Evidence for Mycobacteria in Sarcoidosis

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Despite its recognition as a distinct granulomatous disease for over a century, the etiology of sarcoidosis remains to be defined. Since the early 1900s, infectious agents have been suspected in causing sarcoidosis. For much of this time, mycobacteria were considered a likely culprit, yet until recently, the supporting evidence has been tenuous at best. In this review, we evaluate the reported association between mycobacteria and sarcoidosis. Historically, mycobacterial infection has been investigated using histologic stains, cultures of lesional tissue or blood, and identification of bacterial nucleic acids or bacterial antigens. More recently, advances in biochemical, molecular, and immunological methods have produced a more rigorous analysis of the antigenic drivers of sarcoidosis. The result of these efforts indicates that mycobacterial products likely play a role in at least a subset of sarcoidosis cases. This information, coupled with a better understanding of genetic susceptibility to this complex disease, has therapeutic implications.

Keywords: sarcoidosis; mycobacteria; granulomas; microorganisms; peptides; immune response

Sarcoidosis is an idiopathic, multiorgan, inflammatory disease characterized by the presence of epithelioid cell, noncaseating granulomas (1–3). The lungs, lymph nodes, skin, and eyes are most often affected (4), yet potential involvement of any organ system contributes to its protean clinical manifestations. Heterogeneous presentations and a lack of specific laboratory assays can render the disease a diagnostic challenge. Further complicating diagnosis, the histologic findings of sarcoidosis may be present in other granulomatous conditions and foreign-body reactions. Clinical and radiologic findings in conjunction with histologic studies and the exclusion of other granulomatous conditions are usually required for a definitive diagnosis (4, 5).

The etiology of sarcoidosis remains unknown. Its development is complex, with genetic susceptibility and environmental factors playing important roles (6–8). Certain occupations and environmental exposures have been associated with a higher risk of sarcoidosis (9, 10). Because lungs are the most commonly affected organ, the search for an etiology has focused on airborne antigens, infectious or otherwise (11, 12). The granuloma formation associated with chronic beryllium exposure so closely resembles sarcoidosis that some investigators have suggested that berylliosis defines a sarcoidosis subset (3, 13, 14). It is probable that distinct antigens can precipitate sarcoidosis or sarcoidosis-like diseases in susceptible individuals. An antigen-driven process is also consistent with the oligoclonal restriction of CD4⁺ T cells seen in sarcoidal granulomas (15–17).

An infectious agent has been suspected in sarcoidosis since the early 20th century when the disease was considered to be a manifestation of tuberculosis (2, 3). Supporting this belief, intraperitoneal, intravenous, or footpad injection of sarcoidal homogenates into mice induced granuloma formation (18–22). Yet, Belcher and colleagues reported similar rates of granuloma formation in mice after injections of lymphoid tissue homogenate from patients with sarcoidosis or control subjects (23). Another research team demonstrated that injections of bronchoalveolar lavage (BAL) material from patients with sarcoidosis into rabbits could cause granuloma formation but not if the BAL was first disinfected (24). In case-control studies from the Isle of Man, 40% of sarcoidosis cases reported contact with a patient with sarcoidosis before disease onset, compared with only 1% of control subjects (25, 26). Finally, some patients developed sarcoidosis after receiving a transplant from a patient with sarcoidosis (27–30). These observations strongly support the presence of a transmissible agent in sarcoidosis.

Histopathologically, sarcoidosis granulomas are similar to those caused by other Th₁-dependent responses, including those produced by mycobacteria (5, 31). Investigative efforts to find a communicable trigger for sarcoidosis have focused on infection by *Mycobacterium tuberculosis* (MTB) and atypical mycobacteria. We reviewed the scientific literature that supports or refutes the association between mycobacteria and sarcoidosis. Studies investigating this association have used several techniques: examining histology stains, culturing organisms from lesions or blood of affected individuals, identifying bacterial nucleic acids or bacterial antigens, and detecting immunologic responses to mycobacterial antigenic determinants in patients with sarcoidosis.

