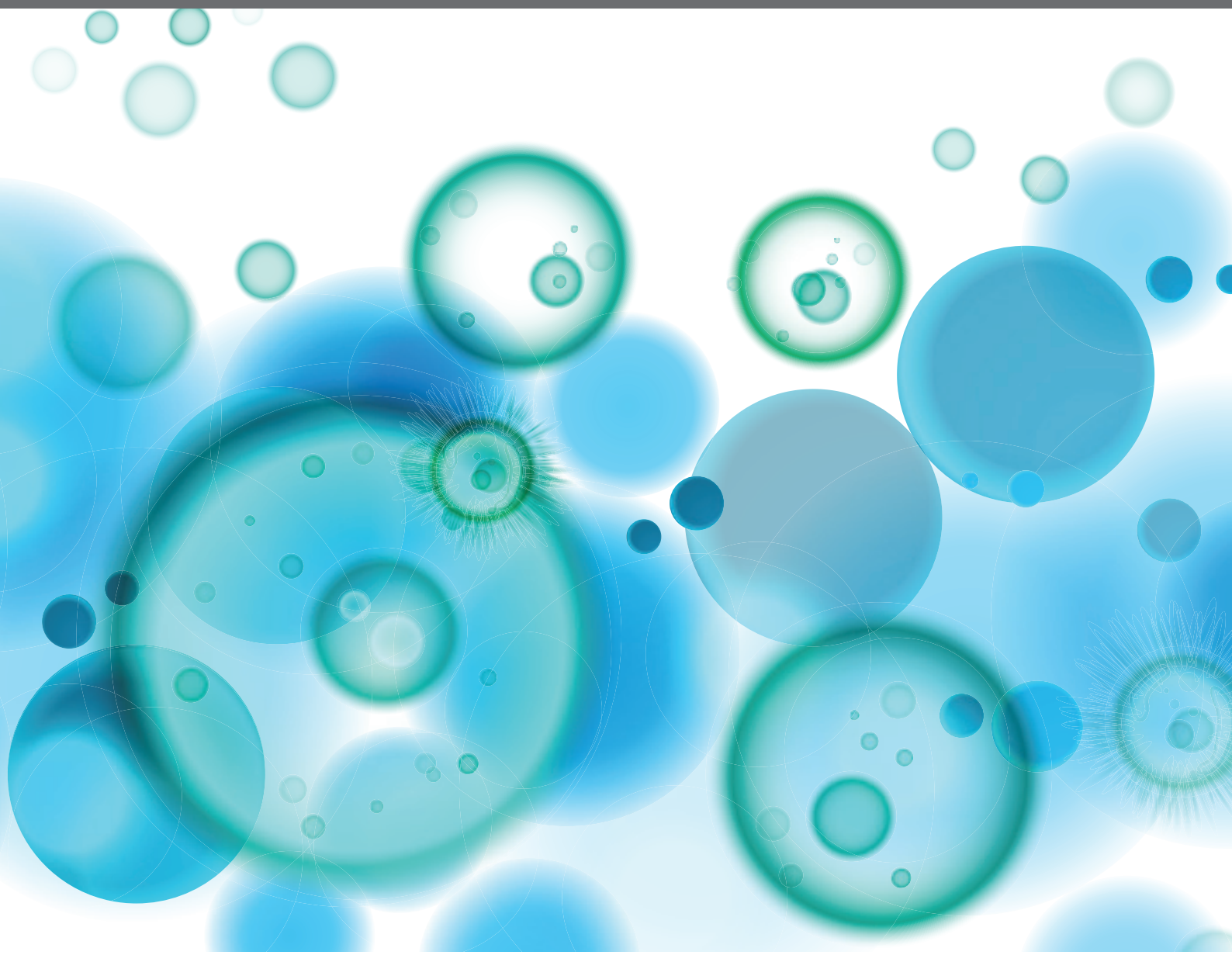
NOVEL INSIGHTS INTO THE IMMUNOLOGY OF PULMONARY GRANULOMATOUS DISEASES

EDITED BY: Mary Jane Thomassen, Marc A. Judson and Barbara P. Barna
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88966-468-9
DOI 10.3389/978-2-88966-468-9

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NOVEL INSIGHTS INTO THE IMMUNOLOGY OF PULMONARY GRANULOMATOUS DISEASES

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Citation: Thomassen, M. J., Judson, M. A., Barna, B. P., eds. (2021). Novel Insights into The Immunology of Pulmonary Granulomatous Diseases. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-468-9

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frontiers in Immunology

EDITORIAL
published: 05 January 2021
doi: 10.3389/fimmu.2020.633103

Editorial: Novel Insights Into the Immunology of Pulmonary Granulomatous Diseases

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Keywords: granulomatous disease, sarcoidosis, Th17, matrix metalloproteinase 12, environmental and occupational exposures

Editorial on the Research Topic

Novel Insights Into the Immunology of Pulmonary Granulomatous Diseases

Pulmonary granulomatous disease encompasses both infectious and non-infectious disease forms. Presently, the immunopathogenesis of granulomatous inflammation is incompletely understood. This issue of Frontiers in Immunology explores recent insights concerning the clinical and immunological aspects of pulmonary granulomatous disease.

Sarcoidosis is a complex multi-system disease of unknown cause which can be related to a diverse collection of environmental signals. The review by Judson summarizes current knowledge of the immunopathogenesis of sarcoidosis as well as the nature of varied environmental and occupational exposures, which could cause or affect the disease. Many infectious agents have been implicated in the disease, especially mycobacterium tuberculosis, and the review summarizes evidence for sarcoid patient immune responsiveness to such agents.

A different perspective on pulmonary tuberculosis, another granulomatous disease, is provided by Muefong and Sutherland in their review of neutrophil activities in this deadliest of infectious diseases in humans. The authors raise the possibility that neutrophils may play a more central role in tuberculosis pathogenesis than previously thought. They propose that some neutrophil-related inflammatory mediators as potential targets for developing novel tuberculosis therapies.

Hena reviews the effects of race on sarcoidosis epidemiology. The author presents data that show that in the United States, black patients with sarcoidosis experience more severe pulmonary disease, more multiorgan involvement, and an overall worse prognosis. The author proposes that epidemiologic concepts can be used to modulate and even prevent sarcoidosis in black Americans at risk of life-threatening disease phenotypes.

Kraaijvanger et al. address the problem of identifying useful biomarkers for sarcoidosis diagnosis and prognosis. As noted by the authors, sarcoidosis is a heterogeneous disease, making treatment decisions problematic. Exploration of signaling pathways in sarcoidosis such as JAK/STAT and mTOR may result in the discovery of more specific biomarkers that may be relevant to prognosis. The authors stress that there is an unmet need for more specific and more sensitive biomarkers in sarcoidosis care.

Gerke focuses on the deficiencies of the current treatment options for sarcoidosis. Sarcoidosis treatment decisions are problematic because of the marked heterogeneity of the disease. Clinical evidence is sparse because the disease is relatively rare and few clinical trials have been performed. Goals of treatment are to protect organ function and to decrease symptoms, but most clinical

Edited and reviewed by:
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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *24 November 2020*
Accepted: *26 November 2020*
Published: *05 January 2021*

Citation:
Thomassen MJ, Judson MA and Barna BP (2021) Editorial: Novel Insights Into the Immunology of Pulmonary Granulomatous Diseases. Front. Immunol. 11:633103. doi: 10.3389/fimmu.2020.633103

sarcoidosis trials have not focused on the impact of the disease on function or quality of life. Gerke emphasizes the lack of knowledge concerning etiology and pathophysiology of the disease and the need to utilize more patient-centered approaches to treatment for exploration of immunosuppression, symptom control, and treatment of co-morbid conditions.

Relevant to the Gerke study, Talreja et al. present original research on alveolar macrophage and blood monocyte gene profiles in response to *in vitro* dexamethasone treatment in sarcoidosis. Effects of glucocorticoids on alveolar macrophages or monocytes have not been well documented. These authors describe profound transcriptosomal changes in lung macrophages relative to cellular metabolism, lysosomal phagosomes and cytoskeleton function. Further studies are proposed to examine proteome profiles in additional cell types relevant to pulmonary sarcoidosis that can correlate with RNA seq data and give a more comprehensive picture of dexamethasone effects within the lung.

Mohan et al.'s study using a multiwall carbon nanotube model of chronic granulomatous disease demonstrated many similarities to sarcoidosis including high expression levels of MMP12 (matrix metalloproteinase). These investigators used MMP12 knock-out mice to investigate the role of MMP12 in granuloma formation and persistence and demonstrated similar acute responses (10 days); however; at day 60, there was a marked reduction in granuloma persistence suggesting that MMP12 is essential for the persistence of granulomas.

Arger et al. observed that T-Bet expression on Th17.0 cells reflected the extent of clinical granuloma burden in sarcoidosis patients. These authors postulated that T-Bet Th17.0 cells represent a transition state leading to interferon-producing Th17.1 cells which are elevated in BAL fluid and mediastinal lymph nodes of sarcoidosis patients with granulomatous inflammation. These studies emphasize the importance of Th17 in sarcoidosis.

Greaves et al. performed a comparative review of adaptive immunity in two pulmonary diseases: pulmonary sarcoidosis and chronic beryllium disease (CBD). The authors note that one of the defining features of both CBD and sarcoidosis is the pulmonary infiltration of activated CD4+ T lymphocytes. Interestingly, sarcoidosis lung also contains a significant population of Th17 cells which are absent in CBD. While beryllium is the antigen that can activate CBD lymphocytes, the antigenic specificity of sarcoidosis lymphocytes is not yet known.

Locke et al. reviewed various *in vitro* and *in vivo* models of sarcoidosis. Each of the current model systems has strengths and weaknesses. The authors suggest that the integrated use of the various preclinical models will accelerate progress toward identifying targets and the testing of new drugs.

We hope that the articles in this issue of Frontiers in Immunology will educate researchers and clinicians concerning the immunology of pulmonary granulomatous disease. Furthermore, we hope that these articles will serve as a stimulus to further exploration of the mechanisms of granuloma formation that will ultimately improve the lives of patients.

AUTHOR CONTRIBUTIONS

All authors contributed equally. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by grants ES025191 to MT and CHHE P30 ES025128.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 18 March 2020
doi: 10.3389/fimmu.2020.00474

Adaptive Immunity in Pulmonary Sarcoidosis and Chronic Beryllium Disease

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Pulmonary sarcoidosis and chronic beryllium disease (CBD) are inflammatory granulomatous lung diseases defined by the presence of non-caseating granulomas in the lung. CBD results from beryllium exposure in the workplace, while the cause of sarcoidosis remains unknown. CBD and sarcoidosis are both immune-mediated diseases that involve Th1-polarized inflammation in the lung. Beryllium exposure induces trafficking of dendritic cells to the lung in a mechanism dependent on MyD88 and IL-1α. B cells are also recruited to the lung in a MyD88 dependent manner after beryllium exposure in order to protect the lung from beryllium-induced injury. Similar to most immune-mediated diseases, disease susceptibility in CBD and sarcoidosis is driven by the expression of certain MHCII molecules, primarily *HLA-DPB1* in CBD and several *HLA-DRB1* alleles in sarcoidosis. One of the defining features of both CBD and sarcoidosis is an infiltration of activated CD4+ T cells in the lung. CD4+ T cells in the bronchoalveolar lavage (BAL) of CBD and sarcoidosis patients are highly Th1 polarized, and there is a significant increase in inflammatory Th1 cytokines present in the BAL fluid. In sarcoidosis, there is also a significant population of Th17 cells in the lungs that is not present in CBD. Due to persistent antigen exposure and chronic inflammation in the lung, these activated CD4+ T cells often display either an exhausted or anergic phenotype. Evidence suggests that these T cells are responding to common antigens in the lung. In CBD there is an expansion of beryllium-responsive TRBV5.1+ TCRs expressed on pathogenic CD4+ T cells derived from the BAL of CBD patients that react with endogenous human peptides derived from the plexin A protein. In an acute form of sarcoidosis, there are expansions of specific TRAV12-1/TRBV2 T cell receptors expressed on BAL CD4+ T cells, indicating that these T cells are trafficking to and expanding in the lung in response to common antigens. The specificity of these pathogenic CD4+T cells in sarcoidosis are currently unknown.

Keywords: human, T cells, MHC, lung, T cell receptors, sarcoidosis

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *06 February 2020*
Accepted: *28 February 2020*
Published: *18 March 2020*

Citation:
Greaves SA, Atif SM and Fontenot AP (2020) Adaptive Immunity in Pulmonary Sarcoidosis and Chronic Beryllium Disease. Front. Immunol. 11:474. doi: 10.3389/fimmu.2020.00474

INTRODUCTION

Granulomatous lung diseases represent a diverse set of disorders that are caused by both infectious and non-infectious agents that induce granuloma formation in the lung. Infectious organisms that induce granulomas include *Mycobacteria* sp. and various fungal organisms, while non-infectious etiologies include hypersensitivities, particulate exposures, and autoimmune reactions, among

others [reviewed in (1)]. Because the clinical manifestations of many of these diseases are similar, the differential diagnosis is broad, and it is sometimes difficult to obtain diagnostic certainty. Sarcoidosis and chronic beryllium disease (CBD) are both non-infectious granulomatous lung diseases defined by the presence of non-caseating granulomas, comprised primarily of lymphocytes, epithelioid cells, giant cells, and macrophages (2–4). Although sarcoidosis can affect many organ systems, it involves the lungs in ∼95% of cases (3), while CBD is typically limited to the pulmonary system.

Sarcoidosis is a disease of unknown etiology that occurs in individuals of all age, sex, and ethnic backgrounds. The highest prevalence of sarcoidosis is found in Sweden and the United States (5). CBD results from exposure to beryllium, which is a rare alkaline earth metal with exposures occurring through inhalation in the workplace, particularly in the United States (6). The adverse health effects of beryllium have long been described (7), and despite the implementation of beryllium exposure standards (8) cases of beryllium-induced disease continue to occur. The clinical manifestations of both CBD and sarcoidosis can be highly variable. In sarcoidosis, symptoms range from coughing to loss of lung function [reviewed in (9)]. Some patients develop an acute form of the disease that is self-resolving, while others develop a chronic condition that can substantially affect their quality of life. The average mortality rate for sarcoidosis patients is 3.6 per million, although this is highly variable depending on age, sex, and ethnicity (5). The first stage of beryllium-induced disease is beryllium sensitization (BeS), which involves beryllium-specific immune responses in the blood with no clinical manifestations (10). A subset of BeS patients eventually develop CBD, depending on quantity of beryllium exposure and genetic susceptibility of the individual (11, 12). If left untreated, CBD can progress to lung fibrosis, with one-third of patients historically progressing to respiratory failure (13).

Both diseases are characterized by an infiltration of activated CD4+ T cells in the lung. In CBD patients, these T cells recognize beryllium, while in sarcoidosis their specificity is unknown. Certain MHCII molecules, primarily *HLA-DPB1* in CBD and several *HLA-DRB1* alleles in sarcoidosis, increase disease susceptibility (14–17). Recently, there has also been evidence to suggest that B cells and antibody responses are involved in the pathogenesis of CBD and sarcoidosis (18–20). This review focuses on how different aspects of the immune system influence the progression of these diseases.

INNATE IMMUNE RESPONSES IN CBD AND SARCOIDOSIS

Innate immune cell populations are involved in propagating inflammation in both CBD and sarcoidosis (**Table 1**). Alveolar macrophages (AMs) comprise a major cell population in a healthy lung and are crucial for maintaining lung immunity (21). AMs are altered in sarcoidosis, with protein profiling revealing 80 differentially-expressed proteins in AMs of sarcoidosis patients compared to control subjects, including increased expression of two major phagocytic pathways (22). Activation of the mTORC1 pathway by deletion of the TSC2 gene in macrophages promoted excessive granuloma formation in mice (23). In sarcoidosis patients, mTORC1 activation and macrophage proliferation are associated with disease progression (23). Additionally, the number of monocytes undergoing phagocytosis in the blood of sarcoidosis patients is heightened compared to control subjects (24), and circulating monocytes also display higher TLR2 and TLR4 expression (25). Bronchoalveolar lavage (BAL) cells from sarcoidosis patients produce more TNF-α and IL-6 in response to TLR2 agonists than healthy controls, while PBMCs from sarcoidosis patients had impaired TLR2 responses (26, 27). Serum amyloid A (SAA) proteins are a group of acute phase inflammatory response proteins that are significantly elevated in the granulomas of sarcoidosis patients and localize to macrophages and giant cells within the granulomas (28). In a murine model of granulomatous lung inflammation SAA amplified inflammatory responses via TLR2 signaling (28). It has also recently been shown that SAAs promote Th17 induced inflammation (29), which will be discussed in the T cell section below. Certain MyD88 polymorphisms are also associated with the development of sarcoidosis (30). While these studies have led some to speculate that the abnormal inflammation in sarcoidosis is caused by aberrant monocyte and macrophage activity and TLR responses, the evidence for specific antigenic stimuli, as discussed below, indicates that there is an interplay between the innate and adaptive immune responses that drives the inflammation and granuloma formation.

Similar to sarcoidosis, beryllium exposure and the development of granulomatous inflammation in CBD patients results from activation of both innate and adaptive immunity. Although beryllium is a part of an antigenic complex that generates an adaptive immune response in genetically-susceptible, beryllium-exposed workers, it also serves as an adjuvant to prime innate immunity (31–33). As compared to mice immunized with parasitic antigens alone, mice immunized with a combination of beryllium and parasitic antigens had enhanced control of the infection through increased production of IFN-γ in lymph nodes and spleen (34). PMBCs and dendritic cells (DCs) exhibit increased production of inflammatory chemokines after exposure to beryllium, and TNF-α was produced by beryllium-stimulated PBMCs independent of disease-associated HLA molecules (35, 36). In murine models of CBD, beryllium exposure promoted trafficking of DCs to lung-draining lymph nodes and enhanced expression of costimulatory molecules, CD80 and CD86, on DCs that was dependent on MyD88 signaling (37). Furthermore, it was recently demonstrated that exposure to beryllium hydroxide ($Be(OH)_2$) resulted in the release of IL-1α and DNA into the airways, which subsequently acted as damage-associated molecular patterns (DAMPS) by engaging IL-1R1 and TLR9 to induce migration of DCs into lung-draining lymph nodes and the subsequent generation of memory CD4+ T cells (38). Thus,

Abbreviations: CBD, chronic beryllium disease; AM, alveolar macrophage; DC, dendritic cell; DAMP, damage associated molecular pattern; BeO, beryllium oxide; BAL, bronchoalveolar lavage; ELA, ectopic lymphoid aggregate; TRAV, TCR alpha variable region; TRBV, TCR beta variable region; SAA, serum amyloid A.

TABLE 1 | Immune characteristics of chronic beryllium disease and sarcoidosis.

	Sarcoidosis	Chronic beryllium disease
Innate Immunity	Increased TLR2 and TLR4 expression on monocytes Alveolar macrophages display increased phagocytosis Increased mTORC1 activation and macrophage proliferation PMBCs show impaired TLR2 responses MyD88 polymorphisms are associated with disease	Beryllium has adjuvant properties that prime innate immunity PMBCs and DCs have increased production of inflammatory cytokines Increased CD80 and CD86 on DCs Beryllium generates DAMPs to induce migration of DCs to lung
B cells/antibody responses	Increased IgA Anti-vimentin antibodies in BAL fluid	B cells recruited to lung dependent on MyD88 and organized into ELAs B cell depletion enhances lung injury
HLA susceptibility	*HLA-DRB1*11:01* (US) *HLA-DRB1*15:01* (US) *HLA-DRB1*03:01* (Sweden)	*HLA-DPB1*02:01* *HLA-DPB1*17:01*
Cytokine profiles	Increased Th1 polarized cytokines including TNF-α, IL-6, IFN-γ, IL-1, IL-2, IL-12	Increased Th1 polarized cytokines including IFN-γ, TNF-α, IL-2, IL-1
CD4+ T cells	CD4+ T cell alveolitis Majority Th1 polarized Th17 and Th17.1 CD4+ T cells also present Elevated PD-1 expression, dysfunctional TCR responses	CD4+ T cell alveolitis Majority Th1 polarized No evidence of Th2 or Th17 CD4+ T cells present Increased PD-1 and CTLA-4 expression Predominantly effector memory phenotype
Public TCRs	TCRs with TRAV12-1/TRBV2 are most frequent in HLA-DR3 Löfgren's syndrome patients	TRBV5.1 beryllium specific TCRs are expressed in majority of HLA-DP2 patients Public TCRs recognize HLA-DP2/beryllium/plexin A peptide complex

beryllium exposure has a crucial effect on innate cell function that contributes to disease development and progression.

B CELL INVOLVEMENT IN CBD AND SARCOIDOSIS

CBD and sarcoidosis have historically been thought of as T cell-driven inflammatory diseases. Until recently, the role of B cells in CBD was completely unknown. Our group recently demonstrated the importance of B cells in protecting the lung from beryllium oxide (BeO)-induced injury (**Table 1**). Using a murine model of CBD that mimics the human disease, we established that activated follicular B cells are recruited to the lung after beryllium exposure and are organized into ectopic lymphoid aggregates (ELAs) (20). B cell depletion eradicated these ELAs and enhanced lung injury, indicating that B cells play an important protective role in BeO-induced lung injury (20). In addition, BeO-induced B cell recruitment to the lung is dependent on MyD88 signaling (20). B cells also accumulate in the lungs of CBD patients, indicating that this protective role may translate to human disease (20). Further studies are needed to determine the exact mechanism of B cell-mediated protection in the lung after BeO exposure.

Although there is no evidence of B cells playing a direct role in disease pathogenesis in sarcoidosis, several studies have reported altered antibody responses in these patients. Particularly in patients with pulmonary sarcoidosis, there is a direct correlation between the number of T cells in the BAL and the proportion of BAL cells that secrete IgG, which is not apparent in control subjects (39). Additionally, increased numbers of memory B cells producing IgA are found in patients with sarcoidosis, raising the possibility that IgA could be involved in granuloma formation (40). Antibodies toward autoantigens have been found in only a small proportion of sarcoidosis patients, making it unclear whether autoimmune responses play a role in disease pathogenesis (18). Recent data show that in patients with an acute, self-resolving form of sarcoidosis, Löfgren's syndrome, there are increased quantities of anti-vimentin antibodies in the BAL fluid (19). Vimentin is a self-antigen that has been found in the lungs of sarcoidosis patients (41), although more validation is required to determine whether immune responses to vimentin are involved in disease initiation and progression. Overall, the role of B cells in sarcoidosis needs to be more thoroughly investigated to establish their role in this disease.

T CELLS IN CBD AND SARCOIDOSIS

A defining feature of both CBD and pulmonary sarcoidosis is an infiltration of pathogenic CD4+ T cells in the lung (42–47) (**Table 1**, **Figure 1**). CD4+ T cells in the BAL fluid of CBD patients are predominantly antigen-experienced T cells expressing CD45RO and lacking CD62L and CCR7 (48). Large numbers of these cells (in some cases, >30%) are responsive to beryllium sulfate ($BeSO_4$) in culture, secreting Th1 cytokines, including IFN-γ and TNF-α and lesser quantities of IL-2 (48). CD4+ T cell responses to beryllium are dependent upon major histocompatibility complex class II (MHCII) molecules, but independent of CD28 costimulation, indicative of an effector memory T cell phenotype (48, 49). There is no evidence that beryllium-responsive Th2 or Th17 CD4+ T cells or CD8+ T cells are present in the lungs of CBD patients or that these cells play a role in beryllium-induced disease (48, 50).

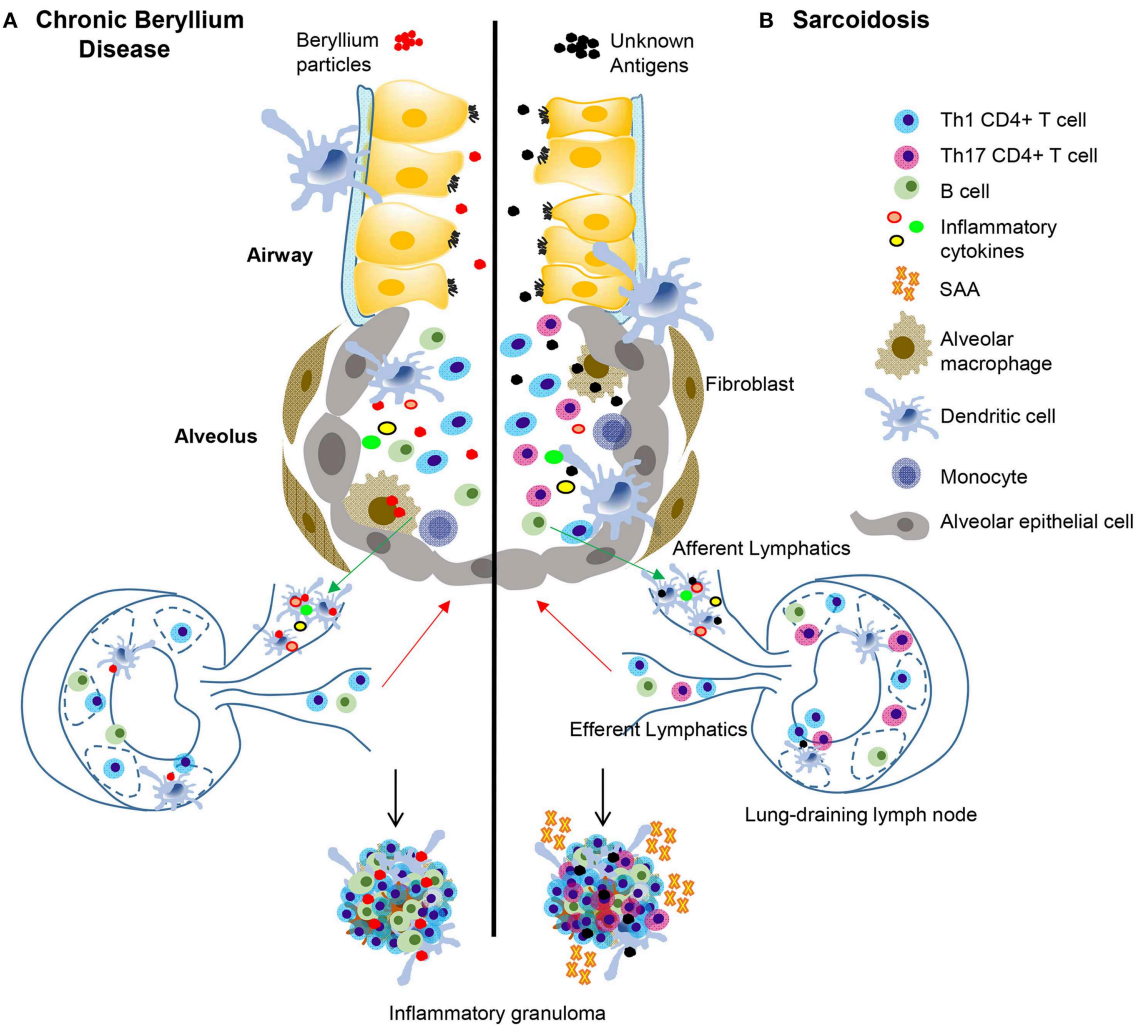

FIGURE 1 | Immunopathogenesis of chronic beryllium disease and sarcoidosis. In CBD **(A)**, beryllium particles enter the lung and generate an immune response. In sarcoidosis **(B)** unknown antigens induce an immune response after entering the lung. In CBD, beryllium particles cause the release of DAMPs in the airways, which promote activation and migration of DCs. Either beryllium **(A)** or unknown antigens **(B)** are taken up by alveolar macrophages and dendritic cells in the lung and are transported to lung-draining lymph nodes. In the lymph nodes, antigen specific CD4+ T cells are stimulated by activated antigen-presenting cells expressing certain HLA molecules dependent on disease (see **Table 1**). T-B cell interactions also occur in the lymph nodes and commitment of B cells to antibody production takes place in the germinal centers. Antibodies and activated B and T cells circulate back to the lung to generate an adaptive immune response against the target antigen. In CBD, Th1 CD4+ T cells that display an effector memory phenotype are the primary cell type, but there is also an infiltration of activated B cells in the lungs that help form granulomatous structures **(A)**. In sarcoidosis and CBD, Th1 CD4+ T cells make up the majority of the cell population in the lungs. In sarcoidosis, but not CBD, there are also Th17 and Th17.1 cells present. SAAs are also present in pulmonary granulomas of sarcoidosis patients, but not CBD, where they contribute to the inflammatory milieu and induce proliferation of pathogenic, inflammatory Th17 cells. In both diseases, there is an abundance of Th1 inflammatory cytokines secreted by T cells and innate cells that propagate the inflammatory environment, including TNF-α, IFN-γ, IL-1, and IL-2.

CD4+ T cells from the BAL of sarcoidosis patients are also highly Th1 polarized. This enhanced Th1 polarization is driven by increased expression of inflammatory cytokines, such as IFN-γ, IL-1, IL-2, IL-6, IL-12, and TNF-α in addition to others, while Th2 cytokines have not been detected (51–53). Unlike CBD, there is no known inciting particle(s) or antigen(s) in sarcoidosis. However, in light of the infiltration of Th1-polarized CD4+ T cells in the lung, it is widely accepted that these T cells are trafficking to and proliferating in the lung in response to an unknown antigen. In contrast to findings in the BAL of sarcoidosis patients where a CD4+ T cell alveolitis (20–60% total cell count) exists (42), T cell lymphopenia in the peripheral blood is characteristic of the disease (54). CD4+ T cells with a Th17 phenotype and T cells expressing both IL-17 and IFN-γ (i.e., Th17.1 cells) have been found in lung tissue and BAL from patients with sarcoidosis (55, 56). A study recently demonstrated that there is a significant increase in CCR6+ Th17.1 cells in both the BAL fluid and mediastinal lymph nodes of sarcoidosis patients compared to controls and that these cells contribute to IFN-γ production (57). Additionally, Th17.1 cells in the BAL fluid are found in higher proportions in sarcoidosis patients with active disease vs. patients undergoing disease resolution,

suggesting that these cells could be playing a role in disease progression (57). Interestingly, a recent study highlighted the importance of SAA in the differentiation of pathogenic Th17 cells in certain inflammatory conditions (29). SAA expression is increased in the lungs of sarcoidosis patients (28), so it is possible that SAAs contribute to the expansion of Th17 cells observed in these patients. Furthermore, SAAs in the lungs of sarcoidosis patients are significantly higher than that of CBD patients (28), which could explain the lack of Th17 cells in CBD.

As a consequence of constant antigen exposure in both sarcoidosis and CBD, pathogenic CD4+ T cells develop an anergic and/or exhausted phenotype as a mechanism to decrease chronic T cell activation and subsequent inflammation. Pulmonary CD4+ T cells from sarcoidosis spontaneously secrete IL-2 *ex vivo*, but when given TCR stimulation, these cells express less IL-2 and IFN-γ relative to CD4+ T cells from various other lung diseases and healthy control subjects, consistent with an anergic/exhausted phenotype (58). Additionally, sarcoidosis CD4+ T cells have a reduced proliferative capacity and higher levels of apoptosis, along with increased PD-1 expression, which is often associated with persistent antigen exposure to limit chronic T cell activation (59, 60). A recent study demonstrated that PD-1 expression was highest on sarcoidosis CD4+ T cells with a Th17 phenotype (61). Furthermore, PD-1+ Th17 CD4+ cells produce high levels of TGFB-1, a key factor in the development in fibrosis (61). PD-1 blockade significantly reduced TGFB-1 produced by Th17 CD4+ T cells from sarcoidosis patients and reduced their ability to induce collagen-1 production in a fibroblast cell line (61). These data demonstrate that T cell dysfunction in sarcoidosis is seemingly due to persistent antigen exposure, thus discovering the etiologic antigens driving this disease will be important for therapies directed at reducing antigen exposure.

BAL CD4+ T cells in CBD also have elevated PD-1 expression and blockade of the PD-1 pathway increases proliferation of beryllium-responsive CD4+ T cells (62). Interestingly, a subsequent study showed that CTLA-4, another T cell co-inhibitory receptor, was upregulated on BAL CD4+ T cells in CBD, but its expression was not capable of reducing beryllium-stimulated T cell proliferation (63). Taken together, these findings show that persistent antigen exposure drives an exhausted T cell phenotype in both CBD and sarcoidosis. Despite the immune systems attempt to dampen T cell-mediated immune activation, the cells are still able to promote chronic inflammation and disease progression in non-infectious granulomatous lung disease.

GENETIC SUSCEPTIBILITY TO CBD AND SARCOIDOSIS

Similar to most immune-mediated diseases, MHCII molecules are strongly associated with both sarcoidosis and CBD (**Table 1**). Saltini et al. (44) demonstrated that BAL CD4+ T cells from CBD patients recognize beryllium in an MHCII-restricted manner. Genetic susceptibility was strongly linked to HLA-DPB1 alleles with a glutamic acid (E) at position 69 of the β-chain (βGlu69), with the most prevalent βGlu69-containing allele being *HLA-DPB1*02:01* (14). Multiple studies have corroborated these findings, documenting the presence of βGlu69-containing *DPB1* alleles in 73–95% of BeS and CBD patients compared to 30–48% of exposed controls [reviewed in (64)]. A differential risk of disease development is also associated with certain rare βGlu69-containing *DPB1* alleles, such as *HLA-DPB1*17:01* (15, 65–67). Thus, CBD is a classic example of a disorder resulting from gene-by-environment interactions, where both components are required for the development of granulomatous inflammation. In this regard, the probability of CBD increases with HLA-DP βGlu69 copy number and increasing workplace exposure to beryllium (12), suggesting that genetic and exposure factors may have an additive effect on the risk of disease development (11).

Similar to CBD, genetics and environmental exposures are involved in the initiation of sarcoidosis. Because sarcoidosis is a more heterogeneous disease that occurs worldwide and the etiologic antigen(s) are currently unknown, genetic studies are important to determine common disease characteristics. However, many genetic studies involving sarcoidosis patients demonstrate that even commonalities among subsets of patients differ between ethnic and regional groups, suggesting that there are multiple factors involved in the progression of sarcoidosis. A large-scale ACCESS study that collected patient data in the United States from 1996 to 1999 demonstrated that siblings of sarcoidosis patients are about 5-fold more likely to develop the disease than unrelated control subjects (68), suggesting that genetic factors influence disease susceptibility. As suspected by the accumulation of CD4+ T cells in the BAL of sarcoidosis subjects, the dominant genetic associations in sarcoidosis are linked to HLA. While both HLA class I and class II genes are associated with the development of sarcoidosis, the strongest correlations are shown with class II genes, although these differ between ethnic and regional groups [reviewed in (69)]. In the United States, *HLA-DRB1*11:01* and *HLA-DRB1*15:01* are both associated with sarcoidosis (16). The most striking genetic association in sarcoidosis is present in Löfgren's syndrome in Scandinavian patients. Löfgren's syndrome is an acute form of sarcoidosis associated with a favorable prognosis that presents with a specific set of inflammatory symptoms including bilateral hilar lymphadenopathy, fever, erythema nodosum, and ankle arthritis (70). The majority of Löfgren's syndrome patients express *HLA-DRB1*03:01*, with most of these patients resolving their disease within 2 years (70). Specific αβTCR variable region genes are also highly associated with Löfgren's syndrome, as discussed below. These studies clearly demonstrate that genetic susceptibility due to expression of certain MHCII molecules in both CBD and sarcoidosis is a crucial aspect of disease pathogenesis.

PUBLIC T CELLS IN BAL OF CBD AND SARCOIDOSIS DRIVING DISEASE PATHOGENESIS

As discussed in the previous section, specific MHCII expression is crucial for disease development in both CBD and sarcoidosis.

CD4+ T cells recognize peptide epitopes in an MHCII-restricted manner (71). In CBD, MHCII molecules require beryllium for antigen presentation to pathogenic T cells (48). The positively-charged beryllium particle is coordinated in the MHCII groove along with specific naturally-occurring peptides to complete the beryllium-dependent T cell ligand (72, 73). BAL CD4+ T cells from CBD patients have unique oligoclonal populations that are enriched for certain TCR β-chain motifs (46, 74). Furthermore, there are public (i.e., expressed in the majority of HLA-DP2-expressing CBD patients) beryllium-responsive TCRβ variable region (TRBV) 5.1+ TCRs expressed on CD4+ T cells derived from the BAL of CBD patients, and the frequency of these public beryllium-responsive TCRs inversely correlate with loss of lung function, suggesting that these public T cells are pathogenic in nature (75). Falta et al. (76) discovered beryllium-dependent mimotopes (i.e., peptides that mimic the naturally-occurring epitope) that bound to HLA-DP2 in the presence of beryllium and are recognized by pathogenic TRBV5.1+ CD4+ T cells expanded in the lungs of HLA-DP2+ CBD patients. Our group further discovered that an endogenous human peptide derived from plexin A proteins binds to the HLA-DP2/Be complex and stimulates these same pathogenic CD4+ T cells from CBD patients (76). CD4+ T cells specific for the HLA-DP2-plexin A4/beryllium complex comprise ∼5% of beryllium-responsive CD4+ T cells in the lung (76).

Although the inciting antigen(s) in sarcoidosis is unknown, there is strong evidence suggesting that the disease is driven by antigen-specific CD4+ T cells in the lungs of patients. The most conclusive evidence comes from Löfgren's syndrome. The majority of Löfgren's syndrome patients in Sweden express *HLA-DRB1*03:01* (HLA-DR3) (17), and expression of HLA-DR3 was strongly associated with disease resolution and an excellent prognosis (70). Additionally, HLA-DR3-expressing Löfgren's syndrome patients exhibit oligoclonal expansions of CD4+ T cells in the BAL that express TCRα variable region (TRAV) 12-1, and the quantity of these cells in the BAL correlates with disease remission (77–79). Importantly, this expansion of TRAV12-1+ CD4+ T cells is only seen in patients with active disease, and upon disease resolution, these cells are greatly reduced, highlighting their importance in disease pathogenesis (80). Using deep sequencing approaches, our group recently demonstrated that TRAV12-1 preferentially pairs with TCRβ variable region (TRBV) 2 in BAL CD4+ T cells from Löfgren's syndrome patients and that TRAV12-1 and TRBV2 are the most expanded variable regions relative to control subjects (81). Additionally, there are specific TRAV12-1/TRBV2 CDR3 motifs expressed on BAL CD4+ T cells of multiple Löfgren's syndrome patients, indicating that these T cells are trafficking to and expanding in the lung in response to the same antigen.

ANTIGENIC DRIVERS OF SARCOIDOSIS

One of the most active areas of sarcoidosis research is focused on determining the etiologic antigens that drive the recruitment of CD4+ T cells to the lungs of sarcoidosis patients. One problem hindering this endeavor is the lack of understanding of the origin of the inciting antigens, whether it is a foreign or self-antigen, or even a product of environmental exposure. Although there is some evidence for each of these categories, nothing has been conclusive enough to determine a cause of disease. The most detailed studies involving an autoantigen focus on vimentin, a filamentous protein of which the cytoskeleton is composed (82). Vimentin has been eluted from HLA-DR molecules expressed on BAL cells from some patients with sarcoidosis, and vimentin-specific autoantibodies are found in BAL fluid of sarcoidosis patients (19, 41, 83). More validation is required to determine whether immune responses to vimentin are involved in disease initiation and progression.

Due to the similarities between the clinical features of sarcoidosis and certain pulmonary infections, it has long been thought that there may be an infectious agent driving sarcoidosis etiology. The most substantial evidence linking a microorganism to sarcoidosis pathogenesis involves *Mycobacteria sp*. Several meta-analyses demonstrate that the odds of finding mycobacterial DNA by PCR in the lungs of sarcoidosis patients is ∼5–20% greater than in control subjects (84, 85). However, the data collected in these studies is very heterogeneous, and *Mycobacteria sp.* are not detected in the majority of sarcoidosis patients.

To investigate whether there were any functional implications that mycobacterial infections induce sarcoidosis, several groups have measured T cell responsiveness to mycobacterial candidate antigens in sarcoidosis patients. A higher number of T cells in the blood of sarcoidosis patients produce IFN-γ after stimulation with a *M. tuberculosis* antigen catalase peroxidase (mKatG) (86). Additionally, T cells derived from the BAL fluid of sarcoidosis patients respond to mKatG along with an additional mycobacterial antigen, early secreted antigen protein (ESAT-6) (87). Furthermore, one study detected protein derived from mKatG in ∼50% of tissue samples from sarcoidosis patients (88), and IgG antibodies were detected in ∼50% of sarcoidosis serum samples (88). Despite this evidence, sarcoidosis patients show no signs active mycobacterial infections even during immunosuppressive treatments. There are also no mycobacterial-specific CD4+ T cell clones identified from sarcoidosis patients to date.

Cutibacterium acnes (previously *Propionibacterium acnes*) infections are also associated with sarcoidosis. *C. acnes* is a commensal bacteria that is commonly found on the skin of healthy individuals. By quantitative PCR, DNA from *C. acnes* is found in the majority of lymph nodes obtained from sarcoidosis patients (89). However, *C. acnes* is also found in a large percentage of lymph nodes isolated from healthy control subjects (90), making it difficult to ascertain whether the presence of *C. acnes* in sarcoidosis patients is a consequence of the disease or normal colonization of commensal bacteria.

In addition to infectious agents, it has also been postulated that there are environmental factors involved in the progression of sarcoidosis. Stage I sarcoidosis occurs more frequently in the Spring, and the lowest incidence of disease diagnosis occurs in the Winter, suggesting that airborne allergens or other seasonal particulates may be involved in disease progression (91). There are positive associations between the development

of sarcoidosis and certain environmental and occupational exposures, such as insecticides and mold/mildew (92). "Sarcoid-like" granulomatous pulmonary disease occurred in rescue workers that were exposed to airborne particulates during the World Trade Center collapse (93). Recently, it was reported that patients exposed to occupational silica during work in the iron production industry in Sweden have a significantly higher incidence of sarcoidosis than unexposed individuals (94). Whether these exposures are driving sarcoidosis by activating specific CD4+ T cells in the lung or are merely inducing an inflammatory environment that makes individuals more susceptible to disease is currently unknown.

CONCLUSIONS

The immunology of sarcoidosis and CBD are quite similar, based on a predominance of CD4+ T cells in the lung, a Th1 polarized immune response, and a pathologic hallmark of granulomatous inflammation. As such, the presence of a known antigen in CBD can further our understanding of the driving factors involved in sarcoidosis pathogenesis. In CBD, our group has performed unbiased antigen discovery approaches that focused on the related and expanded CD4+ T cell subsets in the BAL of patients with active disease and have delineated several beryllium-dependent T cell ligands. It is possible that a similar unbiased antigen discovery approach utilizing expanded CD4+ T cell clones in the lungs of sarcoidosis patients may lead to the discovery of the inciting antigens that drive CD4+ T cell alveolitis and granulomatous inflammation in sarcoidosis.

AUTHOR CONTRIBUTIONS

SG, SA, and AF wrote the article. SA contributed to designing the figure and table for the article. All authors contributed to article revisions.

FUNDING

This work was supported by the following NIH grants: ES025534, HL062410, and HL102245 (to AF).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 12 May 2020
doi: 10.3389/fimmu.2020.00779

Derangement of Metabolic and Lysosomal Gene Profiles in Response to Dexamethasone Treatment in Sarcoidosis

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Glucocorticoids (GCs) play a central role in modulation of inflammation in various diseases, including respiratory diseases such as sarcoidosis. Surprisingly, the specific anti-inflammatory effects of GCs on different myeloid cells especially in macrophages remain poorly understood. Sarcoidosis is a systemic granulomatous disease of unknown etiology that occurs worldwide and is characterized by granuloma formation in different organs. Alveolar macrophages play a role in sarcoidosis granuloma formation and progressive lung disease. The goal of the present study is to identify the effect of GCs on transcriptomic profiles and the cellular pathways in sarcoidosis alveolar macrophages and their corresponding blood myeloid cells. We determined and compared the whole transcriptional signatures of alveolar macrophages from sarcoidosis patients and blood $CD14^+$ monocytes of the same subjects in response to *in vitro* treatment with dexamethasone (DEX) via RNA-sequencing. In response to DEX, we identified 2,834 genes that were differentially expressed in AM. Predominant pathways affected were as following: metabolic pathway (FDR $= 4.1 \times 10^{-10}$), lysosome (FDR $= 6.3 \times 10^{-9}$), phagosome (FDR $= 3.9 \times 10^{-5}$). The DEX effect on AMs is associated with metabolic derangements involving glycolysis, oxidative phosphorylation and lipid metabolisms. In contrast, the top impacted pathways in response to DEX treatment in blood $CD14^+$ monocytes were as following; cytokine-cytokine receptor interaction (FDR $= 6 \times 10^{-6}$) and transcriptional misregulation in cancer (FDR $= 1 \times 10^{-4}$). Pathways similarly affected in both cell types were genes involved in lysosomes, cytoskeleton and transcriptional misregulation in cancer. These data suggest that the different effects of DEX on AMs and peripheral blood monocytes are partly dictated by lineage specific transcriptional programs and their physiological functions.

Keywords: dexamethasone, corticosteroids, gene expression, sarcoidosis, alveolar macrophages, monocytes, RNA-seq

OPEN ACCESS

Edited by:
Barbara P. Barna, East Carolina University, United States

Reviewed by:
Elliott Crouser, The Ohio State University, United States
Krzysztof Guzik, Jagiellonian University, Poland

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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *19 December 2019*
Accepted: *06 April 2020*
Published: *12 May 2020*

Citation:
Talreja J, Bauerfeld C, Sendler E, Pique-Regi R, Luca F and Samavati L (2020) Derangement of Metabolic and Lysosomal Gene Profiles in Response to Dexamethasone Treatment in Sarcoidosis. Front. Immunol. 11:779. doi: 10.3389/fimmu.2020.00779

INTRODUCTION

Pulmonary sarcoidosis is characterized by granuloma formation in the lungs. Pulmonary involvement is the leading cause of morbidity and mortality in sarcoidosis patients in the US, while other organ involvements including eyes, central nervous system, cardiac, and skin may lead to severe functional impairments (1, 2). Lungs are constantly exposed to toxins and microbial products and lung macrophages play important roles in the maintenance of immunological homeostasis and are the cornerstone of granuloma formation (1, 3, 4).

Due to their immune-modulatory actions, glucocorticoids (GCs) are one of the most widely prescribed drugs for the treatment of inflammatory and immune mediated disorders, including sarcoidosis (5). The long-term use of GCs is limited due to profound derangements of glucose metabolism, mineral homeostasis, amino acid metabolism, cognition and effects on the cardiovascular system (6). Several studies questioned the long-term benefits of GCs treatment in several respiratory diseases, including chronic obstructive lung disease (COPD). In idiopathic pulmonary fibrosis GCs treatment has been associated with worsening prognosis (7–9). Similarly, the long term benefits of GC treatments in sarcoidosis is unclear (7). While GCs suppress inflammation by decreasing lymphocyte activation, proliferation, and survival, there is a knowledge gap in how GCs modulate the immune response of human myeloid cells and tissue macrophages, especially in sarcoidosis. Recent studies highlighted that GCs have little effect in controlling inflammation in macrophage-dominated diseases (8, 10).

Because AMs originated ontologically from yolk sac and adapt to maintain pulmonary hemostasis and clearance, the effect of GCs on AMs may be different as compared to myeloid derived macrophages and monocytes (11). Additionally, in sarcoidosis the response to GCs treatment is partly dictated by organ involvement. This posits to question if tissue macrophages, especially AMs as compared to monocytes, are less susceptible to GCs treatment. Dissecting the effect of GCs treatment on cellular responses of two different macrophage lineages may uncover important molecular pathways to discriminate beneficial from detrimental effects of this therapy.

The goal of this study was to identify the differential transcriptional signatures and signaling pathways affected by corticosteroids in two relevant macrophage lineages (AMs and peripheral monocytes) in sarcoidosis. We performed transcriptomic analysis of isolated AMs and $CD14^+$ monocytes from the same sarcoidosis patients in response to *ex-vivo* treatment with DEX. We identified common as well as unique pathways affected by GCs in sarcoidosis AMs and monocytes.

METHODS

Study Subjects

Sarcoidosis diagnosis was based on the ATS/ERS/WASOG statement (12). The enrollment criteria for sarcoidosis patient were as previously described (13–15). A total of 10 patients with sarcoidosis participated in this study.

Isolation of AMs From BAL

AMs were isolated from BAL fluid as previously described (14, 15). Viability of AMs was routinely about 98%. Immunofluorescent staining with CD68 antibody confirmed >99% purity.

Isolation and Purification of $CD14^+$ Monocytes

$CD14^+$ monocytes were purified from PBMCs by using the MACS monocyte isolation kit (Miltenyl Biotech, San Diego, CA) according to the manufacturer's instructions (13, 14). The purity of enriched monocytes was evaluated by flow cytometry using fluorochrome-conjugated CD14 antibody; the purity of monocytes was >98%.

mRNA Isolation

Sarcoidosis AMs or monocytes were cultured in the presence of DEX (100 ng/mL) or vehicle for 16 h. After 16 h incubation, mRNA was isolated from purified AMs and monocytes using the Dynabeads mRNA Direct Kit (Ambion) as described earlier (13).

RNA-seq Library Preparation and Sequencing

RNA-seq libraries were prepared using the NEBNext ultradirectional library preparation protocol (New England BioLabs, Ipswich, MA) as described earlier (13). RNA-seq library quality was assessed using an Agilent Bioanalyzer. Individually barcoded RNA-seq libraries were pooled in equimolar quantities. A pooled library of 40 samples: untreated sarcoidosis AMs ($n = 10$), DEX-treated sarcoidosis AMs ($n = 10$), untreated sarcoidosis monocytes ($n = 10$), and DEX-treated sarcoidosis monocytes ($n = 10$) was sequenced on the Illumina Next-Seq 500 (75 cycles, PE).

RNA-seq Data Analysis, Differential Gene Expression, and Canonical Pathway Analysis

RNA-seq data were analyzed using the Illumina Basespace RNA express application. The sequencing reads were aligned to the reference human genome hg19 using STAR aligner and differentially expressed (DE) genes (FDR < 5%) were identified with the DEseq2 analysis tool (16, 17). Enrichment of cellular pathway and gene ontology category was calculated using iPathwayGuidetool and Gene Trail 2 Over-Representation Analysis (ORA) and comparing the list of DE genes with background expressed genes. Pathway enrichment was determined at FDR < 5%, using Benjamini and Hochberg procedure to control for multiple testing (18).

RESULTS

Effect of Dexamethasone on the Transcriptional Signature of Sarcoidosis AMs

Using CD68 antibody as macrophage marker, the purity of isolated AMs was >99% (**Figure S1**). The subject demographics are displayed in **Table 1**. All subjects were non-smokers, and none were on corticosteroid or other immune-suppressive medication. Isolated AMs were cultured either in the presence of DEX (100 ng/mL) for 16 h or vehicle. RNA-seq libraries were prepared from DEX treated AMs ($n = 10$) and untreated AMs ($n = 10$) from the same subjects serving as controls. RNA-seq libraries were sequenced on one lane of the Illumina Next-Seq 500 as described previously (13). Using DeSeq2, we identified 2834 DE genes ($\log_2$-fold change (FC) > 0.6 and FDR < 5%) between DEX-treated vs. untreated AMs from same sarcoidosis patients (**Figure 1A**). The Gene ontology (GO) enrichment analysis of DE genes (FDR < 5%) showed that the significant biological processes impacted after DEX treatment were neutrophil degranulation (FDR $= 1.7 \times 10^{-7}$), regulation of transcription (FDR $= 3.3 \times 10^{-4}$), and insulin receptor signaling (FDR $= 4.1 \times 10^{-4}$) (**Figure 1B**). Most genes involved in neutrophil degranulation were downregulated. CD14 is a general marker for myeloid cells (macrophages and monocytes), but it has been shown that activated neutrophil exhibit surface CD14 (19). The analysis of biological function enrichment showed lysosome and lysosomal membrane as significant cellular components affected by DEX treatment (**Figure 1C**). **Table 2** summarizes the most up and down regulated genes in response to DEX treatment in sarcoidosis AMs. Increased expression of several of these genes has been reported in THP-1 cell lines or monocyte-derived dendritic cells in response to DEX treatment (20–22). We identified that DEX-treatment induced the expression of two novel genes; Ring finger protein (RNF) 175 and amine oxidase copper containing 2 (AOC2). Other upregulated genes were formyl peptide receptor 1 (FPR1) ($\log_2$ FC = 2.6, FDR $= 1.8 \times 10^{-7}$) and Vanin 1 (VNN1) ($\log_2$ FC = 2.0, FDR $= 7.1 \times 10^{-7}$). FPR1 is a member of the G-protein coupled receptor 1 family encoding protein (21), which mediates the response of phagocytic cells to microorganisms. Vanin 1 belongs to vanin gene family and has pantetheinase activity and participates in hematopoietic cell trafficking (23). Similarly, complement factor properdin (CFP) ($\log_2$ FC = 1.3, FDR $= 7.4 \times 10^{-5}$) was upregulated. This gene positively regulates the alternative complement pathway. DEX-treatment downregulated several small nucleolar RNA box genes, solute carrier families and integrin genes in sarcoid AMs.

Pathway enrichment analysis identified over 50 significant pathways affected by DEX treatment (**Figure 1D**). The most significant pathways were metabolic pathway (FDR $= 4.1 \times 10^{-10}$), lysosome (FDR $= 6.3 \times 10^{-9}$), phagosome (FDR $= 3.9 \times 10^{-5}$), FoxO signaling pathway (FDR $= 7 \times 10^{-6}$), transcriptional misregulation in cancer (FDR $= 1.6 \times 10^{-6}$), and regulation of actin cytoskeleton (FDR $= 5.7 \times 10^{-5}$).

TABLE 1 | Subject demographics.

Characteristic	Patients
Age (year)	46.11 ± 14.4
BMI	29 ± 10.4
Gender, *N* (%)	
Female	5 (50)
Male	5 (50)
Self-reported Race, N (%)	
African American	10(100)
White	0 (0)
CXR stage, *N* (%)	
0	0 (0)
1	0 (0)
2	6 (60)
3	4 (40)
Organ Involvements, *N* (%)	
Neuro-ophthalmologic	2 (10)
Lung	10 (100)
Skin	4 (10)
Multiorgans	15 (75)
PPD	Negative
AFB/culture	Negative
IGRA	Negative

BMI, body mass index; CXR, chest X-ray; PPD, purified protein derivative; AFB, acid-fast bacilli; IGRA, interferon gamma release assay.

