Low Levels of NF-κB/p65 Mark Anergic CD4+ T Cells and Correlate with Disease Severity in Sarcoidosis

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T lymphocytes from patients with sarcoidosis respond weakly when stimulated with mitogen or antigen. However, the mechanisms responsible for this anergy are not fully understood. Here, we investigated the protein levels of nuclear transcription factor NF-κB (p50, p65, and p105), IκB (inhibitor of NF-κB), T-cell receptor (TCR) CD3ε-chain, tyrosine kinase p56Lck, and nuclear factor of activated T cells c2 (NF-ATc2) in peripheral blood CD4+ T cells from patients with sarcoidosis. Baseline expression of p65 in these lymphocytes was reduced in 50% of patients. The reduced levels of p65 in sarcoid CD4+ T cells concurred with decreased levels of p50, p105, CD3ε, p56Lck, IκBα, and NF-ATc2. Polyclonal stimulation of NF-κB-deficient sarcoid T cells resulted in reduced expression of CD69 and CD154, decreased proliferation, and cytokine (i.e., interleukin-2 [IL-2] and gamma interferon [IFN-γ]) production. The clinical significance of these findings is suggested by the association between low p65 levels and the development of more severe and active sarcoidosis. Although correlative, our results support a model in which multiple intrinsic signaling defects contribute to peripheral T-cell anergy and the persistence of chronic inflammation in sarcoidosis.

Sarcoidosis is a multisystem disease of unknown etiology characterized by noncaseating granuloma formation (15, 32). It is associated with anergic responses to skin tests and depressed peripheral T-lymphocyte responses in vitro (16, 34). Several studies have examined the mechanisms of peripheral anergy in sarcoidosis. Early reports concluded that the T-cell anergy in sarcoidosis patients was partly due to a decreased production of interleukin 1 (IL-1) by monocytes (28). It was also shown that monocytes contributed to the suppressed lymphocyte responses by releasing increased amounts of prostaglandins (24). More recently, it was demonstrated that expansion of regulatory T cells (Treg cells) and diminished dendritic cell function could be responsible for the peripheral T-cell anergy observed with sarcoidosis. The proposed mechanisms implicated in this suppression included inhibition of IL-2 production and T-cell proliferation by Treg cells and a decreased ability of myeloid dendritic cells to stimulate T lymphocytes (46, 50). Sarcoid patients, however, do not appear to develop significant clinical evidence of immunosuppression, as they are capable of mounting effective immune responses to bacterial, fungal, and viral infections (70). Compartmentalization of these effective responses to the affected organs (i.e., lungs) could also explain the peripheral anergy associated with this disease (30, 31). Although the T-cell anergy associated with sarcoidosis was recognized long ago, the underlying mechanism and implications of this phenomenon for the pathogenesis of sarcoidosis remain unclear.

A key event in the induction of CD4+ T-cell responses is the stimulation of the T-cell receptor (TCR)/CD3 complex on the membranes of T cells by major histocompatibility complex class II (MHC-II) molecule-peptide conjugates (13). The TCR/CD3 complex consists of six distinct chains. The clonotypic α and β chains of the TCR are responsible for recognizing antigens embedded in the MHC-II molecule expressed on the surfaces of antigen-presenting cells (APC). The remaining invariant subunits, collectively termed the CD3 complex, include the γ, δ, ε, and ζ chains of CD3. Ligation of the TCR with its cognate peptide–MHC-II ligand expressed on APC results in the rapid phosphorylation of tyrosine residues within the tyrosine-based activation motifs of the CD3ζ chain by the Src family kinases p56Lck and p59Fyn. These biochemical events ultimately result in the activation of transcription factors that translocate to the nucleus to initiate cytokine gene transcription, lymphocyte proliferation, and effector responses (10, 13, 64).

Transcription factors that participate in inducing cytokine synthesis in T cells include AP-1, NF-AT, and NF-κB (71). Although these transcription factors all contribute to the activation of human T cells, NF-κB is essential in initiating the transcriptional response to TCR and CD28 ligation, expression of IL-2, and proliferation (29, 40, 44, 49). The NF-κB family of transcription factors comprises five members: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), cRel, and RelB. These factors interact with one another to form homo- or heterodimers which exert important transcriptional activities (66). In resting T cells, the NF-κB subunits are sequestered in the cytoplasm through physical interactions with inhibitors of the IκB family. Following TCR stimulation, a cytoplasmic kinase complex, the...
IκB kinase (IKK) becomes activated and phosphorylates the IκB molecules, leading to their degradation through the ubiquitin-proteosome pathway. NF-κB dimers then translocate to the nucleus and activate their target genes (22, 69).

In autoimmune diseases, chronic infections, and cancer, pathological conditions in which persistent antigenic stimulation of T cells occurs, decreased expression of NF-κB, CD3ζ, and p56Lck in T lymphocytes has been implicated in the T-cell anergy associated with these diseases (9, 20, 47, 48, 71, 75). Ligation of CD152 (CTLA-4) and CD279 (PD-1), two coinhibitory molecules of the CD28 family which are expressed at increased levels on chronically stimulated T cells, can also result in functional exhaustion of T lymphocytes (12, 19). Clonally exhausted T cells were first identified in mice infected with lymphocytic choriomeningitis virus, but exhausted lymphocytes have now been found in humans with chronic infections, autoimmunity, granulomatous diseases, and cancer (17, 23, 38, 56, 72, 74).

Another lymphocyte marker that has been used to differentiate T cells according to their stimulation history is CD27. CD27 is a member of the TNF-R family and is expressed on most peripheral blood T cells. Upon antigenic restimulation, surface expression of CD27 is irreversibly lost in T cells. Thus, lack of CD27 expression is a valid surrogate marker to identify chronically stimulated T lymphocytes (3, 27).

