Reversal of Global CD4+ Subset Dysfunction Is Associated with Spontaneous Clinical Resolution of Pulmonary Sarcoidosis


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Reversal of Global CD4+ Subset Dysfunction Is Associated with Spontaneous Clinical Resolution of Pulmonary Sarcoidosis

Kyra A. Oswald-Richter,* Bradley W. Richmond,† Nicole A. Braun,‡ Joan Isom,§ Susamma Abraham,§ Thyneice R. Taylor,‡ John M. Drake,§ Daniel A. Culver,§ David S. Wilkes,‖ and Wonder P. Drakex*

Sarcoidosis pathogenesis is characterized by peripheral anergy and an exaggerated, pulmonary CD4+ Th1 response. In this study, we demonstrate that CD4+ anergic responses to polyclonal TCR stimulation are present peripherally and within the lungs of sarcoid patients. Consistent with prior observations, spontaneous release of IL-2 was noted in sarcoidosis bronchoalveolar lavage CD4+ T cells. However, in contrast to spontaneous hyperactive responses reported previously, the cells displayed anergic responses to polyclonal TCR stimulation. The anergic responses correlated with diminished expression of the Src kinase Lck, protein kinase C-α, and NF-κB, key mediators of IL-2 transcription. Although T regulatory (Treg) cells were increased in sarcoid patients, Treg depletion from the CD4+ T cell population of sarcoidosis patients did not rescue IL-2 and IFN-γ production, whereas restoration of the IL-2 signaling cascade, via protein kinase C-0 overexpression, did. Furthermore, sarcoidosis Treg cells displayed poor suppressive capacity indicating that T cell dysfunction was a global CD4+ manifestation. Analyses of patients with spontaneous clinical resolution revealed that restoration of CD4+ T cell function was associated with resolution. Conversely, disease progression exhibited decreased Th1 cytokine secretion and proliferative capacity, and reduced Lck expression. These findings implicate normalized CD4+ T cell function as a potential therapeutic target for sarcoidosis resolution.

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Materials and Methods

Subject characterization

We prospectively enrolled patients from the Cleveland Clinic and Vanderbilt University Medical Center, who were undergoing bronchoscopy and for whom sarcoidosis was a diagnostic consideration. BAL cells for all experiments were obtained from the diagnostic bronchoscopy, whereas peripheral blood samples were obtained during the diagnostic bronchoscopy or subsequent to the initial diagnosis. All subjects provided written

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informed consent that was approved by the appropriate Institutional Review Boards. For inclusion in this study, the clinical, histological, and microbiologic criteria used to define sarcoidosis were as previously described (12). Scadding radiographic staging was performed, as previously described (13). Study participant demographics are provided in Table I. Approximately 32% of the subjects were on immunosuppressants at the time of their bronchoscopy; their immunosuppressant regimen was initiated by the referring physician. We noted no distinctions in cytokine expression or proliferative capacity based upon whether patients were on immunosuppressive therapy or not. Disease controls were subjects for whom an alternate diagnosis was obtained after bronchoscopy. Disease control diagnoses were as follows: in 3 of the 10, no clinical diagnosis was determined. The remaining 7 represented the following: ischemic cardiomyopathy (1), organizing pneumonia (1), rheumatoid lung (1), eosinophilic bronchiolitis (1), lung adenocarcinoma (1), and asthma exacerbation due to Stenotrophomonas superinfection (1).

**Cell isolation and culture**

BAL fluid and peripheral blood were processed, as previously described (14, 15). Resting CD4+ T cells were purified from fresh or cryopreserved PBMC by magnetic separation (Dynal CD4 Positive Isolation Kit; Invitrogen). Purified resting CD4+ T cells were activated by cross-linking with plate-bound anti-CD3 Ab (OKT-3; American Type Culture Collection) and soluble anti-CD28 Ab (1 μg/ml; BD Biosciences), as previously described (14). Flow cytometry

T cells were stained with the relevant Ab on ice for 30 min in PBS buffer containing 2% FCS and 0.1% sodium azide. Cells were then washed twice, fixed with 1% paraformaldehyde, and analyzed with a FACS Calibur or LSR-II flow cytometer (BD Biosciences). Live cells were gated based on forward- and side-scatter properties, and analysis was performed using FlowJo software (Tree Star, Ashland, OR). The following anti-human Abs were used for surface staining: CD3, CD4, CD25, CD45RO, and CCR7, all obtained from BD Biosciences. T cell subset staining was performed, as previously described (16). A minimum of 30,000 events was acquired per sample.