Effect of Dexamethasone on Metabolic Pathway

We found that 237 DE genes are involved in metabolic pathway of which 65 genes were upregulated and 172 genes were downregulated. To determine the metabolic pathways impacted by DEX treatment, we performed a pathway analysis of these 237 genes using Gene Trail 2. The most significant pathways were as following; oxidative phosphorylation (FDR $= 1.4 \times 10^{-36}$), carbon (FDR $= 4.7 \times 10^{-34}$), purine metabolism (FDR $= 4.2 \times 10^{-22}$), pyrimidine metabolism (FDR $= 4.8 \times 10^{-18}$), non-alcoholic fatty liver disease (NAFLD) (FDR $= 2.3 \times 10^{-17}$), glycolysis (FDR $= 2.4 \times 10^{-16}$), and fatty acid metabolism (FDR $= 2.1 \times 10^{-12}$). **Figure 2** shows the heatmap of genes involved in fatty acid metabolism (A), oxidative phosphorylation (B), and lysosome pathway (C) in response to DEX-treatment. DEX-treatment led to downregulation of genes involved in glycolysis and oxidative phosphorylation. Specifically, DEX-treatment downregulated the expression of peroxisome proliferator-activated receptor γ (PPARγ) co-activator 1 Beta gene (PPARGC1B) ($\log_2$ FC $= -1.4$, FDR $= 3.08 \times 10^{-9}$). PPARγ co-activator is known to be the master regulator of mitochondrial biogenesis, oxidative phosphorylation and lipid metabolism (24, 25). In contrast, DEX increased expression of genes involved in amino sugar and nucleotide sugar metabolism. Fucose-1-phosphate guanylyltransferase (FPGT) increased in response to DEX treatment by 2 folds. Protein encoded by this gene participates in phosphorylation of L-fucose to form beta-L-fucose-1-phosphate which is a substrate of fucose-1-phosphate

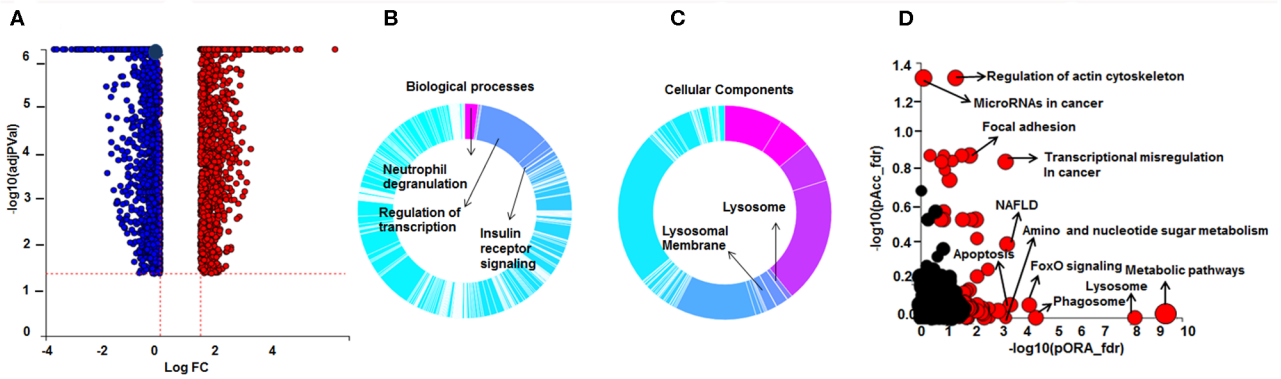


FIGURE 1 | Differential gene expression and Gene Ontology (GO) processes and significant pathways between DEX-treated compared to untreated sarcoid AMs. **(A)** Volcano plot: 2,834 significant DE genes analyzed by DEseq2 analysis tool are represented in terms of their measured expression fold change (log_2FC) on the x-axis and the significance of the change ($-log_{10}$ adj *p*-value) on the y-axis. The dotted lines represent the thresholds used to select the DE genes: log_2-fold change (>0.6) and significance (FDR < 5%). The up-regulated genes (positive log_2FC) are shown in red, while the down-regulated (negative log_2FC) genes are shown in blue. **(B,C)** Gene Ontology (GO) analysis of DE genes. To assess the enrichment of GO terms Elim pruning method was used. GO analysis identified top **(B)** biological processes and **(C)** cellular components. **(D)** Pathway analysis on the DE genes was performed considering a threshold of log_2 FC > 0.6 and FDR < 5% using iPathwayGuide analysis tool that uses two types of evidence: the over-representation on the horizontal axis (pORA) and the perturbation on the vertical axis (pAcc). Significant pathways (FDR < 5%) are shown in red, whereas non-significant are in black. The size of the circle is proportional to the number of genes in that pathway.

guanylyltransferase and GTP to form GDP-beta-L-fucose (26). AOC2 gene (log_2 FC = 3, FDR = 1.1×10^{-13}) catalyzes the oxidative conversion of amines to aldehydes and ammonia in the presence of copper and quinone cofactor and is associated with eye diseases (27). Glutamate-ammonia ligase (GLUL) expression is also increased in response to DEX (log_2 FC = 2, FDR = 3.8×10^{-11}). The protein encoded by this gene catalyzes the synthesis of glutamine from glutamate and ammonia in an ATP-dependent reaction (28). It is a key protein of glutamine synthesis and essential for cellular metabolism including macrophages (29). DEX upregulated the genes involved in insulin receptor signaling. For instance; insulin receptor substrate 2 (IRS2) (log_2 FC = 1.2, FDR = 5.4×10^{-5}), insulin receptor (INSR) (log_2 FC = 1.0, FDR = 2.0×10^{-3}), and phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1) (log_2 FC = 1.0, FDR = 3.4×10^{-6}). AMP-activated protein kinase (AMPK) is an important energy-sensing enzyme that monitors cellular energy status. DEX upregulated the expression of genes that encode AMPK subunits, including catalytic subunit alpha 1 (PRKAA1) (log_2 FC = 0.96, FDR = 1.8×10^{-5}) and non-catalytic subunit beta 2 (PRKAB2) (log_2 FC = 0.95, FDR = 6.2×10^{-11}) (30). The catalytic subunit of AMPK protects cells from ATP depletion by switching off ATP-consuming biosynthetic pathways (30).

Modulation of Phago-Lysosomal Genes by Dexamethasone

The lysosomal pathway was the second most impacted pathway in response to DEX treatment. **Figure 2C** shows the effect of DEX treatment on the gene expression involved in the lysosome pathway in sarcoidosis AMs. Among 44 lysosomal genes only 6 genes were upregulated while the rest were down regulated in response to DEX treatment. Clathrin heavy chain like (CLTCL) 1 expression increased 4 fold (log_2 FC = 2.0, FDR = 8.2×10^{-8}) in response to DEX treatment. Clathrin coated vesicles have been shown to endocytose accumulated protein aggregates and direct them to the lysosome for further degradation (31). Cathepsins represent the major components of the lysosomal proteolytic system. Cathepsin F (CTSF) (log_2 FC = 1.5, FDR = 0.8×10^{-7}) and cathepsin O (CTSO) (log_2 FC = 0.65, FDR= 5.4×10^{-3}) were upregulated. Galactosylceramidase (GALC) hydrolyzes the galactose ester bonds of galactosylceramide and was increased (log_2 FC = 1.0, FDR = 9.1×10^{-5}). Lysosomal related genes involved in V-ATPase dependent organelle acidification such as T Cell Immune Regulator 1 (TCIRG1) (log_2 FC = -1.3, FDR = 1.4×10^{-16}), lysosomal proteases (CTSK) (log_2 FC = -1.1, FDR = 6.3×10^{-4}), sulfatases (ARSB) (log_2 FC = -0.8, FDR = 1.5×10^{-5}), and lysosomal membrane proteins (LAMP) (log_2 FC = -0.7, FDR = 3.5×10^{-3}) were significantly decreased in response to DEX treatment. Cathepsin K (CTSK) expression was downregulated in sarcoid AMs after DEX treatment. CTSK, a lysosomal cysteine protease, is proteolytic for matrix proteins, including gelatin and fibrillar collagens. It has been shown to play a key role in protecting against lung fibrosis (28). The Mucolipin 1 (MCOLN1) gene that encodes a member of the transient receptor potential (TRP) cation channel gene family was downregulated (log_2 FC = -1.1, FDR = 2.6×10^{-10}). MCOLN1 has been suggested to regulate fusion/fission of vesicles in the endocytic pathway (32), and lysosomal ion homeostasis. Mutations in the MCOLN1 gene have been reported with lysosomal storage disease (33). The Transcription factor EB (TFEB) gene was downregulated (log_2 FC = -1.7, FDR = 2.2×10^{-9}). TFEB plays an important role in lysosomal biogenesis, autophagy and cellular metabolism (34). These data show that DEX treatment downregulates genes of the lysosomal pathway and lysosomal biogenesis that are involved in uptake and acidification processes and in the proteolysis of degraded proteins/debris in lysosomes.

TABLE 2 | Top up and down-regulated regulated genes in AMs in response to dexamethasone treatment.

Gene symbol	Gene name	Type	log_2 fold change	FDR *p*-value
pkp2	Plakophilin 2	Cytoskeleton	4.5	7.87E-19
ABLIM3	Actin binding LIM protein family member 3	Cytoskeleton	3.5	1.23E-13
SRPX	Sushi repeat containing protein X-linked	Peroxiredoxin-like domain	3.5	4.03E-10
TSC22D3	TSC22 domain family member 3	Leucine zipper protein	3.4	5.13E-26
METTL7A	Methyltransferase like 7A	Methyltransferase	3.1	6.28E-14
MFGE8	Milk fat globule-EGF factor 8 protein	Membrane glycoprotein	2.9	6.59E-15
RNF175	Ring finger protein 175	E3 ligase	2.9	2.89E-09
AOC2	Amine oxidase copper containing 2	Amine oxidases	2.8	1.1E-13
FMN1	Formin 1	Cytoskeleton	2.7	1.47E-25
MERTK	MER proto-oncogene, tyrosine kinase	Tyrosine kinase	2.7	3.97E-07
FKBP5	FKBP prolyl isomerase 5	Immunophilin protein family	2.7	2.04E-27
RGS2	Regulator of G protein signaling 2	G protein signaling	2.6	3.54E-09
SESN1	Sestrin 1	Sestrin family. P53	2.2	2.20E-19
CD163L1	CD163 molecule like 1	Scavenger receptor	2.7	6.01E-13
FPR1	Formyl peptide receptor 1	G-protein coupled receptor	2.6	1.89E-07
TFPI	Tissue factor pathway inhibitor	Serine protease inhibitor	2.3	3.54E-09
NES	Nestin	Intermediate filament protein family	−3.7	2.74E-12
METTL7B	Methyltransferase like 7B	Methyltransferase	−3.7	1.66E-10
TIE1	Tyrosine kinase with immunoglobulin like and EGF like domains 1	Protein tyrosine kinase	−3.7	1.66E-10
SNORD15B	Small nucleolar RNA, C/D box 15B	Small nucleolar RNAs	−3.7	4.78E-16
SNORA8	Small nucleolar RNA, H/ACA box 8	Small nucleolar RNAs	−3.4	2.04E-11
LGALS2	Galectin 2	Galactoside binding lectin	−3.4	3.58E-13
SNORD97	Small nucleolar RNA, C/D box 97	Small nucleolar RNAs	−2.9	2.04E-12
PRAM1	PML-RARA regulated adaptor molecule 1	Adaptor protein	−2.6	7.21E-25
COL6A1	Collagen type VI alpha 1 chain	Collagen (cytoskeleton)	−2.05	1.52E-06
SLC37A2	Solute carrier family 37 member 2	Solute carrier protein	−2.4	9.65E-07
SLC6A12	Solute carrier family 6 member 12	Solute carrier protein	−2.3	7.21E-15
SLC29A3	Solute carrier family 29 member 3	Solute carrier protein	−1.9	7.68E-13
CASS4	Cas scaffold protein family member 4	Scaffolding protein	−2.4	4.78E-08
UBTD1	Ubiquitin domain containing 1	E3 ligase	−1.7	1.87E-08
ITGB7	Integrin subunit beta 7	Integrin	−2.9	1.92E-11

In response to DEX most genes related to phagosome formation showed decreased expression, only 6 genes were upregulated. Upregulated genes in response to DEX treatment were as following: member RAS oncogene family RAB5A (log_2 FC = 0.7, FDR = 3.39×10^{-11}) that is required for the fusion of plasma membranes and early endosomes, TLR4 (log_2 FC = 1.5, FDR = 2.8×10^{-7}), TLR5 (log_2 FC = 1.1, FDR = 4.1×10^{-2}), TLR8 (log_2 FC = 0.8, FDR = 4.1×10^{-3}), dynein cytoplasmic 1 light intermediate chain 2 (DYNC1L) (log_2 FC = 0.8, FDR = 5.7×10^{-15}) and dynein cytoplasmic 2 heavy chain 1 (DYNC2H1) (log_2 FC = 1.6, FDR = 1.3×10^{-6}). This gene encodes a large cytoplasmic dynein protein involved in retrograde transport in the cilium and intra flagellar transport, a process required for ciliary/flagellar assembly. Cytoplasmic dynein-1 transports cargo into cell interior toward microtubule minus ends (35). Dynein-2, also known as intra flagellar transport dynein, moves cargoes along the axoneme of eukaryotic cilia and flagella (36). They are important for ciliary function, neuron and phagolysosome formation (30, 37). Aberrant function of the protein has been reported in ciliary dysfunction (35). Three genes encoding subunits of NADPH oxidase complex were downregulated. These were CYBA (p22-PHOX) (log_2 FC = −0.7, FDR = 7.2×10^{-9}), CYBB (log_2 FC = −0.8, FDR = 1.0×10^{-3}) and NCF2 (log_2 FC = −0.9, FDR = 1.0×10^{-4}). While NCF-1 gene expression was upregulated (log_2 FC = 1.72, FDR = 3.7×10^{-8}). NADPH oxidase is a key producer of reactive oxygen species (ROS) participating in killing of bacteria in phagosomes. Mutation in this gene is associated with chronic granulomatous disease (38).

Transcriptional Misregulation in Cancer

DEX treatment of sarcoidosis AMs upregulated the expression of 18 genes and downregulated the expression of 20 genes related to transcriptional misregulation in cancer. The expression of

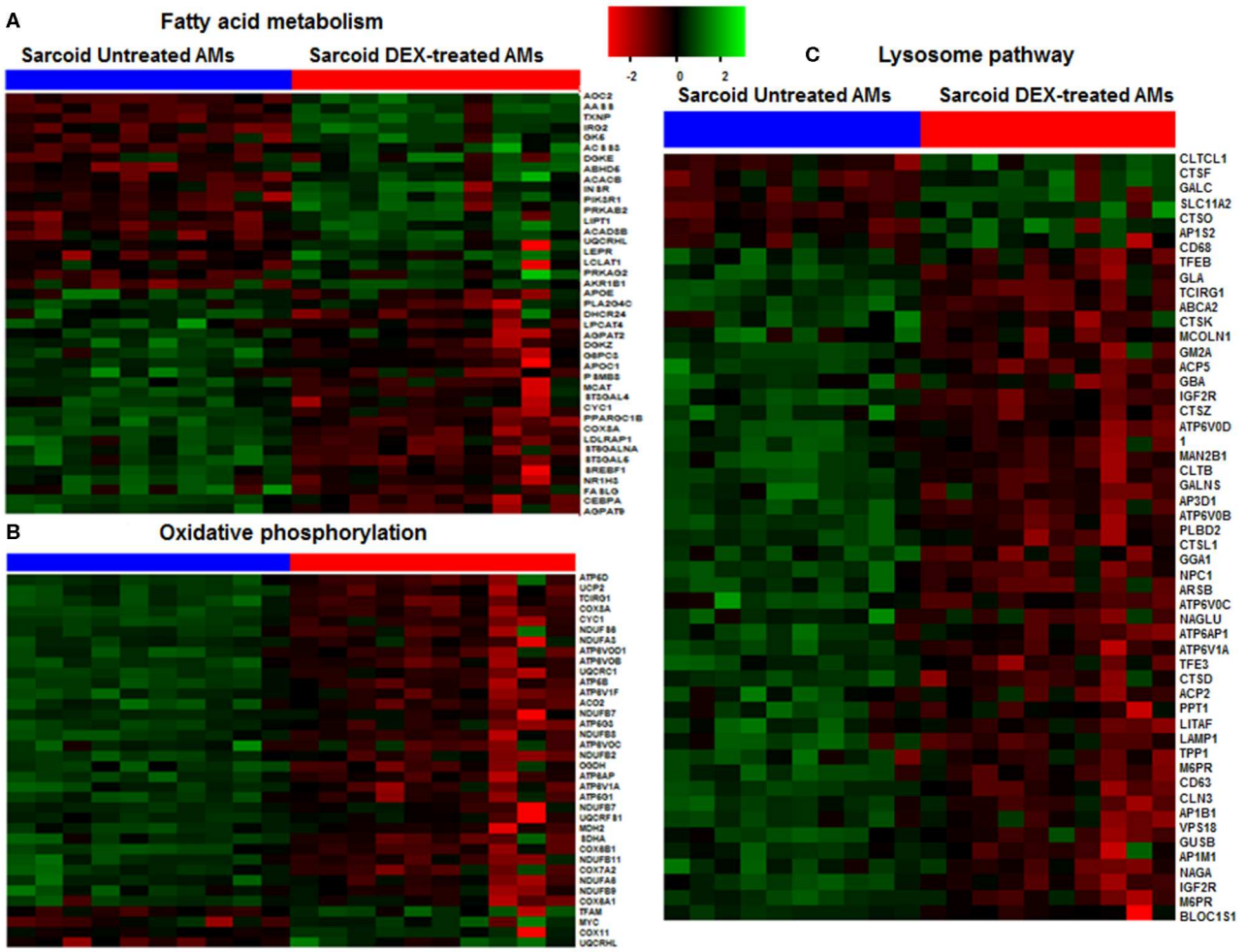


FIGURE 2 | Heat map of DE genes involved in fatty acid metabolism, oxidative phosphorylation and lysosomal pathways. **(A)** Fatty acid metabolism, **(B)** Oxidative phosphorylation, **(C)** Lysosomal pathway. The thresholds used to select the DE genes: $\log_2$ FC > 0.6 and FDR < 5%. Green shade represents high expression and red shade represents low expression.

15-hydroxyprostaglandin dehydrogenase (HPGD) was increased ($\log_2$ FC = 1.5, FDR = 9.3×10^{-4}). HPGD is involved in the degradation of prostaglandin E2 (PGE_2) and hence suppresses tumor formation and inhibits inflammation induced by PGE_2 (39, 40). DEX treatment increased the expression of the FLT3 gene ($\log_2$ FC = 1.5, FDR = 1.4×10^{-3}). This gene encodes a receptor tyrosine kinase promoting Ras signaling, which plays a key role in survival, proliferation and differentiation of hematopoietic cells (41–43). Myocyte enhancer factor 2C (MEF2C) expression was upregulated ($\log_2$ FC = 1.0×10^{-6}, FDR = 3.8×10^{-8}). MEF2C, a member of the MADS family of transcription factors, regulates hematopoietic self-renewal and differentiation, supports the proliferation of leukemias, and is associated with increased risk of relapse in leukemia patients as its phosphorylation leads to chemotherapy resistance (44). The NFKB inhibitor zeta (NFKBIZ) gene that encodes for IkappaB-zeta, a transcriptional regulator for NF-kappaB, was upregulated ($\log_2$ FC = 1.5, FDR = 1.0×10^{-5}) with DEX treatment. It has been shown to induce pro-inflammatory responses and lymphoproliferative disorders and in solid tumors (45). The MDM2 proto-oncogene ($\log_2$ FC = 1.5, FDR = 1.0×10^{-6}) encodes a nuclear-localized E3 ubiquitin ligase, promoting tumor formation by targeting tumor suppressor proteins, such as p53, for proteasomal degradation. The upregulated expression of the FLT3, MEF2C, NFKBIZ, and MDM2 genes in sarcoid AMs suggests the potential side effect of DEX and it may lead to increased risk of tumor cell proliferation. DEX treatment modulated expression of several genes related to focal adhesion and cytoskeleton, including integrin families. Cyclin D2 (CCND2) functions as regulators of CDK kinases important for cell cycle G1/S transition was downregulated ($\log_2$ FC = −1.7, FDR = 1.3×10^{-4}) (46).

Regulation of Actin Cytoskeleton

One of the major pathways affected by DEX treatment was regulation of the actin cytoskeleton. Actins are highly conserved

proteins that are involved in cell motility, structure, integrity, and intercellular signaling (47). Plakophilin 2 (PKP2) encoding a plakophilin protein, was significantly upregulated (log_2 log_2 FC = 4.6, FDR = 7.9×10^{-19}). Plakophilin 2 participates in linking cadherins to intermediate filaments in the cytoskeleton and cardiac muscles. It has been shown to be associated with several types of cancers in humans (48). PKP2 regulates calcium signaling in cardiac muscles (49). The slingshot homolog 2 (SSH2) gene was upregulated (log_2 FC = 1.2, FDR = 1.0×10^{-6}). SSH2 encodes a protein tyrosine phosphatase, which regulates actin filaments by dephosphorylating and activating cofilin which promotes actin filament depolymerization (50). The actin regulatory protein, cofilin/actin depolymerization factor (ADF), serves a vital function in cells by severing filaments, thereby increasing the number of filament ends from which polymerization and depolymerization can occur (47). The cofilin pathway is important for actin dynamics, cell movement, cytoskeleton, distribution of receptors and synapse formation and migration of immune cells. It is critical for phagocytosis, motility, and antigen presentation in macrophages, dendritic cells, and neuronal cells (51). Similarly, we found that DEX treatment downregulated the expression of several integrin membrane protein genes. Few of these genes are: Integrin subunit beta and alpha, ITGB7 and ITGA7 (log_2 FC = −1.2, FDR = 4.6×10^{-5}), ITGAM, ITGA6, ITGB2. These genes code for integrins/adhesion receptors involved in leucocyte migration.

Effect of Dexamethasone on Genes Related to Inflammation

The TSC22 domain family member 3 gene (TSC22D3) was found to be highly upregulated in sarcoid AMs after DEX treatment (**Table 2**). TSC22D3 gene encodes GC-induced leucine zipper transcription factor, whose expression is rapidly induced by GC, IL-10 and TGF-β (52, 53). This protein has been shown to play a key role in immunosuppressive effects of GCs. DEX upregulated the expression of genes involved in the negative regulation of inflammation, including Dual specificity phosphatase (DUSP)1, also known as MKP1, which is a phosphatase involved in the dephosphorylation of MAPKs (15), and suppressor of cytokine signaling (SOCS4 and SOCS6). DEX downregulated the expression of various genes that play a key role in inflammation. Gene expression for interleukin 6 receptor complex (IL6R), TNF-receptor superfamily (TNFRSF4) and MyD88, essential signaling molecules in TLR and IL-1 signaling were decreased in response to dexamethasone treatment.

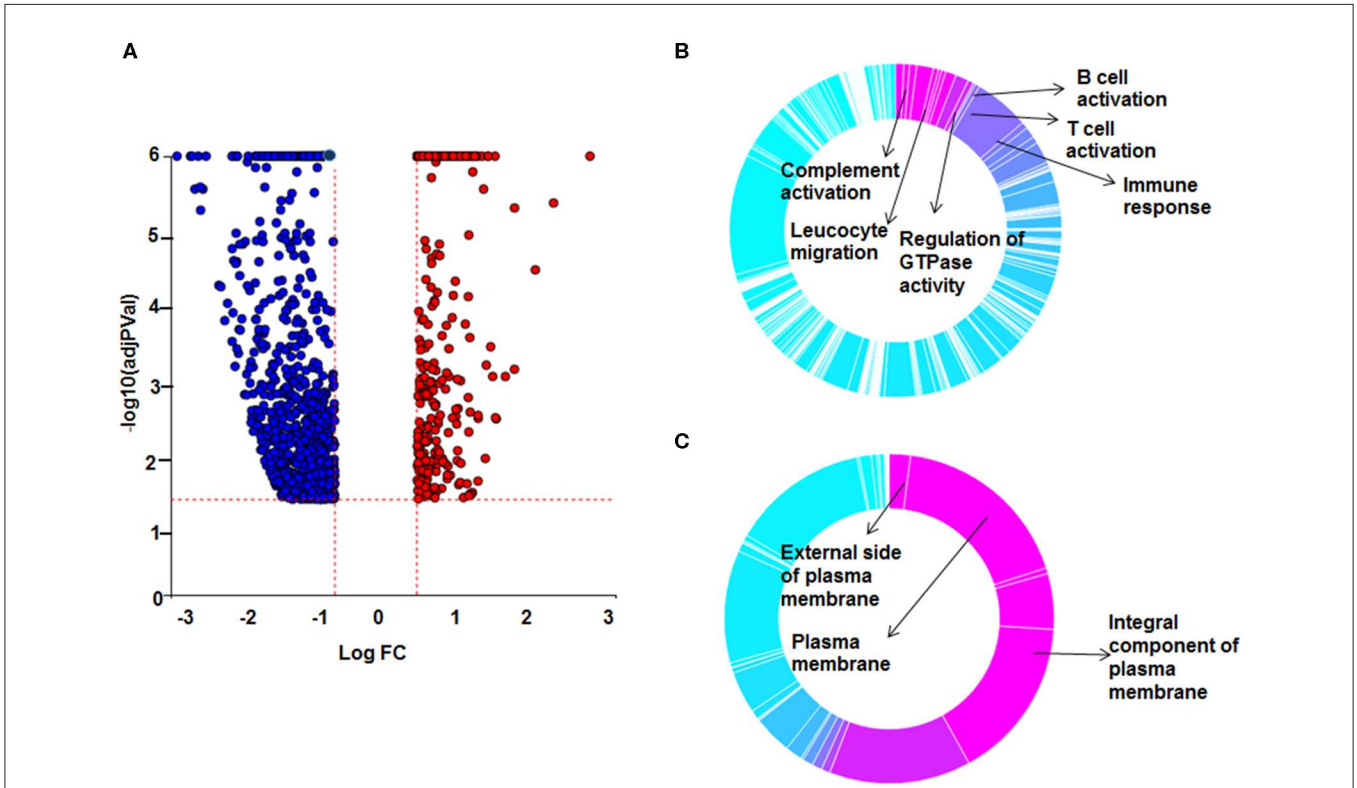


FIGURE 3 | Differential gene expression and Gene Ontology (GO) processes between sarcoid DEX-treated and untreated sarcoid monocytes. **(A)** Volcano plot: All 1,958 significant DE genes analyzed by DEseq2 analysis tool are represented in terms of their measured expression fold change (log_2FC) on x-axis and the significance of the change (–log_{10} adj *p*-value) on y-axis. The dotted lines represent the thresholds used to select the DE genes: log_2 FC > 0.6 and significance FDR<5%. The up-regulated genes (positive log_2FC) are shown in red, while the down-regulated (negative log_2FC) genes are shown in blue. **(B,C)** Gene Ontology (GO) analysis of DE genes was done using ipathwayGuide analysis tool. To assess the enrichment of GO terms Elim pruning method was used. GO analysis identified top **(B)** biological processes and **(C)** cellular components.

Effect of Dexamethasone on Transcriptional Signature of Sarcoidosis CD14$^+$ Monocytes

To decrease the effect of genetic variation, we used the same cohort of patients used for the AMs study. To identify genes responsive to GC on sarcoidosis monocytes, we isolated CD14$^+$ monocytes from PBMCs and treated in a similar fashion with DEX. In response to DEX treatment 1,958 genes were differentially expressed in monocytes (log_2-fold change of 0.6 and FDR < 5%) (**Figure 3A**). The Gene ontology (GO) enrichment analysis showed that the significant biological processes impacted after DEX treatment were classic complement activation, leukocyte, T and B cell activation as well as phagocytosis (**Figure 3B**) and significant cellular components impacted are shown in **Figure 3C**. Next, we performed pathway enrichment analyses. The top impacted pathways in monocytes were as following: cytokine-cytokine receptor interaction (FDR = 6×10^{-6}), transcriptional misregulation in cancer (FDR = 1×10^{-4}), Type I DM (FDR = 2.6×10^{-3}), cell adhesion (FDR = 0.003), cytoskeleton (FDR = 2.0×10^{-3}), RAS signaling (FDR = 2.0×10^{-3}), and JAK-STAT pathway (FDR = 2.0×10^{-3}) (**Figure 4A**). The top most impacted pathway in CD14$^+$ monocytes in response to DEX treatment was related to cytokine-cytokine receptor interaction. DEX treatment decreased expression of numerous cytokine and chemokine genes in sarcoidosis monocytes, while increasing the expression of CXCL10 and IL-10. In contrast, DEX treatment led to a significant decrease in expression of CCL22 (also known as macrophage-derived chemokine) and CCL24. CCL22 is produced by myeloid cells and regulates migration of leukocytes (54). Many genes related to tumor necrosis factor alpha superfamily members, *TNFRSF8, 9, 11, 14, and 15* were down regulated in response to DEX treatment. **Figure 4B** shows the heatmap of genes involved in cytokine pathways. Similarly, gene expression for several TNF receptor associated factors (TRAFs) that are important for the TLR and TNF signaling activation were decreased. In contrast,

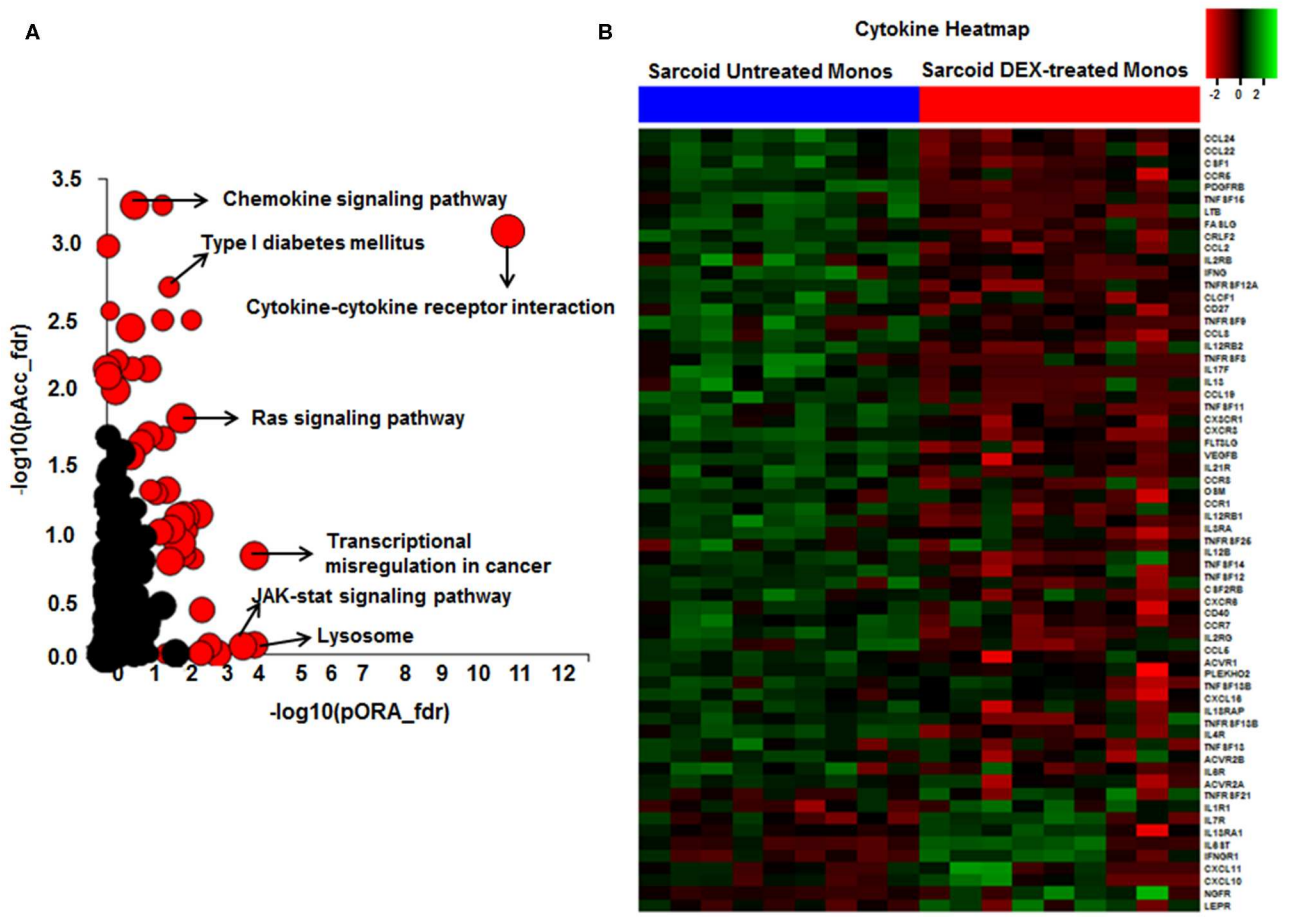


FIGURE 4 | Significant pathways enriched in DEX-treated compared to untreated sarcoid monocytes. **(A)** Pathway analysis was done on the DE genes (log_2FC > 0.6 and FDR < 5%) using iPathwayGuide analysis tool that uses two types of evidence: the over-representation on the horizontal axis (pORA) and the perturbation on the vertical axis (pAcc). Significant pathways (FDR < 5%) are shown in red, whereas non-significant are in black. The size of the circle is proportional to the number of genes in that pathway. **(B)** Heat map of DE genes involved in cytokine-cytokine receptor interaction pathway (log_2 FC > 0.6 with FDR < 5%). Green shade represents high expression and red shade represents low expression.

TABLE 3 | 10 top up and down-regulated genes in monocytes in response to dexamethasone treatment.

Gene symbol	Gene name	Type	Log fold change	FDR *p*-value
ABLIM3	Actin binding LIM protein family member 3	Cytoskeleton	3.1	5.89E-09
SERPINE1	Serpin family E member 1	Serine protease	2.4	1.0E-06
MT1G	Metallothionein 1G		2.5	4.37E-06
CD163L1	CD163 molecule like 1		1.9	1.0E-06
FPGT-TNNI3K	FPGT-TNNI3K	Cytoskeleton	2.7	1.0E-06
SLC6A13	Solute carrier family 6 member 13	Solute carrier protein	2.7	1.0E-06
FKBP5	FKBP prolyl isomerase 5	Immunophilin protein family	2.05	5.11E-06
RGS2	Regulator of G protein signaling 2	G protein signaling	2.4	2.82E-06
HSPE1P18	Heat Shock 10kDa Protein 1 Pseudogene 18	Heat shock protein	2.2	1.0E-06
TUBE1	Tubulin epsilon 1	Cytoskeleton	2.3	1.64E-06
RYR2	Ryanodine receptor 2	ER resident	2.7	3.59E-05
CCL24	C-C motif chemokine ligand 24	Cytokine	−3.0	3.70E-08
CCL22	C-C motif chemokine ligand 22	Cytokine	−3.7	3.70E-08
FPR3	Formyl peptide receptor 3	G-protein-coupled receptors	−3.4	3.25E-09
RAPGEF5	Rap guanine nucleotide exchange factor 5	Ras family member	−2.6	2.12E-04
HSD3B7	Hydroxy-delta-5-steroid dehydrogenase	Member of the short-chain dehydrogenase/reductase superfamily	−2.05	2.65E-06
FEZ1	Fasciculation and elongation protein zeta 1	Zygin family	−2.4	9.40E-07
ADAMTS14	ADAM metallopeptidase with thrombospondin type 1 motif 14	Metallopeptidase	−1.9	2.9E-03
KCNN4	Potassium calcium-activated channel subfamily N member 4	Voltage-independent potassium channel	−2.4	1.20E-06
ATP8B4	ATPase phospholipid transporting 8B4	Phospholipid transport	−1.7	1.43E-06
IL32	Interleukin 32	Cytokine	−1.8	1.43E-06

DEX treatment led to increased expression of negative regulators of TLR signaling, TNF alpha induced protein 3 (TNFAIP3-A20). Similar to AMs, the pathway related to transcription misregulation in cancer was significantly impacted in response to DEX treatment of sarcoidosis monocytes.

The top most upregulated gene in response to DEX treatment in sarcoidosis monocytes was actin binding LIM protein family member 3 (ABLIM3) ($\log_2$ FC = 3.1, FDR = 1.0×10^{-6}) (**Table 3**). The encoded protein interacts with F actin filaments and is a component of adherents junctions in several cell types, including cardiac myocytes (55). Several genes related to retinoid X receptors (RXRs) and retinoic acid receptors (RARs) were downregulated in response to DEX treatment. These are important members of the steroid/thyroid hormone receptors acting predominantly as transcription factors (56).

Concordance Between DEX-Treated Sarcoidosis AMs and Monocytes

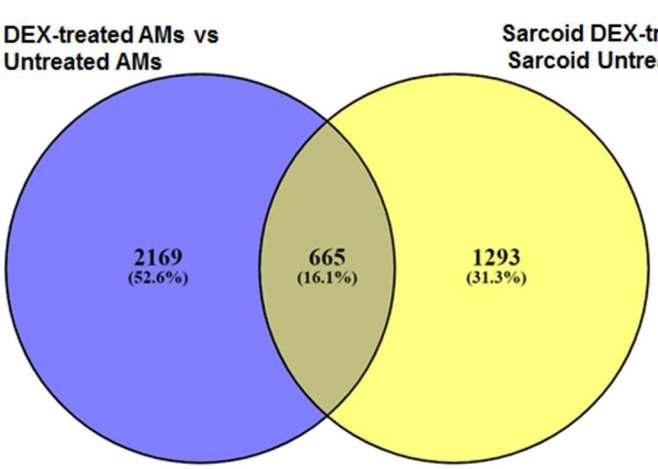
In response to DEX 2169 genes were exclusively differentially expressed in sarcoid AMs, while 1293 DE genes were found in sarcoid monocytes as shown in Venn diagram (**Figure 5A**). These results show that the impact of DEX treatment is more profound on sarcoid AMs as compared to monocytes. **Figure 6** shows 665 DE genes overlapping in DEX-treated sarcoidosis AMs and monocytes. We found that the effect of DEX on overlap DE genes was similar except for a few genes. DEX treatment upregulated the expression of activin A receptor type 2B (ACVR2B), MYC proto-oncogene, bHLH transcription factor (MYC), zinc finger and BTB domain containing 18 (ZBTB18) genes in AMs whereas it downregulated their expression in monocytes. ZBTB18 encodes a C2H2-type zinc finger protein and acts a transcriptional repressor of genes involved in neuronal development. Further, we performed the pathway enrichment analysis on the common DE genes in both cell types. The common pathways that were enriched in both DEX-treated AMs and monocytes were Rap1 signaling pathway, lysosomal pathway, PI3kinase signaling pathway, transcriptional misregulation in cancer, Type I diabetes mellitus (**Figure 5B**).

DISCUSSION

Macrophages have a central role in the maintenance of immunological homeostasis and host-defense. They play a crucial role in granuloma formation and provide metabolic cues for T cell responses in sarcoidosis. Alveolar macrophages, similar to other tissue associated macrophages have evolved to perform phagocytic clearance of pathogens and dying cells in the lungs immune-surveillance of the lung for inhaled pathogens as well as homeostatic regulation of lung tissues (11, 57). Circulating monocytes have the potential to differentiate into monocyte derived tissue macrophages and to migrate and interact with antigen-specific T and B lymphocytes to initiate adaptive immune responses.

Glucocorticoids modulate host immune responses to pathogens. In inflammatory diseases, including sarcoidosis, GCs have been used as an immunosuppressive drug to reduce inflammation (58). It has been shown that GCs suppress the expression of inflammatory genes, including TNF-α and

A



B

Type	Name	Number of hits	Expected score	Adjusted p-value
enriched	Rap1 signaling pathway	17	2.30197	1.21E-006
enriched	Lysosome	13	1.3056	2.28E-006
enriched	Jak-STAT signaling pathway	14	1.67208	2.92E-006
enriched	Insulin signaling pathway	13	1.50029	5.29E-006
enriched	PI3K-Akt signaling pathway	19	3.73354	7.69E-006
enriched	Cytokine-cytokine receptor interaction	16	2.89751	2.52E-005
enriched	Transcriptional misregulation in cancer	13	1.93549	3.76E-005
enriched	MAPK signaling pathway	15	2.72572	4.10E-005
enriched	Ras signaling pathway	14	2.37069	4.22E-005
enriched	Non-alcoholic fatty liver disease (NAFLD)	11	1.61482	1.72E-004
enriched	Regulation of actin cytoskeleton	12	2.24471	5.16E-004
enriched	Glycerophospholipid metabolism	8	0.97347	1.04E-003

FIGURE 5 | Metanalysis of DE genes between two groups: DEX-treated vs. untreated sarcoid AMs and DEX-treated vs. untreated sarcoid monocytes. **(A)** Venn diagram of DE genes showing 2,169 DE genes exclusively expressed in DEX-treated sarcoid AMs whereas 1293 DE genes were exclusively expressed in DEX-treated sarcoid monocytes. 665 DE genes overlap between the two groups **(B)** GeneTrail pathway analysis of common 665 DE genes between DEX treated sarcoid AMs and DEX treated sarcoid monocytes shows top 12 enriched significant pathways.

IFN-γ, and chemokines that are important for the granuloma formation and cell mediated Th1 responses. GCs are the only FDA approved drug to treat pulmonary and extrapulmonary sarcoidosis (59). However, despite their widespread use, there is a lack of evidence for their long term survival benefit in sarcoidosis or prevention of end organ damage such as lung fibrosis (58). In contrast, long term use of GCs has been shown to have significant side effects affecting all systems in the body (60). The most common side effects are excessive weight gain, insomnia, diabetes, osteoporosis, arterial hypertension and depression (60).

To determine the effect of GCs on transcriptome profiling of sarcoid AMs and $CD14^+$ monocytes, we compared the RNA-seq data of sarcoidosis AMs and $CD14^+$ cells treated with dexamethasone *in vitro* vs. untreated sarcoidosis AMs and $CD14^+$ monocytes, respectively. To decrease the confounding factors related to human genetic variation, we isolated the $CD14^+$ peripheral blood monocytes and AMs from the BALs of the same sarcoidosis subjects and compared pairwise RNA seq results. We identified two major pathways impacted after DEX treatment: metabolic and phagolysosomal pathways. Metabolic reprogramming in macrophages is critically important for their effector function (61, 62). **Figure 7** summarizes perturbation of genes related to metabolic pathways. We found that DEX treatment leads to suppression of gene expression related to glycolysis, fatty acid oxidation, TCA cycle and oxidative phosphorylation (**Figure 6**). In contrast, GCs enhance the expression of genes related to amino acid degradation pathways and generation of glutamine from glutamate (GLUL). Dexamethasone effected genes involved in metabolism of glucogenic amino acids (AAs) and ketogenic AAs, suggesting increased glucogenesis and fatty acid precursors (**Figure 6**). These alterations in response to dexamethasone explains some of the adverse effects seen in these patients after treatment with GCs, including loss of muscle mass, and lipodystrophy (63).

Other important dexamethasone effects were its effects on genes involved in lysosome and phagosome functions. Lysosomes are the common platform for endocytosis, phagocytosis, autophagy and biosynthetic routes. They receive both extracellular and intracellular-derived molecular cargo,

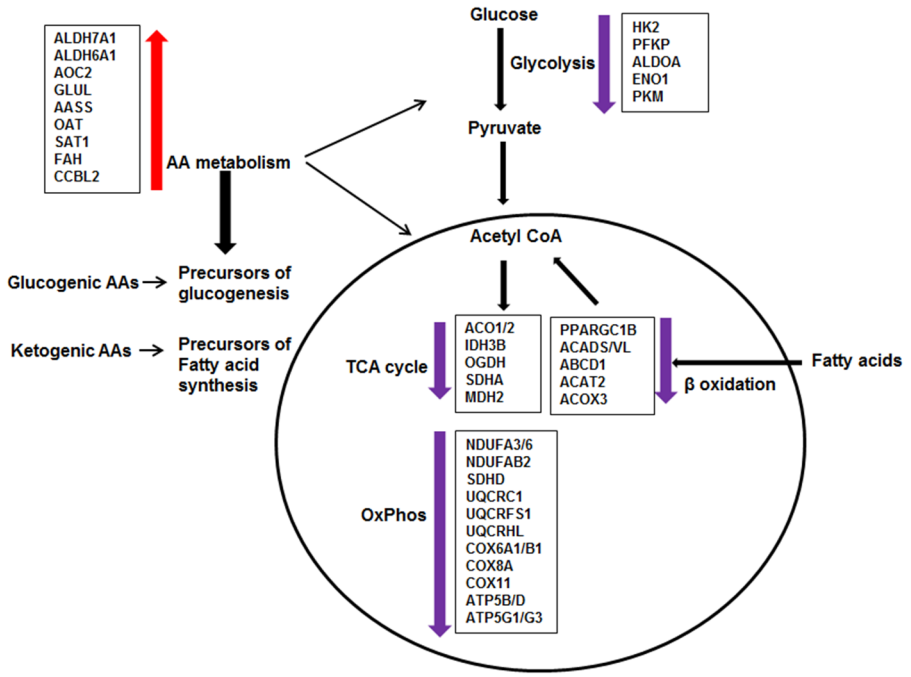


FIGURE 6 | Effect of DEX on metabolic pathways in sarcoid AMs. The pathway analysis of metabolic DE genes showed that the major pathways affected in DEX-treated sarcoid AMs as compared to untreated sarcoid AMS were: Glycolysis, Citric acid cycle (TCA), Oxidative phosphorylation (OxPhos), β-oxidation of fatty acids and Amino acid (AA) metabolism. DEX-treatment resulted in the downregulation of genes involved in glycolysis, TCA cycle, Oxphos and β-oxidation of fatty acids. Alteration in these pathways suggests that glucose metabolism is decreased; glucose is not being utilized completely to generate energy producing molecules ATP and NADH. Reduced β-oxidation of fatty acids suggests that fatty acids are not being transported and degraded optimally in mitochondria to generate energy. Suboptimal fatty acid oxidation may result in the accumulation of long chain fatty acids and metabolites. The upregulation of genes involved in AA metabolism shows that DEX-treated sarcoid AMs may switch to AA catabolism as a source of energy. AAs catabolism results in the conversion of AAs either to pyruvate or intermediates of TCA cycle for energy production or generation of precursors of glucogenesis and fatty acid synthesis.

damaged organelles, engulfed dead cells, and foreign particulates like bacteria for digestion. Tissue macrophages, including AMs, are sentinel cells that are equipped with a series of mechanisms of vesicular trafficking to orchestrate the elimination of bacteria as well as dead cells and debris (60). Clearance of bacteria by macrophages involves internalization of the microorganisms into phagosomes, which are then delivered to endo-lysosomes for enzymatic degradation. Lysosomes are a central organelle in the processing of exogenous and intracellular biomolecules. Thus, the final clearance of pathogens depend on lysosomal function (64). Lysosomes integrate and facilitate cross-talk between nutritional signals such as AA and energy levels, membrane damage and infection, and ultimately enable responses such as autophagy, cell growth, membrane repair and microbe clearance (65, 66). Lysosomes are hubs for cellular signaling and nutritional sensing through interplay with mTOR, AMPK, and GSK signaling (65). DEX-treated AMs show significantly decreased gene expression for the phagosome and lysosome machinery. For instance, transcription factor EB (TFEB), a master regulator of lysosomal biogenesis and fusion (65) was significantly downregulated in sarcoidosis AMs in response to DEX. **Figure 7** shows the downregulation of lysosome pathway genes encoding lysosomal acid hydrolases, membrane proteins, acidification and biogenesis proteins. Our results show that DEX-treatment led to the downregulation of two important genes, TFEB and PPARGC1 (PGC1) that play an important role in lysosomal and mitochondrial biogenesis and cellular metabolism. TFEB has been shown to control lipid catabolism and control the expression of PGC1α (34, 67). The DEX induced downregulation of TFEB, PGC1α, mitochondrial Acyl-CoA Dehydrogenase short chain fatty acid (ACADS) and very long-chain acyl-CoA dehydrogenase (VLCAD), as well as lysosomal genes in sarcoid AMs. This suggests that DEX-treatment decreases fatty acid oxidation and thus may lead to increased lipid deposition. Furthermore, DEX-treatment upregulated the expression of the CLTC1 gene (lysosomal pathway) that may contribute to accumulation of clathrin coated vesicles containing aggregated proteins. In contrast, the downregulation of lysosomal genes involved in proteolysis and acidification suggests that DEX-treatment inhibits the proteolytic degradation. This may result in lysosomal overload with protein aggregates and fatty acid deposits. Decreased lysosomal function may lead to an increase in unfolded protein accumulation and finally to unfolded protein response and ER stress (68). Because pathogens and antigens may play a role in the pathogenesis of sarcoidosis, the downregulation of lysosomes and phagosome by DEX may have negative implications for the disease progression.

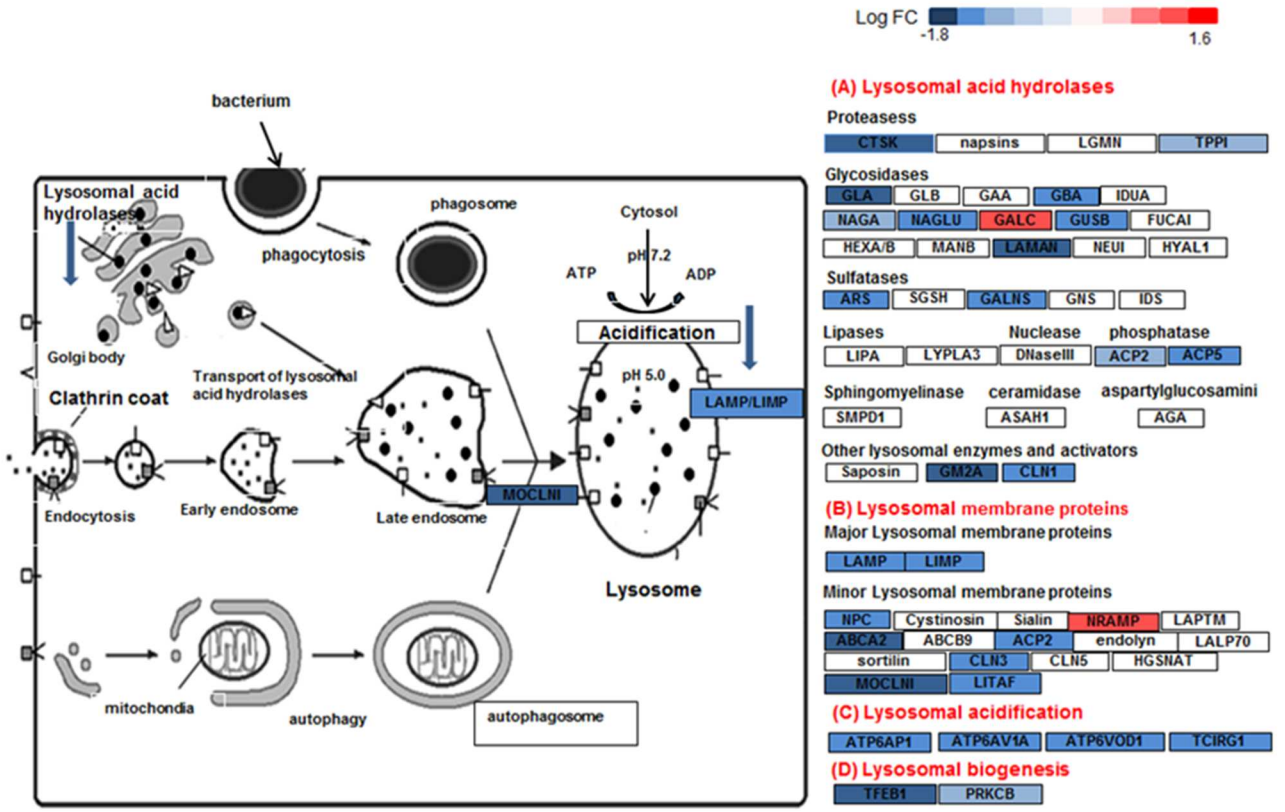


FIGURE 7 | Lysosome pathway in DEX-treated sarcoid AMs. Graphic illustration of pathway analysis of DE (log_2 FC > 0.6 with FDR < 0.05) genes related to lysosome pathway in sarcoid-DEX treated AMs. The pathway diagram is overlaid with the computed perturbation of each gene. The perturbation accounts both for the gene's measured fold changes and for accumulated perturbation propagated from any upstream genes (accumulation). The color intensity corresponds to the level of upregulation (red) or downregulation (blue) of the DE genes in sarcoid-DEX treated AMs vs. sarcoid untreated AMs.

Similar to rheumatoid arthritis, some studies have shown an increased risk of developing cancer in sarcoidosis patients, while others could not confirm such association (69–73). While it is difficult to interpret uncontrolled and retrospective observational studies, the increased risk is partly attributed to uncontrolled inflammation. Our study showed that transcriptional misregulation in cancer was also one of the enriched pathways in response to DEX treatment. We found that DEX treatment upregulated the expression of numerous genes involved in cell proliferation, tumor formation and lymphoproliferative disorders. Interestingly, DEX treatment led to upregulation of this pathway in the monocytes of sarcoidosis subjects, suggesting that this effect is a global effect. These results demonstrate that long-term usage of DEX may lead to uncontrolled proliferation. For example: RAS superfamily gene are proto-oncogene that are involved in the development of various cancers (74, 75). It has been shown that DEX upregulates several members of this family (76). Our data is in line with previous RNA seq data of cultured human pulmonary type II A549 cells showing upregulation of transcriptional misregulation in cancer in response to corticosteroids (77).

In response to DEX treatment, we identified derangement of large numbers of genes regulating the cytoskeleton. Most genes belong to integrin family, but also genes involved in desmosomes such as PKP2 gene encoding a protein which links cadherins to intermediate filaments in the cytoskeleton. Aberrant regulation of these genes, including PKP2, ITGA6, and seven are important for cytoskeleton and cell-cell and cell-matrix interactions and are associated with cardiomyopathy, arrhythmogenic R ventricular cardiomyopathy and hypertrophic cardiomyopathy. Collectively, these data suggest that dexamethasone drives a broad gene expression program promoting matrix stiffening and supporting fibroblast proliferation and selective but coordinated suppression of genes encoding collagen-degrading enzymes.

On the other hand, our results show that DEX treatment altered the expression of genes involved in inflammation and immune responses. DEX treatment led to decreased expression of genes related to inflammation including IL6R, IL-17R, TNFRSF4, and MyD88. Along with this DEX treatment upregulated the expression of several genes that suppress inflammation, including Glucocorticoid-Induced Leucine Zipper Protein (GLIZ, also known as TSC22D3), dual specificity phosphatase 1 (DUSP1, also known as MKP-1), Suppressor of Cytokine Signaling (SOCS) 4 and 6. We have previously shown that DUSP1 expression is decreased in sarcoidosis AMs and monocytes and GCs induced

DUSP1 expression at the protein level (15). Current RNA seq data in an independent cohort of patients is in agreement with our previous results.

CONCLUDING REMARKS

For more than 60 years GCs have been used as anti-inflammatory drugs to treat a variety of inflammatory diseases such as rheumatoid arthritis and pulmonary diseases such as sarcoidosis. Given the fact that macrophages play a critical role in infectious and non-infectious granulomatous diseases (78, 79), it is of surprise that the effects of GCs on macrophages are less well-documented. Here we show that DEX treatment led to profound transcriptomic changes related to cellular metabolisms, lysosomes/phagosomes, and cytoskeleton in lung tissue macrophages. While similar effects were observed after DEX treatment on isolated $CD14^+$ monocytes of the same subjects, the effect on cytokine-cytokine receptor interaction was more prominent in monocytes, suggesting that the effect on lung tissue macrophages are more likely due to lysosomal function metabolic derangement. Further studies need to determine the effects of DEX on proteome profile in different cell types and correlation with RNA seq data.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI BioProject database with accession number PRJNA630000.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Committee for Investigations Involving Human Subjects at Wayne State University. The IRB number for this study is 055208MP4E. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JT conducted the experiments, analyzed the results, and she contributed in writing the manuscript. CB critically reviewed the manuscript. ES performed the statistical analysis of the RNA-seq data. FL and RP-R reviewed the data analysis and the manuscript. LS conceived and designed the study, participated in all areas of the research such as patients' selection and oversaw patient enrollment, data analysis, and writing of the manuscript.

FUNDING

This work was supported by a grant (R01HL113508) (LS) as well as the Department of Medicine and the Center for Molecular Medicine and Genetics, Wayne State University School of Medicine (LS).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00779/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 27 May 2020
doi: 10.3389/fimmu.2020.00962

Neutrophils in Tuberculosis-Associated Inflammation and Lung Pathology

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Protective immunity to *Mycobacterium tuberculosis (Mtb)*—the causative agent of tuberculosis (TB)—is not fully understood but involves immune responses within the pulmonary airways which can lead to exacerbated inflammation and immune pathology. In humans, this inflammation results in lung damage; the extent of which depends on specific host pro-inflammatory processes. Neutrophils, though increasingly linked to the development of inflammatory disorders, have been less well studied in relation to TB-induced lung pathology. Neutrophils mode of action and their specialized functions can be directly linked to TB-specific lung tissue damage observed on patient chest X-rays at diagnosis and contribute to long-term pulmonary sequelae. This review discusses aspects of neutrophil activity associated with active TB, including the resulting inflammation and pulmonary impairment. It highlights the significance of neutrophil function on TB disease outcome and underlines the necessity of monitoring neutrophil function for better assessment of the immune response and severity of lung pathology associated with TB. Finally, we propose that some MMPs, ROS, MPO, S100A8/A9 and Glutathione are neutrophil-related inflammatory mediators with promising potential as targets for developing host-directed therapies for TB.

Keywords: tuberculosis, neutrophils, inflammatory mediators, lung damage, sequelae

OPEN ACCESS

Edited by:
Mary Jane Thomassen, East Carolina University, United States

Reviewed by:
Niaina Rakotosamimanana, Institut Pasteur de Madagascar, Madagascar
Jose Roberto Lapa E. Silva, Federal University of Rio de Janeiro, Brazil

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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *02 March 2020*
Accepted: *23 April 2020*
Published: *27 May 2020*

Citation:
Muefong CN and Sutherland JS (2020) Neutrophils in Tuberculosis-Associated Inflammation and Lung Pathology. Front. Immunol. 11:962. doi: 10.3389/fimmu.2020.00962

INTRODUCTION

Tuberculosis (TB) is the single deadliest infectious disease known to man with 10 million new cases and 1.6 million deaths (including 300,000 HIV coinfected) in 2018 (1). This report does not account for health impairment nor deaths during and following TB treatment; which is suggested to be about three times higher than those observed in the general population or suitably matched controls (2). It is known today that despite being diagnosed as microbiologically cured from TB, about 50% of patients still suffer from some form of pulmonary impairment after tuberculosis (PIAT), irrespective of smoking habits (3). The definition of PIAT encompasses several clinical conditions; which in former TB patients is suggested to result from chronic inflammation, characterized by disrupted pulmonary structure and function (residual lung deficits) (4, 5); a state described as thoracic/TB sequelae (6). These include parenchymal, airway, vascular and mediastinal lesions manifested mainly through structural [cicatrization, calcification, fibrosis and reduction in cavity wall thickness (6)] and functional [deficit in forced expiratory volume (4)] damage; the establishment (7) and severity (8–10) of which, are associated to neutrophil abundance and (hyper-)activity.