A recent theory for the pathogenesis of sarcoidosis postulates that sarcoid granulomas are caused by an effective host response to deposition of mycobacterial antigens aggregated with host proteins. It is believed that these complexes form a poorly soluble nidus for granuloma formation that drives the pathogenic Th1 response characteristic of this disease (52). We hypothesize that failure to remove the aggregates could result in chronic stimulation of T cells and eventually in clonal exhaustion or anergy. As a first step in studying this idea, we investigated the total number and frequency of peripheral CD4+ T-cell populations, the expression profiles of several key proteins involved in TCR signal transduction (i.e., NF-κB, CD3ζ, p56Lck, IκBα, and nuclear factor of activated T cells c2 [NF-ATc2]), the response of T cells to stimulation through the TCR, and the expression profiles of inhibitory receptors in T cells from sarcoid patients and healthy controls. Our results suggest that the anergic state of CD4+ T lymphocytes in sarcoidosis could be a consequence of impaired signal transduction via the TCR/CD3 complex. Furthermore, we propose that by impairing CD4+ T-cell effector functions, these signaling defects exert key regulatory roles that may lead to granuloma susceptibility and persistence in patients with sarcoidosis.

**MATERIALS AND METHODS**

Patients. Twenty-two adult patients who had biopsy-proven sarcoidosis confirmed by ACCESS criteria (54) were recruited for this study. All patients had chronic persistent sarcoidosis (duration of disease, >6 years) and were either on chronic immunosuppressive therapy or not on therapy at the time of peripheral blood collection. The patients were divided into two groups: normal expressers and expressers of low levels of NF-κBp65, based on the levels of NF-κBp65 protein expressed by their peripheral blood CD4+ T cells (see Fig. 2A for the results of a representative immunoblot analysis). Clinical and demographic features of the patients with sarcoidosis are summarized in Table 1. Blood samples from age- and sex-matched healthy donors, who had no history of sarcoidosis, were collected by advertising at the local university and in the hospital employee pool. All subjects participated in the studies after signing an informed consent approved by the local ethics committee.

**TABLE 1. Clinical and demographic features of sarcoid patients**

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Note: The table provides a summary of the clinical and demographic features of sarcoid patients, including age, gender, disease stage, and treatment status. The data are presented as absolute numbers and percentages.
Lymphocyte isolation. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral venous blood by Ficoll-Histopaque (Sigma, St. Louis, MO) density gradient centrifugation (6). Total, naive, and memory CD4+ T cells were purified from PBMC as untouched lymphocytes using CD4+ T-cell isolation kit II and naive and memory CD4+ T-cell isolation kits, respectively (Miltenyi Biotec, Auburn, CA). In some experiments, CD25+ cells were depleted from the CD4+ T-cell population by positive selection using CD25 microbeads (Miltenyi Biotec). The purified lymphocyte fractions routinely contained ≥95% of the total, naive, or memory CD4+ T cells. The CD25-depleted fractions did not contain detectable amounts of CD56+ cells as measured by flow cytometry (FC).

Flow cytometry. PBMC or purified CD4+ T cells (1 × 10^6) were stained for CD3e, CD4, CD25, CD45RA, CD26L, CD60, CD134, CD152, CD273, CD274, CD279 (BD Biosciences, San Diego, CA), CD27, CD45RO, CD86, CD95, CD154, or HLA-DR (eBioscience, San Diego, CA) using marker-specific fluorescent antibodies (Abs). After incubation on ice for 10 min, cells were washed twice with buffer (phosphate-buffered saline [PBS] containing 1% bovine serum albumin [BSA], 0.01% NaN₃) and resuspended in 0.5 ml of the same buffer for FC analysis. Sample acquisition was performed using a FACSscan (Becton Dickinson, San Jose, CA) flow cytometer, and data were analyzed with CellQuest software (Becton Dickinson). An Fc receptor-blocking reagent (human immunoglobulins) was used to reduce nonspecific staining as indicated by CellQuest software (Becton Dickinson). An Fc receptor-blocking reagent (human immunoglobulins) was used to reduce nonspecific staining as indicated by CellQuest software (Becton Dickinson). An Fc receptor-blocking reagent (human immunoglobulins) was used to reduce nonspecific staining as indicated by CellQuest software (Becton Dickinson). An Fc receptor-blocking reagent (human immunoglobulins) was used to reduce nonspecific staining as indicated by CellQuest software (Becton Dickinson).

Lymphocyte activation and proliferation assays. Fractions of peripheral blood CD4+ T cells (total, naive, or memory), CD4+ T cell-depleted PBMC fractions, or Jurkat and K562 cells were extracted in 1% Triton X-100 lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 μM NaCl, 5 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 10 mM β-mercaptoethanol, and a protease inhibitor cocktail (Roche Appligene, Indianapolis, IN). Protein extracts were obtained by centrifugation at 15,000 × g for 15 min at 4°C and stored at −80°C. Protein concentration was determined by standard colorimetric assay (Bio-Rad Laboratories, Richmond, CA). Twenty micrograms of purified total protein was loaded and separated by 10 to 12% SDS-PAGE. Separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and immunoblotted with monoclonal Abs to NF-κB/p65. Twenty micrograms of purified total protein was loaded and separated by 10 to 12% SDS-PAGE. Separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and immunoblotted with monoclonal Abs to NF-κB/p65.

Immunoblotting. Purified fractions of peripheral blood CD4+ T cells (total, naive, or memory), CD4+ T cell-depleted PBMC fractions, or Jurkat and K562 cells were extracted in 1% Triton X-100 lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 μM NaCl, 5 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 10 mM β-mercaptoethanol, and a protease inhibitor cocktail (Roche Appligene, Indianapolis, IN). Protein extracts were obtained by centrifugation at 15,000 × g for 15 min at 4°C and stored at −80°C. Protein concentration was determined by standard colorimetric assay (Bio-Rad Laboratories, Richmond, CA). Twenty micrograms of purified total protein was loaded and separated by 10 to 12% SDS-PAGE. Separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and immunoblotted with monoclonal Abs to NF-κB/p65.