**Proliferation assay**

To determine proliferation and quantitate cell division, purified CD4+ T cells were labeled with CFSE (Molecular Probes). Purified cells were first washed and resuspended in PBS. While vortexing the cells, CFSE was added at a final concentration of 5 μM. The mixture was vortexed for an additional 15 s and incubated at 37°C for 3 min. Labeling was quenched by the addition of 50% PBS in PBS. Cells were washed once more with 50% serum PBS, followed by two washes with RPMI 1640-supplemented medium. CFSE-labeled CD4+ T cells were TCR stimulated in RPMI 1640-supplemented medium, using anti-CD3 and anti-CD28 Abs. At day 5 postactivation, cells were fixed and analyzed for CFSE expression and cell size by flow cytometry.

**Treg depletion of CD4+ T cells**

CD4+ T cells were stained with anti-human CD4, CD25, and CD45RO Abs (BD Biosciences). To deplete CD4+ T cells of Tregs, T cells were flow sorted into the following two groups: 1) CD4+CD45RO+CD25(high) T cells and 2) all other CD4+ cells. FOXP3 intracellular staining was performed on a portion of the sorted cells to confirm cells were Tregs (eBioscience). The Treg-sorted cells were >98% pure as measured by high CD45RO+CD25(high) expression. The Treg-depleted CD4+ cells were >95% pure.

**Treg suppression assay**

Unlabeled purified Tregs were cocultured with CFSE-labeled CD4+ T cells (2 × 10⁵ T cells) at 1:5 ratio in 96-well plates coated with suboptimal anti-CD3 (100 ng/ml) and anti-CD28 (1 μg/ml) Abs. At day 4 postactivation, cells were fixed and analyzed for CFSE expression and cell size by flow cytometry.

**Intracellular cytokine analysis**

Intracellular staining was performed to identify IL-2 and IFN-γ secreting BAL CD4+ T cells in response to TCR stimulation, as previously described (15). Cells were gated on singlets, live CD3+, and CD4+ cells. The IFN-γ frequency was defined as the subject’s percentage of stimulated CD3+ CD4+ T cells minus their unstimulated background frequency.

**Extracellular cytokine assay**

Supernatants were collected from TCR-stimulated cells at 24 h and analyzed for extracellular IL-2 and IFN-γ by cytokine bead array (CBA), according to the manufacturer’s instructions (BD Biosciences). For TCR stimulation, 2 × 10⁵ PBMC or CD4+ T cells were stimulated through the TCR by plate-bound anti-CD3 and soluble anti-CD28 Abs.

**RNA isolation and quantitative real-time PCR**

CD4+ T cells were sorted from sarcoidosis and healthy control PBMC. Total cellular RNA was extracted from 5 × 10⁶ CD4+ T cells using RNasy Mini Kit (Qiagen), according to the manufacturer’s protocol. First-strand cDNA was generated from 200 ng total RNA using oligo-dT primer and the AMV reverse transcriptase (Reverse Transcription System; Promega, Madison, WI). Quantitative real-time PCR amplification was performed with primers for Lck, protein kinase C (PKC)-θ, and NF-κB (TaqMan gene expression assays; Applied Biosystems, Foster City, CA). Expression levels were normalized to β-actin and 18S. Reactions were carried out in a StepOnePlus Real Time PCR System (Applied Biosystems) in a 10 μl vol.

**PKC-θ retroviral vector production and infection**

The human PKC-θ retroviral vector was provided by A. Altman (La Jolla Institute). PKC-θ was subcloned into a HIV-derived vector encoding GFP downstream of an internal ribosome entry site. HIV pseudotyped with vesicular stomatitis virus glycoprotein was generated as previously described (17).