As compelling as these effects of TB may be, PIAT is presently not included in global estimates of TB burden despite increasing scientific interest and evidence of associated morbidity and mortality (3–5, 11–14).

A recent cohort study showed that subjects with a history of fully treated active TB (ATB) lost 3.6 years on average of disability-adjusted life expectancy compared to subjects with latent TB infection (LTBI) who did not progress to the active state (12). Despite the lack of data to support disease burden resulting from long-term sequelae (11), the above reduced life expectancy is a direct result of TB sequelae and suggests that a considerable proportion of the TB disease burden is contributed by subjects who have successfully cleared Mtb. Indeed, a study in Texas, USA reported that the number of years lived with chronic TB disability accounts for 75% of non-fatal health effects of TB (11). This same study suggests that the most health and financial savings may be achieved by preventing rather than shortening therapeutic strategies. Additionally, a recent retrospective study reveals the negative effect of drug-resistance and disease recurrence on PIAT (15). Consequently, early detection of parameters which increase the likelihood of ATB complication into chronic inflammation and long-term sequelae would inform clinicians on the need for case-specific treatment measures and contribute to minimizing the global TB burden (13). Such parameters can be realistically linked to neutrophil function and/or interaction with other immune cell populations in the view of their specific activities described in subsequent sections below.

Generally, protective immunity to *Mycobacterium tuberculosis* (Mtb)—the causative agent of TB—is a combination of innate and adaptive immune responses within the pulmonary airways via which this pathogen gains entrance into the human host (16, 17). This immune response to TB is described as a chronic granulomatous inflammation; caused by close interaction between Mtb bacilli and host immune agents at the infection site (18). Indeed, the term "chronic granulomatous" draws from a condition, chronic granulomatous disease (CGD), with similar inflammatory outcomes; resulting from genetic mutations of reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) oxidase-encoding genes (19, 20). Disruption in the production of this enzyme; which normally catalyzes the synthesis of reactive oxygen species (ROS) used by phagocytes to destroy bacteria during phagocytosis, leads to enhanced susceptibility to infectious pathogens and granuloma formation; particularly in the lungs (21). Despite several gaps in knowledge, the contribution of adaptive immune responses: particularly T-cells [reviewed in Jasenosky et al. (22)] and to a lesser extent B-cells [reviewed in Achkar et al. (23)] have been addressed. Furthermore Dyatlov et al. recently reviewed the role of B cells on reducing neutrophil influx to infection sites (24) and; these Mtb-specific immune responses having been studied extensively and will not form a focus of this review.

Recent studies have revealed that the innate arm of the immune system plays a bigger role in the onset and regulation of inflammatory processes during ATB than previously thought. ROS-generating cells are central to Mtb-induced inflammatory response; and that they are main actors of relevant cell death processes (i.e., apoptosis, necrosis, pyroptosis, necroptosis, pyronecrosis, NETosis, and autophagy) that influence TB disease progression [reviewed by Mohareer et al. (25)], suggests that their activity contributes considerably to destructive immunity to Mtb infection. The aim of this review is to provide an update on the importance of neutrophils during ATB and to identify related immune mediators associated with anti-TB treatment response and lung damage.

TB-INDUCED INFLAMMATORY RESPONSE

Innate immune responses play a central role in the pathology of infectious and inflammatory diseases including acute abdominal inflammation (26), cancers (27, 28) and respiratory tract disorders (29, 30). Phagocytic cells (i.e., neutrophils and macrophages) are the predominant components of this response in TB (17). In collaboration with inflammatory mediators like cytokines (31) and proteases, they are key contributors to the host interaction with Mtb, in a process which generally ends with the destruction of the pathogen and resolution of inflammation (32). In many cases, however, the inflammatory response is relatively ineffective and can lead to destruction of host tissues as reviewed by Fullerton and Gilroy (33). Such an unwanted scenario is characterized by a constant influx of inflammatory mediators and innate immune cells to the site of infection with progressive deterioration of the affected tissue. The end result is the formation of tuberculous granulomas whose structure, immune/pathogen cell balance (34), and intrinsic T-cell activity (35) ultimately determine the degree of formation of tissue lesions (36).

Defining and Assessing Lung Impairment

In order to understand the role of neutrophils in lung pathology, we need consensus on structural versus functional impairment. There are currently no international guidelines describing how to classify levels of structural impairment following TB as well as identifying TB sequelae in general (37). ATB is increasingly further classified with respect to disease severity into the extent of functional and/or structural lung damage, however, a decisive classification of TB patient pathology has not been reached at this time. Nonetheless, certain criteria have allowed the severity of active pulmonary TB to be determined following assessment of impaired pulmonary function via spirometry testing (38) and the observation of lesions and/or lung cavities through chest x-rays (CXR) and computed tomography (CT) (39).

Structural lung abnormalities determined by x-ray or computed topography (CT) scores have been observed to correlate to a degree with lung function in pulmonary TB (40). Reports also suggest that functional pulmonary impairment at diagnosis only begins to improve significantly several months after the end of successful TB therapy (4, 40). Saldana et al. observed that CXR abnormalities are inversely proportional to and more reliable than spirometry evaluations when assessing severity of lung impairment in cured ATB patients (41). An even earlier study by Plit et al. showed that the change in CXR score (pre- vs. post-treatment) is the most reliable predictor of the severity of functional lung impairment in ATB: here too, an inverse proportionality was observed between CXR

TABLE 1 | Clinical studies assessing TB sequelae.

References	Study site	Study type and design post-treatment commencement	ATB sample size, *n*	Nature of residual lung impairment	Associated inflammatory response
Ngahane et al. (46)	Cameroon	Cross-sectional Includes HIV$^+$	269	Structural (CXR lesions) and Functional (dyspnoea and spirometry)	No
Ralph et al. (13)	Indonesia	Longitudinal [Baseline (BL), 6M & over 6M] Includes HIV$^+$	200	Structural (CXR score of lesions and cavitation) and Functional (dyspnoea, SGRQ, spirometry)	No
Kumar et al. (47)	India	Longitudinal (BL & 6M) No HIV$^+$ cases Part of larger study involving patients with co-morbidities	24	Structural (cavitation; no CXR-score)	Yes
Ravimohan et al. (48)	USA	Prospective (over 6M) All TB/HIV$^+$	14	Functional (spirometry)	Yes (MMPs)
Pasipanodya et al. (11)	USA	Longitudinal (BL, 6M & over 6M) Includes HIV$^+$	177	Functional (SGRQ and spirometry)	No
Plit et al. (42)	South Africa	Longitudinal (BL & 6M) Includes HIV$^+$	76	Structural (CXR score of lung infiltrates) Functional (spirometry)	Yes (c-reactive protein (CRP) and serum α1-protease inhibitor (α1-PI)
Cole et al. (43)	South Africa	Cross-sectional Includes HIV$^+$	55	Functional (SGRQ and spirometry)	No
Patil and Patil (44)	India	Longitudinal (6M, 9M, & 12M) No HIV$^+$ cases	118	Functional (dyspnoea and spirometry)	No
Hnizdo et al. (4)	South Africa	Retrospective (BL-–375M) Includes HIV$^+$	2,599	Functional (spirometry)	No
Maguire et al. (45)	Indonesia	Longitudinal (BL, 2M, & 6M) Includes HIV$^+$	115	Functional (dyspnoea, SGRQ, spirometry)	No
Saldana et al. (41)	Mexico	Cross-sectional Includes HIV$^+$	127	Functional (Spirometry) and Structural (CXR abnormalities)	No
Vecino et al. (14)	USA	Longitudinal (BL, 6M, & over 6M)	123	Functional (Spirometry)	No
Chushkin et al. (3)	Russia	Prospective (Over 12M) Undetermined HIV status	214	Functional (Spirometry)	No

scores and forced expiratory volume (FEV1; a spirometric parameter) (42). These suggest that monitoring variations in structural impairment during TB therapy is essential (or at least of significant added value) when attempting to determine the extent of TB sequelae. However, whilst most relevant clinical studies have generally attempted to monitor ATB-linked signs of TB sequelae via assessment of dyspnoea and disrupted lung function by spirometry (3, 4, 11, 14, 43–45), fewer cases have accounted for both structural and functional damage (13, 41, 42, 46), and none focussing on the former exclusively (see **Table 1**). Relevant follow-up parameters, where available (especially involving longitudinal cohort studies), appear to have relied on the researchers' study objective and understanding of TB sequelae—variably assessing different forms of pulmonary damage, lung rehabilitation and even treatment responses but not the potential inflammatory triggers of these events as the Ravimohan group's latest review hints (49). This is probably owing to absence of a referential guideline as mentioned above. At this time, a few studies: Ravimohan et al. (48) and Plit et al. (42) have assessed severity of lung impairment in ATB in relation to the expression of inflammatory mediators: matrix metalloproteinases (MMPs) in the former and; serum c-reactive protein (CRP), serum α1-protease inhibitor (α1-PI) and urine cotinine in the latter. To account for these limitations, a multisite trial is currently underway to monitor host-pathogen and socioeconomic factors that influence the development of pulmonary sequelae in ATB patients (50).

EVIDENCE OF NEUTROPHIL IMPACT ON DESTRUCTIVE TB INFLAMMATION

Neutrophilia and Hyperinflammation

Polymorphonuclear neutrophils are the most abundant type of white blood cells and play a central role in the immune response to bacterial pathogens (51). The protective activity of neutrophils in TB infection is observed during granuloma formation where mycobacteria are phagocytosed from infected macrophages by oxidative killing (52).

Previous work indicates that the levels of granulocytes (neutrophils and eosinophils) in circulation are higher in patients with ATB disease than those with latent TB infection (LTBI); with levels decreasing significantly following successful TB treatment (53). It has also been demonstrated that neutrophilia independently associates not only with increased risk of cavity formation and lung tissue damage (54), but also mortality in patients undergoing TB therapy (55), suggesting that the neutrophil count in tuberculosis positively correlates with bacillary load and disease outcome. Recently, Leem et al. (56) monitored inflammatory markers in TB patients and found that the neutrophil counts and neutrophil to lymphocyte ratios (NLR) were decreased following a 6-months anti-TB drug therapy compared to baseline. These results hint that inflammation might be resolved only following the 6-month treatment completion, suggesting that progress to chronic inflammation and development of pulmonary lesions is a silent process potentially mediated by secondary products of inflammatory response whose activity persist in tissue long after mycobacterial clearance.

Despite the lack of a consensus on neutrophil classification, varying attributes: granule content (cytotoxic species/enzyme concentration), density (low or normal density granulocytes), nuclear segmentation [banded or (hyper)-segmented], tumor suppressive/enhancing functions (N1/N2), to surface antigen expression [CD177 (7, 57, 58); CD16, CD62L and CD11b] and cytokine/chemokine secretion levels have been associated to disease and immunoregulation [reviewed in Hellebrekers et al. (59), Perobelli et al. (60), and Wang (61)]. It is therefore arguable that a combination of these attributes could constitute a neutrophil profile suggestive of disease severity at an early stage as well as anticipated development of sequalae if chronic conditions (in TB potentially) were to be established. However, given the vast discrepancies in markers, experimental conditions and disease models investigated by previous studies as described in the reviews cited above, these functional differences in neutrophil subsets will not constitute a focus here. Nevertheless, the severity of ATB is linked to neutrophilia as discussed above; but also a specific hyperactivated profile of the circulating neutrophils; which has predominantly been associated with immature banded (or non-segmented) neutrophils (8). Interestingly, the bulk of neutrophil cytotoxic (and antibacterial) molecules are concentrated in their granules. Hence, neutrophil degranulation and exocytosis: processes requiring phosphatidylinositol 3-kinase, (PI3-K) (62); are closely related to the severity of neutrophil-mediated inflammation. We therefore anticipate that a potential neutrophil bio-signature of ATB would encompass enhancement/inhibition of some specific chemokines and increased neutrophil-specific enzyme concentrations. In fact, a recent review by Leisching (7) exposes the regulatory role of PI3-K on enhanced neutrophil mobility and hyperactivity and; the effect on neutrophil-driven TB inflammation. This hyperactivity is equally suggested to be at play in chronic periodontis where it is associated with increased migratory capacity as well as pro-inflammatory cytokine (IL-8, TNF, and IL-1, notably) production by circulating neutrophils (63). Taken together, neutrophil relative abundance (in circulation and at infection sites) and cytokine/enzyme release are potentially major agents of hyperinflammatory conditions observed in ATB.

The mechanisms responsible for this inflammatory response mainly result from three neutrophil functions: oxidative burst, necrosis and NETosis.

Oxidative Burst Capacity

Although neutrophils have the capacity to protect against Mtb infection, if left uncontrolled their collective activity may produce pathogenic effects through different functions (64). One such phagocytic function is oxidative burst, which is the release of reactive oxygen species (ROS) mainly by neutrophils and to a lesser extent, macrophages during phagocytosis, a process which is mediated by nicotinamide adenine dinucleotide phosphate (NADP) oxidase (65). This antibacterial activity is performed by a myeloperoxidase system composed mainly of reduced NADP ($NADPH_2$), reduced glutathione (GSH), azide, cyanide, thiocyanate, Tapazole, thiourea, cysteine, ergothioneine, thiosulfate, reduced nicotinamide adenine dinucleotide ($NADH_2$), and tyrosine (66).

GSH levels have been shown to reduce significantly in PMBCs and red blood cells isolated from tuberculosis patients compared to healthy controls (67), while increased GSH levels are reported to enhance T-cell capacity to inhibit Mtb growth inside macrophages (68). Also, ROS produced by neutrophils during oxidative burst have been reported to drive Mtb-induced necrosis; which in turn promotes Mtb growth (69). It has also been suggested that rapid assessment of individual neutrophil oxidative burst capacity could distinguish patients at risk of excessive immune responses and thus could potentially guide therapy (70). Hence, correlating neutrophil oxidative burst capacity with GSH and/or $NADPH_2$ levels in TB patients may provide avenues for novel host-directed therapies.

Neutrophil Extracellular Traps (NETs)

In-vitro studies by Brinkmann (71) revealed that neutrophil activation with lipopolysaccharide (LPS), interleukin 8 or phorbol myristate acetate (PMA) led to the release of cell components, which form an extracellular fibril matrix called neutrophil extracellular traps (NETs). These components are proteins [namely neutrophil elastase (NE) and myeloperoxidase (MPO)], DNA and chromatin-derived fibers; which destroy bacteria extracellularly (71, 72). This process, NETosis, is a powerful neutrophil-mediated response to a range of infections but also acts as a double-edged sword during inflammatory diseases (73). Interestingly, neutrophils can sense pathogen size and can produce more NETs in presence of larger pathogens like *Mycobacterium bovis* (74). Although aggregated NETs are reported to degrade neutrophil-derived inflammatory mediators in an attempt to resolve inflammation (75), NETs also stimulate unwanted immune reactions and trigger tissue injury (73, 76).

In TB pathogenesis, Mtb is reported to induce the formation of NETs, which trap Mycobacteria *in vitro* but are unable to kill them (77). This may be partially explained by the fact that expression of enzyme systems such as those required in inflammatory pathways [i.e., to degrade proteins within

the phagolysosome (e.g., MPO) and for the phagocytic burst, NADPH-oxidase complex and the generation of ROS] are suppressed (78). Furthermore, these Mtb-induced NETs are also associated with macrophage activation in humans (79) and could thus help elucidate specific inflammatory mechanisms of lung damage in TB pathogenesis. Indeed, a recent study by De Melo et al. revealed high levels of citrullinated H3—a common NET marker—in serum samples from TB patients with extensive pulmonary damage (54). Although this marker is usually measured in combination with others (i.e., MPO and NE) to specifically identify NETs, this study suggests that NET formation is centrally linked with severe lung tissue damage in TB patients and could be implicated in subsequent pulmonary pathology.

Neutrophil-mediated lung injury is not just restricted to Tuberculosis. For example, excessive neutrophil recruitment and NETosis was linked to acute lung injury in a mouse model of Influenza pneumonitis (80). Additionally, more recent studies reveal that reduced neutrophil recruitment into infected tissue promotes resolution of inflammation (81). Hence, monitoring NETosis and neutrophil-associated inflammatory mediators within inflamed tissue could be useful in developing therapeutic targets against chronic inflammatory conditions like TB (72).

Metalloproteinases in Destructive TB Immunity

A group of molecules increasingly associated with excessive lung inflammation is the matrix metalloproteinases (MMPs). In the case of cystic fibrosis, which results in loss of pulmonary architecture, Pardo et al. described in a review (82) the essential role played by MMPs in modifying the tissue microenvironment and modulating cell signaling through their ability to degrade constituents of the extracellular matrix. Although the origin of most MMPs cannot be directly linked to neutrophils, MMP-9 is known to be secreted rapidly by neutrophils in whole blood from healthy volunteers following proinflammatory stimulus (83) and is suggested to facilitate transmembrane neutrophil migration (84); also reviewed in Pardo et al. (82). Similar to MMP-9, MMP-8 synthesis in ATB patients is also suspected to be of neutrophil origin (85). In effect, Ravimohan et al. (48) assessed the role of MMPs on TB-immune reconstitution inflammatory syndrome and observed increased MMP-8; whilst MMP-2, -3 and -9 levels reduced (MMP-1 did not vary significantly) in patients with impaired lung function post-TB cure following antiretroviral therapy. MMP-1 and MMP-8 have previously been shown to correlate with pulmonary tissue damage (PTD) in patients with ATB (85, 86) while MMP-14 has been shown to play a central role in TB pathogenesis by provoking collagen degradation and regulating monocyte migration (87). Interestingly, a more recent study by De Melo et al. found lower levels of serum MMP-8 in TB patients with severe PTD showing no radiological improvement after 60 days of anti-TB treatment (54). Whether this change in trend is related to plasma vs serum or due to the presence/absence of HIV infection is unknown, however, the latter observation is inconsistent with that from the previous studies reported above and suggests that MMP regulation in TB patients might differ between the circulation and the lung as well as in the presence of coinfection. Nonetheless, de Melo et al. (54) did find higher MMP-1 levels in these patients compared to those with improved chest-x rays. Hence, whilst there is clearly a role for MMPs in TB-linked tissue damage, more detailed studies, with assessment of coinfections, are required to ascertain which MMPs are predominant mediators. This will help to determine potential host-directed therapeutic strategies.

Furthermore, a few clinical studies on major TB comorbidities have recently emerged. One shows that sputum levels of MMP-1, -2, -3 and -9 are higher in HIV negative TB patients than in TB healthy controls (HC) and HIV positive TB patients with a correlation found between the degree of chest x-ray inflammation and both MMP-1 and MMP-3 levels in HIV negative TB patients (88). Moreover, the clinical severity of TB is known to increase in TB patients with diabetes mellitus (DM). Kumar et al. have shown that circulatory levels of MMP-1, -2, -3, -8, and -13 in these patients decrease following successful TB treatment and that MMP-1 (in sputum) and MMP-1, -2, -3, -9, and -12 levels (in serum) were higher in patients with more severe structural lung damage at baseline (47) as determined from chest x-rays.

These findings suggest that MMPs (MMP-1, -2, -3, -8, and -9, particularly) may promote tissue injury following Mtb infection. Hence, monitoring the correlation of these particular MMPs together with the downregulation of other neutrophil-related inflammatory proteins and pro-inflammatory cytokines associated with intracellular killing pathways during TB infection would increase our understanding of the active inflammatory pathways which enhance susceptibility to development of PIAT and subsequent sequelae. Importantly, natural regulation of MMP activity is performed by tissue inhibitors of metalloproteinases (TIMPs). The levels of TIMPs have not yet been monitored in TB patients; an aspect of TB research which should be addressed for optimal understanding of inflammatory mechanisms involved in development and host control of TB-related PTD.

NEUTROPHIL-RELATED TB HDT

With increasing cases of co-infections, co-morbidities, drug resistance; as well as the cost associated with the relatively long standard antibiotic TB-treatment, new treatment regiments like host directed therapies (HDT) could complement existing Mtb-targeted approaches. Meanwhile biomarkers for efficiently identifying and treating TB disease progressors at an early stage are being actively researched (89), those that could single out individuals who develop unresolving inflammation-induced lung damage are still greatly under-investigated. This means that research on HDTs should ideally focus on diagnosis and prevention of the latter long-lasting condition as well. Recent reviews have highlighted various established as well as promising host directed adjuvant therapies against the development of TB disease (90), TB-linked inflammation (91, 92) and lung damage (93). Drugs that potentially inhibit pulmonary damage and/or promote lung repair range from steroids to nonsteroidal anti-inflammatory drugs, statins, metformin, dietary supplements,

TNF blockers etc. (10). Of these, we observe that those suppressing pro-inflammatory aspects of the disease appear to be potent targets in preclinical and clinical trials. In fact, Young et al. have recently reviewed current targets in TB HDT with some of the most advanced ATB-relevant in clinical trials being modulators of pro-inflammatory mediators which: dampen inflammatory responses, curb immunopathology and resolve lung damage (93). These include the phase 3 drugs: cox-2 inhibitor (Meloxicam) and corticosteroids (Prednisolone and Dexamethasone) amongst others. This HDT potential of inflammatory mediators has also been addressed with inhibitory effects on neutrophil recruitment (Ibuprofen) and neutrophil-derived inflammatory mediators such as ROS and MPO as reviewed by Dallenga et al. (94).

Also, a combination of the anti-inflammatory drug, zileuton (an inhibitor of the synthesis of pro-inflammatory eicosanoid; already approved against asthma) and prostaglandin E2 (95) is reported to reduce bacillary load and TB-induced lung damage in mice. Statins are also interesting HDT targets against destructive lung pathology following ATB (96); with a promising phase 2 trial using pravastatin being investigated in South Africa (ClinicalTrials.gov Identifier: NCT03456102). Moreover, a phase 2b trial testing the effect of atorvastatin against PIAT in patients with or without HIV is about to begin in South Africa (ClinicalTrials.gov Identifier: NCT04147286); underlining the potential of these agents.

Potentially, some mediators of neutrophil function (mentioned in previous sections) could provide suitable HDT targets. Amongst others, these involve: vitamin D which is reported to inhibit Mtb-induced expression of MMP-7 and−10 as well as MMP-9 gene expression, secretion and activity by peripheral blood mononuclear cells (PBMCs) (97). Although the authors reported that the latter inhibition occurs irrespective of infection, MMP-9 is reported to be of neutrophil origin and in-depth investigation may be warranted. Doxycycline is also a known MMP-inhibitor which in TB-HIV co-infection particularly, is shown to suppress the secretion of TNF, MMP-1 and−9 by primary human macrophages while reducing Mtb growth in the guinea pig model of TB (98). Also, Allen et al. reviewed the importance of considering GSH as HDT against TB and TB/HIV co-infection (99). Furthermore, it is important to note that N-acetylated proline glycine proline (ac-PGP) induces neutrophil chemotaxis and neutrophil production of MMP-9 and IL-8 (100, 101) which has led this molecule to be suggested as potential HDT-target against chronic neuroinflammatory diseases (102) and cystic fibrosis (103); which result in MMP activation and result in considerable tissue damage like TB.

Besides these, calprotectin, a hetero-dimer made up of proteins S100A8 and S100A9 is a mediator of inflammatory responses and a potent diagnostic and HDT target against inflammatory diseases (104). Actually, Gopal et al. (9) reported that S100A8/A9 accumulates in TB-induced granulomas. The authors showed that this accumulation was neutrophil-driven (in humans) and the animal models they employed suggested an association between S100A8/A9 and the degree of inflammation and lung pathology during ATB. Recently, it has been shown that these high levels of S100A8/A9 as well as an S100A8/A9-mediated enhanced accumulation of neutrophils in lungs of mice and macaques are associated with Mtb proliferation in chronic TB disease (105). Both studies reveal a close interaction between neutrophils and S100A8/A9 in ATB suggesting that neutrophils and S100A8/A9, particularly could be targeted in TB HDT.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In conclusion, we believe that observation and monitoring of neutrophil subsets and related inflammatory mediators is important not only for studies aiming at developing novel therapeutic targets against TB (72) but also for improved estimation of host immuno-modulatory effects on the severity of TB sequalae. It is foreseeable that the extent of long-term pulmonary injury sustained and potentially resorbed following TB therapy (irrespective of HIV coinfection) could be correlated to a specific neutrophil function. It is also likely that ATB patients who express a specific form of neutrophil-mediated inflammatory response over the period from diagnosis through treatment are more susceptible to developing chronic PIAT than otherwise. A major challenge will be harmonizing the categorization of disease severity (structural and functional) to ease comparison between clinical studies. Moreover, we believe that prediction of treatment response and residual pulmonary impairment in future clinical studies would be made more effective and reproduceable by evaluating inflammatory responses as well as simultaneously monitoring variations in pulmonary structure and function during and months after treatment completion. Finally, prospective HDTs; which rely on inflammatory mediators of neutrophil activity particularly, should be investigated further.

AUTHOR CONTRIBUTIONS

CM was the primary author of the manuscript. JS provided substantial writing support, edits, and suggestions.

FUNDING

CM was supported with a Ph.D student stipend from TB-Sequel (grant number 66.3010.7-002.00) funded by the German Ministry for Education and Research (BMBF).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 26 June 2020
doi: 10.3389/fimmu.2020.01340

Environmental Risk Factors for Sarcoidosis

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Sarcoidosis is a multisystem granulomatous disease that may affect any body organ. Sarcoidosis is associated with many environmental and occupational exposures. Because the exact immunopathogenesis of sarcoidosis is unknown, it is not known whether these exposures are truly causing sarcoidosis, rendering the immune system more susceptible to the development of sarcoidosis, exacerbating subclinical cases of sarcoidosis, or causing a granulomatous condition distinct from sarcoidosis. This manuscript outlines what is known about the immunopathogenesis of sarcoidosis and postulates mechanisms whereby these exposures could cause or exacerbate the disease. We also describe the varied environmental and occupational exposures that have been associated with sarcoidosis. This includes potential infectious exposures such as mycobacteria and Propionibacterium acnes, a skin commensal bacterium, as well as non-infectious environmental exposures including inhaled bioaerosols, metal dusts and products of combustion. Further insights concerning the relationship of environmental exposures to the development of sarcoidosis may have a major impact on the prevention and treatment of this enigmatic disease.

Keywords: sarcoidosis, antigen, environment, infection, immunity

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *06 March 2020*
Accepted: *26 May 2020*
Published: *26 June 2020*

Citation:
Judson MA (2020) Environmental Risk Factors for Sarcoidosis. Front. Immunol. 11:1340. doi: 10.3389/fimmu.2020.01340

INTRODUCTION

Sarcoidosis is a multisystem granulomatous disease of unknown cause. The lung is the most common organ involved with sarcoidosis at a frequency of ∼90 percent (1, 2). The skin, eyes, peripheral lymph nodes and liver are also commonly involved (1, 2). Unlike sarcoidosis, the causes of many granulomatous diseases are known. Exposures that may cause granulomatous inflammation include mycobacteria and fungi that may cause granulomatous infection, (3, 4) bioaerosols including bird antigens that cause hypersensitivity pneumonitis (5) and metals including beryllium that causes chronic beryllium disease (CBD) (6). It is plausible that sarcoidosis is caused by one or several antigen exposures that initiates and possibly perpetuates the granulomatous process. Several environmental exposures have been linked to sarcoidosis. Because these exposures are disparate, they may lead to the development of sarcoidosis via different mechanisms; and in fact, it is possible that sarcoidosis represents a conglomeration of several dissimilar diseases ["the sarcoidoses" (7)]. This manuscript will explore environmental risk factors for sarcoidosis. We will briefly describe the potential role of environment antigens in leading to the granulomatous inflammation of sarcoidosis, and then focus on the available evidence supporting an association of specific environmental exposures with the development of sarcoidosis.

OVERVIEW OF IMMUNOPATHOGENESIS OF SARCOIDOSIS RELATIVE TO ENVIRONMENTAL RISK FACTORS

Environmental exposures are postulated to be associated with the development of sarcoidosis in four general ways. The first mechanism involves the detection and processing of antigen by antigen presenting cells such as macrophages and dendritic cells. These processed antigens are subsequently presented via human leukocyte antigen (HLA) Class II molecules to a restricted set of T-cell receptors on naive T lymphocytes that are primarily of the $CD4^+$ class (8). An interplay of antigen, HLA class II molecules, and T-cell receptors occurs at the HLA molecule binding site and is thought to be essential for sarcoidosis to develop (9). These events induce a polarization of the T lymphocytes to a Th1/Th17 phenotype, (10) followed by cellular recruitment, proliferation, and differentiation leading to formation of the sarcoid granuloma. This mechanism is thought to be common across most granulomatous lung diseases known to be caused by a specific antigen, and therefore it is possible that the immune system may not be dysregulated in this instance.

There is a large body of evidence to support this proposed mechanism for the immunopathogenesis of sarcoidosis. Various HLA gene alleles have been associated with development of sarcoidosis, (11, 12) protection from developing sarcoidosis, (11, 12) and specific disease phenotypes (11, 12). Further analyses have suggested that such HLA gene polymorphisms result in conformational changes in the antigen binding pockets of HLA molecules (13). Additional evidence supporting this proposed mechanism for sarcoidosis relates to CBD, a phenotypic mimic of sarcoidosis both radiographically and pathologically, (14, 15) which is associated with specific amino acid substitutions in the HLA molecule (16, 17) Beryllium-specific oligoclonal CD4+ T lymphocytes recognize beryllium within HLA molecules with these amino acid substitutions and this recognition leads to CD4+ lymphocyte proliferation, recruitment of other T cells and monocytes to the lung, (18) and the production of Th1/Th17 cytokines that eventually results in granuloma formation (18, 19). Further indirect support of this mechanism of sarcoidosis granuloma formation is that the lung and the skin are the two most common organs involved with sarcoidosis when the disease is clinically isolated to one organ (20). The skin and the lung are particularly conductive sites for antigen capture (21) and adaptive immune responses (22). It may be that these two organs are the main "portal of entry" for antigens that elicit the sarcoidosis granulomatous response, with further organ involvement requiring dissemination of antigen and/or other inflammatory mechanisms, (20) such as T-cell homing (23).

Although there is abundant evidence supporting the aforementioned mechanism of antigen-induced granulomatous inflammation in sarcoidosis, this mechanism is inconsistent with several available clinical and epidemiologic data. First, the associations between various HLA alleles and sarcoidosis phenotypes are not universal, but rather ethnicity-specific (11, 12, 24). Second, although sarcoidosis patients with specific phenotypic features of sarcoidosis have statistically higher percentages of certain HLA alleles than sarcoidosis patients without those specific phenotypic features or the general population, a significant percentage of individuals in these latter two groups carry the allele of risk (12, 25). In addition, most of these allele-specific phenotypes explain a small minority of cases (12, 13, 25). Another criticism of this mechanism is that it is problematic to account for the systemic features of sarcoidosis. The granulomas of sarcoidosis are often found in multiple and disparate organs. It is unclear how causative sarcoidosis antigens could disseminate throughout the body.

The second mechanism by which environmental antigens may induce a granulomatous response in sarcoidosis involves dysregulation of the immune system leading to autoimmunity. Evidence is accumulating that autoimmunity may be involved in some forms of sarcoidosis (26–30). Autoimmunity in sarcoidosis may occur via molecular mimicry whereby antigens trigger inflammation leading to exposure of self-peptides (31). Immunologic similarities between the "foreign" trigger and the "self" peptide promote autoreactive T or B cells in a susceptible individual. It is possible that the initial granulomatous reaction in sarcoidosis is a direct consequence of an antigen exposure in a target organ, but that subsequent granulomatous reactions in other organs are the result of molecular mimicry. This mechanism might explain how sarcoidosis manifests as a systemic disease without the need for a putative antigen to disseminate throughout the body. The best evidence for autoimmunity has been demonstrated in Lofgren's syndrome, a self-limiting form of the sarcoidosis where independent groups have identified vimentin as a possible autoantigen using proteomic techniques on lung macrophages and homogenized spleen tissue (32–36). Molecular mimicry may also by alteration of the binding pocket of the HLA molecule causing a granulomatous reaction to self-antigens. This mechanism appears to be relevant in the case of chronic beryllium disease, (37) and may explain other associations of metal exposures to the development of sarcoidosis or sarcoidosis-like reactions (vide infra). Antinuclear antibodies have been found in more than one-quarter of sarcoidosis patients in some cohort, suggesting autoimmunity may occur in sarcoidosis and may cause overlap syndromes with connective tissue diseases (38). Another form of "autoimmunity" could occur from autophagy that has been shown to promote MHC-II (major histocompatibility complex-II) presentation of proteins from intracellular sources (39). Perhaps environmental antigens first stimulate HLA molecules that interact with intracellular proteins as the result of autophagy.

A third mechanism by environmental exposures may induce sarcoidosis is by acting as an adjuvant and/or as a non-specific stimulator/dysregulator of the immune system. Such a mechanism would not directly cause sarcoidosis but would render the immune system more susceptible to another antigen or mechanism that could cause sarcoidosis. Such a mechanism may be analogous to a drug-induced sarcoidosis (DISR) like reaction that occurs with immune checkpoint inhibitor (ICI) therapy (40). ICIs are drugs that not only enhance anti-tumor activity, but also stimulate the immune system resulting in numerous immune-related adverse events (irAEs) One of several of these irAEs is a DISR, although $< 10\%$ of irAEs were DISRs in one series (41). It is therefore plausible that ICIs are not

stimulating the immune system specifically to cause sarcoidosis but enhancing the risk of sarcoidosis in susceptible individuals.

Finally, environmental exposures that are epidemiologically associated with sarcoidosis may not be involved in any mechanism of disease development, as association does not prove causation. **Figure 1** outlines the possible mechanisms to explain the association of environmental exposures to sarcoidosis.

THE ROLE OF GENETICS

The aforementioned discussion concerning the relationship between sarcoidosis and potential environmental exposures is incomplete without a discussion of the importance of genetics. It is hypothesized that a combination of genetic and environmental factors contribute to the development of sarcoidosis (42). A recent large familial aggregation study showed that heritability of the disease was 39%, (43) suggesting that genetic variation is an important contributing factor to the risk of sarcoidosis. Various HLA polymorphisms have been associated with development of sarcoidosis, protection from sarcoidosis and certain phenotypic expressions of sarcoidosis (11, 13). In addition, genome-wise association studies have reported variations numerous non-HLA genes that are associated with sarcoidosis. These include annexin A11 (44) that is involved in cell division and apoptosis, NOTCH4 (45) that regulates the activity of T cell immune responses, and BTNL2 (45, 46) that is involved in T cell activation. We suspect that many of the aforementioned mechanisms described concerning the association of environmental exposures to sarcoidosis depend on specific genetic factors. We envision that in the future, the etiology of sarcoidosis will be personalized whereby certain genetics traits present in an individual will suggest that certain specific exposures will place the subject at risk of developing sarcoidosis.

POTENTIAL INFECTIOUS CAUSES OF SARCOIDOSIS

Infectious agents have been suspected as being a possible cause of sarcoidosis. However, data supporting this conjecture are inconsistent and unconvincing. There is an abundance of indirect evidence that mycobacteria are involved in the development of sarcoidosis. Two meta-analyses of studies evaluating infectious agents as a cause of sarcoidosis have suggested an etiologic link between mycobacteria and sarcoidosis (47, 48). Molecular techniques have identified mycobacterial components in sarcoidosis tissues in some (49–51) but not all (52, 53) studies. Mycobacterial catalase-peroxidase protein (mKatG) has been identified in sarcoidosis tissues. mKatG has similar physicochemical properties to the Kveim-Siltzbach reagent that induces granulomatous inflammation almost exclusively in

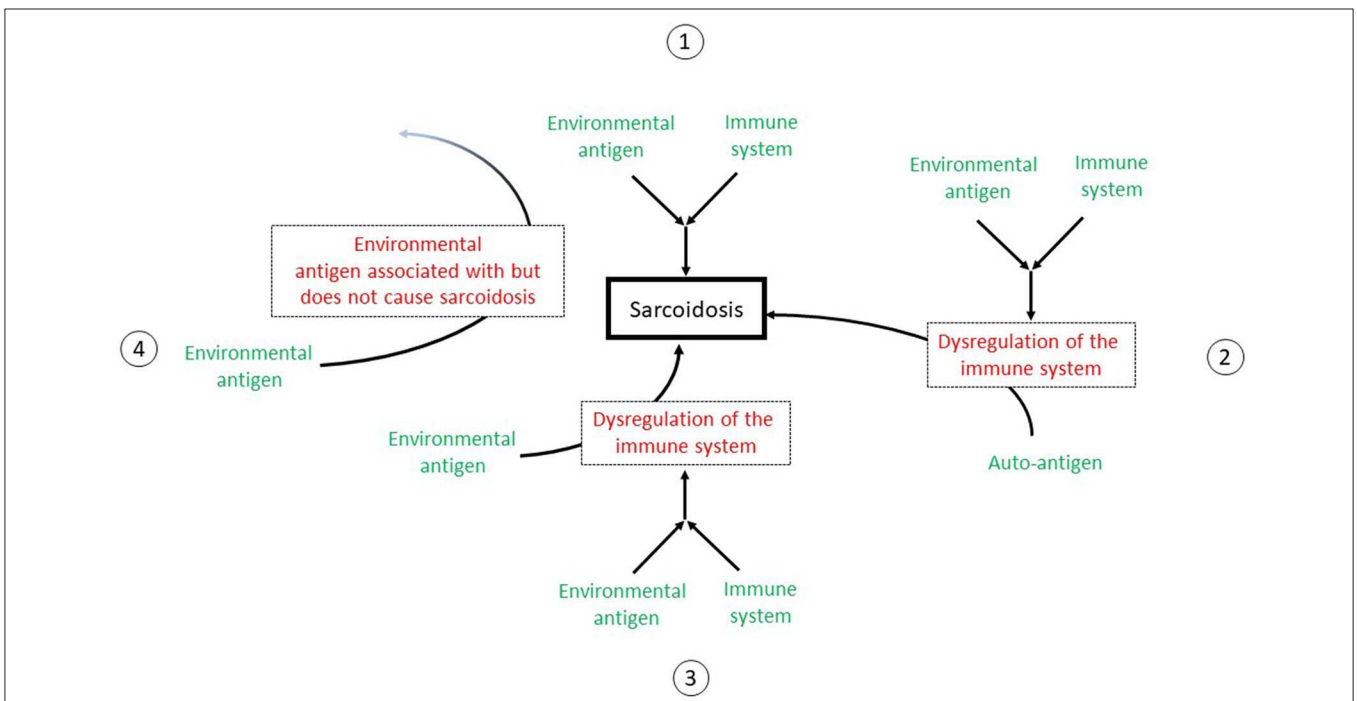

FIGURE 1 | A depiction of the possible mechanisms to explain the association of environmental exposures to sarcoidosis. 1: The environmental exposure may act as an antigen to stimulate the immune system to directly cause sarcoidosis. The immune system is functioning normally with a appropriate response to the antigen. 2: The antigen interacts with the immune system to cause its deregulation. The immune system then acts abnormally to cause sarcoidosis. 3: The antigen acts as an adjuvant that acts as an adjuvant to stimulate or dysregulate the immune system but not directly cause sarcoidosis. However, the immune system is now "primed" such that another antigen or stimulus can now interact with the immune system to cause sarcoidosis. 4: The environmental exposure is a cofounder. Although this exposure is associated with sarcoidosis, it does not cause sarcoidosis.

sarcoidosis patients (54) T-cell responses to mKatG have been demonstrated in peripheral blood monocytes of sarcoidosis patients (55, 56) with even more robust T-cell responses in bronchoalveolar lavage fluid (55, 57) and strongest responses in those with active disease (55, 57) Similar findings have not been demonstrated in other lung diseases (55, 57). It should be noted that the demonstration of mycobacterial antigens in sarcoidosis patients and granulomatous immune responses to mycobacterial antigens does not imply that sarcoidosis is a form of mycobacterial infection. Rather, it may be that some poorly degraded mycobacterial antigens contribute to the immune process of sarcoidosis without the presence of viable invasive mycobacterial organisms (48).

There is abundant evidence that Propionibacterium acnes, a skin commensal bacterium, is associated with sarcoidosis. This microorganism is the only one that has been cultured from sarcoidosis lesions (58, 59). Numerous studies have identified specific immune responses to Propionibacterium acnes in sarcoidosis patients but much less to none in non-sarcoidosis controls (52, 60, 61). Propionibacterium acnes was highly associated with sarcoidosis in a meta-analysis of studies evaluating infectious agents as a cause of the disease (47).

Numerous other infectious agents have been implicated in the immunopathogenesis of sarcoidosis. Several of these infectious agents are listed in **Table 1**. Implicated infectious agents include bacteria, mycobacteria, and fungi.

Several trials of antibiotics for sarcoidosis have targeted suspected infectious pathogens. Therapy with concomitant levofloxacin, ethambutol, erythromycin and rifampin (CLEAR) has been used for the treatment of sarcoidosis by targeting presumed mycobacterial pathogens. An open label trail of CLEAR for pulmonary sarcoidosis showed an improvement in forced vital capacity in 8 of the 15 enrolled patients who were able to tolerate the study drugs for the full 8 weeks of the study (72). A subsequent small ($N = 29$) single-blind placebo-control trial of the CLEAR regimen for cutaneous sarcoidosis showed a statistically significant greater reduction in lesion size with CLEAR than placebo (73). A larger randomized double-blind placebo-controlled trial of CLEAR for pulmonary sarcoidosis is currently underway. Several case series of tetracyclines, including doxycycline and minocycline, have been reported as effective for cutaneous sarcoidosis (74, 75). These reports were unblinded descriptions of treated cases without comparisons with a control group. Furthermore, it is unclear if the mechanism of action of these tetracyclines was antibacterial or anti-inflammatory (76). Case reports and case series have shown a benefit from with clarithromycin for presumed Propionibacterium acnes infection in sarcoidosis patients, (77) as well as a benefit from empiric anti-fungal therapy (78). However, these reports also contained no control patients and/or were poorly designed to rigorously demonstrate a clinically relevant endpoint. In summary, the available evidence does not clearly demonstrate that therapy vs. a specific infectious pathogen is useful for the treatment of sarcoidosis. As previously mentioned, this does not exclude infectious organisms being involved in the immunopathogenesis of sarcoidosis, as an antigen of a microorganism may stimulate the immune system in ways that promote the granulomatous inflammation of sarcoidosis.

Besides examining individual infectious pathogens as causes of sarcoidosis, human microbiotica may have an important role in disease development. Human microbiotica regulate several physiological processes including metabolic functions and immune homeostasis (79). Alterations in the gut and respiratory microbiome have been associated with several inflammatory diseases including autoimmune diseases and cancer (80–83). It is plausible that sarcoidosis may be associated with specific changes in the composition of lung or gut microbiotica. However, a few studies examining changes in the lung microbiome have failed to identify distributions that are specific for sarcoidosis (84, 85). One recent study did suggest that Atopobium and Fusobacterium may be associated with sarcoidosis, (86) and another found that microbiota in bronchoalveolar lavage of sarcoidosis patients was less diverse and abundant compared to healthy controls (87). However, it is unclear whether these changes in the lung microbiome are causing sarcoidosis or are a result of the disease.

TABLE 1 | Selected infectious agents associated with sarcoidosis*.

Infectious agents	Immunologic	Molecular	Culture
Mycobacterium tuberculosis	√(50, 55, 62)	√(49, 56, 63)	
Other Mycobacteria	√(57, 64)	√(49, 51)	
Propionibacterium acnes	√(52, 65, 66)	√(60)	√(58, 59)
Fungi	√(67, 68)		
Borrelia	√(69)	√(70, 71)	

*References are in parentheses.

POTENTIAL NON-INFECTIOUS ENVIRONMENTAL RISK FACTORS FOR SARCOIDOSIS

There are numerous non-infectious environmental risk factors associated with sarcoidosis. These risk factors include working in various occupations, exposure to various substances, and dwelling in particular environments (**Table 2**). Most of these associations are epidemiologic. Numerous epidemiologic studies have demonstrated that sarcoidosis occurs most commonly in the Spring season (88–91). This suggests that some sarcoidosis cases may result from inhalation of an organic bioaerosol that is more abundant in the springtime, possibly analogous to summer-type hypersensitivity which is a form of hypersensitivity pneumonitis in Japan caused by inhalation of certain fungi that reach high concentrations in the summer air (113). Several other epidemiologic analyses have found that the prevalence of sarcoidosis is associated with exposure to other organic bioaerosols such as exposure to musty odors at the workplace (103) and exposure to industrial organic dusts (104, 105).

Sarcoidosis is also associated with inorganic aerosol exposures, particularly with several metal dusts. This association is not surprising, as CBD from beryllium exposure is a clinical mimic of sarcoidosis. Sarcoidosis is not only associated with

TABLE 2 | Non-infectious environmental risk factors associated with sarcoidosis.

General category	Type of study	Study population	Findings	Reference
Space and/or time clustering: Seasonal variation in disease incidence	Space-time analysis	[88]- Rochester, MN; [89]-Turkey; [90]-New Zealand; [91]-Catlonia, Spain	Increased risk in the Spring	(88–91)
	Space-time analysis	USA Veterans	Increased risk in the Summer	(92)
	Space-time analysis	Rochester, MN	Decreased incidence in Autumn	(93)
Space clustering	Space clustering analysis	Ireland	Increased prevalence in certain regions of Ireland	(94)
	Space clustering analysis	Ireland	Higher risks in the North than South	(94)
	Space clustering analysis	Japan	Higher rates of sarcoidosis in Northern than Southern Japan	(95)
	Space clustering analysis	Hospitalized patients US military	Higher frequency in the Southeast US than other US locations	(96)
	Space clustering analysis	South Carolina	Increased prevalence near the coastline of South Carolina	(97)
	Space-time analysis vs. standard incidence and prevalence rates of sarcoidosis	Poland, living in forest of arable land	Increased incidence	(98)
	Co-inertia analysis plus linear model of hospitalized patients	Switzerland, Living near areas with metal industries	Increased prevalence	(99)
	Co-inertia analysis plus linear model of hospitalized patients	Switzerland, living in areas with potato production, artificial meadows, grain production	Increased prevalence	(99)
Occupational exposure	firefighter cohort vs. EMT cohort	NYC, Firefighters	Increased incidence and/or prevalence	(100)
	firefighter cohort vs. police cohort	Prov, RI, Firefighters	Increased incidence and/or prevalence	(101)
	Hospitalizations rates of Blacks in the US Navy	Black US Navy ship servicemen	Increased risk	(102)
	Hospitalizations rates of Blacks in the US Navy	Black US Navy Aviation structural mechanics	Increased risk	(102)
	Hospitalizations rates of Blacks in the US Navy	White US Navy ship culinary specialists	Increased risk	(102)
	Case-control US	Using insecticides	Increased risk	(103)
	Case-control US	Musty odor at work	Increased risk	(103)
	Case-control US	Building materials	Increased risk	(104)
	Case-control US	Hardware	Increased risk	(104)
	Case-control US	Garden supplies	Increased risk	(104)
	Case-control US	Mobile homes	Increased risk	(104)
	Case control US	Industrial organic dusts	Increased risk	(104)
	Case-control*AA Detroit, MI	Education	Increased risk	(105)
	Case-control*AA Detroit, MI	Metal machining	Increased risk	(105)
	Case-control*AA Detroit, MI	Metal working	Increased risk	(105)
	Case-control*AA Detroit, MI	Transportation services	Increased risk	(105)
	Incidence vs. exposure	Sweden: Silica exposure in foundry workers	Increased risk	(106)
	Longitudinal cohort of construction workers, exposed vs. unexposed to silica	Sweden, construction workers	Increased risk	(107)
	Silica in lung and lymph node biopsy, Case series: 2 cases	Silica (metal-halide lamp production)	Increased risk	(108)
	Case-control with sarcoidosis patients and their siblings who did not have sarcoidosis	AAs USA, Photocopier toner exposure	Increased risk	(109)

(Continued)

TABLE 2 | Continued

General category	Type of study	Study population	Findings	Reference
	Tracking sarcoidosis incidence in FDNY workers pre and post WTC disaster	NYC, World Trade Center dust exposure	Increased incidence	(110)
	Case-control*AA Detroit MI	AA Detroit MI, Working in high humidity	Increased risk	(105)
	Case-Control*AA Detroit MI	AA Detroit MI, Working with titanium	Increased risk	(105)
	Case-Control*AA Detroit MI	AA Detroit MI, Working with vegetable dust	Increased risk	(105)
	Elicited history of exposure and analyzed lung biopsy specimens	Man-made mineral fibers	Increased risk	(111)
Environmental exposure	Case-Control with dose response SC	SC, Wood stove use	Increased risk	(112)
	Case-Control with dose response SC	SC, Fireplace use	Increased risk	(112)
	Case-Control*AA Detroit MI	AA Detroit MI, Musty odors	Increased risk	(105)
	Case-control SC	SC, Non-public water use	Increased risk	(112)
	Case-control SC	SC, Living/working on a farm	Increased risk	(112)

**Controls were unaffected siblings of sarcoidosis cases; MN, Minnesota; NYC, New York City; RI, Rhode Island; AA, African American; FDNY, Fire Department of New York City; WTC, World Trade Center; SC, South Carolina.*

several occupations directly involved with manipulations of metals (102, 105) but also more subtle exposures including photocopier toner (109) that contains copper, iron, and silica (114). One report found a significant association of man-made mineral fiber exposure and the development of sarcoidosis, and then went further to perform electron microscopy quantitative analysis on previous lung specimens in the sarcoidosis group and found that half of them silica, aluminum and/or titanium (111).

Exposure to combustible products, especially combustible wood, has been associated with the development of sarcoidosis. A prototypical example of this association is the high incidence and prevalence rates sarcoidosis that is observed in firefighters (100, 101). In one analysis, emergency medical technicians (EMTs) served as a control group to the firefighters because both groups went to fire sites (100). The annual incidence rate of sarcoidosis was extremely high (44/100,000) in the firefighters whereas it was 0 in the EMTs. Wood stove use and fireplace use have also been associated with the development of sarcoidosis (112). The rigor of this association was strengthened by demonstrating a significant dose-response relationship of both wood stove and fireplace use to the frequency of sarcoidosis. Dust from the World Trade Center disaster has been associated with increased rates of developing sarcoidosis within the first 4 years after exposure (110). However, World Trade Center dust was a heterogenous exposure, and it is unclear whether the causative substance(s) was a combustible product, metal or gas.

Higher prevalence rates of sarcoidosis have been observed in Northern latitudes such as Northern Europe and Northern Japan, (95, 115) and it has been postulated that this relates to decreased sunlight exposure causing a deficiency in 1,25-dihydroxy-vitamin D (116). A deficiency in 1,25-dihydroxy-vitamin D is associated with decreased production of the antimicrobial peptide cathelicidin that contributes to the development of infectious granulomatous diseases such as tuberculosis (95, 117). A relative deficiency in 1,25-dihydroxy-vitamin D may also explain the increased frequency of sarcoidosis in Blacks, as the ability to convert 7-dehydrocholesterol to previtamin D is suppressed because of skin pigmentation (118).

Some exposures associated with sarcoidosis are problematic to explain such as working in education (105) or the culinary arts (102). This may relate to the aforementioned concept that sarcoidosis may involve an initial portal of entry where a causative antigen first interacts with the immune system and then requires additional inflammatory modulation to cause disease. In an analysis that focused on mortality from sarcoidosis and not the incidence or prevalence of disease, women with sarcoidosis were more likely to have exposure from person-to-person contact (administration and banking) whereas men who were more likely to have inhalational exposures (119). This may explain why woman are more likely to develop non-pulmonary sarcoidosis than men, (20) and it might also explain how non-respiratory exposures may be mechanistically linked to the development of sarcoidosis.

ANIMAL AND EXPERIMENTAL MODELS OF GRANULOMATOUS DISEASE

Various animal and experimental models of granulomatous have been developed that have involved exposure to environmental substances (120). Carbon nanotube induced granulomatous lung disease has been demonstrated in an animal model and has shown several similarities to sarcoidosis (121, 122). Numerous components of infectious agents, particularly mycobacteria and Propionibacterium acnes have mimicked features of sarcoidosis

in animal models (123–126). These models have demonstrated similar immune responses in terms of T-cell function and the production of cytokines seen in sarcoidosis (120).

SUMMARY

In conclusion, sarcoidosis is associated with several environmental exposures including infectious agents, non-infectious organic antigens, metals, combustible products, and other inorganic substances. These disparate exposures may suggest that sarcoidosis represents a collection of different disorders that all result in the development of a multisystem granulomatous disease. Alternatively, these varied exposures may each stimulate the immune system in different ways such that a specific immune pathway that leads to sarcoidosis is promoted. This could include the induction of autoimmunity. Genetics factors are most probably an important aspect of these mechanisms. Further insights concerning the relationship of environmental exposures to the development of sarcoidosis may have a major impact on the prevention and treatment of this enigmatic disease.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 14 July 2020
doi: 10.3389/fimmu.2020.01443

Biomarkers in the Diagnosis and Prognosis of Sarcoidosis: Current Use and Future Prospects

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Sarcoidosis is a heterogeneous disease in terms of presentation, duration, and severity. Due to this heterogeneity, it is difficult to align treatment decisions. Biomarkers have proved to be useful for the diagnosis and prognosis of many diseases, and over the years, many biomarkers have been proposed to facilitate diagnosis, prognosis, and treatment decisions. Unfortunately, the ideal biomarker for sarcoidosis has not yet been discovered. The most commonly used biomarkers are serum and bronchoalveolar lavage biomarkers, but these lack the necessary specificity and sensitivity. In sarcoidosis, therefore, a combination of these biomarkers is often used to establish a proper diagnosis or detect possible progression. Other potential biomarkers include imaging tools and cell signaling pathways. Fluor-18-deoxyglucose positron emission tomography and high-resolution computed tomography have been proven to be more sensitive for the diagnosis and prognosis of both pulmonary and cardiac sarcoidosis than the serum biomarkers ACE and sIL-2R. There is an upcoming role for exploration of signaling pathways in sarcoidosis pathogenesis. The JAK/STAT and mTOR pathways in particular have been investigated because of their role in granuloma formation. The activation of these signaling pathways also proved to be a specific biomarker for the prognosis of sarcoidosis. Furthermore, both imaging and cell signaling biomarkers also enable patients who might benefit from a particular type of treatment to be distinguished from those who will not. In conclusion, the diagnostic and prognostic path of sarcoidosis involves many different types of existing and new biomarker. Research addressing biomarkers and disease pathology is ongoing in order to find the ideal sensitive and specific biomarker for this disease.

Keywords: biomarkers, serum, bronchoalveolar lavage, imaging biomarkers, future biomarkers, sarcoidosis

OPEN ACCESS

Edited by:
Marc A. Judson,
Albany Medical College, United States

Reviewed by:
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Kunihiro' Yamaoka,
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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *08 April 2020*
Accepted: *03 June 2020*
Published: *14 July 2020*

Citation:
Kraaijvanger R, Janssen Bonás M, Vorselaars ADM and Veltkamp M (2020) Biomarkers in the Diagnosis and Prognosis of Sarcoidosis: Current Use and Future Prospects. Front. Immunol. 11:1443. doi: 10.3389/fimmu.2020.01443

INTRODUCTION

Sarcoidosis is a systemic inflammatory disorder of unknown cause which can lead to a variety of clinical symptoms. It commonly affects the lungs and intrathoracic lymph nodes, and is characterized by the formation of non-caseating epithelioid cell granulomas (1). In case of pulmonary involvement, granuloma formation can result in a decreased lung volume and diffusing capacity, with further shortness of breath (2). In most cases, the inflammation of sarcoidosis resolves within 2–3 years. In about 10–30% of the patients, however, the inflammation persists, leading to a chronic, sometimes progressive and even fibrotic disease for which treatment is

required (3, 4). Mortality attributable to sarcoidosis is estimated between 0.5 and 5%, which is clinically relevant for a disease affecting relatively young people (5).