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RESULTS

Increased number and frequency of blood memory CD4+ T cells in sarcoidosis. Surface staining of CD4+ T cells with monoclonal Abs to CD62L and CD45RO or CD45RO allows the identification of three distinct T-cell subpopulations characterized by different homing, proliferative, and effector functions: naïve T cells (CD62L+CD45RA−CD45RO+), central memory T cells (CD62L+CD45RA−CD45RO−), and effector memory T cells (CD62L−CD45RA−CD45RO+). Compared with healthy controls, patients with sarcoidosis (Fig. 1A and F). Compared with healthy controls, patients with sarcoidosis exhibited significantly increased frequency (33.2 ± 10.3 versus 47 ± 12.5) of CD45RO+ lymphocytes (Fig. 1G). The absolute number (205.1 ± 139.4 versus 400.5 ± 487.6 cells/μl of naïve CD4+ T cells) were significantly decreased in patients with sarcoidosis (Fig. 1C and D). In contrast, the frequency (8.7 ± 2.8 versus 12.8 ± 6.7) and total number (57.1 ± 63.4 versus 207.1 ± 206.9 cells/μl) of CD4+ T cells were significantly increased in sarcoid patients (Fig. 1E and F). Compared with healthy controls, patients with sarcoidosis exhibited a significantly increased frequency (33.2 ± 10.3 versus 47 ± 12.5) of CD45RO+ lymphocytes (Fig. 1G). The absolute number (205.1 ± 139.4 versus 400.5 ± 487.6 cells/μl of CD4+ T cells was also increased in sarcoid patients, but this difference did not reach statistical significance (data not shown). A comparative analysis of CD27 expression in PBMC from sarcoid patients and healthy controls revealed a significantly increased frequency (5.1 ± 3.9 versus 13.6 ± 9.2) of CD27+ lymphocytes within the memory CD4+ T-cell subset of patients with sarcoidosis (Fig. 1H). Additionally, sarcoid patients exhibited a significantly increased proportion (0.7 ± 2.0 versus 7.6 ± 9.8; P = 0.0036) of CD4+ T cells expressing the CD27−CD45RO+/− phenotype. The CD27−CD45RO+/− cells were almost absent from the peripheral CD4+ population of healthy controls (<1%). A representative example of the immunofluorescent staining for CD4, CD45RA, and CD42L is shown in Fig. 1A. Compared with healthy controls, sarcoid patients had a significantly reduced frequency (32.7 ± 6.1 versus 24.3 ± 10.7) (Fig. 1B) but normal absolute numbers (1,623 ± 654.8 versus 1,325 ± 1,389 cells/μl; P = 0.0856) of CD4+ T cells (data not shown). Both the frequency (56.3 ± 12.2 versus 35.1 ± 15.6) and the absolute number (1,005 ± 482.6 versus 436.5 ± 449.3 cells/μl of naïve CD4+ T cells were significantly decreased in patients with sarcoidosis (Fig. 1C and D). In contrast, the frequency (8.7 ± 2.8 versus 12.8 ± 6.7) and total number (57.1 ± 63.4 versus 207.1 ± 206.9 cells/μl) of CD4+ T cells were significantly increased in sarcoid patients (Fig. 1E and F). Compared with healthy controls, patients with sarcoidosis exhibited a significantly increased frequency (33.2 ± 10.3 versus 47 ± 12.5) of CD45RO+ lymphocytes (Fig. 1G). The absolute number (205.1 ± 139.4 versus 400.5 ± 487.6 cells/μl of CD4+ T cells was also increased in sarcoid patients, but this difference did not reach statistical significance (data not shown). A comparative analysis of CD27 expression in PBMC from sarcoid patients and healthy controls revealed a significantly increased frequency (5.1 ± 3.9 versus 13.6 ± 9.2) of CD27+ lymphocytes within the memory CD4+ T-cell subset of patients with sarcoidosis (Fig. 1H). Additionally, sarcoid patients exhibited a significantly increased proportion (0.7 ± 2.0 versus 7.6 ± 9.8; P = 0.0036) of CD4+ T cells expressing the CD27−CD45RO+/− phenotype. The CD27−CD45RO+/− cells were almost absent from the peripheral CD4+ population of healthy controls (<1%). A representative example of the immunofluorescent staining for CD4, CD45RA, and CD42L is shown in Fig. 1I. The accumulation of memory CD4+ T cells lacking CD27 expression suggest accelerated differentiation of naïve T cells into memory cells and persistent antigenic stimulation in sarcoidosis.

Peripheral blood CD4+ T cells from patients with sarcoidosis exhibit decreased levels of NF-κB/p65. To investigate whether signaling defects associated with persistent antigenic stimulation were responsible for the peripheral T-cell anergy observed in sarcoidosis patients, immunoblotting of whole-protein lysates of peripheral blood CD4+ T cells isolated from
sarcoid patients or controls was performed using a monoclonal Ab specific to NF-κB/p65 (Fig. 2A). We found that p65 protein levels were significantly reduced or absent in 50% of the sarcoid patients analyzed. The intensity of the p65 band in the remaining 50% of sarcoid patients was quantitatively comparable to that of normal controls. Using protein extracts from the same patients and controls, we reassessed the levels of p65 with another anti-p65 monoclonal Ab that recognizes the N terminus of the protein (Fig. 2B). We again noted the reduction or absence of the p65 protein in these peripheral blood sarcoid CD4 T cells, indicating that defective p65 expression was not due to a loss or modification of a specific epitope. In contrast, p65 protein was detected, albeit at somewhat reduced levels, in lysates of CD4-depleted PBMC from these sarcoid patients, suggesting that the defect was predominant, but not limited, to peripheral blood CD4 T cells (Fig. 2C). Next, we determined the amounts of NF-κB (p105 and p50), p56LCK, NF-ATc2, PP2A, and CD3ζ in peripheral blood CD4 T cells from sarcoid patients and controls by immunoblotting. As shown in Fig. 2A, D, and E, protein expression levels of p105, p50, p56LCK, NF-ATc2, and CD3ζ were reduced predominantly in the group of sarcoid patients that exhibited decreased levels of p65. The reduced levels of p105, p50, p56LCK, NF-ATc2, and CD3ζ were not the result of a global defect in the expression of signaling molecules in sarcoid T cells, since levels of the protein phosphatase PP2A were comparable in all peripheral blood CD4 T cells analyzed (Fig. 2D). In addition, immunoblot analysis of lysates of naïve and memory CD4 T cells isolated from sarcoid patients and controls revealed that the p65 deficiency was not restricted to the memory population since naïve cells exhibited the exact same defect (Fig. 2F). In one patient, however, lack of p65 was limited to naïve cells, but longer exposure of the same blot revealed the presence of a faint p65 band (data not shown). Finally, association studies demonstrated that p65-deficient sarcoid patients had higher (57.1 ± 63.4 versus 150.5 ± 148.9 cells/μl) absolute numbers of