HISTOLOGY AND BACTERIOLOGY

Microorganisms are generally not detected through conventional staining techniques or cultures of sarcoidal granulomas. However, using special stains or culture methods, several investigators have identified microorganisms in sarcoidosis lesions, most commonly those resembling mycobacteria. During the first half of the 20th century, MTB bacilli were identified in tissue from 8 to 25% of patients with sarcoidosis (3, 32, 33). Those early investigators suggested a “transition” from a sarcoidal to a caseating granuloma (overt tuberculosis), at which point mycobacteria could be readily identified by acid-fast stain (3). Patients with tuberculosis preceding sarcoidosis and patients with concomitant sarcoidosis and tuberculosis were also identified (3, 34–39). However, other investigators failed to demonstrate the presence of bacilli within sarcoidosis lesions (40). Confounding these findings was the high prevalence of MTB infection during the early and mid 20th century when many of these observations were reported. Moreover, patients with sarcoidosis may have immunological defects that render them susceptible to mycobacterial infection (4), and mycobacteria superinfection may preferentially enter existing granulomas (41). Arguments against mycobacteria as a cause

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have centered on reports that patients with sarcoidosis rarely manifest overt tuberculosis infections (42, 43).

Other bacteriological and histological methods have supported a role for mycobacteria in sarcoidosis. Histologically, bacilli-like structures have been observed in sarcoidal tissue using immunofluorescence techniques (44). Schaumann bodies, inclusions found in sarcoidosis granulomas as well as other inflammatory conditions, were regarded as “transformed tuberculous bacilli” by Schaumann himself (45). More recently, these structures were characterized as sites of mycobacterial degradation by demonstrating the colocalization of lysosomal components and mycobacterial antigens in immunohistologically stained sarcoidosis tissues (46).

Several investigators have described the presence of bacterial structures in various samples from patients with sarcoidosis (47–52). Initially, these structures were characterized as cell wall-deficient (CWD) bacteria (50, 51) and later as acid-fast bacilli (AFB). Indeed, mycobacteria can lose their cell walls during their life cycle or in response to inhospitable conditions such as exposure to antibiotics, but the clinical significance of these changes remains controversial (53–55). One group isolated CWD mycobacteria from skin samples and cerebrospinal fluid from patients with sarcoidosis and identified the organisms as belonging to the *M. avium* complex and/or *M. paratuberculosis* (56). The same group had previously shown that CWD bacteria isolated from cutaneous sarcoidosis lesions could revert to AFB (57). However, a larger study failed to show disease specificity, finding bloodborne CWD forms in similar numbers among control subjects and patients with sarcoidosis (58). In the latter study, the organisms were not identified, so it is conceivable that the CWD bacteria differed between the sarcoidosis and control groups.

Non-MTB mycobacteria have received less attention than MTB as candidate agents for sarcoidosis. Nevertheless, several reports have described cases of sarcoidosis preceded by infection with *Mycobacterium avium-intracellulare* complex (MAC) (56, 59), *M. marinum* infection, and other atypical mycobacteria, including Bacille Calmette-Guérin vaccination (59–63). Sarcoidosis is one of the conditions to be considered in the differential diagnosis of *M. marinum* infection, a disease in which AFB are detected in only 22% of active cases (64). The histological similarity between sarcoidosis and infection with atypical mycobacteria raises the possibility that the sarcoidosis diagnosed in the above reports was actually persistent or recurrent infectious disease where the organism evaded detection.

Overall, histological detection and positive cultures have demonstrated that mycobacteria can be identified in a portion of patients with sarcoidosis. *Mycobacterium* sp. are detected at elevated rates among patients with sarcoidosis in many of the reports. However, distinguishing coincident infection from a causal relationship cannot be definitively concluded from these studies.

NUCLEIC ACID IDENTIFICATION

The advent of molecular analysis to detect bacterial DNA reinvigorated the search for pathogens, especially mycobacteria, as a potential cause of sarcoidosis. Using PCR, Saboor and colleagues found MTB DNA in half of sarcoidosis lung samples and identified non-MTB DNA in an additional 20% (65). Similarly, Mitchell and coworkers used liquid-phase hybridization to demonstrate an approximately 5-fold increase in the detection of MTB rRNA in sarcoidosis spleens compared with control subjects (66). Other investigators used similar techniques to find MTB or non-MTB mycobacterial sequences in samples of sarcoidosis tissues (67, 68). In cutaneous lesions of sarcoidosis, PCR evaluation revealed