The diagnostic trajectory of sarcoidosis is long and complicated; it requires invasive methods like bronchoalveolar lavage and evidence of granuloma in lung or other tissue through a biopsy (6, 7). The diagnosis of sarcoidosis is then established by excluding other diseases with similar clinical or histopathological features (8).

The discovery of a specific biomarker for this disease would help diagnose sarcoidosis. According to the National Institutes of Health Biomarker Definitions Working Group, a biomarker is "a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (9). Such predicative biomarkers are already widely used to detect the presence and severity of a variety of inflammatory diseases. However, no specific biomarker for sarcoidosis has so far been identified (2). A predictive biomarker would also be useful for treatment decisions. Because of the heterogeneity of sarcoidosis, it currently remains unclear who will benefit from a specific type of treatment (10).

The ideal biomarker should not be related to other diseases and should be highly sensitive. Moreover, it should not be invasive, and should be reproducible. Also, the ideal biomarker should be inexpensive (11). A combination of measurable biomarkers and cytology would help to understand the changes within the body that are caused by sarcoidosis, as well as the occurrence and progression of the disease, and would enable personalized treatment (2).

In this review, we address current and possible future biomarkers in sarcoidosis. Serum and bronchoalveolar lavage biomarkers are discussed in terms of antigen presentation, granuloma formation, and T-cell activation. As regards imaging, we focus on the value of chest X-ray, fluor-18-deoxyglucose positron emission tomography (^{18}F-FDG PET) and high-resolution computed tomography (HRCT) as diagnostic and prognostic tools in sarcoidosis.

SERUM BIOMARKERS

Most serum biomarkers found in sarcoidosis are produced by inflammatory cells involved in granuloma formation (**Figure 1**). In this overview, biomarkers are discussed for each of the cell types associated with the biomarkers (**Table S1**).

Macrophages

Monocyte-derived macrophages are known to be key players in granuloma formation. In order to regulate granuloma formation, monocytes, and macrophages produce a number of cytokines, chemokines and other signaling proteins. Many of these have been previously described as potential serum biomarkers in sarcoidosis and normally correlate with granuloma burden (12).

Serum Angiotensin-Converting Enzyme

The most well-known serum biomarker in sarcoidosis is serum angiotensin-converting enzyme (sACE). sACE is an acid glycoprotein which converts angiotensin I into angiotensin II. It is mainly produced by activated alveolar macrophages and correlates with granuloma burden and radiological stages II and III (13). Elevated serum levels of ACE have been intensively studied since 1975 and this is currently the most frequently used laboratory test in sarcoidosis (14). Roughly 30–80% of sarcoidosis patients have increased sACE levels, and sensitivity ranges between 22 and 86% and specificity between 54 and 95% (8). Due to the low sensitivity of sACE levels, their value as a diagnostic or prognostic tool remains a matter of debate. The low sensitivity of elevated sACE levels in sarcoidosis patients is partly due to the fact that sACE levels have been found to be increased in several other inflammatory diseases, like tuberculosis, berylliosis, histoplasmosis, Gaucher's disease and many others (15–18).

Furthermore, ACE levels in the normal range could in fact be elevated in specific patients. This is based on the influence of an insertion (I) or deletion (D) polymorphism in the ACE gene on sACE levels (19). In healthy controls, subjects with an II genotype had a significantly lower serum ACE concentration compared to subjects with a DD genotype. In clinical practice, a Z-score has been developed that corrects the ACE activity for the I/D polymorphism (19, 20). Using the correction for this I/D polymorphism results in a different interpretation in 8.5% of measurements (19).

ACE could also have a role in predicting treatment outcome. High serum levels of ACE before treatment correlate significantly with lung function improvement after 6 months of methotrexate treatment (21). What has to be kept in mind is the use of ACE inhibitors in patients with sarcoidosis (22). If patients with sarcoidosis use ACE inhibitors, serum ACE levels cannot be used in diagnosis or disease monitoring, and should be interpreted carefully (23).

Lysozyme

Lysozyme is a bacteriolytic enzyme that hydrolyses glycosidic bonds in order to degrade peptidoglycans in bacterial cell walls. At the site of infection lysozyme activity limits the cause of inflammation by rapidly degrading peptidoglycans (24). In sarcoidosis, lysozyme is produced by monocyte-macrophage systems and epithelioid cells, and is involved in granuloma formation. Increased concentration of lysozyme is mainly observed at onset of disease and has low sensitivity for sarcoidosis. Hence, lysozyme is more suitable as a prognostic rather than a diagnostic tool (25, 26).

Neopterin

Neopterin is a non-specific marker of inflammation produced by activated monocytes, macrophages, dendritic cells, and endothelial cells upon stimulation mainly by interferon gamma (IFN-γ) (27). Neopterin is released in response to cytokines mostly produced by T-cells and natural killer cells. The interaction between T-cells, macrophages, and dendritic cells in the process of granuloma formation could be due to the higher neopterin levels which have been found in sarcoidosis patients with active disease (28, 29). Although serum neopterin levels seem to be increased in sarcoidosis patients, the specificity of neopterin for sarcoidosis is low; therefore, it has little value as

a diagnostic biomarker. Nevertheless, it could be a potential marker for disease activity and predictor of progression, but further research is required (30).

YKL40

The human cartilage glycoprotein-39 or YKL-40 is a growth factor for fibroblasts and vascular endothelial cells and is secreted by macrophages and neutrophils. Increased serum YKL-40 levels have been found in patients with diseases characterized by inflammation, tissue remodeling and ongoing fibrosis (31). In sarcoidosis patients, serum YKL-40 levels have been found to be elevated and to inversely correlate with diffusing capacity of the lung for carbon monoxide (DLCO) at presentation. In addition, serum YKL-40 levels are higher in patients with active sarcoidosis than in patients with inactive sarcoidosis (32, 33). A correlation has been found between YKL-40 levels and both sIL-2R levels and sACE levels in patients with active sarcoidosis, suggesting YKL-40 to be a marker for granuloma burden (33). At this moment, however, little is known about the value of serum YKL-40 levels, and more research is required to determine the value of YKL-40 as a diagnostic or prognostic biomarker in sarcoidosis (28).

sCD163

CD163 is a transmembrane hemoglobin-haptoglobin scavenger receptor expressed selectively on most macrophages in human tissues and on at least 10–30% of monocytes. Expression of CD163 is increased by IL-6, IL-10, glucocorticoids, and most immunomodulatory factors. Expression of CD163 decreases under the influence of tumor necrosis factor (TNF), interferon gamma (IFN-γ), and transforming growth factor beta (TGF-β). These are all cytokines involved in granuloma formation and disease activity. This may suggest a correlation between the expression of CD163 and inflammatory status in sarcoidosis patients. Shortly after activation of the toll-like receptors-2,4, and 5 (TLR), CD163 is shed into the environment as soluble CD163 (sCD163) (34). This quick release of sCD163 into the environment after TLR activation implies a role in inflammation, but no specific function of sCD163 has yet been identified (35). sCD163 may inhibit T-cell proliferation and activation, although the exact mechanism for this effect remains unclear. Nevertheless, the increase of sCD163 levels after stimulation of TLR suggests that sCD163 may play a role in inflammatory disorders mediated by monocyte-macrophage lineage cells. In sarcoidosis patients sCD163 was found to be significantly increased in comparison to healthy controls and correlated with sIL-2R and sACE levels (36). Although little is known about the role of sCD163 in sarcoidosis, it may be a useful biomarker with potentially high sensitivity but low specificity. In other diseases like RA, MS and Crohn's disease, sCD163 has been associated with disease activity and suggested as a useful predictor of progression (35, 37).

CC Chemokine Ligand 18

C-C motive chemokine ligand 18 (CCL18) is a CC chemokine produced primarily by antigen-presenting cells such as macrophages, dendritic cells, and peripheral blood monocytes. CCL18 is chemotactic for both naive and activated T-lymphocytes. Elevated levels of CCL18 have been detected in serum of patients with T-cell helper 2 (Th2) predominant diseases like idiopathic pulmonary fibrosis, bronchial asthma and scleroderma (38). CCL18 can stimulate the mRNA and protein production of collagen, possibly stimulating fibrosis. In sarcoidosis patients, elevated levels of CCL18 have been found in patients with active disease (39). Unfortunately, CCL18 is elevated in most interstitial lung diseases, and also in Gaucher's disease, so it is not suitable as a diagnostic biomarker for sarcoidosis. However, CCL18 has potential to be used as a marker to monitor disease and predict progression (39), as was already suggested by Prasse et al. (40), who demonstrated that CCL18 levels in bronchoalveolar lavage (BAL) correlate with the scadding stage.

Serum Amyloid A

Serum amyloid A (SAA) is produced by the liver during an acute phase reaction. In inflammatory conditions, macrophages produce high levels of SAA, which indicates that elevated levels of SAA are a clinical marker of inflammation. Elevated levels of SAA are found in several inflammatory diseases like RA and Crohn's disease (41). Elevated levels of SAA have also been found in sarcoidosis, and appear to correlate with a decline in lung function (42). Sarcoidosis has been suggested as a heterogeneous disease, with multiple potential triggers such as metals or micro-organisms (43). If in the future patients could be distinguished based on possible triggers, it would be interesting to study SAA as a biomarker in a specific subgroup of sarcoidosis patients with micro-organisms such as mycobacteria or propionic bacteria as suspected triggers (44).

Chitotriosidase

Chitotriosidase (CTO) is an enzyme of the chitinase family. It degrades chitin, a polymer found in cell walls of fungi and the exoskeletons of insects and crustaceans. Pulmonary neutrophils and macrophages can secrete CTO upon stimulation of toll-like receptors (TLRs) by IFN-γ, TNF, and granulocyte/macrophage colony-stimulating factor (GM-CSF). CTO serum levels directly correlate with sACE levels (45). Highest serum levels were found in patients with progressive disease and were found to decrease upon treatment with prednisone or other immunosuppressant therapy. As a diagnostic marker, CTO is less useful due to low specificity. CTO serum levels have been found to be elevated in other diseases like Gaucher's disease, malaria, multiple sclerosis, atherosclerosis, Alzheimer's disease, and tuberculosis (20). However, CTO sensitivity and specificity are higher than those of other serum biomarkers, making CTO a potentially useful biomarker in sarcoidosis. CTO serum levels have prognostic value in sarcoidosis and can be used to monitor disease activity, with the potential to be used as a diagnostic tool for this disease (45, 46).

Monocytes

As mentioned above, macrophages are key players in granuloma formation. However, macrophages are difficult to study or use as diagnostic/prognostic biomarkers, since they are predominantly

found in tissue and granuloma. Monocytes are precursors of macrophages and are found in the bloodstream, making them accessible and interesting as biomarkers in sarcoidosis. There are three subtypes of monocytes, classical monocytes (CD14++/CD16–), intermediate monocytes (CD14+/CD16+) and non-classical monocytes (CD14–/CD16++). Intermediate and non-classical monocytes are more inflammatory type monocytes, and have been found to be elevated in sarcoidosis (47). In addition, treatments with prednisone and infliximab have been shown to have a downregulating effect on intermediate and non-classical monocytes, suggesting a role in disease activity and prognostic value for response to treatment (48, 49). In addition to the CD14 and CD16 surface markers, many different monocyte surface markers have been studied, with promising results (50, 51). However, circulating intermediate and non-classical monocytes have also been found to be elevated in other diseases, like cardiovascular diseases and other interstitial diseases, thus decreasing its specificity (52, 53). Circulating subtypes of monocytes are potentially interesting prognostic biomarkers, but further research is required.

T-Cells

T-cells play an important role in the development of granulomas. Antigen presenting cells like macrophages present peptides to T-helper (Th) cells via MHC class II molecules. This activates T-cells and leads to greater proliferation and recruitment of neutrophils and monocytes (54). Based on T-cell activation, many T-cell cytokines and chemokines have been described as potential biomarkers for sarcoidosis. The activation of T-cells can be seen as a crucial step in the perpetuation of granuloma formation.

Serum Soluble Interleukin 2 Receptor

Serum soluble interleukin 2 receptor (sIL-2R) is the circulating form of the membrane IL-2R, a proposed marker of disease activity in sarcoidosis. Upon activation, Th1 cells upregulate the expression of IL-2R on the cell surface, and are able to shed sIL-2R into circulation (55). Increased sIL-2R levels are therefore considered to be a marker of Th1 cell activation in the formation and perpetuation of granuloma (56). Increased levels of sIL-2R in sarcoidosis patients have been described since 1983 (57). Unfortunately, elevated sIL-2R levels are not specific for sarcoidosis, as elevated serum levels of sIL-2R are found in other granulomatous diseases, hematological malignancies, and various autoimmune disorders (10). Even though the use of sIL-2R as a diagnostic marker for sarcoidosis remains a matter of debate, a recent study performed in patients suspected of sarcoidosis has shown a sensitivity of 88% and a specificity of 85% (58). This indicates that sIL-2R can be a useful tool in the diagnosis of sarcoidosis when combined with other (imaging) biomarkers and clinical features in the process of diagnosis.

sIL-2R seems to correlate with active disease and multiple organ involvement, and can possibly predict progression and relapse after discontinuation of therapy (59–61) Furthermore, sIL-2R can be used as a prognostic tool to determine if therapy is needed and/or to predict relapse after discontinuation of therapy (21, 59). Moreover, sIL-2R can be used in serial measurements during therapy or in follow-up to evaluate treatment effect (11).

It is important to state that patients with impaired renal function have elevated sIL-2R. This can result in high sIL-2R levels in the absence of active disease. Since renal impairment can occur in sarcoidosis patients, this effect has to be kept in mind when sIL-2R is used as a marker to monitor disease activity (59).

B-Cells

Although the innate immune system and T-cell immunity are known to be involved in the pathogenesis of sarcoidosis, it is becoming increasingly evident that B-cells are involved in granuloma formation as well. B-cell accumulation has been shown in granuloma and pulmonary lesions, and a positive effect of B-cell depletion has been reported (62, 63).

B-Cell Activating Factor

B-cell activating factor (BAFF) is a cytokine of the TNF family with a critical role in B-cell development and function. *In vivo* and *in vitro* studies have shown that blocking of BAFF results in reduced follicular and marginal zone B-cell numbers, while overexpression of BAFF in mice resulted in an increase in activated B-cells, activated T-cells, autoantibody production, and hypergammaglobulinemia. In sarcoidosis patients, higher levels of BAFF have been found in serum in comparison to healthy controls (64–66). However, elevated BAFF levels are not specific for sarcoidosis, as these have also been found in other immunomodulatory diseases like systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (67). In addition to elevated serum levels being found in sarcoidosis patients, a correlation between BAFF levels and disease severity has been shown. Highest levels of BAFF in sarcoidosis patients are associated with multiple organ involvement, decline in pulmonary function, and more advanced chest radiographic stages (II/III) (64). In a different study, higher serum levels of BAFF were associated with a higher frequency of eye and skin involvement and with increased levels of sACE, lysozyme, and IFN-γ (66). Although much is still unknown about the mechanisms of BAFF in sarcoidosis patients, results reported so far seem promising enough to further explore the value of BAFF as a prognostic or even diagnostic biomarker in sarcoidosis.

Naïve and Memory B-Cells

The B-cell ablative rituximab targets CD20, a marker expressed on the surface of naïve and memory B-cells, and has been shown to yield clinical improvement in some sarcoidosis patients (63, 68). This observation suggests a role for naïve and memory B-cells in sarcoidosis pathophysiology. When phenotyping B-cells, subpopulations are distributed differently in sarcoidosis patients in comparison to healthy controls (62, 69, 70). Naïve B-cell populations have been found to be elevated in sarcoidosis patients, and memory B-cells have been found to be downregulated in sarcoidosis patients (62). However, not all studies found the memory B-cells to be downregulated (69). When comparing sarcoidosis patients with and without pulmonary involvement, only the naïve mature B-cells are different between the two groups: sarcoidosis patients without pulmonary involvement do not have increased numbers of naïve mature B-cells.

Not only are numbers of naïve and memory B-cells altered, but sarcoid B-cells were found to be anergic in chronic sarcoidosis. This anergy could be partly due to the reduced levels of NF-κB/p65 found in sarcoid B-cells. B-cells with reduced NF-κB have an impaired response to antigens (69, 71). Furthermore, when memory B-cells are reduced, this may lead to a defective antibody response that is not able to eliminate the antigens responsible for granuloma formation. These findings suggest an important and possibly pathogenic role for B-cells in sarcoidosis, but the involvement of the different B-cell populations in granuloma formation remains unclear (62).

Regulatory B-Cells

Regulatory B-cells (Bregs) are IL-10 producing B-cells. This type of B-cell has been found to reduce inflammation through cytoplasmic IL-10 expression (72). Phenotyping B-cell populations in sarcoidosis patients revealed that circulating numbers of IL-10 producing B-cells were elevated in patients

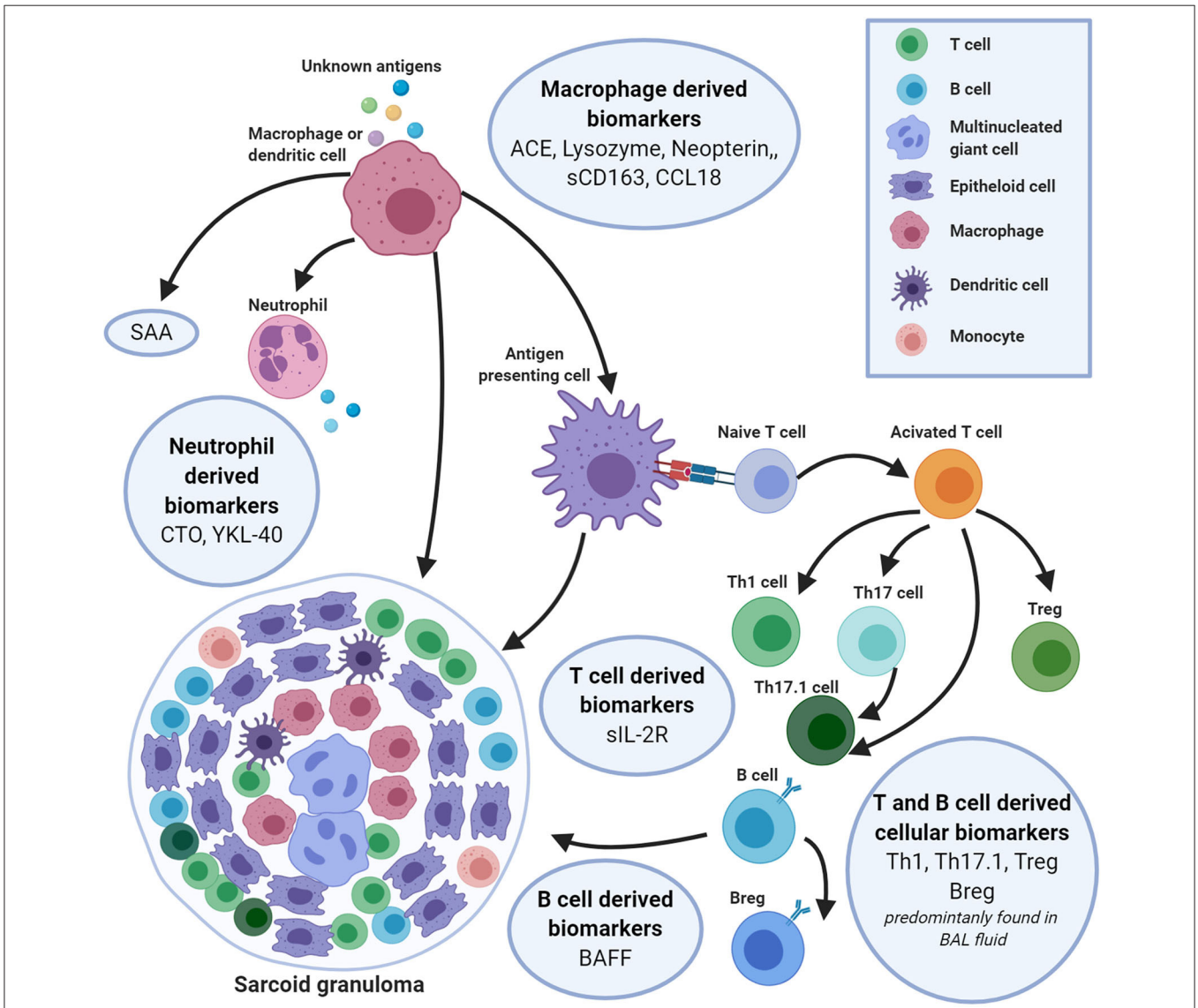

FIGURE 1 | An integrated overview of serum and bronchoalveolar lavage fluid biomarkers produced by cells of the innate and adaptive immune system, involved in the formation of granulomas in sarcoidosis. The sarcoidosis granuloma consists of a tightly formed core of epithelioid and multinucleated-giant cells (MGCs) encircled especially by T helper (Th) cells, but also by B cells, macrophages and dendritic cells (DCs). During this granuloma formation a variety of biomarkers is released by these inflammatory cells. Macrophages are key players in granuloma formation and produce a number of inflammatory biomarkers [e.g., serum angiotensin-converting enzyme (sACE), lysozyme, neopterin, CD163, C-C motive chemokine ligand 18 (CCL18), serum amyloid A (SAA)]. Macrophages activate T-cells by presenting a triggering antigen, which results in an upregulated expression of soluble interleukin 2 receptor (sIL-2R). Apart from T-cells, B-cells also have shown to play a role in granuloma formation. Crucial for the B-cell maturation and function is the biomarker B-cell activating factor (BAFF). All these biomarkers may be useful for the diagnosis and prognosis of sarcoidosis. Figure created with Biorender.com.

with active sarcoidosis. Both the frequency and absolute numbers were significantly higher in active sarcoidosis. Although current knowledge of IL-10 producing B-cells is insufficient to draw any conclusion on the role of Bregs, it seems that there is an altered B-cell homeostasis in active sarcoidosis.

This observation does appear to be in line with findings in other inflammatory diseases like SLE, RA, and multiple sclerosis. Furthermore, since IL-10 is important in the development of fibrosis, this association could justify further research into this population of B-cells (62, 65).

In conclusion, B-cells are relatively new and somewhat unknown cells in sarcoidosis. However, since B-cells seem to be involved in several processes in sarcoidosis pathogenesis, the expectation would be that BAFF levels and memory, naïve, and regulatory B-cell counts could be used to assess disease activity and could possibly be used in the diagnosis of sarcoidosis. However, further research is required before B-cell counts and BAFF levels can be used as biomarkers in sarcoidosis.

Combining Serum Biomarkers

All biomarkers mentioned above as serum biomarkers appear to have the same limitations, namely, insufficient sensitivity and specificity. Since many biomarkers have shown a mutual positive correlation (25, 28, 73), different combinations are being explored. Using a combination of different biomarkers, various studies have already demonstrated an increase of both sensitivity and specificity (74–76).

BRONCHOALVEOLAR LAVAGE FLUID

The diagnosis of sarcoidosis is often confirmed by analyzing bronchoalveolar lavage fluid (BALF) (77). Various cells and soluble components in BALF, such as proteins and cytokines, have been addressed in different immunological studies. These studies have considerably increased our understanding of the immunopathogenesis of sarcoidosis (29, 77) (**Table S2**).

CD4/CD8 Ratio

In sarcoidosis, the percentage of lymphocytes in BALF is often increased. Sensitivity of lymphocytosis was found to range between 68 and 95% (78, 79). This variation is related to the fact that an increase in lymphocytes in BALF is nonspecific for sarcoidosis, as it is also observed in other granulomatous and lung diseases (10, 29, 80). The observation that CD4+ T-cells play an important role in the development of sarcoidosis has helped distinguish sarcoidosis from other diseases (81). Measurement of the ratio of CD4/CD8 T-cells has been used to differentiate sarcoidosis from other diseases since the 1980s (29, 80). Increased CD4/CD8 ratio was observed in sarcoidosis patients, with a sensitivity of between 54 and 80% and a specificity of between 59 and 80% (10, 29, 78, 79). In the clinic, the diagnosis of sarcoidosis is supported by a CD4/CD8 ratio >3.5 and lymphocytosis > 15%. However, BALF lymphocytosis is not a universal finding in sarcoidosis (81). A recent study by Darlington et al. (82) showed that the measurement of the proportion of T-cell receptor (TCR) CD4+ Va2.3+ T-cells in BALF could also be a biomarker in addition to the CD4/CD8 ratio, to support a sarcoidosis diagnosis. TCR Va2.3 gene segments were found to be increased in BALF, especially in patients with an HLA-DRB1*03 genotype, which is usually found in patients with Löfgren's syndrome (LS). This biomarker therefore proved to be more useful for the diagnosis of LS, while the CD4/CD8 ratio is useful for the diagnosis of any type of sarcoidosis. On the other hand, specificity proved to be higher for CD4+ Va2.3+ T-cells compared to the CD4/CD8 ratio in BALF (97 vs. 92%), which will help with the diagnosis of sarcoidosis (82). Despite the frequent use of the CD4/CD8 ratio for the diagnosis of sarcoidosis, this ratio does not reflect the severity of the disease (29).

CD103+CD4+/CD4+ Ratio

While the CD4/CD8 ratio is useful for the diagnosis of sarcoidosis, sensitivity and specificity of this biomarker are low. Therefore the expression of other cellular markers in this subset of T-cells have been explored.

Expression of the aEβ7/CD103β7 integrin (CD103) has been related to the retention of intraepithelial lymphocytes (IEL) in mucosal tissue of the lung. CD103 is expressed by lymphocytes within the bronchial epithelium, some alveolar wall lymphocytes and CD4+ T-cells in the BAL. Expression of CD103 on CD4+ T-cells in BAL fluid is found to be different in ILD patients, depending on the type of disease (83). In patients with sarcoidosis, numbers of CD103 CD4+ T-cells in BAL fluid are significantly lower than in other lung diseases (83). Therefore, differential expression of CD103 on CD4+ T-cells could serve as a diagnostic marker for sarcoidosis. However, there is some controversy on the use of the CD103+CD4+/CD4+ ratio as a diagnostic tool for sarcoidosis. The sensitivity and specificity seems to depend strongly on the population used in the study. Heron et al. (84) and Bretagne et al. (85) show a significant decrease in the CD103+CD4+/CD4+ ratio in sarcoidosis patients in comparison to other ILD while a study by Hyldgaard et al. (79) does not show a similar decrease. The difference in outcome might be explained by the inclusion of patients without alveolar lymphocytosis by Hyldgaard et al. (79).

While the CD103+CD4+/CD4+ appears to be a useful diagnostic tool in sarcoidosis caution should be taken with the interpretation of this ratio. Therefore, a CD103+CD4+/CD4+ ratio 0.2 can be seen as additional evidence pointing towards a diagnosis of sarcoidosis.

T-Helper 17.1 Cells

For a long time, sarcoidosis was seen as a Th1-driven disease, because of the variety of type 1 specific cytokines present in BALF and serum of sarcoidosis patients, including IFN-γ, IL-12, and IL-18. However, nowadays it is accepted that sarcoidosis is driven by a combination of Th1- and Th17-associated factors (86). Th17 cells normally produce IL-17A and IL-17F, but in sarcoidosis Th17 cells were also observed to produce IFN-γ (12, 87). These cells are referred to as Th17.1 cells and are thought to derive from classically polarized Th17 cells. Th17 cells have a high plasticity and were found to be able to differentiate into a Th1-like phenotype, in which the majority mainly produce IFN-γ (87, 88). Both Th17 and

Th17.1 cells were identified in sarcoidosis patients, and these Th-cells were found to express different chemokine receptors, and demonstrated different cytokine effector functions. The number of Th17.1 cells proved to be significantly higher in the BALF, but not the blood, of sarcoidosis patients, compared to healthy controls (88). Th17.1 cells are promising immunological markers in the diagnosis of sarcoidosis, but results are based on small cohorts, so further research is required to determine the diagnostic value. Additionally, it was seen that there is a significant association between the number of Th17.1 cells in BALF and the development of chronic sarcoidosis, making these cells an interesting prognostic marker for the development of (chronic) pulmonary sarcoidosis (87).

Regulatory T-Cells

Regulatory T-cells or Treg cells may also play an important role in the development of sarcoidosis. Treg cells normally suppress immune responses by the host immune system against self and foreign antigens. In the case of sarcoidosis, it is hypothesized that Tregs are functionally defective or exhausted, making them unable to fully suppress the immune responses (89). The dysfunction of Tregs in sarcoidosis, resulting in impaired self-tolerance and failed immune homeostasis, could be an important element in the pathogenesis of sarcoidosis (90). A Polish study showed that the dysfunction of Tregs resulted in an exaggerated Th1 response in sarcoidosis patients (91). The diagnostic value of Tregs in sarcoidosis remains to be determined. A number of possible correlations between Tregs and disease activity have been described, with prognostic values. A Scandinavian study showed that a low Treg level in BALF was associated with a favorable prognosis (92). On the other hand, a German study reported that a decreased Treg level in BALF corresponded with the development of chronic disease (93).

Although there are thus conflicting results on the presence and effect of Tregs in active sarcoidosis, what is clear is that an imbalance exists between Th17 and Tregs. Th17 cells are elevated in the blood of sarcoidosis patients, while the number of Tregs is decreased (90). The Treg/Th17 ratio proved to be inversely related to disease activity, decreasing in those with relapsing pulmonary sarcoidosis and increasing in those with chronic active disease (12, 94).

Neutrophils

Neutrophils are important players in the innate immune system, but their role in sarcoidosis is still little understood. Neutrophils are attracted by chemokines, such as IL-8, which are released by monocytes and macrophages (12). Patients in an advanced radiological stage of sarcoidosis (stage II or III), or with an unfavorable evolution, were reported to have an elevated percentage of neutrophils in BALF compared to healthy controls. Furthermore, BALF neutrophil levels were significantly higher in patients with progressive disease compared to those with stable disease. Therefore, neutrophils may be useful markers of progressive disease (29, 95). A possible origin of this correlation could be the large number of proteases, like collagenase or gelatinase, which may initiate collagen destruction and remodeling, leading to the development of pulmonary fibrosis (12).

Natural Killer (NK) Cells

Other immune cells in BALF which are associated with poor outcome and an advanced radiological stage are natural killer (NK) cells (95). NK cells (CD3–CD16/56+) are part of the first-line defense of the immune system (96). They are suspected to play a role in several inflammatory pulmonary diseases because of their ability to produce cytokines (95). IFN-γ is released by active NK cells and stimulates TNF-α secretion from alveolar macrophages, which is associated with progressive and corticosteroid resistant disease (95). An impaired lung function was shown to be associated with a high level of NK cells in the lungs. Furthermore, sarcoidosis patients requiring steroid treatment also had a higher percentage of these cells in BALF (95).

Natural Killer T (NKT) Cells

Natural killer T (NKT) cells (CD3+CD16/56+) are a regulatory T cell lineage which both express T cell (CD3) and NK (CD16/56+) receptors on their surface membranes, thereby influencing both Th1 and Th2 cytokine systems and stimulating cell-mediated immunity or suppressing autoimmune responses (96, 97). NK cells are able to release a various cytokines determined by antigen signal strength, including IFN-γ, TNF-α, and a variety of interleukins (IL-4, IL-10, IL-13, IL-17, IL-21) (97). In sarcoidosis patients reduced numbers of NKT cells were found in blood and BALF (98–100). However, there are also reports on the accumulation of NKT cell in granulomatous lesions of sarcoidosis patients (99). Deficiency and/or impaired function of NKT cells results in loss of the immunoregulation, which might contribute to the prolonged T-cell activity characteristic for sarcoidosis (101).

CXCL9, CXCL10, and CXCL11

The monocyte-macrophage cell lineage is the origin of the expression of a variety of cytokines and chemokines that play many different roles in the inflammatory phenotype of sarcoidosis, including T-cell attraction and promotion of Th1/Th17 differentiation (8). Some interesting chemokines were found to be elevated in the BALF and serum of sarcoidosis patients. These included chemokines that are part of the CXC chemokine subfamily; CXCL9, CXCL10, and CXCL11 (102, 103). These chemokines bind to the CXCR3 receptor and thereby recruit CD4+ T-cells, monocytes and other inflammatory cells to the site of inflammation (103). Although these chemokines share common functions, there are differences in their biological properties (103, 104). One major difference is the fact that CXCL9 and CXCL11 can solely be induced by IFN-γ, while CXCL10 can also be induced by TNF-α, IFN-α, and LPS (103, 104). These differences may influence the clinical outcomes of these chemokines (104). In tuberculosis, this chemokine subfamily was already found to represent useful biomarkers for the prediction of disease progression and therapy response (105, 106).

In the case of sarcoidosis, a recent study by Arger et al. (103) showed that CXCL10 correlates negatively with lung function, and that elevated CXCL10 also correlates with higher

dyspnea scores in longitudinal analyses. These correlations could not be found for CXCL9. However, CXCL9 proved to be positively associated with the total number of organs involved in sarcoidosis. CXCL11 proved to be negatively associated lung function and correlated positively with the number of organs involved (103). It was speculated that each of these chemokines could be used to help predict the clinical outcome of a sarcoidosis patient, as well as acting as biomarkers for both pulmonary outcomes and response to therapy (103).

Krebs von den Lungen-6

Krebs von den Lungen-6 (KL6) is a human high molecular weight MUC1 mucin protein derived from type II pneumocytes and respiratory bronchiolar epithelial cells (107). An increased level of KL-6 reflects damaged or regenerating type II pneumocytes (108).

Elevated levels of KL-6 have been found in BALF and serum in patients with idiopathic pulmonary fibrosis, sarcoidosis, and other ILDs (109–112).

The fact that it is not a disease specific marker indicates that it has no major role in diagnosing sarcoidosis. However, in sarcoidosis patients both serum and BALF levels of KL-6 correlate with higher serum ACE levels and CD4+/CD8+ ratio (25, 112). Suggesting usefulness as a disease monitoring tool. It is demonstrated that the highest level of KL-6 can be found in patients with radiological stage IV pulmonary sarcoidosis (25, 76, 111) suggesting a potential role as a marker of severity of pulmonary sarcoidosis.

Combining BALF Biomarkers

The biomarkers mentioned above give limited information about pulmonary sarcoidosis as individual biomarkers. However, combining some of these biomarkers may be helpful in the diagnosis and prognosis of pulmonary sarcoidosis. CD4/CD8 ratio and CD103+CD4+/CD4+ ratio appear to be useful diagnostic biomarkers to support the diagnosis of sarcoidosis, while disease severity can be predicted and monitored by a correlation between KL-6 and CD4+/CD8+ ratio. Furthermore, disease progression can be monitored trough the percentage of neutrophils, NK cells, and NKT cells in BALF. In addition, Th17.1 cells and Treg cells are promising prognostic biomarkers, however, further research is required to determine the diagnostic value. The BALF chemokines CXCL9-11 in their turn may be useful biomarkers for treatment decisions.

IMAGING BIOMARKERS

Clinical manifestations of sarcoidosis are highly variable and any organ can be affected. For pulmonary sarcoidosis, chest X-ray, and high-resolution computed tomography (HRCT) are frequently used. However, in sarcoidosis patients with extrapulmonary manifestations, such as cardiac sarcoidosis or neurosarcoidosis, other imaging modalities are warranted. For these patients, magnetic resonance imaging (MRI) and fluor-18-deoxyglucose positron emission tomography (^{18}F-FDG PET) scanning are increasingly recognized as essential imaging techniques for adequately identifying sarcoidosis localization and determining disease management (113).

TABLE 1 | Radiographic staging of sarcoidosis patients at presentation according to the scadding criteria.

Radiographic stage	Chest X-ray	Frequency (%)	Resolution (%)
0	Normal	5–15	
I	BHL	25–65	60–90
II	BHL and pulmonary infiltrates	20–40	40–70
III	Pulmonary infiltrates without BHL	10–15	10–20
1V	Advanced pulmonary fibrosis	5	0

The estimated frequency at presentation is given as well as the probability of spontaneous resolution during disease course (BHL, bilateral hilar lymphadenopathy) (1, 114, 116).

Chest X-ray

Conventional chest radiography is performed in most patients with sarcoidosis and is abnormal in over 90% of cases (114). Sarcoidosis is commonly staged according to its appearance on chest X-ray. This staging system was introduced by Scadding almost six decades ago (115), but still holds a prominent position in assessment of sarcoidosis. Scadding classified chest radiography findings in sarcoidosis into five stages, shown in **Table 1**.

An interesting feature of the abovementioned Scadding stages is the fact that it gives prognostic information both on resolution of disease as well as mortality (1, 114, 116, 117). Spontaneous resolution varies from 60 to 90% in stage I disease to no resolution in stage IV fibrotic disease. Furthermore, Kirkil et al. (117) demonstrated that mortality rates were correlated to Scadding stages with the highest mortality in patients presenting with stage IV disease.

Shortcomings of conventional chest X-ray is the fact that in absence of pathological confirmation, clinical features together with chest X-ray can be diagnostic in stage I and II disease but are less accurate in stage 0, II, and IV disease (1). Furthermore, only moderate agreement on Scadding stages is reported in literature (118). Finally, Judson et al. (119) demonstrated that chest X-ray was inadequate to reliably detect acute exacerbations of pulmonary sarcoidosis. These are some reasons why the HRCT now has a prominent place in the diagnosis and monitoring of sarcoidosis.

High-Resolution Computed Tomography

HRCT has a solid position in the diagnostic approach and follow-up of sarcoidosis, as it provides high-resolution slides of the pulmonary parenchyma. Although widely used, quantification of HRCT results remains a difficult task even for experienced radiologists and pulmonologists. A reliable inter- and intra-patient comparison of results requires standardized quantification of HRCT findings.

In a pioneering approach to objectively quantifying HRCT results, Oberstein et al. developed a scoring system based on the sum of typical patterns of parenchymal involvement, combined with lymph node enlargement and pleural involvement. The

Oberstein score yielded high correlation coefficients with BAL total cell count and sIL-2R (120).

Drent et al., who also found a good interobserver agreement in a retrospective cohort of 80 sarcoidosis patients, later replicated the Oberstein score. They also found a correlation between pulmonary function and HRCT abnormality scores for all subgroups except lymph node enlargement (121). When used together with serum sIL-2R values, the HRCT scoring system can predict disease activity and could replace ^{18}F-FDG PET (122). Although this finding was not replicated, it could be an important finding especially with regard to cost-effectiveness and the fact that ^{18}F-FDG PET is not universally available.

In order to objectively quantify HRCT results regarding the extent and activity of sarcoidosis, Benamore et al. more recently developed the CT activity score (CTAS), which consists of CT extent scores for nodularity, ground-glass opacification, interlobular septal thickening and consolidation. The total CTAS was found to predict response to treatment in FVC after 1 year in their cohort of 100 patients. Furthermore, CTAS had a high degree of interobserver agreement (123).

A retrospective study of a cohort of 57 patient reproduced these results and found significant correlations with lung function changes after six months of therapy (Change in %VC: $r = 0.543$, $P < 0.001$). Furthermore, higher CTAS scores were seen in patients with high sACE levels (124). Even though the results of these HRCT scoring systems are promising, they are still not widely implemented in standard patient care.

Fluor-18-Deoxyglucose Positron Emission Tomography

Over the past decade, ^{18}F-FDG PET has gained territory as an important and reliable imaging biomarker in severe sarcoidosis (125).

Prior to ^{18}F-FDG PET, gallium-67 (^{67}Ga) scintigraphy was the most frequently used form of nuclear imaging in sarcoidosis, but ^{18}F-FDG PET has replaced it, as it was shown to be more accurate in depicting extrathoracic lesions (126) and yields high quality images with superior contrast and spatial resolution compared to ^{67}Ga scintigraphy (127). ^{18}F-FDG PET can be used as a diagnostic marker, but also to predict treatment response and prognosis.

^{18}F-FDG PET as a Diagnostic Marker in Pulmonary Sarcoidosis

Although it is not necessary to obtain an ^{18}F-FDG PET scan of every new sarcoidosis patient, it sometimes provides valuable information for the diagnostic process. In patients in whom the diagnosis of sarcoidosis is suspected, the extent of organ involvement and disease activity can be visualized using ^{18}F-FDG PET. A prospective study of 36 newly diagnosed untreated sarcoidosis patients showed visual activity on ^{18}F-FDG PET in 34 patients (94%). Furthermore, ^{18}F-FDG PET was far more sensitive than ACE and sIL2R in this study (128).

In a small retrospective study of 23 biopsy-proven sarcoidosis patients with an indication to receive corticosteroid therapy, 91.7% had ^{18}F-FDG PET positive findings. Sensitivity improved to 100% after skin involvement was excluded (129).

In a large retrospective study of 158 sarcoidosis patients, 75% had ^{18}F-FDG PET positive findings (130). In some patients, new occult organ localizations such as cardiac or ossal sarcoidosis can be detected with ^{18}F-FDG PET (131). As ^{18}F-FDG PET can reveal occult localizations, it can also be used to determine the most suitable biopsy site (7). In a study of 89 patients with unexplained disabling symptoms, 73% had signs of disease activity on ^{18}F-FDG PET, which were mostly extrathoracic (132).

^{18}F-FDG PET as a Marker of Therapeutic Response and Prognosis in Pulmonary Sarcoidosis

The natural disease course of sarcoidosis is very unpredictable, with not all patients being in need of systemic therapy. It is highly important, but very difficult, to distinguish patients who might benefit from treatment from those who will not.

A reasonable number of reports objectifying ^{18}F-FDG PET as a predictor of treatment response have been published over the past few years. Keijsers et al. studied 43 newly-diagnosed sarcoidosis patients and found that patients with increased lung parenchymal activity on ^{18}F-FDG PET had significantly lower DLCO. Moreover, high parenchymal activity was a predictor of improvement in pulmonary function upon treatment with corticosteroids. Patients without parenchymal activity assessed by ^{18}F-FDG PET did not show decreasing pulmonary function when untreated (133). A prospective study included 56 severe sarcoidosis patients, refractory to first- and second-line treatment, who were treated with the anti-TNF drug infliximab. High ^{18}F-FDG PET activity of the pulmonary parenchyma (SUVmax) at baseline was able to predict lung function improvement after 6 months of treatment ($R = 0.62$, $p = 0.0004$) (134) (**Figure 2**). As anti-TNF therapy is costly and known to cause side-effects, ^{18}F-FDG PET can be very useful in therapeutic decision making, especially for this group of refractory patients.

In a study of 90 sarcoidosis patients with persistent symptoms, 72% had signs of activity on ^{18}F-FDG PET. Furthermore, 38 patients (51%) with positive ^{18}F-FDG PET results had normal ACE levels, suggesting an added value of ^{18}F-FDG PET as an adjunct to serum biomarkers (135).

To date it is unknown what the optimal duration of systemic therapy should be. ^{18}F-FDG PET is a potential tool to determine which patients are at risk for relapse of disease activity and symptoms. In a retrospective study, Vorselaars et al. (59) assessed the risk of relapse after discontinuation of infliximab therapy in severe sarcoidosis patients, and found that high activity ($SUV_{max} > 6$) of mediastinal lymphnodes on ^{18}F-FDG PET is a predictor of relapse after discontinuation of infliximab therapy, with a hazard ratio of 4.33 ($p < 0.001$). This suggests that high activity on ^{18}F-FDG PET reflects the burden of inflammation and therefore the need for prolonged therapy. A small retrospective study of 23 newly diagnosed sarcoidosis patients showed that persistent disease activity on ^{18}F-FDG PET during the first months of corticosteroid treatment is a predictor of relapse (129).

Most studies have used the maximum standard uptake value (SUV_{max}) to quantify disease activity. Recently, a new tool for the quantification of disease activity of the pulmonary parenchyma was proposed. Volumetric ^{18}F-FDG PET analysis of global lung

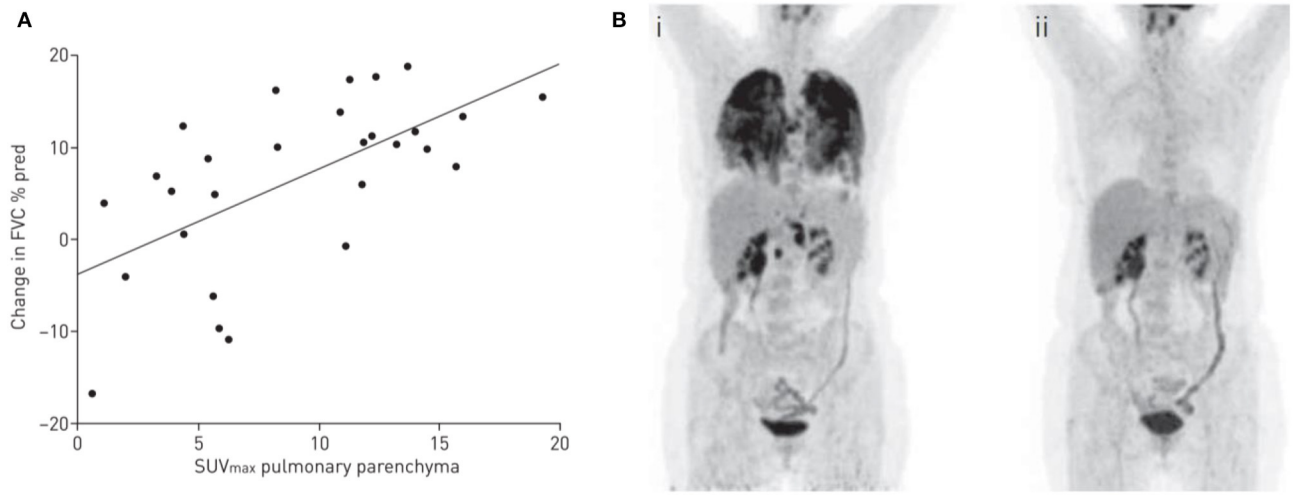

FIGURE 2 | (A) ^{18}F-fluorodeoxyglucose (FDG) by positron emission tomography (PET) activity and improvement in pulmonary function. Correlation between high activity of pulmonary parenchyma on ^{18}F-FDG PET (maximum standardized uptake value [SUV_{max}]) at baseline and improvement in forced vital capacity (FVC) in patients with a pulmonary treatment indication ($R = 0.62$, $p = 0.0004$). Reproduced and modified from (134) with permission. **(B)** Example of a PET-CT scan of a sarcoidosis patient with pulmonary involvement, before (i) and after (ii) 6 months of infliximab therapy.

inflammation was proposed by Adams et al. (136) and possibly correlates better with conventional serum biomarkers ACE and sIL-2R than SUV_{max}. TluG (Total Lung Glycolysis) measures the total activity of the pulmonary parenchyma, and although this derivative from the total lesion glycolysis in oncology studies looks promising, it was not superior to SUV_{max} in predicting lung function change during infliximab therapy (137).

Cardiac Imaging Biomarkers

Current improved imaging techniques are leading to higher rates of incidence and recognition of cardiac sarcoidosis than previously reported (138). Approximately 5% of all sarcoidosis patients will have clinically relevant cardiac involvement (139).

Magnetic Resonance Imaging in Cardiac Sarcoidosis

The diagnosis of cardiac sarcoidosis can be challenging, as the diagnostic yield of endomyocardial biopsy is low and this procedure also has potential hazards (138). Cardiac Magnetic Resonance Imaging (CMR) is acknowledged in the diagnostic guidelines for cardiac sarcoidosis (140). Late gadolinium enhancement (LGE) on CMR (in a pattern consistent with cardiac sarcoidosis) in patients with known biopsy-proven sarcoidosis and no other explanation for cardiac disease can be used as a criterion to diagnose sarcoidosis (140).

Formation of edema or scar tissue in the myocardium or pericardium shows late gadolinium enhancement on CMR, as gadolinium is an extracellular contrast agent. Late gadolinium enhancement can potentially be optimized with the use of T1/T2 mapping, which can potentially discriminate between fibrosis (T1 mapping) and extracellular water/edema (T2 mapping) (141). T1/T2 mapping for this use needs further evaluation.

A large number of studies have found LGE on CMR to be an independent prognostic biomarker (142, 143). A large prospective cohort study of 321 sarcoidosis patients by Kouranos et al. (144) showed that LGE was an independent predictor of the primary endpoint (composite of death, life-threatening arrhythmias, admission for heart failure, and heart transplantation) with a hazard ratio of 5.7 ($P < 0.004$). Moreover, a large meta-analysis including 10 studies and a total of 760 patients concluded that patients with LGE on CMR were at increased risk of events, compared to patients without LGE (11.9 vs. 1.1%; $P < 0.001$), even if they had preserved left ventricle systolic function (11.6 vs. 0.7%; $P < 0.0011$) (145). A study investigating both LGE on CMR and uptake on ^{18}F-FDG PET in 56 sarcoidosis patients with high clinical suspicion of cardiac sarcoidosis showed that LGE alone was the primary risk factor for events (arrhythmia/death). The authors suggest that future events may be driven by the presence of myocardial fibrosis instead of inflammation (146).

^{18}F-FDG PET in Cardiac Sarcoidosis

^{18}F-FDG PET has an important role as a diagnostic marker for cardiac involvement and is included in the Hearth Rhythm Society Guideline for diagnosis of cardiac sarcoidosis. "Patchy uptake on dedicated cardiac PET (in a pattern consistent with Cardiac Sarcoidosis)" in the right context can point towards the diagnosis (140).

Due to the high rate of glucose metabolism in the human heart, cardiac assessment on ^{18}F-FDG PET can be challenging. In a randomized trial of 82 patients, an 18-h fast with low carbohydrate diet preparation significantly reduced diffuse cardiac left ventricle uptake (147). A large meta-review including 559 patients revealed that the diagnostic sensitivity and specificity

were significantly improved by increased fasting time and heparin administration before scanning ($P = 0.01, 0.02$) (148).

The added value of ^{18}F-FDG PET in combination with CMR in the diagnostic process was studied in a cohort of 107 patients suspected of cardiac sarcoidosis. Added value was found in 45% of patients, as they were reclassified as likely to have cardiac sarcoidosis, most of them (80%) being correctly reclassified when compared with the final diagnosis (149).

Combining cardiac ^{18}F-FDG PET with rubidium-82 to evaluate perfusion defects can increase the prognostic value. In a study including 118 consecutive cardiac sarcoidosis patients, the presence of both perfusion defects and increased FDG uptake identified patients at higher risk for VT or death HR 3.9 ($p < 0.01$) (150).

Newer PET/CMR scans are now available offering the possibility to perform cardiac ^{18}F-FDG PET and CMR together, depicting both the pattern of injury and disease activity in a single scan (151).

Furthermore, ^{18}F-FDG PET can be used to replace cardiac MRI in patients in whom the latter is contraindicated (e.g., devices, severe claustrophobia) (138).

Neuroimaging Biomarkers

Neurosarcoidosis (NS) refers to the involvement of the central nervous system (CNS) and is seen in approximately 5% of all sarcoidosis patients. It is a serious and devastating complication which is difficult to diagnose because of the many different ways in which this disease can present itself (152, 153).

Magnetic Resonance Imaging in Neurosarcoidosis

The Zajicek criteria are commonly used to define the diagnosis of NS. In the Zajicek critera the diagnosis of NS is considered definite if there is biopsy confirmation of neural tissue, probable if there is evidence of neurological inflammation together with biopsy proven systemic sarcoidosis and possible when the presentation is typical and other potential causes have been excluded (154). In addition to histopathological tissue confirmation, imaging is used for the diagnosis of NS. Positive gallium-67 scintigraphy, MRI, ^{18}F-FDG PET, and HRCT are used in different steps of the diagnostic process (155).

The gold standard to evaluate involvement of the CNS is the use of contrast enhanced MRI. The most common abnormalities seen in neurosarcoidosis patients at MRI are periventricular white matter lesions, meningitis or meningoencephalitis, solid parenchymal enhancing lesions, cranial neuritis, and hydrocephalus (156).

Unfortunately, NS is very hard to diagnose, especially in the absence of systemic sarcoidosis. In addition to a number of imaging methods the analysis of cerebrospinal fluid and blood are performed, despite the low sensitivity of these tests (157).

^{18}F-FDG PET in Neurosarcoidosis

As mentioned previously, MRI is the gold standard to evaluate CNS involvement in sarcoidosis. However, ^{18}F-FDG PET can occasionally be helpful in demonstrating neurologic involvement not visualized on MRI (158). Furthermore, PET can help in detecting systemic manifestations of sarcoidosis other than neurological involvement (159) or assess disease activity during treatment of NS (160).

FUTURE BIOMARKERS

JAK/STAT Signaling

The accumulation and activation of macrophages that form granulomas in sarcoidosis are driven by IFN-γ secretion. IFN-γ signaling is partly dependent on a specific signaling pathway named the Janus kinase/signaling transducer and activator of transcription (JAK/STAT) pathway (161). This pathway can activate a total of 6 STATs. Gene expression studies showed that the JAK/STAT signaling pathway is differentially expressed in most sarcoidosis patients, and that this pathway is more activated compared to healthy controls. Genes regulated by STAT1 in particular showed to be elevated in the blood of sarcoidosis patients (4, 162, 163). In addition to STAT1, STAT3 was also found to play a role in the granuloma formation. While STAT1 is primarily activated within granuloma macrophages, STAT3 is activated in between the granulomas in lymphocytes (162, 164).

Recently, various case reports have been published in which JAK/STAT inhibitors like tofacitinib and ruxolitinib were successfully used to treat patients with therapy-refractory sarcoidosis (165, 166).

It may be useful in the future to further unravel this pathway and focus on which specific patients may benefit from this therapy and whether JAK/STAT activation markers can act as biomarkers.

mTOR Signaling

The mechanistic target of rapamycin (mTOR) signaling pathway is involved in cellular metabolism as well as proliferation and has been implicated in an increasing number of pathological conditions (167).

It has been demonstrated in a mouse model that granuloma can be formed spontaneously when the mTORC1-inhibitor TSC2 is depleted. It was also found that sarcoid granulomas from patients with active and progressive disease showed active mTORC1 signaling (168). A decreased expression of TSC1 was observed in 33% of the sarcoidosis patients studied, leading to increased mTORC1 activation (168). Interestingly, whole exome sequencing (WES) suggested that the mTOR signaling pathway could be involved in the development of familial sarcoidosis (169). In terms of clinical relevance, successful treatment of a sarcoidosis patient with the mTOR inhibitor rapamycin has been reported (133). As with JAK/STAT inhibition, it may also be useful in the future to further unravel the mTORC1 pathway in sarcoidosis, to see whether markers of mTORC1 activation can be used as therapeutic biomarkers in sarcoidosis. A focus of interest could be whether absence of mTORC1 activation can predict treatment response with rapamycin. Rapamycin has been successfully used to treat a patient suffering from sarcoidosis (89).

Hair Cortisol

In sarcoidosis, 50–70% of the patients experience fatigue, some even experience chronic fatigue. Fatigue can still be present when sarcoidosis has clinically resolved, causing an impaired quality of

life (170). One of the factors which is suspected to play a role in fatigue is stress. When the body is under psychological or physical stress, the hormone cortisol is released. Cortisol levels are commonly measured in serum, saliva, or urine, only acute changes are represented in this way. This could be overcome by measuring multiple samples during the day, but this would be labor-intensive and invasive, which in itself would increase cortisol release (171, 172). An emerging alternative technique is the analysis of scalp hair samples. Hair analysis has been done for decades, mostly to detect drugs of abuse. Hair samples are easy to collect in a non-invasive way and can be stored at room temperature.

The average scalp hair grows by 1 cm/month, so this method can retrospectively measure the production of these hormones over months (171, 172). The first study measuring cortisol levels of sarcoidosis patients in hair samples was performed by Van Manen et al. (171). This study showed that hair cortisol and cortisone levels were significantly higher in sarcoidosis patients compared to healthy controls, and that these increased levels related positively to the reported psychological distress, assessed by questionnaires. However, no differences in hair cortisol levels were found between sarcoidosis patients with and without fatigue.

Hair cortisol levels could be a future long-term biomarker for the measurement of chronic stress in sarcoidosis. A great advantage of this biomarker is that it is non-invasive and easily available, and can be used in a retrospective way. Using only one or a few hair strings, it may be possible to screen for fatigue and physical distress as well as to measure it in follow-up measurements. This can be used as an alternative to the currently used questionnaires, preventing variations in scores as to when patients fill in multiple questionnaires over time (171).

Labeled PET-Tracers

A PET-traceable biomarker specific for sarcoid granulomas would expand the possibilities to diagnose this disease as well as response to treatment (127). ^{18}F-FDG has been shown to be a useful PET-tracer in severe sarcoidosis. Other tracers, such as somatostatin receptors (SSTRs) like ^{68}Ga-DOTA-NaI-octreotide (DOTANOC) or ^{68}GA-DOTA-D-Phe-Tyr-octreotide (DOTATOC), would appear to be reliable alternatives to ^{18}F-FDG. Multiple inflammatory cells that are found in granulomas, including epithelioid cells and macrophages, have SSTRs on their surface, while normal monocytes have not. In the case of cardiac sarcoidosis, DOTANOC is taken up by sarcoid lesions but not by normal myocardium (173). More research is needed to determine the clinical value of these different PET tracers.