**Statistical analysis**

Comparisons of the distribution of T cell frequencies were performed using the Wilcoxon rank sum test. Categorical comparisons, such as immune reactivity to TCR stimulation by individuals within a group, were analyzed using Fisher’s exact test. Multiple comparisons were performed. All performed comparisons are reported, all p values are two sided, and all analyses were performed using R (Version 2.1.1). The p value ≤0.05 was considered statistically significant.

**Results**

**BAL and peripheral CD4+ T cell dysfunction in sarcoidosis**

A key immunological feature of sarcoidosis is the spontaneous production of IFN-γ and IL-2 at local sites of granulomatous inflammation and anergic responses peripherally. This spontaneous release of cytokines is thought to be the result of a local hyperactive immune response. In 90% of sarcoidosis patients, the lungs are the active site of granuloma formation and chronic inflammation. Many studies have demonstrated that CD4+ T cell anergic responses occur peripherally, yet studies are lacking that demonstrate the presence of hyperactive CD4+ T cell responses to polyclonal stimulation in BAL. To better understand the role of the CD4+ T cells in sarcoidosis, CD4+ T cell responses were studied peripherally and in a local site of granulomatous inflammation, the lung, using BAL fluid. BAL CD4+ T cells spontaneously produced IL-2 (Fig. 1A). There was no significant difference in IFN-γ production in the sarcoidosis CD4+ T cells compared with disease controls (Fig. 1B). Interestingly, peripheral CD4+ T cells produced increased levels of IL-2 and IFN-γ spontaneously (Fig. 1C, 1D). In contrast, upon polyclonal TCR stimulation, BAL CD4+ T cells demonstrated a marked reduction in IL-2 and IFN-γ (Fig. 1E, 1F). These anergic responses were mirrored in the peripheral CD4+ T cells (Fig. 1G, 1H). Furthermore, the proliferative capacity of both the BAL and peripheral CD4+ T cells was greatly reduced (Fig. 1I–K). To determine whether differences noted in cytokine and proliferation of the sarcoidosis CD4+ T cells were due to differential distribution of CD4+ T cell subsets as compared with controls, T cell subset analysis was performed on ex vivo BAL and PBMC. There was no significant difference in subset distribution between sarcoidosis and disease control BAL. For peripheral CD4+ T cells, there were fewer naive CD4+ T cells in sarcoidosis as compared with healthy controls (Supplemental
Sarcoidosis CD4+ T cells demonstrate defects in the TCR signaling cascade

To determine the molecular basis for the T cell dysfunction in sarcoidosis, we investigated the signaling pathway upstream of IL-2 induction following TCR activation. We assessed Lck, a Src family kinase, PKC-θ, and NF-κB, key mediators of IL-2 transcription. Upon TCR stimulation, Lck is recruited to the immunologic synapse and subsequently activates PKC-θ through phosphorylation. PKC-θ activation results in the translocation of NF-κB to the nucleus, where it stimulates transcription of variety of genes involved in T cell proliferation and effector responses, including IL-2. Lck, PKC-θ, and NF-κB gene expression levels were dramatically reduced in sarcoidosis CD4+ T cells, compared with

Table I. Demographics of sarcoidosis and control populations

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<th>Sarcoidosis</th>
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<th>Disease Controls</th>
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<tr>
<td>Number</td>
<td>55</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>30; 25</td>
<td>5; 5</td>
<td>6; 4</td>
</tr>
<tr>
<td>Age (y), median (min, max)</td>
<td>50 (34, 68)</td>
<td>44 (22, 59)</td>
<td>56 (42, 68)</td>
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<tr>
<td>Race</td>
<td>13.5 (0, 77)</td>
<td>NA</td>
<td>2 (0, 77)</td>
</tr>
<tr>
<td>BAL lymphocyte %</td>
<td>5.6 (0.81, 27.9)</td>
<td>NA</td>
<td>2.3 (1.02, 5.64)</td>
</tr>
<tr>
<td>BAL CD4/CD8 ratio</td>
<td>median (min, max)</td>
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AA, African-American; AI, Asian Indian; W, white.