MTB or non-MTB mycobacterial DNA in 80% of samples (69). MAC DNA has been recovered from the cerebrospinal fluid of a patient with neurosarcoidosis (70). In a case report of an immunocompetent host with sarcoidosis, *M. marinum* was revealed in a sputum sample by culture techniques and in a lung biopsy specimen by PCR analysis (71). In an interesting case series, three out of four cases of recurrent lung sarcoidosis were positive for identical non-MTB DNA sequences in pre- and posttransplantation lung biopsies despite an absence of clinical mycobacterial infection (72), suggesting a causal relationship between nonpathogenic mycobacteria and sarcoidosis. These and similar studies have been criticized for their small size and high false-positive PCR rate, especially because other investigators failed to find mycobacterial DNA or RNA in sarcoidosis tissues (73–75). Moreover, finding mycobacterial components in a sarcoidal granuloma raises the question of possible misdiagnosis, especially with the atypical mycobacteria.

Some authors detected multiple mycobacterial nucleic acid components in sarcoidosis tissues using distinct PCR techniques and target gene sequences. Drake and coworkers found MTB rRNA in 48%, MTB rpoB (RNA polymerase) DNA in 24%, and the MTB-specific insertion sequence IS6110 in 0% of 25 patients with sarcoidosis, but none of these sequences was found in 25 control subjects (76). Other authors have also reported low levels or the absence of the MTB IS6110 sequence in sarcoidosis samples (77, 78). Whether this finding reflects the differential sensitivity of these tests or the presence of a MTB-related organism that lacks IS6110 is unknown. However, a study in Shanghai using real-time PCR found low levels of MTB-specific IS986 DNA in sarcoidosis and control tissue compared with tuberculosis granulomas (79), arguing against MTB as a common agent in sarcoidosis.

In other recent studies, sensitive techniques such as real-time PCR and sequencing detected several amplicons for the mycobacterial virulence factor superoxide dismutase A (SodA) in 12 of 17 sarcoidosis samples, compared with 2 of 16 negative control subjects (80). Most of these sequences were identified as MTB SodA, but two were non-MTB (80). Using a distinct approach and *in situ* hybridization, Song and coworkers found evidence of mycobacterial catalase-peroxidase antigen (mKatG), another mycobacterial virulence factor, and MTB 16S rRNA DNA in a similar proportion of sarcoidosis tissues (~40%) (81, 82). Of note, 83% of the tissues that reacted with the mKatG probe were positive for MTB 16S rRNA, but none of the control samples was positive for either probe.

In a recent metaanalysis, Gupta and colleagues analyzed 31 series and case control studies published between 1980 and 2006. In a pooled analysis, 231 out of 874 patients with sarcoidosis were positive for mycobacterial (MTB or non-MTB) nucleic acid (83). The authors concluded that, despite evidence of a reporting bias, the odds ratio in their analysis suggested an association between mycobacteria and sarcoidosis (83).

Molecular detection of MTB has been linked to poor prognosis in sarcoidosis (84, 85). Grosser and colleagues found that in patients with sarcoidosis with MTB-reactive PCR results from biopsies, blood samples, or BAL, the disease was more likely to be chronic (85). Fite and coworkers demonstrated that 39% (9/23) of patients with sarcoidosis tested positive for the IS6110 MTB insertion element by PCR and Southern blot hybridization, in contrast to 4% (1/23) in the control group. Remission occurred in 10 of 14 cases of MTB-negative disease but in only three of nine MTB-positive patients with sarcoidosis. None of these patients developed clinical tuberculosis despite positive DNA findings (84).

Taken together, the above studies demonstrate that molecular assays can detect genetic material from mycobacteria in

sarcoidosis samples with high sensitivity and support an association between mycobacterial infection and the development of sarcoidosis. The variable detection of MTB-specific IS6110 DNA sequence, even in sarcoidosis samples positive for other mycobacterial genes, suggests that there is an MTB subtype or a related non-MTB mycobacteria species that is preferentially associated with sarcoidosis.

IMMUNOLOGIC AND ANTIGEN ISOLATION

Whereas molecular methods are quite sensitive, immune assays can be more powerful because trace amounts of antigen are sufficient to trigger an immune activation in a sensitized host. Moreover, the presence of preformed humoral or cellular mediators against an antigen supports prior exposure, even in the absence of active infection. Finally, a host immunologic response provides a direct mechanism by which mycobacterial antigens could drive disease development or progression in sarcoidosis.

