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*J Immunol* 2008; 181:746-755; doi: 10.4049/jimmunol.181.1.746

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The Anergic State in Sarcoidosis Is Associated with Diminished Dendritic Cell Function

Sneha Mathew,* Kristy L. Bauer,* Arne Fischoeder,† Nina Bhardwaj,* and Stephen J. Oliver2*

Sarcoidosis is a chronic inflammatory disease of unknown cause, characterized by granuloma formation similar to tuberculosis, but without clear evidence of a microbial infection. Because sarcoidosis is linked with clinical anergy and other evidence of diminished cellular immunity, we hypothesized that decreased skin delayed-type hypersensitivity (DTH) responses to recall Ags in affected individuals would be associated with decreased function of their blood dendritic cells (DCs). Our study involved ex vivo isolation, phenotyping, and functional testing of myeloid DCs (mDCs), plasmacytoid DCs, and T lymphocytes from blood of normal healthy volunteers and sarcoidosis subjects with active, untreated pulmonary disease. We found mDC function in the allogeneic MLR directly corresponded to the magnitude of skin DTH reactions to recall Ags in both sarcoidosis subjects and normal volunteers. However, both of these outcomes were significantly decreased in the sarcoidosis group. Diminished mDC function occurred despite up-regulated costimulatory and maturation markers. Clinical relevance is suggested by the inverse relationship between both mDC allogeneic responses and skin DTH responses with clinical disease severity as measured by chest radiograms. Because granulomas form when cellular immunity fails to clear antigenic stimuli, attenuated mDC function in sarcoidosis may contribute to susceptibility and persistence of the chronic inflammation characteristic of this disease. The Journal of Immunology, 2008, 181: 746–755.

Granuloma formation results when the cellular immune response fails to eliminate antigenic stimuli such as foreign bodies or microorganisms. Granulomatous inflammation in sarcoidosis, a multisystem disease of unknown etiology, occurs in the absence of clearly defined immunological targets (1). Although both epidemiologic data and histopathology of lesions have been suggestive of an infectious etiology underlying this disease, attempts to link sarcoidosis to a causative pathogen have proven difficult and remain controversial (2, 3).

Despite the presence of locally up-regulated cellular immune activity within inflamed granulomatous tissues, sarcoidosis patients often express paradoxical suppression of their peripheral immune responses, with a high degree of partial or complete clinical anergy to specific skin test Ags (4, 5). In addition to anergic responses to purified protein derivative of tuberculin, between 30 and 70% of patients with active sarcoidosis also fail to respond to Ags from Trichophyton or Candida, and even to chemical haptens such as dinitrochlorobenzene (6). In addition to anergy in vivo, dysfunctional cellular immunity in sarcoidosis is also suggested by in vitro studies demonstrating decreased peripheral blood lymphocyte responses to mitogens and recall Ags (7–9). Thus, sarcoidosis involves foci of chronic inflammation in the setting of attenuated systemic cellular immunity.

The presence of anergy can indicate abnormal functioning in Ag uptake and presentation and/or the effector lymphocytes involved in the delayed-type hypersensitivity (DTH) response. Dendritic cells (DCs), including both myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), are specialized APCs located in the skin and other sentinel positions in the body’s tissues (10). In reaction to danger signals, DCs go through a maturation process, upregulating various costimulatory surface markers and migrating to secondary lymph nodes to present Ags to lymphocytes and inducing both primary and secondary immune responses. Because of the central role played by DCs in organizing effective responses to immunological challenge, any abnormalities in DC maturation and function could lead to aberrant immunity and disease. Indeed, reports of altered DC maturation in human disease have been recorded for a growing number of infectious disorders and malignancies (11–16). However, in sarcoidosis, detailed functional analysis of circulating blood DCs has not been previously reported.

In this study, we analyzed phenotypic markers and function of mDCs and pDCs isolated ex vivo directly from the circulating blood of normal healthy volunteers and individuals with untreated, active pulmonary sarcoidosis. We found for both groups that the allogeneic immune responses induced in vitro by their mDCs were directly correlated with their DTH responses in vivo to skin recall Ags. Furthermore, we found that both of these outcomes were significantly reduced in the sarcoidosis group and were linked with clinical disease severity. Surprisingly, these observed in vitro differences in allogeneic responses by mDCs occurred without relationship to their phenotypic maturation levels. Our results link mDC function with skin anergy and identify this key immune cell as a potential target in sarcoidosis for therapeutic manipulation.

1 This work was supported by the National Institutes of Health grants, including HL077461 (to S.J.O.), and in part by the New York University School of Medicine General Clinical Research Centers Grant M01 RR-00096 from the National Center for Research Resources.