FIGURE 1. CD4+ T cell anergy in sarcoidosis. (A and B) Intracellular cytokine staining was performed on BAL cells from sarcoidosis subjects after 6-h culture to assess spontaneous release of IL-2 and IFN-γ. Representative flow cytometry analysis and cumulative analysis for (A) IL-2 and (B) IFN-γ. For BAL ICS experiments, data represent 45 sarcoidosis subjects and 10 disease controls (DC); median indicated by horizontal bars. (C and D) CD4+ T cells were sorted from PBMC and then cultured for 24 h. Spontaneous release of IL-2 and IFN-γ production was measured by CBA. Representative flow cytometry analysis and cumulative analysis for (C) IL-2 and (D) IFN-γ. For peripheral CD4+ T cytokine analysis, data represent 25 sarcoidosis subjects and 20 healthy controls (HC); median indicated by horizontal bars. (E and F) ICS was performed on BAL cells from sarcoidosis subjects after TCR stimulation using plate-bound anti-CD3 and soluble anti-CD28 Abs. Representative flow cytometry analysis and cumulative analysis for (E) IL-2 and (F) IFN-γ. (G and H) Peripheral CD4+ T cells were TCR stimulated using anti-CD3/anti-CD28. Supernatants were collected 24 h post-TCR activation and measured for (G) IL-2 and (H) IFN-γ production by CBA. (I–K) CD4+ T cells were CFSE labeled and stimulated with anti-CD3/anti-CD28. Cells were collected after 5 d, and proliferation was measured by flow cytometry. (I) Representative proliferation data for one HC and two sarcoidosis subjects. (J) BAL CD4+ proliferation. Data represent eight sarcoidosis subjects and three DC; median indicated by horizontal bars. (K) Combined proliferation data for sarcoidosis and HC CD4+ T cells. Data represent 15 sarcoidosis subjects and 5 HC; median indicated by horizontal bars.
healthy control CD4+ T cells (Fig. 2A). Similar results were observed in total BAL cells (Fig. 2B); however, due to cell limitations, gene expression was not measured in BAL CD4+ T cells.

Treg dysfunction in sarcoidosis

Another potential mechanism for the reduced cytokine production and proliferation of sarcoidosis CD4+ T cells is suppression by Tregs. The key function of Tregs is to suppress T cell activation, which can be measured by both T cell cytokine production and proliferation. Consistent with prior reports (6, 18–20), we observed an increase in Tregs peripherally (Fig. 3A). To investigate whether Tregs contributed to the reduced cytokine production, we depleted Tregs from the CD4+ T cells by flow sorting the CD45RO+CD25high cells from total CD4+ T cells. The Treg-depleted CD4+ T cells were then TCR stimulated and compared with the total CD4+ T cells. In the absence of Tregs, sarcoidosis T cells maintained reduced IL-2 and IFN-γ production (Fig. 3B, 3C). Moreover, proliferation and Lck expression in the CD4+ T cells were not restored in the absence of Tregs (Fig. 3D, 3E, 3G).

The unexpected finding that increased Tregs did not contribute to impaired T cell cytokine production and proliferation suggested that Tregs may also have impaired function in sarcoidosis. To investigate this possibility, the suppressive capacity of Tregs was tested. Sorted Tregs were cocultured with CFSE-labeled CD4+ T cells in the presence of TCR stimulation. At 5 d postactivation, cells were collected and analyzed for proliferation. Healthy control Tregs demonstrated robust suppressive capacity at a 1:5 ratio (Tregs to CD4+ T cells), whereas sarcoidosis Tregs demonstrated minimal suppressive capacity (Fig. 3D, 3F). Furthermore, healthy control Tregs suppressed sarcoidosis CD4+ T cells, whereas sarcoidosis Tregs did not suppress healthy control CD4+ T cells (Fig. 3D). These findings indicate that sarcoidosis CD4+ T cells, including Treg cells, demonstrate reduced functional capacity.

