improvement in seven (70%) of ten participants (211). A potential protective effect of mTOR inhibitors against the development of sarcoidosis has also been observed (212).

GM-CSF inhibitors. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is an important hematopoietic growth factor and immune modulator that has been implicated in several autoimmune and inflammatory diseases (213, 214). Inflammatory cells recovered from BAL fluid of patients with pulmonary sarcoidosis express GM-CSF mRNA (215), and AMs and peripheral monocytes from sarcoidosis patients secrete a significantly greater amount of TNF-α and IL-1β compared to healthy controls following GM-CSF stimulation (216). A phase II randomized, placebo-controlled trial evaluating the safety and efficacy of namilumab, a monoclonal antibody against GM-CSF, in patients with pulmonary sarcoidosis is currently ongoing (NCT05314517).

Induction of immune tolerance. Tregs have a key role in the maintenance of immune tolerance and prevention of autoimmunity, and antigen-specific Tregs can potentially be used to treat autoimmune diseases by restoring immune tolerance (217, 218). Using a mouse model of experimental autoimmune uveitis (EAU), Chen and colleagues developed a system to generate *in vivo* autoantigen-specific Tregs through induction of transient depletion of pathogenic $CD4^+$ T cells coupled with administration of retinal autoantigens (219). This approach led to remission of ocular inflammation and rescue of visual function without impairing the host overall T-cell immunity, and can potentially be transferred to human autoimmune diseases. The application of this approach to sarcoidosis, however, may prove particularly challenging due to the lack of a single antigen (i.e., a variety of antigens, many of which remain unknown, are likely to be involved in disease pathogenesis) and the systemic nature of the disease (i.e., a multiorgan rather than a tissue-specific immune tolerance would need to be induced).

CONCLUSION

In the last few years, several studies have explored the role of various immune cells in the development and evolution of granulomatous inflammation in sarcoidosis. These studies have identified, among others, Th17 cells, Th17.1 cells and Treg cells as effector cells involved in the formation or resolution of sarcoid granuloma, and macrophages and M2 polarization as contributors to the transition from persistent granulomatous inflammation to tissue fibrosis. The role of B cells, DCs, NK cells, and NKT cells in disease pathogenesis has also been better defined. However, more work remains to be done to unravel the precise immunological mechanisms underlying this puzzling disorder. Such research, which will require large databases of

phenotypically well-characterized patients, equipped with biological samples (i.e., peripheral blood, BALF, lymph nodes, biopsies from the lung and other affected tissues) ideally collected both at baseline and at different time points, has the potential to answer fundamental questions, such as how the immune system contributes to sarcoidosis development, clinical manifestations, outcome (resolution vs. progression to fibrosis) and response to treatment. Increased understanding of these aspects of sarcoidosis is instrumental to the identification of predictors of disease behavior and novel therapeutic targets thus to the development of truly efficacious treatments.

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CHAPTER 3

Circulating T cells in sarcoidosis have an aberrantly activated phenotype that correlates with disease outcome

Jelle R. Miedema[#], Lieke J. de Jong[#], Denise van Uden, Ingrid M. Bergen, Mirjam Kool, Caroline E. Broos, Vivienne Kahlmann, Marlies S. Wijsenbeek, Rudi W. Hendriks and Odilia B.J. Corneth

[#] Equal contribution

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ABSTRACT

Rationale

Disease course in sarcoidosis is highly variable. Bronchoalveolar lavage fluid and mediastinal lymph nodes show accumulation of activated T cells with a T-helper (Th)17.1 signature, which correlates with non-resolving sarcoidosis. We hypothesize that the peripheral blood (PB) T cell phenotype may correlate with outcome.

Objectives

To compare frequencies, phenotypes and function of circulating T cell populations in sarcoidosis patients with healthy controls (HCs) and correlate these parameters with outcome.

Methods

We used multi-color flow cytometry to quantify activation marker expression on PB T cell subsets in treatment-naïve patients and HCs. The disease course was determined after 2-year follow-up. Cytokine production was measured after T cell stimulation *in vitro*.

Measurements and Main Results

We observed significant differences between patients and HCs in several T cell populations, including $CD8^+$ and $CD4^+$ T cells, Th1/Th17 subsets, $CD4^+$ T memory stem cells, regulatory T cells (Tregs) and γδ T cells. Decreased frequencies of $CD4^+$ T cells and increased frequencies of Tregs and $CD8^+$ γδ T cells correlated with worse outcome. Naïve $CD4^+$ T cells displayed an activated phenotype with increased CD25 expression in patients with active chronic disease at 2-year follow-up. A distinctive Treg phenotype with increased expression of CD25, CTLA4, CD69, PD-1 and CD95 correlated with chronic sarcoidosis. Upon stimulation, both naïve and memory T cells displayed a different cytokine profile in sarcoidosis compared to HCs.

Conclusions

Circulating T cell subpopulations of sarcoidosis patients display phenotypic abnormalities that correlate with disease outcome, supporting a critical role of aberrant T cell activation in sarcoidosis pathogenesis.

INTRODUCTION

Sarcoidosis is a complex granulomatous disease of unknown etiology with an unpredictable clinical course. Granulomas may affect almost any part of the body but are mostly located in lungs and mediastinal lymph nodes (MLN) (1). Most patients with sarcoidosis show spontaneous disease resolution, but 20-50% have non-resolving disease and may require long term treatment (2, 3). Estimating long-term prognosis at time of diagnosis is challenging and the decision to initiate treatment is based on clinical presentation, potentially dangerous organ involvement and symptom burden (4).

The pathogenesis of sarcoidosis is thought to involve an unknown antigen and complex immunological interactions in a genetically susceptible host, whereby T cells play a central role. Granuloma formation is accompanied by the accumulation of activated CD4+ T helper (Th) cells (5). When compared to healthy controls (HCs), bronchoalveolar lavage (BAL) fluid and MLN of sarcoidosis patients showed a significant increase in Th17.1 cells, defined as Th cells expressing the CCR6 chemokine receptor and producing interferon-g (IFN-γ) or both interleukin-17 (IL-17) and IFN-γ (6-8). Hereby, high proportions of Th17.1 cells in BAL fluid at diagnosis correlated with chronic disease. However, Th17.1 cells were not increased in peripheral blood (PB) (7-9), suggesting tissue-specific inflammatory changes of sarcoidosis-affected organs.

Although the role of regulatory T cells (Tregs) in disease pathogenesis remains controversial, their suppressive capacity and cellular survival in peripheral blood and BAL of patients with sarcoidosis is impaired (10-13). Interestingly, the expression of co-inhibitory receptor cytotoxic T-lymphocyte antigen 4 (CTLA4) is specifically decreased in Th17 cells and Tregs in BAL and MLN of patients with sarcoidosis (14). CTLA4 is critical for immune homeostasis, as both CTLA4 haploinsufficiency and cancer treatment with CTLA4 blockade are associated with immune dysregulation and granulomatous lung disease (15, 16). Decreased CTLA4 expression may therefore contribute to local inflammation by enhancing Th17 cell activation and impairing Treg function (14). In contrast to CD4+ memory cells, other PB T cell populations, including naïve, CD8+ and γδ T cells, are relatively understudied in sarcoidosis (13).

A detailed characterization of PB T cells in sarcoidosis to find new T cell biomarkers is warranted because MLN and BAL sampling are invasive procedures and PB can easily be sampled multiple times during treatment. Furthermore, understanding the phenotypical and functional changes of circulating T cells in chronic sarcoidosis may help to unravel pathogenesis and identify new drug targets. In this report, we hypothesized that in sarcoidosis circulating T cell populations express cell surface markers indicative of cellular activation, which may correlate with long-term prognosis. Furthermore, circulating T cells may respond aberrantly to T cell receptor (TCR) stimulation.

MATERIALS AND METHODS

Study design and subjects

This prospective study was approved by the Medical Ethical Committee of the Erasmus MC (Rotterdam, The Netherlands, MEC-2012-347 and MEC-2013-244) and performed in accordance with the Declaration of Helsinki. Patients were excluded (I) when any immunomodulatory medication (any reason) was used <3 months prior to study inclusion and in case of (II) respiratory tract infection four weeks prior to study inclusion, (III) pulmonary disease other than sarcoidosis, including chronic obstructive pulmonary disorder and asthma, and (IV) autoimmune or malignant diseases, HIV infection, allergy or pregnancy.

All blood samples were obtained during the initial diagnostic phase, all patients were treatment naïve and patients were prospectively followed to register long term outcome. In cohort I we included treatment naïve, newly diagnosed sarcoidosis patients. Sarcoidosis was diagnosed with confirmed granulomatous inflammation in tissue biopsies and/or CD4/CD8 ratio >3.5 in BAL, in accordance with the ATS/ERS/WASOG guidelines (17) or a confident diagnosis of sarcoidosis based on typical clinical and chest radiographic features at diagnosis and additional two years of prospective follow-up without signs of any alternative diagnosis. Follow-up was prospectively registered until 2 years after study inclusion. In line with previous work (7), disease 'resolution' was defined by normal chest radiography or tomography and no need for immunosuppressive treatment. At 2-year follow-up, patients with residual abnormalities on chest radiography but without need for treatment of sarcoidosis were designated 'non-active chronic' and patients who were treated with immune suppression for sarcoidosis at two years from diagnosis were designated 'active chronic'. A second cohort of treatment-naïve sarcoidosis patients in whom prednisone therapy was about to be initiated for a pulmonary indication were included for functional T cell analyses. An age and sex-matched HC group was selected for comparison.

Flow cytometry

Flow cytometric analyses were performed as previously described (18). PBMCs were first stained for chemokine receptors followed by extracellular markers and intracellular markers. Non-specific cell staining was prevented by blocking Fc receptors using human TruStain FcX (Biolegend, San Diego, CA, USA) and dead cells were excluded using BD Horizon^{tm} Fixable Viability stain 575 (BD Biosciences). Stained cells were resuspended in MACS buffer (0.5% BSA and 2mM EDTA in PBS) (22) and measured on the BD FACSymphonyTM A5 Cell analyzer (BD Biosciences, San Jose, CA, USA). Antibodies used for intra- and extracellular staining are listed in **Supplementary Table 1**.

Flow cytometry standard (FCS) files obtained with the BD FACSymphonyTM A5 Cell analyzer were preprocessed and quality control was performed using Peak Extraction And Cleaning Oriented Quality Control (PeacoQC)(19). In short, files were first preprocessed using Flowcore

Package followed by peak detection and outlier removal using PeacoQC package in RStudio (v4.1.2). Clean FCS files were analyzed using FlowJo v10 (Tree Star Inc Software).

Additional criteria were used to improve the quality of the data set. At first, samples that passed the quality control by PeacoQC but in which >50% of events had to be removed because of poor quality were excluded. Secondly, samples that contained >70% debris were excluded from the analysis. Lastly, percentage or geometric mean fluorescent intensity (gMFI) of activation markers was calculated only from cell subsets containing >100 cells.

Statistical analysis

GraphPad Prism 9 software (GraphPad Software Inc; San Diego, CA, USA) was used to design graphs and to calculate statistics. A Mann-Whitney U test was used to calculate significant differences between two groups. In case of multiple group comparisons, statistical analysis was calculated using a Kruskal-Wallis test combined with a Dunn's multiple comparison test. *p* values <0.05 were considered significant.

Principal component analysis (PCA) was performed using R and RStudio (packages FactoMineR and Factoextra) as described previously (20, 21). Statistical evaluation of PCA dimension coordinates for differences between HCs and sarcoidosis patient groups were done by a Kruskal-wallis test and Dunn's multiple comparisons test using Graphpad Prism v8 (Graph Pad Software, San Diego, CA, USA).

RESULTS

We included 57 treatment-naïve sarcoidosis patients during diagnostic workup (cohort I) and 22 healthy controls (HCs). Prospective assessment at two-year follow up showed either active chronic disease (n=22), non-active chronic disease (n=21) or disease resolution (n=14). Baseline subject characteristics at time of diagnosis are shown in **Table 1**. Additionally, 19 treatment-naïve sarcoidosis patients (cohort II) with pulmonary involvement were included for functional T cell analysis (**Table 1**). There were no significant differences between HCs and the two patient cohorts, or between the three sarcoidosis outcome groups regarding age, sex and smoking history.

Circulating T lymphocyte populations are significantly altered in patients with sarcoidosis compared to HCs

First, we compared circulating T-lymphocyte populations between all sarcoidosis patients (cohort 1 and 2 combined) and HCs. Flow cytometry analysis demonstrated a significantly reduced proportion of total $CD3^+$ T lymphocytes in PBMC fractions of patients (**Figure 1A**). In line with previous reports (8), we found a reduced proportion of $CD4^+$ T lymphocytes of total $CD3^+$ cells in sarcoidosis. Proportions of $CD8^+$ T cells were increased and γδ T cells were unaltered (**Figure 1B**).

Table 1: Baseline characteristics of participating subjects

	Control	Sarcoidosis with 2 year follow up (cohort 1)	Pulmonary sarcoidosis (cohort 2)
Subjects	N=22	N=57	N=19
Age	39 (25-56)	44 (24-90)	44 (26-72)
Female/male	11(50) /11(50)	20(35)/37(65)	7(37)/12(63)
Ethnicity			
Black / white / other	0/14(64)/2(9)	10(18)/42(74)/5(8)	5(26)/11(58)/3(16)
Unknown	6(27)	-	-
Smoking			
No/yes/former	12(55)/2(9)/4(18)	35(61)/7(12)/15(27)	12(63)/5(26)/2(11)
Unknown	4(18)	-	-
Diagnosis confirmed by			
clinical- radiological features and tissue biopsy n, (%)	-	47(82)	18(95)
clinical - radiological features and BALF CD4/CD8 ratio >3.5 n, (%)		2(4)	-
clinical- radiological features n, (%)		8(14)	1(5)
Pulmonary function			
Forced vital capacity (FVC) in % predicted	-	99 (51-135)	69 (30-87)
Unknown		5(9)	-
Forced expiratory volume in 1 second (FEV1) in % predicted	-	95 (48-121)	62 (25-84)
Unknown	-	4(7)	-
Diffusion capacity (DLCOc) in % predicted		77 (35-132)	64 (31-98)
Unknown		4(7)	1(6)
Scadding stage chest X-ray			
0	-	8(14)	1(5)
1		23(40)	1(5)
2		15(26)	14(74)
3		1(2)	2(11)
4		1(2)	-
Unknown		9(16)	1(5)
Chest CT stage *			
1		17(30)	1(5)
2	-	37(64)	16(85)
3		1(2)	1(5)
4		1(2)	-
Unknown		1(2)	1(5)
Extra thoracic involvement			
No/yes	-	39(68)/18(32)	11(58)/8(42)
Skin		19(33)	2(10)
Eyes		22(38)	2(11)
Central nervous system		4(7)	-
Joints / arthralgia		22(38)	5(26)

Data presented as median (range) or n(%)
*Thoracic involvement was assessed with chest computed tomography (CT): stage 1 = mediastinal lymphadenopathy, stage 2 = mediastinal lymphadenopathy and pulmonary involvement, stage 3 = pulmonary involvement only, stage 4 = pulmonary fibrosis.

Using the strategy outlined in **Suppl. Figure 1**, Tregs were gated as $CD127^{-}CD25^{high}CD4^{+}$ T cells (which were high in FoxP3 expression) and $CD127^{+}CD25^{low}CD4^{+}$ T cells were divided into $CD27^{+}CD45RA^{+}$ naïve and $CD45RO^{+}$ memory T cell fractions. Whereas the proportions of naïve and memory cells were similar in patients with sarcoidosis and HCs, the frequency of Tregs was significantly increased in patients (**Figure 1C**). A detailed analysis of effector memory, central memory and effector memory re-expressing CD45RA (TEMRA) $CD4^{+}$ and $CD8^{+}$ T cells did not reveal significant differences between patients and HCs.

Although frequencies of total γδ T cells were similar to HCs (**Figure 1B**), sarcoidosis patients showed a significant increase in $CD8^{+}$ γδ T cells, which are thought to be self-reactive and have a regulatory function (22). Very low numbers of $CD4^{+}$ γδ T cells were identified (**Figure 1D**).

Based on CCR6 and CXCR3 expression within memory $CD4^{+}$ T cells, we found significantly decreased fractions of Th1 and Th17.1 cells in sarcoidosis versus HCs, while proportions of non-Th1/17 cells were increased (**Figure 1E**).

Finally, we analyzed the newly defined stem cell-like memory (Tscm) T cell subsets, which show apoptosis resistance, rapid multi-differentiation potential and are elevated in systemic autoimmune disease (23). We gated Tscm cells on the basis of their $CD95^{+}CD45RA^{+}CD45RO^{-}$ phenotype (**Suppl. Figure 1B**) (24) and found that the frequency of $CD4^{+}$ but not $CD8^{+}$ Tscm was elevated in PB of sarcoidosis patients (**Figure 1F, Suppl. Figure 3A**).

Principal component analysis (PCA) of T cell subpopulations revealed a significant difference between sarcoidosis patients and HCs in the second, third and fourth dimensions (DIM2-4) (**Suppl. Figure 2A-D**). The frequencies of $CD4^{+}$ T cells, Tregs, double negative γδ T cells, $CD4^{+}$ γδ T cells, Th17 and Th17.1 cells contributed substantially to DIM2 (**Suppl. Figure 2E**). In DIM3, frequencies of $CD8^{+}$ and $CD4^{+}$ T cells contributed most, while the significant difference in DIM4 was mostly based on double negative, $CD8^{+}$ γδ and $CD4^{+}$ γδ T cells (**Suppl. Figure 2E**).

We next analyzed the distribution of monocyte and dendritic cell (DC) subpopulations, given their role in the activation of T cells (for gating see **Suppl. Figure 4A**). We found few differences between sarcoidosis patients and HCs, except a significant increase in the proportion of monocytes in PB of patients (**Suppl. Figure 4B-G**). Also, the expression of activation markers CD86, CD80, HLA-DR and CD11c was comparable between the two groups (**Suppl. Figure 4H**).

In summary, these findings indicate that patients with sarcoidosis differed from HCs in frequencies of several T cell populations in PB, most prominently in the $CD4^{+}$ T cell lineage.

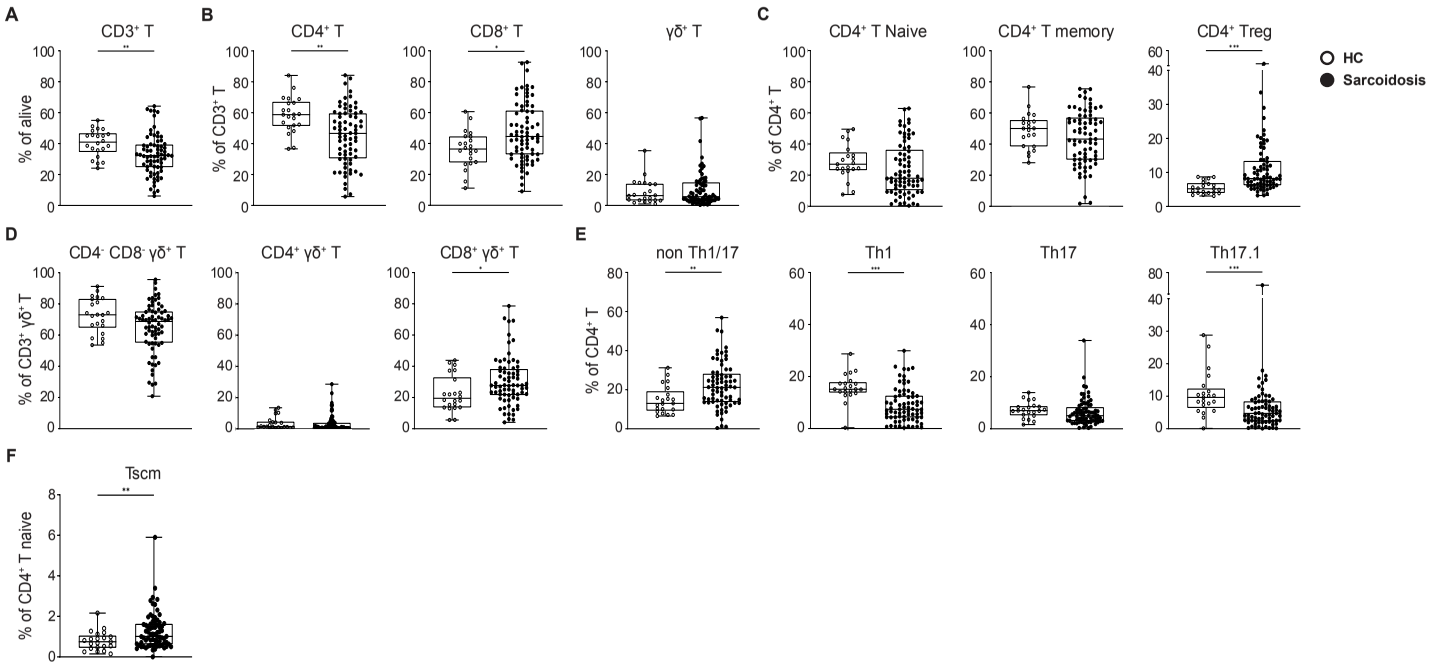

Figure 1: Peripheral blood T lymphocyte populations are altered in sarcoidosis patients.
(A) Proportions of CD3+ T cells of total live cells and (B) proportions of CD4+, CD8+ and γδ+ T cells of total CD3+ T cells; (C) Proportions of CD4+ T naïve, T memory and Treg cells of total CD4+ T cells; (D) Proportions of CD4-CD8-, CD4+ and CD8+γδ T cells of total CD3+γδ+ T cells; (E) Proportions of the indicated Th subsets of total CD4+ T cells of HCs and sarcoidosis patients. (F) Proportions of stem cell memory T cells (Tscm) cells of total CD4+ T naïve cells; Symbols represent individual values in healthy controls (HCs; open circles) and sarcoidosis patients (closed circles). All data were measured by flow cytometry. Mann-Whitney U test was used to calculate significant differences between two groups. In case of multiple group comparisons, statistical analysis was calculated using Kruskal-Wallis test combined with a Dunn's multiple comparison. *p<0.05, **p<0.01 and ***p<0.001.

Distribution of multiple T cell subpopulations correlates with sarcoidosis outcome at 2-year follow-up

In patients with two-year follow-up, we investigated whether frequencies of T cell subpopulations at baseline correlated with disease outcome. Significantly reduced proportions of total $CD3^+$ T lymphocytes were observed in PB of patients with active chronic disease (**Figure 2A**). Whereas the fraction of $CD4^+$ T cells appeared to be decreased in all sarcoidosis patient groups, this only reached significance in the active chronic outcome group, when compared with HCs (**Figure 2B**). No differences were observed in the fractions of $CD8^+$ T cells, total γδ T-cells or naïve to memory ratio of $CD4^+$ T cells across any of the patient groups (**Figure 2B,C**). Significantly increased proportions of circulating Treg cells were found specifically in patients progressing to active or non-active chronic disease compared to HCs and in active chronic disease compared to disease resolution (**Figure 2C**). Proportions of γδ T cells expressing CD8 were increased in active and non-active chronic disease compared to HCs. (**Figure 2D**)

Taken together, decreased proportions of $CD4^+$ T cells and increased proportions of Tregs and $CD8^+$γδ T correlated with worse sarcoidosis outcome at 2-year follow-up.

Increased proportions of $CD25^+CD4^+$ naïve T cells correlate with active chronic sarcoidosis

Using flow cytometry, we quantified the expression of various surface markers that are known to be upregulated upon antigen recognition, including CD25, CTLA4 and the very early activation marker CD69. In the $CD4^+$ naïve T cell population, the frequency of $CD25^+$ cells was significantly increased in active chronic patients, compared to HCs, while the frequency of $CTLA4^+$ cells was increased in all three patient groups (**Figure 3A,B**). CD69 showed yet another profile: significantly enhanced expression in non-active chronic patients compared to HCs and active chronic disease (**Figure 3C**). We found no significant differences in the activation markers on CD8+ naïve T cells and CD4+ or CD8+ T effector or central memory T cells, except that the proportion of patients with CTLA4+ CD4+ T memory cells was increased in active chronic patients, compared to HCs (Suppl. Fig 3B). Additionally, marker expression of CD95, CD137 and the checkpoint molecule programmed death-1 (PD-1) on CD4+ T naïve and memory cells was not significantly different between patients and HCs.

Evaluation of activation marker expression on γδ T cells did not reveal significant differences across disease outcome groups, except for $CD8^+$ γδ T cells: expression of CD69 was significantly increased in patients with active and non-active chronic disease (**Suppl. Fig 3C**).

In summary, these results indicate that in sarcoidosis naïve $CD4^+$ T cells have an aberrantly activated phenotype. This phenotype, particularly increased CD25 expression, was most prominent in the active chronic disease outcome group, while the resolution group only showed minor deviations from HCs.

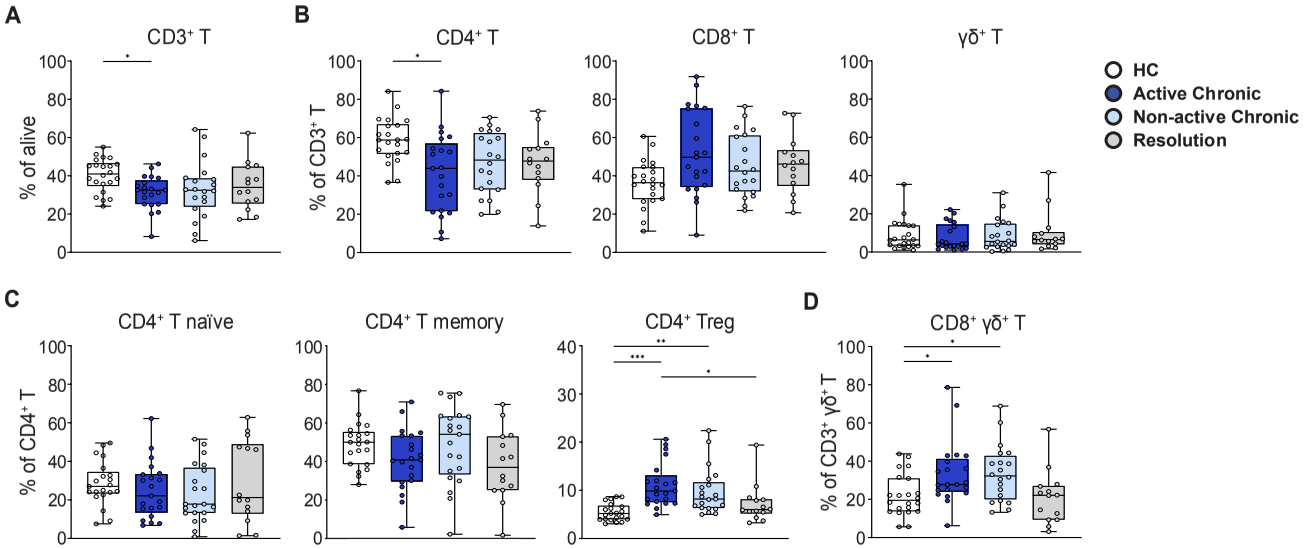

Figure 2: Distribution of peripheral T cell subpopulations correlates with sarcoidosis outcome at 2-year follow up.
(A) Proportions of CD3⁺ T cells of total live cells and **(B)** proportions of CD4⁺, CD8⁺ and γδ⁺ T cells of total CD3⁺ T cells; **(C)** Proportions of CD4⁺ T naïve, T memory and Treg cells of total CD4⁺ T cells; **(D)** Proportions of CD8⁺γδ T cells of total CD3⁺γδ+ T cells. Symbols represent values in individual healthy controls (HCs; open circles) and in individual sarcoidosis patients (colored circles), whereby different colors represent the three different outcome groups at 2-year follow-up. All data were measured by flow cytometry. Mann-Whitney U test was used to calculate significant differences between two groups. In case of multiple group comparisons, statistical analysis was calculated using Kruskal-Wallis test combined with a Dunn's multiple comparison. *p<0.05, **p<0.01 and ***p<0.001.

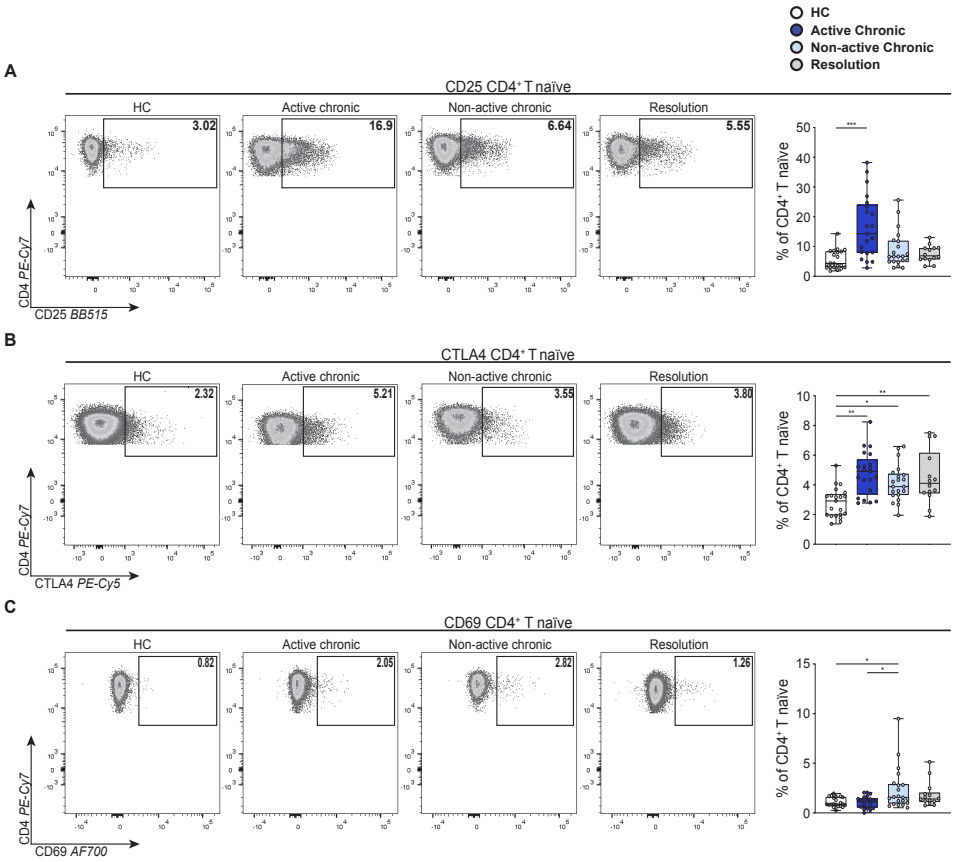

Figure 3: CD25, CTLA4 and CD69 expression on CD4⁺ T naïve differ between patients and the three sarcoidosis outcome groups.

(A-C) Representative flow cytometry dot plot images displaying CD25/CTLA4/CD69 versus CD4 expression in the indicated group of healthy controls (HC) and disease outcome groups (*left*) and quantification of the proportions of CD25⁺, CTLA4⁺, or CD69⁺ cells with the population of CD4⁺ T naïve cells (*right*). All data were measured by flow cytometry. Symbols represent values in individual healthy controls (HCs; open circles) and in individual sarcoidosis patients (colored circles), whereby different colors represent the three different outcome groups at 2-year follow-up. Mann-Whitney U test was used to calculate significant differences between two groups. In case of multiple group comparisons, statistical analysis was calculated using Kruskal-Wallis test combined with a Dunn's multiple comparison. *p<0.05, **p<0.01 and ***p<0.001.

Aberrant phenotype of regulatory T cells correlates with sarcoidosis outcome

Next, we characterized the phenotype of circulating Tregs in relation to long term prognosis. Paralleling our findings in naïve $CD4^+$ T cells, Tregs of sarcoidosis patients displayed increased expression of CD25, CTLA4 and CD69, compared with HCs (**Figure 4**). Tregs in active chronic patients showed significantly increased expression of CD25, CTLA4, CD95 and PD-1. In the non-active chronic, CD25, CD69 and PD-1 were significantly increased. For all these markers, the resolution group was in the normal ranges, except for the proportions of $CD69^+$ T cells (**Figure 4C**).

Together, these analyses demonstrate that the phenotype of Tregs is particularly aberrant in sarcoidosis patients with an active chronic disease outcome. The aberrant phenotype points to enhanced suppressive capacity of Tregs (increased CD25 and CTLA4) but may also indicate that their function is hampered (increased PD-1 and CD95). A summary of the altered proportions and phenotype of T cell subsets in sarcoidosis outcome groups compared to HCs is provided in **Table 2.**

Principal component analysis of T cell subset separates chronic sarcoidosis patients from patients with disease resolution

To investigate if a multivariate analysis would improve the separation of sarcoidosis patient groups and HCs, and to concomitantly identify T cell characteristics that contribute most to the separation, we performed a PCA (**Figure 5**). We included T cell subset distribution, activation marker expression and markers of Treg function. The first dimension (DIM1, capturing ~14.9% of variance) significantly separated both groups of chronic sarcoidosis patients from the HCs and active chronic disease from resolution (**Figure 5A,B**). Many of the T cell characteristics included contributed to DIM1. In this analysis, DIM2 with a major distribution of proportions of total $CD4^+$ memory, Th1 and Th17-lineage cells did not show significant differences between the groups (**Figure 5C**). In DIM3 (dominated by $CTLA4^+$ $CD4^+$ naïve, memory and regulatory T cells), HCs and active chronic disease were comparable and PCA significantly separated both groups from non-active chronic and disease resolution (**Suppl. Figure 5A-C**). We did not find significant differences between the HCs and outcome groups in DIM4. Additionally, PCA of the three outcome groups separated active chronic patients from disease resolution in DIM2. Here, proportions of $CD69^+$ Tregs and CD25 expression on Tregs contributed most (**Suppl. Figure 5D-F).**A PCA that included T cell subset distribution and cell surface markers could not separate patient groups based on radiographic stage at baseline.

Taken together, PCA separated chronic sarcoidosis patients from HCs and, importantly, also from resolution patients. Most T cell compartment parameters contributed to the separation.

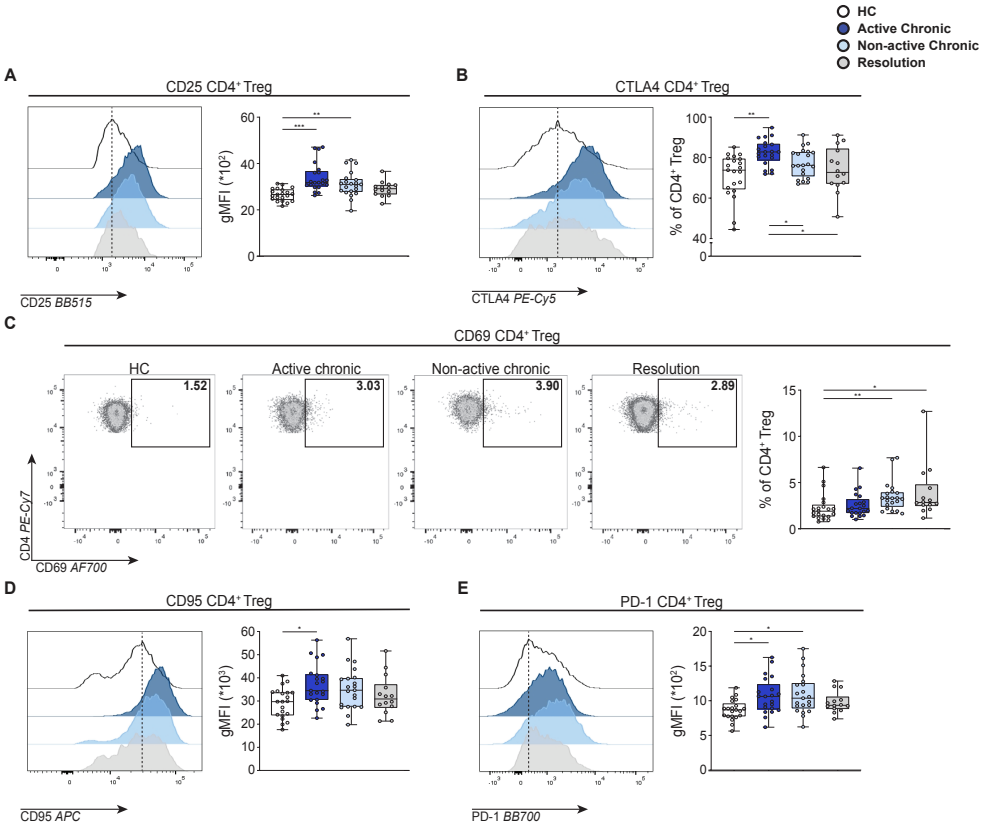

Figure 4: Aberrant phenotype of regulatory T cells in sarcoidosis patients.
(A) Histogram overlays of CD25 expression (*left*) and quantification by geometric mean fluorescence intensity (gMFI) for CD25 (*right*) of Tregs from healthy controls (HC) and the indicated sarcoidosis outcome groups. **(B)** Histogram overlays of CTLA4 expression (*left*) and quantification by gMFI for CTLA4 (*right*) of Tregs from healthy controls (HC) and the indicated sarcoidosis outcome groups. **(C)** Representative dot plot images displaying CD69 versus CD4 and the proportions of CD69+ Tregs within the indicated gate (*left*) and quantification by gMFI for CD69 (*right*) of Tregs from healthy controls (HC) and the indicated sarcoidosis outcome groups. **(D-E)** Histogram of CD95 **(D)** and PD-1 **(E)** expression and the quantification by gMFI of from healthy controls (HC) and the indicated sarcoidosis outcome groups. All data were measured by flow cytometry. Symbols represent values in individual healthy controls (HCs; open circles) and in individual sarcoidosis patients (colored circles), whereby different colors represent the three different outcome groups at 2-year follow-up. Mann-Whitney U test was used to calculate significant differences between two groups. In case of multiple group comparisons, statistical analysis was calculated using Kruskal-Wallis test combined with a Dunn's multiple comparison. *p<0.05, **p<0.01 and ***p<0.001.

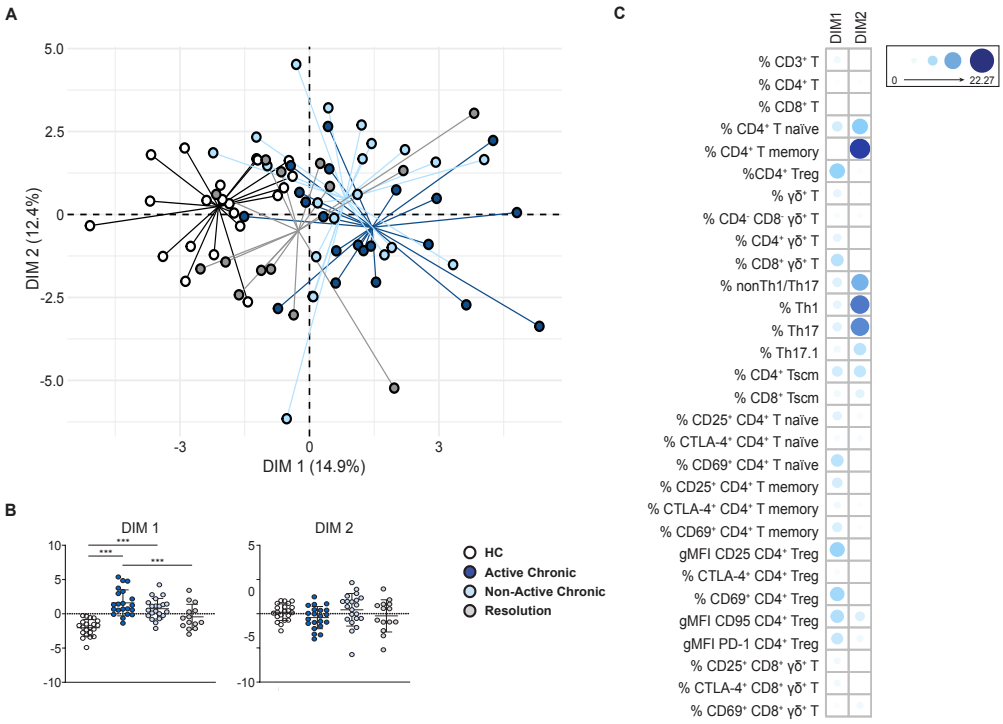

Figure 5. Principal component analysis of T cells from HCs and different sarcoidosis outcome groups. **(A)** Principal component analysis (PCA) of T cell parameters, including subset frequencies and activation markers, of HC and sarcoidosis patients from different outcome groups, as determined by flow cytometry. **(B)** Coordinate values of PCA dimension 1 (DIM1) and DIM2, showing the separation between HCs and sarcoidosis patient groups. **(C)** Contribution (in %) of the parameters listed to the variance in DIM1 and DIM2 of the PCA, indicated by symbols in blue color range of dimension. **(A-B)** Symbols represent values in individual healthy controls (HCs; *open circles*) and in individual sarcoidosis patients (*colored circles*), whereby different colors represent the three different outcome groups at 2-year follow-up. Kruskal-Wallis test and Dunn's multiple comparisons test were used for statistical analysis of coordinates on the dimensions between sarcoidosis patients and HCs, *p<0.05, **p<0.01 and ***p<0.001.

PB naïve and memory T cells in sarcoidosis display aberrant cytokine production and activation marker profiles upon T cell receptor stimulation

Finally, we investigated the functional response of circulating naïve and memory T cells in sarcoidosis (cohort 2) and HCs after overnight stimulation with anti-CD3 and anti-CD28. Importantly, both naïve and memory cells can be activated *in vitro*, as demonstrated by a high proportion of IL-2 positive cells after stimulation **(Figure 6)**. The proportion of tumor necrosis factor alpha (TNF-α) positive naïve CD4⁺ T cells in sarcoidosis was increased, while IFN-γ positive naïve T cells were decreased compared to HCs. In memory T cells, we found decreased proportions of granulocyte macrophage colony-stimulating factor (GM-CSF) and IFN-γ positive cells compared to HCs **(Figure 6)**.

After TCR stimulation, proportions of CD69⁺, but not CD25⁺, naïve T cells were decreased in sarcoidosis patients (**Suppl. Fig. 6A**). Additionally, proportions of both CD25⁺ and CD69⁺ memory T cells were significantly decreased in patients (**Suppl. Fig 6B**).

These results further indicate that PB T cells can be activated in sarcoidosis, but demonstrate both an altered cytokine profile and aberrant phenotype upon TCR stimulation. Interestingly, this includes naïve T cells.

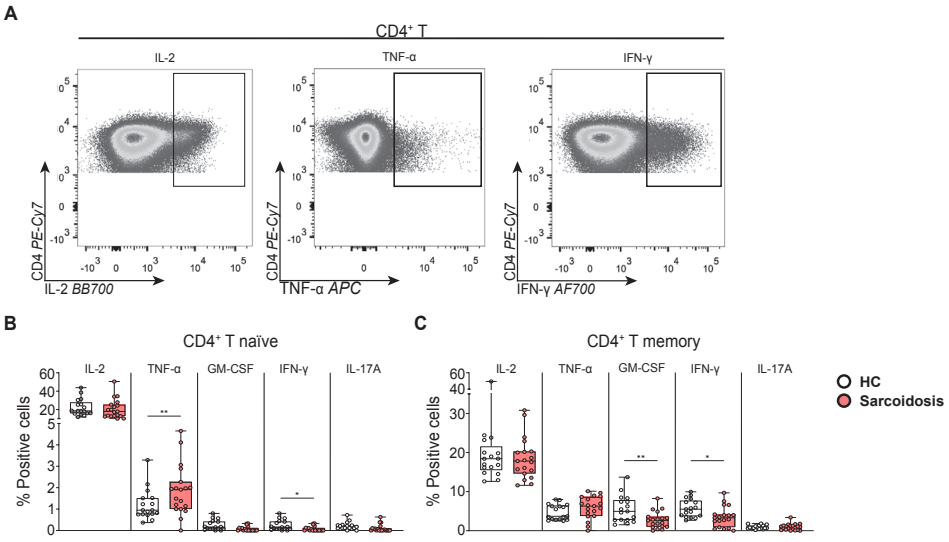

Figure 6. Cytokine staining on naïve and memory T cells after TCR stimulation (A) Representative flow cytometry dot plot images displaying interleukin-2 (IL-2), Tumor necrosis factor alpha (TNF-a) and interferon gamma (IFN-y) versus CD4 expression of total CD4+ T cells. Proportions of positive cells for IL-2, TNF-a, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-y and interleukin IL-17A **(B)** from the population of CD4+ T naïve cells **(C)** from the population of CD4+ T memory cells. Symbols represent individual values in healthy controls (HCs; open circles) and sarcoidosis patients (closed circles). All data were measured by flow cytometry. Mann-Whitney U test was used to calculate significant differences between two groups. *p<0.05, **p<0.01 and ***p<0.001.