Although PET is a useful technique, it might not be able to function as a diagnostic biomarker of sarcoidosis on its own. Nevertheless, this technique provides evidence supporting or refuting the diagnosis and gives additional information about active disease in specific organs.

Another interesting application is specific labeling of the target drug used in sarcoidosis treatment with PET tracers. Radioactively labeling infliximab with technetium-99 m (99mTC) allows responders to infliximab therapy to be distinguished from non-responders. This imaging method is already being used in several TNF-α mediated diseases, such as RA, inflammatory bowel disease (IBD) and spondyloarthropathy (174). In sarcoidosis, Vis et al. (174) quantified serum TNF-α levels with ^{99m}TC-infliximab to evaluate disease activity and to identify responders, partial responders or non-responder priors to infliximab therapy. They showed that ^{99m}TC-infliximab accumulation was highest in patients with an indication for systemic treatment (174). ^{99m}TC-infliximab has not yet found its way into clinical practice for sarcoidosis.

OMICS

Sarcoidosis is a complex systemic disease reflecting immunological responses to different antigens in patients with certain genetic susceptibility (8, 175). Omics is an emerging field of research that encompasses genomics, epigenomics, transcriptomics, proteomics as well as metabolomics and is already widely used to understand polygenic and phenotypically diverse diseases and may also help identify more effective disease biomarkers in sarcoidosis (8, 175, 176).

In sarcoidosis, multiple diagnostic and prognostic biomarkers are already being identified by using single nucleotide polymorphism (SNP) technology, RNA sequencing and pathway analysis (175). Strong associations were found between sarcoidosis and various polymorphisms of the HLA allele and IL-1α. Various SNPs were also found to play a role in sarcoidosis pathogenesis, such as BTNL2, annexin A11, and NOTCH4, but the biological significance of several identified SNPs is unclear (noncoding) (175). A greater understanding of sarcoidosis may be obtained by moving beyond specific biomarkers to unbiased genome-wide analytic approaches. A study comparing lung tissue of sarcoidosis patients with that of healthy controls found that genes regulating macrophage-derived proteases and Th1 immune responses were differentially upregulated in sarcoidosis. Furthermore, IL23 and IL23R of the Th17 pathway and IL21 of STAT3 were also upregulated in sarcoidosis skin lesions compared to normal skin. Analyzing gene expression in peripheral blood proved to be a reliable surrogate for the use of tissue specimens, as it was able to reliably distinguish sarcoidosis patients from healthy controls, making this a useful approach to diagnosing and monitoring sarcoidosis (175). Based on current research, it is to be expected that the field of omics will further identify new promising biomarkers in sarcoidosis in the near future.

CONCLUSION

Although numerous biomarkers have been evaluated for patients with sarcoidosis in recent decades, no gold standard has been set for their use in diagnosis or predicting disease course. Combinations of several serum biomarkers have been explored, with promising results, but the Holy Grail remains to be discovered. Future research should focus on combining serum or BALF biomarkers and further refined imaging techniques such as CT, MRI, and PET. Data on successful treatment of sarcoidosis patients with JAK/STAT and mTORC1 inhibitors suggests that

these new immunological pathways should also be explored for new prognostic biomarkers in the near future.

AUTHOR CONTRIBUTIONS

RK, MJ, and AV wrote the manuscript, contributed to the concept, and designing of the work. AV and MV revised the work critically for important intellectual content. RK and MJ set up the reference database. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01443/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 22 July 2020
doi: 10.3389/fimmu.2020.01129

T-bet Expression in Peripheral Th17.0 Cells Is Associated With Pulmonary Function Changes in Sarcoidosis

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Background: Interferon-gamma (IFN-γ) is a key mediator of sarcoidosis-related granulomatous inflammation. Previous findings of IFN-γ-producing Th17 cells in bronchoalveolar lavage fluid from sarcoidosis patients invokes the transition of Th17.0 cells to Th17.1 cells in the disease's pathogenesis. Since the T-bet transcription factor is crucial for this transition, the goal of this study was to determine if T-bet expression in Th17.0 cells reflects the extent of granulomatous inflammation in sarcoidosis patients as assessed by clinical outcomes.

Methods: Using a case-control study design, we identified two groups of sarcoidosis subjects (total $N = 43$) with pulmonary function tests (PFTs) that either (1) changed (increased or decreased) longitudinally or (2) were stable. We used flow cytometry to measure the transcription factors T-bet and RORγt in Th1, Th17.0, and Th17.1 cell subsets defined by CCR6, CCR4 and CXCR3 in blood samples. We compared the percentages of T-bet$^+$ cells in RORγt$^+$Th17.0 cells (defined as CCR6$^+$CCR4$^+$CXCR3$^-$) based on subjects' PFT group. We also assessed the relationship between the direction of change in PFTs with the changes in %T-bet$^+$ frequencies using mixed effects modeling.

Results: We found that T-bet expression in subjects' RORγt$^+$Th17.0 cells varied based on clinical outcome. The T-bet$^+$ percentage of RORγt$^+$Th17.0 cells was higher in the cases (subject group with PFT changes) as compared to controls (stable group) (27 vs. 16%, $p = 0.0040$). In comparisons before and after subjects' PFT changes, the T-bet$^+$ frequency of RORγt$^+$Th17.0 cells increased or decreased in the opposite direction of the PFT change. The percentage of these T-bet$^+$ cells was also higher in those with greater numbers of involved organs. Serum levels of interferon-γ-induced chemokines, CXCL9, CXCL10, and CXCL11, and whole blood gene expression of IFN-γ-related genes including *GBP1*, *TAP1*, and *JAK2* were independently positively associated with the T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells.

Conclusions: These data suggest that expression of T-bet in Th17.0 cells could reflect the extent of granulomatous inflammation in sarcoidosis patients because they represent a transition state leading to the Th17.1 cell phenotype. These findings indicate that Th17 plasticity may be part of the disease paradigm.

Keywords: sarcoidosis, Th17, Th17.1, Th1, T-bet, RORγt, interferon-gamma, chemokine

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *23 February 2020*
Accepted: *07 May 2020*
Published: *22 July 2020*

Citation:
Arger NK, Machiraju S, Allen IE, Woodruff PG and Koth LL (2020) T-bet Expression in Peripheral Th17.0 Cells Is Associated With Pulmonary Function Changes in Sarcoidosis. Front. Immunol. 11:1129. doi: 10.3389/fimmu.2020.01129

INTRODUCTION

Sarcoidosis is a systemic disease in which granulomatous inflammation affects the lungs in the vast majority of patients (1). Its etiology remains unknown and no cure currently exists. Multiple studies have established the importance of IFN-γ-producing $CD4^+$ T helper (Th) cells in sarcoidosis, which have been traditionally designated as "Th1" cells (2–6). In recent years, additional Th cell populations have been recognized in sarcoidosis, including IL-17-producing "Th17" cells (7–10). We previously identified two different $CD4^+$ Th17 subsets in sarcoidosis patients using the expression of chemokine receptors CCR6, CCR4, and CXCR3 (11). In blood, we observed Th17.0 ($CCR6^+CCR4^+CXCR3^-$) cells with increased frequencies in sarcoidosis compared to health. The majority of these cells produced IL-17 after *ex vivo* stimulation. In contrast, in bronchoalveolar lavage (BAL), we observed "Th17.1" ($CCR6^+CCR4^-CXCR3^+$) cells, with increased frequencies in sarcoidosis compared to health (11, 12). We found that the majority of Th17.1 cells produced IFN-γ while only a small fraction produced IL-17 upon *ex vivo* stimulation (11). The increased proportion of Th17.0 cells in the circulation accompanied by an increased proportion of Th17.1 cells in the BAL led us to consider whether these findings might be the result of Th17 plasticity, whereby circulating Th17.0 effector cells polarize into Th17.1 cells and accumulate in the lung tissue where the granulomatous inflammation is located.

Prior studies have elucidated how Th17.0 cells can polarize or transition into Th17.1 cells. The initial polarization of Th17.0 effector cells from naïve T cells occurs under the control of the orphan nuclear hormone receptor RORγt (13–17). During this polarization, the chemokine receptors CCR6 and CCR4 are upregulated (18–20). In this context, the transcription factor RORγt is used to define Th17.0 cells (15–17). The mechanism by which Th17.0 cells "polarize" into Th17.1 cells has been elucidated through *in vitro* stimulation with IL-12 and IFN-γ. This stimulation causes upregulation of the transcription factor T-bet (21–24). T-bet is the main transcription factor that controls polarization of naïve T cells to Th1 cells (25–29). Once T-bet is activated, several downstream genes are upregulated including those for CXCR3 and IFN-γ (26–28). Based on this collective T cell biology, we speculate that T-bet upregulation in Th17.0 cells in sarcoidosis patients may be initiated by exposure to IL-12 and IFN-γ in lymph nodes or tissues containing granulomatous inflammation (such as the lung). This Th17 plasticity allows them to express both RORγt and T-bet transcription factors and as well as pathogenic cytokines (IFN-γ) and the complement of chemokine receptors including CXCR3 that permit homing from blood to sites of inflammation such as the lung (30). In our study, we hypothesized that the expression of T-bet in circulating Th17.0 cells prior to upregulation of CXCR3 may serve as an indirect measure of the extent of interferon-driven inflammation to which the Th17.0 cells are exposed. To test this, we used flow cytometry to compare the T-bet-expressing frequencies in peripheral blood Th17.0 ($ROR\gamma t^+CCR6^+CCR4^+CXCR3^-$) cells between sarcoidosis subjects with different clinical trajectories defined by longitudinal changes in lung function and immunosuppression use.

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CI, confidence interval; CS&T, cytometer setup and tracking; DLCO, diffusing capacity of the lungs for carbon monoxide; DMARD, disease modifying antirheumatic drug; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FMO, fluorescence minus one; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; FEV1/FVC, forced expiratory volume in 1 S to Forced vital capacity ratio; FSC, forward scatter; IFN-γ, interferon-gamma; IFN factor, interferon factor; MFI, median fluorescence intensity; NonTreg, Non-T regulatory; PBMC, peripheral blood mononuclear cell; %pred, percent predicted; RPMI, roswell park memorial institute; SD, standard deviation; SSC, side scatter; Th, T helper; TLC, total lung capacity; TNF-α, tumor necrosis factor alpha.

MATERIALS AND METHODS

Clinical Cohort

We enrolled subjects who met diagnostic criteria for sarcoidosis per guidelines endorsed by the American Thoracic Society (31) as previously described (32). The study design for this cohort did not require individuals to be newly diagnosed to participate. Follow-up visits were performed every 6–12 months for up to 66 months (∼5 years). At each visit, we performed blood sampling and collected clinical data. These data included: demographics (age, sex, and self-identified race), chest radiography at initial visit, organ involvement at the initial visit (as assessed by physician review of medical records) (32); and pulmonary function tests (PFTs), which included forced expiratory volume in 1 second (FEV1) percent predicted (%pred), forced vital capacity (FVC %pred), diffusing capacity for carbon monoxide (DLCO %pred), and total lung capacity (TLC %pred). We obtained immunosuppression use history, including dosages of oral corticosteroids or disease-modifying antirheumatic drugs (DMARDs), specifically, methotrexate, azathioprine, mycophenolate, colchicine, hydroxychloroquine, or anti-TNF-α therapy that subjects were actively taking at the time of their study visits. For the current analysis, we also included serum protein (chemokine levels for CXCL9, CXCL10, and CXCL11) and whole blood RNA transcript levels of IFN-γ-related genes that were measured in the same blood samples as previously described (33–35).

Selection of Study Subjects

We used a case-control study design with criteria based on (1) lung function changes and (2) immunosuppressive treatment history to compare cell populations between sarcoidosis subjects with different clinical trajectories. Cases were defined by having a change in absolute FVC or DLCO of 10% or 15% (33, 36, 37), respectively, between any two visits separated by 6 months or greater. Cases could have been on or off treatment at the time of the blood draw (**Figure S1**). For all the cases, we identified the two visits between which the PFT change occurred. We then analyzed blood samples from the first of these two visits (before their lung function change occurred). In a subset of cases, we also analyzed blood samples from the second of these two visits (at the visit in which the change in lung function was measured). This subset of subjects with two measurements included subjects not on immunosuppression with a PFT decline (to avoid the effect of immunosuppression on T cell function) and all subjects with a

PFT increase, some of whom were on immunosuppression (since the sample size was small for this group). We used a change in lung function as a surrogate of on-going granulomatous inflammation or "disease activity" for two main reasons: (1) there is a lack of reliable and accurate non-invasive tests for active granulomatous inflammation and (2) we did not have longitudinal radiographic imaging at each follow up visit, which also can be used to infer disease activity. For controls, we used criteria of (1) no change in FVC and DLCO measurements for at least 24 months following the enrollment blood draw used in this analysis and (2) no use of immunosuppression prior to or during the study period. Of note, we did not include FEV1 in our criteria for PFT changes due to the fact that other processes such airways disease independent of sarcoidosis could affect this value over time; plethysmography was not performed at every visit, therefore TLC was also not used as a criterion.

Flow Cytometry

We employed a 12-parameter flow cytometry panel using an LSRII cytometer (BD Biosciences, San Jose, CA) in the UCSF Flow Cytometry Core (www.flow.ucsf.edu). Samples were blinded and randomized to 8 different batches with cases and controls distributed to each batch. Cases with longitudinal samples were analyzed in the same batch. At time of sample collection, we isolated peripheral blood mononuclear cells (PBMCs) using Leucosep^{TM} tubes then froze them in FBS with 10% DMSO for storage in our liquid nitrogen biorepository. For each batch, samples were thawed in RPMI media, counted, and stained on ice in the dark. Details for antibody staining reagents are listed in **Table S1**. We stained with a Fixed Viability Dye (eBioscience) at 1:500 dilution in PBS per manufacturer's instructions. For surface staining, we incubated cells in flow cytometry buffer (PBS with 2% BSA and 2 mM EDTA) along with 50% Brilliant Stain Buffer (BD Horizon) per manufacturer's instructions. Surface antibodies included CD3 APC-R700, CD4 BUV395, CD25 BV786, CCR6 BV421, CCR4 PE-CF594 (BD Horizon); CD127 BV650, CD45RA APC-Cy7 (BioLegend); and CD45RO PerCP-eFluor710, CXCR3 PE-Cy7 (eBioscience). For intracellular staining, we used T-bet PE and RORγt Alexa Fluor 488 (BD Pharmingen) antibodies along with the Transcription Factor Buffer Set (BD Pharmingen) for both fixation and permeabilization. To set gating parameters for each fluorophore, we used fluorescence minus one (FMO) controls (38–41). We analyzed the same internal reference standard of PBMC for all batches to assess for staining variation with each batch acquisition. To standardize voltages across batches, we used Cytometer Setup and Tracking (CS&T) beads (BD Pharmingen) to measure median fluorescence intensities (MFI) and then matched voltages to these MFI values for each batch (42, 43). We used Ultracomp^{TM} beads (Invitrogen) as compensation controls with each batch. We collected at least 2×10^5 events for each sample.

Gating Strategy

We used FlowJo^{TM} software v10.0.07 (Becton, Dickinson and Company: Ashland, OR) to analyze the flow cytometry data. Compensation was performed using Ultracomp^{TM} beads. We employed our previous gating strategy to identify Th populations (11). Gating steps were as follows: (**1**) singlets using forward scatter (FSC—height vs. area), (**2**) live cells (negative for the fixed viability dye), (**3**) lymphocytes based on FSC and side scatter (SSC), (**4**) $CD3^+$ cells, (**5**) $CD4^+$ cells, (**6**) Non-T regulatory (NonTreg) cells defined as $CD25^-$ and either $CD127^{Lo}$ or $CD127^{Hi}$, (**7**) T-effectors ($CD45RA^-$ and $CD45RO^+$), and (**8**) the Th17.0, Th17.1, and Th1 subsets were gated based on staining for CCR6, CCR4, and CXCR3 (**Table 1**). We first determined the RORγt and T-bet distributions among the three Th subsets. Because RORγt is the transcription factor that defines the Th17 lineage (13–17), our primary analyses were based on Th17.0 cells that expressed RORγt and we present data for the proportion of these cells that stained positive for T-bet based on FMO control samples.

TABLE 1 | Definitions of Th subsets based on surface chemokine receptor expression.

	CCR6	CXCR3	CCR4
Th17.0	+	−	+
Th17.1	+	+	−
Th1	−	+	−

Data Analysis

Data were analyzed in Stata/SE 15.1 software (StataCorp LLC: College Station, TX) and figures were constructed in GraphPad Prism 6 software (GraphPad Software, Inc.: La Jolla, CA). To compare demographics data, we used *t*-tests for two group comparisons of means from parametric data, analysis of variance analysis (ANOVA) for comparisons of means between three or more groups, and chi-squared testing to compare proportions between groups. We created several linear regression models where the T-bet^+ frequency (%) of RORγt^+Th17.0 cells was the dependent variable. In these models, we examined case status as a binary predictor with or without adjustment for confounders. In separate models, we compared those with PFT declines and PFT increases along with controls as distinct groups using a categorical predictor with or without adjustment for confounders. Where indicated, we adjusted our regression models for several confounders including age, sex, race, binary designations for immunosuppression use (yes/no), and prior smoking history (yes/no). In a subset of cases with PFT declines (these cases were not on immunosuppression) and PFT increases (these cases were either on or off immunosuppression), we used mixed effects linear regression models to determine if the T-bet^+ frequency of RORγt^+Th17.0 cells was associated with the direction of PFT change. The fixed effects were the PFT change designations as well as the clinical covariates (age, sex, race, immunosuppression use, and smoking history), and the random effects were the subjects. We specified unstructured covariation matrices in these mixed effects models (44, 45).

We used linear regression models to determine how the T-bet^+ frequency of RORγt^+Th17.0 cells varied based on the number of total organs involved, where thoracic adenopathy

and/or lung parenchymal involvement was considered as one organ. These regression models had either binary (one or greater than one organ) or categorical (each organ number as its own group) designations for total organ number. For the categorical model, five or more organs was considered one group to ensure sufficient numbers of subjects in each category and we also performed a *post-hoc* linear trend test.

We used Pearson correlation analyses to determine the association between T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells and previously measured blood markers related to IFN-γ, including serum levels of the IFN-γ-induced chemokines of CXCL9, CXCL10, and CXCL11 (34, 35) as well whole blood RNA transcript levels for IFN-γ-related genes (33). We also used linear regression models to adjust for potential confounders. We calculated correlation r values for these models by taking the square-root of the adjusted R^2 from the linear regression models. For all regression models, we used robust standard errors (46, 47). We considered an $\alpha < 0.05$ as significant and report two significant digits for all analyses.

RESULTS

Characteristics of Sarcoidosis Subjects

We identified 33 subjects who met the PFT change threshold case definition (22 with declines and 11 with increases) and 10 subjects who met the case definition for control subjects; the clinical characteristics of these subjects are shown in **Table 2** and details regarding their immunosuppression use are shown in **Table S2**. For the cases, the average time between visits where a PFT change occurred was 19 months ($SD = 11$). Cases who had PFT increases had lower values of DLCO and FVC at first measurement than the other groups (cases with declines or controls).

TABLE 2 | Sarcoidosis characteristics at enrollment/time of blood draw in cases and controls.

	Cases		Controls	*p*-value*
	PFT Decrease	PFT Increase		
	N = 22	*N* = 11	*N* = 10	
Age (mean years, SD)	56 (12)	50 (13)	46 (9)	0.082
Female (%)	16 (73)	4 (36)	3 (30)	0.089
Race (%)				0.37
African American	1 (5)	2 (18)	0 (0)	
White	19 (86)	7 (64)	8 (80)	
Hispanic	2 (9)	0 (0)	0 (0)	
Other	0 (0)	2 (18)	2 (20)	
Ever Smokers	11 (50)	6 (55)	4 (40)	0.64
Immunosuppression Use (%)	9 (41)	6 (55)	0 (0)	N/A
Extra-thoracic Organ Involvement (%)	75	78	10	0.00056
Initial Visit PFTs: Mean (SD)				
FVC %predicted	97 (16)	80 (18)	99 (13)	0.012†
FEV1 %predicted	89 (18)	75 (24)	89 (17)	0.11
FEV1/FVC	0.72 (0.085)	0.72 (0.14)	0.71 (0.081)	0.95
DLCO %predicted	74 (16)	59 (13)	79 (12)	0.011
TLC %predicted	99 (11)	83 (17)	93 (13)	0.027

**p-values are for comparisons between cases and controls.*
†*p-values for PFTs compare the controls and the cases as two separate groups.*
N/A, not applicable.

Transcription Factor Expression in Th Cells

Figure 1 shows the gating strategy to identify the Th populations of interest (Th17.0, Th17.1, and Th1) among T-effectors cells based on surface expression of chemokine receptors. Because the polarization of each of these Th cell populations is influenced by the RORγt and T-bet transcription factors (16, 17, 26–28, 48, 49), we also measured the expression of these transcription factors as part of our staining panel (**Figures 2A–C**). We used FMO staining for RORγt and T-bet to set gates for positive and negative expressing cells as shown in **Figures 2D,E** (for FMO controls for each of our staining reagents, please refer to **Figure S2**). **Figures 2A–C,F** shows that using chemokine receptors to identify these Th cell subsets is sensitive but not as specific as also including transcription factors staining. As shown, the majority of Th17.0 cells express RORγt, most of the Th1 cells express T-bet, and the majority of Th17.1 cells express both RORγt and T-bet; further data to illustrate the concordance between these chemokine receptor patterns and transcription factor expression are shown in **Figure S3**. Although Tregs were not the focus of this study, we found that CD25^{Hi}CD127^{Lo}CD4$^+$ T cells were present in the blood and represented 6.7% (95% CI 5.4–8.0) of total CD4$^+$ T cells. Because the Th cell population of interest in this study was the Th17.0 subset, to increase the specificity of this population, we defined these cells by including expression of RORγt as shown by the solid blue box in **Figure 2A** and refer to them as RORγt$^+$Th17.0 cells. We found that some of these RORγt$^+$Th17.0 cells also expressed T-bet as shown in **Figure 2A** by the dashed black box. **Figure 2G** shows the range of T-bet expression in RORγt$^+$Th17.0 cells across subjects. This T-bet$^+$ frequency of RORγt$^+$Th17.0 cells was the focus of our subsequent analyses.

Relationship Between the T-bet$^+$ Frequencies of RORγt$^+$Th17.0 Cells and Clinical Outcomes

We determined how the %T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells varied between cases and controls. Cases had higher T-bet$^+$ frequencies compared to controls (27 vs. 16%, $p = 0.0040$) in unadjusted analysis; this difference was also statistically significant in a model adjusted for age, sex, race, immunosuppression use, and prior smoking status as shown in **Figure 3A**. We also found that as separate groups, those with PFT declines and those with PFT increases had higher T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells compared to controls in both unadjusted and adjusted models (**Figure 3A**). Of note, we did not find any association between the PFT groups and any of the T helper populations as defined by chemokine receptors and also using RORγt and/or T-bet to delineate these populations (Th17.0, Th17.1, Th1, or Th2) (see **Table S3**). We used a mixed effects model adjusted for age, race, sex, immunosuppression use,

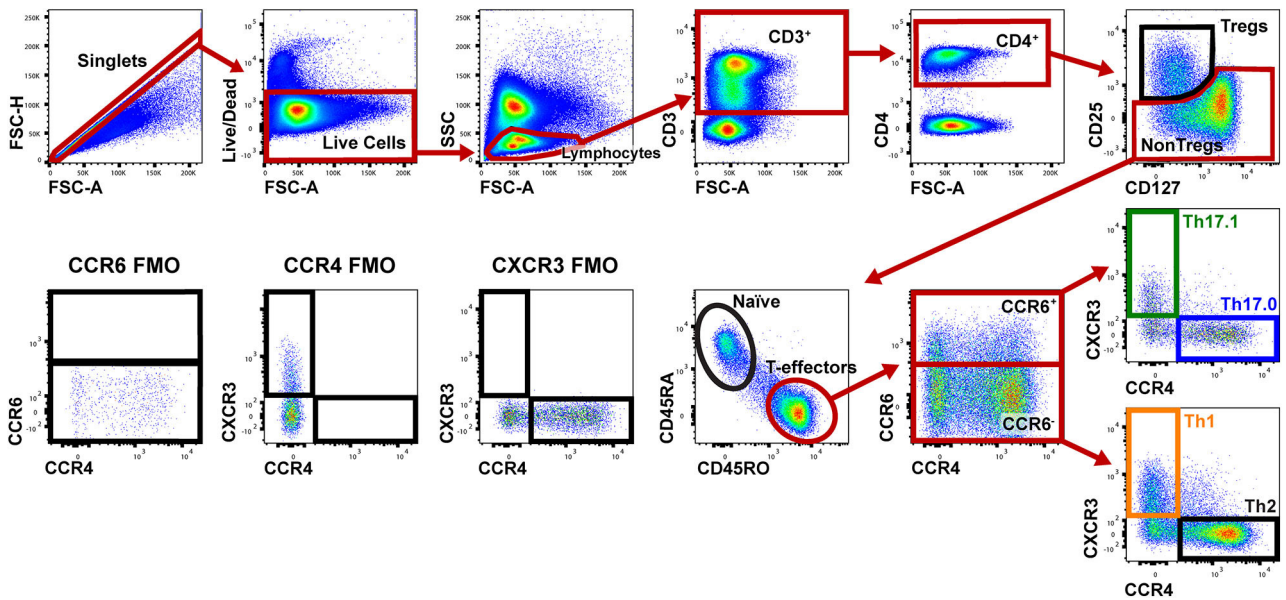

FIGURE 1 | Gating strategy to identify Th populations among T-effector cells. Shown is a representative sarcoidosis subject sample. We gated on singlet cells using FSC-H and FSC-A, then live cells (negative for the fixed viability dye), lymphocytes based on FSC and SSC, then $CD3^+$ and $CD4^+$ cells. We then gated on NonTregs that were $CD25^-$ and either $CD127^{Lo}$ or $CD127^{Hi}$ and then T-effectors ($CD45RA^-$ and $CD45RO^+$). Among these T-effectors, we enriched for Th subsets using CCR6, CCR4, and CXCR3. Th17.0 cells were $CCR6^+CCR4^+CXCR3^-$, Th17.1 cells were $CCR4^-CCR6^+CXCR3^+$ and Th1 cells were $CCR6^-CCR4^-CXCR3^+$. The gating strategy used for fluorescence minus one (FMO) controls for CCR6, CCR4, and CXCR3 are shown in the lower left corner.

and prior smoking, to examine the T-bet$^+$ frequencies of Th17.0 cells in relation to the direction of PFT change. We found that being in the PFT decline group was associated with an increase in T-bet$^+$ frequency (average change = 8.6%, 95% CI 1.5–16, p = 0.017) (**Figure 3B**). Conversely, being in the PFT increase group was associated with a decrease in T-bet$^+$ frequency (average change = −6.0%, 95% CI −14 to 2.4, p = 0.16) (**Figure 3B**). This model included an interaction term between the PFT change group (decline or increase) and the visit variable to assess if the change in T-bet$^+$ frequency between the two visits differed based on the direction of PFT change. We found that there was a statistically significant interaction between the direction of PFT change and the visit variable. The magnitude of this difference was 15 percentage points (95% CI 3.7–26, p = 0.0089).

Association Between T-bet$^+$ Frequencies of RORγt$^+$Th17.0 Cells and Organ Involvement

We considered the total number of organs involved with sarcoidosis at enrollment into the study as a separate manifestation of granulomatous disease burden. To test if T-bet expression in RORγt$^+$Th17.0 cells varied based on the number of organs involved, we used linear regression models and adjusted for age, sex, race, and immunosuppression use. T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells were 11 percentage points higher on average in those with more than one organ involved relative to those with a single organ involved (**Table 3**). We also constructed a model wherein the number of organs involved was a categorical predictor (from 1 organ to >5 organs) and found that the T-bet$^+$ frequencies increased as the number of organs involved increased, especially with ≥ 5 organs involved (trend test, $p < 0.001$) (**Table 3** and **Figure 4**).

Correlations Between the T-bet$^+$ Frequencies of RORγt$^+$Th17.0 Cells and Other IFN-γ-Related Blood Markers

To assess if T-bet expression in RORγt$^+$Th17.0 cells was associated with other markers of IFN-γ-related inflammation, we determined the correlations between these T-bet$^+$ frequencies and serum levels of IFN-γ-induced chemokines and whole blood gene transcript levels of IFN-γ-related genes, which we had previously measured in the same blood samples (33–35). We found that the T-bet$^+$ frequencies positively correlated with all three serum chemokines (CXCL9, CXCL10, and CXCL11) in models adjusted for age, race, sex, and immunosuppression use (**Table 4**). We previously found that a three gene mean of *GBP1*, *STAT1*, and *STAT2* was higher in a larger number of subjects from this cohort who had either PFT declines or flares requiring immunosuppression use (33). This three gene mean (the "IFN Factor") as well as genes identified to be related to IFN-γ using Ingenuity Pathway Analysis (*TAP1* and *JAK2*) were positively correlated with the T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells (**Table 4**).

DISCUSSION

Sarcoidosis is a systemic disease involving granulomatous inflammation with upregulation of immune pathways related

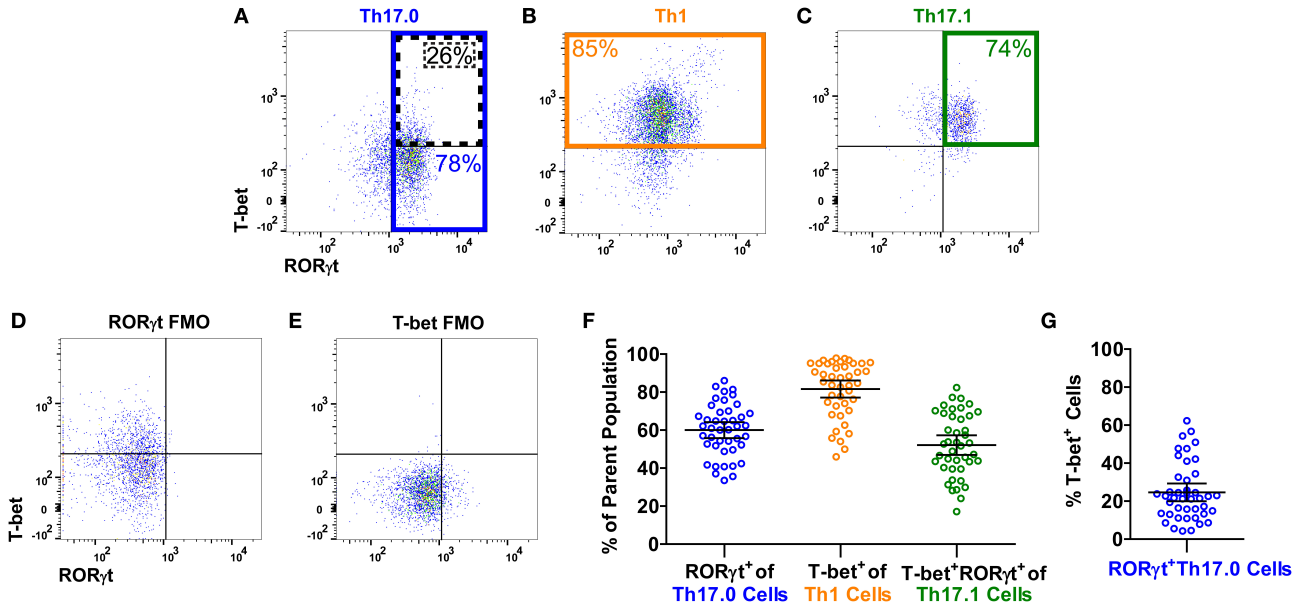

FIGURE 2 | T-bet and RORγt expression in the Th17.0, Th1, and Th17.1 cell populations. For each Th population (as defined in **Figure 1**), we determined the expression of T-bet and RORγt, as shown in a representative sarcoidosis sample. Expression of T-bet and RORγt in **(A)** Th17.0, **(B)** Th1, and **(C)** Th17.1 cells along with fluorescence minus one controls (FMOs) for **(D)** RORγt and **(E)** T-bet are displayed as dot plots. The majority of cells in each Th population had the expected expression pattern of T-bet and RORγt based on its chemokine receptor pattern: **(A)** the majority of Th17.0 cells expressed RORγt (outlined in blue); **(B)** the majority of Th1 cells expressed T-bet (outlined in yellow); and **(C)** the majority of Th17.1 cells expressed both RORγt and T-bet (outlined in green). These frequencies of RORγt$^+$ and/or T-bet$^+$ cells in each of these Th cell populations for this representative subject sample are shown on each plot with corresponding colors. These frequencies are displayed graphically in **(F)** across all subjects where each open circle represents a single subject along with the mean and 95% confidence interval (CI). Our primary population of interest in this study was the Th17.0 subset, so to achieve the highest specificity for the "Th17.0" phenotype, we focused on RORγt$^+$Th17.0 cells as outlined in blue in **(A)**. We found that some of these RORγt$^+$Th17.0 cells also expressed T-bet, as outlined by the dotted black box in **(A)**. There was a range of T-bet$^+$ cells within this RORγt$^+$Th17.0 population across subjects, as shown graphically in **(G)**, where each open circle represents a subject along with the mean and 95% CI. Our subsequent analyses focused on how this T-bet$^+$ frequency among RORγt$^+$Th17.0 cells related to clinical sarcoidosis outcomes.

to IFN-γ (5, 6, 50, 51). In light of recent findings that IFN-γ-producing Th17.1 cells are elevated in the lungs and lymph nodes of sarcoidosis patients with chronic disease (52), the ontogeny and function of these cells may be important in the pathogenesis of sarcoidosis (11, 52). This study was motivated by (1) clinical observations that Th17.0 cells are elevated in blood while Th17.1 cells are elevated in BAL fluid and mediastinal lymph nodes of sarcoidosis patients (11, 52), and (2) scientific evidence from mice and humans demonstrating the plasticity of Th17.0 cells to become Th17.1 cells after exposure to IL-12 and IFN-γ and upregulation of T-bet (21–24). Since granulomas are a source of IL-12 and IFN-γ (53, 54), we hypothesized that T-bet expression in peripheral Th17.0 cells would reflect the extent of granulomatous inflammation in sarcoidosis patients. Therefore, we used transcription factor staining for T-bet and RORγt along with chemokine receptor staining to identify Th17 cell populations, specifically T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells. We found that these T-bet$^+$ frequencies were higher in sarcoidosis subjects with clinical evidence of greater disease burden as manifested by clinically meaningful PFTs changes and organ involvement.

Given the limitations in directly quantifying the degree of inflammation present in our human subjects, our study design utilized clinical features of disease severity as indicators of the extent of granulomatous inflammation. Specifically, we used a case-control study design to compare sarcoidosis subjects with different clinical courses. We assumed that those with any type of PFT change (our cases) had greater amounts of granulomatous inflammation during the study period as compared to those who had stable PFTs and did not require immunosuppression (our controls). The greater T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells in our cases supports our hypothesis that this T-bet$^+$ frequency measure reflects the extent of disease burden. In subgroup analyses of our cases, we found that the T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells were increased following a PFT decline. Conversely, T-bet$^+$ frequencies were decreased following a PFT improvement. We interpret these findings as evidence that these T-bet$^+$ frequencies changed based on disease trajectory and therefore varied based on the extent of granulomatous inflammation over time. We also used the number of organs involved as another indicator of disease burden. Thus, we interpreted the positive association between these T-bet$^+$ frequencies and organ involvement as further evidence that these T-bet$^+$ frequencies were associated with the extent of granulomatous inflammation. As additional support for our findings, we found positive correlations between these T-bet$^+$ frequencies and other measures of IFN-γ-related inflammation, including

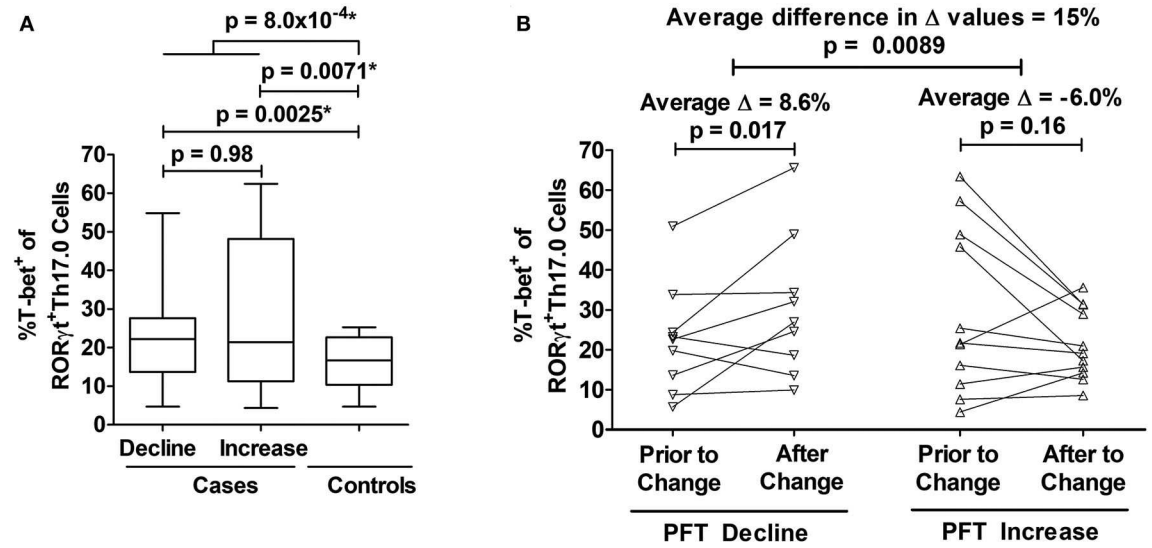

FIGURE 3 | Associations between T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells and pulmonary function changes. **(A)** Cases had higher %T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells compared to controls in either adjusted or unadjusted models (*p-values from model adjusted for age, sex, race, immunosuppression use, and prior smoking). Cases were defined by declines ($n = 22$) or increases ($n = 11$) in either forced vital capacity (FVC) or diffusing capacity (DLCO) of 10 or 15%, respectively during follow up regardless of immunosuppression. Controls ($n = 10$) lacked these same pulmonary function test (PFT) changes and never required immunosuppressive treatment. The upper most p-value represents the result from a regression model that compared all cases to controls. The middle two p-values represent results from a regression model that distinguished cases as separate groups based on either PFT declines or increases and compared these groups to controls; the lower most p-value represents the results from this same regression model where cases with PFT declines were compared to cases with PFT increases. Data are displayed as box-and-whisker plots with median and interquartile ranges. **(B)** As assessed by mixed effects modeling adjusted for age, sex, race, immunosuppression use, and prior smoking, cases had either an increase ($n = 9$) (left panel) or decrease ($n = 11$) (right panel) in T-bet$^+$ frequencies at the visit at which their PFT change occurred. The difference in the magnitude of these changes between those with PFT declines and PFT increases was 15%. In **(B)**, each subject's T-bet$^+$ frequency is represented by an open symbol and are plotted based on when they were sampled relative to the PFT change.

TABLE 3 | Linear regression using two different variables to delineate the number of involved organs†.

Outcome	Predictor	β-Coefficient	95% CI	p-value
%T-bet$^+$ of RORγt$^+$Th17.0 cells	Model (1) Binary: >1 Organ (1 Organ = Ref)	11%	(0.36, 22)	**0.043**
	Model (2) Categorical:		Trend Test:	**1.2×10^{-38}**‡
	1 Organ	(Ref)	(Ref)	(Ref)
	2 Organs	11%	(−2.9, 24)	0.12
	3 Organs	3.5%	(−7, 14)	0.51
	4 Organs	9.4%	(−3.4, 22)	0.15
	≥5 Organs	25%	(16, 34)	**9.8×10^{-8}**

†*Adjusted for age, sex, race, and immunosuppression use.*
‡*p-value for the test of linear trend for the categorical organ variable.*
Ref, Reference.

serum interferon-induced chemokines and whole blood gene transcript levels.

The polarization of Th17.0 cells to Th17.1 cells has been studied both in mice and several human diseases including rheumatoid arthritis, multiple sclerosis, and Crohn's disease (12, 17, 55–62). Mechanisms currently put forth for how Th17.0 can become Th17.1 involve upregulation of T-bet. It has long been established that T-bet expression in naïve T cells leads to the acquisition of a Th1 phenotype after antigenic stimulation in the presence of cytokines such as IL-12 and resulting upregulation of STAT1 (25–29). This process includes T-bet's role in the upregulation of CXCR3 expression, which is essential for trafficking of Th1 cells to sites of inflammation (26, 27, 48). During and post-polarization, T-bet also upregulates IFN-γ through binding to both the promoter and enhancer loci for *IFNG* (29). Mouse models have shown that Th17.0 cells incubated with IFN-γ and IL-12, or TNF-α gain features of Th17.1 cells including CXCR3 expression and the capacity to produce IFN-γ (22–24). This phenomenon of plasticity from IL-17-producing cells to IFN-γ-producing cells has also been observed in mouse models of innate lymphoid cells, where RORγt$^+$ ILC3 cells transition to ILC1 cells through upregulation of T-bet in the presence of specific cytokines such as IL-12 (63–66). A human study of inflammatory bowel disease found that *ex vivo* IL-12 stimulation led to IFN-γ production in Th17 cells that were isolated as CCR6$^+$CXCR3$^-$ cells from mesenteric lymph nodes (17). This observation suggests that upregulation of CXCR3 may occur later in the cellular differentiation of Th17.0 to Th17.1 cell phenotype. Other groups including Cohen et al. (21) showed that polarized Th17.0 cells derived from *ex vivo* human PBMCs could subsequently upregulate the T-bet gene *TBX21* after incubation with IL-12 and IFN-γ. Therefore, T-bet expression in RORγt$^+$Th17.0 cells (CCR6$^+$CXCR3$^-$CCR4$^+$)

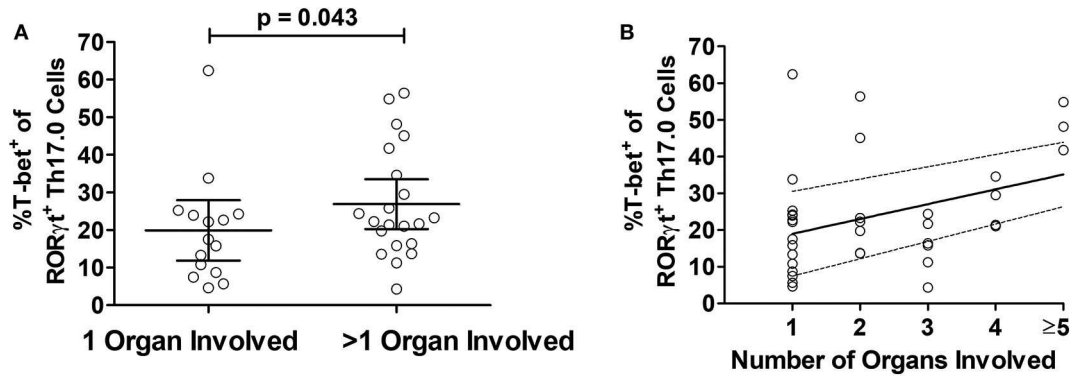

FIGURE 4 | Relationship between T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells and organ involvement. **(A)** The frequencies of %T-bet$^+$ of RORγt$^+$Th17.0 cells were higher in those with greater than one organ involved as compared to only one organ (*p*-value adjusted for age, sex, race, and immunosuppression use). **(B)** The T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells where higher in those with greater number of organs involved. In a linear regression model with total organ involvement as a categorical predicator adjusted for age, sex, race, and immunosuppression use, there was a positive trend toward increasing T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells with greater organ involvement (see **Table 3**). The solid line shows the organ number adjusted for age, sex, race, and immunosuppression use and the dashed lines represent the 95% confidence interval.

TABLE 4 | Results from correlation analyses and regression models for T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells and IFN-γ-related blood markers.

Outcome	Main Predictor	Adj β-coeff[†]	95% CI	Adj *p*-value	Adj r value	Unadj r value[‡]
%T-bet$^{\pm}$ of	Log_{10}[CXCL9][§]	22	(14, 31)	**1.5×10^{-5}**	0.66	0.65
RORγt$^{\pm}$Th17.0 cells	Log_{10}[CXCL10]	23	(3.6, 42)	**0.022**	0.47	0.41
	Log_{10}[CXCL11]	30	(11, 49)	**0.0028**	0.51	0.50
	IFN Factor[††]	6.9	(0.81, 13)	**0.028**	0.37	0.58
	JAK2[‡‡]	4.2	(2.5, 10)	**0.034**	0.29	0.42
	TAP1[‡‡]	4.8	(0.25, 9.3)	**0.041**	0.26	0.42

[†] *β-coefficient is adjusted for age, race, sex, and immunosuppression use.*
[‡] *Unadjusted Pearson r coefficient.*
[§] *Serum chemokine values were log_{10}-transformed.*
[††] *The "IFN Factor" = a three gene mean of GBP1, STAT1, and STAT2 previously measured from whole blood.*
[‡‡] *Whole blood gene expression values in the form of log_2[relative expression].*
Adj, Adjusted; CI, Confidence Interval; Coeff, Coefficient; Unadj, Unadjusted.
Bold values indicates p-values for adjusted β-coeffecients.

could potentially represent a transitional state between the Th17 and Th17.1 immune subsets. Our focus on Th17.0 cells (and not Th17.1 cells that also express CXCR3), was motivated by our goal to identify cells prior to upregulation of CXCR3, since expression of this chemokine receptor could lead to trafficking of cells out of the blood.

Where the transition between Th17.0 and Th17.1 occurs *in vivo* has been an on-going question in studies of T cell biology. In the setting of other granulomatous processes such as pulmonary tuberculosis infection, mouse models have shown that dendritic cells present antigen to naïve T cells in mediastinal lymph nodes (67–69). Therefore, the initial polarization of Th1 cells in this disease model does not occur in the alveoli, but instead these polarized cells must traffic to the initial site of infection after their activation in local lymph nodes. For Th17.0 cells, mouse models studying gastrointestinal T cells have shown that Th17.0 cells initially become polarized in the mesenteric lymph nodes then traffic through the blood to the intestine in models of both healthy and inflammatory states (70, 71). The location of Th17.1 polarization potentially includes both lymph nodes and inflamed tissue. In a mouse model of experimental autoimmune encephalitis, single-cell RNA sequencing analysis showed that Th17 cells isolated from lymph nodes and affected central nervous system tissues have several phenotypes with respect to cytokine production (62). These phenotypes ranged from self-renewing IL-17-producing cells and IFN-γ-producing cells in the lymph nodes to IFN-γ-producing cells in inflamed tissues. In a mouse model of colitis, Harbor et al. (59) showed that naïve T cells polarized to Th17.0 cells *ex vivo* and then injected into the peritoneum were later retrieved and found to be competent to produce IFN-γ. These cells were recovered from both mesenteric lymph nodes and inflamed intestinal tissue, suggesting that Th17.0 cells could be subsequently polarized to Th17.1 cells in either compartment. Taken together, these findings suggest that the transition from Th17.0 to Th17.1 cell may occur in both lymph nodes and inflamed tissues.

These mouse and human studies of Th17 plasticity provide a framework for how the transition from Th17.0 to Th17.1 cells might be occurring in sarcoidosis. Although it is unclear *where* the transition is occurring, we speculate that Th17.0 cells exposed to granuloma-related cytokines (i.e., IFN-γ, IL-12 and TNF-α) in the lymph nodes of affected organs, especially mediastinal lymph nodes, could lead to T-bet upregulation. We theorize that we are detecting this early T-bet$^+$ transition state of Th17.0 cells in the blood as these cells exit the lymph nodes and enter the peripheral circulation. Eventually, increased T-bet expression in these Th17.0 cells results in upregulation of CXCR3, which is a key surface marker for the Th17.1 phenotype. Since CXCR3 is a homing receptor, its expression in Th17.1 cells leads to their accumulation in affected tissues. This conceptual framework is supported by several prior and current observations. Previously, we observed elevated frequencies of Th17.0 cells in the blood associated with elevated frequencies of Th17.1 cells in BAL (11). In the current study we observed a positive correlation between T-bet$^+$ frequency in RORγt$^+$Th17.0 cells and several IFN-γ-related blood markers including serum IFN-induced chemokines and gene transcript levels. These observations along with the associations of T-bet$^+$ frequency in RORγt$^+$Th17.0 cells with clinical outcomes lead us to infer that there is a systemic up-regulation of IFN-γ-related pathways and the degree to which these pathways are upregulated may be related to the burden of granulomatous inflammation in the body. Since these responses can be measured in the blood, they could be used to prognosticate patients as well as further our understanding of the underlying immunopathology.

Our findings share commonalities and differences with a prior publication of RORγt and T-bet expression in sarcoidosis subjects. Kaiser *et al.*, showed strong associations between dual expression of CCR6 and CXCR3 in RORγt$^+$T-bet$^+$ T cells (10); we found similar strong associations between CCR6$^+$ and CXCR3$^+$ co-expressing cells we defined as Th17.1 cells that also had dual expression of RORγt and T-bet (see **Figure 2C** and **Figure S3**). However, our study differed in that we did not have BAL specimens and only focused on blood cells. The Kaiser study found that co-expression of RORγt and T-bet in CD4$^+$ cells was higher in the BAL of patients with Löfgren syndrome as compared to non-Löfgren sarcoidosis, suggesting that co-expression predicted a more favorable phenotype. Our study differed in that we did not have patients with Löfgren syndrome, and we used longitudinal PFT changes, immunosuppression use, and total number of organs involved to phenotype subjects. The other difference was that our main focus was on T-bet-expressing RORγt$^+$Th17.0 cells that did not express CXCR3 in order to identify potential transition cells in the blood, which was not addressed in the Kaiser study.

Some of the limitations of our study included our inability to directly assess the potential pathogenicity and IFN-γ-producing capabilities of the RORγt$^+$Th17.0 cells that also expressed T-bet. Staining for transcription factors involves fixation and permeabilization of the cells, which prevents analyses involving stimulation and cytokine measurements after sorting these cells. Nonetheless, the association of the T-bet$^+$ frequencies with clinically meaningful outcomes of sarcoidosis indicates that this cell population may be important to examine in future studies that further define the Th17.0 to Th17.1 transition. Our study was also limited by the lack of serially measured organ involvement and chest radiography, which precluded us from including these clinical findings in our definitions of sarcoidosis outcomes. Another important limitation relates to generalizability since our cohort was heterogeneous, and therefore our study design did not allow us to extrapolate the prognostic value of T-bet$^+$ frequencies of RORγt$^+$Th17.0 cells at initial diagnosis. Similarly, this study also was not designed to address the question of whether T-bet expression in RORγt$^+$Th17.0 cells can predict the likelihood of spontaneous remission. Some of these limitations can be addressed in future studies that enroll subjects at the time of diagnosis. In terms of our study design, we created case and control definitions to maximize the likelihood of identifying those with active and inactive granulomatous inflammation. The limitation of doing this is that we used lack of immunosuppression use to help identify those who were more likely to have inactive disease, therefore use of immunosuppression was not matched between cases and controls. We dealt with this by including immunosuppression as a covariate in all our regression models to control for possible bias in our point estimates. Moreover, we would assume that immunosuppression use would decrease T-bet expression in controls even further, therefore the results we report here are likely more conservative than if we matched based on immunosuppression use.

CONCLUSIONS AND FUTURE DIRECTIONS

In summary, we provide evidence showing that T-bet expression in RORγt$^+$Th17.0 cells was associated with both pulmonary and systemic organ involvement outcomes. These associations were potentially due to the effects of IFN-γ and IL-12 on Th17.0 cells as they circulate through affected lymph nodes where higher levels of these cytokines might be found. Future goals include determining how these frequencies in the blood relate to those in the lung, how they are associated with outcomes when measured at time of diagnosis, and how they change in response to treatment. With this information, we may be able to leverage biological data taken at the time of sarcoidosis diagnosis to inform patient prognosis and guide clinical decision making.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of California, San Francisco

Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NA and SM conducted the experiments. NA, IA, PW, and LK analyzed the data. All authors contributed to the writing and editing of the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Institutes of Health (R56IO87652 and T32HL007185).

ACKNOWLEDGMENTS

The authors thank the following individuals for their specific contributions: Michelle Nguyen, Joris Ramstein, Christine Nguyen, Melissa Ho, Sara Sun, Zoe Lehman, and Victoria Wang for assistance with sample acquisition, analysis, and management of the database; and Owen Solberg, Ph.D., for database programming. We would also like to thank all the participants who volunteered their time for this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01129/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 04 August 2020
doi: 10.3389/fimmu.2020.01719

Current Sarcoidosis Models and the Importance of Focusing on the Granuloma

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The inability to effectively model sarcoidosis in the laboratory or in animals continues to hinder the discovery and translation of new, targeted treatments. The granuloma is the signature pathological hallmark of sarcoidosis, yet there are significant knowledge gaps that exist with regard to how granulomas form. Significant progress toward improved therapeutic and prognostic strategies in sarcoidosis hinges on tractable experimental models that recapitulate the process of granuloma formation in sarcoidosis and allow for mechanistic insights into the molecular events involved. Through its inherent representation of the complex genetics underpinning immune cell dysregulation in sarcoidosis, a recently developed *in vitro* human granuloma model holds promise in providing detailed mechanistic insight into sarcoidosis–specific disease regulating pathways at play during early stages of granuloma formation. The purpose of this review is to critically evaluate current sarcoidosis models and assess their potential to progress the field toward the goal of improved therapies in this disease. We conclude with the potential integrated use of preclinical models to accelerate progress toward identifying and testing new drugs and drug combinations that can be rapidly brought to clinical trials.

Keywords: sarcoidosis, granuloma, modeling, macrophages, lung

OPEN ACCESS

Edited by:
Mary Jane Thomassen, East Carolina University, United States

Reviewed by:
Matyas Sandor, University of Wisconsin-Madison, United States
Juerg Hamacher, Lindenhofspital, Switzerland

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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *08 April 2020*
Accepted: *29 June 2020*
Published: *04 August 2020*

Citation:
Locke LW, Schlesinger LS and Crouser ED (2020) Current Sarcoidosis Models and the Importance of Focusing on the Granuloma. Front. Immunol. 11:1719. doi: 10.3389/fimmu.2020.01719

INTRODUCTION

Sarcoidosis is a complex immune disease with genetic susceptibility and environmental factors playing important roles. The disease occurs in about 15–40 per 100,000 people in the United States (1). In terms of both frequency and severity, the disease disproportionately affects women, people of African American and Northern European descent, and economically disadvantaged populations (1, 2). Severe clinical phenotypes are associated with progressive lung, heart, or brain damage and are debilitating and potentially fatal (2). The failure to effectively model sarcoidosis in the laboratory or in animals has been a roadblock to gaining crucial insights into the triggers and underlying cellular and molecular mechanisms driving the disease pathogenesis. This has manifested not only in terms of hindering the establishment of disease-specific biomarkers for diagnostic and prognostic purposes, but lack of mechanistic insight into disease regulating targets also impairs our capacity to treat this disease. In order to identify disease-specific treatments, the field needs a tractable model that recapitulates the essential elements of the disease pathogenesis. The granuloma is the signature pathological hallmark of sarcoidosis. Importantly, the architecture and immunology of granuloma formation differ significantly from one granulomatous disorder

to the next. These differences, which are dictated by a highly complex and coordinated interplay of many diverse immune cell populations, are evident even during the early stages of granuloma formation, and they are further amplified as the structures evolve. However, a significant knowledge gap exists with regard to how granulomas form due to the fact that local factors, such as cytokines, chemokines, and direct cell-cell interactions among various immune cells, strongly influence the function of each immune cell during granuloma formation. *Significant progress toward improved therapeutic and prognostic strategies in sarcoidosis hinges on tractable experimental models that recapitulate the process of granuloma formation in sarcoidosis and allow for mechanistic insights into the molecular events involved*. The purpose of this review is to critically evaluate current sarcoidosis models and assess their potential to progress the field toward these goals. Selected sarcoidosis models described in the following sections have been summarized in **Table 1**.