Early observations of a host immune activation in sarcoidosis included an increase in the γ globulin fraction of sarcoidosis serum samples, which was remarkably similar to the spike found in tuberculosis blood samples (86). This elevation in immunoglobulin probably reflects increased B-cell activation and antibody production in the early course of the disease (87). Circulating anti-mycobacterial antibodies have been used to substantiate PCR findings detecting MAC genetic material in patients with sarcoidosis (56). Sera from patients with sarcoidosis tested positive on immunoblots of recombinant mycobacterial antigens p36 and heat shock protein (hsp)65. Sera from normal patients did not react to these antigens, although 53% of patients with ulcerative colitis reacted to the p36 antigen (56). Another study using ELISA showed high levels of anti-hsp70 antibodies in patients with sarcoidosis (88). This antigen increases expression of costimulatory molecules, suggesting that MTB-hsp70 positivity could lead to a break in immune tolerance and subsequent autoimmune disease (89). Consistent with these positive serology observations, mycobacterial hsp16, hsp65, and hsp70 have been detected in sarcoidal tissue by immunostaining (77).

A robust step associating sarcoidosis and mycobacteria occurred with the identification of mKatG as an antigen and a target of the adaptive immune response in patients with sarcoidosis (81). This work relied on identifying antigens in the Kveim reagent. In the Kveim reaction, lymph node or spleen homogenates from a subject with confirmed sarcoidosis injected into a patient with putative sarcoidosis resulted in the formation of granulomas at the inoculation site 80% of the time (90). Song and coworkers reasoned that components of the Kveim reagent could be the antigen(s) responsible for triggering sarcoidal granuloma formation. Previous research on the Kveim reagent suggested that the antigen was protein in nature, given that its reactivity was abrogated by denaturants but was resistant to neutral detergents, acidity, and nucleases (91). Dissociated sarcoidosis tissue produced poorly soluble aggregates that reacted with sera IgG of patients with sarcoidosis. When these aggregates were analyzed by mass spectrometry, the highest match was mKatG. The authors then found mKatG protein in sarcoidosis samples by immunoblotting in 55% of cases but not in control tissues. Furthermore, they discovered serum antibodies against recombinant mKatG in 48% of patients with sarcoidosis but not in control subjects (81). These results demonstrated that a mycobacterial antigen was present in at least a subset of sarcoidosis tissues and that a humoral response against that antigen could be detected.

Subsequently it was shown that mKatG could trigger cellular immune responses in lymphocytes collected from patients with sarcoidosis. Drake and colleagues reported that peripheral blood mononuclear cells (PBMNs) from 15 of 26 patients with

sarcoidosis responded to peptides from the mycobacterial antigens ESAT-6 and/or mKatG, compared with 1 of 24 purified protein derivative (PPD)-negative control subjects (92). The same group observed higher rates of ESAT-6 and mKatG-dependent T-cell activation and proliferation in BAL samples from patients with sarcoidosis versus control subjects (93). It was further shown that CD4⁺ T cells mediated a Th₁ response in patients with sarcoidosis when these peptide antigens were presented by DRB1*1101-expressing antigen-presenting cells (94). This finding links mycobacterial antigens to a typical sarcoidal Th₁ response in the context of a well established sarcoidosis susceptibility MHC allele.

In similar studies, the group that originally identified mKatG in the Kveim reagent demonstrated that mKatG protein was able to activate PBMNs from patients with sarcoidosis (95). Compared with PPD-negative control subjects, 67% of patients with sarcoidosis had significantly more IFN- γ -secreting, mKatG-reactive PBMNs. Moreover, 85% of sarcoidosis cases had high responses to mKatG or PPD components, suggesting prior exposure to mycobacteria. IFN- γ secretion from BAL cells was much higher than from PBMNs, demonstrating that mKatG-specific cells concentrate in the region of active disease. Finally, a higher percentage of BAL and PBMN CD4⁺ cells from patients with sarcoidosis proliferated in response to mKatG, and the level of mKatG responsiveness correlated to disease course. The authors concluded that mKatG behaves as a pathogenic antigen at least in a subset of sarcoidosis cases (95).