DISCUSSION

In this study, we found that several PB T cell subpopulations differ between sarcoidosis patients and HCs, both in frequency and in phenotype. To the best of our knowledge, we are the first to report that many of these phenotypical differences, particularly in naive $CD4^+$ T cells and Tregs, are specifically observed in patients with chronic disease, and not resolution, at 2-year follow-up. When integrated in a PCA analysis, we observed a significant separation between HCs and the chronic disease outcome groups, as well as between active chronic and disease resolution.

First, we found that naïve T cells displayed increased CD25, CTLA4 and CD69 expression, whereby CD25 expression was most prominent in the active chronic disease outcome group. Naïve T cells are regarded as a quiescent population that are antigen-naive. However, it was recently shown that pre-differentiation of naïve T cells may result in phenotypical heterogeneity, including activation state, cytokine production and survival (25). Interestingly, $CD25^+$ naïve T cells clearly correlate with worse disease outcome in our study. IL-2 is an essential cytokine for T cell proliferation and differentiation (26) and increased expression of its receptor CD25 on PB T cells in sarcoidosis patients has previously been demonstrated (26). A recent study using single-cell RNA sequencing of PB immune cells in sarcoidosis provided evidence of general naïve T-cell activation with upregulation of several pathways prior to antigen recognition, including IL-2R signaling, but no evidence of antigen driven T-cell stimulation (27). Upon TCR stimulation, the proportion of TNF-α-producing naïve T cells is increased in sarcoidosis. Our data support the concept of a general, antigen-independent pre-activated state of naïve $CD4^+$ T cells with an altered cytokine profile in sarcoidosis patients.

Secondly, Tregs were increased in patients with worse prognosis. Hereby, expression of CD25 and CD69 was enhanced in all outcome groups, and expression of CTLA4, PD-1 and CD95 in patients progressing to chronic disease. Contradictory studies reported either decreased (28, 29) or increased (10, 11) proportions of Tregs in sarcoidosis. Additionally, a reduced regulatory capacity and survival of Tregs was found, whereby restoration of their function was associated with clinical resolution of sarcoidosis (10, 11). We observed an activated Treg phenotype in all sarcoidosis outcome groups, but a remarkable concomitant increase in surface expression of CD95 and PD-1 on Tregs of patients with worse prognosis. Mechanistically, increased CD95 expression predisposes Tregs to CD95 ligand-mediated apoptosis (10) and high PD-1 expression impairs their suppressive function (30). It is therefore conceivable that the aberrantly activated phenotype of multiple circulating T cell subpopulations is the consequence of reduced immunosuppressive capacity of Tregs, most prominently in patients with non-resolving disease. Alternatively, sarcoidosis may be associated with generalized T cell abnormalities, affecting both Tregs and other T cell subsets.

Thirdly, total proportions of $CD4^+$ T cells were reduced, whereby the fractions of Th1 and Th17.1 subsets were decreased and non-Th1/17 subsets and $CD4^+$ Tscm were increased in

sarcoidosis patients. Given the long-term persistence and immunological memory characteristics of Tscm (24), an increase of aberrantly activated Tscm might induce long-lasting T cell-mediated inflammation.

Finally, γδ T cells were unchanged in their frequencies, but showed a relative increase in $CD8^+$ γδ T cells, which correlated with active and non-active chronic disease. The literature on γδ T-cells in sarcoidosis is remarkably scarce. Previous studies only investigated the classic $CD4^-CD8^-$ γδ T-cells, which were found either unchanged (31) or elevated in patients with active Scadding stage III or long-standing disease, but not in inactive disease (32). Although $CD8^+$ γδ T cells have been linked to self-reactivity (22), their function remains largely unknown and the potential role of these cells in sarcoidosis pathogenesis requires further research.

A pre-activated state and altered cytokine profile of multiple circulating T cell populations likely contributes to ongoing systemic inflammation and non-resolving local disease, when recruited from the circulation. Previously, substantial differences in proportions and phenotype of T cells between PB and chronically inflamed sites such as the MLN or lung were demonstrated (14). In a study with unsupervised sarcoidosis cluster analysis it was found that decreased peripheral $CD4^+$ T cells significantly related to disease activity on 18-FDG positron emission tomography (PET) (33). It is conceivable that T cell trafficking between the circulation and different body compartments in sarcoidosis may not only result in decreased proportions of circulating T CD4+ T cells, but also in an aberrantly activated phenotype of these cells. Together, these data argue for consideration of PB as an immunological compartment with distinctive T cell characteristics in sarcoidosis. In addition to T cells, alveolar macrophages are also central to disease pathogenesis and some studies suggest their potential prognostic value in sarcoidosis (34, 35). However, we did not detect significant differences in the expression of activation markers on circulating myeloid cell populations.

Our study has some limitations to address. Characteristics of T cells as biomarkers in sarcoidosis need to be validated before they can be used for outcome prediction and clinical decision making. Our PCA analyses revealed a significant separation between HCs and the chronic disease outcome groups as well as a separation between active chronic and disease resolution on the basis of aberrant phenotypes of multiple T cell subsets. However, we could not identify a single PB marker or T cell subset that would independently predict disease outcome.

In conclusion, the current study shows altered proportions and phenotypic characteristics of multiple T-cell subsets in peripheral blood of sarcoidosis patients. Several of these parameters correlated with disease outcome. Remarkably, naïve $CD4^+$ T cells of patients displayed an aberrantly activated phenotype, whereby particularly the proportions of CD25+ cells correlated with sarcoidosis outcome. Moreover, in vitro stimulation of both naïve and memory T cells resulted in an altered cytokine profile in sarcoidosis.

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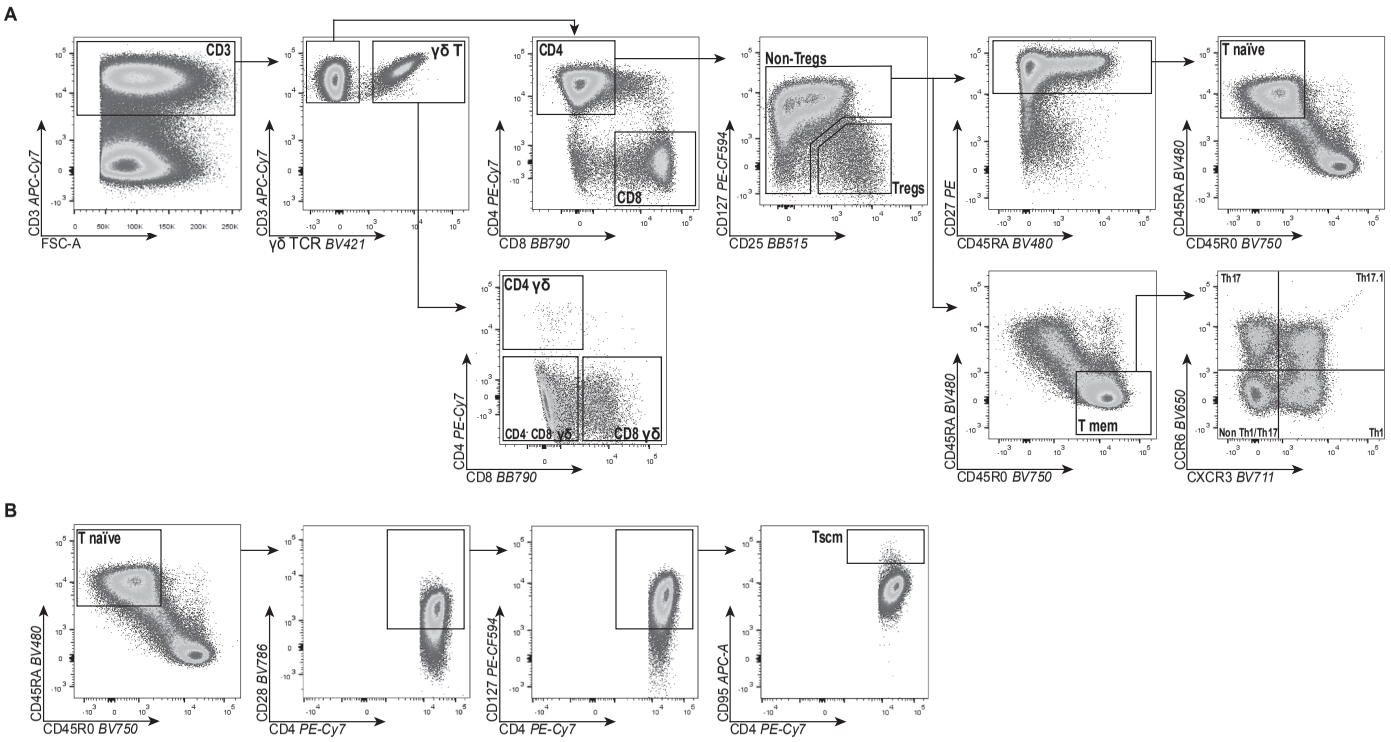
SUPPLEMENTARY MATERIAL

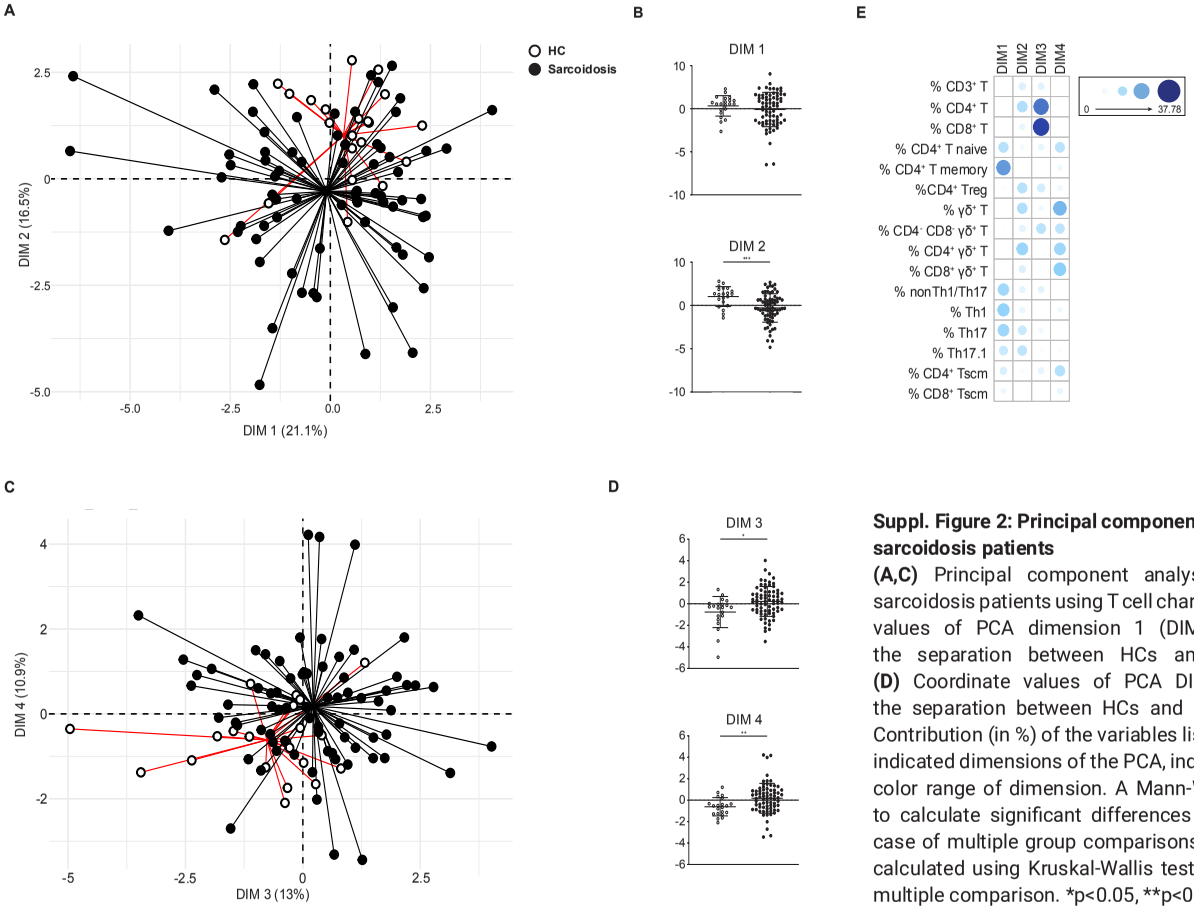
Supplemental Table 1: Antibodies used for flow cytometry

Antibody	Alternative name	Function / expressed on	Conjugate	Clone	Manufacturere
CD3			APC-Cy™7	SK7	BD Biosciences
			AF700	UCHT1	eBioscience
CD4			PE-Cy™7	SK3	BD Biosciences
CD8			BB790-P	SK1	BD Biosciences
CD11c			BV605	3.9	Biolegend
CD14		Activation marker innate immune cells	BV785	M5E2	BD Biosciences
CD16	FcγRIII	Fc receptor	FITC	3G8	BD Biosciences
CD19		B-cell	AF700	HIB19	eBioscience
CD20		B-cell	AF700	2H7	BD Biosciences
CD25	IL-2Rα	Early activation marker	BB515	2A3	BD Biosciences
CD27		Activation marker	PE	O323	BD Biosciences
			BV605	O323	Biolegend
CD28		Costimulation	BV786	CD28.2	BD Biosciences
CD45RA		Naive T-cell	BV480	5H9	BD Biosciences
			Pe-Cy5	5H9	BD Biosciences
CD45RO		Memory T-cell	BV750	UCHL1	BD Biosciences
CD56		NK cell	PE-Cy™7	B159	BD Biosciences
CD69		Early activation marker	AF700	FN50	BD Biosciences
CD80	B7-1	Costimulation	BV421	L307.4	BD Biosciences
CD86	B7-2	Costimulation	Biotin	FUN-1	BD Biosciences
CD95	Fas	Apoptosis marker	APC	DX2	BD Biosciences
CD123	IL-3Rα		BV650	7G3	BD Biosciences
CD127	IL-7Rα		PE-CF594	HIL-7R-M21	BD Biosciences
CD137		Activation marker	BV605	4B4-1	BD Biosciences
CD152	CTLA4	Co-Inhibitory marker	PE-Cy™5	BNI3	BD Biosciences
CD196	CCR6		BV650	11A9	BD Biosciences
CD183	CXCR3		BV711	1C6	BD Biosciences
CD278	ICOS	Activation marker	BV421	DX29	BD Biosciences
CD279	PD-1	Co-Inhibitory marker	BB700	Eh12.1	BD Biosciences
AXL			APC	FAB154A	R&D system
FoxP3		Regulatory T-cell	AF647	259D/C7	BD Biosciences
GM-CSF			PE	BVD2-21C11	BD Biosciences
Helios		Activation marker	PE	22F6	BD Biosciences
HLA-DR			BV711	G46-6	BD Biosciences
IL-2			BB700	MQ1-17H12	BD Biosciences
IL-17A			BV650	TC11-18H10	BD Biosciences
IFN-γ			AF700	B27	Biolegend
IRF4			PE	3E4	eBioscience
IRF8			BB700	V3GYWCH	eBioscience
PD-L1			PE-CF594	M1H1	BD Biosciences
Streptavidin			APC-Cy™7	-	eBioscience
TNF-α			APC	6401.1111	BD Biosciences
γδ TCR		γδ T-cell	BV421	11F2	BD Biosciences



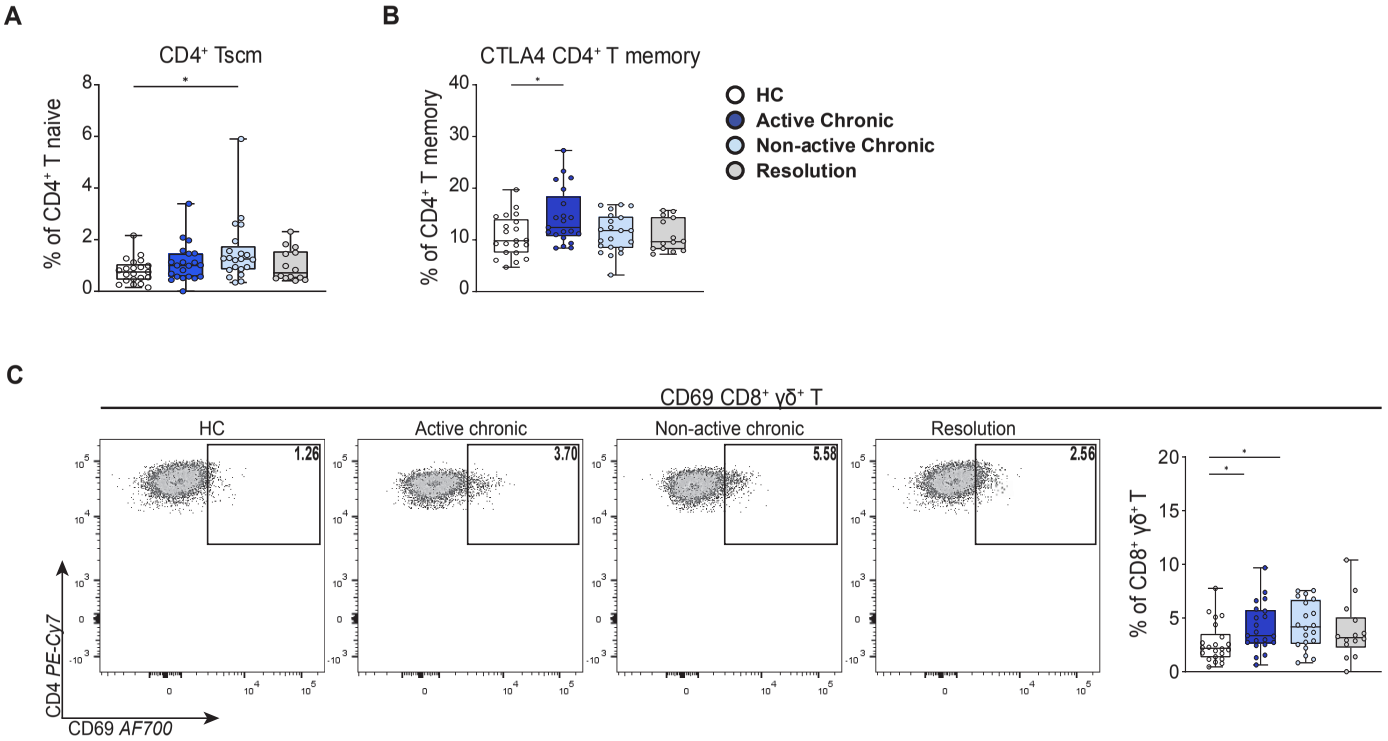
Suppl. Figure 1: Gating strategy of human T cell subsets

(A) Gating strategy for human peripheral T cells, starting by gating CD3$^+$ T cells. Subsequently, cells were gated for various T cell subsets, such as T naïve (CD27$^+$ CD45RA$^+$ CD4$^+$), T memory (CD127$^{+/-}$CD45RO$^+$CD4) and Treg (CD25$^+$CD127$^-$CD4$^+$). Th cell subsets were defined following gating CXCR3 and CCR6, selecting for nonTh1/Th17 (CXCR3$^-$ CCR6$^-$), Th1 (CXCR3$^+$), Th17 (CCR6$^+$) and Th17.1 (CXCR3$^+$CCR6$^+$). γδ T cell subsets were further defined for CD4$^-$ CD8$^-$, CD4$^+$ and CD8$^+$ γδ T cells. **(B)** Gating strategy for human CD4$^+$ stem cell memory T (Tscm) cells (CD95$^+$CD127$^+$CD28$^+$CD45RA$^+$) gated from the naïve T cell population.

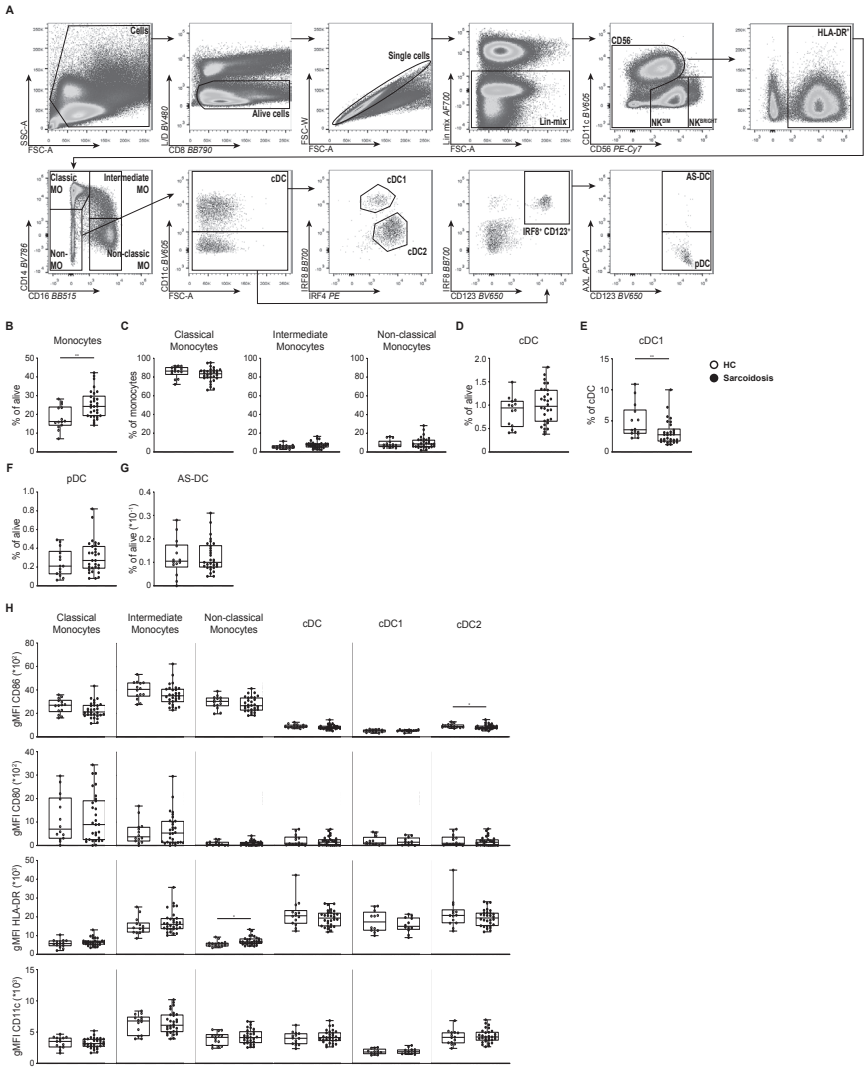


Suppl. Figure 2: Principal component analysis of controls and sarcoidosis patients

(A,C) Principal component analysis (PCA) of HCs and sarcoidosis patients using T cell characteristics **(B)** Coordinate values of PCA dimension 1 (DIM1) and DIM2, showing the separation between HCs and sarcoidosis patients. **(D)** Coordinate values of PCA DIM3 and DIM2, showing the separation between HCs and sarcoidosis patients. **(E)** Contribution (in %) of the variables listed to the variance in the indicated dimensions of the PCA, indicated by symbols in blue color range of dimension. A Mann-Whitney U test was used to calculate significant differences between two groups. In case of multiple group comparisons, statistical analysis was calculated using Kruskal-Wallis test combined with a Dunn's multiple comparison. *p<0.05, **p<0.01 and ***p<0.001.

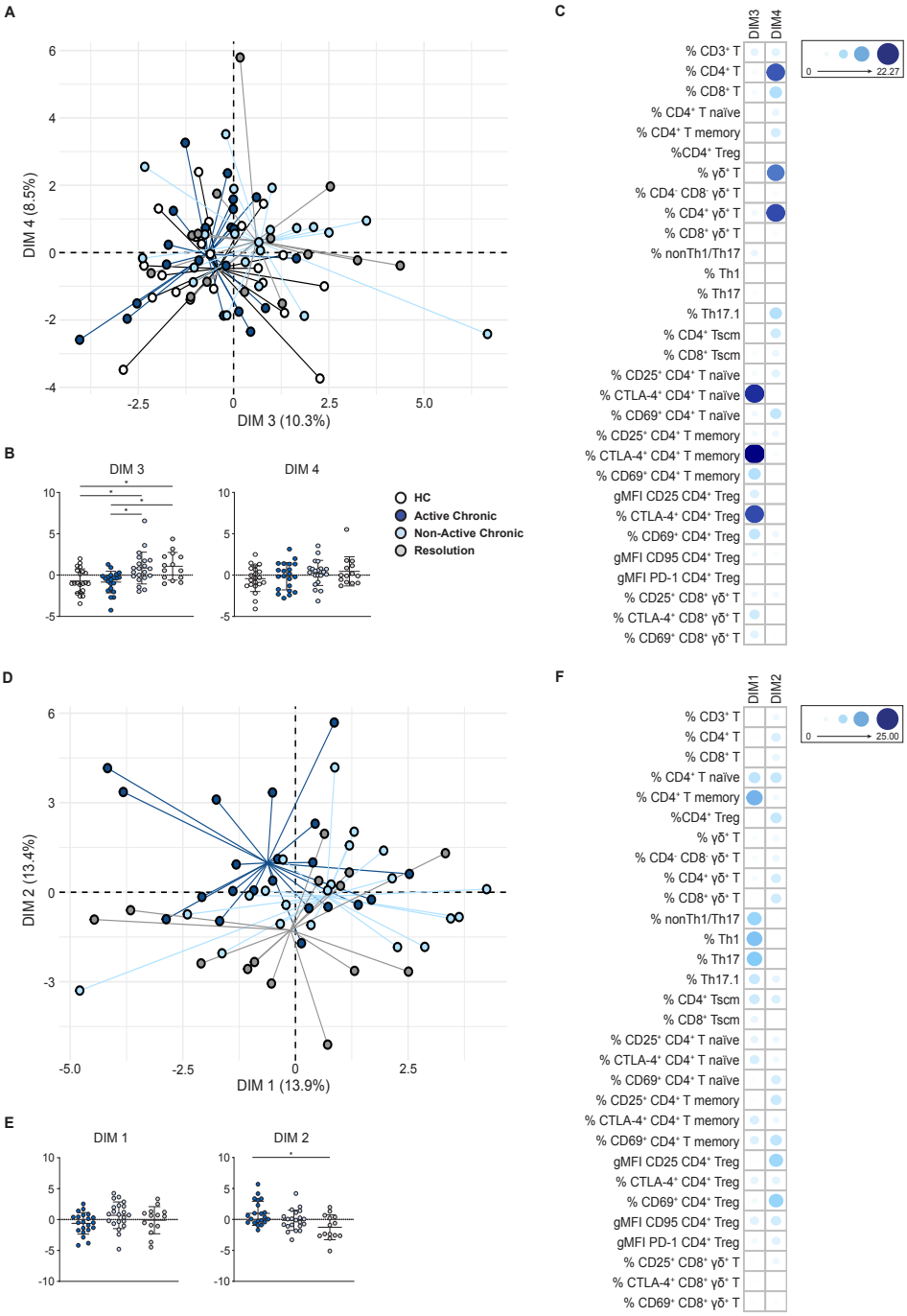


Suppl. Figure 3: Distribution of Tscm and proportions of CTLA4⁺ CD4⁺ T memory cells and CD69⁺ CD8⁺ γδ T cells in sarcoidosis outcome groups and HCs
(A) Proportions of memory stem cells (Tscm) from total CD4⁺ T naïve cells. **(B)** Quantification of the proportions of CTLA4⁺ cells with the population of CD4⁺ T memory cells. **(C)** Representative flow cytometry dot plot images displaying CD69 versus CD4 expression in the indicated group of healthy controls (HCs) and disease outcome groups (*left*) and quantification of the proportions of CD69⁺ cells within the population of CD8⁺ γδ T cells (*right*). Symbols represent values in individual healthy controls (HCs; open circles) and in individual sarcoidosis patients (colored circles), whereby different colors represent the three different outcome groups at 2-year follow-up. A Mann-Whitney U test was used to calculate significant differences between two groups. In case of multiple group comparisons, statistical analysis was calculated using Kruskal-Wallis test combined with a Dunn's multiple comparison. *p<0.05, **p<0.01 and ***p<0.001.



Suppl. Figure 4: Gating strategy of human monocytes and dendritic cells

(A) Gating strategy for human classical, intermediate and non-classical monocytes, cDCs, cDC1 cDC2s, pDC and AS-DC. **(B)** Proportions of total monocytes as fraction of alive cells in HCs and sarcoidosis **(C)** Proportions of classical, intermediate and non-classical monocytes as percentage of total monocytes in HCs and sarcoidosis **(D)** Proportions of cDC as percentage of alive cells in HC and sarcoidosis **(E)** Proportions of cDC1 as percentage of cDC in HC and sarcoidosis **(F)** Proportions of pDC as percentage of alive cells in HC and sarcoidosis. **(G)** Proportions of AS-DC as percentage of alive cells in HC and sarcoidosis. **(H)** Expression of markers CD86, CD80, HLA-DR and CD11c measured by geometric MFI on classical, intermediate and non-classical monocytes and cDC, cDC1 en cDC2 in the group of HC and sarcoidosis patients. (**A-H**) Symbols represent individual values in healthy controls (HCs; open circles) and sarcoidosis patients (closed circles). All data were measured by flow cytometry. Mann-Whitney U test was used to calculate significant differences between two groups. *p<0.05, **p<0.01 and ***p<0.001.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DIM 4
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% CD4⁺ T naïve
% CD4⁺ T memory
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% CD4⁺ γδ⁺ T
% CD8⁺ γδ⁺ T
% nonTh1/Th17
% Th1
% Th17
% Th17.1
% CD4⁺ Tscm
% CD8⁺ Tscm
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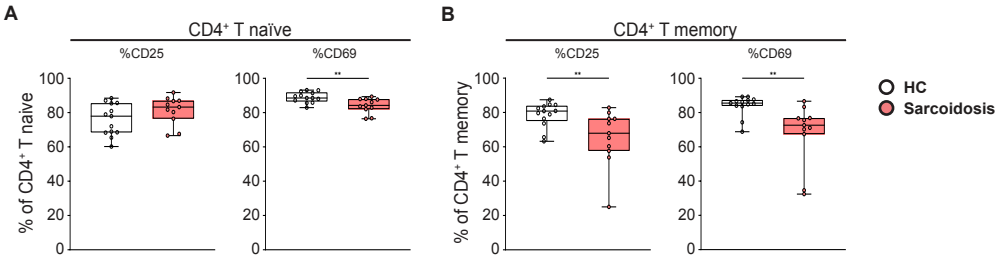
gMFI PD-1 CD4⁺ Treg
% CD25⁺ CD8⁺ γδ⁺ T
% CTLA-4⁺ CD8⁺ γδ⁺ T
% CD69⁺ CD8⁺ γδ⁺ T

◀ Suppl. Figure 5: Principal component analysis of T cells from HCs and different sarcoidosis outcome groups

(A) Dimensions 3 (DIM3) and DIM4 of a principal component analysis (PCA) of T cell parameters, including subset frequencies and activation markers, of HC and sarcoidosis patients from different outcome groups, as determined by flow cytometry. **(B)** Coordinate values of PCA dimension 3 (DIM3) and DIM4, showing the separation between HCs and sarcoidosis patient groups. **(C)** Contribution (in %) of the parameters listed to the variance in DIM3 and DIM4 of the PCA, indicated by symbols in blue color range of dimension. **(D)** Dimensions 1 (DIM1) and DIM2 of a principal component analysis (PCA) of T cell parameters, including subset frequencies and activation markers, of sarcoidosis patients from different outcome groups, as determined by flow cytometry. **(E)** Coordinate values of PCA dimension 1 (DIM1) and DIM2, showing the separation between active chronic and disease resolution sarcoidosis patient groups. **(F)** Contribution (in %) of the parameters listed to the variance in DIM1 and DIM2 of the PCA, indicated by symbols in blue color range of dimension. Symbols represent values in individual healthy controls (HC; *open circles*) and in individual sarcoidosis patients (*colored circles*), whereby different colors represent the three different outcome groups at 2-year follow-up. Kruskal-Wallis test and Dunn's multiple comparisons test were used for statistical analysis of coordinates on the dimensions between sarcoidosis patients and HCs, *p<0.05, **p<0.01 and ***p<0.001.



Suppl. Figure 6: Activation marker expression on naïve and memory T cells after TCR activation

Proportions of positive cells for CD25 and CD69 in HCs and sarcoidosis patients **(A)** from the population of CD4⁺ T naïve cells **(B)** from the population of CD4⁺ memory T cells. All data were measured by flow cytometry. Symbols represent individual values in healthy controls (HCs; open circles) and sarcoidosis patients (closed circles). Mann-Whitney U test was used to calculate significant differences between two groups. *p<0.05, **p<0.01 and ***p<0.001.

CHAPTER 4

Increased proportions of circulating PD-1$^+$ CD4$^+$ memory T cells and PD-1$^+$ regulatory T cells associate with good response to prednisone in pulmonary sarcoidosis

Jelle R. Miedema, Lieke J. de Jong, Vivienne Kahlmann, Ingrid M. Bergen, Caroline E. Broos, Marlies S. Wijsenbeek, Rudi W. Hendriks and Odilia B.J. Corneth.

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ABSTRACT

Background

The treatment response to corticosteroids in patients with sarcoidosis is highly variable. $CD4^+$ T cells are central in sarcoid pathogenesis and their phenotype in peripheral blood (PB) associates with disease course. We hypothesized that the phenotype of circulating T cells in patients with sarcoidosis may correlate with the response to prednisone treatment. Therefore, we aimed to correlate frequencies and phenotypes of circulating T cells at baseline with the pulmonary function response at three and twelve months during prednisone treatment in patients with pulmonary sarcoidosis.

Methods

We used multi-color flow cytometry to quantify activation marker expression on PB T cell populations in 22 treatment-naïve patients and 21 healthy controls (HCs). Pulmonary function tests at baseline, three and twelve months were used to measure treatment effect.

Results

Patients with sarcoidosis showed an absolute forced vital capacity (FVC) increase of 14.2% predicted (±10.6, $p<0.0001$) between baseline and three months. Good response to prednisone (defined as absolute FVC increase of ≥10% predicted) was observed in 12 patients. $CD4^+$ memory T cells and regulatory T cells from patients with sarcoidosis displayed an aberrant phenotype at baseline, compared to HCs. Good responders at three months had significantly increased baseline proportions of $PD\text{-}1^+CD4^+$ memory T cells and $PD\text{-}1^+$ regulatory T cells, compared to poor responders and HCs. Moreover, decreased fractions of $CD25^+$ cells and increased fractions of $PD\text{-}1^+$ cells within the $CD4^+$ memory T cell population correlated with ≥10% FVC increase at twelve months. During treatment, the aberrantly activated phenotype of memory and regulatory T cells reversed.

Conclusions

Increased proportions of circulating $PD\text{-}1^+CD4^+$ memory T cells and $PD\text{-}1^+$ regulatory T cells and decreased proportions of $CD25^+CD4^+$ memory T cells associate with good FVC response to prednisone in pulmonary sarcoidosis, representing promising new blood biomarkers for prednisone efficacy.

BACKGROUND

Sarcoidosis is a granulomatous multi-organ disease with variable disease course. The majority of patients presents with pulmonary involvement(1). The decision to initiate treatment for sarcoidosis is based on an individual assessment of dangerous or progressive organ involvement and quality of life(2). Glucocorticoids are recommended as first line choice(2). However, the response to prednisone is highly variable and some patients do not respond to treatment(3). Estimating the response to glucocorticoids can be challenging and prolonged prednisone use can have significant toxicity and side effects(2-4). There is an unmet need to develop biomarkers that predict therapeutic response(5).

Granuloma formation in sarcoidosis results from an exaggerated immune response to an unidentified antigen in genetically susceptible individuals. Several disease-triggering antigens have been suggested that may initiate the inflammatory cascade(6). Convincing evidence has been obtained that cluster of differentiation (CD)4^+ T cell immunology is central to sarcoidosis disease course(6-8). In bronchoalveolar lavage fluid (BALF) and mediastinal lymph nodes (MLN) of patients with sarcoidosis, the frequencies of IL-17^+ or IL-17^+IFNγ^+ memory T cells are increased(7-9). Moreover, aberrant activation of T helper (Th)17 and regulatory T (Treg) cells was found, characterized by decreased expression of co-inhibitory receptor cytotoxic T-lymphocyte antigen 4 (CTLA4) in BALF and MLN(10). Additionally, increased proportions of circulating $CD4^+$ T cells with expression of checkpoint molecule programmed death-1 (PD-1) and an exhausted phenotype were found in progressive sarcoidosis relative to patients with disease resolution and healthy controls (HCs)(11). Proportions of circulating PD-1^+CD4 central- and effector memory T cells in sarcoidosis were increased and these cells showed decreased proliferative capacity(12). During resolution of sarcoidosis, $PD1^+CD4^+$ T cells in peripheral blood (PB) decreased and normalization of immune function was observed, indicating that the phenotypical and functional T cell changes can be reversible(11). Interestingly, cancer treatment with anti-PD-1 or CTLA4-immune checkpoint blockade can cause a drug-induced sarcoidosis-like disease, further highlighting the link between aberrant T cell activation and sarcoidosis(13).

Early $CD4^+$ T cell activation leads to upregulation of the interleukin-2 receptor IL-2R/CD25(14). Subsequently, CD25 is released into the circulation as soluble IL-2R (sIL2R), which can be used to assess sarcoidosis disease activity(15). It correlates with proportions of $CD4^+$ T cells in the BALF and acute disease(16-18). However, multiple studies failed to demonstrate a correlation between sIL2R and baseline pulmonary function, disease progression or response to glucocorticoid treatment(16, 19, 20). Thus, despite strong evidence for critical involvement of T cells in sarcoidosis, it remains unknown whether the phenotype of circulating T cells can be used as therapeutic biomarker in sarcoidosis.

In this study, we hypothesized that the PB $CD4^+$ T cell phenotype of patients with sarcoidosis might correlate with response to prednisone treatment. We evaluated frequencies and phenotypes of PB T cell populations in newly diagnosed patients with pulmonary sarcoidosis before

prednisone treatment started, as well as in HCs. We correlated these parameters with lung function response, and we investigated the effect of prednisone treatment on circulating T cells of patients.

METHODS

Study design and subjects

In this prospective multicenter study, treatment-naïve patients with a diagnosis of pulmonary sarcoidosis according to the ATS/ERS/WASOG criteria were included(21). Ethics approval was obtained from the Erasmus Medical Center (MEC-2013-244). All patients and healthy control (HC) subjects provided written informed consent. Previously, we reported on short-term response in hospital and home-based spirometry during prednisone treatment in this cohort (Trial Registration: NL44805.078.13) (22).

All patients had a baseline forced vital capacity (FVC)<85% and parenchymal lung abnormalities. Prednisone treatment was standardized for the first three months: 4 weeks 40 milligrams (mg)/day, 2 weeks 30mg/day, 2 weeks 20mg/day, 2 weeks 15mg/day, 2 weeks 10mg/day. Hereafter, a maintenance dose of 5-10mg/day was continued up until one year. At any time, the treating physician could decide to deviate from this treatment schedule if the clinical situation demanded so. In-hospital pulmonary function measurements of FVC and diffusion capacity for carbon monoxide (DLCO) were conducted at baseline, month 1, 3, 6, 9 and 12 and peripheral blood for immunological analysis was sampled at baseline, month 3 and 12. We defined good initial lung function responders at 3 months as patients with an absolute FVC predicted increase of ≥10% and poor responders as patients with <10% FVC predicted increase during treatment, based on previously reported lung function response in sarcoidosis(22).

Sample processing and flow cytometry

Blood samples were collected in EDTA tubes and PB mononuclear cells (PBMCs) were obtained by ficoll density separation and stored at -180°C in RPMI medium containing 10% fetal calf serum and 10% dimethyl sulphoxide, according to standard procedures. Flow cytometric analyses were performed as previously described(23). Antibodies used for intra- and extracellular staining are listed in **Supplementary Table 1**.

Flow cytometry standard (FCS) files obtained with the BD FACSymphonyTM A5 Cell analyzer were preprocessed and quality control was performed using peak extraction and cleaning oriented quality control (PeacoQC)(24). Files were first preprocessed using Flowcore Package followed by peak detection and outlier removal using PeacoQC package in RStudio (v4.1.2). Clean FCS files were analyzed using FlowJo v10 (Tree Star Inc Software). Samples that contained >70% debris or passed the PeacoQC quality control but >50% of events had to be removed

because of poor quality, were excluded. Percentage or geometric mean fluorescent intensity (gMFI) of activation markers were only calculated from gated fractions containing >100 events.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software Inc; San Diego, CA, USA), which was also used to design graphs and to calculate statistics on flow cytometric analyses. The comparison of means of continued variables measured at different time points were tested with the paired student t-test. A Mann-Whitney U test was used to calculate significant differences between two unpaired groups. Wilcoxon matched-pairs signed rank test was used for paired samples. In case of multiple group comparisons, statistical analyses were performed by Kruskal-Wallis test combined with a Dunn's multiple comparison test. Spearman correlation was used to calculate significance of the strength of linear correlations between paired data represented in a scatterplot. *p* values <0.05 were considered significant. In the figures, significant p values are displayed as *p<0.05, **p<0.01 and ***p<0.001.

RESULTS

In this study 25 patients with pulmonary sarcoidosis were enrolled, three patients were excluded from our analyses. One patient had been using immunosuppressive therapy, one patient was not able to perform a technical correct pulmonary function test and one patient did not have paired blood samples available. No patients with Löfgren's syndrome were included. The baseline demographic and clinical characteristics are shown in **Table 1**. Additionally, 21 age- and sex-matched HC subjects were included for comparison.

Patients with sarcoidosis show variable increase in FVC during prednisone treatment

During the first three months, all patients were treated according to the predefined prednisone treatment schedule outlined in the Materials and Methods section. At 12 months, 15 patients used prednisone ≤10 mg, one patient had missing data, one patient used 15 mg prednisone daily and five patients were not using prednisone anymore. Glucocorticoid-sparing therapy with methotrexate was initiated in five patients and azathioprine in one patient. During treatment with prednisone, FVC improved significantly with a mean increase of 14.2% (±10.6, p<0.0001) between baseline and three months (n=20), and 13.5% (±12.6, p<0.0001) between baseline and 12 months (n=22) **(Figure 1A)**. The DLCO did not change significantly between baseline and three months, but we observed a mean increase of 9.5% (±16.8, p=0.01) between baseline and 12 months **(Figure 1B).** These data show that the increase in FVC during the first three months had a wide range. Using the ≥10% FVC predicted cut-off, 12 patients had a good treatment response with a significant median FVC increase of 14.8% (p=0.0024) while the remaining eight patients were

poor responders and had a non-significant median FVC increase of 4.2% (p=0.1518) at three months follow up compared with baseline **(Figure 1C)**. Of the good responders at 3 months, 10 (83.3%) also had a good FVC response at 12 months, while 1 patient (8.3%) had a poor response and 1 patient (8.3%) had missing FVC data at 12 months. There was no significant difference in baseline FVC or DLCO between patients with good and poor FVC response at three months.