THE SARCOIDOSIS GRANULOMA

Studies of diseased patient biopsy tissue are the foundation of sarcoidosis research. The original description of sarcoidosis in 1877 was based on the characteristic appearance of biopsied skin lesions and histological features that discriminated sarcoidosis from tuberculosis (TB) and other infectious granulomatous disorders (3). Despite the legitimacy of conducting research based on human sarcoidosis tissue samples, such research has yielded limited information regarding disease pathogenesis over the past 143 years. The information that can be obtained from sarcoidosis tissues is limited to a snapshot view of the disease in the form of established granulomas, a perspective that provides very limited insights into the critical and dynamic mechanisms underpinning the pathogenic formation of granulomas.

Sarcoidosis granuloma formation is considered to be environmental antigen-mediated (4), and the lungs are the primary interface between environmental antigens and the host's immune surveillance system. This premise has led many researchers to investigate cells derived from bronchoalveolar lavage (BAL) fluid, particularly alveolar macrophages (AMs) and lymphocytes, to gain insight into sarcoidosis pathogenesis. Such studies indicated that sarcoidosis AMs exhibit enhanced antigen processing capacity and promote greater T cell activation (5), and as such, AMs likely play an important role during the initiation of the granulomatous response in the lungs. While it is likely that AMs interface with sarcoidosis-promoting environmental antigens and may be involved in the initial phases of the granulomatous response, they are not the primary source of macrophages involved in granuloma formation and are therefore insufficient for modeling their complexities. Cells in the peripheral blood, on the other hand, are directly involved in the formation of granulomas in the lungs (6) (**Figure 1**), and laboratory models based on peripheral blood mononuclear cells (PBMCs) have successfully replicated many of the histological and molecular features of human granulomatous diseases, including TB (7, 8), leprosy (9), and sarcoidosis (10). Along with tissue resident antigen-presenting cells (APCs) such as dendritic cells or AMs, blood monocytes act as sentinels for the detection of potential threats to the host and exit the vascular space to "patrol" within the interstitial space of the lungs (6). These monocytes will migrate back into the vascular space if no danger is sensed. When activated by a danger signal (e.g., bacterial antigens) (**Figure 1A**), the monocytes establish residency in the lung tissue (6, 11). The subsequent presentation of antigens by monocytes or tissue resident APCs to T cells (**Figure 1B**) leads to a localized immune response that drives the recruitment and activation of circulating monocytes and lymphocytes into the tissue (12, 13) (**Figure 1C**). The early granuloma begins to get assembled by these infiltrated cells along with tissue-resident cells to wall off the pathogen and protect the host (**Figure 1D**). Thus, PBMCs are essential for granuloma formation in the lungs and in other tissues as well.

ANIMAL MODELS OF SARCOIDOSIS

Animal models are commonly relied upon to understand human disease mechanisms, and they offer potential strengths related to therapeutic manipulation and temporal information pertaining to disease progression. Currently there is no universally accepted animal model of human sarcoidosis, largely because animals [other than horses (14)] do not develop sarcoidosis and the link between human gene polymorphisms and disease prevalence has not been established to the degree that it can be recapitulated in the genetic manipulation of animals (e.g., mice) (15). While pulmonary granuloma formation can be achieved in rodents via pre-sensitization to putative antigens followed by pulmonary administration of the same antigens (i.e., a hypersensitivity response), injection and pulmonary embolization of pathogen-coated beads, or bronchial administration of foreign body particles, the granuloma cellular composition, morphology, local inflammatory milieu, and sustainability of these granulomas are poorly reflective of human sarcoidosis. For example, pulmonary granulomas that form in response to repeated exposures to the bacterium *Propionibacterium acnes* are consistent with hypersensitivity pneumonitis in that, unlike sarcoidosis granulomas, they are poorly formed, transient, and surrounded by prominent lymphocytic pneumonitis (16). Other models that instill carbon nanotubes into the airways of mice induce granulomas with features distinct from sarcoidosis, including foamy lipid-laden macrophages and a paucity of surrounding lymphocytes (17). The immunology of foreign body granulomas fundamentally differs from sarcoidosis because foreign bodies do not promenently feature adaptive immune responses (18, 19). Given the limited ability to recapitulate critical aspects of human sarcoidosis granulomas, animal models have not yielded

Abbreviations: AM, alveolar macrophage; BAL, bronchoalveolar lavage; PBMC, peripheral blood mononuclear cells; JAK, Janus kinase; STAT6, signal transducer and activator of transcription 6; V-ATPase, vacuolar H+-ATPase; *M.tb*, *Mycobacterium tuberculosis*; mTORc1, mammalian target of rapamycin complex 1; Tsc2, tuberous sclerosis-2 gene; DE, differentially expressed; TB, tuberculosis; LTBI, individuals with latent TB infection; PPD, purified protein derivative.

TABLE 1 | The strengths and limitations of current sarcoidosis research models.

Research models of sarcoidosis granulomas	Strengths	Limitations
Animal models	• Allow for therapeutic manipulation and preclinical testing of new therapeutics • Temporal information of disease progression • Genetically tractable	• Do not spontaneously form sarcoidosis (except for horses) • Granulomas are poorly reflective of human sarcoidosis • Not been well-validated against human diseased tissues
BAL cell-based models	• Cells interface with sarcoidosis-promoting environmental antigens • Cells are likely involved in the initial phases of the granulomatous response • Can establish disease biomarkers	• Cells are not engaged physically in granuloma formation • Continuous access to patients needed • Unstimulated immune cells do not recapitulate human sarcoidosis • Invasive to the patient
Unstimulated PBMC-based models	• Cells are directly involved in the formation of granulomas in the lungs • Can establish disease biomarkers	• Unstimulated immune cells do not recapitulate human sarcoidosis • Continuous access to patients needed
In vitro human granuloma model	• Accounts for the complex genetics dictating disease • Captures immune cell populations that engage in granuloma formation as well as cross-talk of these cells • Allows for pre-clinical testing of new therapies • Can accommodate the testing of potential disease-promoting pathogens and triggers • Can establish disease biomarkers	• Continuous access to patients needed • Limited ability to model fibrotic changes due to absence of tissue stromal elements • Lack of cell replenishment limits ability to track granulomas temporally (resolution vs. self-propagation)
Diseased tissues	• Gold standard for characterizing human sarcoidosis that is fully established in tissues	• Snapshots of established (i.e., late stage) disease • Not amenable to manipulation, limited insights into the early mechanisms of granuloma-genesis • Does not represent dynamic changes over time during evolution of sarcoidosis granulomas
Computational models	• Quickly and powerfully elucidate the dynamics and complex interplay of cells and mediators over time • Can be continuously improved as data becomes available • Can manipulate drug targets to model effects on granuloma formation and maintenance	• Needs companion cell-based models and human tissue validation to confirm model-derived hypothesis

major breakthroughs in the field of sarcoidosis, albeit with one exception.

A serendipitous link to human sarcoidosis granuloma formation was recently discovered based on a murine *Tsc2* knockout model (20). The *Tsc2* gene encodes for tuberous sclerosis protein-2 (TSC2), which normally represses the activity of mammalian target of rapamycin complex 1 (mTORc1) (21). The *Tsc2* knockout mice exhibit spontaneous systemic tissue granuloma formation with features resembling those of sarcoidosis patients with active disease progression, including epithelioid-like macrophages, M2 macrophage polarization, and mTORc1 pathway activation (20). However, genome wide association studies have not identified variations of the *Tsc2* gene as a risk factor for sarcoidosis, and the upstream regulators of mTORc1 in humans with sarcoidosis are unknown. Additionally, it is not known if abnormal regulation of mTORc1 alone is sufficient to cause disease in humans. In this regard, rapamycin, an mTORc1 inhibitor, is currently under investigation as a treatment for sarcoidosis and these studies may provide a more definitive answer to this question. It is reasonable to conclude based on available evidence that the murine *Tsc2* knockout model identifies an important immunological pathway that contributes to sarcoidosis disease progression. As such, the model could be leveraged for pre-clinical testing of new sarcoidosis therapeutics targeting mTORc1 regulation pathways.

A major barrier to developing a viable sarcoidosis model is the inability to identify a specific sarcoidosis disease-causing antigen. However, pulmonary sarcoidosis is clinically and histologically very similar to berylliosis, a human disease linked to genetic variability of T cell receptors for divalent beryllium molecules (22). Given the clinical similarities, it has been proposed that sarcoidosis and berylliosis may have similar mechanistic underpinnings (23, 24). Indeed, the berylliosis animal model produces multicellular aggregates of mononuclear cells in the lung consistent with human sarcoidosis (22). However, sarcoidosis patients do not exhibit abnormal T cell-mediated responses to beryllium (25). New data, discussed below, suggest that abnormal macrophage antigen presentation, as opposed to abnormal T cell-mediated antigen recognition, plays a primary role in sarcoidosis pathogenesis.

HUMAN CELL-BASED MODELS OF SARCOIDOSIS

Given that the polygenic nature of sarcoidosis renders it difficult to model using conventional murine knockout approaches,

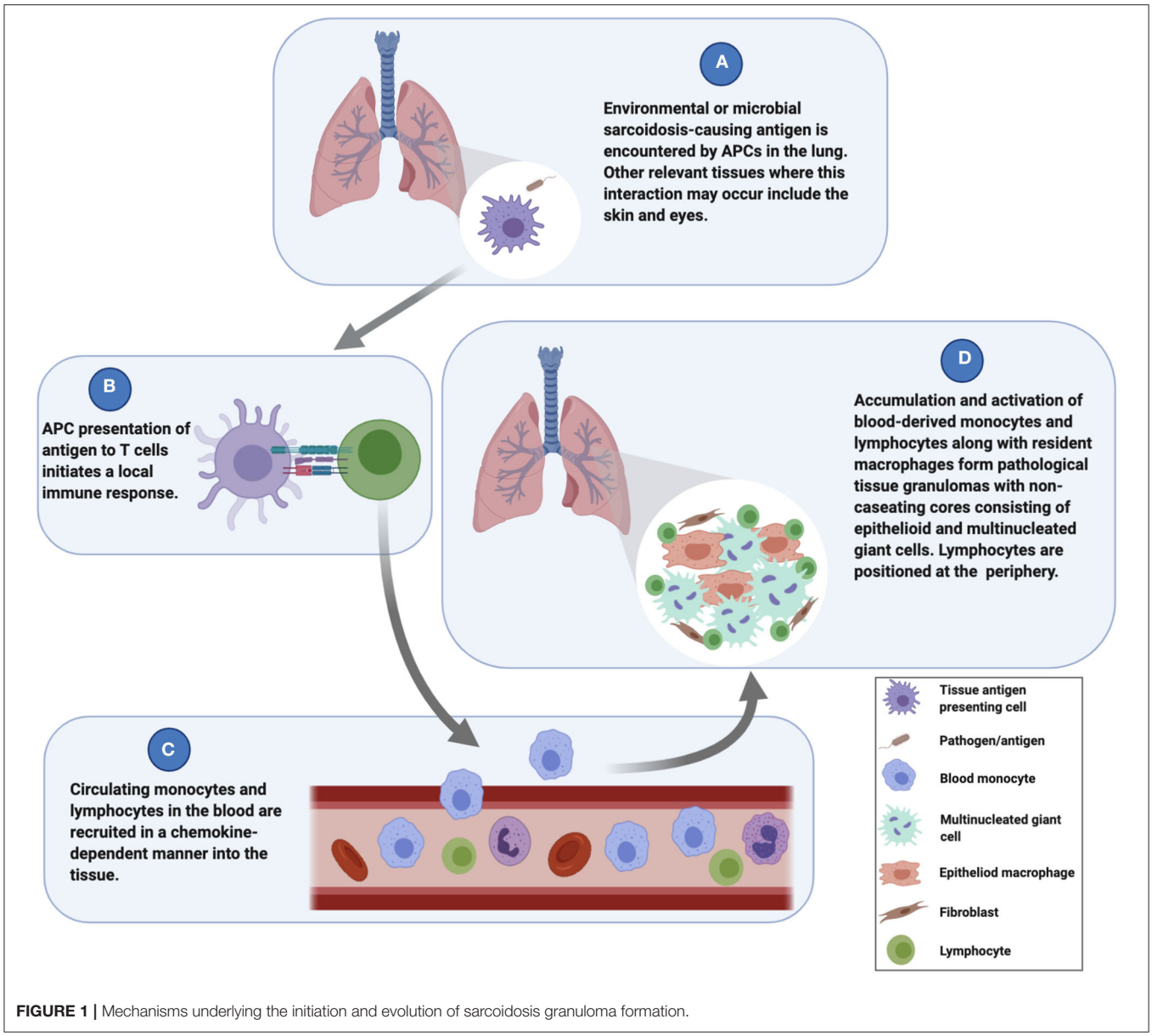

FIGURE 1 | Mechanisms underlying the initiation and evolution of sarcoidosis granuloma formation.

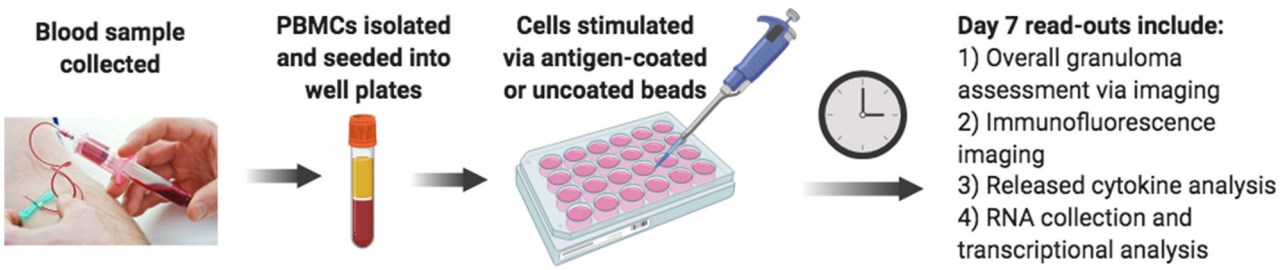

FIGURE 2 | Schematic diagram of the *in vitro* human granuloma model.

the field continues to rely on human subjects to conduct meaningful mechanistic research to understand sarcoidosis disease pathogenesis. There have been several studies that have examined single cell populations from BAL or blood of sarcoidosis patients, yielding new insights into the disease and potential disease trajectories. Drake et al. showed that

$CD4^+$ T cells derived from sarcoidosis BAL samples induced fibroblasts to produce increased amounts of collagen in a STAT3-IL17A dependent manner, thus clarifying signaling events upstream of fibrotic tissue changes (26). Given its ability to modify immune cell function, Yang et al. sought to identify DNA methylation changes in BAL cells from sarcoidosis patients ranging from remitting to severe phenotypes (27). While this study demonstrated an increased variability in DNA methylation in pooled sarcoidosis BAL cells, further insights into DNA methylation patterns associated with different sarcoidosis phenotypes are likely to emerge in the future from a larger patient sample size. In order to better clarify inciting antigens in sarcoidosis, Grunewald et al. used elegant molecular simulations based on $CD4^+$ T cells derived from BAL to demonstrate a potential role for an autoantigen in triggering sarcoidosis (28). Other studies have similarly implicated an autoantigen in sarcoidosis. Chen et al. showed increased levels of serum amyloid A, an amyloid precursor protein, in biopsied sarcoidosis tissues, specifically showing it to be localized in macrophages and giant cells in granulomas (29). The authors postulated that this autoantigen might act to sustain granulomatous inflammation even if the inciting antigen was microbial in nature. In all, studies that have focused on single cell populations or diseased tissues have revealed factors that are likely important modifiers of disease but possess shortcomings with respect to determining the complex and multi-faceted mechanisms underlying pathogenic granuloma formation.

Considering the limitations of current laboratory models, we sought to develop an improved lab model of human sarcoidosis, an *in vitro* human granuloma model (**Figure 2**), with the purpose of gaining mechanistic insights into the molecular events specifically involved in pathological granuloma formation. We posit that there are three key criteria of a viable model to recapitulate the process of granuloma formation in sarcoidosis. First, the model must account for the complex genetics and related unique immune features of sarcoidosis patients. Second, the model must allow for antigen-immune cell interplay, capturing the currently poorly defined immune cell populations that become dysregulated and engaged in granuloma formation. Third, the model must account for the immune cell microenvironment consisting of cytokines and chemokines that promote antigen processing, presentation, and immune cell phenotype changes that occur during the evolution of granulomas. The use of a stimulating antigen is crucial as there is a wealth of evidence supporting an evolution from active granulomatous infection phase to sterile sarcoidosis granulomas (30–32) often harboring non-viable microbial remnants of infectious organisms (33, 34). Recent *in vitro* granuloma models for TB have been developed that appear to exhibit many of these features (35).

The importance of the criteria described above, including accounting for the complex genetics, immune cell cross-talk, and antigen-immune cell interplay dictating granuloma formation was demonstrated in the *in vitro* granuloma model by comparing PBMC responses between *Mycobacterium tuberculosis* (*M.tb*) naïve sarcoidosis patients and healthy controls. No difference in PBMC gene expression profiles was observed between *M.tb* naïve sarcoidosis patients and healthy controls at baseline (**Figure 3A**) (i.e., in the absence of antigen stimulation) in the model, but a vastly divergent response was noted in sarcoidosis PBMCs following challenge with immunogenic *M.tb* antigens (purified protein derivative, PPD) as reflected by >1,000 differentially expressed (DE) genes (**Figure 3B**). While the lack of DE gene expression observed in the absence of antigen likely speaks to variability of gene expression among humans, it provides confidence that the DE expression of >1,000 genes observed after antigen stimulation is not simply due to differences that were already apparent at baseline. Compared to healthy controls, *M.tb* antigen-stimulated sarcoidosis PBMCs also formed more robust granuloma-like aggregates composed of $CD11b^+$ macrophages and $CD3^+$ lymphocytes physically arranged in a manner typical of sarcoidosis granulomas (**Figure 4**) and produced a very different cytokine profile (10). These results challenge the relevance of studying immune cells in an unstimulated fashion and are consistent with the notion that sarcoidosis is a disease incited by exposure to infectious antigen (36).

MODELING DIVERGENT MECHANISMS OF GRANULOMA FORMATION BETWEEN SARCOIDOSIS AND TB

Through its inherent representation of the complex genetics underpinning immune cell dysregulation, the *in vitro* granuloma model holds promise in providing detailed mechanistic insight into sarcoidosis–specific disease regulating pathways at play during early stages of granuloma formation. This was demonstrated through the common stimulation of PBMCs derived from *M.tb* naïve sarcoidosis patients and individuals with latent TB infection (LTBI) with *M.tb*-derived antigens. Both groups produced granuloma-like structures following stimulation, albeit the structures derived from LTBI individuals had a denser lymphocytic cuff. However, gene expression differences were vast between the groups revealing divergent mechanisms of granuloma formation that once again were dependent on antigen stimulation (37). Specifically, we found >5,000 unique DE genes in sarcoidosis compared to LTBI, with many of these DE genes being associated with macrophages (37). Ingenuity Pathway Analysis showed that one distinct pathway in sarcoidosis involves enhanced and prolonged antigen uptake, processing, and presentation (37). Featured in this pathway is the increased expression of vacuolar H^+-ATPase (V-ATPase), a multisubunit proton pump that actively acidifies lysosomal compartments. In addition to this role, V-ATPase forms a signaling complex with mTORc1 that promotes its activation when phagolysosomes are acidified (38). This finding in the *in vitro* granuloma model links macrophage antimicrobial activities with abnormally sustained mTORc1 signaling shown to lead to unchecked granuloma formation in the *Tsc2* knockout mouse model and raises the speculation that evolution of sarcoidosis involves unchecked development of a granulomatous response as a result of enhanced microbial killing. The abnormal immune response in sarcoidosis featuring enhanced intracellular microbial killing via phagolysosomes is unlikely to be confined to

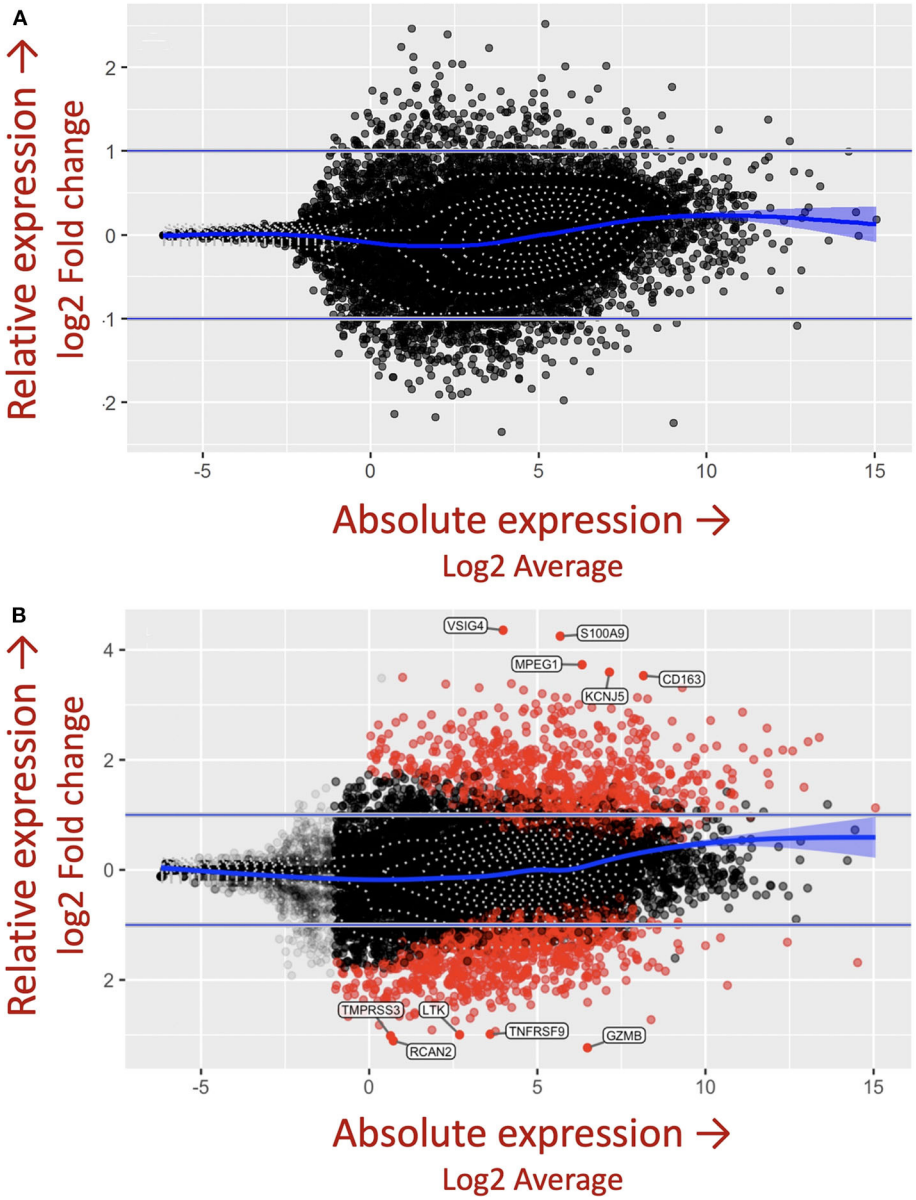

FIGURE 3 | *M.tb* antigen stimulation induces a unique and divergent transcriptional response in sarcoidosis. Differential gene expression in PBMCs derived from patients with sarcoidosis and healthy control subjects after **(A)** uncoated and **(B)** *M.tb* antigen-coated bead stimulation shown as mva style plots. The x-axis is the log2 average of the gene expression level. All genes with an adjusted *P*-value of 0.05 and at least a log2-fold change in the magnitude of gene expression (indicated by the two horizontal blue lines) between *M.tb* antigen and uncoated beads are shaded red.

M.tb and its antigens produced, as other intracellular pathogens, such as *Histoplasmosis capsulatum* and *P. acnes* have been incriminated in the pathogenesis of sarcoidosis (39).

MODELING MACROPHAGE RESPONSES IN SARCOIDOSIS

Given that macrophages are the dominant cell type observed in sarcoidosis granulomas, there have been many different efforts aimed at modeling the macrophage response in sarcoidosis. Macrophage polarization is thought to play a major role in inflammatory diseases including diseases infectious in nature (7), and a growing body of evidence points to a functional imbalance in sarcoidosis in favor of a Th2-biased immune response. Macrophages display enormous plasticity in their phenotypes and appear along a continuum (40). One polar subset identified early on by defined agonists is the so-called M2-type macrophage that takes on immunoregulatory and tissue maintenance and reparative properties (41). Elevated expression

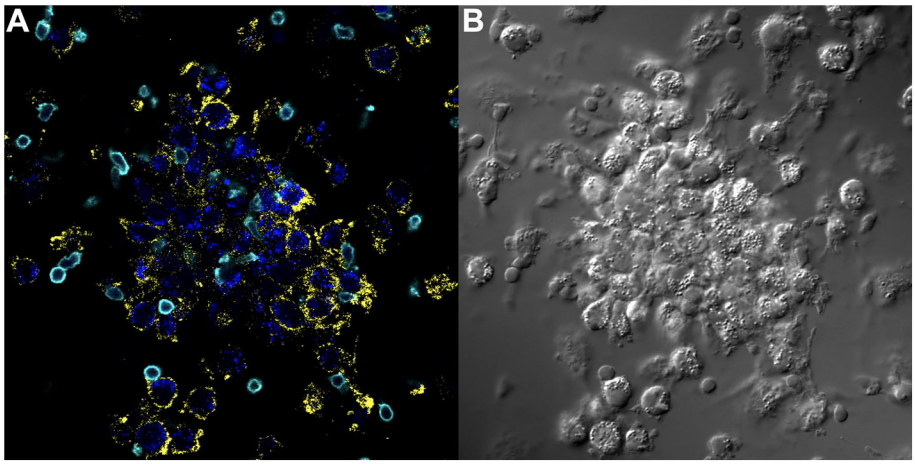

FIGURE 4 | Key immune cell populations are represented in the *in vitro* human granuloma model. **(A)** Immunofluorescence microscopy image showing $CD11b^+$ macrophages and $CD3^+$ lymphocytes are present in a representative granuloma-like structure formed by *M.tb.* antigen-simulated PBMCs from a *M.tb.* naïve sarcoidosis patient. The image is a composite of 3 fluorescent channels: blue, yellow, and cyan channels represent the *M.tb.* antigen-coated beads, CD11b staining, and CD3 staining, respectively. **(B)** A differential interference contrast image of the same granuloma-like structure.

of M2 macrophage-associated markers have been noted in diseased sarcoidosis tissues, including CD206 and CD163, and their expression correlated with disease severity (42–44). Macrophage features observed in our *in vitro* human granuloma model strongly align with this theory, including a significant increase in >50 different M2-like macrophage gene transcripts and elevated expression of CD163 in sarcoidosis granulomas at the protein level (**Figure 5**). Pathway analysis predicted the cytokine IL-13 as being an important upstream regulator of the observed gene expression changes in sarcoidosis compared to healthy controls after antigen stimulation, and we verified this gene network to be highly overrepresented in human sarcoidosis lung and lymph node tissues (45) (**Figure 6**). IL-13 signaling is associated with a Th2-biased immune response and is a known promoter of alternative or M2-like macrophage activation (46, 47). Furthermore, characteristic features of sarcoidosis such as multinucleated giant cell formation (48, 49) and fibrotic tissue changes (8, 39) associated with severe disease phenotypes are signature behaviors of M2-like macrophages.

Sarcoidosis and TB appear to have similar triggers, potentially occupying extremes of a common disease spectrum. Histologically, important differences between sarcoidosis and TB granulomas have been described with a subset of TB granulomas featuring lipid-containing, necrotic macrophages leading to caseation while sarcoidosis granulomas do not present with necrosis or caseation. CD163-expressing macrophages, which were recently shown to be highly abundant in sarcoidosis pulmonary granulomas but not in TB granulomas (43), do not accumulate lipids (50) and therefore their presence in sarcoidosis may explain why sarcoidosis granulomas are "non-caseating." Examination of TB granulomas from infected macaques similarly revealed an absence of CD163 expression in caseating central cores of these structures containing lipid-filled macrophages (51). There is undoubtedly some overlap, however, in granuloma phenotypes between sarcoidosis and TB which is expected given the heterogeneity of these diseases. Ultimately, the connection between macrophage polarization, granuloma characteristics, and antimicrobial capacities including the restriction of *M.tb* growth remains incompletely understood, with the classic picture of M1 macrophages associated with improved protection likely an oversimplification.

Other data from the model that point to a functional imbalance in favor of Th2-immune signaling in sarcoidosis was the relatively blunted INF-γ production associated with sarcoidosis following *M.tb* antigen stimulation and the significant inhibition of granuloma formation in sarcoidosis through pharmacologic signal transducer and activator of transcription 6 (STAT6) inhibition (45). IL-13–mediated activation of STAT6 is a well-established pathway for M2-like macrophage polarization (52). It has also been shown that pharmacologic inhibition of STAT3 via upstream Janus kinase (JAK) inhibition leads to complete resolution of skin lesions in sarcoidosis patients (53). Activation of STAT3 is associated with M2-like macrophage polarization (54). In the *in vitro* human granuloma model we also observed a subset of STAT1-regulated genes that were DE between sarcoidosis and LTBI following *M.tb* antigen stimulation, suggesting a more complex signaling landscape. Indeed, the observed co-existence of both non-CD163-expressing macrophages and CD163-expressing macrophages in the granuloma-like structures (**Figure 5**) supports heterogeneous signaling at the level of the granuloma. Collectively, data from the model with respect to transcriptome-based network characteristics, immune signaling dysfunction, and macrophage immunohistological protein expression patterns reflective of diseased tissues indicate that antigen-stimulated PBMCs derived from patients with sarcoidosis has the ability to manifest the complex and, as yet, undefined genetic features that predispose to sarcoidosis.

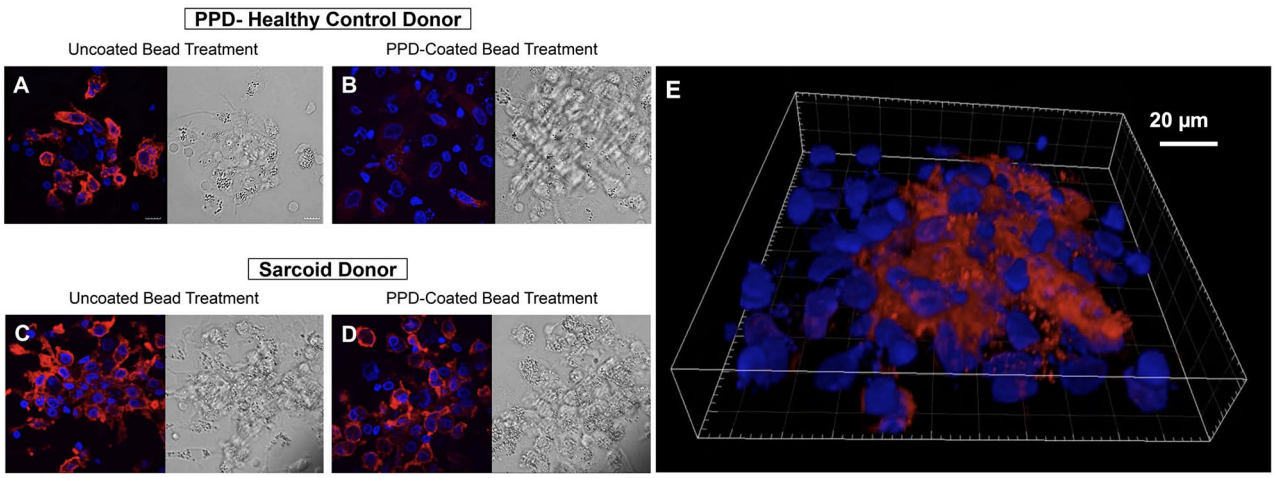

FIGURE 5 | CD163 expression is increased in granuloma-like structures formed by antigen-stimulated sarcoidosis PBMCs in the *in vitro* human granuloma model. Immunofluorescence microscopy imaging demonstrating macrophage CD163 expression upon stimulating healthy control PBMCs with uncoated beads **(A)** with dramatic loss of expression following 7-days *M.tb* antigen-stimulation **(B)**. In contrast, abundant CD163 expression was observed on sarcoidosis macrophages after uncoated bead treatment **(C)** that persisted after *M.tb* antigen-stimulation **(D)**. A 3D rendered volume of a sarcoidosis granuloma-like structure 7 days after *M.tb* antigen-stimulation showing centrally clustered CD163-expressing macrophages **(E)**. DAPI and CD163 staining shown in blue and red, respectively.

MATHEMATICAL MODELING OF SARCOIDOSIS

As cell-based laboratory models continue to advance, the use of mathematical models will act in concert with them to accelerate the pace of discovery (55, 56). A computational model of sarcoidosis has been established and is capable of modeling physical measurements and parameters based on input from companion *in vitro* models and human studies to model the interconnectedness of the cytokine, chemokine, and growth factor proteins contributing to granuloma formation over time (57). We envision that the mathematical model can be used to rapidly interrogate the complex interplay of cells and inflammatory mediators on the evolution of granuloma formation over time through the manipulation of a variable (cytokine for example) or combination of variables, thus informing on specific druggable targets in sarcoidosis. Targets identified in this manner can be rapidly tested in the *in vitro* model for efficacy and thus be combined in a synergistic fashion to bring new therapies (mono or in combo with another) into human trials to effectively inhibit the cycle of pathological granuloma formation (**Figure 7**).

FUTURE DIRECTIONS AND APPLICATIONS OF PRECLINICAL MODELS

As we look toward the future of therapeutic development in sarcoidosis, the role of animal models is currently unclear. Undoubtedly animal models have proven valuable for defining new aspects of disease pathogenesis and pre-clinical testing of new therapeutics. Relevant to sarcoidosis, novel imaging techniques can be performed in mice to enable powerful visual insights into the interplay of immune cells and tissue stroma (58). The future development of new transgenic animal models of sarcoidosis, however, will be contingent upon identifying disease-causing genetic factors in humans. An alternative approach would be to "humanize" animals, a process involving the replacement of the native bone marrow with stem cells from human sarcoidosis donors. There are inherent limitations of this approach, including impaired adaptive immunity in the humanized mice (59), which likely hinder these models from accurately modeling the full cellular and molecular determinants of granuloma formation in sarcoidosis. A humanized model of TB demonstrated pathology that closely modeled human disease, lending promise to this approach in the future (60). We envision a future role of humanized animal models of sarcoidosis that are explored in parallel with *in vitro* human granuloma models as complementary platforms for testing new therapies and evaluating pharmacokinetic and toxicity aspects ahead of human trials (**Figure 7**).

In addition to the providing greater insights into granuloma formation in sarcoidosis, the *in vitro* human granuloma model could be leveraged to screen novel therapies and determine the contribution of specific genes to granuloma formation using siRNA or CRISPR technology (**Figure 7**). Because the model accounts for the inherent genetic variables that predispose to disease, it could be used to gain insights into the ethnic/racial and gender disparities characteristic of this disease. Given that approximately one-third of patients have a progressive form of the disease (61), the model may be able to clarify the unique mechanisms driving granuloma formation in this severe phenotype. An additional strength of the *in vitro* human granuloma model is that it can be used to better understand the dynamic, multicellular mechanisms underpinning distinct

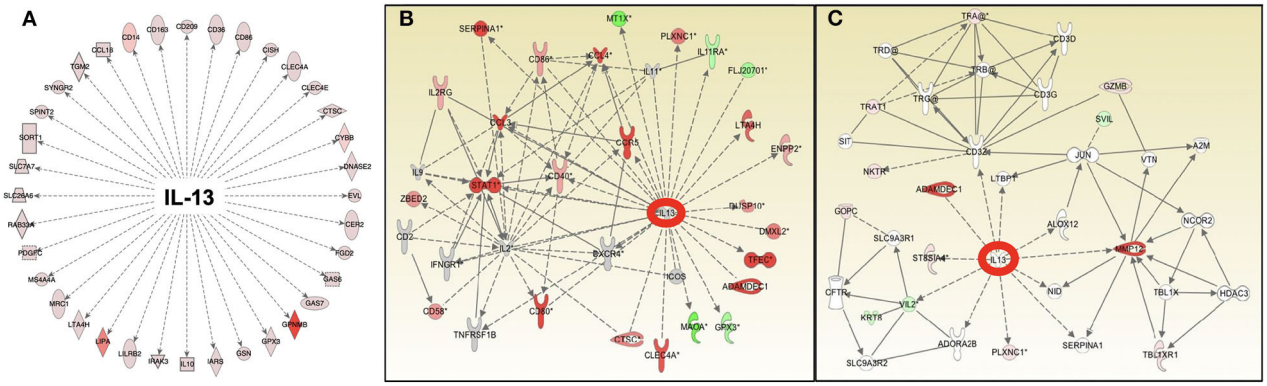

FIGURE 6 | Pathway analysis of gene expression identifies IL-13 as an important and common upstream mediator of the antigen-dependent sarcoidosis granulomatous response in the *in vitro* granuloma model **(A)**, human sarcoidosis lymph node tissue **(B)**, and lung biopsies **(C)**.

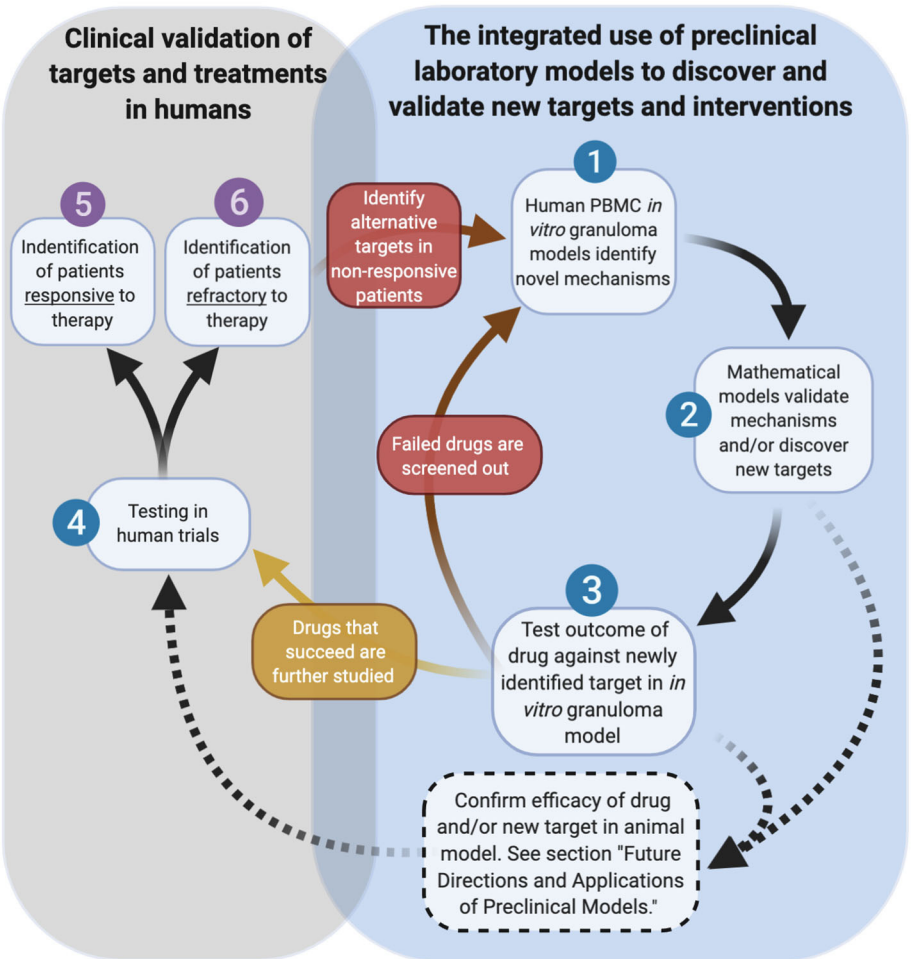

FIGURE 7 | Integrated and synergistic use of sarcoidosis models. Dashed arrows represent future pathways dependent on animal model advancements.

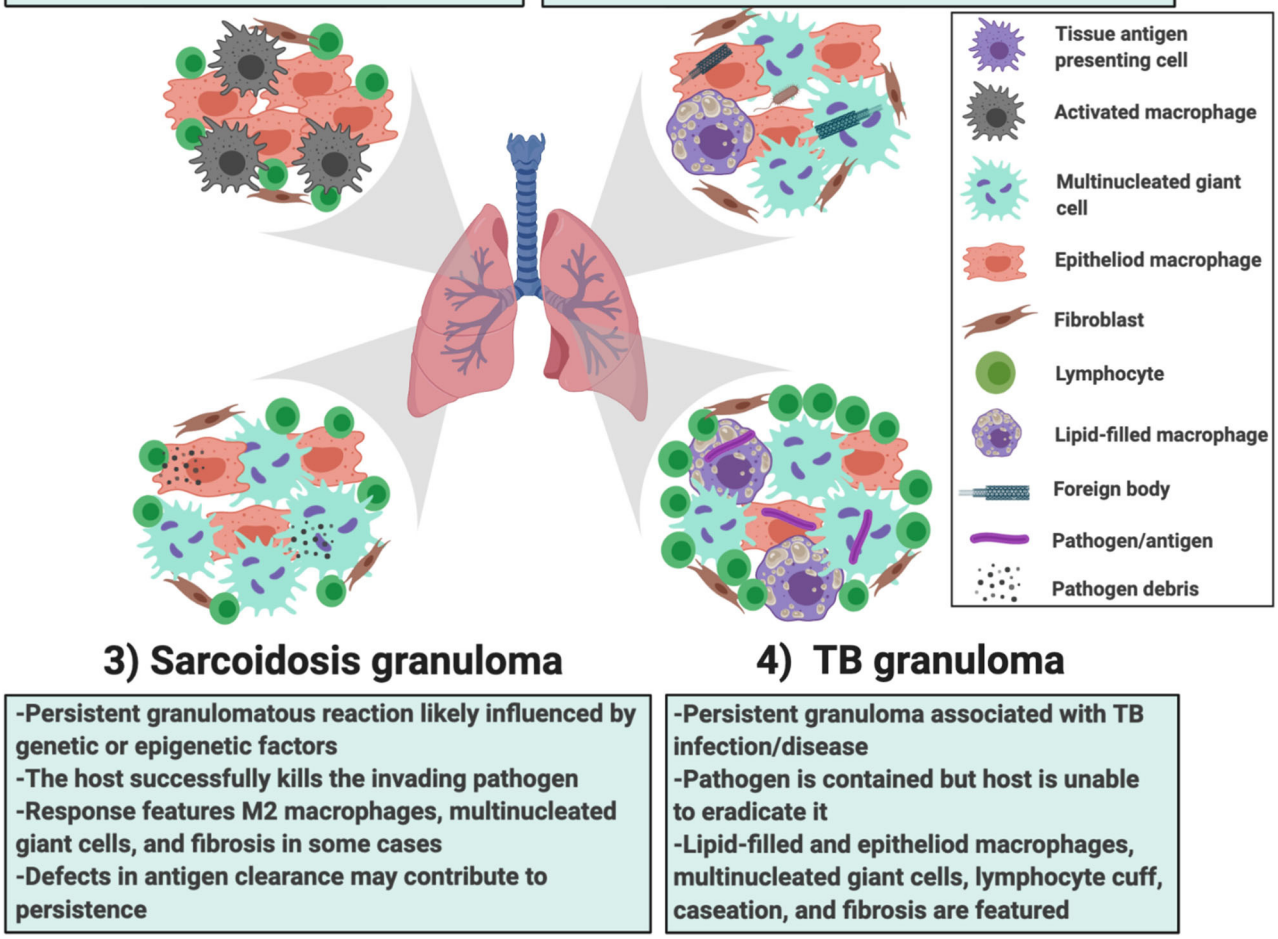

FIGURE 8 | Different scenarios in which the *in vitro* human granuloma model can be used to better understand the dynamic, multicellular mechanisms underpinning distinct granulomatous immune responses that occur in humans ranging from (1) self-limited immune reactions; (2) foreign body immune reactions; (3) sarcoidosis granulomas; and (4) TB granulomas. The granuloma model can be used to test mechanistic hypotheses as well as further our understanding of the importance of environmental (e.g., the type of antigen used to promote granulomas) and host factors (e.g., genetics/race, epigenetic factors/sex, etc.) in these reactions. They can also be used for throughput testing of new potential therapies.

granulomatous immune responses that occur in humans and is not restricted to sarcoidosis (**Figure 8**). For example, the model can be used to explore mechanisms driving early granulomatous responses to etiologic factors other than TB antigens, including exposures to foreign bodies.

There are some caveats of current *in vitro* cell culture models to address. For example, the *in vitro* human granuloma model is likely not capturing the entire spectrum of disease due to the absence of a tissue-mimicking environment. Recent advances in organ-on-a-chip technology are beginning to provide new opportunities in *in vitro* cell culture models such as the modeling of more complex cell interactions involving extracellular matrix components and stromal cells, although to date most of these models lack immune cells (62). Enabling the cross-talk between immune cells and matrix cells such as fibroblasts may allow for mechanisms driving fibrotic changes to be modeled in sarcoidosis, a signature feature of severe disease phenotypes (63). Organ-on-a-chip technology also allows for the continuous perfusion of cells, such as PBMCs, through a vascular-like system, thus potentially modeling cellular recruitment processes and enabling a more prolonged study of the eventual fate of granulomas (i.e., self-perpetuating vs. disaggregating). As with any modeling endeavor, it is challenging to know when a model is "good enough." In the case of sarcoidosis, any

modeling advancement must be weighed against the added cost, throughput potential, and the burden of validation.

CONCLUSION

The failure to effectively model sarcoidosis in the laboratory or in animals continues to hinder the discovery and translation of new, targeted treatments. Preclinical models that are capable of clarifying the significant knowledge gap surrounding the triggers and mechanisms driving granuloma formation and self-perpetuation in sarcoidosis will be key to this cause. Compared to modeling approaches that study only a single cell type, the patient-derived PBMC-based *in vitro* granuloma model allows for the interrogation of antigen-immune cell interplay and related cell-cell interactions driving the various stages of granuloma formation in sarcoidosis and underpinned by the complex and, as yet, undefined genetic features of this disease. The *in vitro* human granuloma model provides a powerful approach to addressing critical knowledge gaps in sarcoidosis, and we envision its complementary use with other pre-clinical models will likely accelerate progress toward identifying and testing new drugs and drug combinations that can be rapidly brought to clinical trials.

AUTHOR CONTRIBUTIONS

EC, LS, and LL wrote the article. EC and LL contributed to designing the figures and table for the article. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the following grants: NIH through The Ohio State University Center for Clinical and Translational Science (Clinical and Translational Science Award UL1TR001070 [EC and LS]), ATS Foundation/Mallinckrodt Pharmaceuticals, Inc. Research Fellowship, and a Sarcoidosis Disease Model Grant from the Foundation for Sarcoidosis Research.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 15 September 2020
doi: 10.3389/fimmu.2020.537382

Sarcoidosis Epidemiology: Race Matters

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Rather than a single disease entity, sarcoidosis may be a constellation of "sarcoidoses" with a characteristic pattern of organ involvement and clinic course, depending upon the triggering exposure and underlying epidemiologic factors such as race. This review examines the racial disparities inherent to sarcoidosis disease course and mortality and discusses factors that may be responsible for these findings. In the United States, black patients with sarcoidosis experience more severe pulmonary disease, more multiorgan involvement, and an overall worse prognosis with higher rates of hospitalization and mortality. Beyond inherent genotype, ascertainment and access to medical care, physician implicit bias, and patient perceived discrimination likely play a role. Moving forward, epidemiologic concepts can be used to formulate strategies for control, treatment, and even prevention of disease in black Americans at risk for developing life-altering or life-threatening sarcoidosis phenotypes. Identification and rectification of modifiable risk factors such as socioeconomic status, lack of insurance, and financial barriers to care as well as the incorporation of implicit bias training for physician will likely lead to improvement in discordant outcomes.

Keywords: sarcoidosis, black race/ethnicity, sarcoidosis epidemiology, sarcoidosis mortality, implicit bias, health equity

OPEN ACCESS

Edited by:
Marc A. Judson,
Albany Medical College, United States

Reviewed by:
Robert Campbell,
The University of Utah, United States
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The Johns Hopkins Hospital, United States
Karen Patterson,
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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *23 February 2020*
Accepted: *17 August 2020*
Published: *15 September 2020*

Citation:
Hena KM (2020) Sarcoidosis Epidemiology: Race Matters. Front. Immunol. 11:537382. doi: 10.3389/fimmu.2020.537382

INTRODUCTION

Sarcoidosis is a multisystem disease characterized by granulomatous inflammation. Although its etiology remains unknown, sarcoidosis is believed to represent a genetically primed abnormal immune response to an antigenic exposure (1). Rather than a single disease entity, sarcoidosis may be a constellation of "sarcoidoses" with a characteristic phenotype, depending upon the triggering exposure and underlying genotype. As such, race plays an important role in sarcoidosis epidemiology, disease presentation, and clinical outcomes (2).

In the United States, in particular, different phenotypic expressions are found between black and white subjects, likely resulting from genetics, environment, socioeconomics, implicit or racial biases, or a combination thereof. Given the current political climate and impetus for change in the United States, it is important to shed light on discordant clinical outcomes in vulnerable patient populations. This article examines the racial disparities inherent to sarcoidosis disease course and mortality and discusses factors that may be responsible for these findings.

INCIDENCE AND PREVALENCE

Consistently, black subjects are reported to be most affected by sarcoidosis, black females even more so. Studies reporting incidence and prevalence of sarcoidosis, broken down by race and sex if available, are presented in **Table 1**.

Dating back to the 1960's, researcher Andrew Z. Keller reported significantly different frequency distributions of sarcoidosis with black patients being more commonly diagnosed and treated than whites (12). In the 1990's, an analysis of race in patients from Detroit, Michigan suggested that the relative rates of sarcoidosis in black compared to white patients was as high as 4:1 (10.9/100,000 per year in whites and 35.5/100,000 in blacks) (10). In this study by Rybicki and colleagues, the highest annual age-specific incidence was found in black females aged 30–39 years (107/100,000) (10). A later analysis of sarcoidosis based on U.S. managed healthcare use from 2009 to 2013 showed that the incidence and prevalence of sarcoidosis was significantly higher for black (17.8 and 141.4 per 100,000) than for white individuals (8.1 and 49.8); the highest prevalence for sarcoidosis was again noted in black females (178.5) (4). Of note, additional minority populations, such as Hispanic and Asian patients, were significantly less likely to have sarcoidosis than black or white patients, consistent with what has been suggested in the past (4, 13). More recently, amongst participants in the Black Women's Health Study, incidence rates were observed as high as 71 per 100,000 per year in African American females (6).

In summary, sarcoidosis incidence and prevalence vary greatly based on race with a strong predilection for black populations in the United States, which may be the result of race-specific genetic associations and environmental socioeconomic factors.

GENETIC PREDISPOSITION

As stated previously, sarcoidosis likely results from an environmental insult in a genetically susceptible host. The high incidence and prevalence of sarcoidosis in black subjects suggests an inherent genetic susceptibility. In fact, first-degree relatives of black sarcoidosis cases have about a 3-fold disease risk increase (14).

Multiple sarcoidosis susceptibility loci have been explored in candidate gene and genome-wide association studies. These include the highly polymorphic human leukocyte antigen (HLA) genes, residing in the class II major histocompatibility complex (MHC) on chromosome 6, as well as other genes that influence antigen processing and presentation, T-cell activation and recruitment, and granulomatous inflammation at the core of sarcoidosis immunopathogenesis. A particular HLA allele, DQB1*0602, was shown to confer both increased susceptibility to sarcoidosis and a predilection for radiographic progression of disease in a family-based genetic association analysis of sarcoidosis in U.S. black families (15). Conversely, Butyrophilin-like 2 (BTNL2), which also resides in the class II MHC region on chromosome 6 and probably functions as a T-cell co-stimulatory molecule, has been shown to have a more modest association with sarcoidosis in black compared to white populations; this may be due to greater allelic diversity or an antagonistic effect of HLA class II risk alleles on BTNL2-associated risk in black samples (16). Amongst black siblings enrolled into the Sarcoidosis Genetic Analysis Consortium (SAGA), a genome scan found the strongest linkage signal on chromosome 5 (17). Subsequent fine mapping studies indicated a sarcoidosis susceptibility gene on chromosome 5q11.2 and a gene protective effect on 5p15.2 (18). Further stratification of black families by genetically determined ancestry revealed linkage differences by subpopulation, with previously reported linkage signals at 1p22, 3p21-14, 11p15, and 17q21 specific to ancestral heritage (19).

In all, given the identification of multiple candidate genes and suggestive regions for linkage, it is likely that more than one gene influences sarcoidosis susceptibility and disease presentation in black populations. This is further confounded by ancestral differences in admixed black communities residing in the United States.

DISEASE PRESENTATION

In sarcoidosis the granulomatous inflammation can affect virtually all organs and tissues. However, disease presentation, including extent and severity of disease, has been shown to vary based on demographic variables such as race. A Case-Control Etiologic Study of Sarcoidosis (ACCESS)—a prospective, multicenter study of sarcoidosis patients enrolled within 6 months of diagnosis—provided an opportunity to examine the association of demographic measures, socioeconomic status and barriers to care with severity of disease at presentation, as defined by extent of organ involvement, chest radiographic staging and basic spirometry (20, 21).

As far as organ involvement at presentation, ACCESS showed that black patients tend to experience more extrapulmonary sarcoidosis, affecting the skin (other than erythema nodosum), bone marrow, liver, extrathoracic lymph nodes and eyes (20). Calcium dysmetabolism, on the other hand, was more common in white patients (20). Only pulmonary involvement was independent of age, sex and race (20). Later ACCESS analyses demonstrated that lower income, the absence of private or Medicare health insurance, and other barriers to care were associated with sarcoidosis severity at presentation, as were black race and female sex (21). In comparison to ACCESS whites, ACCESS blacks were more likely to have more organs involved and lower FVC % predicted as well as family income of <US$20,000 and to have other public insurance such as Medicaid (21).

Therefore, in addition to a suspected genetic predisposition for more severe pulmonary and extrapulmonary disease, the association of black race with socioeconomic status (low income, public insurance) and severity of disease at presentation suggests that black patients are more likely to have financial barriers to care and resultant delay in seeking care than their white counterparts (21). Additionally, implicit bias—any unconsciously-held set of associations about a social group—impacts the behavior of both physicians and black patients, with physicians potentially discounting symptoms of sarcoidosis in

TABLE 1 | Sarcoidosis incidence estimates reported in the literature.

References	Country	Sex, race/ethnicity	Time period	Incidence per 100,000	Data source
Arkema et al. (3)	Sweden	Male and female, race not reported	2003–2013	11.5	National Patient Register
Baughman et al. (4)	USA	Male and female, multiracial	2010–2013	Black: 17.8 White: 8.1 Hispanic: 4.3 Asian: 3.2	Optum Health Care Database
Coquart et al. (5)	Guadeloupe, West Indies	Male and female, Afro Caribbean	2003–2010	2.3	Hospital and laboratory databases
Cozier et al. (6)	USA	Female, black	1995–2007	71	Black Women's Health Study, self-reported sarcoidosis
Duchemann et al. (7)	France, Greater Paris	Male and female, multiracial	2012	Overall: 4.9 Afro Caribbean: 16.9 North African: 9.7 Other: 6.4 European: 2.4	Hospitals in Seine-Saint-Denise County; social security
Dumas et al. (8)	USA	Female, multiracial	1989–2011	Overall: 11 Black: 43 White: 11	Nurses' Health Study II, self-reported
Hillerdal et al. (9)	Sweden, Uppsala County	Male and female, race not reported	1966–1980	Overall: 19 Male: 16.5 Female: 21.7	General Health Screening
Rybicki et al. (10)	USA, Detroit, MI	Male and female, multiracial	1990–1994	Black: 35.5 White: 10.9	Health Alliance Plan HMO
Ungprasert et al. (11)	USA, Olmsted County, MN	Male and female, 90% white	1976–2013	Male: 10.5 Female: 11	Rochester Epidemiology Project

black patients until more severe and/or black patients delaying care out of medical mistrust or perceived discrimination (22–25).

CLINICAL COURSE

While sarcoidosis tends to improve or remain stable in the majority of patients, longitudinal studies have shown worse outcomes are associated with black race and/or lower annual family income (26, 27). In the ACCESS 2 years follow-up study, black patients had a higher likelihood of decreasing FVC and developing new organ involvement over time (26). Similarly, clinical data from a large cohort of sarcoidosis patients followed at Medical University of South Carolina over a 12-years period, showed that black patients had more advanced radiographic stages of sarcoidosis, more organ involvement, and more frequently required anti-sarcoidosis medication compared to white patients (27).