FIG. 1. Increased numbers and frequency of memory CD4 T cells in sarcoidosis patients. (A) Representative example of CD45RA and CD62L expression on CD4 T cells, phenotypic classification of CD4 T-cell subsets according to CD45RA and CD62L expression, and comparison of the frequencies of CD4 T-cell subsets between patients and healthy controls. Results are given as percentages (means ± standard deviations). Frequencies of total (B), naïve (C), TEM (E), TCM (G), or CD27+ CD45RO− (H) cells and absolute numbers of naïve (D) and effector memory (F) cells were determined by dual-platform FC of peripheral blood CD4 T cells from patients with sarcoidosis and healthy controls. (I) Analysis of CD27 and CD45RO expression within the blood CD4 subset of sarcoidosis patients and healthy controls (CD27+ CD45RO+ cells are shown within the dotted square). The median percentages and median absolute numbers of the different populations are shown as solid lines. Dot plots were gated on CD4+ events.
TEM cells than healthy controls, but this difference was not statistically significant (Fig. 2G). Thus, a considerable proportion of sarcoid patients showed an abnormal expression of p65 in naïve and memory CD4\(^+\) T cells, and the p65 defect was associated with a specific decrease in the levels of various signaling molecules (i.e., p105, p50, p56LK, NF-ATc2, and CD3\(\varepsilon\)).

**Impaired responsiveness of NF-kB/p65-deficient sarcoid CD4\(^+\) T cells to TCR stimulation.** In T lymphocytes, NF-kB function is critical for the control of a variety of cellular processes, including activation, proliferation, and cytokine production (22, 66, 69). It was hypothesized, therefore, that decreased levels of p65 in peripheral blood sarcoid T lymphocytes would render these cells functionally impaired. To investigate this, the effect of p65 deficiency on CD4\(^+\) T-cell activation, proliferation, and cytokine production was examined. Purified preparations of p65-deficient or p65-competent sarcoid CD4\(^+\) T cells and CD4\(^+\) T cells from healthy controls were stimulated for 24 h with CD3\(\varepsilon\)/CD28 beads, and the cells were then analyzed by FC for levels of expression of CD25, CD69, CD134, CD154, and HLA-DR. Each of these surface markers is known to be upregulated by CD4\(^+\) T cells upon antigenic stimulation (35, 59, 68). In comparison to p65-competent CD4\(^+\) T cells, stimulation of p65-deficient sarcoid CD4\(^+\) T cells with CD3\(\varepsilon\)/CD28 beads resulted in significantly reduced expression of CD69 and CD154 (Fig. 3A and B). In contrast, stimulation with CD3\(\varepsilon\)/CD28 beads significantly increased the expression of HLA-DR in p65-deficient sarcoid CD4\(^+\) T cells (Fig. 3C). After CD3\(\varepsilon\)/CD28 stimulation, no significant difference in the levels of expression of CD25 and CD134 were observed between p65-deficient sarcoid CD4\(^+\) T cells and p65-competent CD4\(^+\) T cells from sarcoid patients and healthy controls (see Fig. S1A and B in the supplemental material). After 4 days of culture, the CD4\(^+\) T cells were

**FIG. 2.** Reduced levels of NF-kB (p65, p105, and p50), p56LK, NF-ATc2, and CD3\(\varepsilon\) protein in sarcoid CD4\(^+\) T cells, analysis of p65 expression in naïve and memory sarcoid CD4\(^+\) T cells, and increased absolute numbers of TEM cells in patients with sarcoidosis. Purified CD4\(^+\) T cells (A, B, D, and E), CD4\(^+\) T-cell-depleted PBMC fractions (C), or purified naïve and memory phenotype CD4\(^+\) T cells (F) isolated from sarcoid patients and healthy controls by magnet-assisted cell sorting (MACS) were lysed and protein lysates electrophoretically separated, transferred to membranes, and immunoblotted with monoclonal Ab to p65, p105, p50, and actin (A), p65 (N-terminal epitope), p65 (C-terminal epitope), and actin (B), p65 and actin (C), p56LK, NF-ATc2, p65, PP2A, and actin (D), CD3\(\varepsilon\), p65, and actin (E), and p65 and actin (F). (G) Relationship between levels of p65 protein in CD4\(^+\) T cells and the absolute number of TEM cells. The various NF-ATc2 bands shown represent either differences in phosphorylation state of the protein (*, phosphorylated NF-ATc2) or the presence of different isoforms of NF-ATc2, as has been previously described (37). The optical density (OD) of each protein band was quantified by densitometry, and results are expressed in arbitrary units as the ratio of target protein OD to actin OD. The median ODs of the protein bands are shown as solid lines. The numbers of TEM cells were determined by dual-platform FC analysis as indicated in the legend to Fig. 1. Lysates from Jurkat and/or K562 cells were used as controls.
analyzed by FC for proliferation by use of CFSE staining. Cells which have divided exhibit less CFSE fluorescence due to intracellular dilution of the stain (43). Stimulation of p65-deficient sarcoid CD4\(^+\) T cells with CD3\(\varepsilon\)/CD28 beads was associated with substantially less cell proliferation than what was observed for p65-competent CD4\(^+\) T cells (Fig. 3D). The reduced proliferation of p65-deficient sarcoid CD4\(^+\) T cells was not due to a higher fraction of T EM cells, which are incapable of vigorous proliferation; we did not observe any significant difference between the frequencies of T EM cells within the populations of p65-deficient and p65-competent CD4\(^+\) T cells (Fig. 3E). Moreover, cultures of p65-deficient CD4\(^+\) T cells that were depleted of Treg cells exhibited levels of proliferation equivalent to those seen in nondepleted cultures, suggesting that the reduced lymphoproliferation observed in cultures of p65-deficient sarcoid CD4\(^+\) T cells was not the result of the suppressive actions of Treg cells that possibly contaminated these cultures. In accordance with this effect, levels of IL-2 and IFN-\(\gamma\) mRNA were significantly reduced in p65-deficient sarcoid CD4\(^+\) T cells after stimulation of these cells with CD3\(\varepsilon\)/CD28 beads for 6 h. (H) Levels of CD95 were determined by FC in the separated CD4\(^+\) T cells after stimulation of these lymphocytes with CD3\(\varepsilon\)/CD28 beads for 24 h. The median MFI values for CD69, CD154, HLA-DR, CD95, and CFSE staining are shown as solid lines. Representative histograms are shown. (A to D, bottom) Thin line, isotype control (A, B, and C) or CFSE-labeled unstimulated cells (D); thick line, healthy control cells; black, NF-\(\kappa B\) cells; gray, NF-\(\kappa B\) cells.