In patients with sarcoidosis, circulating CD4+ memory and regulatory T cells display an aberrantly activated phenotype

Using flow cytometry, we measured the proportions of PB T cell subsets of patients with sarcoidosis and HCs. The gating strategy is outlined in **Suppl. Figure 1**. Tregs were gated as

Table 1: Baseline characteristics of participating subjects

	Control	**Pulmonary sarcoidosis**
Subjects	N=21	N=22
Age (mean, ±SD)	47.1 (±9.7)	43.9 (±10.2)
Female/male	10(48) / 11(52)	8(36) / 14(64)
Ethnicity Black / white / other		6(27)/12(55)/ 4(18)
Smoking No/yes/former		15(68)/ 2(9)/ 5(23)
Diagnosis confirmed by clinical- radiological features and granuloma's in tissue biopsy n, (%) clinical - radiological features and BALF CD4/CD8 ratio >3.5 n, (%) clinical- radiological features n, (%)		 18(82) 3(14) 1(4)
Pulmonary function Forced vital capacity (FVC) in % predicted (mean, ±SD) Diffusion capacity (DLCOc) in % predicted (mean, ±SD)		 70.6 (±13.3) 62.5 (±19.0)
Scadding stage chest X-ray, n, (%) 0 1 2 3 4 Unknown		 1 (5)* 1 (5) 15 (68) 4 (18) 0 1 (5)
Extra thoracic involvement, n, (%) Skin Eyes Liver Spleen Central nervous system Cardiac		 3 (14) 3 (14) 3 (14) 2 (9) 0 0
Biomarkers ACE (n=21) mmol/L (median, IQR) Soluble interleukin receptor (sIL2R; n=15) U/L (median, IQR)		 75(13-284) 9423(3000-30400)

*confirmed pulmonary parenchymal involvement on chest computed tomography (CT)

$CD127^{-}CD25^{high}CD4^{+}$ T cells (which were high in FoxP3 expression, **Suppl.Figure 1B**). The populations of $CD127^{+}CD25^{low}CD4^{+}$ T cells were divided into $CD27^{+}CD45RA^{+}CD45RO^{-}$ naïve T cells (Tn) and $CD45RA^{-}CD45RO^{+}$ memory T cell (Tm) fractions. Proportions of $CD4^{+}$, $CD8^{+}$ T cells and γδ T cells (of $CD3^{+}$ T cells) were unaltered in patients (**Suppl. Figure 2AB**). Within the $CD4^{+}$ T cell subset, proportions of Tn and Tm cells were also unaltered. In line with previous results(25), increased proportions of Tregs were found in sarcoidosis compared with HCs (**Suppl. Figure 2C**).

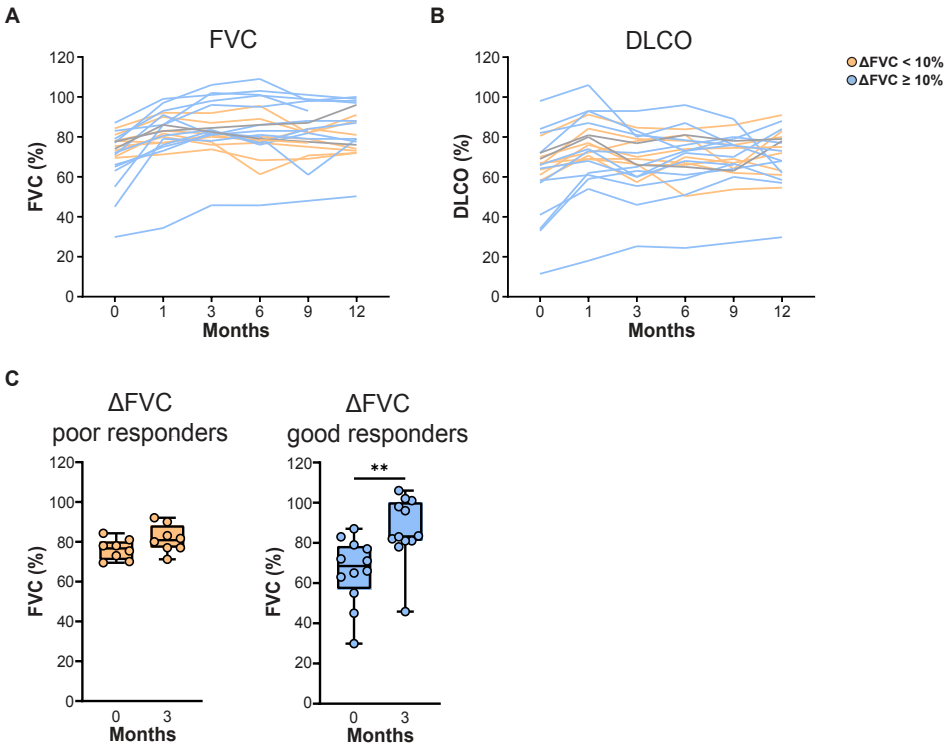


Figure 1: Pulmonary function of patients with sarcoidosis during 12 months of treatment
(A,B) In hospital measurement of Forced Vital Capacity (FVC) in % predicted **(A)** and diffusion capacity of the lungs for carbon monoxide corrected for haemoglobin (DLCOc) in % predicted **(B).** Each line represents one patient. Blue lines represent patients with ≥10% absolute FVC increase between 0 and 3 months. Orange lines represent patients with <10% FVC increase in 3 months. Two patients had missing FVC data at the 3 months measurement (grey lines). **(C)** Boxplots indicating measured FVC in % predicted before and after 3 months of prednisone in two treatment outcome groups. Depicted are poor responders, defined as patients with < 10% absolute FVC increase between 0 and 3 months (*left*) and good responders, defined as patients with ≥ 10% absolute FVC increase between 0 and 3 months (*right*). Wilcoxon matched-pairs signed rank test was used to calculate significant differences between 0 and 3 months and 0 and 12 months. Mann-Whitney U test was used to calculate significant differences between two groups.

We subsequently evaluated the expression of multiple surface markers that are known to be upregulated upon T cell activation **(Suppl. Table 1)**. Activation marker expression on the surface of $CD8^+$ T cells and $CD4^+$ Tn cells was not significantly different between patients and HCs or across treatment response groups. In contrast, within the fraction of circulating $CD4^+$ Tm cells, we found decreased proportions of $CD25^+$ and $CD28^+$ Tm cells in sarcoidosis compared with HCs, only reaching significance for $CD25^+$ Tm cells (Suppl. Figure 3A). Hereby, the $CD25^+$ $CD4^+$ Tm cells showed high levels of CD127 expression, and could therefore readily be distinguished from $CD25^{hi}CD127^-$ Tregs, as shown in **suppl. Figure 1.**

Additionally, proportions of $CTLA4^+$ and $PD\text{-}1^+CD4^+$ Tm were significantly increased in patients. Compared with HCs, regulatory T cells in patients with sarcoidosis displayed increased expression of CD25 and CD95/Fas **(Suppl. Figure 3B)**. Whereas proportions of Tregs expressing CD28 were high and unaltered, the fractions of $CTLA^+$ and $PD\text{-}1^+$Tregs were increased in sarcoidosis, compared with HCs.

Taken together, we conclude that in PB of treatment-naïve patients with sarcoidosis $CD4^+$ Tm and Tregs displayed an aberrantly activated phenotype, compared with HCs.

Increased proportions of circulating PD-1+CD4+ memory T cells in sarcoidosis patients with good response to prednisone

Next, we investigated whether expression of surface markers on sarcoidosis $CD4^+$ Tm cells at baseline correlated with the response to prednisone. Hereby, we compared patients with sarcoidosis and good response to prednisone to poor responders and HCs **(Figure 2A)**. While both treatment response groups had decreased proportions of circulating $CD25^+$ and $CD28^+$ Tm compared with HCs, this only reached significance in the good prednisone responders. Both outcome groups had increased proportions of $CTLA4^+$ Tm cells compared to HCs. Interestingly, only good responders had significantly increased baseline proportions of $PD\text{-}1^+$ Tm cells, compared to other patients with sarcoidosis and HCs **(Figure 2A)**. We found that the surface expression of PD-1 in the good responder group was higher than in the poor responder group in all T cell subsets analysed, including Th1, Th17 and Th17.1, as well as non-Th1/Th17 cells (based on CXCR3 and CCR6 expression).

We subsequently correlated for each patient the proportions of circulating $CD25^+$ and $PD\text{-}1^+CD4^+$ Tm cells with the values for the absolute increase in FVC (% predicted) between baseline and 3 months. Whereas the FVC increase did not correlate with the proportions of $CD25^+$ $CD4^+$ Tm cells, we found a significant positive correlation between FVC increase and $PD\text{-}1^+CD4^+$ Tm cells ($r=0.58$, $p<0.001$) **(Figure 2B)**. Proportions of $CD25^+$ and $PD\text{-}1^+CD4^+$ Tm cells showed a significant inverse correlation ($r= -0.65$ $p<0.001$) **(Figure 2C).** Hereby, the ratio of the proportions of $PD\text{-}1^+$ over $CD25^+$ $CD4^+$ Tm cells was significantly higher in good responders than in poor responders (**Figure 2D**).

In summary, increased baseline proportions of circulating $PD\text{-}1^+CD4^+$ Tm cells significantly associated with good FVC response to prednisone in patients with sarcoidosis.

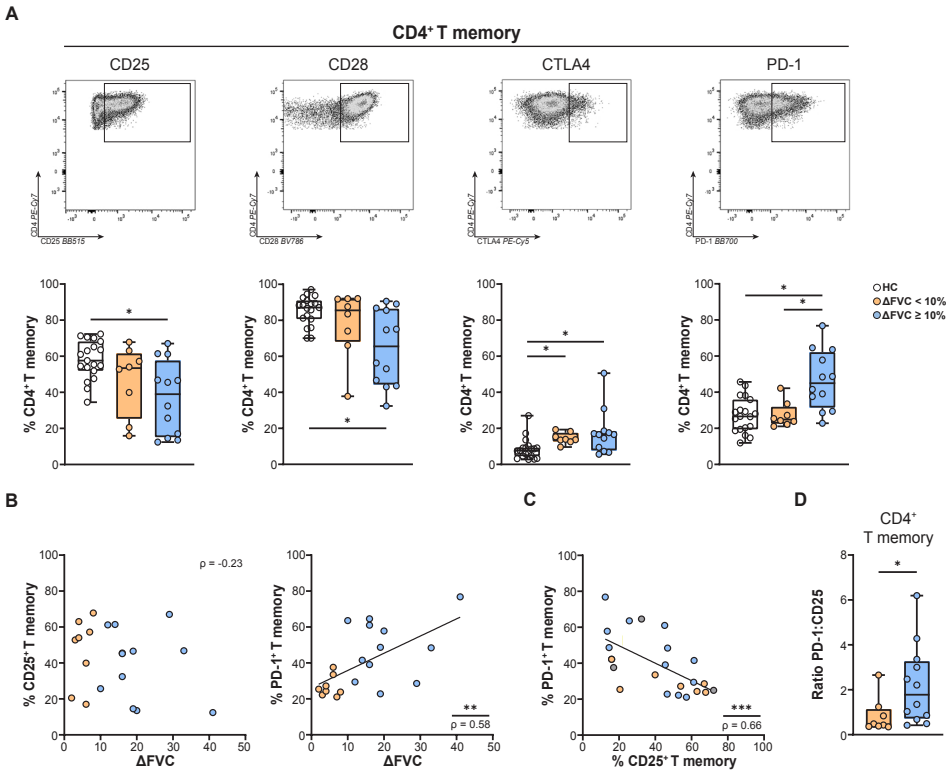


Figure 2: Activation marker expression on CD4⁺ memory T cells in sarcoidosis and correlation with prednisone response

(A) Representative flow cytometry dot plots (*top*) and proportions of CD4⁺ memory T cells positive for CD25, CD28, CTLA4 and PD-1 expression (*bottom*) in healthy controls (HCs), sarcoidosis patients with <10% (poor response) and ≥10% (good response) absolute FVC % predicted increase in 3 months. **(B)** Scatter plots depicting correlation coefficients with p-value between absolute increase in FVC % predicted (Δ FVC) between baseline and 3 months and proportions of CD4⁺memory T cells (in %) positive for CD25 *(left)* or PD-1 *(right)*. **(C)** Scatter plot depicting correlation coefficient with p-value between proportions of CD4⁺memory T cells positive for CD25 (in %) and for PD-1. **(D)** Ratio of the proportions of CD4⁺ memory T cells positive for PD-1 over the proportions positive for CD25 in patients with poor and good response. Symbols represent individual values in HCs (open circles) and patients with sarcoidosis with Δ FVC% predicted of <10% (orange circles) and Δ FVC% predicted of ≥10% (blue circles) between baseline and 3 months. All data were measured by flow cytometry. Mann-Whitney U test was used to calculate significant differences between two groups. In case of multiple group comparisons, statistical analysis was calculated using Kruskal-Wallis test combined with a Dunn's multiple comparison. Spearman correlation was used to calculate significance of linear correlations between paired data in the scatterplots.

Increased proportions of circulating PD-1⁺ regulatory T cells in patients with sarcoidosis and good response to prednisone

We subsequently investigated the phenotype of circulating Tregs in the two treatment outcome groups and HCs. Tregs displayed increased expression of CD25 and CD95 in both sarcoidosis outcome groups compared to HCs. Proportions of CD28⁺ Tregs were high in all groups and not significantly different between patients and HCs (**Figure 3A**). Proportions of CTLA4⁺ Tregs were increased in sarcoidosis compared with HCs. Similar to our findings for PD-1⁺CD4⁺ Tm cells, we found significantly increased proportions of PD-1⁺ Tregs in good responders compared with poor responders and HCs (**Figure 3A**). No correlation was found between proportions of CD25⁺ or PD-1⁺ Tregs and absolute increase in FVC % predicted after 3 months (Shown for PD-1⁺ Tregs in **Figure 3B**).

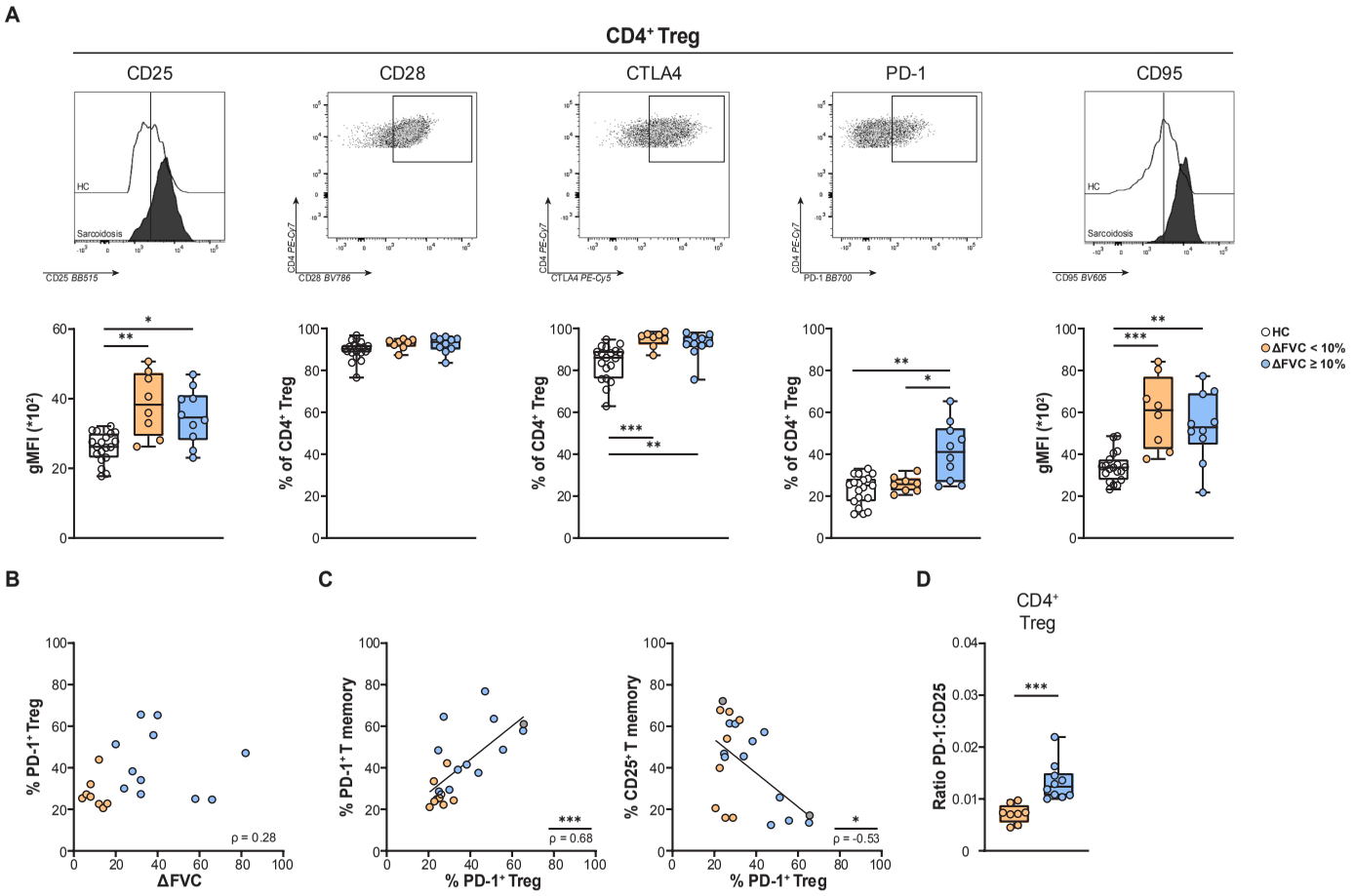
Within sarcoidosis patients, the proportions of PD-1⁺ Tregs showed a positive correlation with the proportions of PD-1⁺CD4⁺ Tm cells (r=0.68 p<0.001), and an inverse correlation with CD25⁺CD4⁺ Tm cells (r= -0.53 p<0.05) **(Figure 3C)**. Interestingly, the PD-1⁺ to CD25⁺ ratio in Tregs markedly separated patients with good glucocorticoid response from patients with poor response (p< 0.001) (**Figure 3D**).

We also tested the correlation between the levels of sIL2R in the serum of patients (n=15) and the FVC response during 3 months of prednisone treatment. In line with published data indicating poor sIL2R biomarker performance(16), we found no significant correlation **(Suppl. Figure 4A)**. Additionally, sIL2R did not significantly correlate with baseline proportions PD-1⁺ or CD25⁺ Tm and Tregs **(Suppl. Figure 4B).**

Collectively, these results indicate that PB Tregs in sarcoidosis have an activated phenotype. The proportions of PD-1⁺ Tregs at baseline correlated with good FVC response at 3 months of prednisone treatment.

Figure 3: Activation marker expression on regulatory T cells in sarcoidosis and correlation with prednisone response ▶

(A) Representative flow cytometry histograms of CD25 and CD95/Fas (gMFI) expression and dot plots of gated CD4⁺ regulatory T cells showing the expression of CD28, CTLA4 and PD-1 (*top*), and box plots showing the expression in healthy controls (HCs), sarcoidosis patients with <10% and ≥10% increase in FVC % predicted (poor and good responders, respectively) in 3 months (*bottom*). **(B)** Scatter plot depicting correlation between FVC % predicted and proportions of cells within Tregs that are positive for PD-1, with ρ-value. **(C)** Scatter plot depicting correlation between proportions of Tregs positive for PD-1 and proportions of CD4⁺ memory T cells positive for PD-1 *(left)* or CD25 *(right).* **(D)** Ratio of the proportions of Tregs expressing PD-1 (in %) over the CD25 expression level (gMFI) on Tregs patients with a poor and a good response (ΔFVC of <10% and ≥10% in 3 months, respectively). Symbols represent individual values in HCs (open circles) and sarcoidosis patients with ΔFVC of <10% (orange circles) and Δ FVC ≥10% (blue circles) between baseline and 3 months. All data were measured by flow cytometry. Mann-Whitney U test was used to calculate significant differences between two groups. In case of multiple group comparisons, statistical analysis was calculated using Kruskal-Wallis test combined with a Dunn's multiple comparison. Spearman correlation was used to calculate significance of linear correlations between paired data in the scatterplots.


A
CD4⁺ Treg
CD25
CD28
CTLA4
PD-1
CD95
HC
Sarcoidosis
CD25 BB515
CD28 BV786
CD4 PE-Cy7
CTLA4 PE-Cy5
PD-1 BB700
CD95 BV605
gMFI (*10²)
% of CD4⁺ Treg
HC
ΔFVC < 10%
ΔFVC ≥ 10%
B
% PD-1⁺ Treg
ΔFVC
ρ = 0.28
C
% PD-1⁺ T memory
% PD-1⁺ Treg
ρ = 0.68
% CD25⁺ T memory
ρ = -0.53
D
CD4⁺ Treg
Ratio PD-1:CD25

Prednisone treatment reverses the aberrantly activated phenotype of circulating CD4⁺ memory T cells and regulatory T cells in sarcoidosis

To elucidate treatment effects on PD-1 and CD25 surface expression on CD4⁺ Tm cells and Tregs in sarcoidosis, we compared baseline blood samples with paired samples at 3 and 12 months. During the first 3 months of prednisone treatment (which was standardized, see Materials and Methods), proportions of CD25⁺ CD4⁺ Tm cells significantly increased, while proportions of PD-1⁺CD4⁺ Tm cells significantly decreased, compared with baseline values **(Figure 4A).** Also, proportions of CD25⁺ and PD-1⁺ Tregs decreased, but this only reached significance for CD25⁺ Tregs **(Figure 4B)**. At 12 months of treatment (in which prednisone was differentially tapered across patients, see Materials and Methods), proportions of Tm cells and Tregs expressing either CD25 or PD-1 were not different from baseline.

Together, these findings indicate that high dose prednisone treatment reversed the aberrant baseline expression of CD25 and PD-1 on CD4⁺ Tm and Treg, but this effect waned after tapering of prednisone at 12 months.

Baseline proportions of CD25⁺ and PD-1⁺CD4⁺ memory T cells predict good lung function response after 1 year

Finally, we investigated if the baseline proportions of CD25⁺ and PD-1⁺CD4⁺ Tm cells and Tregs correlated with good long-term treatment response, defined as ≥10% absolute FVC increase between baseline and 12 months. At one year follow up, there was no significant difference in

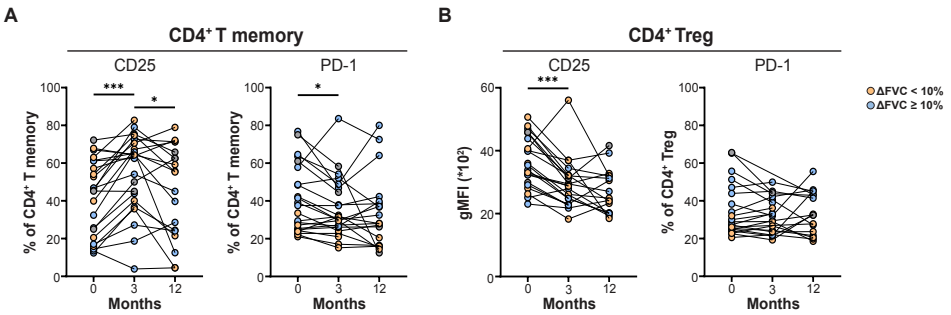


Figure 4: Proportions of CD4⁺PD-1⁺ and CD25⁺memory and regulatory T cells in patients with sarcoidosis during treatment

(A) Paired measurements of proportions of CD4⁺ memory T cells positive for CD25 (*left*) and PD-1 (*right*) at baseline, 3 and 12 months. **(B)** Paired measurement of gMFI values for CD25 (*left*) and the proportions of PD-1-expressing cells in the Treg population at baseline, 3 and 12 months. Each connecting line represents one patient. Symbols represent individual values of patients with sarcoidosis in the two treatment outcome groups with Δ FVC% predicted of <10% (poor response, orange circles) and Δ FVC% predicted of ≥10% (good response, blue circles) in three months. All data were measured by flow cytometry. Wilcoxon matched-pairs signed rank test was used to calculate significant differences between 0 and 3 months, 3 and 12 months and 0 and 12 months.

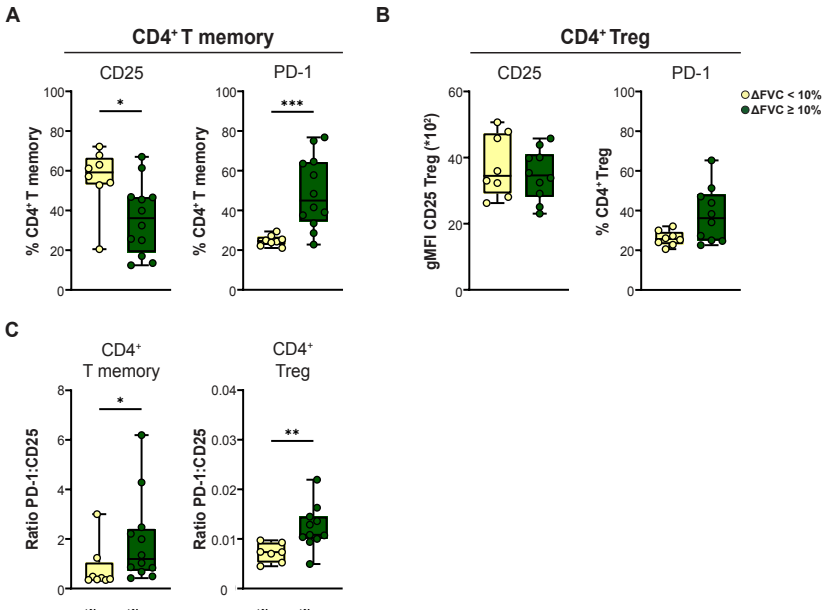


Figure 5: Baseline proportions of PD-1⁺ or CD25⁺ CD4⁺ memory and Treg and correlation with 12 month FVC response

(A) baseline proportions of CD4⁺ memory T cells positive for CD25 (*left*) and PD-1 (*right*) in patients with sarcoidosis in the two treatment outcome groups defined by <10% (poor response) and ≥10% (good response) FVC (% predicted) increase at 12 months compared to baseline. **(B)** Expression of CD25 on Tregs (gMFI) (*left*) and proportions of CD4⁺ regulatory T cells positive for PD-1 (*right*) in sarcoidosis treatment outcome groups with <10% and ≥10% absolute FVC (% predicted) increase at 12 months compared to baseline. **(C)** Ratio of the proportion of CD4⁺ memory T cells expressing PD-1 over de proportions that express CD25 in the two response groups (*left*) and the ratio of the proportion of Tregs that are PD-1⁺ over the expression level of CD25 (in gMFI). Symbols represent individual values for patients in the indicated response groups. All data were measured by flow cytometry. Mann-Whitney U test was used to calculate significant differences between two groups.

steroid dose between the two treatment outcome groups. Good and poor responders used median 7.5 mg and 5.0 mg prednisone daily, respectively (p=0.995). We found significantly decreased proportions of CD25⁺ and significantly increased proportions of PD-1⁺CD4⁺ Tm cells at baseline in patients with good FVC response, compared with poor responders at 12 months **(Figure 5A**). We observed a trend of increased proportions of PD-1⁺ Tregs in patients with good FVC response compared with poor responders, but this did not reach significance **(Figure 5B).** The ratio between PD-1⁺and CD25⁺ proportions for both Tm and Treg at baseline correlated significantly with good response to prednisone at 12 months compared with poor responders (**Figure 5C**). No correlation between activation marker expression on T cell subsets or FVC and DLCOc between baseline and 12 months was found.

In summary, these data show that high baseline ratios of PD-1 to CD25 expression, both on $CD4^+$ Tm cells and on Tregs correlated significantly with FVC response at 12 months.

DISCUSSION

Stratifying patients with a high likelihood to benefit from glucocorticoid treatment is essential to optimize personalized sarcoidosis care. Currently, no predictive biomarker is available and first-line prednisone is recommended for all patients with sarcoidosis with a treatment indication(2). Here, we show for the first time significant correlations with the phenotype of circulating T cells at baseline and ≥10% absolute FVC increase during prednisone treatment in pulmonary sarcoidosis. In particular, increased proportions of $PD\text{-}1^+$ cells and decreased proportions of $CD25^+$ cells within the $CD4^+$ Tm cell population, as well as increased fractions of $PD\text{-}1^+$ cells in the Treg population, correlated with a good treatment response. Importantly, for Tm cells as well as Tregs the ratio of $PD\text{-}1^+$ to $CD25^+$ cells correlated with treatment response, both at 3 and at 12 months. These findings indicate the potential of $PD\text{-}1^+$ and $CD25^+$ expression levels on circulating T cell populations as new biomarkers to predict glucocorticoid therapy response in pulmonary sarcoidosis. In contrast, we did not detect an association with treatment outcome for serum sIL2R, a biomarker currently used in clinical care.

PD-1 is upregulated on all T cells following T-cell receptor-mediated activation and can remain elevated during persistent antigen presentation(26). Increased expression of PD-1 negatively regulates T cell proliferation and effector function and contributes to peripheral tolerance and T cell exhaustion, as observed in tumor microenvironments(26). In progressive sarcoidosis, circulating $CD4^+$ T cells display an exhausted phenotype with high $PD\text{-}1^+$ proportions(11). We previously described comparable baseline proportions of circulating $PD\text{-}1^+$ $CD4^+$ Tm cells in patients with spontaneous disease resolution and progressive sarcoidosis(27). This finding suggests the Tm phenotype itself does not contribute to disease resolution. In the current study, the remarkable $PD\text{-}1^{high}CD25^{low}$ phenotype of $CD4^+$ Tm cell population in good responders may indicate a selection of patients with less successful antigen clearance due to aberrant T cell activation.

In line with previous work, we found an aberrantly activated phenotype of PB Tregs in patients with sarcoidosis, characterized by increased levels of CD25 and CD95 expression and increased proportions of CTLA4-expressing cells(25). Considering Tregs, only increased proportions of $PD\text{-}1^+$ cells associate with good treatment efficacy. Circulating $PD\text{-}1^{high}$ Tregs have impaired suppressive function and signs of exhaustion, as shown in patients with malignant glioma(28). In sarcoidosis, a reduced regulatory capacity and survival of Tregs was described previously, whereby restoration of their function was associated with disease resolution(25, 29). Here, we demonstrate a correlation between proportions of $PD\text{-}1^+$ Tregs and $PD\text{-}1^+CD4^+$ Tm cells.

Mechanistically, it is conceivable that the impaired immunosuppressive function of exhausted PD-1^{high} Tregs contributes to ongoing activation of circulating CD4^{+} Tm cells. However, to elucidate the function and possible exhaustion of circulating PD-1^{high} Tregs in sarcoidosis, additional research is needed.

Given the lack of correlation between sIL2R levels and FVC response to prednisone in this study, overall T cell activation in sarcoidosis probably does not predict glucocorticoid efficacy. Prednisone has broad immunosuppressive effects on immune cells implicated in sarcoidosis pathogenesis(6, 30). Importantly, it inhibits the activation and differentiation of effector T cells and supresses the production of pro-inflammatory cytokines involved in granulomatous inflammation(30). In this study, prednisone treatment was associated with reversal of the aberrantly activated phenotype of PB memory and regulatory T cells in sarcoidosis**.** However, the exact mechanisms by which specifically PD-1^{+}CD4^{+} Tm cells and PD-1^{+} Tregs predispose to good prednisone sensitivity in sarcoidosis need to be elucidated. Additional research is needed to explore whether the association is specific for sarcoid granulomatous inflammation or reflects general mechanisms that fuel glucocorticoid sensitivity. In cancer treatment, the balance of PD-1 expression between CD8^{+} effector T cells and regulatory T cells in the tumor microenvironment associates with the therapeutic effect of PD-1 blocking immunotherapy(31).

Our study has some limitations to address. First, our findings need to be validated in a larger patient cohort before clinical application is possible. Second, we were able to correlate T cell phenotype with FVC but not with the DLCO response. This may be explained by high DLCO variability in response to treatment and therefore it will be valuable to explore this in larger groups of patients. Third, the number of patients included in this study was limited and prospective follow-up was one year. We were therefore not able to correlate T cell phenotypes with clinically relevant disease relapse upon prednisone withdrawal, which is estimated to occur in 20-80% of patients after initial therapy(2, 4, 32).

CONCLUSION

The current study shows increased baseline proportions of circulating PD-1^{+}CD4^{+} memory and regulatory T cells in patients with sarcoidosis and good initial FVC response to prednisone. Additionally, increased PD-1^{+} and decreased CD25^{+} CD4^{+} memory T cell proportions associate with favorable long term FVC response. These findings reveal a promising blood biomarker for prednisone efficacy in treatment-naïve patients with pulmonary sarcoidosis.

LIST OF ABBREVIATIONS

BALF	bronchoalveolar lavage fluid
CD	cluster of differentiation
CTLA4	cytotoxic T-lymphocyte antigen 4
DLCO	diffusion capacity for carbon monoxide
FVC	forced vital capacity
HCs	healthy controls
MLN	mediastinal lymph node
PB	peripheral blood
PD-1	programmed death-1
sIL2R	soluble IL-2 receptor
Th	T helper cell
Treg	regulatory T cell

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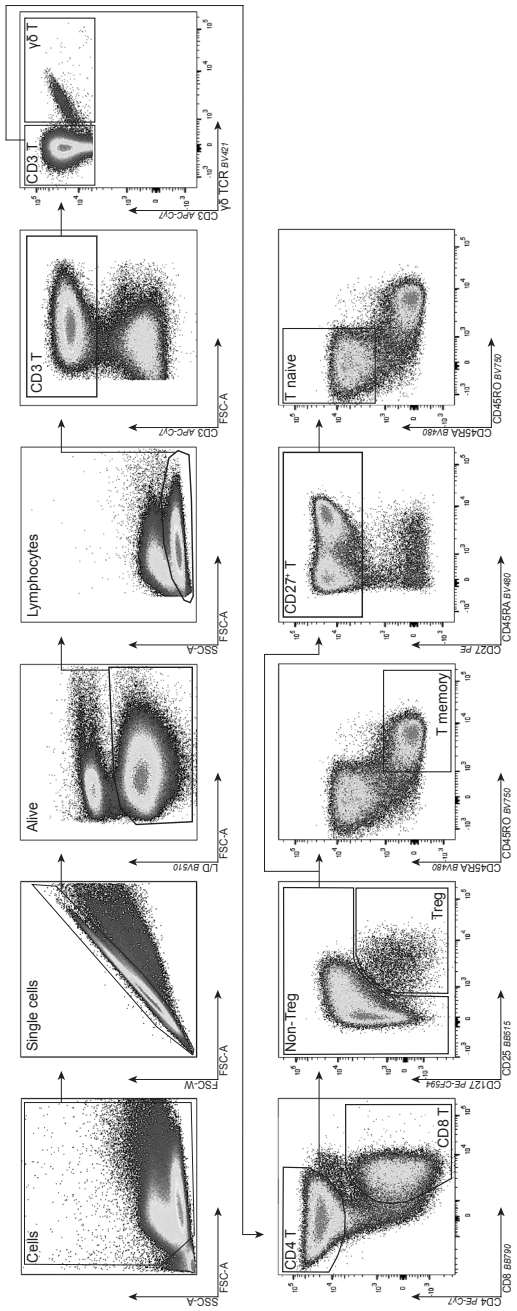
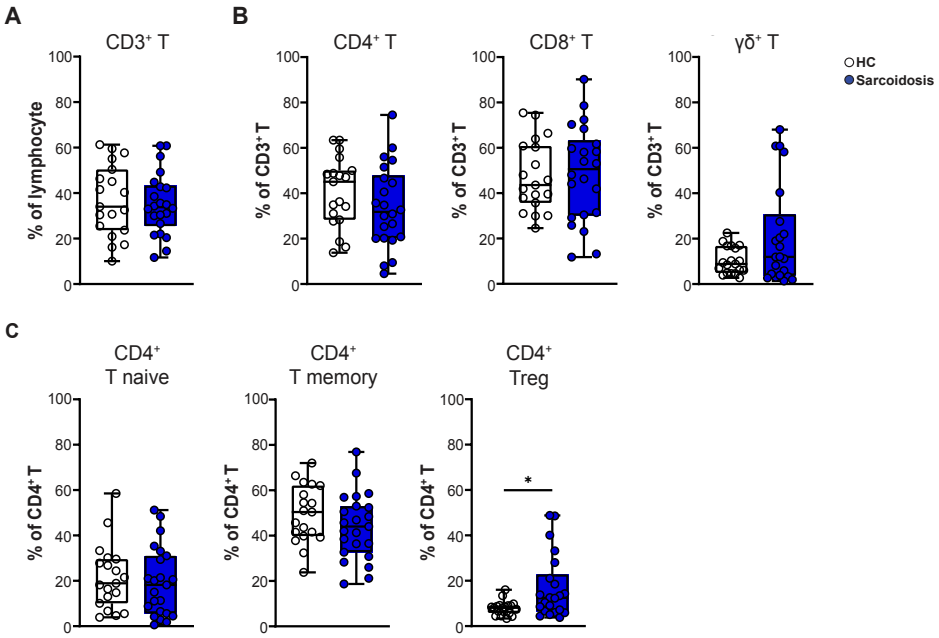
SUPPLEMENTARY MATERIAL



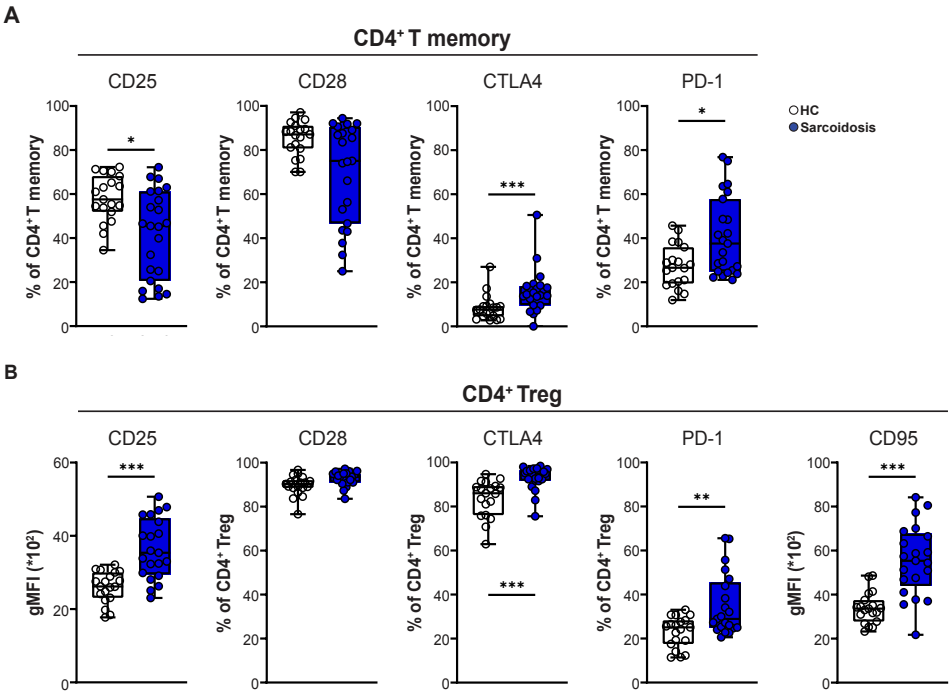
Suppl. Figure 1: Gating strategy of human T cell subsets
Gating strategy for the indicated human T cell populations in peripheral blood mononuclear cell fractions. Resting T cells: $CD127^{+/-}CD25^{-}$, Activated T cells: $CD127^{+}$ $CD25^{+}$, Tregs: $CD127^{-}$ $CD25^{high}$

Supplementary Table 1.

Antibody	Alternative name	Function / expressed on	Conjugate	Clone	Manufacturere
CD3			APC-Cy™7	SK7	BD Biosciences
CD4			PE-Cy™7	SK3	BD Biosciences
CD8			BB790-P	SK1	BD Biosciences
CD25	IL-2Rα	Early activation marker	BB515	2A3	BD Biosciences
CD27		Activation marker	PE	O323	BD Biosciences
CD28		Costimulation	BV786	CD28.2	BD Biosciences
CD45RA		Naive T-cell	BV480	5H9	BD Biosciences
CD45RO		Memory T-cell	BV750	UCHL1	BD Biosciences
CD95	Fas	Apoptosis marker	APC	DX2	BD Biosciences
CD127	IL-7Rα		PE-CF594	HIL-7R-M21	BD Biosciences
CD152	CTLA4	Co-Inhibitory marker	PE-Cy™5	BNI3	BD Biosciences
CD279	PD-1	Co-Inhibitory marker	BB700	Eh12.1	BD Biosciences
γδ TCR		γδ T-cell	BV421	11F2	BD Biosciences

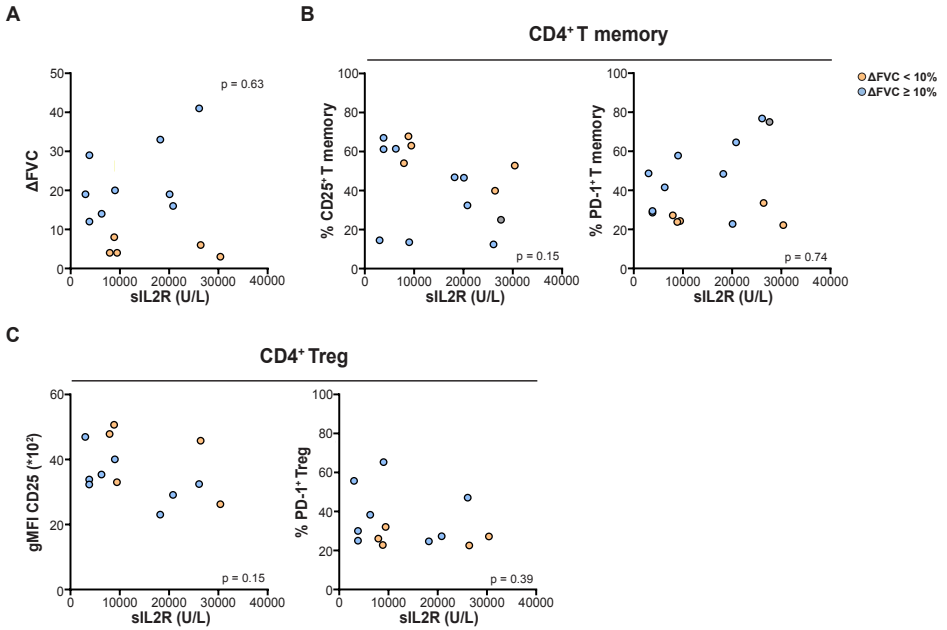


Suppl. Figure 2: Proportions of T cell subsets in healthy controls and patients with sarcoidosis
(**A**) Proportions of CD3⁺ T cells of total lymphocytes in peripheral blood mononuclear cell fractions. (**B**) Proportions of CD4⁺, CD8⁺ and γδ⁺ T cells of total CD3⁺ T cells (**C**) Proportions of CD4⁺ T naïve, T memory and Treg cells of total CD4⁺ T cells. Symbols represent individual values in healthy controls (HCs; open circles) and total sarcoidosis patients (Sarcoidosis; blue circles). All data were measured by flow cytometry. Mann-Whitney U test was used to calculate significant differences between two groups. *p<0.05, **p<0.01 and ***p<0.001.