If mKatG is driving an immune response in sarcoidosis, one would predict an antigenic difference between mycobacterial KatG protein and KatG from other bacterial species that can infect humans. To test if the antigenic KatG peptide associated with sarcoidosis was specific to mycobacteria, we performed a sequence analysis (F. Ramírez-Valle and S. Prystowsky, unpublished data). Whereas KatG protein is approximately 70% identical across several species of mycobacteria as well as other bacteria, including nocardia, the mKatG peptide 13 epitope tested by Drake and colleagues (92, 96) is approximately 90% identical among most mycobacteria (using the SIM protein alignment tool; <http://ca.expasy.org/tools/sim-prot.html>). Restriction of this epitope sequence to mycobacterial species is evidenced by KatG peptide 13 having less than 60% homology between MTB and nocardia or other bacterial species. Figure 1 shows phylogenetic trees of the entire KatG and the KatG peptide 13 among several mycobacteria and other intracellular bacteria, demonstrating that the antigenic peptide 13 is relatively conserved among mycobacteria. Thus, mKatG peptide 13 appears to be an immunodominant peptide specific to mycobacteria that, in conjunction with HLA susceptibility, can induce a Th1 cellular immune response in patients with sarcoidosis.

Cellular responses to several additional mycobacterial antigens have also been reported. In one study, patients with sarcoidosis responded to the mycobacterial virulence factor antigen 85A (Ag85A), a mycolyl transferase (97). The authors reported high IFN- γ production from PBMNs stimulated with whole recombinant Ag85A in 15 of 25 patients with sarcoidosis and in 2 of 22 PPD-negative healthy control subjects. The same group reported Th₁ responses to antigens from mycobacterial ESAT-6, mKatG, and SodA (96). In this study, BAL cells from pulmonary sarcoidosis responded more robustly to the mycobacterial antigens than did peripheral cells, adding to the evidence that antigen and antigen-responsive cells may cluster in areas of active disease. Dubaniewicz and colleagues found that PBMNs from patients with sarcoidosis and patients with tuberculosis were similarly activated by *in vitro* exposure to recombinant MTB heat-shock proteins relative to cells from healthy control subjects (98). Together these data imply that many different mycobacterial

Figure 1. The most antigenic epitope of mycobacterial catalase-peroxidase antigen (mKatG) in patients with sarcoidosis is conserved among mycobacterial species. (A) Phylogenetic diagram of full-length KatG protein showing high homology of KatG homologs across multiple bacterial species. (B) Phylogenetic diagram of the KatG peptide 13, shown by Drake and colleagues (92) to induce maximal Th₁ responses in patients with sarcoidosis. This potentially pathogenic epitope is specific to mycobacterial species. Protein and peptide sequences from different bacterial species were obtained from Genbank, aligned using ClustalX 2.0, and analyzed using DRAWTREE neighbor-joining analysis. Bootstrap values were based on 1,000 replicates, and the branch length index is represented below the tree. Alignments were computed with full-length KatG (A) or KatG peptide 13 sequences (B).

antigens may contribute to pathogenic responses in sarcoidosis. In contrast, PBMNs and BAL mononuclear cells collected from 17 German patients with sarcoidosis showed similar IFN- γ production compared with 35 PPD-negative control subjects with nongranulomatous lung disease when stimulated with mycobacterial antigens ESAT-6 or CFP-10 (78). These findings suggest the possibility that immune cells isolated from diseased lungs possess a general hyperreactivity that is not specific to mycobacterial antigens. However, Drake and colleagues detected mycobacterial antigen-evoked cellular immune responses in cells from patients with sarcoidosis who did not react to *Trypanosoma brucei* lysates (96) or the neoantigen keyhole limpet hemocyanin (93), demonstrating antigen specificity in the immune response.

Because mycobacterial genes and proteins can be found in sarcoidosis samples from patients with specific immune responses to the same proteins, one would expect that mycobacterial peptides might be bound to HLA on antigen-presenting cells in sarcoidal tissues. Yet when peptides bound to sarcoidosis-associated HLA protein DRB1*0301 were isolated in a Swedish population (99), no mycobacterial antigens were observed. It is possible that these antigens are found in limited concentrations or are not amenable to elution and sequencing. Nonetheless, a follow-up study found that the cellular response of PBMNs and BAL cells to mKatG was stronger in Swedish patients with the DRB1*0301

HLA allele (95), suggesting that it may be involved in initiating a pathogenic immune response to this mycobacterial antigen.