FIG. 3. Impaired activation, proliferation, and cytokine production of NF-\(\kappa B/p65\)-deficient sarcoid T cells. CD4\(^+\) T lymphocytes, purified from PBMC of sarcoid patients and healthy controls by MACS, were incubated for 24 h with CD3\(\varepsilon\)/CD28 beads, and the cells were then analyzed by FC for levels of expression of CD69 (A), CD154 (B), and HLA-DR (C). (D) Blood CD4\(^+\) T cells, purified either from sarcoid patients or from healthy controls, were labeled with CFSE and stimulated with CD3\(\varepsilon\)/CD28 beads for 4 days, and the mean fluorescence intensity (MFI) of CFSE staining was determined by FC in the CD4\(^+\) T-cell population. (E) Relationship between p65 status and frequency of T EM cells in sarcoid patients and controls. Levels of IL-2 (F) and IFN-\(\gamma\) (G) mRNA were determined by real-time PCR in purified CD4\(^+\) T cells isolated from sarcoid patients and controls after stimulation of these cells with CD3\(\varepsilon\)/CD28 beads for 6 h. (H) Levels of CD95 were determined by FC in the separated CD4\(^+\) T cells after stimulation of these lymphocytes with CD3\(\varepsilon\)/CD28 beads for 24 h. The median MFI values for CD69, CD154, HLA-DR, CD95, and CFSE staining are shown as solid lines. Representative histograms are shown. (A to D, bottom) Thin line, isotype control (A, B, and C) or CFSE-labeled unstimulated cells (D); thick line, healthy control cells; black, NF-\(\kappa B\) cells; gray, NF-\(\kappa B\) cells.
cells (Fig. 3F and G). In contrast, levels of IL-4 mRNA in p65-deficient sarcoid CD4+ T cells were higher than in p65-competent CD4+ T cells, although the difference was not statistically significant (data not shown). The impaired responsiveness of p65-deficient sarcoid CD4+ T cells could not be attributed to diminished expression of the coreceptors CD3ε and CD28 on these cells; p65-deficient and p65-competent CD4+ T cells expressed equivalent levels of these two molecules (see Fig. S3 in the supplemental material). After stimulation with CD3ε/CD28 beads, significantly increased levels of CD95 were observed in p65-deficient sarcoid CD4+ T cells in comparison to those seen in p65-competent CD4+ T cells (Fig. 3H). No significant differences in cell viability, as determined by trypan blue exclusion assay, were found between cultures of p65-deficient and p65-competent CD4+ T cells expressed equivalent levels of these two molecules (see Fig. S3 in the supplemental material). After stimulation with CD3ε/CD28 beads, significantly increased levels of CD95 were observed in p65-deficient sarcoid CD4+ T cells in comparison to those seen in p65-competent CD4+ T cells (Fig. 3H). No significant differences in cell viability, as determined by trypan blue exclusion assay, were found between cultures of p65-deficient and p65-competent CD4+ T cells for up to 4 days of observation (see Fig. S4 in the supplemental material). Collectively, these results suggest that the deficient expression of various signaling molecules (i.e., p50, p65, p105, p56LCK, NF-ATc2, and CD3ζ) is likely responsible for the impaired activation, proliferation, and cytokine production of peripheral blood CD4+ T cells from patients with sarcoidosis.