Suppl. Figure 3: Expression of activation markers on CD4⁺ memory T cells and Tregs in healthy controls and patients with sarcoidosis

(**A**) Proportions of CD4⁺ memory T cells expressing CD25, CD28, CTLA and PD-1. **(B)** Proportions of Tregs expressing CD28, CTLA and PD-1. Expression of CD25 and CD95 on Tregs is depicted in gMFI. Symbols represent individual values in healthy controls (HCs; open circles) and total sarcoidosis patients (Sarcoidosis; blue circles). All data were measured by flow cytometry. Mann-Whitney U test was used to calculate significant differences between two groups. *p<0.05, **p<0.01 and ***p<0.001.



Suppl. Figure 4: Serum concentration of soluble IL-2 receptor does not correlate with FVC response or T cell activation marker expression

(A) Scatter plots depicting correlation coefficients with p-value between absolute increase in FVC % predicted (Δ FVC) between baseline and 3 months and serum concentration of soluble IL-2 receptor (sIL2R) in U/L in sarcoidosis patients with <10% and ≥10% absolute FVC % predicted increase in 3 months **(B)** Scatter plot depicting correlation coefficient between proportions of CD4+memory T cells positive for CD25 (in %; *left)* and for PD-1 (*right)* and serum concentration of soluble IL-2 receptor (sIL2R) in U/L in sarcoidosis patients with <10% and ≥10% absolute FVC % predicted increase in 3 months (**C**) Scatter plot depicting correlation coefficient between baseline expression level of CD25 (in gMFI) (*left*) or the proportions of Tregs that express PD-1 (*right*) and serum concentration of soluble IL-2 receptor (sIL2R) in U/L in sarcoidosis patients with <10% and ≥10% absolute FVC % predicted increase in 3 months. Symbols represent individual values in patients with sarcoidosis in the two response groups, as indicated.

CHAPTER 5

Drug-induced sarcoidosis-like reactions

Jelle R. Miedema, Hilario Nunes

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ABSTRACT

Purpose of review

Sarcoidosis is a complex granulomatous disease of unknown cause. Several drug categories are able to induce a systemic granulomatous indistinguishable from sarcoidosis, known as *drug-induced sarcoidosis-like reaction* (DISR). This granulomatous inflammation can resolve if the medication is discontinued. In this review, we discuss recent literature on medication associated with DISR, possible pathophysiology, clinical features and treatment.

Recent findings

Recently, increasing reports on DISR have expanded the list of drugs associated with the systemic granulomatous eruption. Most reported drugs can be categorized as combination antiretroviral therapy, tumor necrosis factor-α antagonist, interferons and immune checkpoint inhibitors, but reports on other drugs are also published. The proposed mechanism is enhancement of the aberrant immune response which results in systemic granuloma formation. It is currently not possible to know whether DISR represents a separate entity or is a triggered but "true" sarcoidosis. As DISRs may cause minimal symptoms, treatment is not always necessary and the benefits of continuing the offending drug should be weighed against clinical symptoms and organ dysfunction. Treatment may involve immunosuppressive medication that is used for sarcoidosis treatment.

Summary

In this article, we review recent insights in DISR: associated drug categories, clinical presentation, diagnosis and treatment. Additionally, we discuss possible mechanisms of DISR which can add to our knowledge of sarcoidosis pathophysiology.

INTRODUCTION

Sarcoidosis is a systemic granulomatous disease of unknown cause, which can manifest with many different clinical phenotypes(1). The lungs and mediastinal lymph nodes are frequently involved, but granulomas can affect almost every organ. Genetic factors contribute to disease risk, and several environmental factors including non- degradable parts of (myco-) bacteria, inorganic foreign material and autoantigens, have been proposed as possible disease triggers or antigens(1). The pathological hallmark of sarcoidosis, which is the epithelioid non-necrotizing granuloma, results from a complex interplay between the innate and adaptive immune response in a genetically susceptible individual. Several common drug categories are able to induce a systemic granulomatous syndrome that is clinically indistinguishable from sarcoidosis, known as *drug-induced sarcoidosis-like reaction* (DISR). It occurs in temporal relationship with the offending drug and can improve or resolve if the drug is discontinued(2). The only definite way to distinguish the two entities is the resolution of DISR after discontinuation and recurrence after rechallenge with the offending drug (2, 3). In this paper, we review the current literature about DISRs, associated drug classes, diagnosis and treatment options. Furthermore, we discuss whether DISR should be seen as separate syndrome or "true" sarcoidosis.

Drugs associated with DISR

Several drug classes have been associated with the development of clinical signs similar to sarcoidosis (https://www.pneumotox.com). A comprehensive review about the subject was recently performed(2). An extensive search in the pharmacovigilance database from the world health organization additionally identified 2425 cases of DISR and 55 potential drugs between 1967 and 2019(3). This database contains individual drug case safety reports from 130 countries. Together with several other published cohorts and case reports, the literature on DISR provides an expanding list of associated drug categories. Most reported drugs can be categorized as combination anti-retroviral therapy (cART), Tumor necrosis factor alpha (TNF-α) antagonist, Interferons (IFNs) and immune checkpoint inhibitors (ICIs)(**Table 1**).

Combination anti-retroviral therapy

Human Immunodeficiency virus (HIV) depletes the CD4+ T-cell pool(4). Low CD4+T cell counts in patients with HIV lead to remission of preexisting sarcoidosis (5). In contrast, when HIV positive patients are treated with cART and CD4+T cell counts rise to > 150-200 cells/ microliter, a DISR indistinguishable from sarcoidosis may develop as part of immune reconstitution(2, 5). Additionally, cART may also exacerbate prior sarcoidosis(5-7). Various cART regimens are associated with DISR, indicating that the granulomatous inflammation is not a drug specific effect (2). DISRs are on average reported 9-20 months after cART initiation(2, 6). However, many cases of sarcoidosis occur outside the period of immunological recovery. A recent study of a

Table 1: Drugs classes associated with sarcoid-like reaction

Drug class	Drugs	Proposed mechanisms
Combination anti-retroviral therapy (cART)	Variable	Increased CD4+ T cell immunity / immune reconstitution
Immune checkpoint inhibitors (PD1)	Nivolumab Pembrolizumab	Increased immune activation Altered T cell polarization Neoantigen exposure
Immune checkpoint inhibitors (PD-L1)	Atezolizumab Avelumab Durvalumab	Increased immune activation Altered T cell polarization Neoantigen exposure
Immune checkpoint inhibitors (CTLA4)	ipilimumab	Increased immune activation Altered T cell polarization Neoantigen exposure
Tumor necrosis factor-α antagonist	Etanercept adalimumab, infliximab	Cytokine disbalance Altered T cell polarization
Interferons	Interferon α, interferon β	Cytokine disbalance Altered T cell polarization

nationwide HIV cohort including 18431 patients in active follow up found a relatively high sarcoidosis prevalence of 141/100.000(8). Of these patients, 11% were diagnosed with sarcoidosis before HIV infection and 84% were diagnosed with sarcoidosis after HIV diagnosis during good immune-virological control. The mean interval between HIV diagnosis and sarcoidosis in this study was 11.6 (±7.5) years(8). This indicates that some cases may represent cART-DISR, while others may be unrelated to the immune reconstitution and considered “true” sarcoidosis. Because early treatment is now recommended for all HIV patients regardless of CD4+T cell count, the incidence of cART-DISR may decrease in the future.

Immune checkpoint-inhibitors

Immune checkpoint inhibitors are increasingly used in the treatment of numerous malignancies. This therapy enhances the antitumor response by blocking down regulators of T-cell immunity. Common ICIs are antibodies targeting programmed cell death protein 1 (PD-1: Nivolumab, Pembrolizumab), the PD-1 ligand (PD-L1: Atezolizumab, Avelumab, Durvalumab) or cytotoxic T-lymphocyte antigen 4 (CTLA-4: Ipilimumab)(2). Several side effects including DISR, called immune related adverse events (irAEs), may occur due to the desired immune activation. The ICI most commonly associated with DISR is Ipilimumab(2, 9, 10), which explains why DISR has mainly been described in melanoma patients(2). In a recent systematic review describing DISR related to ICI for advanced melanoma, 35 patients received CTLA-4 inhibitors, 45 PD-1 inhibitors and 21 BRAF/MEK inhibitors(10). The mean time from immunotherapy initiation to DISR onset was 7.1± 9 months. In clinical melanoma trials, DISR incidence is low, with 1.4% in a phase III trial of adjuvant pembrolizumab(11). A remarkably high rate of DISRs was however recently

reported in 10 out of 45 (22%) melanoma patients treated with ICIs (either nivolumab alone or in combination with ipilimumab)(12). The authors speculate that this might be due to the adjuvant clinical trial-setting, compared to the advanced setting in earlier trials. Another possible explanation might be that the combination of anti-CTLA-4 and anti-PD1 therapy in the adjuvant/ neoadjuvant setting increases the risk of DISR(12). The experience with ICI treatment in patients with preexisting sarcoidosis is limited. In a series of 32 patients with pre-existing sarcoidosis who received ICI, only one case with a 20-year remote history had a symptomatic exacerbation requiring systemic corticosteroids(13). Because ICIs are increasingly used, reports on DISRs in this drug category will probably expand in the near future.

TNFα antagonists

Anti-TNF-α drugs are commonly used in the treatment of rheumatoid arthritis, ankylosing spondylitis and psoriasis, and well established as third line sarcoidosis therapy(1). A systematic literature review on publications with DISRs related to anti-TNFα therapy was recently conducted, excluding cases with a previous sarcoidosis diagnosis(14). Over 100 DISRs were identified: 49% due to treatment with Etanercept, 25% received adalimumab and 20% of patients were treated with infliximab. Most patients were on anti-TNFα therapy for more than two years with a mean treatment duration of 25.6 months (range 1-132 months)(14). Other reviews found a mean treatment duration of 18-24 months(2, 15). Certolizumab(15) and golimumab(3) have also been reported to cause DISR, but reports are sparse and could reflect the use of TNFα blockers in clinical practice. The WHO pharmacovigilance database search resulted in 882 suspected cases of TNFα-antagonist-DISRs(3).

Interferon

Over a 100 cases describing DISR secondary to IFN therapy have been published in literature (2). This drug class is used for the treatment of several diseases, including multiple sclerosis, hepatitis C and malignancies(2). IFN-α is much more frequently responsible for DISR than IFN-β, with ten times more cases published(2). Amongst patients with DISR and HCV infection, 40% have IFN-α monotherapy and 60% have combined therapy with ribavirin, supporting a possible additional role of this agent(16). DISR appears during the first 6 months after start of therapy in 66%, between 6 and 12 months in 19%, and more than 12 months after in 15% of patients, sometimes even after completion of antiviral therapy(16). In 9 cases IFN reactivated pre-existing sarcoidosis after 2-15 months of therapy(16, 17), but IFNs have been used in several patients with pre-existing sarcoidosis without evidence of disease exacerbation(18).

Miscellaneous drugs: DISR or no DISR?

Less reported drug categories associated with DISR frequently target different parts of the immune system, such as anti-CD20 (Rituximab)(19), anti-CD25 (Daclizumab)(20), anti- IL6

(Tocilizumab)(21) and anti -L12/23 (ustekinumab)(22). In a cohort with DISR patients on treatment for melanoma with ICI or BRAF/MEK inhibitors, the majority of patients on BRAF/MEK inhibitors had a granulomatous skin lesion and no pulmonary or lymph node involvement(10). Noteworthy, only the minority of the reported patients were screened for pulmonary involvement. It is not possible to differentiate which patients only had a cutaneous granulomatous drug eruption or multisystem involvement. It is important to mention that several drugs are associated with a granulomatous reaction in a single organ such as the skin, without the multisystem involvement which defines a DISR. The differential diagnosis of a granulomatous cutaneous drug eruption is broad, including interstitial granulomatous drug reaction, drug induced accelerated rheumatoid nodulosis, granuloma annulare and DISR(23). If no multisystem disease can be demonstrated, it is questionable whether the drug eruption truly represent DISR. Drugs associated with granulomatous inflammation in a single organ, and therefore not classified as DISR, are Nitrofurantoin, Etretinate, Thalidomide and Quetiapine(2). A systemic granulomatous reaction after BCG instillation into the bladder is also not considered DISR, but a granulomatous response to the live attenuated strain of Mycobacterium Bovis(24, 25). Last, several drugs for the treatment of pulmonary hypertension were recently associated with DISR (3), which is hard to explain while taking the effects of the drug class into account. As pulmonary hypertension is a well-known complication of sarcoidosis(26), the association may be the result of cases in which pulmonary hypertension preceded the diagnosis of an unrecognized preexisting sarcoidosis(27).

Is DISR due to immune checkpoint inhibition a good sign?

Patients diagnosed with cancer can develop granulomas located in the tumour or draining lymph nodes and less commonly in distant organs. This regional sarcoidosis-like reaction signifies better outcomes, including reduced metastasis and increased survival (28-30). It has been postulated that this prognostic advantage reflects a robust immune response. Additionally, an association of irAEs and improved survival has been demonstrated in patients with advanced melanoma receiving adjuvant ICI(31, 32). Patients who experience ICI-DISR also seem to have a favourable or at least similar outcome compared to those without DISR(12, 33, 34). In a review on 26 patients with ICI-DISR, therapeutic response, remission, or stable disease of primary malignancy was reported in 71% of cases over a median follow-up of 11.5 months(34). In a clinical trial enrolling 45 melanoma patients receiving adjuvant ICI, the 10 patients who developed DISR showed no difference in terms of relapse rate compared to the others (20% vs 20% after a median follow-up of 16.4 and 15.3 months, respectively)(12).

Clinical presentation and diagnosis

DISR is a systemic granulomatous reaction that is indistinguishable from sarcoidosis in terms of clinical, biological, radiological and pathological presentation(2). Immunotherapy-DISR is typically associated with respiratory and skin involvement (**Figure 1**) (10). Dermatological symptoms may precede the discovery of pulmonary abnormalities. Conversely, clinically silent

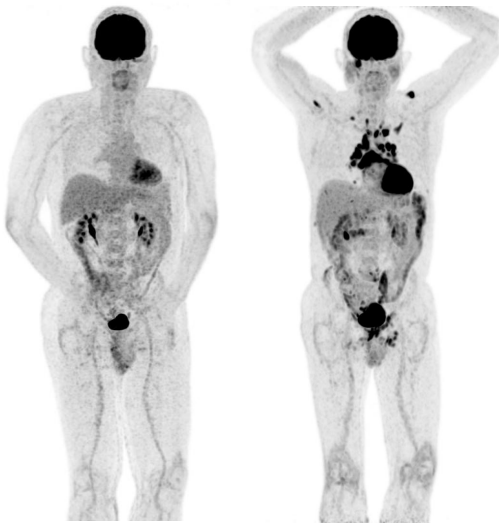

Figure 1: DISR related to immune checkpoint inhibition
FDG -PET CT before (left) and after 12 months of adjuvant ipilimumab (right) for melanoma, demonstrating high FDG uptake in several mediastinal, hilar- and right clavicular lymph nodes (right). Non-necrotizing granulomatous inflammation was demonstrated in multiple mediastinal lymph nodes and bone marrow aspirate. The diagnosis of DISR related to ipilimumab was made after exclusion of alternative causes of granulomatous inflammation.

intra-thoracic lymphadenopathy may be detected in 5% of patients treated with ICIs. Other clinical features include fever, extra-thoracic lymphadenopathy, uveitis, hypercalcemia, nervous system, hepato-splenic, muscle, and osteo-articular involvement(2, 10). The most common sites of cART-, TNFα-antagonist and IFN-related DISR are the respiratory system and the skin, while severe clinical manifestations are rare (5-7, 15, 16). Whatever the responsible drug, the radiological picture of DISR completely resembles sarcoidosis, with typical bilateral hilar lymphadenopathy and/or perilymphatic pulmonary micronodules, and 18F-FDG uptake in involved organs on PET scan(2). Serum angiotensin-converting enzyme levels can also be increased(2). The pathological pattern of DISR is the same as sarcoid-granulomas and the most common procedures to obtain pathological confirmation are skin biopsy and endobronchial ultrasound (EBUS) aspiration of mediastinal lymph nodes(2). DISRs can mimic infection or malignancy, which need to be thoroughly excluded before the diagnosis can be made. Granulomatous inflammation found in HIV patients, especially with lower CD4+T cell counts, should primarily raise suspicion of (myco-) bacterial and fungal infections. In HIV patients with suspected DISR, specific etiologies for granulomatous inflammation were found in all patients with a CD4+T-cell count of < 200 cells/mm3, with infection found in 80% of cases(5). In patients treated with immunotherapy, DISR can be hard to distinguish from metastasis or alternative manifestations of irAEs. Thus, suspected

DISR should lead to strict individualized assessment and rigorous approach. In general, a tissue specimen is imperative, and once granulomas have been confirmed, differential diagnoses have to be ruled out, including mycobacterial and fungal infections (**Figure 2**) (2).

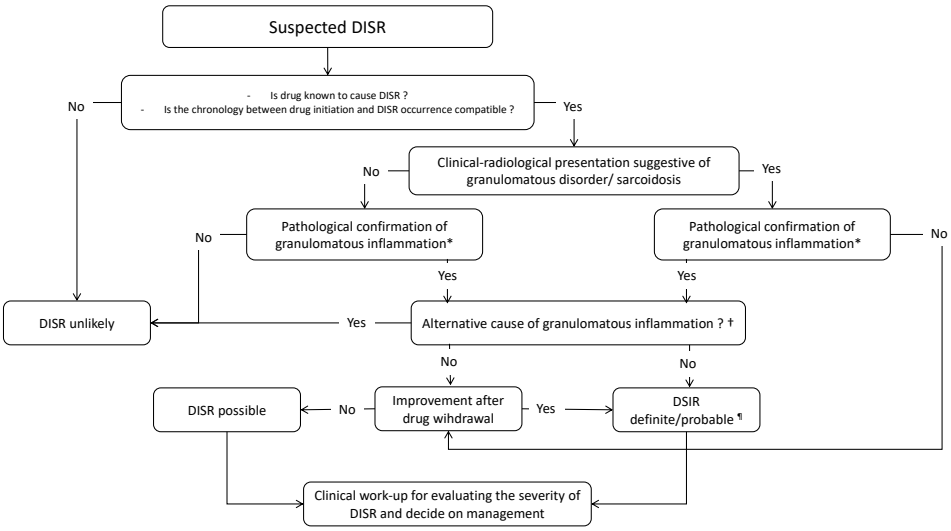

Figure 2: Proposed diagnostic approach of drug-induced sarcoid-like reaction

Abbreviations: ICI: immune checkpoint inhibitor, DISR: drug-induced sarcoid-like reaction,

* Biopsy of an organ or lymph node is necessary in order to rule out differential diagnoses, in particular cancer localization and other immune related adverse events in patients with ICI-DISR. In case of negative biopsy, it should be repeated and may be guided by ^{18}FDG-PET/CT imaging.

† Other causes of granulomatous inflammation, including mycobacterial and fungal infections, have to be excluded thoroughly, in particular in HIV patients with CD4 lymphocyte count < 200/mm3

¶ A definite diagnosis of DISR can only be based on the resolution of disease after drug discontinuation and/ or recurrence after rechallenge, but drug maintenance is feasible in selected cases (see Figure proposed management).

Treatment and outcome of DISR

The therapeutic approach of patients with DISR should be multi-disciplinary and based on three main questions:

1. is it necessary to interrupt the offending drug?
2. is it possible to switch to another agent of the same family?
3. is DISR treatment required?

Decisions must take into account the prognosis of the underlying disease, the severity of DISR presentation and treatment alternatives for the underlying disease (**Figure 3**). The majority of DISRs are characterized by a relatively indolent course with resolution in most cases, either spontaneously or after withdrawal of pharmacological triggers (2, 3).

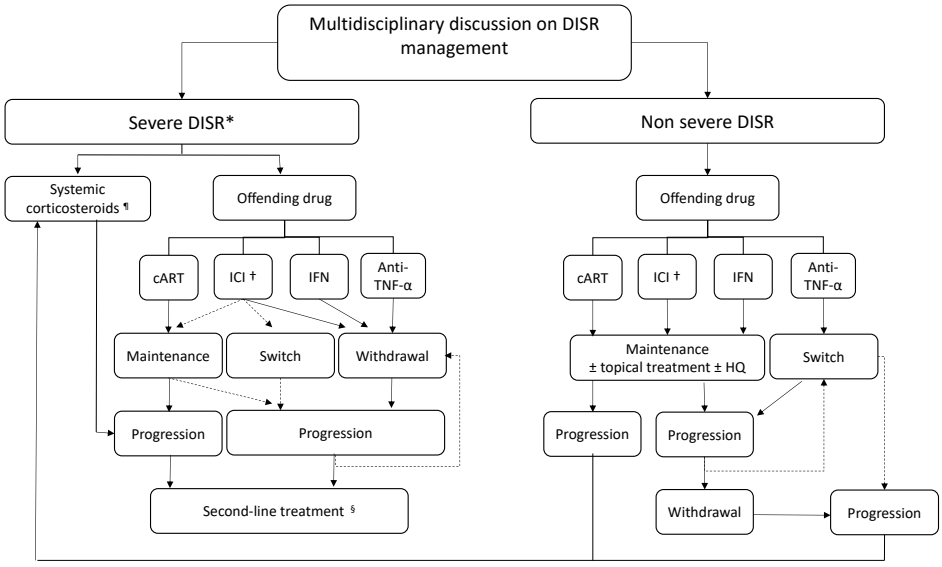

Figure 3: Proposed management of drug-induced sarcoid-like reaction

Abbreviations: cART: combination anti-retroviral therapy, ICI : immune checkpoint inhibitor, IFN : interferon ; TNF: tumor necrosis factor, HQ: hydroxychloroquine; DISR: drug-induced sarcoid-like reaction

* There is no validated definition of severe DISR.

† In patients with ICI-DISR, treatment of the underlying cancer should be prioritized in the large majority of cases. Moreover, DISR may reflect a beneficial effect of immunotherapy. The maintenance or switch of the drug should be favoured whenever possible, depending on the degree of severity of clinical manifestations.

¶ There is no validated protocol for systemic corticosteroids. In patients with ICI-DISR, systemic corticosteroids may have a negative impact on the effect of immunotherapy. If possible, they should be used at low doses and for short periods of time.

§ Progression of DISR during treatment with systemic corticosteroids is very unusual. There is no validated algorithm for second-line therapy and decisions could follow therapeutic guidelines for sarcoidosis. In patients with ICI-DISR, several authors have advocated the use of infliximab, as it may not reduce the effect of immunotherapy.

The prognosis of cART-DISR is good, with unfavorable evolution and poor response to systemic corticosteroids in only 7.8% (6). cART should not be discontinued when a DISR develops. Irrespective of cART, DISR in HIV patients and sarcoidosis in uninfected patients show no differences in terms of organ involvement, disease severity or use of systemic glucocorticoids(35). The evaluation of risk-benefit balance is critical for immunotherapy-DISR. New ICIs have revolutionised the field of oncology and changed the prognosis of patients. Moreover, ICI-DISR is generally a benign reaction and possible sign of a potent anti-tumor response(2). In the systematic review by Rubio Rivas (10), immunotherapy was discontinued in 50% of patients, continued in 37.2% and switched in 12.8%. Specific treatment was required in 58.8% of cases, including topical corticosteroids (12.9%), systemic corticosteroids (43.5%), methotrexate (2.4%),

infliximab (3.5%) and hydroxychloroquine (1.2%). After a mean follow-up of 8.3 ± 9.9 months, DISR was stable in 12% of cases, improved in 38.7% and resolved in 49.3%. Importantly, the decision made on immunotherapy seemed to have no impact on DSIR outcomes(10). Obviously, the treatment of malignancy should be the priority and unless the DISR is severe, immunotherapy should be continued. Because corticosteroids may influence the efficacy of ICI, conservative management is preferred if possible. Several authors have advocated the use of TNF-α-antagonists for treating irAEs(36, 37). Infliximab has been occasionally given for refractory DISR(38-40).

In most cases, the prognosis of TNF-α-antagonist-DISR is good and resolves spontaneously or under corticosteroids(15). Remission usually occurs within 12 months after drug cessation(41). Given these observations, TNF-α-antagonist-DISR appears to be easily manageable with discontinuation or switch of the drug, with or without systemic corticosteroids.

IFN-DISR usually regresses with interruption of the drug or even despite its continuation. This suggests that IFN may trigger DISR but has no significant role in its maintenance(2). In a review on 50 cases with IFN-α-DISR, therapy was discontinued in 60% of cases and continued or adjusted in 14%. Nearly 85% of patients improved or remitted, and outcomes were clearly related to discontinuation of antiviral therapy. Only 35% of patients required systemic corticosteroids(16). Antimalarians and infliximab have been helpful in several cases(16, 17, 42). If systemic corticosteroids or immunosuppressive treatment are necessary, liver function and HCV-RNA levels must be monitored carefully.

Unraveling sarcoidosis pathogenesis: can DISRs help us?

Drugs associated with DISR have important therapeutic effects, but may have unintended downstream effects on the immune system. Immune reconstitution during CD4+T cell increase is, possibly in combination with post-infectious antigen exposure, crucial for cART-DISR. This demonstrates CD4+T cells are key players in pathophysiology. Concerning ICI and DISR occurrence, several possible mechanisms have been postulated. First, ICI can lead to tumor destruction with increased exposure to neoantigens. Second, T cells are activated by inhibition of co-inhibitory signals(43). CTLA-4 is essential for immune tolerance and attenuates T-cell activation at a more proximal step in the immune response compared to PD-1 (43, 44) This may explain the difference in irAEs and DISR observed in patients treated with anti-CTLA-4 compared to anti-PD(L)-1(36). Third, one could hypothesize that in susceptible individuals, ICI alters a delicate balance of T-cell differentiation. Although sarcoidosis has historically been considered a T-helper (Th) 1 disease with cytokines such as IFN-γ, TNF-α, IL-2, IL-12, and IL-18 contributing to granuloma formation, recent data demonstrate Th17 and Th17.1 polarization and disturbed regulatory T cell (Treg) functions(45, 46). A significant decrease in CTLA4 expression on Th17 and Treg cells in lung draining lymph nodes was found in sarcoidosis patients(47). This may lead to what the authors called ‘double trouble’: enhanced Th17 priming and effector function,

while Treg mediated suppression of inflammation is impaired. Th17 polarization is also associated with some types of irAEs in ICI(48). The occurrence of a DISR related to PD-1 inhibitors seems more paradoxical because increased CD4+T cells with high PD1 expression were found in sarcoidosis bronchoalveolar lavage compared to resolved sarcoidosis(49). However, PD-1 blockade in cancer patients increases the production of Th1/Th17 cytokines in response to antigen(50). It is possible that ICI related Th1/Th17 polarization is part of DISR pathophysiology and resembles T-cell subset changes found in sarcoidosis(46).

TNF-α-antagonists can result in several inflammatory side effects including DISR, psoriasiform lesions and arthralgia(51). These inflammatory side effects of TNF-α blockade are incompletely understood, but several mechanisms have been proposed based on the biological properties of the cytokine. While TNF-α stimulates multiple signaling pathways, it inhibits IFN-α and plasmacytoid dendritic cell maturation. Imbalance in this cytokine pathway may increase opposing IFN-α production, alter DC maturation and increase antigen presentation(51). Single nucleotide polymorphisms (SNPs), including in the IL12B and IL23R gene regions, have been identified to increase the risk of paradoxal skin lesions following anti-TNF-α therapy for IBD(51). Interleukin-12 and 23 are important cytokines involved in Th1 and Th17 differentiation. Blocking TNF-α has been associated with increase in IFN-γ and TNF-α positive CD4+ T-cells in human peripheral blood (52) and enhanced Th17 cell function and decreased expansion of Treg in a mouse model(53). These findings support the hypothesis that TNF-α antagonists can alter cytokine balance, favoring Th1 and Th17 cell differentiation, which could be part of DISR pathophysiology in susceptible patients. The pathogenesis of Interferon induced DISR is, like other DISRs, not completely understood. Interferon- α is associated with increased cytokines that contribute to Th1 polarization, granuloma formation and maintenance(2).

To conclude, many aspects of the DISR pathophysiology are not completely understood and several possible mechanisms are postulated for different drug classes. These hypotheses are currently unproven and this complex pathophysiology continues to challenge researchers. Increased understanding of the mechanisms involved may eventually lead to novel therapeutic strategies in both DISR and sarcoidosis.

DISR or sarcoidoses: what's in a name?

It can currently only be speculated whether DISRs represent a separate granulomatous syndrome with distinctive pathogenesis or a "true" sarcoidosis. Because of the striking clinical and histopathological similarity between the two entities, the immunopathogenesis of DISR probably closely resembles sarcoidosis(2). The recent finding of T-cell sensitization to aluminium, beryllium, silica or zirconium in 28% of sarcoidosis patients, underscores the fact that "true" sarcoidosis probably has multiple identifiable triggers in addition to different clinical phenotypes(54). Similarly, DISR associated drug classes may act in different ways on the immune system, varying from cytokine imbalance to altered polarization and T-cell activation, eventually

resulting in systemic granulomatous eruption. The field may increasingly argue for "true" sarcoidosis to include several trigger related sarcoidoses, including different types of drug related granulomatous reactions. DISR or sarcoidoses: what's in a name?

CONCLUSION

Recent publications about DISR provide an expanding list of associated drug categories and increase our knowledge on diagnosis and treatment options. Understanding DISR pathophysiology may lead to novel therapeutic strategies in sarcoidosis.

Key points

- *Drug-induced sarcoidosis-like reaction* (DISR) occurs in temporal relationship with offending drugs and can improve or resolve if the drug is discontinued
- Recent publications have expanded the list of drugs associated with DISR, mostly categorized as antiretroviral therapy, TNF-α antagonist, interferon therapy and immune checkpoint inhibitors.
- DISR treatment is not always necessary and the benefits of continuing the offending drug should be weighed against clinical symptoms and organ dysfunction.
- Increased understanding how several drug classes are able to cause DISR contributes to our understanding of sarcoidosis pathophysiology and might add to the development of new disease models and treatment.

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CHAPTER 6

(Data not visible yet due to embargo)

Decreased circulating CTLA-4^{+} T cell subsets identify sarcoidosis patients with favorable lung function response during abatacept treatment

Jelle R. Miedema, Lieke J. de Jong, Bjorn Christian Frye, Gernot Zissel, Ingrid M. Bergen, Mirjam Kool, Marlies S. Wijsenbeek, Rudi W. Hendriks, Joachim Muller Quernheim and Odilia B.J. Corneth

Submission awaiting publication of ABASARC clinical trial

CHAPTER 7

Expression of CTLA-4 in IL-17A-producing T cells and regulatory T cells is critical for immune homeostasis: Implications for sarcoidosis pathogenesis

Lieke J. de Jong, **Jelle R. Miedema**, Ingrid M. Bergen, Jennifer A.C. van Hulst, Menno van Nimwegen, Anne-Marie Mus, Alex Kleinjan, Rudi W. Hendriks, Odilia B.J. Corneth

Manuscript in preparation

ABSTRACT

Objective

Sarcoidosis is a granulomatous disease of unknown origin that primarily affects the lung. We previously found decreased expression of the co-inhibitory marker cytotoxic T-lymphocyte antigen 4 (CTLA-4) in Th17 cells and regulatory T cells (Tregs) in lung-draining mediastinal lymph nodes (medLN) of patients. However, the effects of low CTLA-4 expression in Th17 cells or Tregs are unknown.

Methods

Using the Cre-LoxP system, we generated mice in which the *Ctla4* gene was specifically targeted in IL-17A-producing cells on one (CTLA-4$^{IL17A\text{-}Hz}$) or both alleles (CTLA-4$^{IL17A\text{-}KO}$), as well as mice haplo-insufficient for CTLA-4 exclusively in Tregs (CTLA-4$^{FoxP3\text{-}Hz}$). The immune status of the mice was evaluated by multicolor flow cytometry at 8 and 30 weeks of age and during an immune response to collagen immunization.

Results

We did not find evidence for immune dysregulation in CTLA-4$^{IL17A\text{-}Hz}$ mice. In contrast, spleen and mesenteric LN of CTLA-4$^{IL17A\text{-}KO}$ mice showed a significant increase in the proportions of CCR6^{+} T cells. Moreover, increased fractions of cells expressing the ICOS activation marker were found within total CD4^{+} memory T (Tm) cells, CCR6^{+} T cells and Tregs. Signs of substantial B cell activation were detected. Nevertheless, these immune alterations did not lead to any histopathological changes in tissues from CTLA-4$^{IL17A\text{-}KO}$ mice. Upon collagen immunization splenic CD4^{+} T cells exhibited increased ICOS^{+} cell proportions and cytokine production, but this was not associated with enhanced collagen-induced arthritis symptoms in CTLA-4$^{IL17A\text{-}KO}$ mice. In CTLA-4$^{FoxP3\text{-}Hz}$ mice, Tregs were expanded and, interestingly, the fractions of ICOS^{+} cells were increased in the Tm, CCR6^{+} T cell and Treg populations.

Conclusions

This study shows that expression levels of CTLA-4 in Tregs are critical for the maintenance of immune homeostasis. In IL-17A-producing T cells CTLA-4 expression levels appears less critical and only a complete deficiency induces immune dysregulation. We observed a reciprocal influence between CTLA-4 expression levels on Th17 cells and Tregs. This would be consistent with a model in which both Tregs and Th17-like cell subsets with reduced CTLA-4 expression in sarcoidosis patients may support increased T cell activation and inflammation.

INTRODUCTION

Sarcoidosis is a granulomatous multi-organ disease with variable disease course. The majority of patients demonstrate spontaneous resolution within five years, while approximately 25% will have non-resolving or progressive disease. Granulomas, the histopathological hallmark of sarcoidosis, are most often present in the lungs and mediastinal lymph nodes (MedLN), but can also affect various organs such as the skin, eyes, liver, heart and nervous system (1). Sarcoid granulomas are well-formed non-caseating granulomas composed of epithelioid- and multinucleated-giant cells surrounded by a small rim of lymphocytes, especially $CD4^+$ T helper (Th) cells (2, 3). The pathogenesis of sarcoidosis is only partially unraveled. It is believed that sarcoidosis is initiated by an unknown antigen in a genetic susceptible individual (4). Murine models to unravel the immunopathogenesis and test potential therapy in the context of chronic disease are scarce (5). New disease models that recapitulate clinical features of the disease will help discover the mechanisms involved in chronic disease and development of treatment resistance are highly warranted in the field.

There is substantial evidence that activated Th cells play a central role in the development and progression of the disease (6-8). In sarcoidosis patients, a significant increase in the proportions of interferon- γ (IFN-γ) and interleukin-17 (IL-17) double-producing Th cells was found in bronchoalveolar lavage (BAL) fluid (6, 7, 9). Accordingly, Th17.1 cells (defined as Th cells expressing the CCR6 chemokine receptor and producing IFN-γ or both IL-17 and IFN-γ) were increased in BAL fluid and MedLN of patients, compared to controls (6, 7). Interestingly, the proportions of Th17.1 cells observed in the BAL fluid of individuals who eventually developed chronic disease was much higher than in patients who showed spontaneous resolution (7). Th17.1 cells are highly plastic and presumed to originate from Th17 cells (8, 10). They are found in the context of many autoimmune diseases and are known for their pathogenic potential (7, 8, 10). However, the precise origin of the exaggerated Th17-Th17.1 cell response and its contribution to the pathogenesis of sarcoidosis are currently unknown.

Th cell effector functions are tightly regulated by regulatory T cells (Tregs), which are critical for dampening T cell immune responses. Circulating Tregs from patients with sarcoidosis exhibit reduced immunosuppressive capacity and impaired survival (11-13). We have shown previously that both Th17 and Treg cells in BAL fluid and MedLN of sarcoidosis patients have decreased expression of Cytotoxic T-lymphocyte associate protein 4 (CTLA-4) (14). CTLA-4 is an important co-inhibitory molecule that is highly and constitutively expressed on Tregs and to a lesser extent on Th17 cells. CTLA-4 functions as an immune checkpoint and competes with the co-stimulatory receptor CD28 on T cells for interaction with their shared ligands CD80/86 on antigen presenting cells (APCs). This competition favors CTLA-4 due to its higher affinity, resulting in T cell suppression upon binding (15). In sarcoidosis, decreased CTLA-4 expression likely contributes to ongoing granulomatous inflammation by enhancing Th17 activation and concomitantly impairing Treg function (14).

In addition to its co-inhibitory effect, CTLA-4 is also known to play an important role in Th subset differentiation. Studies have shown that blocking the CTLA-4:CD80/86 interaction enhances Th17 cell differentiation (16). Interestingly, cancer patients receiving immunotherapy with CTLA-4 blockade have increased proportions of circulating IL-17A^{+} Th cells (17). Moreover, CTLA-4 blockade can trigger a systemic granulomatous disease indistinguishable from sarcoidosis (18).Together, these data suggest a pathogenic, perhaps even central role for aberrantly activated Th17 cells with low CTLA-4 expression in sarcoidosis. However, the precise function of CTLA-4 on Th17 cells and their potential contribution to granuloma formation remains unknown.

Therefore, in the current study we examined the immune system of mice with CTLA-4 haplo-insufficiency (expression from only one allele), as well as complete absence of CTLA-4 specifically on IL-17A-producing cells. Additionally, the impact of Treg-specific CTLA-4 downregulation on the in vivo immune phenotype was studied in mice that were haplo-insufficient for CTLA-4 on Tregs. To this end, we made use of the Cre-LoxP system and crossed *Ctla4*$^{fl/fl}$ mice with IL17A-cre transgenic mice (targeting IL-17A producing cells) and FoxP3-cre transgenic mice (targeting Tregs). Hereby, we hypothesize that these mice may serve as animal models to study disease pathogenesis and treatment interventions in sarcoidosis upon induction of granulomas with an antigenic trigger.

METHODS

Mice

CTLA-4$^{fl/fl}$ mice (available through collaboration with prof. S. Sakaguchi, Osaka University, Japan) were crossed with either IL-17A inducible cre (icre) mice (available at Jax mice; reference 016879) or transgenic (tg) FoxP3-EGFB/icre mice to generate CTLA-4$^{IL17A\text{-}Hz}$, CTLA-4$^{IL17A\text{-}KO}$ and CTLA-4$^{FoxP3\text{-}Hz}$ mice respectively. Littermates expressing Cre and wild-type (WT) CTLA-4 were used as WT control mice. Mice were genotyped by Polymerase Chain Reaction (PCR) of genomic DNA isolated from mouse toes.

All mice were bred and maintained under specific pathogen-free (SPF) conditions in the experimental animal facility (EDC) of the Erasmus Medical Centre (EMC). Experimental protocols were reviewed and approved by the EMC Committee for Animal Experiments (DEC).
Mice were sacrificed at 8 and 30 weeks of age, and organs were harvested for flow cytometric analysis and histology.

Collagen-induced arthritis

Collagen-induced arthritis (CIA) was conducted in CTLA-4$^{IL17A\text{-}KO}$, CTLA-4$^{IL17A\text{-}Hz}$ and WT mice through immunization with chicken collagen type II (CII). CII was emulsified in an equal volume

of complete Freund's adjuvant (CFA) containing 1mg/mL *Mycobacterium Tuberculosis* (strain H37Ra) to obtain a CII/CFA emulsion. Mice aged 10-15 weeks were immunized intradermally at the base of the tail with 100 uL CII/CFA emulsion. On day 21, a booster injection of 100 uL CII/CFA emulsion was given subcutaneously between the shoulder blades.

Arthritis development was monitored three days a week beginning on day 13. Mice were scored positive for arthritis if significant macroscopic changes in erythema and/or edema were observed in any of the four paws. The maximum score for arthritis is 8, but mice scoring 6 or higher are sacrificed for ethical reasons. All mice were sacrificed on day 52 and popliteal LN (pLN) and spleen were collected for flow cytometric analysis.

Flow Cytometry procedures

Single cell suspensions of mouse tissues in RPMI 1640 medium supplemented with 5% FCS (RPMI-5% FCS) were prepared by mechanical disruption using a Falcon® 100 µm Cell Strainer (Corning, NY, USA). Cells were stained for extracellular markers for 1 hour at 4°C followed by live/dead marker staining for 20 min at 4°C. Cells were then were fixed and permeabilized with BD CytoFix/CytoPerm (BD Biosciences) for 20 min at 4°C before staining for intracellular markers for 1 hour at 4°C. Antibodies used are listed in Supplementary table 1.

For intracellular cytokine staining, single cell suspensions of mouse tissue were stimulated with phorbol myristate acetate (PMA) (0.05 µg/mL) and ionomycin (0.5 µg/mL) in the presence of GolgiStop (BD Biosciences) for 4 hours at 37°C. After stimulation, a similar staining procedure was performed for extracellular and intracellular staining, except that stimulated cells were fixed with 2% paraformaldehyde (PFA) for 20 min at room temperature and permeabilized with 0.5% saponin buffer.

All samples were measured in phosphate-buffered saline (PBS) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 1% bovine serum albumin (BSA) on the BD FACSymphony™ A5 Cell Analyzer (BD Biosciences, San Jose, CA, USA), and analyzed using Flowjo v10.

Hemotoxylin and Eosin staining

Mouse lungs were carefully inflated with O.C.T. diluted in PBS until they are fully expanded. Lungs were then harvested and snap frozen in liquid nitrogen. A proportion of the spleen was used for histology and embedded in Tissue-Tek II O.C.T. compound (Miles Inc.) and immediately frozen. Tissues were stored at -80°C until further processing. Biopsy slides were generated using a cryostat microtome.

For hemotoxylin and eosin (HE) staining, slides were first fixed in 4% formalin and placed in acetone for 10 min. Slides were then stained by immersion in Gills' Hematoxylin for 30 second, washed with tap water, immersed in 2% eosin B acetified with 150 µL glacial acetic acid for 1 min, and washed again with tap water. For dehydration, the slides were first rinsed in 70% ethanol,

followed by 95% ethanol, and the slides were placed in 100% ethanol for 2 min. Finally, the slides were placed in xylene for 2 min and mounted with Entallan. Histology of mouse tissue slides was visualized using confocal microscopy.

HEp-2 Assessment of Autoreactive Antibodies

25 µL serum (1:100 in PBS) was added to Hep2 slides and incubated for 1 hour at room temperature (RT) in the dark. Slides were carefully washed with PBS and stained with 25 µL of Cy3- and AF488-labeled antibodies against IgG and IgM, respectively (1:200) (Invitrogen). Slides were covered to keep them in the dark and kept at RT for 20 min. Slides were gently washed with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI) (1:5000) for 5 min in the dark at RT. Slides were washed again with PBS, mounted with VECTASHIELD HardSet Antifade Mounting Medium and coverslipped. Slides were dried for 15 min and analyzed by confocal microscopy.

Statistics

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc; San Diego, CA, USA). Statistical differences between two or more groups were calculated using ordinary one-way ANOVA, and an unpaired t-test was performed to test for significant differences between two groups. *p* values <0.05 were considered significant.