DISCUSSION

Since its designation, an infectious agent has been hypothesized as an etiologic factor in sarcoidosis. Evidence implicating HLA alleles and other genes involved in T-cell activation suggests that sarcoidosis carries a genetic susceptibility. However, because genetic susceptibility alone is insufficient to cause sarcoidosis, it is just as likely that antigen exposure drives the disease process in genetically predisposed individuals. Exposure to one antigen, beryllium, appears to promote sarcoidal disease in a susceptible subset of patients (100). Although mycobacteria infection has long been a candidate source for causative antigens in sarcoidosis, only recently has strong evidence supported a causal connection.

In addition to mycobacteria, microbial agents such as propionibacteria have been associated with sarcoidosis. A group in Greece reported that 72% of sarcoidosis tissues contained amplifiable MTB DNA, yet they also isolated propionibacteria DNA from 43% of sarcoidosis samples (101). Moreover, studies in Japan have found high levels of propionibacteria DNA but no mycobacterial DNA in sarcoidosis samples (74). However, because propionibacteria are the most common commensal bacteria in the lungs of Japanese patients, the high frequency of propionibacteria nucleic acid in sarcoidosis lung tissue may not reflect causality (102). Nonetheless, in a screen of a *P. acnes* expression library, the protein RP35 was identified as reacting with serum and mononuclear cells from a subset of Japanese patients with sarcoidosis (103), with a higher frequency of immune reactions in patients than in control subjects. Pulmonary and hepatic granulomas formed in 25 to 50% of mice sensitized with killed *P. acnes* or recombinant RP35 protein, supporting the possibility that propionibacteria antigens are capable of triggering sarcoidal type inflammation (104). Thus, like mycobacteria, there is a growing body of evidence suggesting an association between propionibacteria and sarcoidosis. It may be that in the susceptible host, sensitization to the right microbial antigens (mycobacterial, propionibacterial, or other) may be sufficient to trigger sarcoidosis.

Advances in immunologic and molecular techniques have strengthened the association between mycobacteria and sarcoidosis. The cumulative evidence (Table 1) suggests that mycobacterial antigens are responsible for initiating and/or maintaining granulomas in some patients with sarcoidosis. The inconsistent detection of mycobacteria by histological stains or microbial culture indicates that sarcoidosis is associated with mycobacteria that are present below the detection threshold for these techniques. It is possible that mycobacterial antigen(s) only need to be present to elicit the original immune reaction and break immune tolerance in a susceptible host. The antigen may remain in the tissue (i.e., protected from degradation or clearance by the

TABLE 1. SUMMARY OF REVIEWED REFERENCES (BY REFERENCE NUMBER) SUPPORTING OR REFUTING AN ASSOCIATION BETWEEN MYCOBACTERIA AND SARCOIDOSIS.

	Mycobacteria in Sarcoidosis	
	Evidence for	Evidence against
Histology	3, 32, 33, 44, 46–52, 77	40
Culture	56, 57	58
Clinical infection	25, 26, 59–64, 71	42, 43
Bacterial nucleic acids	39, 56, 65–72, 76, 80–82, 83*, 84, 85, 101	73–75, 77–79
Immunologic response	56, 80, 81, 86–88, 92–98	78, 99
Evidence for a transmissible agent in sarcoidosis		
18–22, 24–26, 27–30, 72, 90, 91		

*Metaanalysis.

granuloma), leading to chronic or advancing disease, or it may clear and lead to disease remission. In this scenario, a living organism may not be found because its presence would not be needed to maintain disease. Consistent with this concept, mice injected with only synthetic mycobacterial SodA peptide can develop a Th1 immune response and noncaseating lung granulomas (105).

Molecular techniques demonstrating genomic or protein material of mycobacterial origin in sarcoidosis tissues along with elevated humoral and cellular immune response to mycobacterial antigens (particularly at sites of disease involvement) support the hypothesis that mycobacterial antigens may drive some cases of sarcoidosis. The advancement of molecular and immunologic tools, along with other methods such as genomics and proteomics, should help in further isolating and authenticating relevant antigens in sarcoidosis, including candidate mycobacterial proteins. These techniques will also help increase our understanding of the immune response caused by these agents and, in turn, improve therapeutic options.

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