NF-κB/p65-deficient sarcoid CD4+ T cells exhibit phenotypic features of exhaustion. To investigate whether the impaired proliferation of p65-deficient sarcoid CD4+ T cells was the result of T-cell exhaustion, the expression of CD152 and CD279 in peripheral blood sarcoid T cells was examined. Purified preparations of CD4+ T cells from sarcoid patients or healthy controls were analyzed by FC for levels of expression of CD152 and CD279 after these cells were cultured with and without CD3ε/CD28 beads. Unstimulated CD4+ T cells from sarcoid patients and healthy controls expressed equivalent levels of CD279 (Fig. 4A). However, levels of CD152 expressed by unstimulated p65-deficient sarcoid CD4+ T cells were significantly higher than those expressed by unstimulated p65-competent CD4+ T cells (Fig. 4B). In comparison with no stimulation of CD4+ T lymphocytes, stimulation with CD3ε/CD28 beads increased the levels of CD279 and CD152 expressed by CD4+ T cells from both sarcoid patients and healthy controls (Fig. 4A and B). Levels of CD279 in stimulated sarcoid CD4+ T cells were not significantly different than those observed in stimulated CD4+ T cells from healthy controls (Fig. 4A). When sarcoid CD4+ T cells were stimulated with CD3ε/CD28 beads, however, significantly increased levels of CD152 were detected in these cells in comparison to levels in identically stimulated CD4+ T cells from healthy controls (Fig. 4B). In sarcoid CD4+ T cells that were either stimulated or not with CD3ε/CD28 beads, levels of CD273 and CD274 (two CD279 ligands) were low (CD274) or negative (CD273) and did not significantly differ from those observed in control CD4+ T cells (data not shown). Compared with levels in control CD4+ T lymphocytes, significantly increased levels of CD86 (a ligand for CD152) were found in both resting and activated p65-deficient sarcoid CD4+ T cells (Fig. 4C). Since stimulation with CD3ε/CD28 beads significantly enhanced the levels of CD86 and CD152 expressed on p65-deficient sarcoid CD4+ T cells, it was possible that increased levels of these molecules could facilitate direct interactions between T cells through CD86-CD152, thus inhibiting subsequent responses of these lymphocytes. However, blocking of CD86-CD152 interactions with an anti-CD152 Ab did not significantly enhance the proliferation of p65-deficient sarcoid CD4+ T cells in comparison to proliferation in cultures treated with a control Ab. Similar results were observed when anti-CD274 was used to block CD274-CD279 interactions (see Fig. S5A and B in the supplemental material). Taken together, these data suggest that p65-deficient sarcoid CD4+ T cells are exhausted lymphocytes but

FIG. 4. Expression levels of CD279, CD152, and CD86 in sarcoid CD4+ T cells. CD4+ T lymphocytes, purified from PBMC of sarcoid patients and healthy controls by MACS, were stimulated or not for 24 h with CD3ε/CD28 beads, and the cells were then analyzed by FC for levels of expression of CD279 (A), CD152 (B), and CD86 (C). The median MFI for each of the indicated markers is shown as a solid line. Representative histograms are shown. Thin line, isotype control cells; thick line, healthy control cells; black, NF-κB− cells; gray, NF-κB+ cells.
that the reduced proliferation exhibited by these cells was not the result of negative signals delivered by the coinhibitory receptor CD152.

NF-κB/p65 levels and clinical variables of disease activity. Immunoblot analysis revealed that half of the sarcoid patients exhibited deficient p65 expression in their peripheral blood CD4+ T cells. To examine the clinical relevance of this laboratory finding, we investigated possible associations between p65 protein levels and clinical variables of disease activity in sarcoidosis (Table 1). Compared to what we observed in p65-competent patients, multiple (≥3)-organ involvement (9% versus 36%) and stage IV pulmonary sarcoidosis (18% versus 55%) were seen predominantly in p65-deficient patients. p65-deficient patients also had more active sarcoidosis (36% versus 82%) and worsening disease (0% versus 55%) and were more likely to take corticosteroids (36% versus 73%) or combination therapy (45% versus 64%) than p65-competent patients. Lastly, p65-deficient patients exhibited significantly lower absolute CD4+ (1,623 ± 654.8 versus 703.4 ± 875.4 cells/μl) and CD19+ (2,346 ± 1,178 versus 845.9 ± 941.3 cells/μl) cell counts in comparison to p65-competent patients or healthy controls (Fig. 5A and B). Thus, reduced levels of p65 in peripheral blood CD4+ T cells identify a subset of sarcoid patients who exhibited more active/severe disease and significantly reduced absolute B-cell and T-cell counts.

NF-κB (p65, p105, and p50), IκBα, and CD3ζ levels and treatment status. Because corticosteroids can affect various aspects of T-cell function (53, 61, 67), it is essential to determine whether steroid therapy is associated (i) with the observed deficiency of NF-κB (p65, p105, and p50) and CD3ζ, (ii) with the CD4+ T-cell population imbalance found in sarcoidosis patients, or (iii) with variations of IκBα protein levels in sarcoid CD4+ T cells. Levels of p65 were significantly reduced in patients that were taking prednisone (Fig. 6A). Three patients on steroids showed levels of p65 that were equivalent to those seen in healthy controls and in patients that were off prednisone. Three patients that were not taking prednisone displayed reduced p65 protein levels that were similar to those observed in patients that were on prednisone. There were no associations between the use of prednisone and deficiency of p50 (Fig. 6B), CD3ζ (Fig. 6C), and p105 (data not shown).

Western blot analysis revealed that levels of IκBα protein in p65-deficient sarcoid CD4+ T cells were significantly lower than those found in p65-competent CD4+ T cells and that levels of IκBα protein in sarcoid CD4+ T lymphocytes did not correlate with steroid treatment (Fig. 6D). We did not find any associations between the use of prednisone and increased frequency and numbers of T_{CM} (Fig. 6E and F) and T_{EM} (Fig. 6G and H) cells or between the use of prednisone and decreased frequency (data not shown) and numbers of naïve CD4+ T cells (Fig. 6I). Furthermore, no associations were found between the use of hydroxychloroquine or methotrexate and reduced levels of NF-κB (p50, p65, and p105) and CD3ζ, or between the use of hydroxychloroquine or methotrexate and increased frequency and numbers of T_{CM} and T_{EM} cells (data not shown). These results suggest that therapy, for the most part, cannot be held accountable for the molecular and cellular dysfunctions observed in p65-deficient sarcoid T cells.

DISCUSSION

 Patients with sarcoidosis exhibit a depressed systemic cellular immunity manifested by impaired T-cell responses to mitogens and recall antigens (16, 34). Here, we demonstrate that this anergic state correlates with multiple intrinsic defects in peripheral blood CD4+ T cells. To our knowledge, these results represent the first analysis of the expression of NF-κB, IκBα, CD3ζ, p56LCK, and NF-ATc2 in highly purified preparations of sarcoid CD4+ T lymphocytes.