RESULTS

IL-17A-Cre-mediated CTLA-4 deletion results in a decrease of CTLA-4$^+$ cells in IL-17A$^+$ and CCR6$^+$ Th cell fractions

To verify the introduced genetic alteration, a flow cytometric analysis was conducted on lymphocytes derived from the spleen and mesenteric lymph nodes (mesLN) of CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice aged 8 and 30 weeks to assess CTLA-4 expression on IL-17A-expressing cells. To identify IL-17A-producing cells, cell fractions were stimulated with PMA/ionomycine for 4 hrs. We observed decreased proportions of CTLA-4$^+$ cells within the population of IL-17A$^+$ CD4$^+$ T cells in the spleen and mesLN of CTLA-4$^{IL17A\text{-}KO}$ mice compared to WT mice (**Figure 1A-B**). The decrease reached significance in 30-week-old mice only, while in 8-week-old CTLA-4 was expressed only in a small fraction of IL17A-producing cells. The presence of the *Il17A-Cre* allele did not appear to affect IL-17A expression, because the frequencies of IL-17A$^+$ cells within the CD4$^+$ T cell population did not vary across the three mouse groups at 8 or 30 weeks of age (**Figure 1C**). No differences were observed in the proportion of CTLA-4$^+$ cells in the IL-17A$^+$ CD4$^+$ T cell population in the haplo-insufficient CTLA-4$^{IL17A\text{-}Hz}$ mice.

Considering that IL-17A is the signature cytokine produced by Th17 cells (19), the genetic alteration likely affects the expression of CTLA-4 on Th17-lineage cells, which are characterized

by the expression of the chemokine receptor CCR6. Therefore, we analyzed the proportions of CTLA-4$^+$ cells within the CCR6$^+$ CD4$^+$ T cell subset. The proportions of CTLA-4$^+$ cells within the CCR6$^+$ CD4$^+$ T cell fraction were significantly decreased in the spleen and mesLN from 30-week-old CTLA-4$^{IL17A\text{-}KO}$ mice compared to WT and CTLA-4$^{IL17A\text{-}Hz}$ mice (**Figure 1D-E**). Again, we found no such differences in CTLA-4$^{IL17A\text{-}Hz}$ mice.

In addition to the naïve mice, we next analyzed the activated IL-17A$^+$ CD4$^+$ T cells present in the spleens of 21-week-old WT, CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice that were subjected to an IL-17-dependent collagen-induced arthritis immune response. The experimental set-up is shown in **Suppl Figure 1A**. Here, we could confirm a reduction of CTLA-4$^+$ cells in the splenic IL-17A$^+$ and the CCR6$^+$ CD4$^+$ T cell population of CTLA-4$^{IL17A\text{-}KO}$ mice, compared to WT and CTLA-4$^{IL17A\text{-}Hz}$ mice (shown in **Figure 1F** and **Figure 1G,** respectively).

In addition to CD4$^+$ Th cells, also CD8$^+$ T cells and B cells may produce IL-17A. However, we found that the proportions of IL-17A$^+$ cells in these two cell types were very low in all three genotypes (<1%; **Suppl Figure 1B,C,E,F).** These few IL-17A$^+$ expressed very low levels of CTLA-4, without significant differences across the genotypes (**Suppl Figure 1D,G**).

Conclusively, this data confirms that the genetic modification selectively targeted the expression of CTLA-4 on IL-17A-expressing cells, in both naive and collagen-immunized CTLA-4$^{IL17A\text{-}KO}$ mice. Given the expression profiles of IL-17A and CTLA-4, targeting of the *Ctla4* gene essentially affected the Th17 lineage and not CD8$^+$ T cells or B cells. Reduced CTLA-4 expression was only observed in CTLA-4$^{IL17A\ KO}$ mice and not in CTLA-4$^{IL17A\text{-}Hz}$ mice, indicating that a single intact *Ctla4* allele is sufficient to reach wild-type CTLA-4 protein expression levels.

CTLA-4$^{IL17A\text{-}KO}$ mice display altered CD4$^+$ T cell activation characterized by increased ICOS$^+$ cells

Having established that the genetic modification specifically targets IL-17A-expressing cells, our next objective was to elucidate the immune phenotype of 8- and 30-week-old CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice by flow cytometry (**Figure 2,** for gating strategy: see **Suppl Figure 2A-C**). We observed no differences in the absolute counts of CD3$^+$ and CD4$^+$ T cells in the spleens of the three mouse groups (**Supplementary Figure 2D)**.

First, we focused on CCR6$^+$ CD4$^+$ T cells, because they were predominantly affected by decreased CTLA-4 expression. At 30 weeks of age, the absolute numbers of these cells were significantly increased in the spleens of CTLA-4$^{IL17A\text{-}KO}$ mice, compared to WT and CTLA-4$^{IL17A\text{-}Hz}$ mice (**Figure 2B**). Within this splenic T cell population, the proportions of ICOS$^+$ cells and cycling Ki67$^+$ cells were significantly higher – but the proportions of CD25$^+$ cells were lower - in 8- and 30-week-old CTLA-4$^{IL17A\text{-}KO}$ mice than in WT and CTLA-4$^{IL17A\text{-}Hz}$ mice.

Next, we investigated CD44$^-$ naive T (Tn) cells, CD44$^+$ memory T (Tm) cells and FoxP3 Treg. The numbers of splenic Tn, Tm and Treg cells remained largely consistent across the groups (shown in **Figure 3C-E**). Analysis of their activation status showed increased proportions of ICOS$^+$ Tn, Tm and Treg cells of CTLA-4$^{IL17A\text{-}KO}$ mice, compared to WT and CTLA-4$^{IL17A\text{-}Hz}$ mice at both 8 and

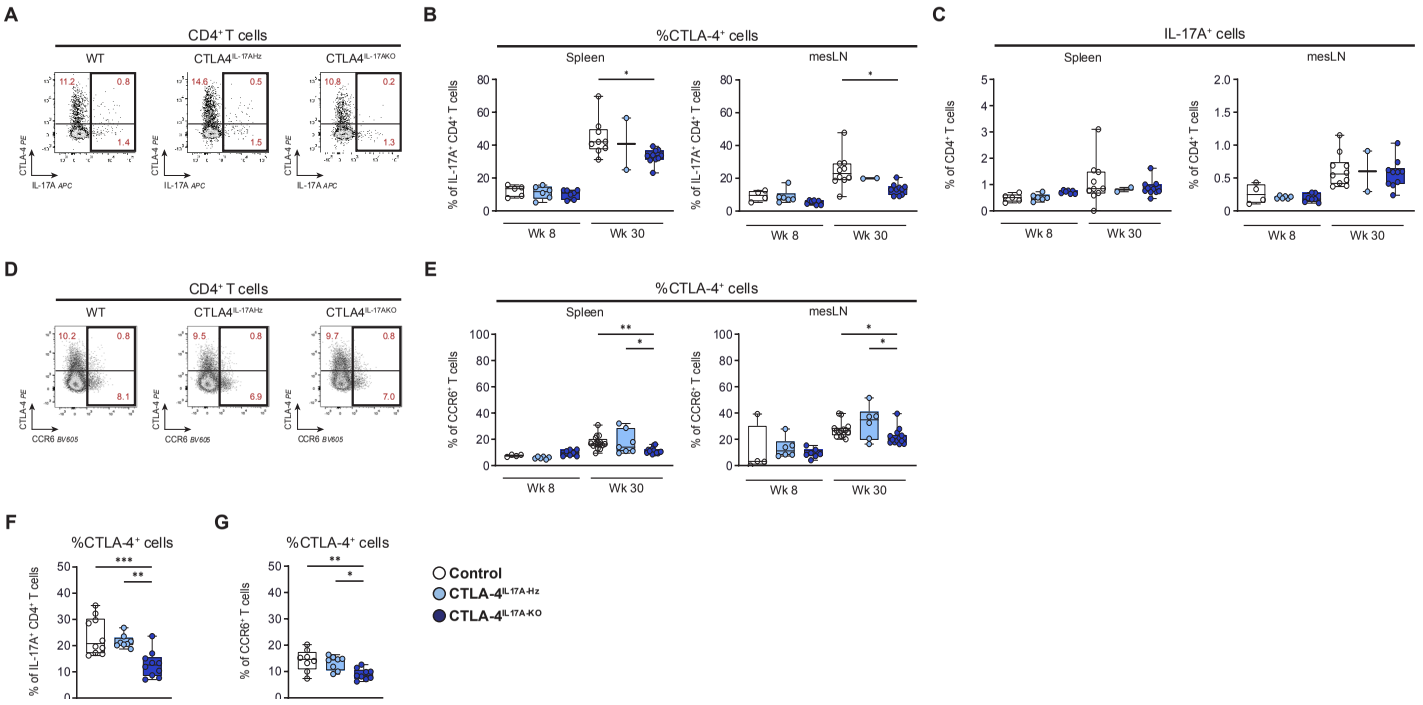

Figure 1: CTLA-4^{+} cells within the IL-17A^{+} CD4^{+} and CCR6^{+} CD4^{+} T cell subsets in CTLA-4$^{IL\text{-}17A\text{-}KO}$ mice

(A) Representative flow cytometry dot plots of CTLA-4^{+} IL-17A^{+} CD4^{+} T cells in the spleen of 30-week-old WT, CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice. **(B)** Proportions of CTLA-4^{+} IL-17A^{+} CD4^{+} T cells, and **(C)** IL-17A^{+} CD4^{+} T cells. **(D)** Representative flow cytometry dot plots of CTLA-4^{+} CCR6^{+} T cells, and **(E)** proportions of CTLA-4^{+} CCR6^{+} T cells in the spleen and mesLN of 8- and 30-week-old WT, CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice. **(F)** CTLA-4^{+} IL-17A^{+} T cells, and **(G)** CTLA-4^{+} CCR6^{+} T cells in the spleen of CII/CFA-immunized WT, CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice. White dots represent WT mice, light blue dots represent CTLA-4$^{IL17A\text{-}Hz}$ mice and dark blue dots represent CTLA-4$^{IL17A\text{-}KO}$ mice. Intracellular cytokine production was detected after 4 hours of PMA/ionomycin stimulation of cells from the spleen and mesLN. All data were measured by flow cytometry. Statistical analysis was performed using ANOVA. *p<0.05, **p<0.01, and ***p<0.001.

30 weeks of age (**Figure 3C-E**). The proportions of CTLA-4^{+}, Ki67^{+} and CD25^{+} Tn cells, Tm and Tregs in the spleen were comparable between CTLA-4$^{IL17A\text{-}KO}$ and WT mice at 8 and 30 weeks of age, except that the frequencies of CTLA-4^{+} Tregs in 8-week-old mice were reduced in the CTLA-4$^{IL17A\text{-}KO}$ mice (**Figure 2C-E**). Furthermore, Ki67^{+} and CD25^{+} Tn and Tm cells were decreased and increased, respectively, in 30-week-old CTLA-4$^{IL17A\text{-}HZ}$ compared to CTLA-4$^{IL17A\text{-}KO}$ mice.

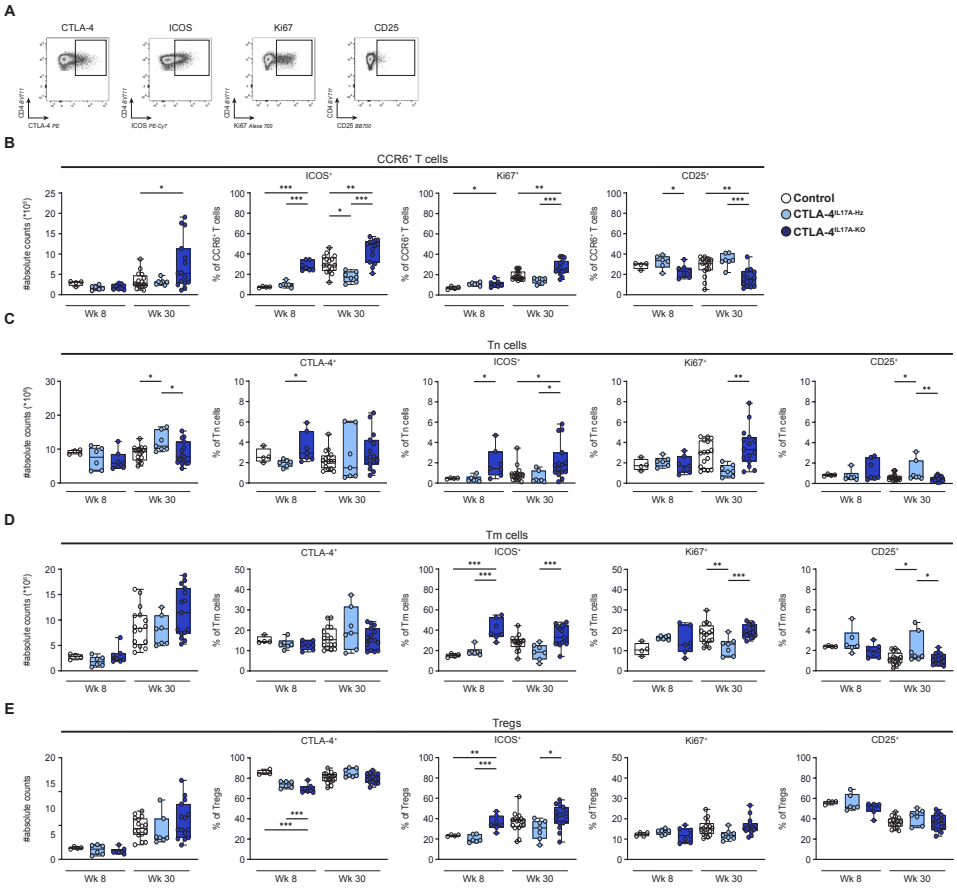

Figure 2: Splenic CD4^{+} T cell proportions and activation in 8- and 30-week-old CTLA-4$^{IL\text{-}17A\text{-}KO}$ mice
(A) Representative flow cytometry dot plots of CTLA-4, ICOS, Ki67 and CD25 expression on CD4^{+} Tm cells. **(B)** The absolute number of CCR6^{+} T cells and the percentage of CCR6^{+} T cells positive for ICOS, Ki67 and CD25 in 8- and 30-week-old WT, CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice. Absolute cell number and the percentage of cells positive for CTLA-4, ICOS, Ki67 and CD25 within the subset of **(C)** Tn, **(D)** Tm, and **(E)** and Treg. White dots represent WT mice, light blue dots represent CTLA-4$^{IL17A\text{-}Hz}$ mice and dark blue dots represent CTLA-4$^{IL17A\text{-}KO}$ mice. All data were measured by flow cytometry. Statistical analysis was performed using ANOVA. *p<0.05, **p<0.01, and ***p<0.001.

Parallel results were obtained for the MesLN: also the fractions of ICOS$^+$ cells in the Tn, Tm and Treg populations were increased (**Supplementary Figure 3B-D**). However, reduced frequencies of CTLA-4$^+$ Tregs were found in MesLN of 30-week-old, but not 8-week-old, CTLA-4$^{IL17A\text{-}KO}$ mice. Other exceptions were increases in the proportions of Ki67$^+$ Tm cells at 8 weeks of age, and the proportions of CD25$^+$ Tm cells at 8 and 30 weeks of age in CTLA-4$^{IL17A\text{-}KO}$ mice. Taken together, IL-17A-Cre-mediated CTLA-4 deletion primarily affected the activation status of CCR6$^+$ T cells, as evidenced by significantly increased proportions of ICOS$^+$ CCR6$^+$ T cells in CTLA-4$^{IL17A\text{-}KO}$ mice. Such an increase was not found in CTLA-4$^{IL17A\text{-}Hz}$ mice. Remarkably, also secondary effects were observed: proportions of ICOS$^+$ Tn, Tm and Tregs cells were increased, and the proportions of CTLA-4$^+$ Tregs were reduced in CTLA-4$^{IL17A\text{-}KO}$ mice. It remains unclear whether this would reflect a reduced inhibitory capacity of in CTLA-4$^{IL17A\text{-}KO}$ Tregs, given their increased ICOS expression.

Spleens of CTLA-4$^{IL17A\text{-}KO}$ mice display enhanced B cell activation and increased numbers of germinal center B cells

Because the altered activation status of T cells in CTLA-4$^{IL17A\text{-}KO}$ mice may engage B cells, we next investigated their phenotype in the spleen by flow cytometry (**Figure 3;** for gating strategy: see **Suppl Figure 2)**. The total number of B cells in the spleen were similar in WT, CTLA-4$^{IL17A\text{-}HZ}$ and CTLA-4$^{IL17A\text{-}KO}$ mice at 8 and 30 weeks of age.

Strikingly, 8-week-old CTLA-4$^{IL17A\text{-}KO}$ mice exhibited higher proportions of B cells expressing the activation marker CD86, compared to CTLA-4$^{IL17A\text{-}Hz}$ mice (at 8 weeks of age) or to WT mice (at 30 weeks) (**Figure 3A-B**). Further examination of the B cell compartment revealed increased absolute numbers of germinal center (GC) B cells (defined as IgD$^-$CD95$^+$B220$^+$CD19$^+$) in the spleens of 30-week-old CTLA-4$^{IL17A\text{-}KO}$ mice. The absolute numbers of memory B (Bm) cells (defined as PDL-2$^+$CD86$^+$B220$^+$CD19$^+$) and plasma cells (defined as CD128$^+$CD19$^{+/-}$) were unaltered. CD86 expression was increased on GC and Bm cells, but not on plasma cells in 30-week-old CTLA-4$^{IL17A\text{-}KO}$ mice (**Figure 3C-E**). Spleens of CTLA-4$^{IL17A\text{-}KO}$ mice displayed significantly increased proportions of Ki67$^+$ Bm cells at 30-weeks of age, but other B cell populations did not differ in Ki67$^+$ expression between the three groups of mice (**Suppl Figure 4A-B**). Absolute numbers of splenic follicular T helper (Tfh) cells (defined as PD1^{hi}CXCR5$^+$CD4$^+$) were comparable between the three groups, although increased proportions of ICOS$^+$ cells were present in the Tfh cell population of 8-week-old CTLA-4$^{IL17A\text{-}KO}$ mice (**Suppl Figure 4C**).

To investigate whether the activated phenotype of T and B cells in CTLA-4$^{IL17A\text{-}KO}$ mice was associated with autoantibody formation, we measured anti-nuclear antibodies in the serum from 30-week-old WT, CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice. No significant differences in the amount of total IgG anti-nuclear antibodies were found between mice (**Figure 3F**). Additionally, histological analysis of the lungs of 30-week-old mice revealed no signs of spontaneous inflammation, including granuloma formation, nor other structural changes in the lungs of CTLA-4$^{IL17A\text{-}KO}$ mice compared to WT mice (**Figure 3G**).

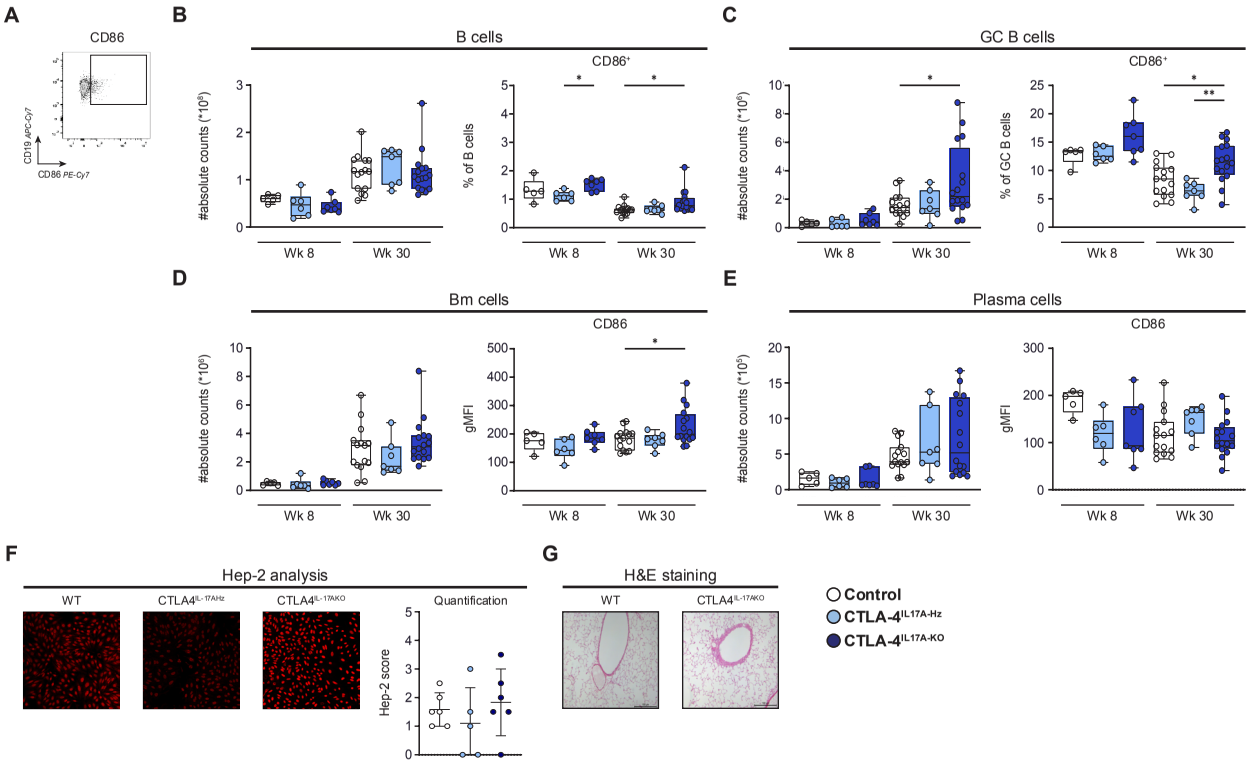

Figure 3: B cell proportions and activation in the spleen of 8- and 30-week-old CTLA-4$^{IL\text{-}17A\text{-}KO}$ mice

(A) Representative flow cytometry plot of CD86 expression on B cells. The absolute number of cells and the proportion of cells positive for CD86 within the subset of B cells **(B),** GC B cells **(C)**, Bm cells **(D)**, and plasma cells **(E)** in the spleen of 8- and 30-week-old WT, CTLA-4$^{IL17A\text{-}Hz}$, and CTLA-4$^{IL17A\text{-}KO}$ mice. **(F)** HEp-2 analysis of total IgG in the serum of 30-week-old WT, CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice. 0; no staining or similar to negative control, 1; weak staining, 2; strong staining, 3; overexposed. **(G)** Hematoxylin and eosin (H&E) staining of lung sections from 24-week-old WT and CTLA-4$^{IL17A\text{-}KO}$ mice. White dots represent WT mice, light blue dots represent CTLA-4$^{IL17A\text{-}Hz}$ mice and dark blue dots represent CTLA-4$^{IL17A\text{-}KO}$ mice. All data were measured by flow cytometry. Statistical analysis was performed using ANOVA. *p<0.05, **p<0.01, and ***p<0.001.

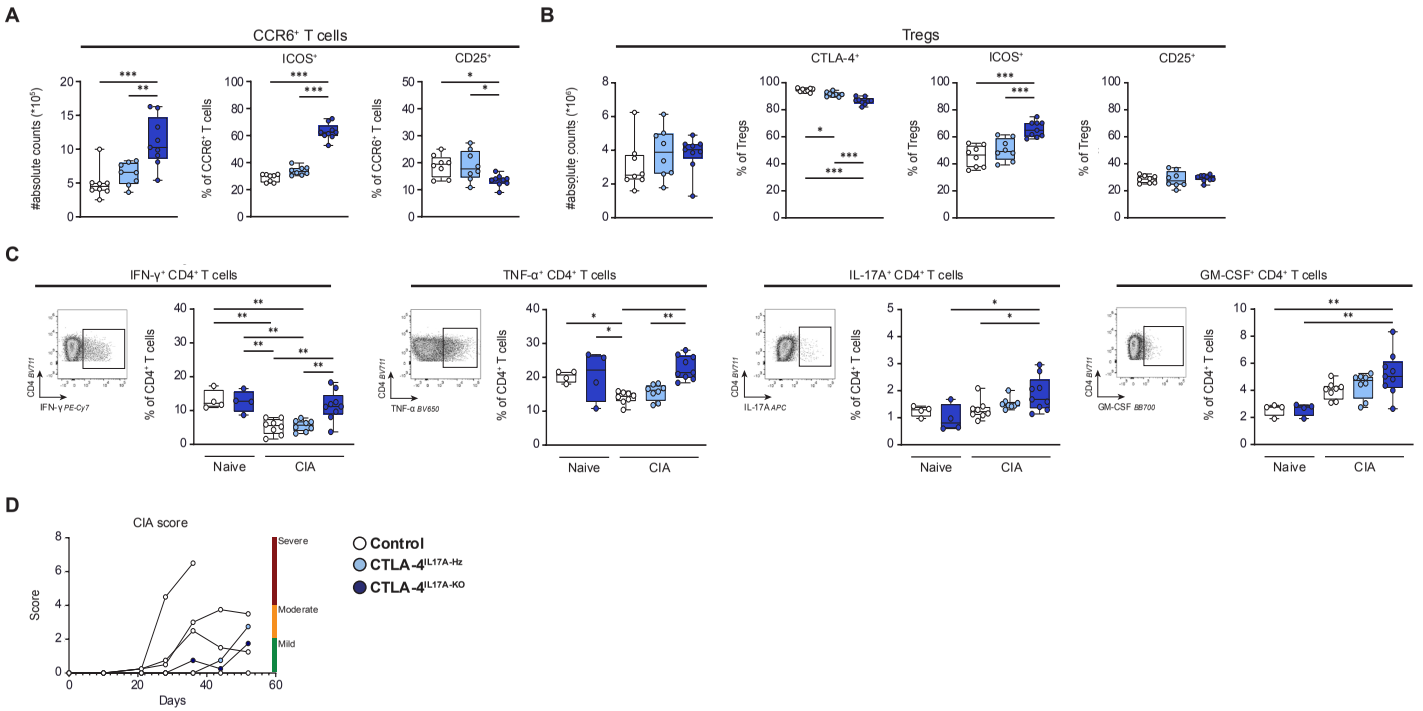

Figure 4: T cell phenotype and cytokine expression in naive and CII/CFA-immunized CTLA-4$^{IL\text{-}17A\text{-}KO}$ mice
(A) The absolute number of CCR6$^+$ T cells and the proportion of CCR6$^+$ T cells positive for ICOS and CD25 in CII/CFA-immunized WT, CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice. **(B)** The absolute number of Tregs and the percentage of Tregs positive for CTLA-4, ICOS and CD25. **(C)** Representative flow cytometry plots of the expression and the proportion of IFN-γ$^+$, TNF-α$^+$, IL-17A$^+$ and GM-CSF$^+$ CD4$^+$ T cells in naïve and CII/CFA-immunized mice. **(D)** CIA score. White dots represent WT mice, light blue dots represent CTLA-4$^{IL17A\text{-}Hz}$ mice and dark blue dots represent CTLA-4$^{IL17A\text{-}KO}$ mice. Intracellular cytokine production was detected after 4 hours of PMA/ionomycin stimulation of cells from the spleen and mesLN. All data were measured by flow cytometry. Statistical analysis was performed using ANOVA. *p<0.05, **p<0.01, and ***p<0.001.

In conclusion, IL-17A-Cre-mediated CTLA-4 deletion resulted in an increase in GC B cells and substantial B cell activation characterized by high proportions of CD86$^+$ B, GC B and Bm cells. This B cell phenotype, most likely induced indirectly through T cell activation, was not associated with anti-nuclear autoantibody formation or any histopathological changes.

CTLA-4$^{IL17A\text{-}KO}$ mice exhibit increased ICOS expression and increased cytokine production in a collagen-induced arthritis model

Next, we studied the effect of reduced CTLA-4 levels on IL-17A-expressing cells on the immune phenotype of T cells during a collagen-induced arthritis (CIA) response, which is known to be IL-17-dependent (20). To this end, we exposed WT, CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice to Collagen type II (CII) with Complete Freund's Adjuvant (CFA). The experimental set-up is illustrated in **Suppl Figure 1G**.

Absolute numbers of CCR6$^+$ T cells were significantly increased in the spleens of CII/CFA-immunized CTLA-4$^{IL17A\text{-}KO}$ mice compared to WT and CTLA-4$^{IL17A\text{-}Hz}$ mice (**Figure 4A**). Further analysis of their activation status revealed a significant increase in the proportions of ICOS$^+$ CCR6$^+$ T cells in CII/CFA-immunized CTLA-4$^{IL17A\text{-}KO}$ mice compared to WT mice, and these proportions were higher than in naive CTLA-4$^{IL17A\text{-}KO}$ mice (compare **Figure 4A and 2B**). Conversely, CD25$^+$ CCR6$^+$ T cells were decreased in CII/CFA-immunized CTLA-4$^{IL17A\text{-}KO}$ mice, mirroring findings observed in 30-week-old naive CTLA-4$^{IL17A\text{-}KO}$ mice (compare **Figure 4A and 2B**).

Similar to 8-week-old naïve mice, the proportions of CTLA-4$^+$ Tregs in CII/CFA-immunized CTLA-4$^{IL17A\text{-}KO}$ mice were decreased compared to WT and CTLA-4$^{IL17A\text{-}Hz}$ mice, pointing to indirect effects of CTLA-4-deficient IL-17$^+$ T cells on Tregs. Interestingly, proportions of ICOS$^+$ Tregs in the spleens of CII/CFA-immunized CTLA-4$^{IL17A\text{-}KO}$ mice were also increased compared to WT and CTLA-4$^{IL17A\text{-}Hz}$ mice, while no changes were observed in the proportion of CD25$^+$ Tregs, similar to observations in naïve mice (**Figure 4B and 2E**).

Subsequently, we evaluated the cytokine expression profile of *in vitro* PMA/ionomycin-stimulated CD4$^+$ T cells and CD19$^+$ B cells in naive and CII/CFA-immunized mice. Intracellular cytokine staining showed increased proportions of CD4$^+$ T cells expressing the inflammatory cytokines IFN-γ, Tumor Necrosis Factor (TNF)-α and IL-17A in CII/CFA-immunized CTLA-4$^{IL17A\text{-}KO}$ mice compared to WT and CTLA-4$^{IL17A\text{-}Hz}$ mice (**Figure 4C**). However, no significant differences were found in the production of IFN-γ and IL-6 by B cells (**Suppl Figure 4D**).

Lastly, we scored mice for the development of arthritis to determine their susceptibility to arthritis upon CII/CFA immunization. Despite the presence of highly activated T cells associated with increased inflammatory cytokine production, CTLA-4$^{IL17A\text{-}KO}$ mice did not demonstrate a higher incidence rate or exhibit more severe disease compared to WT mice (**Figure 4D**), although the overall arthritis incidence in this experiment was very low.

Together, these data indicate that upon immunization, T cells in CTLA-4$^{IL17A\text{-}KO}$ mice show an increased activated phenotype compared to WT and CTLA-4$^{IL17A\text{-}Hz}$ mice, illustrated by increased

ICOS expression in CCR6⁺ Th and Treg cells, as well as increased pro-inflammatory cytokine expression. Interestingly, the genetic alteration in IL17A⁺ T cells affected all other CD4⁺ T cell subsets, including naïve T cells, non-IL17A expressing Th cells and Tregs.

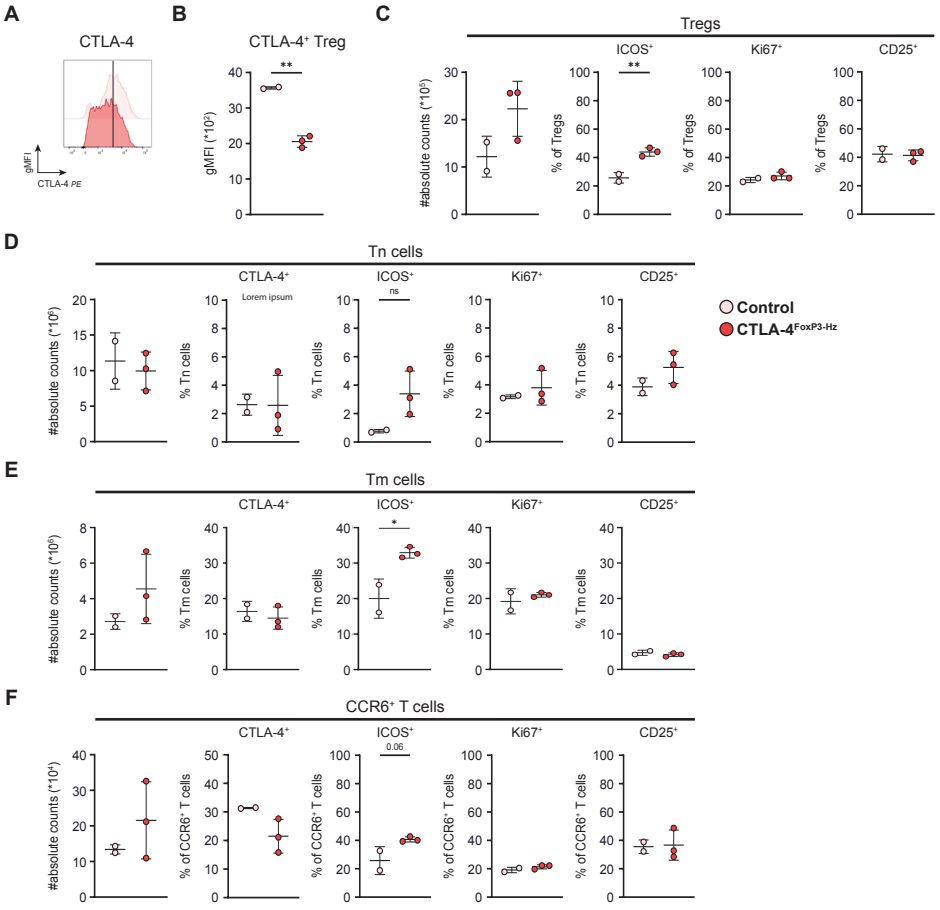

Figure 5: Phenotype of CCR6⁺ T cells Tregs in CTLA-4$^{FoxP3\text{-}Hz}$ mice
(A) Representative flow cytometry plot of CTLA-4 expression on Tregs in the spleen of WT and CTLA-4$^{FoxP3\text{-}Hz}$ mice. **(B)** The percentage of CTLA-4⁺ Tregs. **(C)** Absolute number of Tregs and the proportion of Tregs positive for ICOS, Ki67 and CD25. The absolute number of cells and the proportion of cells positive for CTLA-4, ICOS, Ki67 and CD25 within the subset of **(D)** Tn, **(E)** Tm and **(F)** Treg. Light pink dots represent WT mice and red dots represent CTLA-4$^{FoxP3\text{-}tg}$ mice. All data were measured by flow cytometry. Statistical analysis was performed using unpaired T-test. *p<0.05, **p<0.01, and ***p<0.001.

CTLA-4 haplo-insufficiency in Tregs leads to expansion of Tregs and increased proportions of ICOS$^+$ cells in major T cell populations

The finding that absence of CTLA-4 on IL-17A expressing cells affected CTLA-4 expression on Tregs (**Figure 2A and 4B**), prompted us to generate mice in which one *Ctla4* allele was targeted by FoxP3-Cre-mediated deletion. CTLA-4 is crucial for the inhibitory function of Tregs (21, 22). Complete CTLA-4-deficiency on FoxP3-expressing cells results in severe autoimmune disease in young mice and was therefore not included in this study (22).

Thus, the observed increased activation of various T cell populations in CTLA-4$^{IL17A\text{-}KO}$ mice could be induced by increased activation of IL-17-producing Th cells, decreased regulatory capacity of Tregs or a combination of both. This is very relevant in the context of sarcoidosis, given that in patients with sarcoidosis, Treg function is often compromised, accompanied by reduced CTLA-4 expression observed in Tregs isolated from lung-draining MedLN (11, 14).

To explore the effects of reduced CTLA-4 expression on Tregs *in vivo*, flow cytometric analysis was performed to characterize the phenotype of Tregs in the spleen of 24-week-old CTLA-4$^{FoxP3\text{-}Hz}$ mice, whereby FoxP3-Cre *Ctla-4$^{+/+}$* mice served as controls. The expression of CTLA-4 in Tregs was significantly decreased in CTLA-4$^{FoxP3\text{-}Hz}$ mice compared to WT mice, confirming our model (**Figure 5A-B**). Furthermore, we observed a trend towards increased proportions of Tregs and a significant increase in ICOS$^+$ cells in the Treg population in the spleens of CTLA-4$^{FoxP3\text{-}Hz}$ mice (**Figure 5C**).

We subsequently analyzed the proportions and activation status of other CD4$^+$ T cell subsets in CTLA-4$^{FoxP3\text{-}Hz}$ mice. The absolute numbers of Tn, Tm and CCR6$^+$ T cells remained unchanged between the two groups of mice (**Figure 5D-F**). Although no differences were found in the proportions of CTLA-4$^+$ Tn and Tm cells, there was a trend toward decreased proportions of CTLA-4$^+$ CCR6$^+$ CD4$^+$ T cells in CTLA-4$^{FoxP3\text{-}Hz}$ mice. Increased proportions of ICOS$^+$ cell in the populations of Tn, Tm and CCR6$^+$ T cells were found, but significant differences were reached only in the Tm subset. The expression of Ki67 and CD25 was not different in all T cell populations analyzed (**Figure 5C-F**).

Given the significant B cell activation in CTLA-4$^{IL17A\text{-}KO}$ mice, we additionally analyzed B cell proportions and activation status in the spleen of CTLA-4$^{FoxP3\text{-}Hz}$ mice. We found a trend toward increased absolute numbers of GC B cells and plasma B cells in CTLA-4$^{FoxP3\text{-}Hz}$ mice compared to WT mice, as well as a trend toward increased expression of CD86 on B and Bm cells (data not shown).

Taken together, these data show that reduced CTLA-4 expression in Tregs does not only lead to increased activation of these cells (characterized by increased ICOS expression), but also affects ICOS expression on Tn, Tm and CCR6$^+$ T cells. Thus, remarkably, reduced expression of CTLA-4 in Tregs induced activation of CCR6$^+$ CD4$^+$ T cell subsets (characterized by increased ICOS and decreased CTLA-4 expression), and vice versa as observed in mice with IL-17A-Cre-mediated CTLA-4 deletion.

DISCUSSION

The current study aimed to describe the immunological phenotype of CTLA-4-deficiency in IL-17A-expressing T cells and in Tregs, as potential mouse models to study the disease pathogenesis of sarcoidosis.

Our results show that IL-17A-Cre-mediated *Ctla-4* deletion affected the expression of CTLA-4 on Th17 cells, which are the main producers of IL-17A (19). CTLA-4-deficiency leads to significant T cell activation, characterized by increased proportions of $ICOS^+$ T cells. It is crucial to note that the complete absence of CTLA-4 on IL-17A expressing cells was necessary for the observed phenotype, as haplo-insufficiency of CTLA-4 on IL-17A-expressing cells did not result in any phenotype. Additionally, IL-17A-Cre-mediated CTLA-4 deletion led to a decrease in the proportions of $CTLA\text{-}4^+$ Tregs, both in 8-week-old naïve mice and in our CIA model. Conversely, reducing the proportion of $CTLA\text{-}4^+$ Tregs in mice resulted in increased T cell activation, characterized by an increase in $ICOS^+$ $CCR6^+$ T cells and Tregs. Furthermore, a trend towards a decrease in the fraction of $CTLA\text{-}4^+$ cells in the $CCR6^+$ T cell population was observed in $CTLA\text{-}4^{FoxP3\text{-}Hz}$ mice. Our study therefore reveals a reciprocal influence between CTLA-4 expression levels on Th17 cells and Tregs. This suggests a potential link between CTLA-4 expression on $CCR6^+$ T cells and Treg cells and would be consistent with a model in which both Tregs and Th17-like cell subsets with reduced CTLA-4 expression in sarcoidosis patients may support increased T cell activation and inflammation.

It is conceivable that CTLA-4 haplo-insufficiency would already lead to dysregulation of T cell homeostasis, given the observed reduced CTLA-4 expression in patients with sarcoidosis and CVID (14, 23). However, immune dysregulation was observed solely in IL-17A-Cre mice with complete CTLA-4 deficiency and not in $CTLA\text{-}4^{IL\text{-}17A\text{-}Hz}$ mice. Because $CTLA\text{-}4^{FoxP3\text{-}Hz}$ mice did show significant changes in the immune phenotype, it is likely that the phenotype observed in sarcoidosis patients primarily results from reduced CTLA-4 levels on Tregs, subsequently affecting CTLA-4 levels on Th17 cells. We therefore conclude that IL-17A-Cre-mediated CTLA-4 deletion might therefore be a model more valuable for investigating the function of CTLA-4 in Th17 cells, whereas FoxP3cre-mediated CTLA-4 reduction is promising for a better understanding of the immune pathogenesis of sarcoidosis.

Both $CTLA\text{-}4^{IL\text{-}17A\text{-}KO}$ and $CTLA\text{-}4^{FoxP3\text{-}Hz}$ mice displayed enhanced T cell activation, characterized by increased ICOS expression. Similar findings were observed in patients with pulmonary sarcoidosis, particularly in Löfgren syndrome, where $ICOS^+$ Tregs were significantly increased in the lung [27]. Recent studies have shown that $ICOS^+$ Tregs possess greater suppressive capacity and enhanced survival compared to $ICOS^-$ Tregs (24). Moreover, the activation marker ICOS was implicated in the maintenance of Th17 cells (25, 26). It is conceivable that in $CTLA\text{-}4^{IL\text{-}17A\text{-}KO}$ mice $ICOS^+$ Treg will develop in an environment containing highly activated T and B lymphocytes. Additionally, upregulation of ICOS on Tregs may compensate for reduced CTLA-4 levels on Tregs

in CTLA-4$^{FoxP3\text{-}Hz}$ mice. Moreover, we found activated Tn cells, characterized by increased ICOS expression, both in CTLA-4$^{IL\text{-}17A\text{-}KO}$ and in CTLA-4$^{FoxP3\text{-}Hz}$ mice. This parallels findings in sarcoidosis patients, where we observed activated Tn cells in the circulation (27). On the contrary, we found a lower percentage of CD25$^+$ CCR6$^+$ T cells in naive and CII/CFA-immunized CTLA-4$^{IL\text{-}17A\text{-}KO}$ mice. CD25 is a marker for T cell activation and is upregulated after T cell receptor signaling. However, CD25 can be cleaved from the cell surface during excessive T cell activation (28), which might explain the lower expression of CD25 on activated CCR6$^+$ T cells in CTLA-4$^{IL\text{-}17A\text{-}KO}$ mice.

Sarcoidosis is mainly regarded as a T cell-driven disease and studies addressing B cell involvement in disease pathogenesis is limited (29). However, B cells are found in granulomatous tissues from patients and increased frequencies of B cell subsets were found in the circulation along with higher levels of autoantibodies compared to healthy controls (30-32). Interestingly, CTLA-4$^{IL17A\text{-}KO}$ mice showed spontaneous B cell activation, but this phenotype did not lead to the production of autoantibodies or increased susceptibility to CIA the development. Similar findings were demonstrated in CTLA-4$^{FoxP3\text{-}Hz}$ mice, but less pronounced due to the small number of mice. Humoral responses are often induced via Tfh cells. Previous studies have reported significantly higher proportion of CXCR5$^+$ Tfh(-like) cells in blood from patients with sarcoidosis (32, 33). Here we found no alterations in the numbers of Tfh cells, but only in the proportions of ICOS$^+$ Tfh cells. Activated Tfh cells likely serve as pivotal players in B cell activation. On the other hand, Th17 cells can have properties comparable to Tfh cells, including the ability to induce B cell proliferation (34). Alternatively, CTLA-4 on Th17 cells and the interaction with CD80/86 on B cells, may directly influence B cell activation. Interactions between Th17 and B cells and their activation remains to be elucidated, as does the generation of activated Tfh cells and the role of CTLA-4 on Th17 cells in both processes.