Modulation of IL-2 rescues diminished CD4+ T cell responses

To further confirm that poor activation of the IL-2 induction cascade contributes to decreased IL-2 and IFN-γ production, we cultured sarcoidosis CD4+ T cells overnight in exogenous IL-2 and then washed away the IL-2 prior to T cell activation. Addition of exogenous IL-2 restored the IL-2 and IFN-γ secretion levels similar to healthy controls (Supplemental Fig. 2). To further delineate the contribution of TCR activation to the diminished CD4+ T cell responses, we modulated PKC-θ expression in sarcoidosis CD4+ T cells. PKC-θ plays a critical role in IL-2 production and proliferation of T cells (21, 22). We exposed sarcoidosis CD4+ T cells to the PKC-θ inhibitor, rottlerin, to assess its effects on IL-2 and IFN-γ production. PKC-θ inhibition resulted in the dose-dependent abrogation of IL-2 and IFN-γ production, whereas the addition of a PKC-θ inhibitor resulted in no change (Supplemental Fig. 2). Inhibitor treatment of the cells did not affect viability (data not shown). With the goal of restoring cytokine production in sarcoidosis CD4+ T cells, we overexpressed PKC-θ by retroviral transduction in the CD4+ T cells. PKC-θ overexpression was confirmed by real-time RT-PCR. PKC-θ overexpression increased IL-2 and IFN-γ levels similar to levels induced by TCR stimulation in healthy controls (Fig. 4). These results suggest that PKC-θ may be a viable target for immunomodulation in sarcoidosis.

Sarcoidosis subjects with clinical resolution have restored Th and Treg function

Sarcoidosis patients may spontaneously resolve their disease (23). In other granulomatous diseases such as tuberculosis, restoration of T cell function is associated with disease resolution. Therefore, we investigated T cell function among sarcoidosis subjects during active disease and after disease resolution (active disease was defined by abnormal chest X-ray and evidence of impaired pulmonary function of >10%; resolution was ascertained at >2-y period postdisease diagnosis, defined by normalization of chest X-ray and restoration of lung function). CD4+ T cells were TCR stimulated, and then supernatants were collected at 24 h and assessed for IL-2 and IFN-γ production (Fig. 5A, 5B). T cells during active disease displayed reduced T cell responses to TCR stimulation, whereas T cells demonstrated a significant increase in IL-2 and IFN-γ expression with disease resolution. The IL-2 and IFN-γ levels in patients with spontaneously resolved disease approached that of healthy controls. For six of the subjects, we had peripheral blood samples from prior to disease resolution and

FIGURE 2. Decreased TCR-responsive genes in sarcoidosis CD4+ T cells. (A) RNA was isolated from sorted peripheral CD4+ T cells, followed by cDNA synthesis. Lck, PKC-θ, and NF-κB gene expression were normalized to a healthy control (HC). Data represent 25 sarcoidosis subjects and 20 HC. (B) Total BAL Lck, PKC-θ, and NF-κB gene expression. Gene expression normalized to a disease control. Data represent 8 sarcoidosis subjects and 3 disease controls (DC). Median indicated by horizontal bars.
postdisease resolution. All six subjects demonstrated a significant increase in IL-2 and IFN-γ production postdisease resolution (Fig. 5C, 5D). T cell cytokine production was not restored in subjects with disease progression (Fig. 5C, 5D). CD4+ T cell proliferative capacity increased among those with clinical resolution, but was not restored in subjects with disease progression (Fig. 5E, 5F). The restored functional parameters in the disease resolvers corresponded with normalized expression of Lck, PKC-θ, and NF-kB to healthy controls (Fig. 5G). Furthermore, analysis of Treg function demonstrated that Tregs regained suppressive capacity with disease resolution (Fig. 6). Clinically, restored T cell cytokine production correlated with radiographic improvement by Scadding stage criteria (Table II).