Biochemical analysis of sarcoid CD4+ T cells revealed reduced p65 protein levels in 50% of the patients. The reduced p65 levels concurred with deficient expression of NF-κB (p50 and p105), IκBα, CD3ζ, p56LCK, and NF-ATc2 and with decreased responses of these lymphocytes to TCR stimulation. Compared to p65-competent CD4+ T cells, p65-deficient sarcoid CD4+ T cells proliferated less and expressed lower levels of IL-2 and IFN-γ mRNA following activation. The presence of κB binding sites in the promoters of IL-2 and IFN-γ mRNA suggests that IL-2 and IFN-γ play a role in the regulation of CD4+ T cell activation. The reduced levels of p65, p56LCK, NF-ATc2, or CD3ζ in sarcoid T cells were directly responsible for the anergy of these lymphocytes. However, the functional defects observed in transgenic mice in which p65/p50 complexes were selectively inhibited in T cells demonstrates that NF-κB deficiency may induce an anergic state in T cells (1, 14).

Restoration experiments are under way in our laboratory to demon-
strate a cause-effect relationship between reduced levels of p65, p50, CD3, and IκBα proteins in sarcoid CD4+ T cells and between the use of prednisone and the frequency of TCM and TEM cells (E and G, respectively) and numbers of TCM, TEM, and naïve CD4+ T cells (F, H, and I, respectively) in patients with sarcoidosis. The levels of p65, p50, CD3, and IκBα protein and the frequencies and numbers of T-cell subpopulations were determined by Western blotting and dual-platform FC analysis, respectively, as indicated in the legends to Fig. 1 and 2. The ODs of p65, p50, CD3, and IκBα protein bands were determined by densitometry (data for IκBα are not shown). The median percentages or absolute numbers of TCM, TEM, and naïve populations are shown as solid lines. Lysates from Jurkat and K562 cells were used as controls.

FIG. 6. Relationship between lymphocyte variables and corticosteroid therapy. Associations were investigated between the use of prednisone (Predn) and levels of p65 (A), p50 (B), CD3 (C), and IκBα (D) proteins in sarcoid CD4+ T cells and between the use of prednisone and the frequency of TCM and TEM cells (E and G, respectively) and numbers of TCM, TEM, and naïve CD4+ T cells (F, H, and I, respectively) in patients with sarcoidosis. The levels of p65, p50, CD3, and IκBα protein and the frequencies and numbers of T-cell subpopulations were determined by Western blotting and dual-platform FC analysis, respectively, as indicated in the legends to Fig. 1 and 2. The ODs of p65, p50, CD3, and IκBα protein bands were determined by densitometry (data for IκBα are not shown). The median percentages or absolute numbers of TCM, TEM, and naïve populations are shown as solid lines. Lysates from Jurkat and K562 cells were used as controls.

The magnitude of T-cell division is partially determined by the relative proportions of memory T-cell subsets in the blood. In chronic beryllium disease and AIDS, a reduced lymphoproliferation correlated with an increased frequency of circulating TEM cells (21, 73). However, reduced division of p65-deficient sarcoid T cells was not the result of an increased proportion of TEM cells, which are incapable of vigorous proliferation; the frequencies of blood TEM cells were similar in all sarcoid patients analyzed regardless of levels of p65 protein in their CD4+ T lymphocytes. Because Treg cells accumulate in the peripheral blood of patients with sarcoidosis and chronic exposure to antigen upregulates the expression of inhibitory receptors in T cells (17, 36, 50, 56), it is possible that Treg-cell-mediated suppression or reception of negative signals through inhibitory receptors could have induced T-cell anergy in sarcoidosis patients. None of these mechanisms were responsible for the observed anergy, since depletion of Treg cells and blockage of CD152-CD80/CD86 or CD279-CD274 interactions did not restore the proliferation of p65-deficient sarcoid T lymphocytes. Since NF-κB is a critical factor involved in the protection of T cells from apoptosis, p65 deficiency could have caused increased apoptosis in stimulated T cells. None of these mechanisms were responsible for the observed anergy, since depletion of Treg cells and blockage of CD152-CD80/CD86 or CD279-CD274 interactions did not restore the proliferation of p65-deficient sarcoid T lymphocytes. Since NF-κB is a critical factor involved in the protection of T cells from apoptosis, p65 deficiency could have caused increased apoptosis in stimulated T cells. None of these mechanisms were responsible for the observed anergy, since depletion of Treg cells and blockage of CD152-CD80/CD86 or CD279-CD274 interactions did not restore the proliferation of p65-deficient sarcoid T lymphocytes.
CD152 expression, it is possible that upregulation of CD152 in p65-deficient sarcoid T cells might have protected these lymphocytes from apoptosis.

Corticosteroids suppress NF-κB activity by increasing the level of IκBα (2, 61). However, the possibility of a role for steroids in the observed decrease in p65 levels can be excluded on the basis of the following observations. First, steroids have no effect on the total amount of cellular p65 protein (61). Second, the use of whole-protein lysates and reducing conditions in the immunoblot assay ruled out sequestration of p65 in the cytoplasm by the inhibitor IκBα. Third, p65-deficient sarcoid T cells expressed significantly reduced levels of IκBα protein, and levels of IκBα protein did not correlate with steroid treatment. Fourth, low p65 levels were found in some patients who were off steroids. Fifth, some patients who were taking prednisone displayed normal p65 levels. Interestingly, sarcoid patients who exhibited reduced p65 levels required more immunosuppression (i.e., prednisone, hydroxychloroquine, and methotrexate) than those with normal levels of that same protein, perhaps as a result of increased disease activity or severity (i.e., radiographic evidence of pulmonary fibrosis [stage IV pulmonary sarcoidosis], worsening disease, need for combination therapy, and greater extrapulmonary involvement). This may explain the association between low p65 protein levels and steroid therapy found in this study. Since steroids inhibit NF-κB signaling and since our p65-deficient sarcoid CD4+ T cells already had low IκBα and NF-κB protein levels, it is possible that these lymphocytes are insensitive to the effects of steroids because they use alternative pathways to activate their effector functions. Consequently, low p65 protein levels in blood CD4+ T cells could possibly be used as a marker to identify a subset of sarcoid patients who will probably not benefit from steroid therapy. Hydroxychloroquine and methotrexate, on the other hand, suppress T-cell responses by inhibiting antigen presentation and cell proliferation, respectively, but these two disease-modifying antirheumatic drugs have no effects on the basal level of p65 protein in T lymphocytes (51).