The model has some limitations to address. IL-17A can be produced by many immune cells, including γδ$^+$ T cells, CD8$^+$ T cells, B cells and different innate immune cells (35-37). However, the expression levels of CTLA-4 in these immune cells are low compared to CD4$^+$ T cells (38-40). As a consequence, it is reasonable to assume that the genetic alteration will predominantly target Th17 cells, although it can also affect other cells with limited consequences for the model. In any case, we found that IL-17A-cre-mediated CTLA-4 deletion predominantly affected Th17 cells as they are major producers of IL-17A. Second, our mice did not spontaneously develop sarcoidosis. Nevertheless, in this study we describe the phenotype of mice with genetic alterations that would mimic immunological features seen in patients with sarcoidosis (14). It is thought that a genetic predisposition combined with an external antigenic trigger are crucial in the disease pathogenesis of sarcoidosis. As a next step, we therefore aim to investigate the effects of exposure to trehalose 6,6'-dimycolate (cord factor) of mycobacterium tuberculosis in CTLA-4$^{IL17A\text{-}KO}$, CTLA-4$^{IL\text{-}17\text{-}Hz}$ or CTLA-4$^{FoxP3\text{-}Hz}$ mice, which is an existing murine model to induce granulomas in the lungs (41). It would be very interesting to see whether these mice would be more susceptible to sarcoidosis-like symptoms, underscoring the potential to serve as a sarcoidosis model when granulomas are induced with an antigenic trigger.

In summary, our study shows that expression levels of CTLA-4 in Tregs are critical for the maintenance of immune homeostasis. In IL-17-producing T cells CTLA-4 expression levels appears less critical and only a complete deficiency induces immune dysregulation. We observed a reciprocal influence between CTLA-4 expression levels on Th17 cells and Tregs. This would be consistent with a model in which both Tregs and Th17-like cell subsets with reduced CTLA-4 expression in sarcoidosis patients may support increased T cell activation and inflammation.

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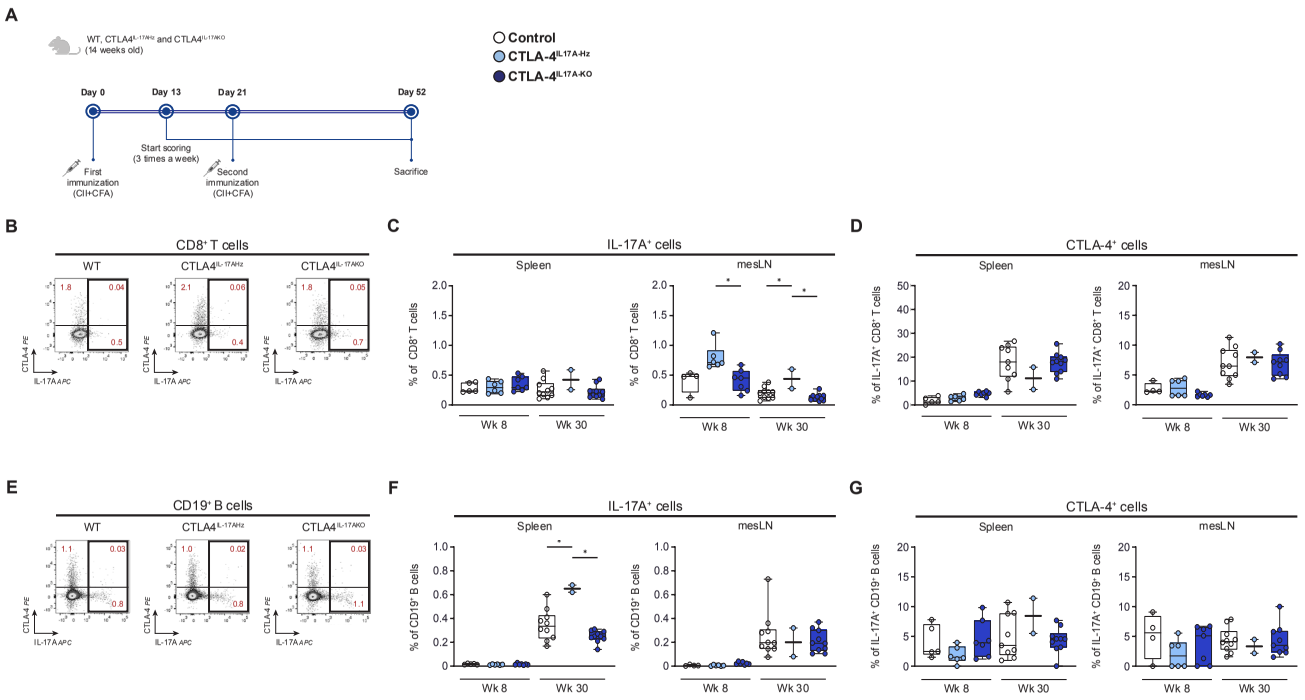
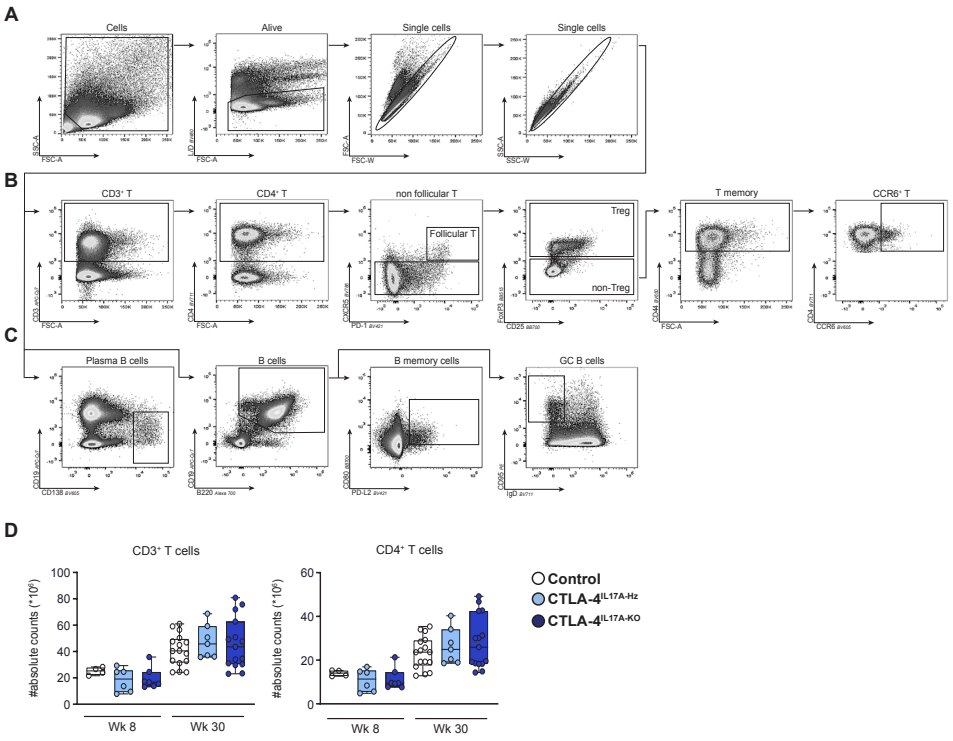


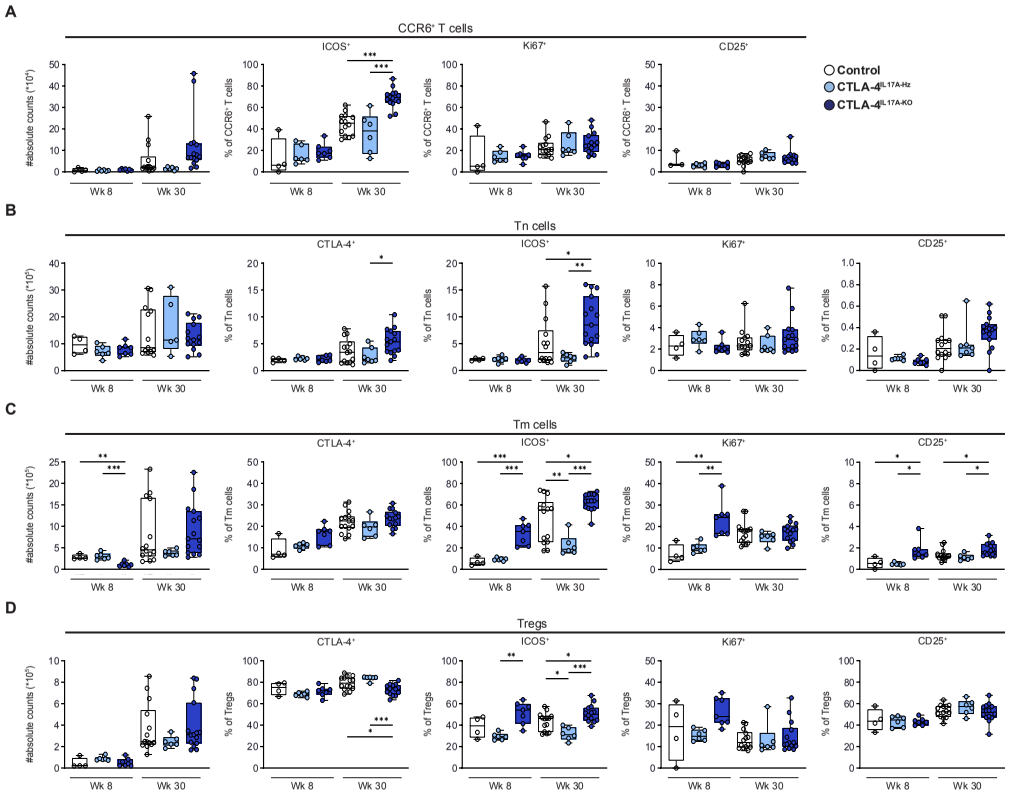
Suppl Figure 1: CTLA-4^{+} cells within the CD8^{+} T and CD19^{+} B cell subsets in CTLA-4$^{IL\text{-}17A\text{-}KO}$ mice

(A) Experimental setup of CIA. **(B)** Representative flow cytometry dot plots of CTLA-4^{+} IL-17A^{+} CD8^{+} T cells in the spleen of 30-week-old WT, CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice. **(C)** Proportions of IL-17A^{+} CD8^{+} T cells, and **(D)** CTLA-4^{+} IL-17A^{+} CD8^{+} T cells in spleen and mesLN of 8- and 30-week-old mice. **(E)** Representative flow cytometry dot plots of CTLA-4^{+} IL-17A^{+} CD19^{+} T cells in the spleen of 30-week-old WT, CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice. **(F)** Proportions of IL-17A^{+} CD19^{+} T cells, and **(G)** CTLA-4^{+} IL-17A^{+} CD19^{+} T cells. White dots represent WT mice, light blue dots represent CTLA-4$^{IL17A\text{-}Hz}$ mice and dark blue dots represent CTLA-4$^{IL17A\text{-}KO}$ mice. Intracellular cytokine production was detected after 4 hours of PMA/ionomycin stimulation of cells from the spleen and mesLN. All data were measured by flow cytometry. Statistical analysis was performed using ANOVA. *p<0.05, **p<0.01, and ***p<0.001.



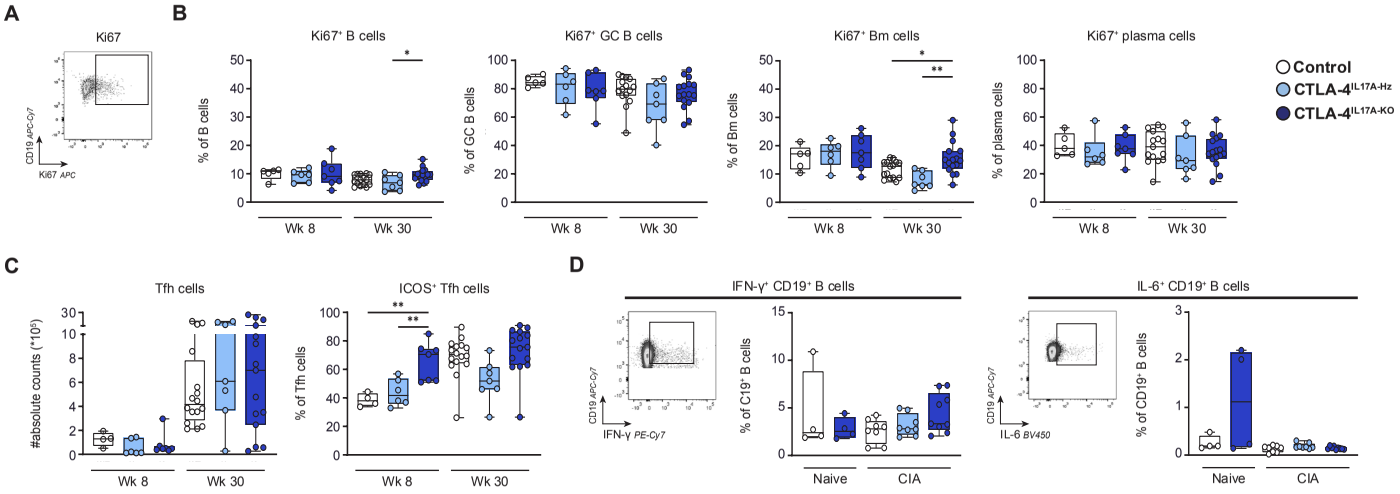
Suppl figure 2: Gating strategy of T and B cell subsets

Gating strategy of **(A)** single cells, **(B)** T cell subsets, and **(C)** B cell subsets. **(D)** Absolute cell counts of $CD3^+$ and $CD4^+$ T cells in the spleen of 30-week-old WT, CTLA-$4^{IL17A\text{-}Hz}$ and CTLA-$4^{IL17A\text{-}KO}$ mice. White dots represent WT mice, light blue dots represent CTLA-$4^{IL17A\text{-}Hz}$ mice and dark blue dots represent CTLA-$4^{IL17A\text{-}KO}$ mice. All data were measured by flow cytometry. Statistical analysis was performed using ANOVA. *p<0.05, **p<0.01, and ***p<0.001.



Suppl Figure 3: CD4⁺ T cell proportions and activation in the mesLN of CTLA-4$^{IL\text{-}17A\text{-}KO}$ mice

(A) The absolute number of CCR6⁺ T cells and the percentage of CCR6⁺ T cells positive for ICOS, Ki67 and CD25 in 8- and 30-week-old WT, CTLA-4$^{IL17A\text{-}Hz}$ and CTLA-4$^{IL17A\text{-}KO}$ mice. Absolute cell number and the percentage of cells positive for CTLA-4, ICOS, Ki67 and CD25 within the subset of **(B)** Tn, **(C)** Tm, and **(D)** Treg. White dots represent WT mice, light blue dots represent CTLA-4$^{IL17A\text{-}Hz}$ mice and dark blue dots represent CTLA-4$^{IL17A\text{-}KO}$ mice. All data were measured by flow cytometry. Statistical analysis was performed using ANOVA. *p<0.05, **p<0.01, and ***p<0.001.



Suppl Figure 4: B cell and Tfh cell activation and cytokine production in the spleen of 8- and 30-week-old CTLA-4$^{IL-17A-KO}$ mice

(A) Representative flow cytometry dot plot of Ki67 expression on CD19⁺ B cells. **(B)** Proportions of B, GC B, Bm and plasma cells positive for Ki67 in 8- and 30-week-old WT, CTLA-4$^{IL17A-Hz}$ and CTLA-4$^{IL17A-KO}$ mice. **(C)** The absolute number of Tfh cells and the percentage of Tfh cells positive for ICOS. **(D)** Representative flow cytometry dot plots of IFN-γ and IL-6 expression on CD19⁺ B cells and the percentage of CD19⁺ B cells positive for IFN-γ and IL-6. White dots represent WT mice, light blue dots represent CTLA-4$^{IL17A-Hz}$ mice and dark blue dots represent CTLA-4$^{IL17A-KO}$ mice. All data were measured by flow cytometry. Statistical analysis was performed using ANOVA. *p<0.05, **p<0.01, and ***p<0.001.

CHAPTER 8

Looking into the future of sarcoidosis: what is next for treatment?

Jelle R. Miedema, Francesco Bonella, Johan Grunewald, Paolo Spagnolo

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ABSTRACT

Purpose of review

Sarcoidosis is a complex granulomatous disease of unknown cause. Corticosteroids and immune suppressants are often given long term in chronic disease, which may result in substantial toxicity. Validated strategies for selecting patients at risk for disease progression, who might benefit from early and targeted treatment, are lacking. Consequently, the unmet need for new treatment options in sarcoidosis is high. In this review, we critically discuss potential therapeutic targets and ongoing clinical trials in sarcoidosis.

Recent findings

Despite the heterogeneous clinical manifestations and the lack of a reliable animal model, our knowledge and understanding of the pathogenesis of sarcoidosis has improved in recent years, which has resulted in the identification of several potential therapeutic strategies. They include the inhibition of cytokines involved in maturation of macrophages, activation of dendritic cells, and maturation and activation of pathogenic T-lymphocytes. The inflammasome and the autophagy are additional areas for future research. Anti-fibrotic therapy might also be a reasonable choice in selected patients, although the best treatment strategy in progressive fibrotic sarcoidosis remains undetermined.

Summary

In this article, we review novel approaches to sarcoidosis treatment and potential therapeutic targets.

INTRODUCTION

Sarcoidosis is a chronic inflammatory disease of unknown cause characterized by noncaseating epithelioid granulomas at affected organs, mostly the lungs (1). Disease immunopathogenesis is only partially understood and intriguingly complex. With these limitations, sarcoidosis is believed to develop in genetically susceptible individuals following an uncontrolled immune response to organic, inorganic or auto-antigens (2). Historically, sarcoidosis has been considered a benign disease; however, recent data indicate a significant disease burden and increasing mortality, highlighting the need for more efficacious and better tolerated treatment strategies (3-5). The decision to start treatment with first line corticosteroids, often combined with immunosuppressants in relapsing or chronic disease, is made on an individual basis, taking into account symptom burden, organ involvement and functional impairment (2). Current therapy, which is given long-term in chronic sarcoidosis, frequently results in substantial toxicities. Validated strategies to select patients at risk for progression who might benefit most from early treatment are lacking. Progress in our understanding of disease pathogenesis has resulted in the identification of promising new therapeutic targets. So, to get to the bottom line of this review: *what's next for treatment* of sarcoidosis?

Unravelling sarcoidosis immunology for future therapeutic targets

Unraveling pathways in a quest for novel therapies faces a number of challenges. Firstly, the complexity of the immune mechanisms involved in granuloma formation cannot be unraveled without taking into account the disease spectrum. At one end, there is the mostly self-limiting Löfgren syndrome (LS), especially in patients carrying the HLA-DRB1*03 allele, which may even represent a separate disease (6, 7). At the other end of the spectrum, there is non-Löfgren (non-LS) sarcoidosis, with a variety of clinical phenotypes and approximately 50% of patients developing chronic disease (6, 8). Disease stratification by clinical phenotypes will allow for more thorough understanding of factors driving disease progression. Secondly, within the same sarcoidosis patient, differences in cellular content across peripheral blood, mediastinal lymph nodes and lung parenchyma have been demonstrated (9, 10). As such, blood sampling is not adequate to "capture the whole picture". Peripheral blood and affected tissues should probably be sampled simultaneously and preferably throughout disease course to improve insight in the complex immunological interplay. Genetic factors should also be taken in the (pathogenetic) equation.

Lastly, as with other pulmonary diseases, there is a lack of valid and widely accepted experimental models that accurately simulate human disease (11, 12). However, a single model is unlikely to reproduce all aspects of the complex pathobiology of sarcoidosis (e.g., granuloma formation, development of pulmonary fibrosis, multiorgan involvement). Simultaneous (or sequential) use of more than one model (i.e., animal models, *in vivo* and *in silico* models)

represents, in our view, the approach most likely to be successful in answering some of the outstanding research questions about pathogenesis and treatment of sarcoidosis.

Potential immunologic targets

Sarcoid granulomas consist of a core of macrophages differentiated into epithelioid cells and surrounded by a rim of lymphocytes and dendritic cells (DCs) (13, 14). Immunologic research focussed on CD4+ T-cells, with an increasing appreciation of their plasticity (8). Major cytokines involved in sarcoidosis are tumor necrosis factor alpha (TNF)-α and interferon (IFN)-γ (2). Historically, the typical IFN-γ production by $CD4^+$ T cells in bronchoalveolar lavage (BAL) was ascribed to T helper 1 (Th1)-cells. However, increased proportions of IFN-γ-producing T helper 17 cells, called Th17.1-cells, were found to be the major producer of IFN-γ in sarcoidosis patients, and high levels of Th17.1 cells in the BAL correlate with chronic disease (9). Recently, a **$CD4^+Vα2.3^+$** T cell subset **in the BAL of LS and non-LS sarcoidosis patients was found to correlate with favorable prognosis, further implicating specific T-cells as important factors in disease pathogenesis (8, 15). Therefore, b**locking cytokines involved in activation and polarization of unfavorable T-cells or effector functions is an appealing therapeutic strategy.

While inhibition of TNF-α has proven efficacious in a subset of sarcoidosis patients (16), blocking IFN-γ has never been tried. Inhibition of interleukin (IL)-12/IL-23, cytokines involved in Th1 and Th17 polarization, did not show clinical benefit in a randomized controlled trial with background corticosteroids (17, 18). Many cytokines involved in T-cell proliferation and polarization mediate signaling via the Janus kinase (JAK) pathway (8, 19, 20), which therefore represents a potential therapeutic target in sarcoidosis (**figure 1**). Activated JAK complexes promote phosphorylation of signal transducers and activators of transcription (STATs), which translocate to the nucleus where they bind to regulatory elements of target genes (19). To date, four JAKs (JAK 1, 2, and 3, and TYK2) and seven STATs (STAT 1, 2, 3, 4, 5A, 5B, and 6) have been identified with pleiotropic and redundant effects across different cell types (21, 22). Several recent reports describe a positive clinical effect of JAK inhibitors in refractory sarcoidosis (23-25). Two small phase I, open-label, non-randomized trials are currently evaluating whether the JAK inhibitor Tofacitinib may represent an effective treatment option for cutaneous sarcoidosis (ClinicalTrials.gov Identifier: NCT03910543) and a steroid sparing agent for pulmonary sarcoidosis (ClinicalTrials.gov Identifier: NCT03793439) (**Table 1**).

Dendritic cells (DCs) are potent inducers of T-cell activation and differentiation. Conflicting results on DCs have been obtained in sarcoidosis, probably because of the different methodologies used (10, 14, 26). Exploring their role in sarcoidosis could identify new therapeutic targets that interfere with T cell polarization, aiming to skew these cells towards *resolving* phenotypes. Additionally, B-cells are of interest to study in more detail, as their functional role as antigen-presenting cells has likely been underestimated in sarcoidosis(27). Rituximab, a chimeric monoclonal antibody that induces depletion of CD20+ B-cells, has proven efficacious in refractory

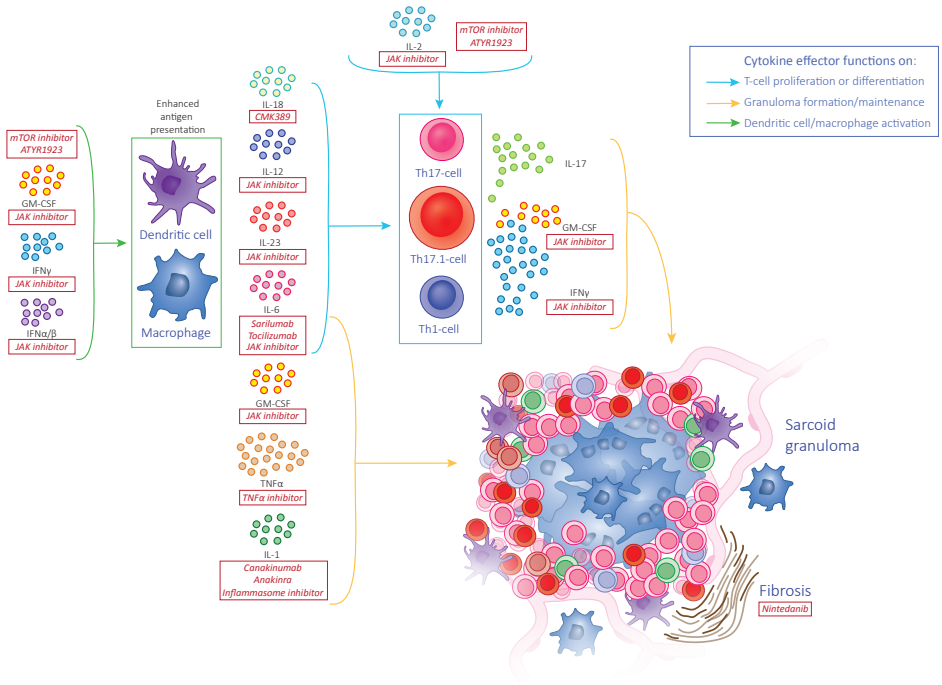

Figure 1: Potential treatment in sarcoidosis
Schematic overview of possible therapeutic approaches in sarcoidosis and their effect on cytokines and immune cells, involved in granuloma formation. Courtesy of M. Kool.

pulmonary sarcoidosis (28). The TNF family ligand B-cell-activating factor (BAFF), which plays a crucial role in B cell survival and differentiation, may also be implicated in disease pathogenesis (29), suggesting that targeting BAFF may represent a novel therapeutic strategy for sarcoidosis. Another intriguing pathway to explore is the NLRP3 inflammasome, which is involved in activation and release of IL-1β by macrophages in response to danger signals (11, 30). Upregulation of this pathway was recently demonstrated in sarcoidosis biopsies (11). Sarcoidosis BAL cells showed increased IL-1β release, and treatment with anti-IL-1β and a direct inhibitor of the NLRP3 inflammasome significantly reduced granulomatous inflammation in a mouse model of sarcoidosis (11). These data warrant further study on inhibitors of the inflammasome in sarcoidosis (11, 30). To this end, a recently completed phase II study has evaluated the efficacy, safety and tolerability of the human anti-IL-1b antibody ACZ885 (Canakinumab) 300 mg/2 mL administered subcutaneously once monthly for 6 months in 40 patients with pulmonary sarcoidosis (ClinicalTrials.gov Identifier: NCT02888080). The results of this trial are expected to be published soon.

Macrophages with increased activation of mammalian target of rapamycin (mTOR) 1, which is involved in Th17 cell differentiation and autophagy, demonstrate spontaneous granuloma formation in mice (31). Moreover, mTOR1 activation is demonstrated in sarcoidosis patients and correlates with progressive disease (23, 31, 32). Interestingly, CD44 expressed on CD4+ T cells which is known to stimulate mTOR, was found downregulted in LS patients, who have a favorable prognosis(33). Genes enriched in the mTOR pathway have recently been identified in five families with familial sarcoidosis (34). Dysfunctional autophagy caused by mutations in mTOR1 and Ras-related C3 botulinum toxin substrate (Rac1) have been postulated as contributors to disease pathogenesis, through ineffective clearance of antigens (34, 35). Future research should investigate the strategy of autophagy enhancement in sarcoidosis.

Novel approaches to treatment

A number of novel potential treatments for sarcoidosis are currently being developed (Table 1). Below, we describe mechanism of action and potential therapeutic targets based on disease pathogenesis for a selection of these compounds.

Anakinra. IL-1 is a pro-inflammatory cytokine that plays an important role in mediating several immunopathogenetic events observed in sarcoidosis(36). Anakinra is a recombinant human IL-1 receptor antagonist that has been approved by the FDA in 2001 for the treatment of moderate-severe rheumatoid arthritis. Specifically, anakinra acts by competitively inhibiting IL-1 binding to the IL-1 receptor I, a signal transmitting receptor that is expressed by a variety of cells including T cells, endothelial cells, and fibroblasts (37). A phase II, double-blind, randomized, placebo controlled trial of anakinra is currently being conducted in patients with cardiac sarcoidosis (ClinicalTrials.gov Identifier: NCT04017936).

Sarilumab. IL-6 is a pleomorphic pro-inflammatory cytokine that is highly expressed in tissues affected by sarcoidosis (38). IL-6 promotes the differentiation of Th17 effector cells, which have been implicated in the pathogenesis of sarcoidosis(39). In addition, IL-6 upregulates serum amyloid A (SAA), an amyloid precursor protein that has been implicated in the development and maintenance of sarcoidosis granulomatous inflammation(40). Recently, tocilizumab, a recombinant, humanized, anti-IL-6 receptor monoclonal antibody that is approved for treatment of rheumatoid arthritis, has been used successfully in four patients with chronic, severe sarcoidosis who had failed multiple steroid-sparing therapies(41). While anecdotal, these data suggest that the IL-6 pathway may represent a potential therapeutic target in sarcoidosis. The safety and efficacy of sarilumab, a human monoclonal antibody against IL-6R, is currently being evaluated in patients with steroid-dependent sarcoidosis (ClinicalTrials.gov Identifier: NCT04008069).

CMK389. IL-18 is a pleiotropic pro-inflammatory cytokine that plays an important regulatory role in both innate and acquired immune response against infection. Levels of IL-18 are increased in the lungs of patients with sarcoidosis(42), and IL-18 is believed to contribute to disease pathogenesis by, together with IL-12, stimulating IFN-γ production from CD4+ Th1 cells and macrophages. CMK389 is an IL-18 inhibitor that is currently being investigated in a phase II clinical trial of patients with chronic pulmonary sarcoidosis (ClinicalTrials.gov Identifier: NCT04064242).

Repository corticotropin injection (RCI). RCI is a natural mixture of prolonged-release adrenocorticotropic hormone (ACTH) analogs and other pituitary peptides that exerts anti-inflammatory and immunomodulatory properties (43). RCI is approved for the treatment of sarcoidosis in the U.S. A retrospective medical record study of patients with advanced symptomatic sarcoidosis treated with RCI was recently performed(44). The study included 302 patients, three quarters of whom had extrapulmonary involvement, mainly skin, joints, heart and eyes. The percentage of patients who used corticosteroids decreased from 61.3% during the 3 months before initiation of RCI to 12.9% 3 months after RCI therapy, and the mean daily dose of corticosteroids decreased from 18.2 mg to 9.9 mg. Moreover, overall status improved in 95% of patients, overall symptoms in 73% and lung function in 38%. However, due to the exploratory and hypothesis-generating nature of the study, RCI was not compared with placebo or other treatments. In addition, the effectiveness of RCI was based on physicians' assessments, which may have introduced bias. An ongoing phase IV, double-blind randomized placebo-controlled clinical trial will examine the safety and efficacy of RCI in patients with pulmonary sarcoidosis (ClinicalTrials.gov Identifier: NCT03320070).

ATYR1923. ATYR1923 is a selective modulator of Neuropilin 2 (NRP2), a transmembrane receptor that is involved in a number of immune processes such as maturation of macrophages, activation of dendritic cells, and maturation and activation of T-lymphocytes (45). By modulating NRP2, ATYR1923 downregulates the innate and adaptive immune response in inflammatory disease states, and may potentially be beneficial in sarcoidosis granulomatous inflammation. Indeed, in a mouse model of sarcoidosis induced by *Propionibacterium acnes*, ATYR1923 reduced lung protein levels of several cytokines or chemokines such as INF-gamma, MCP-1/CCL2, IL-6 and CXCL10 (46). In a phase I study in healthy volunteers, ATYR1923 was well tolerated at all doses tested. The safety and efficacy of ATYR1923 in sarcoidosis will be tested in a phase I/II study that is currently recruiting patients (ClinicalTrials.gov Identifier: NCT03824392).

Table 1: Overview of selected ongoing clinical trials of pharmacological interventions in sarcoidosis

Target population	Intervention	Study Design	Status	Primary outcome	Estimated enrolment/main inclusion criteria	Trial identifier
Sarcoidosis	Prednisolone (low vs. medium dose); oral	Phase IV, randomized, open label	Recruiting	Relapse or treatment failure	86 patients; cytological/histological diagnosis of sarcoidosis; chest CT consistent with sarcoidosis; symptoms or extrathoracic manifestations requiring treatment; reduced lung function; onset of symptoms within two years of study entry	NCT03265405
Hepatic sarcoidosis	Ursodeoxycholic acid; oral	Phase II, non-randomized, single-centre, open label, cross-over	Recruiting	Reduction in alkaline phosphatase and/or gamma glutamyl transferase	10 patients; systemic sarcoidosis with liver involvement; stable dose of treatment (if taken)	NCT03602976
Cardiac sarcoidosis	Low dose prednisone/ methotrexate; oral/ oral, subcutaneous or intramuscular vs. prednisone standard dose; oral	Phase III, randomized, open label, non-inferiority	Recruiting	Summed perfusion rest score on FDG-PET scan	194 patients; cardiac sarcoidosis AND FDG-PET uptake suggestive of active disease AND one or both of the following biopsy-proven sarcoidosis; chest CT consistent with pulmonary disease	NCT03593759
Chronic pulmonary sarcoidosis	CMK389 (IL-18 inhibitor); intravenous	Phase II, multicentre, randomized, placebo-controlled	Not yet recruiting	Change in FVC at week 16	66 patients; biopsy proven pulmonary sarcoidosis; extent of reticular change on HRCT <15%; Scadding stage II-IV on chest X-ray; treatment with 5-15 mg/day prednisone (or equivalent) and co-medication with methotrexate or azathioprine for ≥6 months before screening	NCT04064242
Severe extrathoracic sarcoidosis	Infliximab (chimeric monoclonal antibody against TNF-α); intravenous	Phase III; initial blinded period (2 injections of infliximab or placebo) followed by an open label period (all patients receive infliximab at week 6, 10 and 14)	Recruiting	Proportion of patients with a severity score (ePOST score) <1 in all organs and absence of hypercalcemia at week 6	30 patients; at least one extrathoracic localization (including hypercalcemia); serious organ involvement/ relapse of a new localization despite a first-line immunosuppressive drug.	NCT03704610
Glucocorticoid-dependent sarcoidosis	Sarilumab (human monoclonal antibody against IL-6R); subcutaneous	Phase II, monocentric, randomized, placebo-controlled, withdrawal study	Recruiting	Flare-free survival	15 patients; Scadding stage II-III on chest X-ray; involvement of the lymph nodes, liver, kidneys, spleen, bone, soft tissue, skin and/or eyes; prednisone (or equivalent) dose ≥10 mg and ≤60 mg daily; DMARDs permitted if at a stable dose for ≥28 days prior to baseline	NCT04008069

Table 1: Continued

Central Nervous System Sarcoidosis	Repository Corticotropin (adrenocorticotropin hormone); intramuscular or subcutaneous	Phase IV; single group; open label	Recruiting	Proportion of patients with clinically significant improvement/ successful glucocorticoid tapering	20 patients; moderate to severe neurosarcoidosis; stable dose of immunosuppressive drugs at baseline	NCT02920710
Pulmonary sarcoidosis	Repository Corticotropin (adrenocorticotropin hormone); subcutaneous	Phase IV; randomized, double-blind, placebo-controlled	Recruiting	Number of "responders" using the Sarcoidosis Treatment Score at 24 and 48 weeks	100 patients; symptomatic pulmonary disease; stable dose of prednisone (or equivalent) or another disease-modifying anti-sarcoidosis drug	NCT03320070
Ocular sarcoidosis	Repository Corticotropin (adrenocorticotropin hormone); subcutaneous	Single group, open label	Recruiting	Proportion of patients with clinically significant improvement in visual acuity	20 patients; any anterior, posterior, intermediate or panuveitis requiring therapy; active uveitis at the time of screening	NCT02725177
Pulmonary sarcoidosis	ATYR1923 (selective NRP2 modulator); intravenous	Phase I/phase II, randomized, double-blind, placebo-controlled, multiple ascending dose	Recruiting	Incidence of treatment-emergent adverse events and serious adverse events	36 patients (12 patients in each cohort); symptomatic and/or active pulmonary sarcoidosis; prednisone (or equivalent) dose ≥10 mg and ≤25 mg daily	NCT03824392
Cardiac sarcoidosis	Anakinra (IL-1R antagonist); subcutaneous	Phase II, randomized, double-blind, placebo-controlled	Recruiting	Change in inflammation marker (C-reactive protein)	28 patients; cardiac sarcoidosis AND cardiac FDG uptake on recent PET AND CRP >2 mg/l	NCT04017936
Pulmonary sarcoidosis	ACZ885 (canakinumab) (anti-IL-1 β monoclonal antibody); subcutaneous	Phase II, randomized, double-blind, placebo-controlled, multiple dose	Awaiting results	Change in pulmonary function from baseline through week 24	40 patients between 18 and 80 years of age; pulmonary sarcoidosis with disease duration ≥1 year; clinically active disease on either biopsy of any organ or BAL features; parenchymal lung involvement on chest CT; FVC 50-90% predicted; MMRC dyspnoea scale ≥1	NCT02888080
Pulmonary sarcoidosis	Azithromycin; oral	Phase II, single-arm, open-label, exploratory	Recruiting	Change in 24h cough counts from baseline	30 patients between 18 and 80 years of age; pulmonary sarcoidosis	NCT04020380
Progressive fibrotic pulmonary sarcoidosis	Pirfenidone; oral	Phase IV, randomized, double-blind, placebo-controlled	Unknown	Time to clinical worsening	60 patients; pulmonary function testing with a Composite Physiologic Index score >40; >20% fibrosis on chest HRCT; stable dose of prednisone and/or immunosuppressive drugs	NCT03260556

Table 1: Continued

Corticosteroid-dependent sarcoidosis	Tofacitinib (JAK inhibitor); oral	Phase I; non-randomized, single group, open-label, proof-of-concept	Recruiting	50% reduction in corticosteroid requirement in at least 60% of subjects at week 16	5 patients; histologically-proven sarcoidosis; pulmonary involvement; FVC >50% of predicted; prednisone (or equivalent) dose ≥15 mg and ≤30 mg daily; stable dose of treatment for 4 weeks prior to enrolment	NCT03793439
Cutaneous sarcoidosis	Tofacitinib (JAK inhibitor); oral	Phase I; non-randomized, single group, open-label	Recruiting	Change in Cutaneous Sarcoidosis Activity and Morphology Instrument (CSAMI) score	15 patients; histologically confirmed cutaneous sarcoidosis with either CSAMI ≥10 or any CSAMI score and sarcoidosis involvement causing functional impairment; stable dose of treatment for 3 months prior to enrolment	NCT03910543
Sarcoidosis	Ergocalciferol (vitamin D2); oral	Phase IV; randomized, parallel assignment; vitamin-D deficient subjects will receive either vitamin D2 repletion or placebo while vitamin-D sufficient subjects will be observed without treatment.	Recruiting	Change in lung function from baseline to week 24	90 patients; stable medical condition.	NCT03621553
Sarcoidosis-associated precapillary pulmonary hypertension	Selexipag (prostacyclin receptor agonist); oral	Phase II; multicentre, randomized, double-blind,	Not yet recruiting	Pulmonary vascular resistance up to week 26	74 patients; sarcoidosis-associated PH confirmed by RHC; PH severity II-IV according to modified WHO Functional Classification; stable sarcoidosis treatment; FVC >50%; 6MWD between 50 and 450 m; FEV1/FVC ≥60% or, if FEV1/FVC <60% FEV1 ≥60% of predicted	NCT03942211
Sarcoidosis-associated precapillary pulmonary hypertension	Treprostinil (prostacyclin analog); inhaled	Phase II; single-group, open label	Recruiting	PVR and mPAP by RHC at week 16	10 patients; RHC-confirmed precapillary PH; Scadding stage IV or extensive pulmonary fibrosis on HRCT; stable therapy for 3 months prior to screening; 6MWD >100m	NCT03814317
Sarcoidosis-associated pulmonary hypertension	Nitric oxide; inhaled	Phase IIb; dose escalation, non-randomized, open label	Recruiting	Measurement of mPAP, PVR, PCWP and CO by RHC; change in 6MWD from baseline wo week 16	16 patients; RHC-confirmed precapillary PH; Scadding stage IV or extensive pulmonary fibrosis on HRCT; stable therapy for 3 months prior to screening; 6MWD >100m	NCT03727451

Abbreviations: BAL: bronchoalveolar lavage; CO: cardiac output; CRP: C-reactive protein; DMARDs: disease-modifying anti-rheumatic drugs; FDG: fluoro-deoxyglucose; FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; HRCT: high-resolution CT; MMRC: modified Medical Research Council; mPAP: mean pulmonary artery pressure; 6MWD: 6-minute walking distance; NRP: Neuropilin; PCWP: pulmonary capillary wedge pressure; PET: positron emission tomography; PH: pulmonary hypertension; PVR: Pulmonary vascular resistance; RHC: right heart catheterization;

Targeting fibrosis in sarcoidosis – the potential of IPF-specific therapies

Pulmonary fibrosis is a threatening complication of sarcoidosis in up to 20 % of cases (47). The amount of fibrosis that develops is highly variable and unpredictable (48). In general, mechanisms driving the transition from inflammation to fibrosis have been poorly investigated if compared, for instance, to idiopathic pulmonary fibrosis (IPF). Transforming growth factor-β activity, macrophage phenotype switching from M1 to M2, Th1 to Th2 transition and expansion of Th17 lymphocytes are all regarded as drivers of fibrosis development (49-52). Data from nuclear imaging, especially FDG-PET scans, show that at least in some patients, fibrosis and inflammation may coexist (53, 54). It is currently unknown whether and to what extent immunosuppression can prevent fibrosis. Evidence that systemic corticosteroids can prevent development or halt progression of pulmonary fibrosis is limited (55, 56), and data on other immunosuppressive treatments are lacking. Sarcoidosis patients with progressive pulmonary fibrosis have been included in a clinical trial with the antifibrotic drug nintedanib, a tyrosine kinase inhibitor, for non-IPF progressive lung fibrosis, but the efficacy in this specific group is still unclear due to the low number of patients (3% of the whole cohort) (57, 58). A double-blind, placebo-controlled trial will assess the efficacy of pirfenidone, another antifibrotic drug approved for the treatment of IPF, in sarcoidosis patients with >20% fibrosis on high resolution computed tomography scan (ClinicalTrials.gov Identifier: NCT03260556).

The best treatment strategy in progressive fibrotic sarcoidosis is currently unknown. As with autoimmunity-driven interstitial lung diseases, the coexistence of Inflammation and fibrosis should be carefully taken into account. Theoretically, combination of anti-inflammatory and antifibrotic drugs might be reasonable in selected patients, but further research on the management of fibrosis in sarcoidosis is highly warranted.

The future of clinical trials and patient care

Increasing understanding of pathogenetic mechanisms underlying sarcoidosis, including genetic variants, phenotypic traits and environmental factors that contribute to the phenotypical heterogeneity of the disease, will enable a better patient stratification for targeted treatment. Identifying patient subsets according to organ involvement or disease course is the first and simple step to recruit homogenous cohorts in future clinical trials. Recently, 2163 Caucasian patients with sarcoidosis recruited from 31 study centres were clustered into five distinct subgroups according to predominant organ involvement, namely abdominal organ involvement; ocular-cardiac-cutaneous-central nervous system involvement; musculoskeletal-cutaneous involvement; pulmonary and intrathoracic lymph node involvement; and extrapulmonary involvement (59). However, due to the highly heterogeneous nature of sarcoidosis, patient subtyping based on clinical manifestations has inherent limitations. A molecular-based approach, incorporating genetic and proteomic data, may identify distinct molecular disease phenotypes (i.e., endotypes), and this should also be addressed in future studies.