Furthermore, black patients tend to have a higher rate of hospitalization for sarcoidosis. An analysis of the National Hospital Discharge Survey (NHDS) from 1979 to 2000 showed that mean rates of hospitalization were nine time higher for black patients (28). A retrospective study of the Nationwide Inpatient Sample supported this trend, demonstrating a near doubling of hospitalizations among sarcoidosis patients, with disproportionate rate increases in black, female, and older patients from 1998 to 2008 (29).

As such, black patients with sarcoidosis experience worse prognosis, more multiorgan involvement, and a higher rate of hospitalization. Beyond inherent genotype, ascertainment and access to preventative and primary care may also be playing a role in this disparity of outcomes for black individuals, as is implicit bias.

MORTALITY

Many patients with sarcoidosis have a benign clinical course, but for some, it is a chronic, life-altering, and even fatal disease. While overall mortality rates from sarcoidosis have been shown to be increasing, significant differences exist based on race. Data from the National Center for Health Statistics (NCHS), demonstrated a 3% average yearly increase in mortality rate for all-comers with sarcoidosis from 1988 to 2007 (30). However, the greatest absolute increase in sarcoidosis-related deaths was among black females (10 deaths per million) followed by black males (three deaths per million), in comparison to at most one death per million in white males or females (30). The most common cause of death was sarcoidosis itself; younger sarcoidosis decedents with pulmonary fibrosis were more likely to be black than white and have a cardiac involvement contribute to death. A later study using NCHS data from 1999 to 2010 with a focus on racial and sex disparities, showed a 12 times higher age-adjusted mortality rate for black patients compared with white patients

(16 vs. 1.3 per million, respectively) (31). Again, black sarcoidosis decedents died at an earlier age and more often had associated pulmonary hypertension (31). An early study by Gideon and Mannino reported a U.S. sarcoidosis mortality ratio, black: white, of 14:1 (32).

This race-specific trend of higher mortality from sarcoidosis at a younger age in black patients has multiple potential explanations, some more alarming than others. Hypotheses include increased incidence and severity of disease, a predilection for the lethal complications of pulmonary fibrosis and pulmonary hypertension, as well as long-standing systemic health and social inequities.

RACISM

As the Covid-19 pandemic has so powerfully illustrated, race plays a significant role as a determinant of health in the United States (33). Beyond socioeconomic factors, perceived discrimination has been linked to adverse health outcomes in mental and physical health domains (23–25). In addition to implicit or racial biases, the perception of discrimination or stigma of inferiority appears to induce physiological and psychological arousal with direct deleterious health consequences (34). While this has not been elucidated specifically in sarcoidosis, the association of perceived discrimination with poorer health outcomes has been explored across a broad range of outcomes, including cardiac disease, renal insufficiency and subclinical cerebrovascular disease (23–25).

FUTURE DIRECTIONS

Despite its nineteenth century origin and more than 100 years of inquiry, much about sarcoidosis is still unknown. Perhaps this is because of its many points of divergence—its variant epidemiology, varied triggering exposures and ultimately variable clinical phenotypes or "sarcoidoses." Regardless, its higher contraction and death rates in black Americans deserves further attention.

Ideally, future studies performed will improve our understating of the relationships among exposure, race and other epidemiologic factors, with current clinical phenotypes and outcomes. Such endeavors should include large, matched epidemiologic studies conducted in racially diverse populations, facilitated by large population data sets and disease registries. In addition to facilitating etiologic hypotheses of the exposure-host interaction at the core of sarcoidosis pathophysiology, epidemiologic concepts should be used to formulate strategies for control, treatment, and even prevention of disease in Black Americans who are at the greatest risk for developing life-altering or life-threatening disease. Identification and rectification of modifiable risk factors such as socioeconomic status, lack of insurance, and financial barriers to care will likely lead to improved outcomes. Making anti-racism or implicit bias training an essential professional competency would equip physicians with the tools needed to address racism and its adverse health effects (35).

Finally, these findings should influence research in the areas of health disparities and disease processes among various racial groups and between the sexes throughout the world. Achieving health equity requires valuing all individuals and populations equally, recognizing and rectifying historical injustices, and providing resources according to need. Health disparities will be eliminated *only* when health equity is achieved (36).

AUTHOR CONTRIBUTIONS

KH contributed a race-centered review of sarcoidosis epidemiology, highlighting racial disparities inherent to sarcoidosis disease course and mortality and discusses factors that may be responsible for these findings. She was the sole contributing author for this section.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 18 September 2020
doi: 10.3389/fimmu.2020.553949

Matrix Metalloproteinase-12 Is Required for Granuloma Progression

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Background: Sarcoidosis is a chronic inflammatory disease of unknown cause characterized by granuloma formation. Mechanisms for chronic persistence of granulomas are unknown. Matrix Metalloproteinase-12 (MMP12) degrades extracellular matrix elastin and enables infiltration of immune cells responsible for inflammation and granuloma formation. Previous studies report increased MMP12 in sarcoidosis patients and association between MMP12 expression and disease severity. We also observed elevated MMP12 in our multiwall carbon nanotube (MWCNT) murine model of granulomatous inflammation. Here we hypothesized that MMP12 is important to acute and late phases of granuloma pathogenesis. To test this hypothesis, we analyzed granulomatous and inflammatory responses of *Mmp12 knock-out* (KO) mice at 10 (acute) and 60 days (late) after MWCNT instillation.

Methods: C57BL/6 (wildtype) and *Mmp12* KO mice underwent oropharyngeal instillation of MWCNT. Lungs were harvested at 3, 10, 20, and 60 days post instillation for evaluation of MMP12 expression and granulomatous changes. Bronchoalveolar lavage (BAL) cells were analyzed 60 days after MWCNT instillation for expression of mediators thought to play a role in sarcoid granulomatosis: peroxisome proliferator-activated receptor-gamma (PPARγ), interferon-gamma (IFN-γ), and CCL2 (MCP-1).

Results: Pulmonary granuloma appearance at 10 days after MWCNT instillation showed no differences between wildtype and *Mmp12* KO mice. In contrast, by 60 days after MWCNT instillation, *Mmp12* KO mice revealed markedly attenuated granuloma formation together with elevated PPARγ and reduced IFNγ expression in BAL cells compared to wildtype. Unexpectedly, *Mmp12* KO mice further demonstrated increased alveolar macrophages with increased CCL2 at 60 days.

Conclusions: The striking reduction of granuloma formation at day 60 in *Mmp12* KO mice suggests that MMP12 is required to maintain chronic granuloma pathophysiology. The increased PPARγ and decreased IFNγ findings suggest that these mediators also

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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *20 April 2020*
Accepted: *14 August 2020*
Published: *18 September 2020*

Citation:
Mohan A, Neequaye N, Malur A, Soliman E, McPeek M, Leffler N, Ogburn D, Tokarz DA, Knudson W, Gharib SA, Schnapp LM, Barna BP and Thomassen MJ (2020) Matrix Metalloproteinase-12 Is Required for Granuloma Progression. Front. Immunol. 11:553949. doi: 10.3389/fimmu.2020.553949

may be involved since previous studies have shown that PPARγ suppresses IFNγ and PPARγ deficiency amplifies granuloma formation. Interestingly, a role of MMP12 in granuloma resolution is also suggested by increases in both macrophage influx and CCL2. Overall, our results strongly implicate MMP12 as a key factor in granuloma persistence and as a possible therapeutic target in chronic pulmonary sarcoidosis.

Keywords: sarcoidosis, MMP12, PPARγ, MWCNT, granuloma, inflammation

INTRODUCTION

Sarcoidosis is a prototypic granulomatous inflammatory disorder which predominantly affects the lungs and thoracic lymph nodes (1). Recent studies show that average sarcoidosis-associated mortality has increased by approximately 3% per year in the United States (2, 3). Unfortunately, large gaps in our understanding of sarcoidosis pathogenesis have hindered research and development of novel therapies. Animal models may be helpful for exploring select pathways and directing research toward higher yield mechanisms. Our multiwall carbon nanotube (MWCNT) based murine model of granulomatous inflammation was first described in 2011 (4). The model replicates human disease at multiple biological levels including key mediators such as IFN-γ and PPARγ. The transcription factor, PPARγ is a regulator of glucose and lipid metabolism but is also recognized as a negative regulator of macrophage activation (5). Alveolar macrophages from healthy individuals express constitutively high PPARγ levels but PPARγ is deficient in alveolar macrophages from sarcoid patients (6). Our previous studies with the MWCNT model indicated decreased PPARγ (7). Further studies with macrophage-specific *Pparγ* knock out (KO) mice revealed enhanced granulomatous disease as evidenced by increased granuloma size and incidence (7). Interestingly, previous studies indicated that IFNγ represses PPARγ in human alveolar macrophages (8), suggesting a reciprocal relationship.

MMP12, also known as macrophage metalloelastase is a member of a family of extracellular endopeptidases (9, 10). MMPs were originally thought to be mainly responsible for turnover and degradation of extracellular matrix components. However, in recent years it has become clear that MMPs mediate many crucial functions in immunity and repair including cell migration, leukocyte activation and anti-microbial defense (9, 10). Furthermore, many of the earlier *in vitro* studies may not accurately reflect the *in vivo* situation (11). As Giannandrea and Parks note in their review, degradation studies with individual substrates show that isolated MMPs have redundancy *in vitro*, but *in vivo* functions of specific MMPs are limited and unique (11). MMP12 was first implicated as a mediator in sarcoidosis pathogenesis in 2009 (12). In those studies, lung tissues from sarcoidosis patients showed increased (>25-fold) *Mmp12* gene expression. Interestingly, MMP12 expression was highest near areas of active granulomatous inflammation, and MMP12 levels in bronchoalveolar fluid (BALF) correlated with disease severity. These findings make MMP12 biology an area of acute interest in sarcoidosis pathogenesis.

Using gene network analysis we previously demonstrated that MMP12 was one of the most highly expressed genes in MWCNT-exposed mice as well as in sarcoidosis patients, suggesting that MMP12 is a putative driver of granulomatous disease (detailed microarray information and raw data have been deposited in Gene Expression Omnibus[1] [GSE 100500 and GSE75023 (13, 14)]. We therefore hypothesized that MMP12 is critical to granuloma formation. Because conclusions made from *in vitro* studies have proven to be poor predicators of *in vivo* pathogenesis, an animal model which replicates many of the features of sarcoidosis is essential. To test this hypothesis, we compared *in vivo* granuloma genesis in *Mmp12* gene KO versus wild type mice after exposure to MWCNT.

MATERIALS AND METHODS

Multiwall Carbon Nanotube Model

All studies were conducted in conformity with Public Health Service (PHS) Policy on humane care and use of laboratory animals and were approved by the institutional animal care and use committee. C57BL/6J wild-type, *Mmp12* KO mice (Jackson Laboratories, Bar Harbor, ME, United States) and macrophage-specific *Pparγ* KO (15) received a single oropharyngeal instillation of MWCNT (100 μg) in PBS/35%surfactant (Ony Inc, Amherst, NY, United States) (4). Briefly, mice were sedated with isofluorane and by gently pulling forward the mouse tongue the epiglottis was exposed and a 50 μl volume was instilled using a pipette. MWCNTs (900–1201, lot-GS1802, SES Research, Houston, TX, United States) were freshly prepared and have been described previously (16). Sham controls received vehicle alone. Animals were sacrificed at 3, 10, 20, and 60 days post instillation and evaluated as previously described (4).

Histological Analysis

Lungs were dissected and fixed in PBS-buffered 10% formalin. Paraffin embedded slides were sectioned at 7 μm, and stained with hematoxylin and eosin (H&E) or Gomori's trichrome stain as previously described (4, 16). A previously described semiquantitative scoring system (7, 16, 17) was used to calculate a relative comparison of the numbers and quality of granulomas formed in MWCNT-instilled mice. Trichrome stain was evaluated using modified Ashcroft method (16, 18).

Lymph Nodes

Tracheobronchial lymph node volume, identified based on Van den Broeck et al., was determined using the formula

[1]https://www.ncbi.nlm.nih.gov/geo

(Length × Width^2)π/6, as previously described (16). Lymph nodes were fixed overnight in PBS-buffered 10% formalin and paraffin embedded. Representative Hemotoxylin/Eosin histological images were taken for each condition, time point and mouse strain using a Zeiss Axio Imager A1.

Characterization of Bronchoalveolar Lavage Cells

BAL cells were obtained as previously described (15, 16). Total cell counts and differential counts were evaluated (**Table 1**). Cells were stored at −80°C for gene expression, and BAL fluid was aliquoted and frozen for protein analysis.

RNA Purification and Gene Expression From BAL Cells

Total RNA was extracted from frozen BAL cells using miRNeasy Micro kit, (217084) (Qiagen, Germantown, MD, United States), according to manufacturer's protocol. Mouse specific primers and probes were obtained from Qiagen, for *Mmp12* (PPM03619F), *Ccl2* (PPM03151G), *Pparγ* (PPM05108B), and

TABLE 1 | BAL cell characteristics of C57Bl/6 and *Mmp12* KO mice 60 day.

	Treatment	*N*	Total cell count (×10^5)	AM (X10^5)	LYM (×10^5)	PMN (×10^5)
C57Bl/6	PBS/Surf	10	8.0 ± 2.9	7.7 ± 2.8 [97]	0.3 ± 0.3 [3]	0.06 ± 0.09 [1]
C57Bl/6	MWCNT	10	9.8 ± 2.5	8.9 ± 2.0 [92]	0.7 ± 0.5 [6]	0.2 ± 0.2 [2]
Mmp12 KO	PBS/Surf	10	7.7 ± 2.5	7.5 ± 2.5 [97]	0.1 ± 0.1 [2]	0.05 ± 0.1 [1]
Mmp12 KO	MWCNT	10	12.0 ± 4.1*	11.1 ± 4.0 [92]*	0.4 ± 0.2 [4]	0.5 ± 0.4 [4]*

*AM, alveolar macrophages; LYM, lymphocytes; PMN, neutrophils. *Means ± SD, $p \leq 0.05$ compared to PBS/Surf vs MWCNT in MMP12 KO. [ ] Percentage.*

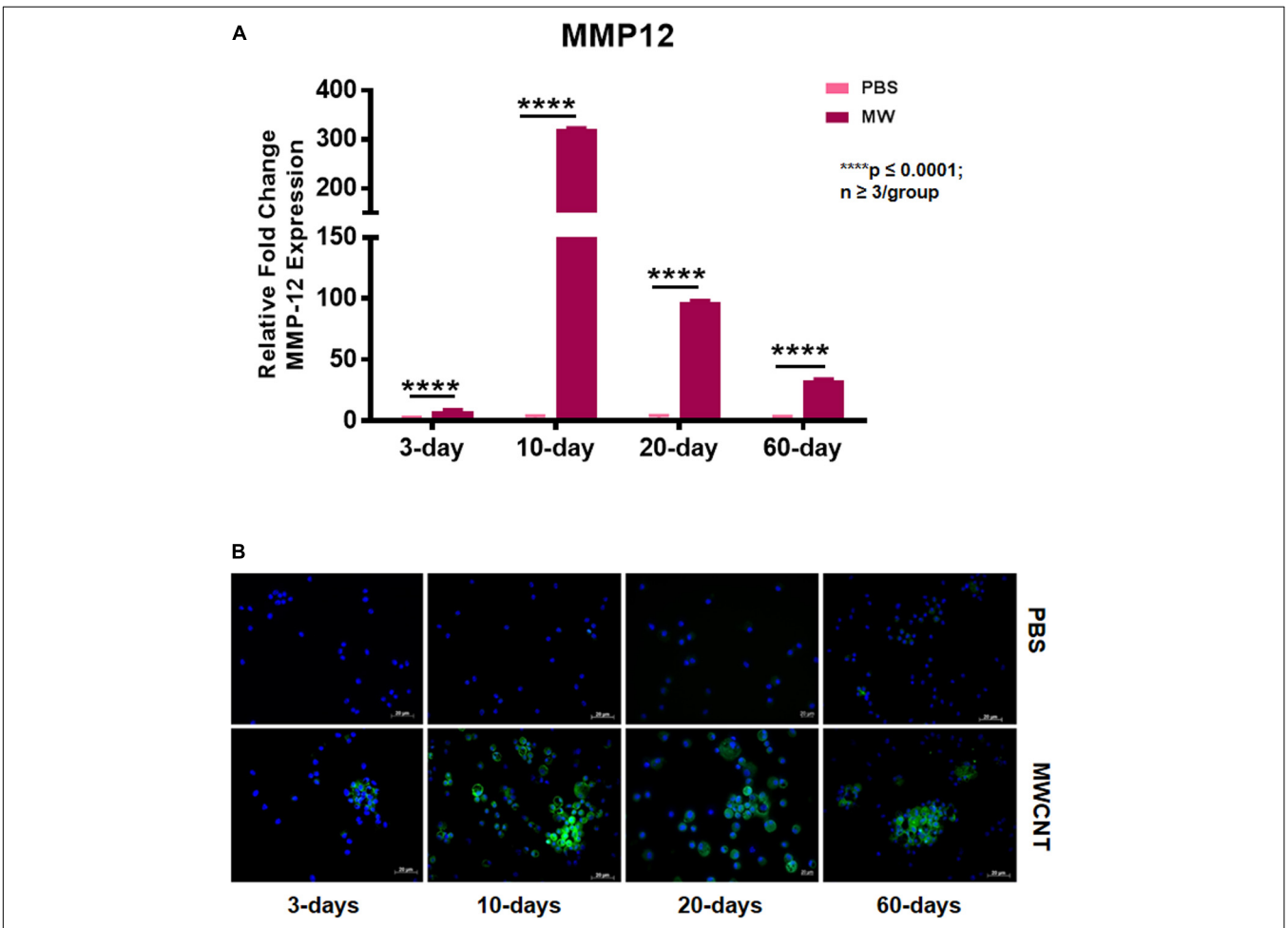

FIGURE 1 | *Mmp12* gene expression and protein are upregulated in BAL cells of MWCNT-instilled C57BL/6. **(A)** *Mmp12* gene expression is increased at 3 days, with a peak at 10 days, and remains significantly upregulated at 60 days post MWCNT-instillation ($p \leq 0.05$; $n \geq 3$ per group). **(B)** Immunofluorescent anti-MMP12 staining was minimal at 3 days, with a peak at 10 days and persistence through 60 days (representative of $n = 3$).

IFNγ (PPM03121A). GAPDH (PPM02946E) was used as a housekeeping gene. Quantitative-PCR was performed on complementary DNA synthesized with the RT2 First Strand Kit and evaluated on the StepOnePlus PCR system (Thermo Fisher Scientific, Waltham, MA, United States) in comparison to GAPDH using the $2^{-\Delta\Delta CT}$ method (19).

Protein Analyses of BAL Fluid

CCL2 was assayed in BALF by ELISA ([MJE00B] R&D systems Minneapolis, MN, United States), as per manufacturer's protocol.

Immunostaining of BAL Cells and Frozen Tissue

Cytospin slides of BAL cells were fixed with 4% paraformaldehyde-PBS, permeabilized with Triton X-100, blocked and stained with anti-PPARγ at 1:250 dilution (Sc-7196) (Santa Cruz Biotechnology, Dallas, TX, United States), and Alexa 488 (1:1000) (Invitrogen, Carlsbad, CA, United States) or anti-IFNγ 1:200 (sc-57207) (Santa Cruz Biotechnology, Dallas, TX, United States) with secondary antibody Alexa Texas Red 569 (15).

Frozen lung tissue sections (7 μm) were fixed with 4% paraformaldehyde–PBS, permeabilized with Triton X-100, blocked with normal goat serum in PBS/Triton X-100 for nonspecific binding and stained with anti-MMP12 antibody (Sc-390863) (Santa Cruz Biotechnology, Dallas, TX, United States), 1:250 dilution, followed by Alexa conjugated goat anti-rabbit IgG 488 (Invitrogen, Carlsbad, CA, United States). Slides were counter-stained with DAPI (Vector Laboratories, Burlingame, CA, United States) to facilitate nuclear localization. Slides were imaged on Zeiss confocal LSM700.

Statistical Analyses

Data were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) using Prism 7 software (GraphPad, Inc., San Diego, CA, United States).

RESULTS

MWCNT Instillation Increases MMP12 Expression in BAL Cells of Wild-Type Mice

To explore MMP12 involvement in granuloma development, we examined the time course of *Mmp12* expression. C57BL/6J wildtype mice were instilled with MWCNT and BAL cells were collected after 3, 10, 20, and 60 days. Quantitative RT-PCR (qRT-PCR) of BAL cells revealed significant increases in *Mmp12* mRNA expression at 3 days (7.6-fold) with a peak at 10 days (322-fold) and sustained elevation at 60 days (33-fold) when compared to PBS-instilled mice (sham controls) (**Figure 1A**). Similarly, immunofluorescence showed MMP12 protein expression to be upregulated in wildtype BAL cells (**Figure 1B**). MMP12 appeared most prominent at 10 days post instillation and persisted to 60 days. In lung tissues from wildtype animals, expression of MMP12 protein was also upregulated around granulomas at 60-days after MWCNT instillation (**Figure 2**).

Progression of Granuloma Formation Is Attenuated in MWCNT-Instilled *Mmp12* KO Mice

The effects of MMP12 on granuloma formation were examined in *Mmp12* KO and wildtype mice instilled with MWCNT. Pulmonary histological changes were observed in both *Mmp12* KO and wildtype mice at 3, 10, 20, and 60 days post MWCNT instillation compared to PBS controls (**Figure 3A**). Granulomas formed in wildtype mice were detected as early as 10 days post instillation. These early granulomas were poorly formed, but by 60 days post instillation, granulomas appeared to be well defined. Surprisingly, no histological differences in granuloma formation were noted acutely in *Mmp12* KO mice compared to wildtype at days 3 and 10. In contrast, by 20 days after MWCNT instillation, granulomas in *Mmp12* KO mice appeared to be resolving and by 60 days were smaller and less well-formed. None of the time points showed evidence of necrosis or caseation. Histological analyses at 60-days post instillation (**Figure 3B**) were scored based upon size and frequency of granulomas. Scores were significantly ($p = 0.01$) less in *Mmp12* KO mice. Trichrome staining revealed no fibrosis in MWCNT-instilled *Mmp12* KO mice (data not shown).

Mediastinal Lymphadenopathy Is Attenuated in *Mmp12* KO Mice at 60 Days

MWCNT promoted an exacerbated lymphadenopathy in wildtype mice (**Figure 4A**). We hypothesized that as granulomas

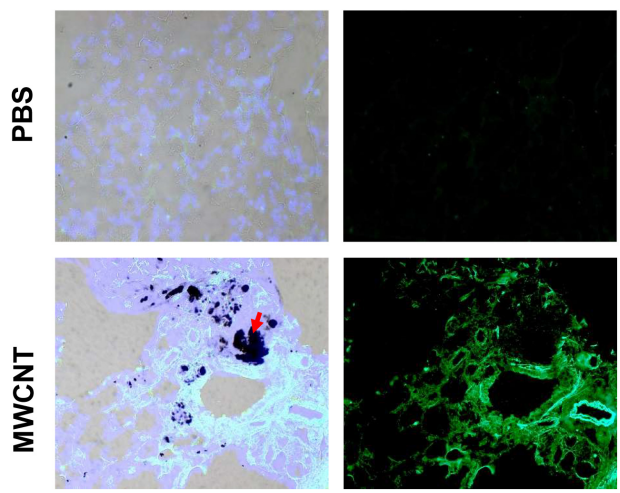

FIGURE 2 | MMP12 protein is upregulated in pulmonary granulomas of 60-day MWCNT-instilled C57BL/6 mice. Representative bright-field and immunofluorescence images for MMP12 protein indicate very minimal to no staining in PBS instilled mice, but increased protein around granulomas in MWCNT-instilled C57BL/6 at 60-days. Black aggregates in (lower left, red arrows) bright-field images are MWCNT accumulations in the tissue.

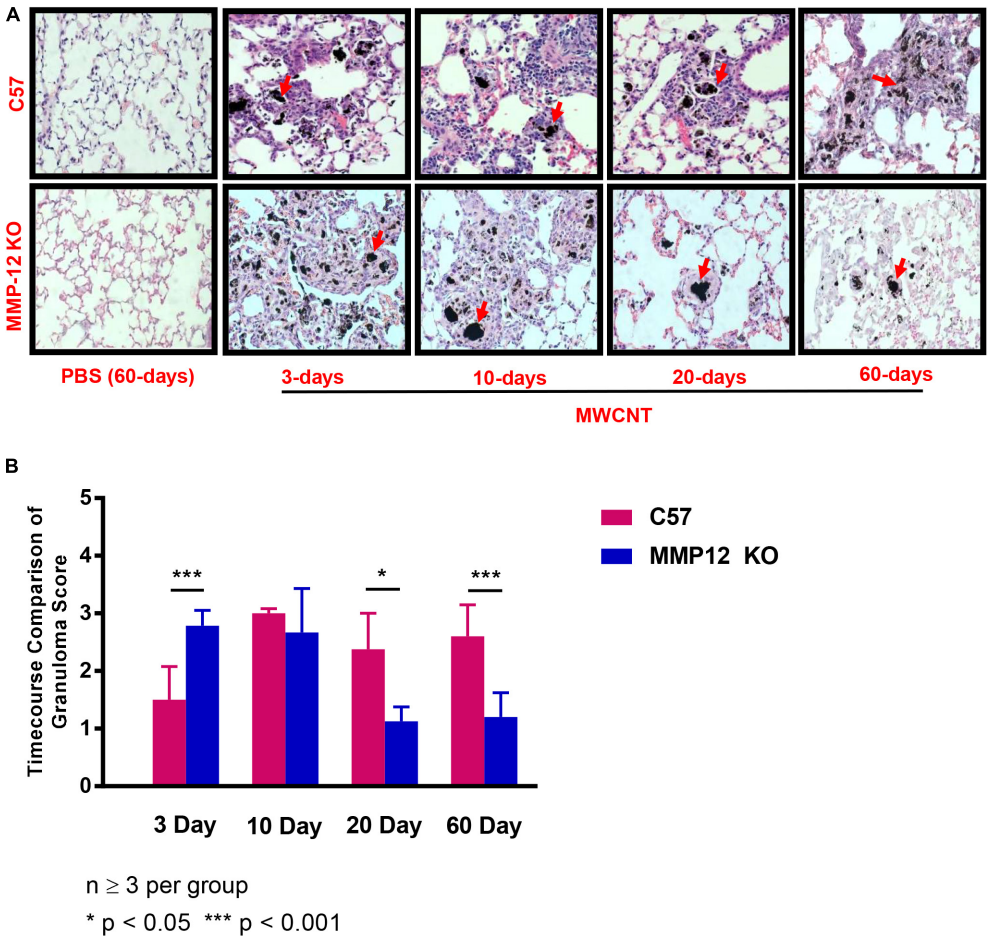

FIGURE 3 | Granulomas from *Mmp12* KO mice are resolving at 60 days. **(A)** Histological examination of H&E-stained lung tissue sections from MMCNT-instilled C57BL/6 and *Mmp12* KO mice indicate loosely formed granulomas as early as 3 days in *Mmp12* KO mice with resolution beginning at 20 days in contrast to C57BL/6. Representative bright field images illustrating granuloma formation in MWCNT-instilled mice at 60 days show decreased size and number of granulomas in *Mmp12* KO compared to C57BL/6 mice. Red arrows indicate deposition of MWCNT in the lung. **(B)** Granuloma scoring (mean ± SEM) of *Mmp12* KO as compared to C57BL/6. *Mmp12* KO lung scores were significantly less than those of C57BL/6 at 60 days (***$p < 0.001$). Sections were evaluated by two independent observers from $n = 6$ lungs for each group.

in *Mmp12* KO mice were diminished at 60 days, mediastinal lymphadenopathy would also be attenuated in these mice. As predicted, MWCNT-instilled *Mmp12* KO mice exhibited reduced mediastinal lymph node sizes compared to wildtype at 60 days after instillation (**Figure 4**). It should be noted that at 10 days, there were no differences in mediastinal lymph node volume between wildtype and *Mmp12* KO mice. Granulomas were not present in the lymph nodes of either wildtype or *Mmp12* KO mice (**Figure 4B**). However, MWCNT are present in wildtype and *Mmp12* KO mice at 10 days and MWCNT deposition is increased in both at 60 days.

MWCNT-Instilled *Mmp12* KO Mice at 60 Days Have Increased Macrophage Influx in BAL

Surprisingly, despite the resolution of granulomatous inflammation at 60 days, MWCNT-instilled *Mmp12* KO mice had significantly ($p \leq 0.05$) increased numbers of macrophages in BAL fluid compared to *Mmp12* PBS controls or MWCNT-instilled wildtypes (**Table 1**). These results suggest that macrophage influx may be involved in the resolution of granulomatous inflammation.

MMP12 Deficiency Does Not Affect CCL2 Expression in BAL Cells and Fluids

Because of the increased number of macrophages in BAL fluid of MWCNT-instilled *Mmp12* KO mice at 60 days despite the granuloma resolution, we investigated the monocyte/macrophage chemokine CCL2. Unexpectedly, *Ccl2* gene expression in BAL cells was not different in MWCNT-instilled *Mmp12* KO mice compared to wildtype despite the histological resolution (**Figure 5A**). In order to determine whether CCL2 was elevated in alveolar spaces, BAL fluid was analyzed. CCL2 protein was elevated in BAL fluids from

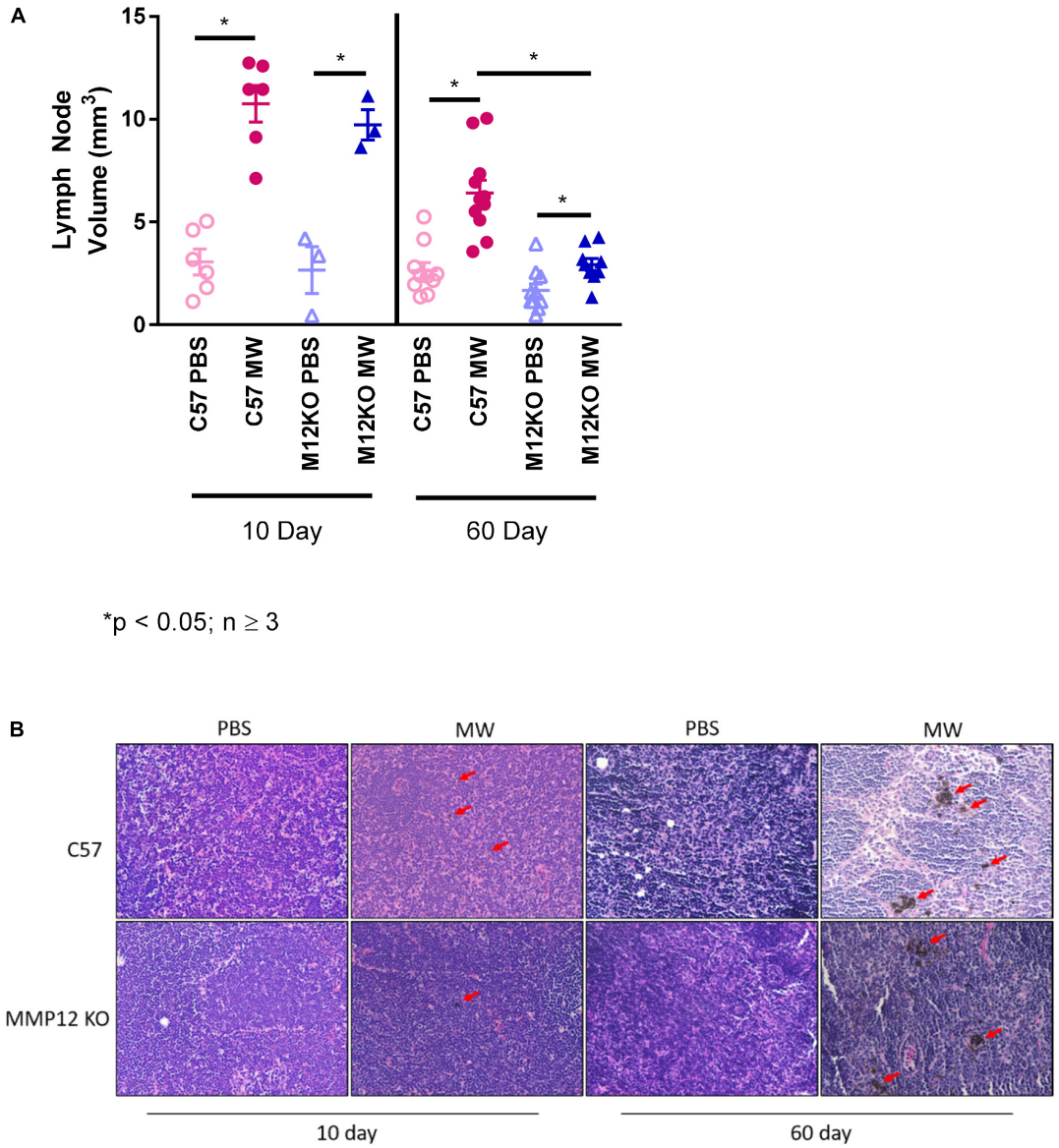

FIGURE 4 | Mediastinal Lymph node volume is decreased at 60-days in MWCNT-instilled *Mmp12* KO mice. **(A)** At 10 days lymph node volumes were not significantly different between MWCNT-instilled C57BL/6 and *Mmp12* KO mice. However, at 60 days, lymph node volumes from MWCNT-instilled *Mmp12* KO mice were significantly decreased compared to C57BL/6 (*$p \leq 0.05$, $n \geq 3$/group). **(B)** Carbon particle deposition can be seen in both C57BL/6 and *Mmp12* KO mice at 10 days (red arrows) and is more prominent at 60-day (red arrows) as compared to respective lymph nodes from PBS-instilled mice.

MWCNT-instilled *Mmp12* KO and wildtype mice compared to PBS-instilled controls and increased levels did not differ between the two mouse strains (**Figure 5B**).

PPARγ Expression Is Increased in *Mmp12* KO Mice Compared to Wild Type

We postulated that MMP12 levels might be higher in PPARγ KO mice. As shown in **Figure 6**, MMP12 expression was significantly elevated in BAL cells of PBS-instilled *Pparγ* KO mice and further increased after MWCNT instillation compared to wildtype. These findings suggested a PPARγ regulatory role in MMP12 expression. Based on these data, we hypothesized that PPARγ might be elevated in BAL cells from *Mmp12* KO mice since granuloma formation was decreased. At 60 days, *Mmp12* KO BAL cells from MWCNT-instilled mice exhibited elevated PPARγ expression in contrast to wild-type where PPARγ was decreased (**Figure 7A**). In order to confirm whether PPARγ was active in *Mmp12* KO mice, BAL cells were stained with anti-PPARγ antibody. Wildtype mice instilled with MWCNT exhibited decreased PPARγ protein. PBS-instilled *Mmp12* KO mice showed increased PPARγ protein, which further increased after MWCNT instillation (**Figure 7B**).

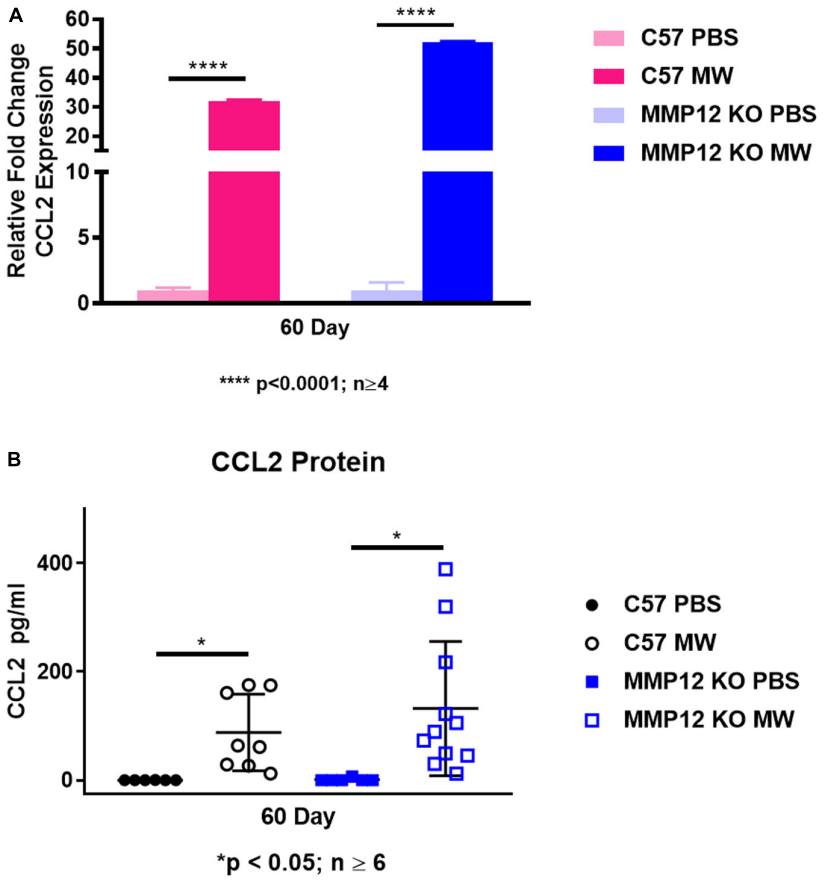

FIGURE 5 | *Ccl2* gene expression and protein are elevated in both C57BL/6 and *Mmp12* KO mice at 60 days after MWCNT instillation. **(A)** *Ccl2* gene expression is increased in BAL cells of both C57BL/6 and *Mmp12* KO MWCNT-instilled mice compared to PBS controls (***$p < 0.0001$; $n \geq 4$). **(B)** CCL2 protein from the BAL fluid is increased in both C57BL/6 and *Mmp12* KO MWCNT-instilled mice compared to PBS (*$p \leq 0.05$; $n \geq 6$).

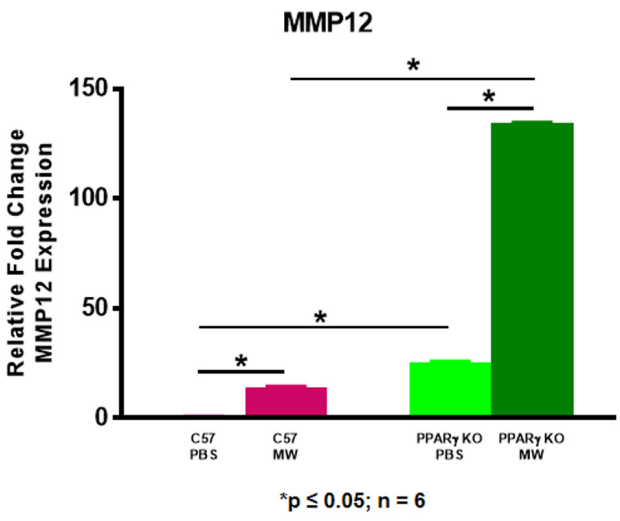

FIGURE 6 | MMP12 is elevated in MWCNT-instilled *Pparγ* KO BAL cells. *Mmp12* gene expression in *Pparγ* KO mice is significantly increased intrinsically, and further increased after MWCNT instillation compared to C57BL/6 (*$p \leq 0.05$; $n = 6$/group).

IFN-γ Gene and Protein Expression Are Not Increased in MWCNT-Instilled *Mmp12* KO Compared to Wild Type

We investigated IFN-γ expression in the MWCNT murine model and found that IFN-γ gene expression was decreased in MWCNT-instilled *Mmp12* KO mice compared to wild type (**Figure 8A**). IFN-γ protein was evaluated by immunostaining of BAL cytospins to confirm the differences. MWCNT-instilled wildtype mice demonstrated prominent IFN-γ protein expression. In contrast MWCNT-instilled *Mmp12* KO mice exhibited almost no detectable IFN-γ (**Figure 8B**).

DISCUSSION

The current findings in the murine MWCNT granuloma model highlight the importance of MMP12 in granuloma pathophysiology and complement findings in sarcoidosis. Our previous and current reports demonstrate that inflammation and granuloma formation in the MWCNT model are associated with a marked increase in MMP12 expression as early as 10 days post instillation with a significant increase persisting

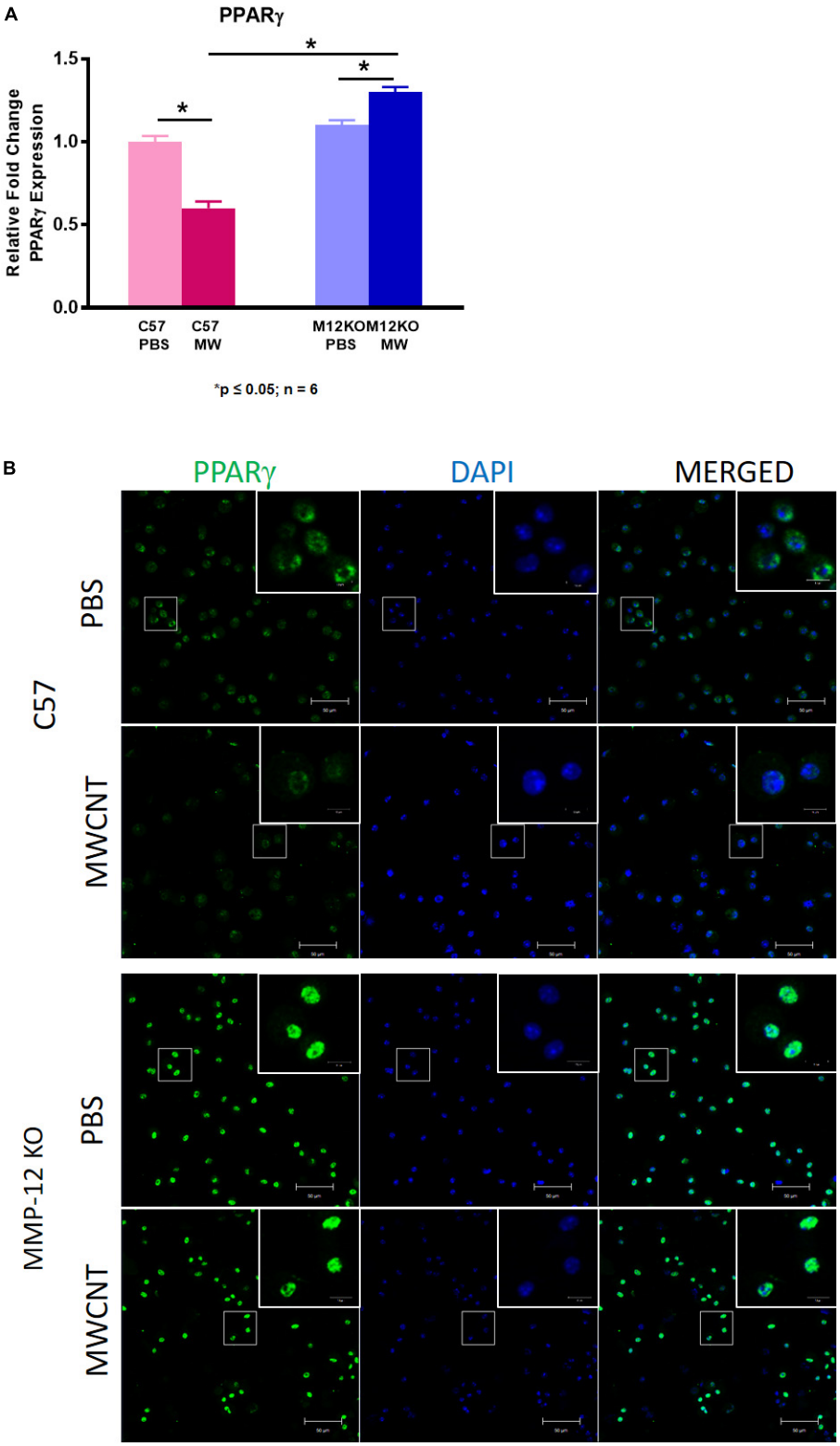

FIGURE 7 | *Pparγ* gene expression and protein are increased in 60-day MWCNT-instilled *Mmp12* KO mice compared to C57BL/6. **(A)** *Pparγ* gene expression is decreased in MWCNT-instilled C57BL/6 mice compared to PBS controls. In contrast, *Pparγ* is significantly increased in MWCNT-instilled *Mmp12* KO mice compared to PBS controls (*$p \leq 0.05$; $n \geq 6$ /group). **(B)** Immunofluorescent anti-PPARγ staining of BAL cells from PBS-instilled mice show constitutive PPARγ protein as indicated by green fluorescence, while MWCNT-instilled C57BL/6 show decreased expression. PBS instilled *Mmp12* KO mice show intrinsically high levels of PPARγ protein, and with MWCNT, PPARγ protein remains highly expressed (representative figure of $n = 3$).

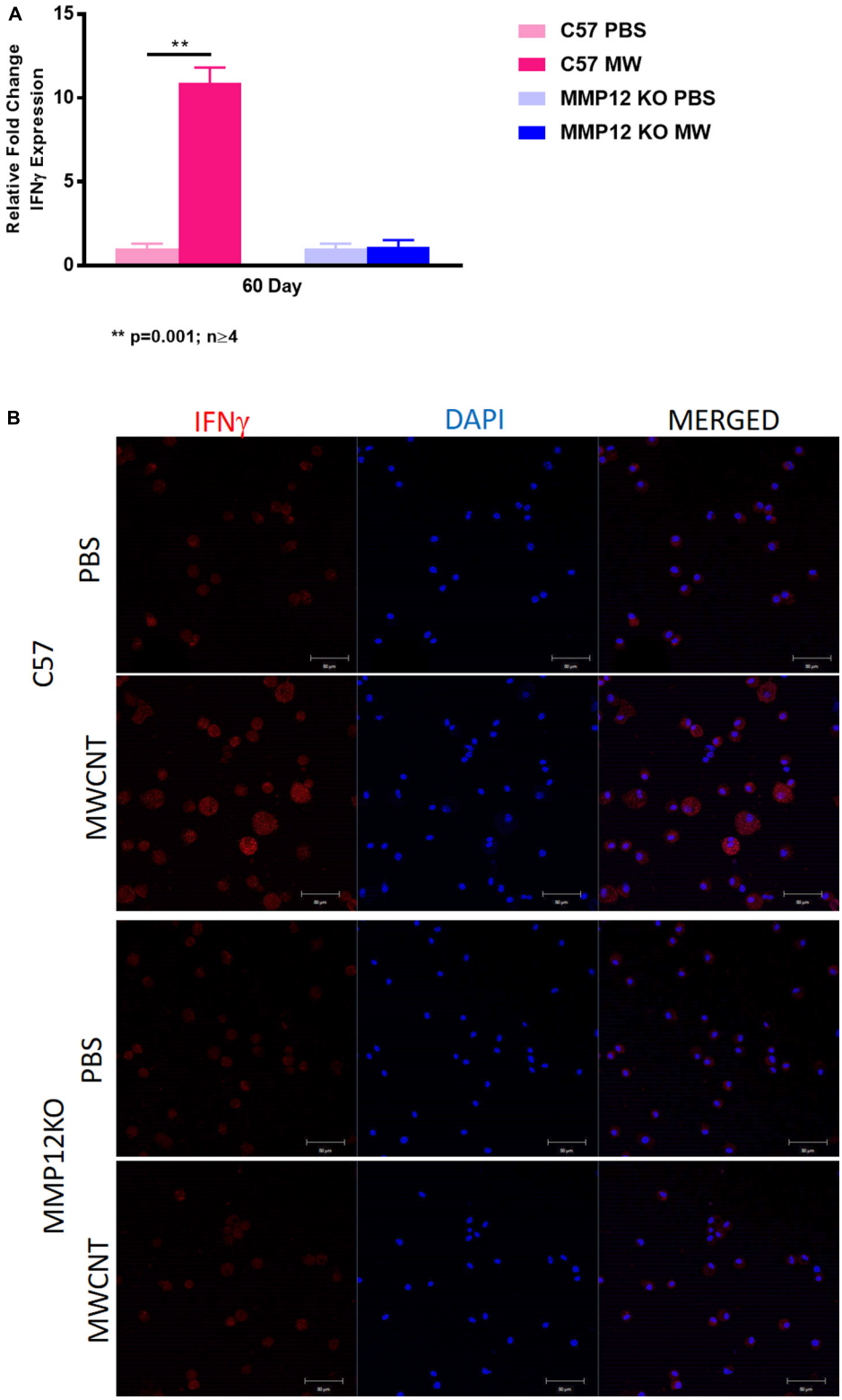

FIGURE 8 | IFNγ gene expression and protein are not increased in MWCNT-instilled *Mmp12* KO compared to C57BL/6. **(A)** *Ifn*γ gene expression is significantly (**$p = 0.001$; $n \geq 4$) increased in C57BL/6 mice instilled with MWCNT compared to PBS controls. In *Mmp12* KO mice, *Ifn*γ gene expression is not increased by MWCNT instillation compared to PBS controls. **(B)** Immunofluorescent anti-IFNγ staining of BAL cells from PBS-instilled mice show minimal IFNγ protein, while MWCNT-instilled C57BL/6 show marked IFNγ protein as indicated by red fluorescence. Both PBS and MWCNT-instilled *Mmp12* KO mice showed minimal staining for IFNγ protein (representative figure of $n = 3$).

to 60 days (4). These data suggest that MMP12 is required for the chronic stages of inflammation associated with granuloma formation. In sarcoidosis, MMP12 constitutes one of the most highly expressed genes in granulomatous lung tissues (12). Our previous comparative transcriptional survey of alveolar macrophages from sarcoidosis patients and MWCNT-instilled mice also revealed marked MMP12 elevation in both species (13). Numerous previous studies have also shown that MMP12, an elastase enzyme predominantly produced by macrophages, is an important mediator of both acute and chronic lung injury and directly involved in development of inflammatory responses (9, 10). MMP12 mechanisms involve degradation of the extracellular matrix protein, elastin, into fragments which can act as a chemoattractant for macrophage recruitment (20).

The monocyte/macrophage chemokine CCL2 has been linked to granuloma formation in other animal model systems (21) and transcriptional studies also indicated elevated expression in both MWCNT instilled mice and sarcoidosis patients (13, 14). Elevation of CCL2 in BAL cells was unexpected in *Mmp12* KO mice at 60 days after MWCNT instillation when granulomas had resolved. In our previous studies, CCL2, which is produced by macrophages, was consistently elevated in parallel with MWCNT-induced granuloma formation (4, 7). Upregulation of CCL2 in BAL fluid is also characteristic of patients with pulmonary sarcoidosis (22, 23). We noted that an influx of BAL macrophages accompanied the granuloma resolution in *Mmp12* KO mice at 60 days. Whether this is driven by CCL2 is unclear. Recent studies have suggested that CCL2 functions may extend beyond its original characterization as a chemoattractant [reviewed in Gschwandtner et al. (24)]. Additional functions attributed to CCL2 have included adhesion, polarization, and effector molecule secretion, and many are context-dependent and may be synergistic with other inflammatory stimuli (24). Gene expression analyses in both MWCNT-instilled mice and sarcoidosis indicated a multitude of elevated inflammatory mediators that may modify CCL2 effects (12, 13). Additional studies will be required to define the complex role of CCL2 in MMP12 regulation.

The current findings also noted a lack of IFN-γ upregulation in 60-day MWCNT-instilled *Mmp12* KO mice in contrast with wildtypes in which IFN-γ was significantly increased as noted in previous MWCNT studies (7, 25). Elevated IFN-γ in sarcoidosis patients with pulmonary disease has been well-reported in the

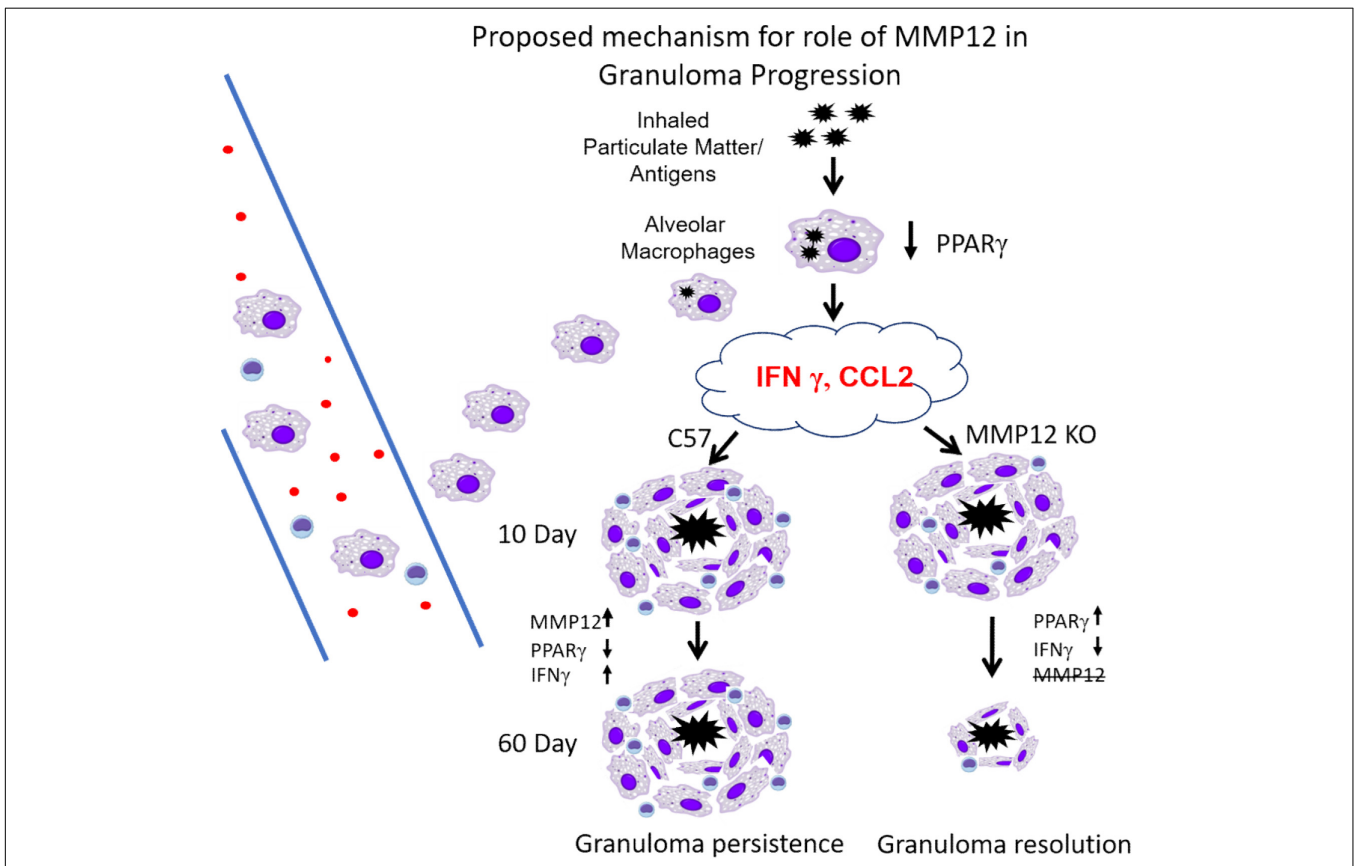

FIGURE 9 | Proposed mechanism for role of MMP12 in granuloma progression. Initial exposure to inhaled particulate matter/and or antigens triggers secretion of several cytokines (CCL2, IFNγ), with further recruitment of alveolar macrophages/monocytes and T cells. Once on site, macrophages produce additional cytokines which promote the formation of multinucleated giant cells and retention of T cells. With MMP12 upregulation (C57/Bl6) persistent PPARγ decrease and IFNγ increase results in granuloma persistence. Whereas as in the absence of MMP12 (*Mmp12 KO*) PPARγ increases and IFNγ decreases resulting in granulomas resolution.

literature (26, 27, 28). Upregulated expression of IFN-γ signaling pathways was also found in both MWCNT-instilled wildtype mice and sarcoidosis patients in our recent transcriptional survey of alveolar macrophages from both groups (13, 14). Thus, the current studies emphasize an association between IFN-γ and granulomatous changes in the lung. In contrast to absence of IFN-γ, expression of PPARγ was elevated in *Mmp12* KO mice at 60 days post MWCNT instillation. PPARγ is a nuclear receptor that regulates expression of genes involved in lipid homeostasis and inflammation in immune cells especially macrophages (29, 30). Interestingly, PPARγ and IFN-γ exhibit mutually antagonistic properties (8, 31) which may explain, in part, our observations. However, further studies are needed to better elucidate the interconnected role of IFN-γ – PPARγ pathways in *Mmp12* KO mice.