Interestingly, a recent report indicated that absolute lymphopenia in sarcoid patients correlates with severe disease manifestations and was not related to medical therapy (65). In accordance with these results, we found that sarcoid patients who exhibited low p65 levels also had absolute lymphopenia that was not associated with any specific medical treatment. Our results suggest that low p65 protein levels and reduced CD4+ and CD19+ lymphocyte counts could be used as novel surrogate markers for disease severity in sarcoidosis.

The CD3ζ chain plays a key role in TCR assembly, expression, and signaling (4, 13). In spite of the reduced CD3ζ protein levels observed in p65-deficient sarcoid CD4+ T cells, CD3ζ surface expression in these lymphocytes was unaffected. These results indicate that down-modulation of the TCR-CD3 complex was not responsible for the anergy exhibited by p65-deficient sarcoid T cells following CD3ζ ligation. Because sustained exposure of mice to bacterial antigens induces CD3ζ down-modulation and impairs T-cell function (8), reduced expression of CD3ζ in sarcoid T cells could be a direct consequence of persistent antigenic stimulation of these lymphocytes in the granulomas. The predominant memory phenotype and increased frequency of differentiated (CD27+) effector cells observed within the peripheral blood CD4+ subset of patients with sarcoidosis, as well as the increased levels of CD95, CD152, and HLA-DR expressed by these lymphocytes, support this notion. However, since the frequency of antigen-specific T cells in patients with sarcoidosis may be low (11), it is unlikely that a general decrease in CD3ζ expression in blood lymphocytes from patients with sarcoidosis arises only from T-cell populations which have encountered their specific antigens. In support of this idea, we found that reduced levels of p65 were not restricted to the population of memory CD4+ T cells but that naive CD4+ T cells also exhibited the same defect. Because chronic exposure of T cells to TNF-α and hydrogen peroxide induce down-modulation of CD3ζ expression and T-cell anergy (33, 55), it is possible that production of these factors within the granulomas could have induced CD3ζ down-modulation in nonspecific T cells that have been recruited to the areas of granulomatous inflammation.

The engagement of the B-cell surface molecule CD40 by its T-cell counter receptor CD154 has a central role in the activation, expansion, and differentiation of B cells participating in T-cell-dependent responses (41, 58). Since NF-ATc2 levels were significantly reduced in p65-deficient sarcoid T cells, and because NF-ATc2 is essential for activation of CD154 gene expression (39, 62), reduced levels of NF-ATc2 could be directly responsible for the down-modulation of CD154 expression observed in these lymphocytes. If an effective B-cell response is essential for clearing pathogenic antigens in sarcoidosis, then reduced expression of CD154 in sarcoid CD4+ T cells may induce a defective Ab response that cannot eliminate antigens that induce granuloma formation. Similarly, engagement of CD40 on macrophages by CD154 results in the production of nitric oxide, TNF-α, and IL-12, three critical mediators involved in the elimination of intracellular antigens and the promotion of Th1-mediated inflammation (26). Thus, deficient expression of NF-ATc2 by sarcoid T cells has the potential to impair macrophage activation and elicitation of cell-mediated immune responses, resulting in antigen persistence and chronic inflammation.

It is difficult to envision how the observed dysfunction of T cells can coexist with local (i.e., lung, skin, etc.) excessive responses that occur in sarcoidosis patients. It is possible that in order to control the inflammation, the immune system works toward a state of suppression of T-cell function by down-modulating the expression of signaling molecules in T lymphocytes but fails to do so adequately (7). Consequently, a low level of T-cell function can still be detrimental. The signaling defects could induce a disturbed balance between Th-cell subpopulations. If such balances are critical for sarcoidosis, then a preponderance of dysfunctional Th1 cells at target organs may be an important determinant of susceptibility and the persistence of inflammation in patients with sarcoidosis.

Preliminary studies in our laboratory showed that some sarcoid patients who had p65 deficiency in their peripheral blood T cells exhibited the exact same defect in their bronchoalveolar lavage T cells. These results suggest that the altered signal transduction phenotype observed in peripheral blood T cells of patients with sarcoidosis is not merely an epiphenomenon but also a characteristic of T cells found at the primary target organ (i.e., lungs). We hypothesize that these signaling defects could be responsible for a selective immune deficiency state that might predispose patients with chronic pulmonary sarcoid-
osis to an increased risk of *Aspergillus* infection and development of mycetomas (70).

In summary, our study identified multiple intrinsic defects in blood CD4+ T cells from patients with sarcoidosis. The biochemical and functional status of these lymphocytes resembles the unresponsive state of T cells in patients with AIDS, cancer, and autoimmunity, and indicates features suggestive of anergy. The alteration of T-cell signaling is probably related, at least in part, to conditions of chronic antigenic stimulation, perhaps by persistent sarcoid antigens, superantigens, and/or autoantigens in the granulomas. Chronic exposure of CD4+ T lymphocytes to elevated levels of TNF-α and hydrogen peroxide may also play a role in the signaling defects. The significance of the T-cell signaling defects for the pathogenesis of sarcoidosis is unclear, but we believe that reduced, rather than overactive, cellular immunity in sarcoidosis is associated with more severe and active forms of the disease. To further clarify this important issue, it will be necessary to correlate the signaling status and active forms of the disease. To further clarify this important issue, it will be necessary to correlate the signaling status and active forms of the disease. To further clarify this important issue, it will be necessary to correlate the signaling status and active forms of the disease.

T-cell signaling defects and anergy in sarcoidosis

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