Choosing relevant endpoints in sarcoidosis clinical trials can be challenging because of variable disease phenotype and disease course, treatment goals and multiple factors causing quality of life impairment. For instance, due to variable physiologic impairment, Forced Vital Capacity (FVC) does not accurately reflect changes in a subset of patients with airflow obstruction. However, FVC and mortality can be relevant endpoints in a sarcoidosis cohort with progressive pulmonary fibrosis, because of restrictive lung disease and worse survival (60). Additionally, clinical endpoints used in trials using first line anti-granulomatous drugs may differ substantially from endpoints to investigate chronic steroid sparing- or anti-fibrotic treatment(61). Clinical trials should therefore select endpoints while taking these differences into account. Importantly, quality of life issues and patients related outcome measures (PROMs) should be used as endpoints to assess the effectiveness of therapies as well. This could lead to development of treatment protocols encompassing physiotherapy and psychological rehabilitation (62).
A marked heterogeneity in treatment response is a well-known problem in sarcoidosis management across all phenotypes (63). Changes at genomic and transcriptomic level, if predictive of clinical response to a specific drug, would be extremely useful. One example of pharmacogenomics in sarcoidosis is the impact of the TNF-α 308-allele polymorphism in response to infliximab. Sarcoidosis patients homozygous for the TNF-α – 308 G allele had a threefold higher response to TNF inhibitors than carriers of the AA- or AG-genotypes (64), although it is difficult to tease out whether this association is *primary* or *driven* by HLA alleles, due to the proximity and high degree of linkage disequilibrium existing between HLA class II alleles and TNF-α. Beside the more precise and refined use of anti-granulomatous therapies, non-granulomatous aspects of sarcoidosis, including chronic fatigue, small fiber neuropathy, pulmonary hypertension and fibrotic complications will require a systematic assessment and care.

Conclusion. Are we going in the right direction?

Clinical phenotypes within sarcoidosis might differ in terms of trigger but also type of immunological response to disease initiating factors. Studying these differences in relation to disease heterogeneity should be prioritized, as they are pivotal for improving our knowledge of the disease and for the development of future therapies. A significant number of novel potential treatments for sarcoidosis are currently being investigated. Technological advances will surely allow for more detailed categorization of immune cells and subsequent detection of new pathways involved. The concept of early-targeted intervention in selected sarcoidosis patients to alter disease course is very appealing as future strategy. To this end, future studies should identify patients at increased risk of morbidity and mortality and interventional studies should address the issue of whether we are actually altering disease course or merely suppressing inflammation.

Key points

- Improved understanding of sarcoidosis pathogenesis has resulted in the identification of several druggable targets
- Treatment may include inhibition of cytokines involved in maturation and activation of macrophages, dendritic cells and pathogenic T-lymphocytes
- The inflammasome, autophagy and the role of anti-fibrotic therapy are areas for further research in sarcoidosis
- An increased understanding of factors contributing to the phenotypical heterogeneity of sarcoidosis is invaluable for the development of personalized treatment

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PART II

Treatment of progressive pulmonary fibrosis

CHAPTER 9

Safety and tolerability of pirfenidone in asbestosis – a prospective multicenter study

Jelle R. Miedema, Catharina C. Moor, Marcel Veltkamp, Sara Baart, Natascha S.L. Lie, Jan C. Grutters, Marlies S. Wijsenbeek, Rémy L.M. Mostard

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ABSTRACT

Background

Pirfenidone slows down disease progression in idiopathic pulmonary fibrosis (IPF). Recent studies suggest a treatment effect in progressive pulmonary fibrosis other than IPF. However, the safety and effectiveness of pirfenidone in asbestosis patients remain unclear. In this study, we aimed to investigate the safety, tolerability and efficacy of pirfenidone in asbestosis patients with a progressive phenotype.

Methods

This was a multicenter prospective study in asbestosis patients with progressive lung function decline. After a 12-week observational period, patients were treated with pirfenidone 801 mg three times a day. Symptoms and adverse events were evaluated weekly and patients completed online patient-reported outcomes measures. At baseline, start of therapy, 12 and 24 weeks, in hospital measurement of lung function and a 6 minute walking test were performed. Additionally, patients performed daily home spirometry measurements.

Results

In total, 10 patients were included of whom 6 patients (66.7%) experienced any adverse events during the study period. Most frequently reported adverse events were fatigue, rash, anorexia and cough, which mostly occurred intermittently and were reported as not very bothersome. No significant changes in hospital pulmonary function (forced vital capacity (FVC), diffusion capacity of the lung for carbon monoxide (DLCO), six minute walking test or patient-reported outcomes measures before and after start of pirfenidone were found. Home spirometry demonstrated a FVC decline in 12 weeks before start of pirfenidone, while FVC did not decline during the 24 week treatment phase, but this difference was not statistically significant.

Conclusions

Treatment with pirfenidone in asbestosis has an acceptable safety and tolerability profile and home spirometry data suggest this antifibrotic treatment might attenuate FVC decline in progressive asbestosis.

BACKGROUND

Asbestosis is a rare occupational interstitial lung disease, caused by inhalation of asbestos fibers(1). Although the use of asbestos has been restricted or banned in many countries, global incidence of asbestosis has increased, including in Western Europe and North America (2). The disease course is variable, but a significant subgroup of asbestosis patients have a progressive fibrotic phenotype (3, 4). Recently, a retrospective study in Germany described the course of lung function in asbestosis patients and found progressive disease, defined as an annual forced vital capacity (FVC) loss of 100 ml or more in 20% of subjects (3). Another study assessed the gender, age and physiologic (GAP) variables model to predict survival and found a high 3-year mortality risk of 33.3% and 60.0% for GAP stage II and III asbestosis respectively(4). The use of immunosuppressive drugs is not recommended and there is no specific treatment available for these patients (1). Therefore, new therapeutic strategies in progressive asbestosis are highly warranted.

Pirfenidone is an antifibrotic drug that has been used extensively in idiopathic pulmonary fibrosis (IPF). It slows down lung function decline and post-hoc analyses also show a positive effect on mortality and risk of respiratory related hospitalizations (5, 6). Asbestosis shows many similarities with idiopathic pulmonary fibrosis (IPF), including demographic characteristics of patients and genetic risk factors (7, 8). Overlapping pathways and mechanisms in IPF and other diseases that manifest with progressive pulmonary fibrosis have been hypothesized, independent of the underlying disease(9). It has been demonstrated that nintedanib is effective in slowing down disease progression in patients with progressive pulmonary fibrosis other than IPF(10). Additionally, two phase 2 studies on the treatment of pulmonary fibrosis suggested that pirfenidone slows down decline of FVC in unclassifiable pulmonary fibrosis and progressive fibrotic ILD other than IPF (11, 12). Therefore, pirfenidone could be a promising treatment strategy in progressive asbestosis. Antifibrotic therapy has not been specifically evaluated in asbestosis patients and only three asbestosis patients were treated with pirfenidone in one of these studies (11).

In this small exploratory study, we aimed to prospectively investigate the safety and tolerability of pirfenidone in asbestosis patients with a progressive phenotype. Secondary objectives were to evaluate the efficacy of pirfenidone in asbestosis measured by home and hospital spirometry, and to assess changes in (health-related) quality of life during treatment.

METHODS

Study design and participants

This was a multicenter prospective study at three sites in the Netherlands performed by the Dutch Association of Pulmonologists (NVALT). Ethics approval was obtained in all participating

sites (MEC-2018-1392). All patients provided written informed consent before start of the study. To be eligible for inclusion, a combination of (1) previous asbestos exposure with a proper latency period, (2) pulmonary fibrosis and (3) pleural plaques or confirmation of asbestos fibers in lung biopsy or bronchoalveolar lavage was defined, in line with the American Thoracic Society statement on diagnosis of nonmalignant asbestos related disease(1). The diagnosis of asbestosis was subsequently confirmed by central review expert panel of the NVALT. Further inclusion criteria were: age between 40 and 85 years, FVC ≥ 50% of predicted, diffusion capacity of the lung for carbon monoxide (DLCO) ≥25% of predicted, a FEV1/FVC ratio of >0.7, a minimal 6 minute walking distance of 150 meter, >10% interstitial fibrosis on HRCT by visual scoring of an experienced thoracic radiologist, and finally, documented disease progression within 6 months prior to the study. Disease progression was defined as FVC decline >5% or DLCO decline of >10% or decrease of >25 meter on 6 minute walking test during the last 6 months. Patients who were treated with immunosupressants except prednisone ≤ 10 mg per day were excluded. Other exclusion criteria can be found in Supplementary File 1.

Study procedures

After inclusion in the study, a 12-week observational period without the study drug was started. During this period patients were asked to perform daily home spirometry (FVC) measurements. At the baseline study visit, patients received a Bluetooth-enabled handheld spirometer (Spirobank Smart, MIR, Italy), connected with the CE-marked online application "ILD online" (Gezondheidsmeter, Curavista, the Netherlands). The application was pre-installed on a password-protected tablet computer. All results were directly transmitted to the hospital via a secure encrypted connection, which enables patients and investigators to access and review data directly. Next to home spirometry measurements, patients were asked to report weekly symptoms and adverse events in the online application.

After 12 weeks of observation, all included patients were started on pirfenidone (Esbriet capsules 267 mg or 801 mg), starting with 267 mg three times a day for 1 week, 534 mg three times a day for 1 week, followed by 801 mg three times a day as a maintenance dose for a total treatment period of 24 weeks. Study visits were planned at baseline, start of treatment, 12, and 24 weeks after start of treatment with pirfenidone. At study visits, patients performed lung function measurements (FVC and DLCO) and a 6 minute walking test (6MWD). During the treatment period patients continued the daily home spirometry measurements. Routine laboratory test were performed monthly during the treatment period. During the treatment phase, patients were actively asked whether they experienced any (pre-specified) side-effects via the online application every week. In addition, patients could also report other side-effects.

At baseline, start of therapy, and 12 and 24 weeks after start of study treatment, patients completed two validated patient-reported outcomes measures online. The King's Brief Interstitial Lung Disease (K-BILD) is a 15-item validated health status questionnaire with three domains (psychological, breathlessness and activities, and chest symptoms). Scores range from 0-100

with higher scores representing a better health status. The Leicester Cough Questionnaire (LCQ) is a 19-item questionnaire on cough-related quality of life. Total scores range from 3 to 21 with higher scores indicating a better cough-related quality of life.

Statistical analysis

The primary outcome of safety and tolerability was recorded descriptively (number and percentage). Home spirometry data were analyzed with a piecewise linear mixed model, to examine differences in lung function decline between the observation and treatment period. In-hospital lung function measurements were analyzed descriptively (median, interquartile range). As this was a descriptive safety study, a formal power calculation was not possible. Based on feasibility, we aimed to include 10 patients.

RESULTS

Between April 2019 and June 2020, 10 patients were included. Two patients died during the study. One patient died during the observation period due to progression of his asbestosis, the other patients died as a result of euthanasia during the treatment period of the study. All patients were male, ex-smoker, mean age was 74.7 years (SD 7.2). Median FVC at baseline was 2.97L (IQR 2.85-3.69) or 73.0% of predicted (IQR 68.8-96.0) , and median DLCO was 42.5% of predicted (IQR 40-49) (*n* = 8). One patient used supplemental oxygen at baseline. Most common comorbidity was cardiovascular disease in 50% of patients.

Safety and tolerability

In total, 6 patients (66.7%) experienced any intermittent adverse event during the study period. Most frequently reported adverse events were fatigue, rash, anorexia and cough (**Table 1**). All patients were able to continue treatment during the study. However, in four patients pirfenidone dosage was reduced due to gastrointestinal side effects (n=2) , skin rash (n=1) and dizziness (n=1) with good clinical effect, enabling patients to continue the treatment. In two patients, pirfenidone was temporarily reduced due to skin rash, which resolved after treatment and the patients were able to continue with 801 mg three times a day. No dose adjustments had to be made due to elevation of aminotransferases, bilirubin or other abnormalities in laboratory tests. Two patients were hospitalized during the study, one due to pneumonia, and one to angina, not related to the treatment.

In-hospital pulmonary function

In-hospital pulmonary function (FVC and DLCO), measured at baseline, start of pirfenidone, 12 and 24 weeks after start of treatment in 8 patients, remained stable before and during treatment

(**figure 1**). Baseline six minute walking test showed a median distance of 405 meters (IQR 335-448) and did not change significantly during the study period.

Table 1: weekly self-reported adverse events

Adverse events	Number of patients (%)
Fatigue	5 (55.6)
Rash	4 (44.4)
Anorexia	4 (44.4)
Cough	4 (44.4)
Headache	3 (33.3)
Insomnia	3 (33.3)
Dizziness	3 (33.3)
Nausea	3 (33.3)
Dyspepsia	3 (33.3)
Decrease in weight	2 (22.2)
Flatulence	1 (11.1)

Home spirometry

In the observation period, FVC significantly declined (slope -0.0017, SE 0.009, p=0.047). After start of treatment, FVC remained stable (slope -0.00021, SE 0.0007, p=0.76). The slopes before and after start of treatment did not significantly differ (p=0.14). Slopes of FVC (in liters) over time and corresponding 95% CI are displayed in **figure 2**. Data from the patient who died before start of treatment were excluded from this analysis.

Patient reported outcome measures (PROMs)

At baseline, mean K-BILD score was 46.8 (SD 7.5) and mean LCQ score was 14.6 (SD 3.0). During the study period, these PROMs did not demonstrate significant changes. Mean difference in K-BILD score between baseline and 24 weeks after start of medication was 2.3 points (95% CI -17.5 – 13.0), with a higher score after 24 weeks. Mean LCQ score decreased with 0.9 points (95% CI -4.7-6.4).

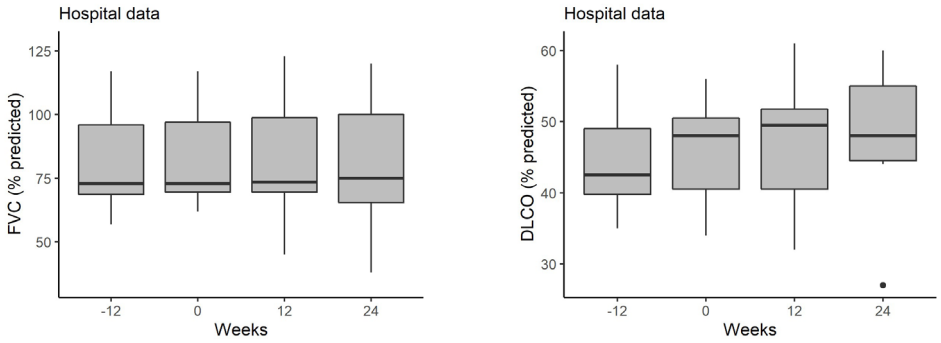

Figure 1: Boxplots of forced vital capacity (FVC) % predicted and diffusion capacity of the lungs for carbon monoxide (DLCO) % predicted, measured in hospital at start of the study, start of treatment with pirfenidone, 12 and 24 weeks after start of treatment. In total, n = 8 patients were included in the analysis of in-hospital measurements. At t = 0 and t = 24 weeks, pulmonary function data from n = 7 patients were available for analysis

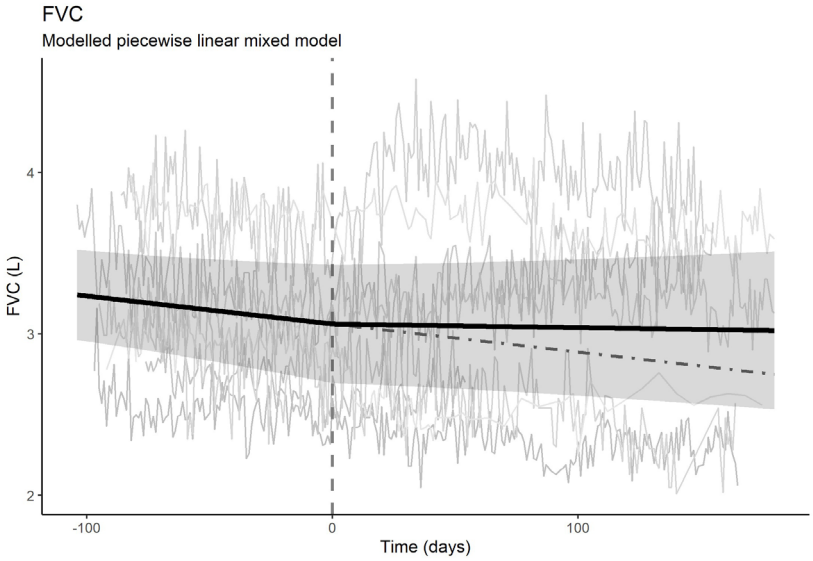

Figure 2: Repeated home spirometry FVC measurements in n=9 individual patients (grey lines) and measured median FVC slope (black line) with 95% CI (grey area), before and after initiation of pirfenidone (time=0). The dotted black line demonstrates the estimated continued FVC slope line without treatment.

DISCUSSION

The current study was the first to evaluate the safety and efficacy of pirfenidone in patients with asbestosis. No novel or unexpected adverse events were noted. Home spirometry demonstrated a decline in median FVC slope before start of pirfenidone and no decline during the treatment phase. This indicates that pirfenidone might attenuate FVC decline in progressive asbestosis. During the study, 6 patients (66.7%) reported any –mostly mild- adverse events, which is in line with real-world data in patients with IPF(13). In a recent phase 2 trial of pirfenidone in unclassifiable ILD, the most common adverse events related to the treatment were gastrointestinal (47% in the pirfenidone group compared to 26% in the placebo group), fatigue (13% versus 10%) and rash (10% versus 7%). A large post authorization study on long-term safety of pirfenidone in IPF patients describing adverse drug reactions of special interest, reported fatigue and photosensitivity reactions/skin rashes in 24.2% and 29.0% of patients respectively (13). In the current study, fatigue (55.6%) and rash (44.4%) were more frequently reported. A reason for this increase might be the way and frequency of AE reporting. Adverse events were actively collected using a weekly electronic questionnaire. In the current study, six patients were (temporarily) treated with a reduced dose of pirfenidone. The patients included in the current study were older (mean age of 74.7) compared to the post authorization study (mean age 69.6) and the mean baseline K-BILD score was lower than other clinical studies in IPF and progressive pulmonary fibrosis, which may reflect increased vulnerability of the current patient group(13-15). Older age is a known risk factor associated with discontinuation of pirfenidone due to adverse drug reactions(13). Importantly, six patients needed a dose reduction due to side effects, but all were able to continue treatment after (temporary) dose adjustment. This demonstrates the importance of patient guidance and pirfenidone dose adjustments as effective strategy to reduce side effects, so patients can continue their treatment.

Two patients died during the trial, not related to the treatment. One patient died of a respiratory cause before starting pirfenidone. The second patient discontinued pirfenidone because of worsening of his clinical situation with increasing shortness of breath despite optimal palliative care. He later died due to euthanasia. Both cases demonstrate the high vulnerability of patients with progressive asbestosis, highlighting the need for new and effective treatment.

Because asbestosis is a very rare occupational disease and only a subgroup of patients demonstrate a progressive decline in pulmonary function, inclusion of a large patient group was considered not feasible, and the study was not powered to detect any treatment effect with pulmonary function measurements. As expected, no significant changes in hospital pulmonary function (FVC, DLCO), six minute walking test or PROMs before and after start of pirfenidone could be demonstrated in the current trial. However, we also used daily home spirometry measurements to obtain a more granular overview of FVC change over time. Recent studies showed that daily home monitoring of FVC in pulmonary fibrosis provided a sensitive prediction

of disease behavior and correlated well with hospital-based measurements of pulmonary function(14, 16). In the current study, we compared slopes of home-based FVC before and after start of pirfenidone. A significant FVC decline was found before start of pirfenidone, while FVC did not decline during the six month treatment phase with pirfenidone. Although the FVC slopes before and after treatment did not significantly differ in this small group size (p=0.14), the home monitoring FVC data suggest pirfenidone reduces FVC decline in the current study with asbestosis patients with a progressive phenotype. These changes could not be captured by in-hospital measurements due to the limited sample size, which illustrates the potential value of frequent home-based measurements as exploratory endpoint in clinical trials, especially in rare diseases. Our findings are in contrast with the U-ILD study where home-spirometry failed as an endpoint due to technical and analytical reasons(12). Nevertheless, our current study as well as previous studies show that with good instructions and technical support reliable home-spirometry can be feasible. Our findings are in line with a previous study which showed that the use of home spirometry could reduce sample sizes for future trials (17). Besides, home monitoring can be used as a safety endpoint, as we did in the current study, with patients reporting symptoms and side-effects in an online home monitoring program. Home monitoring results were sent to the hospital in real-time, which allowed us to safely monitor at a distance, with a low burden for patients.

The main limitation of this study is the planned low number of included patients. Therefore, the primary endpoint of the study could only be descriptive, assessing safety and adverse events of pirfenidone in patients with asbestosis. Nevertheless, we believe this study adds valuable information to the field as it is the first to report prospectively on antifibrotic treatment specifically in this patient group. Moreover, it highlights the potential of online home monitoring as efficacy and safety endpoint in trials with a limited number of participants.

CONCLUSIONS

Our study demonstrates an acceptable safety and tolerability of pirfenidone in patients with asbestosis . Additionally, this study supports the concept that antifibrotic treatment with pirfenidone may slow down disease progression in patients with asbestosis and progressive lung function decline.

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List of abbreviations

CI – confidence interval
DLCO - diffusion capacity of the lung for carbon monoxide
FVC - forced vital capacity
6MWD - six minute walking test
ILD - interstitial lung disease
IPF - idiopathic pulmonary fibrosis
K-BUILD - King's Brief Interstitial Lung Disease
LCQ - Leicester Cough Questionnaire
NVALT - the Dutch Association of Pulmonologists
PROMs - Patient reported outcome measures

SUPPLEMENTARY MATERIAL

Inclusion criteria

Patients (40-85 years) with confirmed asbestosis by Dutch NVALT IPF expertise-panel AND a history of asbestos exposition with 15-30 years latency AND
pleural plaques OR asbestos fibers in pulmonary lavage OR asbestos fibers confirmed in lung biopsy)
AND

1. written informed consent
2. FVC ≥ 50% predicted, DLCO ≥ 25%
3. Minimal 6 minute walk test distance 150 meter
4. FEV1/FVC > 0.70
5. Documented disease progression in 3-6 months (FVC decrease > 5% in < 6 months or DLCOc decrease > 10% in 3-6 months, or decrease ≥ 25 meter on 6 minute walk test in < 3-6 months)
6. > 10% interstitial fibrosis on HRCT by visual scoring

Exclusion criteria

1. Current smoker
2. > 15% emphysema on HRCT thorax
3. Use of immunosuppressant prednisone> 10mg or other: methotrexate, azathioprine, cyclophosphamide
4. Malignancy
5. Renal impairment (GFR < 30 ml/min or dialysis)
6. Pregnancy
7. Concomitant use (<28 days) of a strong and selective inhibitor of CYP1A2 (Fluvoxamin, enoxacin)
8. History of hepatic impairment, elevation of transaminase enzymes, or the confirmation of any of the following liver function test criteria above the specified limits: Total bilirubin above the upper limit of normal (ULN), Aspartate aminotransferase (AST) or alanine aminotransferase (ALT) >1.5 × ULN, Alkaline phosphatase > 2.0 × ULN

CHAPTER 10

General discussion

GENERAL DISCUSSION

ILDs encompass a broad spectrum of ultra-rare to more common pulmonary disorders, classified together because most – but not all - affect the lung interstitium with a variable degree of inflammation and fibrosis (1). In this thesis, we focused on two groups of ILD, representing two sides of the inflammation-to-fibrosis disease spectrum. In **part 1**, we investigated the adaptive immune response in sarcoidosis, an inflammatory ILD with mostly favorable outcome. In **part 2**, we focused on pulmonary fibrosis, which is an irreversible ILD and usually has a poor prognosis (1). In both lung diseases, the pathogenesis is only partly understood and current therapeutic options are limited (1). Improved understanding of the pathogenesis of these ILDs may result in the identification of novel drug targets. In clinical care, the decision to start specific treatment for these lung diseases is particularly challenging given the scarcity of clinical trial data and the heterogeneity of disease behavior. Major unmet needs for people with sarcoidosis and pulmonary fibrosis include new and personalized treatment, taking prognosis and estimated treatment response into account. These limitations in clinical care underscore the need for biomarkers to estimate prognosis and guide therapeutic decision making at time of diagnosis, as well as novel techniques to measure treatment outcome and select patients with beneficial treatment effect in an early phase. Furthermore, novel animal models that accurately capture the biology of sarcoidosis and pulmonary fibrosis will increase our knowledge on pathophysiology and can be used to find therapeutic targets.

In this thesis, we focused on the adaptive immune responses and new personalized treatment in sarcoidosis and pulmonary fibrosis. We aimed to (**I**) identify new T cell biomarkers in sarcoidosis that may correlate with long-term prognosis or favorable response to specific treatment and (**II**) unravel the role of aberrantly activated T cells in sarcoidosis pathogenesis using a novel mouse model. Furthermore, we aimed to (**III**) investigate the safety and efficacy of antifibrotic therapy in progressive pulmonary fibrosis due to asbestosis in a small sample size study, using daily home monitoring of forced vital capacity (FVC).

In this discussion, the aims and results will be placed in the context of scientific literature and their contribution to our understanding of disease pathogenesis and improvement of personalized treatment is discussed. Furthermore, we will summarize outstanding research questions and suggest additional scientific studies to address them.

MAIN FINDINGS IN THIS THESIS

In **chapter 3**, we described our findings on the phenotype of peripheral blood (PB) T cell populations in treatment-naïve patients with sarcoidosis and healthy controls. We observed altered proportions and phenotypic characteristics of multiple PB T-cell subsets of patients. Several of these parameters correlated with 2-year disease outcome, particularly in naive $CD4^+$ T cells and regulatory T cells (Treg) (**Table 1**), but we did not identify a single T cell subset that would independently predict disease outcome. Additionally, we demonstrated an altered activation marker profile and cytokine production of naïve and memory PB T cells upon in vitro stimulation in sarcoidosis.

Stratifying patients with a high likelihood to benefit from first line glucocorticoid treatment is essential to optimize personalized care. In **chapter 4**, we evaluated frequencies and phenotypes of PB T cell populations in healthy controls and in newly diagnosed patients with pulmonary sarcoidosis before prednisone treatment started. We found significant correlations between the phenotype of PB T cells at baseline and ≥10% absolute forced vital capacity (FVC) increase at 3 and 12 months during prednisone treatment. In this study, increased proportions of programmed cell death protein 1 (PD-1)-positive cells and decreased proportions of $CD25^+$ cells within the $CD4^+$ memory T cell population, as well as increased fractions of PD-1^+ cells in the Treg population, correlated with a good treatment response (**Table 1**).

Importantly, several drugs including cancer treatment with antibodies that block co-inhibitory receptor cytotoxic T-lymphocyte antigen 4 (CTLA-4), can induce a granulomatous disease indistinguishable from sarcoidosis. CTLA-4 is an important suppressor of T-cell-mediated immune responses. In **chapter 5**, we reviewed the potential mechanisms involved in these drug-induced "sarcoidoses", which link aberrant T cell activation to sarcoidosis pathogenesis.

We previously implicated dysregulated expression of CTLA-4 on T cells in sarcoidosis pathogenesis, which provided the rationale for a phase II trial in chronic pulmonary sarcoidosis (ABASARC), investigating safety and efficacy of CTLA-4-immunoglobulin (Abatacept) treatment In **chapter 6**, we described increased baseline proportions of circulating T cell subsets that express the co-inhibitory receptor CTLA-4 in patients with therapy-refractory pulmonary sarcoidosis included in the ABASARC trial. Importantly, low proportions of CTLA-4^+ $CD4^+$ Tm, Treg and $CD8^+$ Tm and effector memory T cells re-expressing CD45RA (TEMRA) cell subsets correlated with favorable lung function response after one year (**Table 1**).

In **chapter 7**, we described our current findings on novel murine models with CTLA4 haplo-insufficiency as well as complete absence of CTLA-4 specifically on IL17-producing T cells or Tregs, whereby wild-type littermates served as controls. These experiments were prompted by earlier findings of reduced expression of CTLA4 in Th17 cells and Tregs in the BAL fluid and mediastinal lymph nodes of patients with sarcoidosis. Whereas the absence of CTLA4 on IL-17 producing cells was associated with spontaneous T cell activation, this was essentially not the

case for mice that were haplo-insufficient for CTLA4 in IL-17 producing cells. However, CTLA-4 haplo-insufficiency in Tregs was associated with increased proportions of $ICOS^+$ cells in major T cell populations. In our study, we demonstrated a reciprocal influence between CTLA-4 expression levels on Th17 cells and Tregs. Importantly, mice deficient for CTLA4 in IL-17-producing T cells did not show signs of sarcoidosis, nor autoimmunity during aging or in a collagen-induced arthritis model. We concluded that IL-17A-Cre-mediated CTLA-4 deletion might be a model valuable for investigating the function of CTLA-4 in Th17 cells, whereas FoxP3cre-mediated CTLA-4 reduction is a promising model to investigate the immune pathogenesis of sarcoidosis.

In **part 2** of this thesis, we focused on pulmonary fibrosis. Although the efficacy of antifibrotic treatment was demonstrated in a combined group of patients with a broad range of fibrosing lung diseases, the safety and effectiveness of antifibrotic treatment in selected ILD diagnoses remains less clear. In **chapter 9,** we described the results of a clinical trial investigating the safety and efficacy of antifibrotic treatment with pirfenidone in progressive asbestosis, a rare occupational ILD due to inhalation of asbestosis fibers. We found this treatment was safe and tolerability was acceptable. Furthermore, we demonstrated that daily home monitoring of FVC was able to suggest treatment efficacy in a small sample size trial by obtaining a more granular overview of lung function change before and after start of pirfenidone.

IN SUMMARY, OUR FINDINGS DEMONSTRATE

1. Altered proportions and phenotypic characteristics of multiple T-cell subsets in peripheral blood of sarcoidosis patients. Several of these parameters correlate with 2-year disease outcome.
2. Naïve and memory CD4 T cells in PB of sarcoidosis patients display aberrant cytokine production and activation marker profiles upon T cell receptor stimulation.
3. Increased baseline proportions of circulating $PD\text{-}1^+CD4^+$ memory and regulatory T cells in patients with sarcoidosis correlate with a favorable response to prednisone treatment at 3 months.
4. Increased baseline $PD\text{-}1^+$ and decreased $CD25^+$ $CD4^+$ memory T cell proportions in patients with sarcoidosis correlate with favorable treatment response to prednisone at 12 months.
5. Decreased CTLA-4 expression on multiple PB $CD4^+$and $CD8^+$ T cell subsets may identify therapy refractory sarcoidosis patients with favorable response to CTLA-4-Ig (abatacept).
6. A novel mouse model with CTLA-4-deficiency in IL17-producing T cells or CTLA-4 haplo-insufficiency in Tregs exhibits spontaneous immune activation. These mice may serve as a future sarcoidosis disease model.
7. Treatment with the antifibrotic drug pirfenidone in progressive asbestosis has an acceptable safety and tolerability profile and home spirometry data suggest this antifibrotic treatment might attenuate FVC decline.

PART 1

T CELL PHENOTYPE: FROM DISEASE PATHOGENESIS TO PERSONALIZED MEDICINE IN SARCOIDOSIS

The first hurdle on the road towards personalized treatment in sarcoidosis is the lack of a reliable prognostic biomarker to predict disease progression (2). Recent years have seen a growing amount of evidence indicating a central role of $CD4^+$ T helper (Th) cells in sarcoidosis pathogenesis (3). Our research group and others contributed to the shift from the perception of sarcoidosis as a Th1-cell driven disease to a Th17.1-dominated lung microenvironment that contributes to granulomatous inflammation (4-7). Previously, Th17.1 cell proportions in the lung were found to correlate with long term outcome (7), but fractions were not increased in peripheral blood (4). Moreover, substantial differences in the expression of activation markers on $CD4^+$ T cells in sarcoidosis BAL and blood were demonstrated (8), and reduced protein levels of CTLA-4 on Th17-cells and Tregs were specifically found in the mediastinal lymph node (MLN) compared to healthy individuals (9). These differences between the peripheral blood and inflamed organs in sarcoidosis argue for considering the circulation as an immunological compartment with distinctive Th cell characteristics. Tissue resident Th cells could potentially serve as prognostic biomarkers, but sampling from the MLN or lung are invasive procedures. It is currently unknown if circulating T cells from the peripheral blood can be used as clinical or therapeutic biomarkers. In our study described in **chapter 3**, we used technology that enables simultaneous detection of multiple cell surface or intracellular proteins to accurately subgroup PB T-cells and define their phenotype using specific markers indicative of cellular activation. This allowed for a more detailed characterization of PB T cells in sarcoidosis with the aim to find new prognostic T cell biomarkers. We demonstrated that many of these phenotypical T cell differences, particularly aberrantly activated naive $CD4^+$ T cells and Tregs, are observed in patients who develop chronic disease, and not resolution, at 2-year prospective follow-up. These findings have several implications for the field.

First, although we could not identify a single T cell marker that would predict disease outcome with high accuracy, a unique profile comprising aberrant phenotypes of multiple PB T cell subsets may serve as prognostic biomarker in the future. Because the high degree of clinical heterogeneity among sarcoidosis patients complicates the quest for prognostic PB biomarkers, a precision medicine approach may be further improved by preselecting patient subpopulations for future studies on PB T cell biomarkers. Aberrantly activated naïve PB $CD4^+$ T cells, particularly the proportions of CD25+ naïve T cells, correlated with sarcoidosis outcome in our study. We also found a remarkable concomitant increase in surface expression of CD95 and PD-1 on Tregs of patients with worse prognosis. Our research group has previously demonstrated that increased expression of CD95, the receptor for apoptosis inducer CD95-ligand,

on circulating Tregs makes them more susceptible to apoptosis in sarcoidosis (10). It is conceivable that these Treg characteristics will contribute to the impaired suppressive function that has been reported by various research groups (10). Interestingly, a previous study described restoration of Treg function associated with disease resolution (11).

Second, in vitro stimulation of both PB naïve and memory T cells resulted in an altered cytokine profile in sarcoidosis. An antigen-independent pre-activated state and altered cytokine profile of multiple circulating T cell populations, including naïve T cells, in combination with a reduced immunosuppressive capacity of circulating Tregs, likely contributes to ongoing systemic inflammation and non-resolving local disease in patients with worse prognosis. These aberrantly activated T cells may serve as valuable treatment targets in the future to decrease overall granulomatous inflammation or alter long term outcome.

The next hurdle on the road towards personalized treatment in sarcoidosis, is the lack of a reliable therapeutic biomarker for corticosteroid efficacy. Despite high incidence of prednisone side effects, glucocorticoids are currently recommended as first line therapy for all patients with a treatment indication (12). This one-size-fits-all approach can have significant toxicity, while therapeutic response is variable and unpredictable (12, 13). We addressed this clinical problem in **chapter 4**, in which we describe a prospective clinical biomarker study in treatment-naïve patients with pulmonary sarcoidosis, and found increased baseline proportions of circulating PD-1^{+}CD4^{+} memory and regulatory T cells in patients with sarcoidosis and good FVC response to prednisone at 3 months. Additionally, increased PD-1^{+} and decreased CD25^{+} CD4^{+} memory T cell proportions associate with favorable FVC response at 12 months. These findings indicate the potential of PD-1^{+} and CD25^{+} expression levels on circulating T cell populations as new biomarkers to predict glucocorticoid therapy response in pulmonary sarcoidosis. In contrast, we did not detect an association with treatment outcome for serum sIL2R, a biomarker currently used in clinical care, which is in line with a previous study that also found no relation between sIL2R and treatment response, radiographic evolution or lung function outcome (14). Another serum biomarker used in clinical practise is angiotensin converting enzyme (ACE), which has been proposed to assess the diagnosis and granulomatous burden in sarcoidosis. However, serum ACE has inadequate sensitivity and specificity to allow therapeutic decisions (15). The limitations of established biomarkers underscore the need for improved markers to facilitate personalized treatment strategies in sarcoidosis.

The remarkable PD-1^{high}CD25^{low} phenotype of CD4^{+} Tm cell population in good prednisone responders in our study may reflect a high antigen burden or indicate a selection of patients with less successful antigen clearance due to aberrant T cell activation, while the impaired immunosuppressive function of exhausted PD-1^{high} Tregs contributes to ongoing activation of circulating CD4^{+} Tm cells. However, the exact mechanisms by which specifically PD-1^{+}CD4^{+} Tm cells and PD-1^{+} Tregs predispose to good prednisone sensitivity in sarcoidosis need to be elucidated.

We suggest further research to (I) validate these findings in a second, larger cohort, (II) explore whether the association is specific for sarcoid granulomatous inflammation or reflects general mechanisms that associate with glucocorticoid sensitivity using inflammatory disease control groups and (III) correlate these markers with other relevant outcome parameters such as diffusion capacity, patient reported outcome measures and disease relapse at long term.

A complex feature of T cells is their phenotypical and functional plasticity, which provides them with the capacity to shift between multiple activation or differentiation states, even under physiological conditions. It is plausible that Th-cells undergo a switch during sarcoidosis disease course, gradually transitioning from pro- to anti-inflammatory phenotype. Our research outlined in **chapter 4** primarily focused on *baseline* T cell phenotypes as therapeutic biomarker, as well as the effects of therapy over time. It would be interesting to investigate if changes in T cell phenotype during the disease course enable the identification of patients who need early add-on treatment, before clinical worsening occurs. In order to investigate the correlation of phenotypical and functional changes of circulating T cells over time to other outcome measures, a large cohort size is needed.

Currently, a large randomized controlled trial to evaluate the effectiveness of methotrexate versus prednisone as first-line treatment for pulmonary sarcoidosis is carried out in the Netherlands (16). With the possibility to obtain measurements on various peripheral blood immune cells of patients within this well-designed longitudinal cohort, we will be in the unique position to address many outstanding research questions. Our flow cytometry analyses revealed that in sarcoidosis multiple T cell subpopulations in the circulation have aberrant expression of many molecules critically involved in cellular activation, whereby expression levels are typically heterogeneous within distinct T cell subsets. In this context, a logical next step will be to apply new genome-wide technology such as (single-cell) transcriptome analysis to identify novel biomarkers for prognostic and therapeutic purposes in a clinical trial setting. Such a line of research will likely be complicated, since the heterogeneity among individual immune cells is likely to be higher than the heterogeneity across sarcoidosis patients. Despite these difficulties, it will be invaluable to investigate (single-cell) transcriptome data of circulating immune cells in sarcoidosis to identify reliable diagnostic or treatment-outcome predictive biomarkers.

Previously, our research group demonstrated aberrant activation of Th17 and Treg cells in sarcoidosis, characterized by decreased expression of CTLA4 in the MLN and lung (9). CTLA-4 gene mutations are associated with immune dysregulation and granulomatous lung disease (17). Additionally, as highlighted in **chapter 5**, cancer treatment with antibodies that block CTLA-4 can induce a granulomatous disease indistinguishable from sarcoidosis, implicating the involvement of the CD28/CTLA-4 pathway in sarcoidosis pathogenesis. Tregs constitutively express CTLA-4 to occupy the CD28 ligands CD80/CD86 on antigen-presenting cells and dampen the T cell immune response (18). It is conceivable that decreased CTLA-4 expression on both Th17 and Treg cell subsets contributes to sustained local granulomatous inflammation in

sarcoidosis by enhancing Th17 activation and impairing Treg function (9). This hypothesis provided the rationale to investigate the safety and efficacy of recombinant CTLA-4-immunoglobulin (Ig) fusion protein (abatacept), which inhibits T cell activation by binding to CD80/CD86 on APCs and thereby depleting the CD28 ligand, in chronic sarcoidosis (19).

In **chapter 6**, we described that decreased proportions of CTLA-4-expressing T cell subsets correlate with therapeutic response to CTLA-4-Ig. We feel that this finding contributes to future personalized medicine, as phenotypic analysis of PB T cells may identify treatment-refractory patients with sarcoidosis that benefit most from this treatment. It is conceivable that in sarcoidosis, a disease primarily driven by antigen presentation, low CTLA-4 expression on T cells reflects higher CD80/CD86 ligand availability (18). We hypothesize that CTLA-4-Ig will have increased ligand-capturing potency particularly in this patient subgroup and therefore better clinical effect. Because of the substantial differences in proportions and phenotype of T cells between PB and chronically inflamed sites such as the MLN or lung (9), it would be interesting to explore correlations between CTLA-4-Ig treatment response and baseline expression of CTLA-4 on Th17 cells and Tregs in lung and draining lymph nodes. Along the same lines, future experiments should show whether treatment with CTLA4-Ig primarily dampens Th17 cell activity to restore Treg function, or acts on both cell populations.

CTLA-4 has a critical role in immune regulation, as demonstrated by the development of fatal autoimmune disease in full CTLA-4 knockout mice (20) and Treg-specific complete deficiency of CTLA-4 (21). To further elucidate the role of the CD28/CTLA-4 pathway in sarcoidosis pathogenesis, we made use of the Cre-loxP system to develop a murine model with a selective absence of CTLA-4 specifically on IL-17-producing T cells, as well as CTLA4 haplo-insufficiency for either IL-17-producing T cells or Tregs. Hereby, a single-Cre recombinase recognized two directly repeated LoxP sequences, and subsequently excises the DNA that is flanked by the LoxP sites, also referred to as 'floxed' DNA. $CTLA4^{fl/fl}$ mice were crossed with IL17A-cre transgenic mice (targeting IL-17A-producing cells) or foxP3-cre transgenic mice (targeting Treg cells). In **chapter 7**, we described that homozygous *IL17A-Cre*$^{+}$$CTLA4^{fl/fl}$ mice with complete absence of CTLA-4 on IL-17-producing T cells show signs of spontaneous T cell activation compared to heterozygous *IL17A-Cre*$^{+}$$CTLA4^{fl/+}$ mice and wild-type littermates, as well as increased Th17 cell numbers. This was not demonstrated in mice that were haplo-insufficient for CTLA4 in IL-17 producing cells. Importantly, CTLA-4 haplo-insufficiency in Tregs showed signs of spontaneous T cell activation, as demonstrated by increased proportions of $ICOS^{+}$ cells in major T cell populations. We demonstrated a reciprocal influence between CTLA-4 expression levels on Th17 cells and Tregs.

CTLA-4 is highly expressed on Tregs and their engagement with CD80/86 on APCs will lead to an environment less conducive to T cell activation (22). Deficiency of CTLA-4 specifically in Tregs will promote CD28 co-stimulation, which will exaggerate the immune response and lead to fatal T cell mediated autoimmune disease (21). A reduction of CTLA-4 on Tregs is therefore likely

to induce T cell activation in CTLA-4 deficient FoxP3cre mice. While the role of CTLA-4 on Tregs has been extensively studied, its role on Th17 cells remains less understood. Th17 cells express the highest CTLA-4 levels on their surface compared to other Th cell subsets (23). Additionally, CTLA-4 on Th17 cells was found to be involved in Th17 cell differentiation and proliferation, and disrupting the interaction between CTLA-4 and CD80/86 on APCs will enhance Th17 cell differentiation (24). Hence, it is understandable that elevated Th17 cell numbers were observed in IL-17A-Cre-mediated CTLA-4 deficient mice. The precise mechanism driving the immune phenotype observed in IL-17A-Cre-mediated CTLA-4 deletion remains elusive, with questions surrounding whether reduced CTLA-4 levels on Tregs or Th17 cells are responsible (25).