**Discussion**

Sarcoidosis immunopathogenesis has been characterized by localized accumulation of activated Th1 CD4+ cells. By secreting IL-2, TNF-α, and IFN-γ, Th1 cells play an important role in granuloma formation and maintenance. To our knowledge, this study demonstrates for the first time that polyclonal TCR stimulation of BAL CD4+ T cells results in reduced Th1 cytokine expression and proliferative capacity. We do not dispute the pivotal observations that BAL CD4+ T cells spontaneously secrete IL-2 and IFN-γ (1–3); our investigation noted spontaneous IL-2 production by local CD4+ T cells that exceeded that of disease controls and spontaneous IL-2 and IFN-γ by systemic CD4+ T cells that exceeded that of healthy controls (Fig. 1). Both sarcoidosis and disease control BAL CD4+ T cells spontaneously produced IFN-γ; this observation was not unexpected, as all the disease controls exhibited lung inflammation. It has also been observed that sarcoidosis BAL CD4+ T cells demonstrate evidence of recent activation through the TCR (24). Surprisingly, we noted a significant reduction in IL-2 and IFN-γ expression, following polyclonal TCR stimulation of BAL and systemic CD4+ T cells (Fig. 1). These studies in concert support the observation of spontaneous IL-2 cytokine expression by sarcoidosis BAL CD4+ T cells, while demonstrating that stimulation through the TCR yields functionally reduced IL-2 and IFN-γ expression that is not quantitatively equivalent to that of

**FIGURE 3.** Treg dysfunction in sarcoidosis. CD4+CD45RO+CD25+ cells were flow sorted and removed from sarcoidosis and healthy control (HC) CD4+ T cells. (A) Increased Tregs in sarcoidosis peripheral blood. Data represent the mean (horizontal bars) percentage from 15 sarcoidosis subjects and 5 HC. (B) IL-2 and (C) IFN-γ levels were similar in the total CD4+ and Treg-depleted CD4+ populations after 24-h TCR stimulation using plate-bound anti-CD3 and soluble anti-CD28 Abs as measured by CBA. Data are presented as the mean normalized expression ± SD from 5 sarcoidosis subjects and 2 HC. (D) Proliferation and suppression data for a representative sarcoidosis subject and a HC. CD4+ T cells were labeled with CFSE and then activated through suboptimal anti-CD3 (100 ng/ml) and anti-CD28 (1 μg/ml) Abs. Day 5 postactivation, cells were fixed and CFSE expression was analyzed by flow cytometry. Right panel (suppression assay), unlabeled purified Tregs were cocultured with CFSE-labeled CD4+ T cells (1.5 × 10^5 T cells) at 1:5 ratio in 96-well plates coated with suboptimal anti-CD3 (100 ng/ml) and anti-CD28 (1 μg/ml) Abs. At 4 d postactivation, cells were fixed and analyzed for CFSE expression and cell size by flow cytometry. Representative data for 5 sarcoidosis subjects and 2 HC. (E) Combined proliferation and (F) suppression data for 5 sarcoidosis subjects and 3 HC. (G) Lck was similarly low in the CD4+ and Treg-depleted CD4+ cell populations. Data are presented as the mean normalized expression ± SD from 3 sarcoidosis subjects and 3 HC.

**FIGURE 4.** PKC-θ overexpression in sarcoidosis CD4+ T cells restores cytokine production. CD4+ T cells were purified from PBMC, activated through the TCR, and transduced with PKC-θ–expressing retroviral vector or control vector. PKC-θ–transduced and control cells were expanded in IL-2 media for 7 d, and then GFP-positive cells were sorted out. GFP+ cells were then activated using plate-bound anti-CD3 and soluble anti-CD28 Abs. Supernatants were collected at 24 h and measured for (A) IL-2 and (B) IFN-γ production. Data presented for five sarcoidosis subjects and three healthy controls (HC).
healthy controls (Fig. 1). This finding is particularly important because IL-2 and IFN-γ have crucial roles in adaptive immunity. IL-2− and IL-2R−deficient mice portray the critical role of IL-2 in protective immunity, such as the differentiation, proliferation, and fate of CD4+ T cells (25–27). This inadequate cytokine response of BAL CD4+ T cells would be an important contributor to the perpetuation of sarcoidosis pulmonary pathogenesis.