Our previous studies demonstrated that PPARγ deficiency exacerbates granuloma formation in the MWCNT murine granuloma model (7). The relationship between PPARγ and MMP12 in granuloma formation has not been previously described, and the current data show for the first time an inverse relationship between PPARγ and MMP12 in mediating pulmonary granulomatous inflammation. MMP12 expression is increased by PPARγ deficiency as shown by the MWCNT experiments in PPARγ KO mice. In *Mmp12* KO mice, both PPARγ expression and activity increased with MWCNT instillation, suggesting that MMP12 deficiency enhances PPARγ. Overall, our data suggest that PPARγ pathways may contribute to the reduction of granuloma formation in *Mmp12* KO mice.

Evidence from the present study and previous studies (4, 7, 16, 25) is summarized in **Figure 9**. We propose that an initial exposure to inhaled particulate matter and/or various antigens (which may include bacterial components such as mycobacterial peptides) triggers the alveolar macrophage secretion of several cytokines (CCL2, IFNγ) with further recruitment of alveolar macrophages/monocytes and T cells. Once on site, macrophages produce additional cytokines which promote the formation of multinucleated giant cells and retention of T cells. With MMP12 upregulation (C57/Bl6), persistent PPARγ decrease and IFNγ increase results in granuloma persistence. Whereas in the absence of MMP12 (*Mmp12 KO*), PPARγ increases and IFNγ decreases, resulting in granuloma resolution.

Decades of research suggest that the etiology of sarcoidosis may be multifactorial and complex, as illustrated by the multiple environmental factors which have been associated with sarcoidosis disease development [reviewed by Judson (32)]. Studies of lung tissues from sarcoid patients have found silica, aluminum, and titanium as well as carbon nanotubes (33). Fibrotic granulomatous lung disease together with elevated CCL2 and MMP12 gene expression as we have described in the wildtype murine MWCNT model have been reported in a rat model of chronic silicosis (34). This model, however, lacks some key sarcoidosis features such as elevated IFN-γ and also does not exhibit any MMP12 protein, unlike the MWCNT model in which high levels of MMP12 protein were detected in both alveolar macrophages and lung tissues from MWCNT-instilled mice. A recent review of available animal models of sarcoidosis concluded that no single model faithfully reproduces all aspects of sarcoidosis pathology (35), but the models may help in evaluating selective sarcoidosis pathways that can be reproduced in the models.

CONCLUSION

This study demonstrates that MMP12 deficiency reduces pulmonary granuloma progression and highlights a critical role for MMP12 in the chronicity of granulomatous inflammation. Potential mechanisms involved in granuloma resolution require further exploration, including identifying how the down-regulation of IFN-γ results in elevated PPARγ, and a better understanding of how CCL2 promotes macrophage recruitment. Deciphering MMP12–orchestrated mechanisms in granuloma formation can lead to novel approaches for treating sarcoidosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC of East Carolina University.

AUTHOR CONTRIBUTIONS

NN, ES, MM, NL, DO, and DT: acquisition of data. AMo, AMa, WK, BB, SG, LS, and MJT: concept and design. BB, AMa, and MJT: analysis, interpretation, and drafting of manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

FUNDING

This article was supported by the BSOM-Internal Seed/Bridge Grant Program, ECU (AMo), NIH grant ES025191 to MJT and CHHE P30 ES025128.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 19 November 2020
doi: 10.3389/fimmu.2020.545413

Treatment of Sarcoidosis: A Multidisciplinary Approach

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Sarcoidosis is a systemic disease of unknown etiology defined by the presence of noncaseating granulomatous inflammation that can cause organ damage and diminished quality of life. Treatment is indicated to protect organ function and decrease symptomatic burden. Current treatment options focus on interruption of granuloma formation and propagation. Clinical trials guiding evidence for treatment are lacking due to the rarity of disease, heterogeneous clinical course, and lack of prognostic biomarkers, all of which contribute to difficulty in clinical trial design and implementation. In this review, a multidisciplinary treatment approach is summarized, addressing immunuosuppressive drugs, managing complications of chronic granulomatous inflammation, and assessing treatment toxicity. Discovery of new therapies will depend on research into pathogenesis of antigen presentation and granulomatous inflammation. Future treatment approaches may also include personalized decisions based on pharmacogenomics and sarcoidosis phenotype, as well as patient-centered approaches to manage immunosuppression, symptom control, and treatment of comorbid conditions.

Keywords: therapeutics, immunosuppression, sarcoidosis, drug-related side effects and adverse reactions, treatment outcome, granuloma

OPEN ACCESS

Edited by:
Mary Jane Thomassen, East Carolina University, United States

Reviewed by:
Juerg Hamacher, Lindenhofspital, Switzerland
Nadera J. Sweiss, University of Illinois at Chicago, United States

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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *24 March 2020*
Accepted: *22 October 2020*
Published: *19 November 2020*

Citation:
Gerke AK (2020) Treatment of Sarcoidosis: A Multidisciplinary Approach. Front. Immunol. 11:545413. doi: 10.3389/fimmu.2020.545413

INTRODUCTION

Sarcoidosis is a systemic disease of unknown etiology that can cause organ dysfunction and diminished quality of life. The disease is diagnosed by a constellation of radiographic, clinical and histopathologic findings; it is most often defined by the presence of noncaseating granulomatous inflammation that occurs in the absence of infection, exposures, malignancy, or alternative immune-related disease. Lung and thoracic lymph nodes are most often involved, but any organ can be affected, with multi-system involvement having a worse prognosis. Treatment is directed at alleviating organ dysfunction, preventing irreversible scarring, and improving quality of life. Herein, we review the indications for treatment, pharmacotherapy, treatment duration, side effects, adjunct non-pharmacologic therapies, and outcomes for patients.

BRIEF PATHOGENESIS

The sarcoidosis granuloma is formed by a distinct conglomeration of multinucleated giant cells and epithelioid macrophages surrounded by a rim of CD4+ T cells (1). Less abundantly, CD8+ T cells and B cells can be found in the surrounding rim. The granulomatous inflammation seen in sarcoidosis is thought to be a dysregulated antigenic response due to an unknown environmental exposure in a genetically susceptible individual. Loci that house antigen presentation genes such as HLA class II and BTNL-2 have been linked to development of sarcoidosis, as well as certain disease phenotypes (2–4). Mycobacterial antigens have been proposed based on studies showing heightened immune responses of both peripheral macrophages and bronchoalveolar (BAL) fluid of patients with sarcoidosis to mycobacterial proteins including mKatG and ESAT-6 (5, 6). Similarly, *Propionibacterium acnes* has also been proposed as an etiologic agent given the higher frequency of genetic material found in sarcoidosis granulomas compared to controls, as well as similar exaggerated immune response to *Priopionibacteria* in sarcoidosis T cells compared to normals (7). It is also likely that dendritic cells play an important role in the presentation of antigen and continued immune response, although the mechanisms are not well-understood (8). More recent data would also suggest that not only is sarcoidosis a disease of heightened Th1 immune response, but also potential dysfunction of regulatory immune cells and immune 'exhaustion' with failure to clear an antigenic agent (9, 10). Therefore, therapeutic targets currently include suppression of inflammation, improvement of the regulatory capacity of the immune system, and modulating the antigen or antigen presenting capacity of the immune system.

The basis of treatment of sarcoidosis is regulation of the heightened immune response and suppression of granulomatous inflammation in order to prevent dangerous interference with organ function (as seen in the eye or the heart) and to prevent eventual scarring and fibrosis as seen in the lungs. Current standard of care focuses on suppressing highly activated macrophages and T cells and their production of cytokines such as TNF-α, IL-1, IFN-γ, and IL-6. More recent study has also suggested a possible role of anti-B cell therapy given the presence of B cells in the granuloma and increased B cell activating factor (BAFF) in sarcoidosis patients (11). Additionally, the increasing evidence of a Th17 response (as seen in autoimmune disease) in addition to a strong Th1 mediated response potentially suggests more therapeutic targets (12). Last, cytokine-specific biologics and treatment of mycobacterial infection have emerged as potential future alternatives. **Figure 1** illustrates current and investigational therapies for sarcoidosis based upon pathogenesis.

TREATMENT APPROACH

To date, treatment of sarcoidosis is largely guided by small, uncontrolled trials and expert consensus (13–19). A few randomized controlled trials (RCTs) have been performed, but trials are limited by the rarity of disease, heterogeneity of disease presentation and progression, and lack of standardized, responsive outcome measures (20, 21). Additionally, even past negative trials may not completely negate the efficacy of certain compounds, as study design is difficult due to the idiosyncrasy of the disease itself (21). A number of suggested treatment algorithms have been published, most of which are based upon initiation of corticosteroids in a symptomatic patient with abnormal function or imaging studies, followed by tapering of steroids over a minimum of 1 year (22, 23). Second and third-line agents can be added based on lack of response to therapy, toxic side effects, or inability to taper corticosteroids (**Table 1**). Importantly, outcomes of symptomatic response and toxicity profile can vary from patient to patient, emphasizing the need for a patient-centered approach. Objective outcomes can include imaging studies (e.g., chest X-rays, computed tomography (CT), positron emission tomography (PET) scans, MRI), pulmonary function tests, walk distance, and laboratory testing (e.g., liver function, blood counts, chemistries, calcium). Subjective assessments can include (but are not limited to) dyspnea, cough, fatigue, cardiac symptoms, neurologic symptoms, and pain. In some patients, the objective and subjective outcomes may not correlate, adding to the complexity of management decisions (24). Therefore, management of patients with sarcoidosis often requires a three-pronged approach: treatment of symptomatic granulomatous inflammation, assessment of comorbid conditions, and tempering of immunosuppressive toxicities (**Figure 2**).

INDICATIONS FOR TREATMENT

Over half of patients with sarcoidosis will incur spontaneous resolution or never have clinical manifestations of the disease, whereas the remaining half will experience a more chronic course, often requiring treatment. Mortality appears to be increasing over time, and burden of disease can be substantial due to complications of the disease or its treatments (25). It is unclear if treatment with corticosteroids alters the natural history of disease; therefore, treatment is recommended only for those with high symptom burden and/or evidence of organ damage (26). For those requiring treatment due to symptoms or suspicion of injury to organs, a stepwise approach to therapy is recommended, although more aggressive therapeutic management can be considered in particularly severe cases of neurologic, ophthalmic, or cardiac involvement (23). In general, there are very few large clinical trials for treatment in sarcoidosis (27). Therefore, in clinical practice, use of corticosteroids and other immunomodulatory agents is often modeled by use on other autoimmune and inflammatory diseases in which suppression of the immune system is desired.

IMMUNOSUPPRESSIVE TREATMENT

Corticosteroids

Corticosteroids are considered first-line treatment by consensus of sarcoidosis providers (28, 29). Multiple uncontrolled studies have shown that corticosteroids suppress production of cytokines that contribute to persistent granuloma formation including TNF-α and

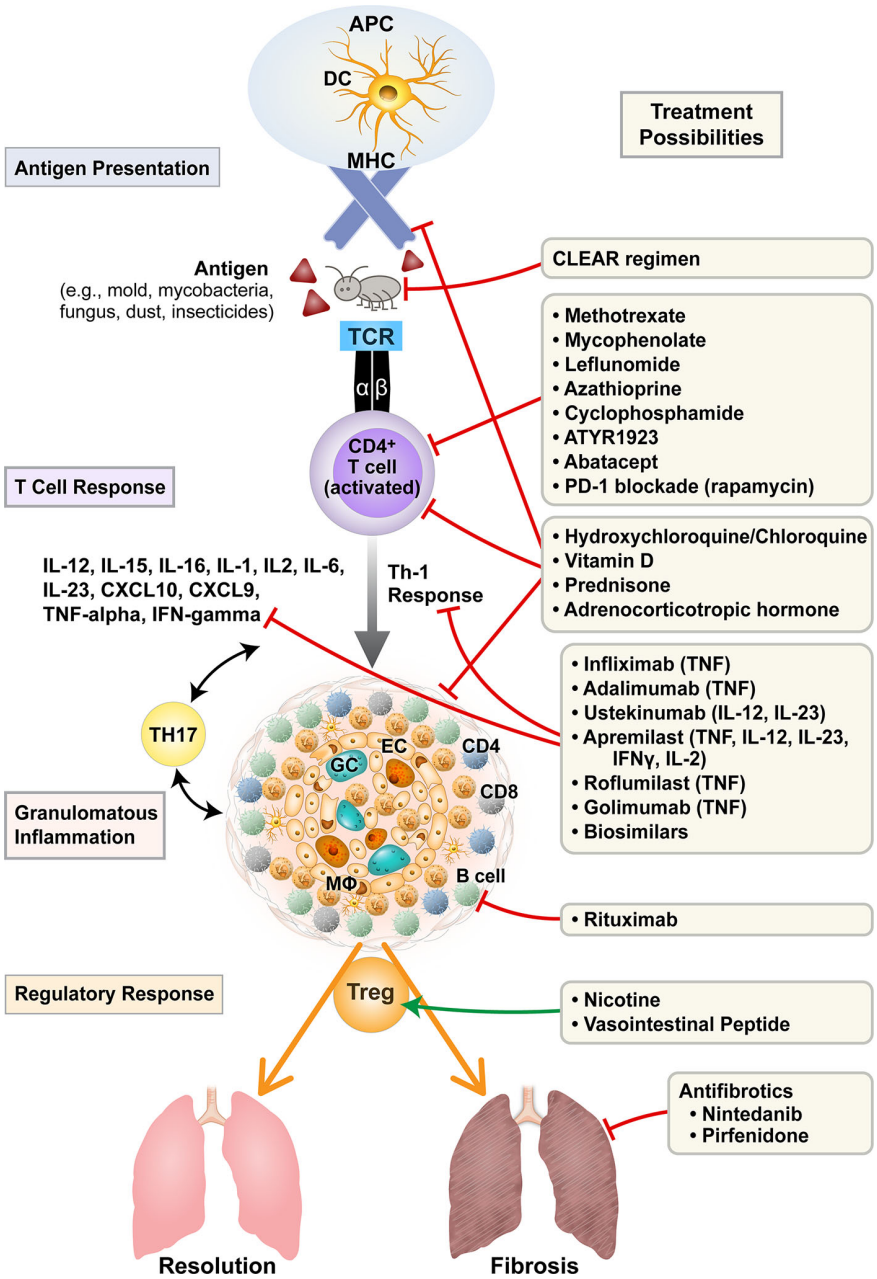

FIGURE 1 | Current and investigational treatments for sarcoidosis based on pathogenesis. Treatments for sarcoidosis target antigen presentation, T cell activation, cytokine/chemokine profiles, propagation of granulomatous inflammation, T-regulatory balance, and the fibrotic response. APC, antigen presenting cell; DC, dendritic cell; MHC, major histocompatibility complex; TCR, T cell receptor; GC, multinucleated giant cell; EC, epitheloid cell; Mφ, macrophage; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; PD-1, programmed cell death protein-1; CLEAR, Combined Levofloxacin; Ethambutol, Azithromycin and Rifampin.

IFN-γ (30, 31). However, only a small number of RCTs with corticosteroids have been performed (26). Although patients have shown improvement in symptoms and biomarkers in the short-term, current evidence is yet unable to show long-term benefit, alteration of natural history or improved mortality (32). Accordingly, the ideal dosing and length of therapy are unknown. A starting dosage of 20–40 milligrams (mg) per day is generally recommended, although a few select patients with severe disease may be initiated on much higher doses in cases of severe neurosarcoidosis, refractory arrhythmias, ophthalmic disease threatening vision loss, or other severe organ damage (29). Lower induction doses may also be reasonable, particularly in pulmonary sarcoidosis, as one early clinical trial of prednisone established improvement in pulmonary infiltrates with 15 mg per day (33). Similarly, a Finnish study from 2002 in pulmonary sarcoidosis used a protocol of 20 mg of prednisolone per day for eight weeks, 15 mg

TABLE 1 | Common therapeutics for treatment of sarcoidosis*.

	Drug name	Suggested dose range	Special treatment issues/monitoring
First-Line Agents	Corticosteroids (Prednisone)	20–40 mg/day initial Dose, tapered to 7.5–15 mg/day	Bone density Eye exams (glaucoma and cataracts) Body Mass Index
Second-Line Agents	Methotrexate	7.5–25 mg/week orally or subcutaneously	Concurrent need for folic acid. Liver function, kidney function, CBC. Can cause hepatotoxicity, GI distress, pneumonitis, mouth ulcers, bone marrow suppression.
	Hydroxychloroquine	200–400 mg/day	Eye exams for retinopathy. Rarely associated with QT elongation (consider drug interactions).
	Leflunomide	10–20 mg/day	Liver function, kidney function, CBC. Can cause neuropathy, hepatotoxicity, GI distress, pneumonitis, bone marrow suppression. In cases of toxicity, can clear more urgently with cholestyramine.
	Azathioprine	50–200 mg/day	Liver function, kidney function, CBC. Consider TPMT level. Can cause hepatotoxicity, GI distress, hypersensitivity reaction, bone marrow suppression.
	Mycophenolate	500–3,000 mg/day	Liver function, kidney function, CBC. Associated with GI distress, bone marrow suppression. Enteric coated option available (different dose range).
Third-Line Agents	Infliximab	3–5 mg/kg intravenously at weeks 0, 2, and every 4–8 weeks thereafter	Tuberculosis Testing Caution in heart failure. Allergic reactions possible with injections. Associated with demyelination syndrome, malignancy, and sarcoid-like reactions.
	Adalimumab	40 mg subcutaneous every 1–2 weeks	Similar precautions and adverse reactions as infliximab.

Other therapeutic options can be considered in some cases (e.g., cyclophosphamide, rituximab, adrenocorticotropic hormone, pentoxifylline).
CBC, complete blood count; GI, gastrointestinal; mg, milligrams; kg, kilograms; TPMT, thiopurine methyltransferase.

per day for 2 weeks, 10 mg per day for 2 weeks, and then inhaled budesonide for 15 months. The results showed improved pulmonary function over a five-year period in patients with Stage II-III disease (34). Long-term doses above 40 mg per day are not recommended for most cases of sarcoidosis due to high risk of corticosteroid-induced toxicity and little added physiologic benefit. For example, a study in Japan revealed increased morbidity and mortality in patients with cardiac sarcoidosis who were treated with higher doses of corticosteroids (greater than 40 mg per day) compared to lower doses (less than 30 mg per day) (35). Similarly, higher cumulative doses of prednisone in sarcoidosis have been associated with decreased quality of life and increased frequency of emergency department visits (36). Another analysis of differing starting dosages for patients with pulmonary sarcoidosis did not show a strong correlation between outcome and initial dose, indicating that lower doses may be used with similar effect. On the other hand, there was a strong correlation between starting dose and weight gain over a two-year period (37). Taken in total, these studies suggest that toxicity of higher dose corticosteroids is prominent, whereas there appears to be no discernible benefits in disease outcomes on higher doses versus lower doses, particularly for maintenance therapy.

Corticosteroid-Sparing Medications

Although corticosteroids are most often used first-line for sarcoidosis, patients with chronic sarcoidosis requiring prolonged therapy, those with particularly severe disease, and those with significant corticosteroid toxicity may require a corticosteroid-sparing medication. A Delphi consensus of sarcoidosis experts noted that doses of greater than 10 mg of prednisone per day were generally considered too high for long-term therapy and a steroid-sparing agent is often considered in these patients (29). Methotrexate, azathioprine, leflunomide, and mycophenolate are the most common steroid-sparing alternatives applied to treat sarcoidosis. Methotrexate is the most frequently recommended second-line therapy, based on its well-established side effect profile and efficacy in autoimmune disease such as rheumatoid arthritis and psoriasis (29). It is a folic acid antagonist that, through a series of steps, inhibits purine and pyrimidine metabolism, as well as amino acid and polyamine synthesis. The drug may also have other anti-inflammatory effects on T cells, suppressing formation of contributing cytokines by these cells *via* the adenosine a2a receptors (38). Methotrexate has been shown to be an effective steroid-sparing agent in two small clinical intervention studies (one randomized and one non-randomized), where patients showed improvement in vital capacity or symptomatic organ dysfunction while concurrently tapering down on steroids (39, 40). These data are supported by multiple case series and retrospective studies, both in pulmonary and extrapulmonary sarcoidosis (19). A dosage of 7.5 mg to 15 mg per week appears to be effective for most cases, with an overall response rate up to 55%, which may be higher if used in combination with corticosteroids and lower if used as monotherapy (19, 41). However, a significant percentage of patients both in sarcoidosis and rheumatoid arthritis do not respond or have toxicity to methotrexate, which may be reflective of certain pharmacogenomic profiles or genetic predisposition to drug efficacy and/or toxicity (42, 43). The most common adverse events seen with treatment with methotrexate include infections,

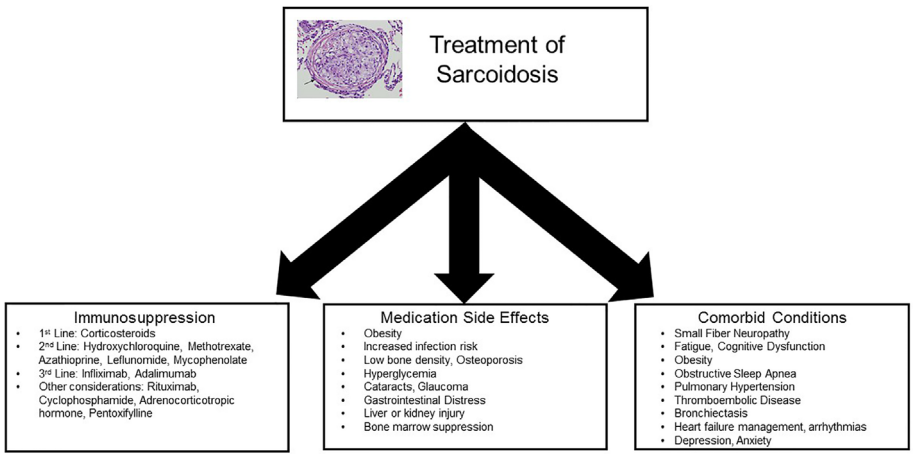

FIGURE 2 | Concepts for treatment of sarcoidosis. Management of patients with sarcoidosis often requires a multidisciplinary approach: treatment of symptomatic granulomatous inflammation, assessment of comorbid conditions, and tempering of immunosuppressive toxicities.

hepatotoxicity, gastrointestinal distress, malaise, and leukopenia. Patients should be started on folic acid concurrently with methotrexate and regular monitoring of liver function, blood counts, and kidney function should be performed.

Similarly, leflunomide, a dihydroorotase inhibitor that inhibits dividing lymphocytes, is often used an alternative (or even additionally, in some cases) to methotrexate in sarcoidosis. Its safety and efficacy are supported by a retrospective study of 76 patients who had progressive sarcoidosis or had failed alternative immunosuppressive medications. A small, but significant improvement was seen in forced vital capacity (FVC) for pulmonary involvement, and a partial or good response was seen in 83% of the patients with extrapulmonary sarcoidosis (44). A steroid-sparing effect was also seen. The side effect and tolerability profiles were similar to studies in other rheumatologic diseases, with diarrhea and liver enzyme elevation being the most common side effects. Respiratory infections and neuropathy were the more serious, but less frequent side effects. A smaller study of 32 patients from 2004 also supported efficacy, with 78% of the patients showing improvement, including in patients who failed to respond to methotrexate (45). Leflunomide has similar lab monitoring requirements to methotrexate. Interestingly, both drugs have been associated with pneumonitis in rheumatoid arthritis, although leflunomide is less common than methotrexate for this confounding reaction (46).

Azathioprine, an inhibitor of purine metabolism, can also be considered as a steroid-sparing agent in patients with sarcoidosis, with similar efficacy compared to methotrexate to improve FVC and diffusing capacity (DLCO), and to taper corticosteroids (47). Azathioprine acts to decrease production circulating T cells and B cells, as well as increase apoptosis of circulating lymphocytes. Toxicity of azathioprine is affected by an individual's thiopurine S-methyltransferase (TPMT) activity which plays a large role in drug metabolism. Genetic deficiency of this enzyme can increase the risk of myelosuppression and hepatotoxicity, although the role for routine testing prior to therapy is unclear given that most people who have myelosuppression do not have deficiency of TPMT (48, 49). The main side effects of azathioprine are gastrointestinal upset, myelosuppression, infections, and potential increased risk of malignancy (47). Side effects are comparable to methotrexate for use in sarcoidosis, although azathioprine did show a higher rate of infections (34.6% vs 18.1%, p=0.01) in one retrospective analysis (47). Doses of 2 mg per kilogram body weight per day were utilized in one study (50), whereas 100–150 mg per day in another (51).

Mycophenolate mofetil (MMF) is another second-line option that acts by inhibiting purine nucleotide synthesis specifically in lymphocytes and decreases production of autoantibodies by B cells; therefore, the drug is used commonly for immunosuppression in a variety of rheumatologic diseases and interstitial lung diseases (52). The largest series reported for use of MMF in sarcoidosis included 37 patients with primarily pulmonary sarcoidosis treated with MMF due to intolerance of an immunosuppressive regimen or treatment failure (53). A trend towards improved DLCO was seen in patients started on MMF, and a steroid-sparing effect was also noted. In a series of patients with neurosarcoidosis, 7 of 8 patients with central nervous system involvement showed remission of disease after 21 months of therapy (54). Three of four patients who had failed alternative immunosuppressive regimens appeared to respond to MMF. However, two patients with sarcoid myopathy did not appear to benefit from MMF. In all patients, no significant side effects were noted, concluding that this drug had a better tolerability profile than other immunosuppressive options and was effective for neurosarcoidosis with central involvement. Another small series of ten patients with chronic pulmonary sarcoidosis treated with MMF found similar steroid-sparing effect and little to no side effects, supporting its utility and tolerability in this patient population (55).

Antimalarials

Chloroquine and hydroxychloroquine have been frequently used in treatment of sarcoidosis based on early randomized trials that showed

a long-term benefit with chloroquine (56). However, based on the better safety profile, hydroxychloroquine is most often preferred. The mechanisms of action for hydroxychloroquine are varied; it can interfere with antigen presentation, prevent T cell activation, inhibit toll-like receptor signaling, and reduce inflammatory cytokines by T cells and B cells (57). Hydroxychloroquine has been particularly useful in cutaneous disease, hypercalcemia, and in some cases of neurosarcoidosis (58–60). Although gastrointestinal side effects are commonly reported with use of hydroxychloroquine, they are generally mild and well-tolerated (61).

Inhaled Therapies

Inhaled corticosteroids have not been shown to have clear benefit in patients with sarcoidosis in a RCT (62), but given their low side effect profile and plausible mechanism of decreasing airway inflammation, they may play a role in maintenance, acute airway exacerbations, or cough (63, 64). However, given the lack of data and the likely small effects, consensus of sarcoidosis experts do not recommend inhaled corticosteroids for initial treatment of sarcoidosis (29). Another inhaled therapy, vasoactive intestinal peptide (VIP), has shown some potential effects; a single trial in 20 patients with sarcoidosis revealed that inhaled VIP decreased TNF-α production by alveolar macrophages and increased regulatory T cells in the lung. Reduced cough was seen in 75% of trial subjects, indicating its possible use for symptomatic relief (65). However, subsequent studies are needed to further dictate use of these inhaled therapies in clinical practice.

Tumor Necrosis Factor (TNF) Antagonists and Biosimilars

TNF-α is a predominant cytokine, consistently elevated in active sarcoidosis as a product of macrophage activation, particularly at sites of granuloma formation (31). It is a major contributor to propagation of granulomatous inflammation. TNF-α by is elevated in progressive or steroid-resistant disease (66), and soluble TNF-α receptors are higher in BAL fluid of patients with active disease, signaling a role in pathogenesis (67). For this reason, the use of TNF inhibition has been studied as a therapeutic target. The use of TNF antagonists is supported by numerous compelling case reports and series in refractory sarcoidosis. Both infliximab and adalimumab are monoclonal antibodies targeted against TNF itself, with infliximab being chimeric and adalimumab being a humanized monoclonal antibody. In one double-blind, placebo-controlled Phase II trial of 138 patients with chronic pulmonary sarcoidosis, infliximab significantly increased the percent of predicted FVC compared to baseline by 2.5%, whereas placebo did not (68). The drug was not approved based on the perceived lack of clinical significance. Interestingly, however, post-hoc analysis suggested that patients with more severe disease and those with extrapulmonary disease may have derived the greatest benefit (68, 69). In addition, there were some improvements in reticular opacities on chest x-rays and decreases in inflammatory cytokines. A later examination of the data showed that infliximab had some benefit over placebo as measured by a novel extrapulmonary severity scoring tool, but the effect was not sustained after 24 weeks of therapy (70). However, a more recent long-term retrospective review of patients treated with infliximab for pulmonary and extrapulmonary sarcoidosis disputes this finding, as 58.5% of patients showed improvement of disease on pulmonary imaging up to 85 months of follow-up (71). Currently, infliximab is the most well-studied third-line agent and dosage recommendations are 3–5 mg/kg with maintenance therapy every 4–8 weeks after initial loading. The role of concurrent immunosuppression (e.g., methotrexate) to decrease formation of anti-drug antibodies for TNF inhibitors is unclear in sarcoidosis; its use is based on studies performed in rheumatoid arthritis and Crohn's disease (72–74).

Increasing data for adalumimab would suggest a role for this TNF-α antagonist in sarcoidosis (75, 76). In one double-blind RCT of 16 patients with cutaneous sarcoidosis, adalimumab was associated with improved skin lesions (77). An open-label single-center study of 11 patients with refractory pulmonary sarcoidosis treated with 40 mg weekly of adalimumab showed that four patients had at least a 5% improvement in percent-predicted FVC (seven had stable FVC) and five had an improvement of at least 50 meters in 6-min walk distance (6MWD), with a total of eight who had improvement in one or the other (76). Another prospective observational study of ten patients with sarcoidosis refractory steroids and cytolytics, the PET avid activity seen with active inflammation was reduced in nine patients with the addition of adalumimab to the existing regimen, indicating responsive disease (78). In sarcoidosis patients with refractory posterior uveitis, adalumimab was associated with improvement in 85% of patients and stabilization in the remaining, supporting its use for ophthalmic sarcoidosis (79). Similarly, a recent series of 17 patients with refractory ocular sarcoidosis (predominately chronic relapsing panuveitis) showed efficacy of both adalimumab (40 mg subcutaneously every 2 weeks) and infliximab (5 mg per kg every 4–8 weeks) (80). The drugs were associated with an improvement in cells in the ocular anterior chamber, vitritis, macular thickness, and visual acuity, with a mean follow-up of 34 months. Corticosteroids were able to be tapered off in these cases. Adalimumab may also be effective for a proportion of patients who develop antibodies or resistance to infliximab therapy (81).

On the other hand, etanercept, a fusion protein that antagonizes the TNF receptor, has not shown clear efficacy in treatment of sarcoidosis. Therefore, this drug is not recommended for use in sarcoidosis. An open-label Phase II study in stages 2 and 3 pulmonary sarcoidosis was stopped early due to excessive treatment failures in patients treated with etanercept 25 mg subcutaneously twice weekly (82). Although five of seventeen patients appeared to respond to the drug, there were no clinical predictors of response that could be found, including TNF-α levels in the serum or BAL fluid. Similarly, in a double-blind randomized trial of 18 patients with refractory chronic ocular sarcoidosis, only three of the patients in the etanercept arm were able to decrease corticosteroid use, which was similar to the placebo group (83). This lack of efficacy may imply an inability for the drug to penetrate the vitreous cavity or may reflect the different mechanism of action by targeting the receptor, as compared to inhibition of the cytokine directly or lysing the cells that produce it, as infliximab does (83).

Similarly, ustekinumab and golimumab were tested in a three-arm double-blind RCT in chronic pulmonary sarcoidosis patients with a primary endpoint of FVC change (84). Golimumab is a fully human monoclonal IgG1 antibody specific for TNF-α, administered subcutaneously every month. Ustekinumab is an IL-12 and IL-23 inhibitor. After 16 weeks of therapy, no differences were observed between the intervention groups and placebo for lung function, although trends were seen in improvement of skin sarcoidosis. Interestingly, in this trial, the placebo group had improved lung function also, indicating that perhaps patient selection of spontaneously resolving patients could have biased results toward the null.

The advent of biosimilars has provided another, potentially less expensive, option for clinicians who desire an anti-TNF therapy. The biosimilar to infliximab has been tested in rheumatoid arthritis and found to have equivalent efficacy to its original form (PLANETRA trial) (85). A retrospective cohort study in 29 patients with sarcoidosis patients who received the infliximab biosimilar at a dose of 5 mg/kg/month showed improvement in FVC, health-related quality of life, reduction of standardized uptake value (SUV) on PET scans, and decreased sIL-2R biomarker (86). Another retrospective review of 20 neurosarcoidosis patients showed good efficacy and tolerable adverse events with infliximab biosimilar (87). Although promising, it will be important to follow patients closely who are treated with biosimilars; it is unclear if there are differences in immunogenicity that could complicate treatment dosages or switching of therapies (88).

Anti-B Cell Therapy

Sarcoidosis has been associated with altered B cell homeostasis (89), high BAFF (11, 90), hypergammaglobulinemia, the presence of IgA-producing plasma cells near the granuloma (91), and autoimmune antibodies, suggesting that the humoral immunity may be playing a role. Consequently, it has been suggested that targeting of the B cells may influence the disease process (90). Rituximab, a chimeric monoclonal antibody against CD20+ B cells that reduce the mature circulating population, has been investigated in small studies (92–94). In one prospective phase I/II trial in ten patients with refractory pulmonary disease, five patients had a greater than 5% absolute improvement in FVC and five patients improved their 6MWD by 30 meters, with a total of seven patients having a response of one or the other (94). The results did not correlate with a patient's pre-treatment immunoglobulin levels. Interestingly, two patients died of respiratory failure (thought to be due to progressive sarcoidosis) and there was one hospitalization for infectious pneumonia during the study follow-up. Lower et al. retrospectively assessed patients with ocular sarcoidosis (n=4) who were treated with rituximab and found that the therapy was effective as a steroid-sparing agent for three of the four, and well-tolerated except for neutropenia in two patients which resolved with lower doses and Staphylococcus aureus skin infections in another (95). Two of the sarcoidosis patients also had concurrent lung disease and incurred symptomatic pulmonary improvement. At this time, the role of rituximab as a third or fourth-line agent in sarcoidosis remains unclear, but future elucidation of the B cell actions in sarcoidosis will likely help in clarifying the use of this drug.

Antifibrotics

Antifibrotics, now approved for treatment of idiopathic pulmonary fibrosis and progressive fibrotic interstitial lung diseases (ILDs), are also an enticing possibility for fibrotic sarcoidosis. The INBUILD trial, a positive RCT of nintadenib in various progressive fibrotic interstitial lung diseases included a few patients with fibrotic sarcoidosis (96). In the overall population of all patients, there was less decline in FVC (a difference of 107 ml) over 52 weeks as compared to placebo. The sarcoidosis patients were included in a group termed "other fibrosing ILDs" which included patients with sarcoidosis and exposure-related ILDs. This group made up approximately 12% of the study population, and therefore, the effects on purely sarcoidosis are not entirely clear. A trial of pirfenidone specifically for fibrotic sarcoidosis is also ongoing (NCT03260556). Future studies will be necessary to determine how antifibrotics may be incorporated into the management of sarcoidosis patients.

Other Third-Line Agents

A few other immunosuppressive drugs have been reported as potential options in sarcoidosis, but lack a significant body of evidence in the form of RCTs or larger series. For example, cyclophosphamide has been reported as an effective treatment in corticosteroid-resistant disease in both neurosarcoidosis and cardiac sarcoidosis, and has been associated with a lower relapse rate for patients with neurosarcoidosis (97–99). However, with more responsive disease, the risk profile is less desirable than other steroid-sparing agents, making cyclophosphamide harder to justify for long-term treatment in milder disease. Adrenocorticotropic hormone analogue is also undergoing evaluation based on a multicenter RCT in chronic pulmonary sarcoidosis that showed improvement in lung function, imaging, and quality of life, combined with a steroid sparing effect (100). Although the drug holds historical FDA approval, there are little prior data to support its use in sarcoidosis; therefore, ongoing clinical trials will inform future use of this drug. Although less commonly used due to side effects and pill burden, pentoxifylline is an oral non-selective phosphodiesterase inhibitor that decreases cytokine production by suppression of macrophages. Its use is supported by one small RCT with 27 patients supporting a steroid-sparing effect, and one observational study in newly treated patients showing improvement or stability of disease with use of pentoxifylline (101, 102).

ADDITIONAL THERAPIES UNDER INVESTIGATION

Additional therapies targeting a variety of pathogenic mechanisms are also undergoing further study, but do not have enough evidence yet to be incorporated into treatment recommendations. For example, nicotine acts upon NFκB in macrophages to decrease cytokine production and acts to decrease the Th17/T-reg ratio by its effects on CD4+ lymphocytes, leading to evaluation of this compound as a treatment for

sarcoidosis. A small RCT of 13 patients with sarcoidosis treated with transdermal nicotine versus standard treatment alone showed that those treated with nicotine had normalization of their TLR-2 and TLR-9 responsiveness and increased the T-reg response (103). Based on these preliminary data, a clinical trial is ensuing investigating the effect of nicotine on inflammatory biomarkers in patients with sarcoidosis (NCT02265874).

Another trial, entitled the CLEAR trial (Combined Levofloxacin, Ethambutol, Azithromycin and Rifampin), is targeting the hypothesis that mycobacteria are the elusive antigen in some cases of sarcoidosis. A pilot study of thirty patients with cutaneous sarcoidosis showed decrease in size of skin lesions and granulomatous burden with the CLEAR regimen (104). In a similar trial of 15 patients with chronic pulmonary sarcoidosis treated with CLEAR showed an improvement in walk distance, dyspnea (measured by St. George's Respiratory Questionnaire), and FVC, although half of patients had to stop therapy due to intolerance (105). Results from a Phase II RCT in patients with progressive pulmonary sarcoidosis are awaited (NCT02024555).

Other drugs are being repurposed for treatment of sarcoidosis and are actively being investigated. For example, roflumilast, an oral anti-TNF agent approved for asthmatics with frequent exacerbations, is being evaluated for acute exacerbations of fibrotic sarcoidosis (NCT01830959). Apremilast, a phosphodiesterase-4 inhibitor approved for psoriasis that decreases production of TNF-α, interferon γ, IL-2, IL-12, and IL-23 was recently investigated in 15 patients with cutaneous sarcoidosis. In this study, skin lesions improved significantly after 12 weeks of therapy (106). A Phase 2a trial testing abatacept, a CTLA-4–Ig fusion protein that interferes with T-cell activation (currently approved for rheumatoid arthritis), is underway to understand safety and efficacy in chronic sarcoidosis (107). Similarly, a phase I/II trial using ATYR1923 (NCT03824392), a compound that downregulates T cell responses, cytokines and inflammatory fibrosis *via* modulation of neuropilin-2, is also ongoing in pulmonary sarcoidosis (108). Results from these studies may increase options for clinicians treating sarcoidosis.

Additionally, evolving research in granuloma formation and propagation has suggested new potential therapeutic targets. For example, mTORC1 activation of macrophages has been associated with disease progression, and alveolar macrophages from sarcoidosis patients have upregulation of interleukin-1 receptor associated kinases (IRAK1 and IRAK-M) and receptor interacting protein 2 (Rip2) (109, 110). Interference with these pathways may result in decrease of granuloma formation.

The role of vitamin D in prevention or treatment of sarcoidosis has also been debated, based on the potential immunomodulatory role in Th1 inflammation. Vitamin D has been shown *in vitro* to inhibit proliferation of Th1 lymphocytes, as well as suppress antigen presentation and activation of macrophages, all of which could potentially diminish granuloma formation and propagation (111, 112). Vitamin D may also modulate dendritic cell differentiation, decreasing the antigen presenting capabilities of the immune response (113). Clinically, low serum levels of 25-hydroxyvitaminD have been associated with active disease (114). However, treatment of sarcoidosis is complicated by the dysregulated calcium metabolism that occurs with granulomatous inflammation. The macrophage converts vitamin D to its active form, 1,25-dihydroxyvitaminD, *via* 1-alpha-hydroxylase, which is activated in sarcoidosis irregardless of the parathyroid feedback mechanisms which normally control calcium levels. Thereby, up to 10% of patients with sarcoidosis will have hypercalcemia and even a higher percentage will have hypercalciuria, making vitamin D supplementation increasingly risky if not closely monitored. A small clinical trial of 16 sarcoidosis subjects with normal serum ionized calcium levels and vitamin D deficiency showed that treatment with ergocalciferol increased the storage form of vitamin D, whereas decreased the active form (1,25-dihydroxyvitaminD) and angiotensin converting enzyme levels, suggesting an effect on granulomatous inflammation (115). Asymptomatic increases in serum calcium levels were seen in three of the patients in this trial. Larger clinical trials will be necessary for future recommendations regarding vitamin D as a treatment modality regarding both safety and efficacy.

CHOOSING A TREATMENT OPTION

Deciding on the most appropriate treatment regimen for a patient is often a complex interplay of disease characteristics (such as immediate risk of severe organ damage), familiarity of therapies by the clinician, side effect profiles, and patient preferences. The choice can be influenced by a patient's age, alcohol intake, likelihood of pregnancy, concurrent medications, or comorbidities such as diabetes, liver or kidney dysfunction. Given the polypharmacy often involved in the patient regimen, drug interactions should be considered in medication choice. Corticosteroids are most often first line therapy for any type of sarcoidosis, but second or third-line therapies can be considered in cases where corticosteroids are risky (decompensated heart failure, uncontrolled diabetes, severe obesity, uncontrolled hypertension, glaucoma) or these drugs can be added in the more aggressive treatment of life-threatening or severe organ derangement such as can be seen in neurosarcoidosis, ophthalmic injury, or severe infiltrative heart disease. On a more chronic, outpatient regimen, steroid-sparing agents are often used when corticosteroids cannot be reduced to reasonable doses (less than 10–15 mg per day), there is corticosteroid toxicity, or the anticipated course of treatment is lengthy.

Length of Treatment

Duration of therapy, whether with corticosteroids or other immunosuppressive treatment, is generally considered to be approximately 1 year, based on data suggesting an increased risk of relapse with shorter courses (23, 116–118). The British Thoracic Society Sarcoidosis Study found that in patients with asymptomatic, but radiographically evident pulmonary sarcoidosis, who were treated empirically with long-term therapy of 18 months versus "selective" therapy based on development of symptoms or radiographic progression, the patients who received long-term therapy had greater

improvements in symptoms, respiratory function, and radiographic appearances than those in the selectively treated group (119). At this point, there are no sensitive or specific biomarkers to predict relapse, leading to blanket generalization of longer duration (120); however, shorter tapers can be considered based on symptoms and overall response to therapy on an individual basis. This is supported by recent data from a prospective study of 21 patients with pulmonary sarcoidosis showing that most of the improvement in pulmonary function (measured by home spirometry), was seen in the first month after initiation of treatment (121). A smaller additional improvement was seen by three months, indicating that steroids may be able to be tapered within the first three months to a tolerable, long-term dose that would lessen the toxicity for patients. Additionally, this study showed that the improvement in fatigue as measured by the Fatigue Assessment Scale (FAS) and dyspnea as measured by the Medical Research Council dyspnea scale also occurred within the first month of treatment (121).

Organ-Specific Therapy

The question of whether there are organ systems which benefit from a particular steroid sparing agent is not wholly clear, and generally, extrapulmonary sarcoidosis is treated with the same algorithms as pulmonary sarcoidosis (28). However, some extrapolations from prior study have suggested preferences for certain therapies depending on sarcoidosis phenotype. For example, hydroxychloroquine has been suggested as quite effective for cutaneous sarcoidosis, but not considered highly effective for pulmonary disease (60). Hydroxychloroquine also impairs 25(OH)D3-1-α-hydroxylase, and therefore, it is often chosen to treat hypercalcemia (59). Similarly, leflunomide seemed to be more effective for lung, skin, eye, and sinus disease, and less so for neurosarcoidosis and musculoskeletal disease in one retrospective study (44). In another series, MMF was quite effective for neurosarcoidosis, resulting in some favoritism of this drug for treatment of neurosarcoidosis (54). However, a more recent review of long-term (median follow-up 8 years) outcomes of a large series of 234 patients from France, found that a lower risk of neurologic relapse was seen in patients treated cyclophosphamide (HR 0.26, 95%CI 0.11–0.59), methotrexate (HR 0.47; 95%CI 0.25–0.87), and hydroxychloroquine (HR 0.37, 95% CI 0.15–0.92) (97). This effect was also seen in risk of overall relapse rate, in addition to neurologic relapse. On the other hand, mycophenolate and azathioprine were not associated with a decreased relapse rate, either overall or specifically in the neurologic system. Infliximab was associated with decreased overall relapse rate (neurologic or other organ), and a trend was seen for decrease in neurologic relapses (HR 0.16, CI 0.021–1.24, p=0.08) Conversely, glucocorticoids alone were associated with a decrease in any organ relapse rate, but not specifically for neurologic relapse, perhaps suggesting a need for dual therapy in these cases. In current management, there are few comparative effectiveness studies that can dictate choice of agent based on organ involvement, and therefore, decisions are often made on tolerability, response, and physician experience.

Predictors of Clinical Course and Treatment Response

Although some demographic and clinical characteristics have been associated with worse outcomes and increased disease severity in the broader sarcoidosis population, there are no patient-specific biomarkers used in clinical practice that can directly predict who will progress and necessitate treatment, nor are there clear biomarkers that will predict relapse in any one individual patient. However, increasing data shows promise for potential biomarkers. Multiple differing HLA haplotypes seem to track with both disease onset and differing presentations, implying that the genetic profile of the immune system can account for some of the varied clinical manifestations (122). For instance, the HLA-A1 and HLA-B8 have been associated with acute onset of sarcoidosis, and HLA-DR14(6) and DR15(2) are associated with chronic disease (123, 124). With further insights into abnormal T-regulatory function, recent data has shown an increased proportion of 'exhausted' T-reg cells is associated with chronic sarcoidosis (125). Conversely, the presence of high levels of functioning T-regs in tissues are associated with more self-limited disease (126). The presence of B cells and aberrant proportions of B cells may also signal poorer outcomes. BAFF has also correlated with multi-system disease, and low NFKβ p65 protein on T cells and B cells has been associated with increased severity of disease (90, 127, 128).

For treatment response, genetic polymorphisms may account for some of the variability in treatment response by therapy. For example, a study of 111 refractory sarcoidosis patients who were started on infliximab or adalimumab showed that patients without the TNF-alpha-308A variant allele (GG genotype) had a three -fold higher response to the TNF antagonists compared to those with the allele (129). Higher soluble TNF-receptor-2 expression levels also seem to correlate with response to infliximab (130). Additionally, a small study of five patients with CD4+ lymphopenia showed both clinical improvement in and an increase in CD4+ T cell counts, suggesting that this phenotype may be particularly responsive to infliximab (131). Increasing research and understanding of the intricate mechanisms of pathophysiology may yield future personalized prognostic markers.

TREATMENT-ASSOCIATED ISSUES

Although immunosuppression dominates the forefront of sarcoidosis therapy, in reality, holistic treatment of patients with sarcoidosis often involves addressing the comorbid conditions associated with the disease and mitigating side effects of immunosuppression. Sarcoidosis can cause a number of "danger situations", including fibrocystic sarcoidosis, sarcoidosis-associated pulmonary hypertension (SAPH), bronchiectasis, and mycetomas (132). Additionally, "parasarcoidosis" syndromes are associated with the disease, including small fiber neuropathy, cognitive dysfunction, chronic pain, and fatigue; each of these issues can cause significant disability and burden upon patients,

and often, do not respond desirably to traditional immunosuppressive therapies (25). Last, the immunosuppressive treatments themselves contribute to predictable comorbidities, including infections, fatigue, and malaise. Corticosteroids are highly associated with obesity, malaise, decreased bone density, cataracts, hyperglycemia and edema (133, 134). Additionally, each steroid-sparing agent can be associated with organ toxicities in the liver, kidney, or bone marrow. One cross-sectional study assessing gastrointestinal (GI) side effects in patients from the United States, United Kingdom, and the Netherlands found that the most important GI side effect was weight gain related to corticosteroid use; methotrexate was associated with nausea and diarrhea. Vomiting and weight loss were most associated with azathioprine and mycophenolate (61). Last, paradoxically, some of the drugs used to treat sarcoidosis (e.g., the TNF-antagonists) have been associated with development of a sarcoid-like reaction (135).

Strategies to circumvent detrimental toxicities of both corticosteroids and alternative immunosuppression are important to incorporate into the multidisciplinary management of patients. For example, maintaining routine recommended vaccinations for infections such as influenza or pneumococcus should be considered (136, 137). Additionally, bone health should be addressed upon initiation of corticosteroids and regularly thereafter; clinical risk assessment (yearly) and intermittent bone mineral density testing can be incorporated based on duration of corticosteroid use, age, and risk scores (138). Treatment often includes bisphosphonates or other anti-fracture medications. Bone health management can be somewhat complicated given the need for calcium and vitamin D supplementation, but can be done safely with close monitoring. Similarly, patients should adhere to recommended laboratory monitoring intervals specific to their immunosuppressive medication to avoid irreversible organ injury (139). Regular eye exams can be helpful to evaluate for glaucoma or cataracts that can result from corticosteroid use.

Cavitary Lung Disease and Bronchiectasis

Fibrotic sarcoidosis has been historically difficult to treat given lack of treatment options and resultant complications of fibrosis (140). Pulmonary cavities can occur in patients with sarcoidosis related to progressive fibrosis of the lung (141). Mycetomas, caused by *Aspergillus* within lung cavities, can cause hemoptysis or lead to invasive infection. Similarly, bronchiectasis resultant of fibrotic sarcoidosis can also complicate treatment. In these cases, use of antibiotics and pulmonary therapies including bronchodilators, mucolytics, and chest physiotherapy is often most effective (140). Immunosuppression may be counterproductive in these cases, exacerbating chronic or repeated infection.

Pulmonary Hypertension

Pulmonary arterial hypertension can be a complication of sarcoidosis. It is associated with increased mortality, especially among those individuals awaiting lung transplantation (142, 143). Pulmonary hypertension in sarcoidosis may be due to granulomatous vasculitis, pulmonary artery compression by lymphadenopathy, left heart dysfunction due to myocardial involvement, porto-pulmonary hypertension in those with associated liver disease, parenchymal and vascular destruction due to fibrosis, or hypoxic vasoconstriction related to parenchymal abnormalities. Additionally, the associated risk of thromboembolic disease can lead to pulmonary emboli (and subsequent pulmonary hypertension), requiring a high index of suspicion (144). Pulmonary hypertension should be suspected if there is a drop in functional status or DLCO in cases of stable pulmonary parenchymal disease. Upon CT evaluation, pulmonary artery diameter indexed to body surface area correlates with the presence of sarcoidosis-associated pulmonary hypertension (SAPH) and may raise also suspicion (145).

Because of the varied contributing pathophysiology (some of which may be overlapping), the treatment of pulmonary hypertension in sarcoidosis can be complex. Corticosteroid treatment may result in the improvement of pulmonary pressures in some, but not all cases, reflecting the differing mechanisms of pulmonary hypertension. Case series suggest benefit of prostacyclin analogues in some patients and endothelin receptor antagonists (ERAs) have conflicting data reports (146–148). Retrospective series have shown that ERAs may decrease pulmonary pressures and improve functional and exercise capacity in some individuals (149). However, a RCT of 35 patients randomized to bosentan 125 mg twice daily versus placebo failed to show a functional improvement in walk distance, although this study did show small, but statistically significant, improvement in mean pulmonary artery pressure and pulmonary vascular resistance (PVR) (150). Two of the treated patients had increased need for oxygen, possibly indicating a deleterious effect on the compensatory mechanisms of hypoxic vasoconstriction seen in patients with concomitant parenchymal lung disease. Another study, an open-label proof of concept trial of 21 SAPH patients treated with ambrisentan, showed that, in the patients who completed therapy over 24 weeks, there was a non-statistical improvement in walk distance and dyspnea as measured by the St. Georges Respiratory Questionnaire (146). In this trial, drop-out rate was 52% primarily driven by intolerance of the drug. A more recent retrospective review of the French Pulmonary Hypertension Registry between 2004–2015 showed that patients on pulmonary vasodilator therapy (including phosphodiesterase-5 inhibitors, prostacyclin analogues, and ERAs) appeared to have hemodynamic benefit, but lacked a functional benefit from PAH therapies, similar to results from the bosentan RCT (142). Clinical trials with selexipag (a non-prostanoid prostacyclin receptor agonist) and inhaled treprostinil in patients with SAPH is have recently been initiated (NCT03942211, NCT03814317) with the primary outcome of hemodynamic measurements, including PVR. Given the multiple potential causes of pulmonary hypertension in this population, current optimal management for SAPH is unclear. Vasodilator treatments should be used with caution and careful patient selection is advised.

Lung Transplantation

In case of progressive organ failure, transplantation can be considered in patients with sarcoidosis. Approximately 3% of lung transplants done in the United States are due to sarcoidosis (151). Successful outcomes have been reported for lung, heart, and liver disease, comparable to transplantation for alternative

causes (151, 152). Interestingly, despite post-transplant immunosuppression, recurrence of non-necrotizing granulomas in the transplanted organ is common, and thought to be derived from the recipient (153, 154). One analysis of DNA from transbronchial biopsies of lung transplant recipients with recurrent granulomatous inflammation found increased percentage of recipient DNA in the epitheloid clusters suggesting repopulation by host macrophages (154). Granulomas can be found on routine transbronchial biopsies in follow-up and often resolve spontaneously (155). Generally, the presence of granulomatous inflammation does not seem to affect overall survival in most patients (151, 156).

Small Fiber Neuropathy, Cognitive Dysfunction, and Fatigue

Small Fiber Neuropathy in sarcoidosis causes chronic pain, autonomic dysfunction, and altered sensation (157). Treatment for small-fiber neuropathy has been notoriously difficult, with failure to respond to most traditional therapies such as corticosteroids, MTX, AZA, MMF, and therefore, symptomatic treatment for neuropathic pain is usually considered (158). Additionally, the anti-TNF agents, both infliximab and adalumimab, have some suggestion of improving sarcoidosis-related fatigue and cognitive difficulties, as compared to other immunosuppressive agents (159). Intravenous immunoglobulin has also been suggested and has shown efficacy in a proportion of patients (160). ARA 290, a peptide that targets the innate repair receptor to decrease cytokine production and tissue inflammation, is also currently under study given supporting preliminary data in a small RCT of 22 patients showing a reduction in small fiber neuropathy symptoms (including pain) when treated with 28 days of ARA 290 (161).

Fatigue in sarcoidosis is extremely common. Its presence is an interplay of inflammation, musculoskeletal disease, mental health, treatment side effects, and sleep issues (162, 163). Given the association of sarcoidosis and sleep apnea, sleep evaluation is recommended in the workup of fatigue in this population (164). Both armodafinil and dexmethylphenidate have shown some benefit for sarcoid-related fatigue in very small studies of 15 patients or less when added to their immunosuppressive regimen (165, 166). Given the above potential benefits seen with the TNF antagonists, further study with these drugs should include fatigue as an endpoint. Treatment of depression and anxiety, both common confounding ailments, should also be evaluated and considered (167).

Physical Training/Pulmonary Rehabilitation

Physical training studies in sarcoidosis have suggested that a structured physical activity program can improve exercise capacity, muscle strength, and fatigue in sarcoidosis (168, 169). Additionally, physical training regimens appear to improve overall physical and psychological well-being, suggesting a role in the treatment of sarcoidosis. Pulmonary rehabilitation and training have been incorporated into expert consensus recommendations (170).

CONCLUSIONS

Treatment of sarcoidosis is complex and non-standardized for clinicians and patients. Further research is necessary to inform clinical guidelines and provide higher quality evidence for treatment regimens. Drug development is challenging due to the lack of animal model and rudimentary understanding of pathogenesis. Additionally, research should involve clinical trial design and prognostic biomarkers to appropriately select and evaluate those patients who will require therapy. Advancing the knowledge regarding etiology and pathophysiology may one day lead to prevention or cure. Future treatments will likely involve prevention of exposure, treatment of an antigen, mitigation of granulomatous inflammation, and interrupting fibrotic pathways. Management decisions should evolve to include personalized medicine based on pharmacogenomics and sarcoidosis phenotype, as well as patient-centered approaches to incorporate immunosuppression, symptom control, and treatment of comorbid conditions.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

AG is funded, in part, by the Federal Drug Administration Grant#FD-R-0005993 to study the natural history of sarcoidosis.

ACKNOWLEDGMENTS

The author would like to acknowledge Dr. Nabeel Hamzeh for his thoughtful edits and suggestions on the final draft.

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