Experimental transgenic murine models to study pathways implicated in sarcoidosis are scarce. Unfortunately, many limitations remain to existing murine disease models as they do not fully capture clinical aspects of sarcoidosis (26). Previously, spontaneous granulomas were found in lung, liver and lymph nodes in a mouse model, in which deletion of the gene encoding tuberous sclerosis 2 (*Tsc2*) activated the metabolic checkpoint kinase mTORC1 in myeloid cells (27). It is conceivable that macrophage-intrinsic pathways initiate granuloma formation, but the contribution of spontaneous T cell activation and Th17 polarization to sarcoidosis phenotype and chronicity remain elusive in this model. Therefore, the *Tsc2* mouse does not appear to be a good tool to study T cell dysfunction in sarcoidosis. In contrast, our in CTLA-4 deficient IL17A and FoxP3cre murine model provides unique opportunities to address critical research questions centered around T cells, particularly when combined with an existing murine pulmonary granuloma model using exposure to trehalose 6,6'-dimycolate (TDM, cord factor) of mycobacterium tuberculosis (28, 29). In our study, mice deficient for CTLA4 in IL-17-producing T cells did not show signs of sarcoidosis, nor autoimmunity during aging or in a collagen-induced arthritis model. We are currently investigating the effects of TDM exposure in *IL17A-Cre*$^{+}$CTLA4$^{fl/fl}$ and *FoxP3Cre*$^{+}$CTLA4$^{fl/+}$ mice. Based on the data described in **chapter 7**, we hypothesized that TDM exposure in our murine model will lead to more pronounced and chronic granulomatous inflammation compared to controls. If our hypothesis is confirmed, the disease model may be suitable for sarcoidosis drug discovery, which is an unmet need in the field of sarcoidosis research.

Additionally, if an exaggerated antigen-driven immune response induces the formation of disease-typical granulomas, we will be able to investigate other proposed disease triggers in our murine model (30). Biopersistent and inorganic antigen exposure is implicated as a disease trigger (31) and organs with direct contact to the external environment including lungs, skin and eyes are most commonly affected in sarcoidosis (32). Interestingly, sarcoid skin granuloma's have been reported in relation to exposition to acrylic and nylon fibers (33). We therefore hypothesize that microplastics (MP), which are found in abundance in the human environment (34), may lead to granulomatous inflammation in susceptible patients. Importantly, it is now clear that MP can persist in human lungs and the cytokines expressed by human monocytes and DCs

ex vivo in response to MP exposure, including TNF-α, are also central in sarcoid granulomatous inflammation (35). We currently aim to investigate the role of inhaled MP using this murine model. More specifically, we will explore whether exposure to inorganic MP triggers an increased $CD4^+$ Th cell response and granuloma formation in the lungs of in *IL17a-Cre*$^+$CTLA4$^{fl/fl}$ mice that resembles sarcoidosis.

Outstanding questions

1. Does the aberrant phenotype of multiple T cell subsets in sarcoidosis reflect primary defects in this cell lineage or does it result from pro-inflammatory external signals?
2. Can the PB T cell phenotype of patients be used as a powerful biomarker to predict favorable response to prednisone specifically in sarcoidosis or can it be applied to predict glucocorticoid sensitivity in other diseases?
3. Does PB T cell phenotype correlate with patient reported outcome measures and long-term disease relapse in sarcoidosis?
4. Are changes in PB T cell phenotype during sarcoidosis disease course able to identify patients who benefit most from treatment?
5. Does the expression of CTLA-4 on Th17 cells and Tregs in lung and draining lymph nodes identify sarcoidosis patients with favorable response CTLA-4-Ig treatment?

		Relative baseline proportions and surface marker expression in sarcoidosis				
		$CD4^+$ cell	Regulatory T cell	$CD8^+$ TEMRA	$CD8^+$ γδ T cell	$CD4^+$ Naive T cell
2 year disease outcome	active chronic sarcoidosis	↓proportions ↑ CTLA4	↑ proportions ↑ CD25 ↑ CTLA4 ↑ CD95 ↑ PD1	=	↑ proportions ↑ CD69	↑ CD25 ↑ CTLA4
	non-active chronic sarcoidosis	=	↑ CD25 ↑ CD69 ↑ PD1	=	↑ proportions ↑ CD69	↑ CTLA4 ↑ CD69
	resolution	=	↑ CD69	=	=	↑ CTLA4
Therapeutic response	favorable response to prednisone	↑ PD1 ↑ PD1/CD25	↑ PD1 ↑ PD1 /CD25			
	favorable response to CTLA-4 Ig	↓ CTLA4 ↓ CTLA-4/CD28	↓ CTLA4 ↓ CTLA-4/CD28	↓ CTLA4 ↓ CTLA-4/CD28		

Table 1: Summary of our reported findings on the association of circulating T cell proportions and phenotype with long term prognosis and therapeutic response in patients with sarcoidosis.

6. Will exposure to trehalose 6,6'-dimycolate in mice with altered CTLA4 expression in Th17 cells or Tregs lead to more pronounced and chronic granulomatous inflammation compared to controls?
7. Can exposure to microplastics via the airways induce sarcoidosis in humans?
8. Will exposure to microplastics lead to granuloma formation in mice, and will the disease burden be more severe in mice with altered CTLA4 expression in T cells?

PART 2

TREATMENT OF PROGRESSIVE PULMONARY FIBROSIS

Besides the development of new antifibrotic treatments, strategies to improve and optimize timing of current therapy in selected ILD patients are also warranted (36). Clinical heterogeneity and small patient numbers pose a challenge to generate evidence for rare diseases such as sarcoidosis and pulmonary fibrosis. Daily home monitoring of FVC has the potential for early recognition of disease progression or response to new treatment in an individual patient, as well as demonstrating treatment efficacy in small sample size clinical trials (37). In **chapter 9,** we described the results of a clinical trial investigating antifibrotic treatment with pirfenidone in progressive asbestosis, which was found safe with acceptable tolerability. Previously, the analytical impact of weekly home monitoring of FVC as clinical endpoint in IPF patients was assessed (38). Based on the obtained data during this 24-week study, it was estimated that a study in IPF using FVC as primary endpoint would require 951 patients using weekly FVC home measurement, while 3840 patients would be needed using hospital based FVC measurement at week 1 and 24 only. Enhanced power and reduction in sample size with FVC home monitoring is possible due to the ability to obtain multiple data points and improvement of analytical efficiency (38). In our small sample size study in asbestosis, daily home monitoring of FVC was indeed able to suggest treatment efficacy by obtaining a more granular overview of lung function change before and after start of pirfenidone. In contrast, in-hospital measurements of pulmonary function did not detect significant treatment effect. Future design of trials in pulmonary fibrosis should incorporate new endpoints that are meaningful to patients to enhance quality of life and survival (39). However, despite international discussion on the future of clinical trial in pulmonary fibrosis (39), home monitoring of FVC is currently not regarded feasible to replace hospital-based FVC measurement due to lack on agreement of analytical issues. Despite technical challenges that need to be overcome before home monitoring can be widely implemented in clinical care (37), it might be applied in the near future to improve personalized treatment in patients with ILDs such as sarcoidosis and pulmonary fibrosis. Currently, a nationwide study is investigating the implementation and impact on health outcomes of home monitoring in clinical ILD care in the Netherlands (40).

Outstanding questions

1. Does home monitoring of FVC identify patients with progressive pulmonary fibrosis in an early stage to guide timely antifibrotic treatment?
2. Can home monitoring of FVC provide data that reveal efficacy of antifibrotic treatment in progressive fibrotic ILDs, such as pulmonary fibrosis due to sarcoidosis?

CONCLUDING REMARKS

In this thesis, we focused on the adaptive immune responses and novel personalized treatment in sarcoidosis and pulmonary fibrosis. Increased understanding of disease pathogenesis enables development and evaluation of new targeted treatments. Although many aspects of the role of the adaptive immune system in sarcoidosis are still being unraveled, this thesis showed that in-depth phenotyping circulating T cells results in the identification of candidate biomarkers for prognosis and response to treatment. Preclinical murine models incorporating important features of ILDs, such as aberrantly activated T cells in sarcoidosis, improve our knowledge on disease pathogenesis and may accelerate drug discovery research. Combining preclinical data with new strategies to accurately measure response to treatment such as home spirometry, is expected to transform a *one-size-fits-all* approach to a more detailed patient stratification and robust *personalized treatment* of patients with sarcoidosis and pulmonary fibrosis in the near future.

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10

APPENDICES

English summary
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ENGLISH SUMMARY

Interstitial lung diseases (ILDs) comprise a broad spectrum of ultra-rare to more common pulmonary disorders. They are classified together because most – but not all - affect the lung interstitium with a variable degree of inflammation and fibrosis. In this thesis, we focus on the adaptive immune responses and new personalized treatment in two relatively common groups of ILD: sarcoidosis (**part 1**) and pulmonary fibrosis (**part 2**).

Part 1

Sarcoidosis is a systemic granulomatous disease of unknown cause, mostly affecting the lungs and lymph nodes, with an unpredictable disease course and variable response to therapy. The disease-typical granulomas contain a core of epithelioid cells and multinucleated giant cells and a rim of T cells and a few B cells (3). No definite cure for sarcoidosis exists and treatment with prednisone is initiated in case of (risk of) organ dysfunction or functional impairment with high symptomatic burden. However, evidence on the best treatment strategy is lacking and prolonged courses of steroids can lead to deleterious side effects. Reliable biomarkers to estimate long-term prognosis or response to therapy are an unmet need to guide the initiation and steroid tapering and/or switching to additional treatment strategies, reducing side effects and improving quality of life for patients.

The complex immunopathogenesis of sarcoidosis is described in **chapter 2**. The development of sarcoid granulomas involves close interactions between T cells and antigen-presenting cells such as macrophages and dendritic cells. The exact cause and triggers of sarcoidosis are still unknown, but the current concept is an exaggerated antigen-driven immune response. The increase of $CD4^+$ T helper (Th) 17.1 cells in the lungs and lymph nodes, specifically in patients with worse prognosis, implicates these cells as the primary drivers of inflammation. An increase in Th17.1 proportions is also found in peripheral blood in sarcoidosis, but it is currently unknown if circulating T cells from the peripheral blood can be used as clinical or therapeutic biomarkers.

Our first aim was to identify characteristics of circulating T cells that would distinguish patients with sarcoidosis from healthy controls and would correlate with long-term prognosis. In a prospective study described in **chapter 3**, we performed an in-depth phenotypic characterization of PB T cells in treatment-naïve sarcoidosis patients and healthy controls (HCs), with a specific focus on surface expression of activation makers. We observed altered proportions and phenotypic characteristics of multiple PB T-cell subsets of patients at study inclusion. In particular naïve $CD4^+$ T cells displayed an activated phenotype with increased CD25 expression in patients with active chronic disease at 2-year follow-up. A distinctive Treg phenotype with increased expression of CD25, CTLA4, CD69, PD-1 and CD95 correlated with development of chronic sarcoidosis. However, we did not identify a single T cell subset that would independently predict disease outcome. Additionally, we demonstrated altered activation marker profiles and

cytokine production of naïve and memory PB T cells upon in vitro stimulation in sarcoidosis.

Next, we investigated the potential of PB T cell parameters as therapeutic biomarkers, as described in **chapter 4**. Prednisone is recommended as first line therapy in sarcoidosis with a treatment indication. However, the treatment response is highly variable and prolonged prednisone use can have significant toxicity and side effects. There is an unmet need to develop biomarkers that predict therapeutic response. We evaluated baseline frequencies and phenotypes of circulating T cell populations in newly diagnosed patients with pulmonary sarcoidosis before standardized prednisone treatment was initiated and correlated these T cell parameters with lung function response at 3 and 12 months. We demonstrated significant correlations with the phenotype of PB T cells at baseline and ≥10% absolute forced vital capacity (FVC) increase during prednisone treatment in pulmonary sarcoidosis. In this study, increased proportions of PD-1$^+$ cells and decreased proportions of CD25$^+$ cells within the CD4$^+$ memory T cell (Tm) population, as well as increased fractions of PD-1$^+$ cells in the regulatory T (Treg) cell population, correlated with a good treatment response.

Another link between aberrantly activated T cells and sarcoidosis is highlighted by the rare occurrence of a drug-induced systemic granulomatous disease that is clinically and histopathologically indistinguishable from sarcoidosis. These drug-induced sarcoid-like reactions (DISRs) occur after initiation of the offending drug and usually resolve after its discontinuation. Most of the associated classes of drugs are able to alter a delicate immune balance in susceptible patients. An example is the treatment with immune checkpoint inhibitors targeting expression of the co-inhibitory surface marker cytotoxic T-lymphocyte antigen 4 (CTLA-4). Anti-tumor T cell responses are enhanced through blockage of co-inhibitory signals, but the immune activation may also lead to immune-related adverse events. Expansion of pre-existing Th17 subsets or altered Th17 polarization during treatment with the pharmacological trigger probably contribute to DISR immunopathogenesis. An in-depth overview of drug categories associated with DISRs is given in **chapter 5**. Unraveling the immunological mechanisms involved greatly improves our understanding of sarcoidosis pathogenesis and may potentially contribute to the prevention of DISRs.

T cell activation requires a signal from the antigen-specific T cell receptor in combination with a co-stimulatory signal, provided by the binding of cluster of differentiation (CD)28 on T cells and CD80 and CD86 on antigen presenting cells (APC). Upon activation, T cells express CTLA-4, which competes with CD28 for binding to CD80/CD86 on APCs with higher affinity. Abatacept, a recombinant CTLA-4-immunoglobulin fusion protein, inhibits T cell activation by binding to CD80/CD86 on APCs and thereby occupying the CD28 ligand. In **chapter 6**, we describe our findings in a subset of participants of the prospective open-labelled phase II trial to assess the safety and efficacy of abatacept treatment in chronic, steroid-refractory sarcoidosis. Baseline T cell phenotypes and activation marker expression were analyzed in relation to one-year pulmonary function response during this novel treatment. We found increased baseline proportions of circulating T cell subsets that express the co-inhibitory receptor CTLA-4 in patients

with therapy-refractory pulmonary sarcoidosis. Interestingly, low proportions of CTLA-4$^+$ T cells subsets correlated with favorable lung function response after one year treatment.

Together, our data from **chapter 3, 4 and 6** demonstrate that phenotypic characteristics of PB T cells hold promise as potential biomarkers in sarcoidosis that may associate with long term prognosis and response to specific treatment and therefore contribute to personalized medicine in the future.

Although many aspects of the complex immunopathogenesis still need to be unraveled, identification of crucial immune reactions in sarcoidosis will help identify new treatment targets. Transgenic mouse models to study pathways implicated in sarcoidosis are scarce, and new models that capture important clinical aspects of the disease are warranted for drug discovery. Previously, we implicated aberrantly activated Th17 and Treg cell subsets with dysregulated CTLA-4 expression in lymph nodes and lungs of patients with sarcoidosis. CTLA-4 has a critical role in immune regulation and full CTLA-4 knockout mice (166) as well as mice with Treg-specific deficiency of CTLA-4 develop fatal autoimmune disease (167). To further elucidate the role of the CD28/CTLA-4 pathway in sarcoidosis pathogenesis, we made use of the Cre-loxP system to develop murine models with a selective absence of CTLA-4 specifically on IL-17-producing T cells, as well as CTLA4 haplo-insufficiency for either IL-17-producing T cells or Tregs (**chapter 7**). Mice with a complete deficiency of CTLA4 in their IL-17-expressing cells showed features of spontaneous T cell activation, compared to CTLA-4 haplo-insufficient and wild-type littermates. However, they did not show signs of sarcoidosis, nor autoimmunity during aging or in a collagen-induced arthritis model. CTLA-4 haplo-insufficiency in Tregs showed signs of spontaneous T cell activation, as demonstrated by increased proportions of ICOS$^+$ cells in major T cell populations. We demonstrated a reciprocal influence between CTLA-4 expression levels on Th17 cells and Tregs. Based on these data, we hypothesize that exposure to trehalose 6,6'-dimycolate, an existing trigger for the induction of sarcoid granulomas in mice, will lead to more pronounced and chronic granulomatous inflammation in mice with a selective CTLA4-deficiency in IL-17-producing T cells and CTLA-4 haplo-insufficiency in Tregs, compared to controls. We are currently investigating if these mice may serve as future sarcoidosis disease model.

There is an unmet need for new treatment options in sarcoidosis. In **chapter 8**, we therefore discuss potential therapy and future strategies based on the latest immunological findings.

PART 2

Interstitial lung diseases (ILDs) are categorized based on underlying cause and many ILDs can eventually lead to progressive pulmonary fibrosis, which is characterized by unopposed deposition of collagen and extracellular matrix in the lung, progressive clinical worsening and a very poor prognosis (143). The archetypal progressive fibrotic ILD is idiopathic pulmonary fibrosis (IPF). Currently, two drugs are available for the treatment of pulmonary fibrosis, which are nintedanib for all forms of progressive pulmonary fibrosis and pirfenidone for idiopathic

pulmonary fibrosis (IPF) (144). Antifibrotic treatment will slow down disease progression but does not stop or reverse the process (144).

We aimed to investigate the safety, tolerability and efficacy of the antifibrotic drug pirfenidone in asbestosis patients with a progressive phenotype in a multicenter prospective study performed by the Dutch Association of Pulmonologists (NVALT). Pirfenidone slows down disease progression in IPF, but its efficacy and safety in other forms of pulmonary fibrosis is less known. In **chapter 9**, we describe our study in which we used daily home monitoring of FVC before and after the star of antifibrotic treatment to obtain a more granular overview of lung function change over time which suggested treatment efficacy in this trial. We demonstrated that daily home monitoring of lung function can identify treatment efficacy in pulmonary fibrosis earlier, despite a small sample size and has the potential to make clinical trials more robust.

Finally, we integrated the findings of this thesis in a general discussion in **chapter 10.** We showed that in-depth phenotyping circulating T cells in sarcoidosis results in the identification of potential biomarkers for prognosis and response to treatment. Preclinical murine models incorporating important features of ILDs, such as aberrantly activated T cells in sarcoidosis, improve our knowledge on disease pathogenesis and may accelerate drug discovery research. Together, new biomarkers, increased knowledge on disease pathogenesis and alternative strategies to measure response to treatment such as home spirometry, will transform a *one-size-fits-all* approach to a more detailed patient stratification and robust *personalized treatment* of patients with sarcoidosis and pulmonary fibrosis in the near future.

NEDERLANDSE SAMENVATTING

Interstitiële longziekten (ILDs) omvatten een breed spectrum van uiterst zeldzame tot vaker voorkomende longziekten. Ze worden samen geclassificeerd omdat de meeste – maar niet allemaal– het interstitium van de long beïnvloeden met een variabele mate van ontsteking en verlittekening (fibrose). In dit proefschrift richten we ons op de immuunrespons van het verworven afweersysteem en nieuwe gepersonaliseerde behandelingen van twee relatief veelvoorkomende ILDs: sarcoïdose (**deel 1**) en longfibrose (**deel 2**).

DEEL 1

Sarcoïdose is een systemische granulomateuze ziekte waarbij de longen en lymfeklieren veelal aangedaan zijn. De voor deze ziekte typische granulomen bevatten een kern van epithelioïde cellen, antigeen presenterende cellen (APCs), meerkernige reuscellen en een rand van T cellen en enkele B-cellen. De oorzaak van sarcoïdose is niet precies bekend en de ziekte wordt gekenmerkt door een onvoorspelbaar ziekteverloop en variabele respons op therapie. Er bestaat geen behandeling voor sarcoïdose die de ziekte geneest en prednison wordt gestart bij (risico op) orgaandysfunctie of klachten met een functionele beperking. Er is een grote behoefte aan betrouwbare biomarkers om de langetermijnprognose of respons op therapie in te schatten. Bijvoorbeeld om de start van behandeling, het afbouwen van steroïden en/of aanvullende behandelingsstrategieën te begeleiden, bijwerkingen te verminderen en de kwaliteit van leven voor patiënten te verbeteren.

De complexe immunopathogenese van sarcoïdose wordt beschreven in **hoofdstuk 2**. De ontwikkeling van granulomateuze ontsteking bij sarcoïdose omvat nauwe interacties tussen T cellen en APCs, zoals macrofagen en dendritische cellen. De exacte oorzaak of triggers van sarcoïdose zijn nog onbekend, maar het huidige concept is een antigeen-gedreven immuunrespons. De toename van $CD4^+$ T-helper (Th) 17.1-cellen die in de longen en lymfeklieren van patiënten met sarcoïdose wordt gezien, specifiek bij een slechtere prognose, wijst er op dat deze cellen de ontsteking stimuleren. In het perifere bloed (PB) wordt ook een toename van Th17.1 cellen gevonden bij patiënten, maar het is momenteel nog onbekend of eigenschappen van T cellen in het PB kunnen worden gebruikt als klinische of therapeutische biomarkers.

Ons eerste doel was om circulerende T cellen te identificeren die patiënten met sarcoïdose kunnen onderscheiden van gezonde controles en die correleren met de prognose van de ziekte. In een prospectieve studie, beschreven in **hoofdstuk 3**, voerden we een diepgaande fenotypische karakterisering uit van PB T cellen bij onbehandelde patiënten met sarcoïdose en gezonde controles, waarbij we specifiek de expressie van T cel activatiemarkers bekeken. In dit onderzoek vonden we dat fenotypische kenmerken van meerdere PB T-cel subsets van patiënten bij de inclusie van de studie anders waren dan die van de gezonde controles. Naïeve $CD4^+$ T cellen vertoonden bij diagnose al een geactiveerd fenotype met verhoogde CD25-expressie, vooral bij

patiënten die na twee jaar nog actieve chronische ziekte hadden. Een onderscheidend fenotype van regulatoire T cellen (Treg), met verhoogde expressie van CD25, CTLA4, CD69, PD-1 en CD95, correleerde met het ontwikkelen van chronische sarcoïdose. We konden echter geen geen enkele T-cel subset identificeren die de ziekteuitkomst betrouwbaar kan voorspellen. Daarnaast vonden we veranderde activatiemarkers en cytokineproductie van naïeve en memory T cellen uit het bloed van patiënten ten opzichte van controles bij in vitro stimulatie van deze cellen.

Vervolgens onderzochten we PB T cellen en hun fenotype als therapeutische biomarkers, zoals beschreven in **hoofdstuk 4**. Prednison wordt aanbevolen als eerstelijnstherapie bij sarcoïdose met een behandelindicatie. De respons op deze behandeling is echter zeer variabel en langdurig prednisongebruik kan significante toxiciteit en bijwerkingen geven. Er is behoefte aan biomarkers die de kans op therapeutische respons voorspellen. We evalueerden daarom percentages en fenotypes van circulerende T cel populaties bij nieuw gediagnosticeerde patiënten met pulmonaire sarcoïdose voordat de behandeling met prednison werd gestart. Vervolgens correleerden we deze T cel parameters met de longfunctie respons na 3 en 12 maanden. We vonden significante correlaties tussen het fenotype van PB T cellen bij diagnose en ≥10% absolute toename in geforceerde vitale capaciteit (FVC) na 3 maanden behandeling met prednison. In deze studie correleerde de verhouding tussen verhoogde PD-1$^+$ cellen en verlaagde CD25$^+$ cellen binnen de CD4$^+$ memory T-cel (Tm) populatie, evenals verhoogde fracties van PD-1$^+$ cellen in de Treg cel populatie met een goede respons op behandeling met prednison.

Geneesmiddelen kunnen een systemische granulomateuze ziekte veroorzaken die klinisch en histopathologisch niet te onderscheiden is van sarcoïdose. Deze door geneesmiddelen geïnduceerde sarcoïde-achtige reacties (DISRs) treden op na de start van het betreffende geneesmiddel en verdwijnen meestal na het staken ervan. De geneesmiddelen die geassocieerd zijn met DISR kunnen een delicate balans van het immuunsysteem bij gevoelige patiënten verstoren. Een voorbeeld hiervan is de behandeling met immunotherapie bij kanker die zich richt op co-inhiberende signalen zoals cytotoxische T lymfocyt antigeen 4 (CTLA-4). T cellen die zich richten tegen een tumor worden geactiveerd door blokkering van deze co-inhiberende signalen, maar deze immuunactivatie kan ook leiden tot immuungerelateerde bijwerkingen. Een toename van Th17 cellen of veranderde Th17 polarisatie tijdens de behandeling met geneesmiddelen dragen waarschijnlijk bij aan het ontstaan van DISRs. Een uitgebreid overzicht van geneesmiddelen die geassocieerd zijn met het ontstaan van DISRs wordt besproken in **hoofdstuk 5**. Het ontrafelen van de immunologische mechanismen die betrokken zijn bij het ontstaan van DISRs, verbetert ook ons begrip van de pathogenese van sarcoïdose en kan mogelijk bijdragen aan de preventie van DISRs.

Het activeren van een T cel vereist een signaal van de antigeenspecifieke T cel receptor in combinatie met een co-stimulerend signaal, geleverd door de binding van CD28 op T cellen, en CD80 en CD86 op APCs. Na activatie van T cellen wordt CTLA-4 tot expressie gebracht, dat met hogere bindingscapaciteit concurreert met CD28 voor binding aan CD80/CD86 op APCs.

Abatacept, een recombinant CTLA-4-immunoglobuline fusie-eiwit, remt T cel activatie door binding aan CD80/CD86 op APCs waardoor CD28 op T cellen niet meer goed kan binden. In **hoofdstuk 6** beschrijven we onze onderzoeksbevindingen in een subgroep van deelnemers aan de prospectieve open-label fase II studie naar de veiligheid en werkzaamheid van abatacept voor chronische, steroïd-refractaire sarcoïdose. T cel fenotypes en activatiemarkers werden geanalyseerd bij start van de studie en gecorreleerd met de longfunctie respons na 1 jaar behandeling. We vonden dat verschillende circulerende T cel subsets bij patiënten met therapieresistente pulmonaire sarcoïdose meer CTLA-4 tot expressie brengen dan controles. Daarnaast correleerde een lage expressie CTLA-4 op verschillende T cel subsets in sarcoïdose met een gunstige longfunctierespons na een jaar behandeling met abatacept.

Onze gegevens uit **hoofdstukken 3, 4 en 6** tonen aan dat fenotypische kenmerken van circulerende T cellen potentieel hebben als biomarkers in sarcoïdose, zowel met betrekking tot prognose alsmede de respons op specifieke behandeling. T cellen uit het bloed kunnen mogelijk bijdragen aan *gepersonaliseerde behandeling* in de toekomst.

Hoewel veel aspecten van de complexe immunopathogenese van sarcoïdose nog moeten worden ontrafeld, zal de identificatie van onderdelen van deze pathogenese helpen om nieuwe aangrijpingspunten voor behandeling te vinden. Transgene muismodellen om de immunopathogenese van sarcoïdose te bestuderen zijn schaars. Nieuwe modellen die belangrijke klinische aspecten van de ziekte omvatten zijn nodig voor het ontwikkelen van nieuwe geneesmiddelen. Onze onderzoeksgroep heeft eerder al laten zien dat abnormaal geactiveerde Th17 en Treg cel subsets met ontregelde CTLA-4 expressie aanwezig zijn in lymfeklieren en longen van patiënten met sarcoïdose. CTLA-4 speelt een cruciale rol in immuunregulatie en CTLA-4 *knockout* muizen, evenals muizen met Treg-specifieke deficiëntie van CTLA-4, ontwikkelen een fatale auto-immuunziekte. Om de rol van de CD28/CTLA-4 pathway in de pathogenese van sarcoïdose verder te onderzoeken hebben we gebruik gemaakt van het Cre-loxP systeem om muismodellen te ontwikkelen met een selectieve afwezigheid van CTLA-4 op IL-17-producerende T cellen, evenals CTLA4 haplo-insufficiëntie voor zowel IL-17-producerende T cellen als Tregs (**hoofdstuk 7**). Muizen met een volledige deficiëntie van CTLA-4 in hun IL-17-producerende cellen vertoonden kenmerken van spontane T-cel activatie, in vergelijking met CTLA-4 haplo-insufficiënte en controle muizen. Ze vertoonden echter geen tekenen van sarcoïdose, noch auto-immuniteit tijdens veroudering of in een collageen-geïnduceerd artritis model. Muizen met CTLA-4 haplo-insufficiëntie in Tregs vertoonde ook tekenen van spontane T cel activatie met toegenomen aantallen $ICOS^+$ cellen in T-cel subpopulaties. We vonden een wederzijdse associatie tussen CTLA-4 expressieniveaus op Th17 cellen en Tregs. Op basis van deze gegevens uit het muismodel denken we dat blootstelling aan trehalose 6,6'-dimycolaat, een bestaande trigger voor de inductie van sarcoïde granulomen in muizen, zal leiden tot meer uitgesproken en chronische granulomateuze ontsteking in muizen met een selectieve CTLA-4 deficiëntie in IL-17-producerende T cellen of CTLA-4 haplo-insufficiëntie in Tregs, vergeleken met controles. We onderzoeken momenteel of deze muizen als toekomstig sarcoïdose ziektemodel kunnen dienen.

Er zijn betere en nieuwe behandelingsopties nodig voor sarcoïdose. In **hoofdstuk 8** bespreken we daarom potentiële therapieën en toekomstige behandelstrategieën op basis van de nieuwste immunologische inzichten.

DEEL 2

Interstitiële longziekten (ILDs) worden ingedeeld op basis van onderliggende oorzaak en veel ILDs kunnen uiteindelijk leiden tot progressieve longfibrose. Dit wordt gekenmerkt door voortdurende collageen- en extracellulaire matrixdepositie in de long, progressieve klinische verslechtering en een zeer slechte prognose. De meest klassieke progressieve fibrotische ILD is idiopathische pulmonale fibrose (IPF). Momenteel zijn er twee geneesmiddelen beschikbaar voor de behandeling van longfibrose, namelijk nintedanib voor alle vormen van progressieve longfibrose en pirfenidon voor IPF. Antifibrotische behandeling vertraagt de ziekteprogressie, maar zal dit niet stoppen of omkeren.

In een multicenter prospectieve studie, uitgevoerd door de Nederlandse Vereniging van Artsen voor Longziekten en Tuberculose (NVALT), onderzochten we de veiligheid, tolerantie en werkzaamheid van het antifibrotische geneesmiddel pirfenidon bij asbestosepatiënten met een progressief fenotype. Pirfenidon vertraagt de ziekteprogressie bij IPF, maar de werkzaamheid en veiligheid bij andere vormen van longfibrose zijn minder bekend. In **hoofdstuk 9** beschrijven we onze studie, waarin we dagelijks thuis monitoring van FVC gebruiken om een meer gedetailleerd overzicht van longfunctieveranderingen te verkrijgen vóór en na de start van antifibrotische behandeling. In deze studie vonden we aanwijzingen voor een positief effect van pirfenidon. We lieten verder zien dat dagelijkse thuismonitoring van longfunctie het effect van de behandeling op FVC bij longfibrose kan identificeren, ondanks een kleine groep patiënten in de studie.

Tot slot hebben we de bevindingen van dit proefschrift samengebracht in een algemene discussie in **hoofdstuk 10**. We hebben in dit proefschrift aangetoond dat diepgaande fenotypering van circulerende T cellen potentiële biomarkers voor prognose en respons op behandeling bij patiënten met sarcoïdose kan opleveren. Preklinische muismodellen die belangrijke kenmerken van ILDs omvatten, zoals geactiveerde T cellen bij sarcoïdose, verbeteren onze kennis over pathogenese en kunnen het onderzoek naar nieuwe geneesmiddelen versnellen. Betere biomarkers, toegenomen kennis van pathogenese en nieuwe manieren om de behandelrespons te meten zorgen hopelijk voor de transformatie van een *one-size-fits-all* benadering naar een *gepersonaliseerde behandeling* van patiënten met sarcoïdose en longfibrose in de nabije toekomst.

PHD PORTFOLIO

Summary of PhD training and teaching
Name PhD student: J.R (Jelle) Miedema
Erasmus MC department: Pulmonary Medicine
Research School: Molecular Medicine
PhD period: 2018 - 2023
Promotor: Prof. Dr. R.W. Hendriks / Prof. Dr. M. Wijsenbeek-Lourens
Co-promotor: Dr. O. Corneth

1. PhD training	**Year**	**Workload** (ECTS)
General academy skills and in-depth courses		
- Basisdidactiek docenten Erasmus MC (2 days)	12-2018	0.6
- BROK course	08-2019	2.0
- Introduction course on SPSS (3 days)	06-2020	1.0
- Research integrity course	07-2021	0.3
- Good clinical practice (2019 & 2022)	11-2022	0.6
- BROK (refresher) course	08-2023	0.3
(Inter)national scientific presentations		
- Moderate ILD session NRS National Lung course	06-2019	0.6
- Oral presentation WASOG (Yokohama, Japan)	10-2019	0.3
- Poster presentation WASOG (Yokohama, Japan)	10-2019	0.3
- Oral presentation Rotterdams Longsymposium	11-2019	0.3
- Oral presentation lung symposium cells&signals (Rotterdam)	11-2021	0.1
- Oral presentation NVALT study symposium (NVALT27)	12-2021	0.1
- Regionale refereeravond Amphia ziekenhuis ILD	03-2022	0.1
- Oral presentation International lung symposium (Barcelona)	07-2022	0.3
- Oral presentation regional scientific evening (Rotterdam)	10-2022	0.1
- Fibrosis NRS national Lung course	02-2023	0.1
- Oral presentation Immunology Meeting Ruhrlandclinic DU	04-2023	0.3
- Oral presentation NVALT longartsendagen	05-2023	0.3
- Moderated poster presentation ATS congress Washington DC	05-2023	0.6
- Oral presentation ATS congress Washington DC	05-2023	0.3
- Oral presentation WASOG (Sweden)	06-2023	0.3
- Poster presentation WASOG (Sweden)	06-2023	0.1

(Inter)national scientific conferences and seminars

- WASOG (4 days, Yokohama, Japan)	10-2019	1.2
- ERS conference (5 days, Madrid)	09-2019	1.5
- International summit for ILD (4 days, Erice, Italy)	12-2019	1.2
- ILD course Davos Switzerland (4 days)	01-2020	1.2
- National clinical NVALT conference in ILD (CPC)	03-2020	0.3
- ERS congress, digital due to covid19 pandemic (3 days)	09-2020	0.9
- Cardiac sarcoidosis, digital due to covid19 pandemic	10-2021	0.3
- Longartsenweek, digital due to covid19 pandemic (2 days)	04-2021	0.6
- ILD course Davos Switzerland (4 days)	01-2022	1.2
- Seminar on target identification and tissue omics in ILD	02-2022	0.3
- Brainfeed Masterclass Ars Moriendi (2 days)	05-2022	0.6
- ICLAF Lung and Airway Fibrosis (5 days)	10-2022	1.5
- Brainfeed Masterclass Darwinism in Medicine (2 days)	11-2022	0.6
- ILD course Davos Switzerland (4 days)	01-2023	1.2
- WASOG (3 days, Sweden)	06-2023	0.9
- ATS congress Washington DC (4 days)	05-2023	1.2

2. Teaching activities	**Year**	**Workload** (ECTS)
Lecturing		
- Lung physiology (Radboud University Nijmegen, 4x)	2020-23	1.2
- International ILD preceptorship, 3 days, 6x 2019-2023	2019-23	6.0
- Lecture pathogenesis of pulmonary fibrosis (bachelor)	2019-23	0.5
- Lecture pathogenesis of sarcoidosis (ILD course Davos)	2019-23	0.5
- Lecture pathogenesis of lung fibrosis (ILD course Davos)	2019-23	0.5
- Lecture connective tissue disease and ILD (ILD course Davos)	2019-23	0.5
- National sarcoidosis patient symposium	10-2020	0.1
- Online Webinar antifibrotic treatment PPF	01-2021	0.1
- Lecture pathogenesis of sarcoidosis (Master I&I)	2021-23	0.3
- Online lecture diagnostics in CTD-ILD	04-2021	0.3
- Online Lecture Sarcoidosis and COVID	01-2021	0.3
- Lecture pro-con use of biopsy in ILD (den Haag)	10-2021	0.3
- National medclass lecture Pulmonary fibrosis	11-2021	0.3
- Lecture Lung physiology Querido course Rotterdam 4x	2020-23	1.2
- ILD course Davos Switzerland (3 lectures in course)	01-2022	0.9
- Lecture ILD Lung conference Amsterdam	04-2022	0.3

- Online National Patient Symposium pulmonary fibrosis	10-2022	0.1
- Medclass Nurse academy ILD MDT	11-2022	0.3
- ILD course Davos Switzerland (3 lectures in course)	01-2023	0.9
- National Lecture CTD-ILD NVR (Amersfoort)	05-2023	0.3
- ILD symposium: Overview ATS,WASOG, ERS 2023	10-2023	0.1
- National patient symposium pulmonary fibrosis	10-2023	0.1
- Online virtual school nurses rheumatology: ILD	10-2023	0.1
- National patient symposium sarcoidosis: microplastics	11-2023	0.1

Supervising students

- Supervising ILD fellowship	2018-2023	5.0
- Supervising Specialized nurse in training (VS ILD) (3 years)	2019-2021	5.0

Total ECTS: 49/30

3. Awards & Grant	**Year**
- Bouwhuis award on sarcoidosis research	2021
- Erasmus MC2 Grant Microplastics in sarcoidosis	2022
- Sarcoidosis.nl patient organization funding	2023
- Erasmus MC Synergy Grant (co-applicant)	2024

LIST OF PUBLICATIONS

Miedema JR, Lieke J. de Jong, Vivienne Kahlmann, Ingrid M. Bergen, Caroline E. Broos, Rudi W. Hendriks, Marlies S. Wijsenbeek, Odilia B.J. Corneth. Increased proportions of circulating PD-1$^+$ CD4$^+$ memory T cells and PD-1$^+$ regulatory T cells associate with good response to prednisone in pulmonary sarcoidosis, Respir Res. 2024 May 7;25(1):196

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De beste manier om dit wetenschappelijk, systematisch en grafisch weer te geven is een Forest plot: hierbij worden de gemiddelde effecten van factoren die een succesvolle PhD stimuleren of tegenwerken samengebracht tot een gewogen gemiddeld effect. Zoals u kunt zien is een succesvol afronden van het proefschrift mede te danken aan velen om mij heen, zowel in de kliniek als het lab, evenals een goede koptelefoon en koffie (**Fig.1**).

Om geen mensen tekort te doen wil ik beginnen met het uitspreken van mijn dank aan *iedereen* die op wat voor manier dan ook betrokken was bij de totstandkoming van dit proefschrift, direct of indirect! Daarnaast wil ik een aantal mensen in het bijzonder noemen in dit dankwoord.

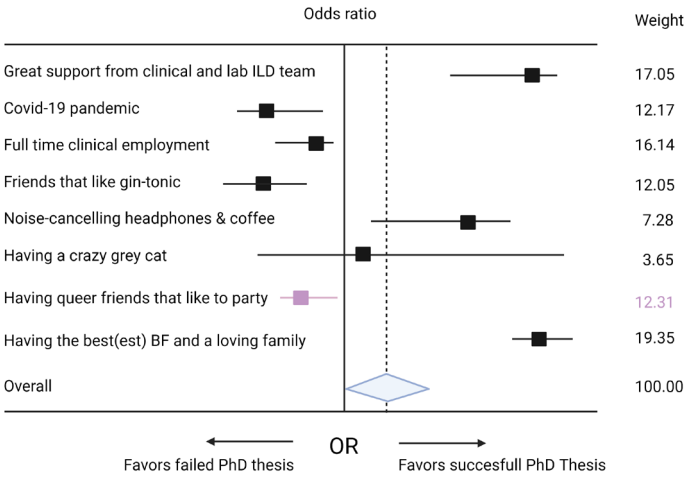

Figure 1: Forest plot depicting odds ratios of influencing factors for finalization of successfull PhD thesis

Patiënten

Onze wetenschappelijke onderzoeken zijn alleen mogelijk door deelname en inzet van patiënten en hun naasten. Hoewel zij in veel gevallen zelf geen baat hebben gehad bij de studies, waren ze toch bereid om deel te nemen. Ik wil dan ook alle patiënten, naasten, Sarcoidose.nl en de longfibrose patiëntenvereniging hartelijk danken voor de onmisbare steun en deelname aan wetenschappelijk onderzoek.

Promotie team

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dan werken in het ziekenhuis. Dank dat je altijd voor me klaarstond, je enthousiasme, voor al het overleg samen en de leuke projecten die we inmiddels opgebouwd hebben. Ik ga er van uit dat mijn promotie traject slechts het begin is van een heel fijne samenwerking met veel waardevol wetenschappelijk onderzoek, waarmee we de zorg voor patiënten verder kunnen verbeteren.

Kleine en Grote Commissie

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Paranymfen

Het woord paranymf komt van de Griekse woorden *para* en *numfios*: *voor de bruidegom*. In het verleden werd promoveren gezien als het sluiten van een huwelijk met de universiteit, vandaar dat een paranymf, ofwel bruidsjonker/bruidsmeisje, onderdeel is van de ceremonie. Voor mijn "universitair huwelijk" heb ik twee belangrijke, fijne mensen gevraagd om deel te nemen.

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Het lab ILD team

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ILD en sarcoïdose team

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Sarcoïdose en ILD expertiseteam

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ABOUT THE AUTHOR

Jelle Rindert Miedema was born on May 21st 1982 in Leek, The Netherlands. He attended primary school at the *Morgenster* and graduated from *Scholengemeenschap Stevensbeek* in in 2001. After graduating medical school at the Radboud University in Nijmegen in 2007, he worked in the medical field as physician at the department of internal medicine of Rijnstate hospital in Arnhem for one year. Jelle started his residency program to become a *respiratory physician* in 2009 at the *Radboud University Medical Center* in Nijmegen under the supervision of dr.Y. Heijdra and prof. dr. R. Dekhuijzen. In 2014, he moved to Rotterdam to finish the residency and join a fellowship program at the *Erasmus University Medical Center*, which is an international expert center for interstitial lung diseases (ILD) and sarcoidosis. He became a permanent staff member in 2016.

Currently, Jelle works as a pulmonary physician and ILD consultant. In clinical care, he is involved in daily care for ILD patients, with a specific interest in connective tissue disease related ILD and sarcoidosis. The clinical ILD and sarcoidosis team at the Erasmus Medical Center, under the dedicated leadership of Prof. Dr. M.S. Wijsenbeek-Lourens, is highly ambitious. Together, the team participates in collaborative projects involving ILD experts from around the world, multiple preclinical and clinical research projects, patients organizations and industry, to improve patient quality of life and treatment outcomes for patients with these rare but devastating lung diseases. Currently, Jelle is chair of the ILD group of the Dutch Pulmonary Society (NVALT) and aims to facilitate national collaboration between health professionals involved in the treatment and management of ILD. An intended PhD was generously supported by the ILD team and Prof. Dr. J.G.J.V. Aerts, head of the pulmonary department. In close collaboration with the translational ILD team, Jelle had the opportunity to work on several translational research projects under dedicated supervision of Prof. Dr. R. Hendriks, head of the Research Laboratory of the Department of Pulmonary Medicine, Prof. Dr. M.S. Wijsenbeek-Lourens and co-promotor dr. O. Corneth, that resulted in the current thesis. The studies included in this PhD thesis were presented at international conferences, including the American Thoracic Society (*ATS)*, European Respiratory Society (ERS) and World Association of Sarcoidosis and Other Granulomatous Diseases (*WASOG*). In 2022, Jelle was awarded the Erasmus MC^2 Research Innovation Grant for sarcoidosis research. In 2023, he was granted the *Bouwhuis award* and received a generous contribution to ongoing research by the *Dutch Sarcoidosis Society* (Sarcoidose.nl). Jelle is dedicated to continue the research on pathogenesis and improved personalized treatment of complex lung diseases such as sarcoidosis and pulmonary fibrosis, with the clinical and translational ILD team of the *Erasmus University Medical Center.*