The observations of spontaneous cytokine expression yet anergic responses upon TCR stimulation in sarcoidosis CD4+ T cells suggest dysfunction of the CD4+ T cell compartment. During states of Ag persistence, T cell dysfunction may occur. Ag-specific CD4+ and CD8+ T cells progress to states of stepwise and progressive impairment of T cell effector function and proliferative capacity (28). The original report of spontaneous IL-2 production from sarcoidosis BAL-derived T cells attributed that expression to an unknown stimulus (1). A growing body of independent literature demonstrates proteins or signals consistent with mycobacterial proteins, such as katG or early secreted antigenic target-6, within sarcoidosis granulomas. These proteins are poorly degradable and persist within sarcoidosis specimens, driving the spontaneous expression of Th1 cytokines from Ag-specific bronchoalveolar-derived CD4+ T cells (15, 29). Sarcoidosis BAL-derived T cells also demonstrate evidence of recent triggering of the αβ TCR (4). It has been demonstrated that myco-

FIGURE 5. Spontaneous sarcoidosis disease resolution reverses T cell anergy. T cell anergy was assessed predisease resolution and postdisease resolution; sarcoidosis subjects were not treated with immunosuppressants. CD4+ T cells were sorted from PBMC, and then TCR stimulated using anti-CD3/anti-CD28 Abs. Supernatants were collected 24 h post-TCR activation and measured for (A) IL-2 and (B) IFN-γ production by CBA. Data represent the mean (horizontal bars) percentage from 11 subjects predisease resolution, 8 subjects postdisease resolution, and 8 healthy controls (HC). (C–G) T cell anergy was assessed in 6 subjects for whom we had peripheral blood cells from predisease resolution and postdisease resolution and for 5 disease progressors. For (C) and (D), cytokine production was also measured at two time points spanning 1–3 y. (C) IL-2 and (D) IFN-γ production by CBA. (E) Representative proliferation data after 5-d TCR stimulation. (F) Combined proliferation data. (G) Lck, PKC-θ, and NF-κB gene normalized expression. Expression levels were compared with HC.

FIGURE 6. Treg function restored with spontaneous sarcoidosis disease resolution. CD4+ T cells were labeled with CFSE and then activated through suboptimal anti-CD3 (100 ng/ml) and anti-CD28 (1 μg/ml) Abs. Day 5 post-activation, cells were fixed and CFSE expression was analyzed by flow cytometry. (A) Representative proliferation and suppression data for a sarcoidosis subject preresorlution and postresolution and a healthy control (HC). (B) Combined suppression data for five sarcoidosis subjects and three HC.
bacterial Ags are targets of the adaptive immune response within systemic and BAL-derived sarcoidosis T cells (15, 28, 38). Persistent TCR stimulation by these Ags drives reductions in IL-2 expression and proliferative capacity following TCR stimulation, analogous to what has been reported by other microbial Ags (30).

Additional proposed mechanisms to explain an anergic phenotype include increased numbers of Tregs (6), defective CD4+ T cell signaling (31), and impaired dendritic cell function (32). Sarcoidosis BAL and periphery have been demonstrated to contain Treg populations above that of healthy controls (6). To determine the contributors of sarcoidosis anergy, we began with the Treg population. Tregs dampen the proliferative capacity and cytokine release of effector T cells. Consistent with a prior report by Miyara and coworkers (33), we noted that sarcoidosis peripheral Tregs failed to effectively reduce IFN-γ expression. In contrast to the report by Miyara and coworkers (33), we found that the increased number of Tregs did not contribute to the anergic phenotype as demonstrated by the result that there was no increase in CD4+ IL-2 expression once Tregs were removed (Fig. 3B). Importantly, we demonstrate that peripheral sarcoidosis Tregs lacked the capacity to reduce the proliferative capacity of effector CD4+ T cells (Fig. 3D, 3E). An earlier study by Miyara et al. (6) found that Tregs maintained suppressive capacity at a ratio of 1:1. In our study, we assessed the suppressive capacity of Tregs to CD4+ T cells (Fig. 3D, 3E). A recent study by Miyara et al. (6) found that Tregs maintained suppressive capacity at a ratio of 1:1. In our study, we assessed the suppressive capacity of Tregs to CD4+ T cells at a 1:5 ratio to better represent physiologic conditions. Healthy control Tregs demonstrated robust suppressive capacity at this concentration, whereas sarcoidosis Tregs demonstrated minimal suppressive capacity (Fig. 3D, 3F). It was notable that the sarcoidosis CD4+ effector cells remain susceptible to suppression, as evidenced by their reduced proliferation when exposed to healthy control Tregs (Fig. 3D, 3F). It is possible that these dysfunctional Tregs drive sarcoidosis pulmonary pathogenesis because the inability to suppress granuloma formation has been attributed to Treg dysfunction within sarcoidosis subjects (33). We attempted to assess local Treg function within sarcoidosis BAL cells, but were unable to due to insufficient numbers. This study demonstrates that the molecular basis of reduced cytokine expression and proliferative capacity is defective expression of key mediators of IL-2 transcription, specifically Lck, PKC-θ, and NF-κB. Prior reports have indicated an association between reduced expression of p56Lck and NF-κB with sarcoidosis severity (31). It was not appreciated that PKC-θ was also contributing to this pathway (Fig. 2). Our work demonstrates that not only reduced Lck, PKC-θ, and NF-κB are associated with sarcoidosis disease progression, but normalized expression of all three mediators of IL-2 transcription are present in sarcoidosis subjects who spontaneously recover from their pulmonary disease (Fig. 5). It is possible that expression of other cytokines is affected; additional studies to assess are in process.

The mechanisms driving reductions in Lck expression in sarcoidosis pathogenesis are unknown. Decreased expression of Lck has been described in other granulomatous diseases, such as tuberculosis and Cryptococcus. Modification of Lck by pathogenic microbial Ags has been shown to be a molecular target to suppress the development of protective immune responses in pulmonary granulomatous diseases (34–36). Mycobacterium tuberculosis ManLAM inhibits TCR signaling by interference with ZAP-70, Lck, and LAT phosphorylation (37). Likewise, the cryptococcal galacto-oxylomannan inactivates Lck through hyperphosphorylation, leading to loss of T cell function (38). It is possible that microbial Ags associated with sarcoidosis pathogenesis drive reductions in Lck expression. Several reports have demonstrated that microbial Ags are the targets of the adaptive immune response within sarcoidosis BAL (15, 29, 39). Also, mycobacterial Ags or signals consistent with mycobacterial Ags, such as the catalase-peroxidase, KatG, or the early secreted antigenic target-6, have been identified using mass spectrometry (15, 39, 40). This detection of local reduced Th1 cytokine expression following TCR stimulation, as well as Treg dysfunction, suggests that a contributor to antigenic persistence is inadequate localized adaptive immunity. These persistent microbial Ags could be responsible for the reduction in Lck expression; future studies will focus on possible mechanisms responsible for reduced Lck expression.

One of the most striking observations of this work is that spontaneous clinical resolution correlated with restoration of Th1 and Treg function, whereas continued T cell anergy is a feature of disease progression (Fig. 5). This is a hotly debated area, and this study contributes to the controversy by demonstrating that restoration of T cell function is associated with resolution of pulmonary sarcoidosis. It is consistent with other studies demonstrating immunosuppression is negatively associated with spontaneous resolution of acute sarcoidosis presentations (41). The improvement in T cell function among those with spontaneous disease resolution suggests that a delicate balance between immunosuppression, to control clinical symptoms, and restoration of T cell function, to augment disease resolution, should be the clinical endpoint. It is important to define the mechanisms driving T cell dysfunction to better design therapies to restore T cell function. Cytokine therapy is not a treatment option to restore CD4+ T cell function in sarcoidosis. Exacerbation of disease has occurred in a patient undergoing IL-2 therapy (42), and numerous case reports indicate that IFN therapy both induces and exacerbates sarcoidosis. The best approach to reverse T cell dysfunction in sarcoidosis may be to directly target the pathways driving the dysfunction. In conclusion, to our knowledge, this study demonstrates for the first time reduced CD4+ cytokine expression and proliferative capacity in local sites of sarcoidosis granulomatous inflammation, following TCR stimulation. This
T-cell dysfunction is present not only in the Th1 subset, but also Tregs. Th17 cells also demonstrate reduced function (43). Moreover, clinical resolution of sarcoidosis granulomatous inflammation accompanies restoration of sarcoidosis CD4+ subset proliferative capacity and cytokine expression, through normalized expression of key mediators of IL-2 transcription. Future investigations will entail delineation of the mechanisms responsible for reduced Lck expression, as well as to determine whether restoration of T-cell function can serve as a biomarker for sarcoidosis clinical recovery in a larger cohort.

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Disclosures
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References