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3 Abbreviations used in this paper: DTH, delayed-type hypersensitivity; DC, dendritic cell; lin−, lineage-negative; mDC, myeloid dendritic cell; Mo-DC, dendritic cell derived in vitro from monocyte precursors; pDC, plasmacytoid dendritic cell.
Materials and Methods

Patients

Twenty-seven consecutive adults (median age 45 years, range 29–63 years) with biopsy-proven, active and untreated sarcoidosis involving the lung were recruited for this study along with 33 normal healthy volunteers (median age 42 years, range 22–65 years). Each group included 19 women. Median disease duration for sarcoidosis patients was 4 mo (range 0.3–35 mo), and extrapulmonary disease was evident in 10 (dermatologic), 7 (ocular), 4 (intraarticular), and 1 (hepatic) patient. Ten sarcoidosis subjects had previous corticosteroid exposure, but received no immune suppressive therapy for ≥1 year before study enrollment. Six untreated patients with the fibrosing tissue disease, systemic sclerosis, were included as a rheumatic disease control for mDC function. This protocol was approved by the institutional review boards of New York University School of Medicine and the Bellevue Hospital Medical Center.

Specimen collection

Seventy-five cubic centimeters of heparinized blood was obtained for DC isolation, along with additional EDTA-treated blood for flow cytometry and plasma cytokine analysis. Clinical laboratory values obtained included a complete blood count, chemistry panel, urinalysis, and erythrocyte sedimentation rate. Protocol items specific for sarcoidosis patients included serum angiotensin-converting enzyme level and chest x-ray. Chest x-ray score was determined by established convention (17) using the radiologist’s report as follows: stage 0, normal radiograph; stage 1, hilar lymphadenopathy; stage 2, hilar lymphadenopathy and interstitial involvement of the lung fields; stage 3, interstitial changes without hilar lymphadenopathy. Additional measures of sarcoid clinical disease activity included a self-administered questionnaire (Short Form-36 from QualityMetric).

Skin testing

Each patient received 0.1 cc intradermal injections of sterile clinical grade Ag suspensions derived from Candida albicans (Allermed Laboratories), Trichophyton species (Meridian Bio-Medical), and tuberculin purified protein derivative (Sanofi Pasteur) at three separate sites on their arms. Forty-eight hours later, each injection site was digitally imaged (Coolpix 990, Nikon) with a calibration marker in each field of view. Diameters of resulting erythematous reactions were measured by digital image analysis (ImageJ, http://rsb.info.nih.gov/ij/).

Whole blood DC phenotyping

EDTA-treated blood samples were stained with FITC-conjugated mAb mixture for non-DC lineage leukocytes or lineage-negative (lin−) cells with specificity for CD3, CD14, CD16, CD19, CD20, and CD56, PerCP-conjugated mAbs against HLA-DR, and PE-conjugated mAbs against either the surface markers CD11c or CD123 (BD Biosciences). Following RBC lysis and wash, the stained leukocytes were acquired by flow cytometry (FACSCalibur, BD Biosciences) with generated data analyzed by FlowJo software version 6.3 (Tree Star). The lin−, HLA-DRhigh cells were identified as either CD11c+ pDCs or CD123+ mDCs, with results expressed as percentage of the acquired leukocytes. Final quantification of blood DCs was adjusted to subject’s total white blood cell count as determined by hospital clinical laboratory testing of a concurrently drawn blood sample.

Ex vivo DC isolation

Plasmacytoid DCs. Forty percent of recovered PBMCs obtained by Ficoll gradient centrifugation were used to directly isolated pDCs by anti-bone DC Ag-4 mAb-conjugated ferromagnetic beads according to the manufacturer’s protocol (Miltenyi Biotec), using two passages through positive selection magnetic columns (Miltenyi Biotec). Median recovery rate of pDCs from PBMCs from both subject groups was 0.23% (range 0.07–0.70%) with a purity of ≥76%. Isolated live pDCs were plated at 2 × 10⁷ cells/well in round-bottom 96-well tissue culture plates, followed by 18 h incubation with 2 μg/ml unmethylated CpG-A DNA (Integrated DNA Technologies). Harvested supernatants from quadruplicate wells were individually stored at −80°C for later IFN-α level determination by ELISA (Pierce-Endogen).

Myeloid DCs. The remaining 60% of subjects’ PBMCs were plated overnight in culture with complete RPMI 1640 (supplemented with 5% heat-inactivated human AB serum). The following day, nonadherent cells were removed from either CD14+ PBMC culture, with a portion examined by flow cytometry for surface expression of CD40, CD80, CD83, and CD86 on lin−, HLA-DRhighCD11c+ cells. Remaining nonadherent cells were depleted of CD19+ B cells, followed by selection for blood DC Ag-1+ cells using magnetized columns (Miltenyi Biotec) according to manufacturer’s instructions. Median mDC recovery rate from PBMCs in both subject groups was 0.26% (range 0.10–0.58%) with a purity of ≥74%. Isolated mDCs were transferred to 96-well round-bottom culture plate for functional testing by allogeneic MLR.

In vitro, monocyte-derived mDCs (Mo-DCs)

A single donor’s PBMCs were plated at 1 × 10⁷ cells/well in 6-well tissue culture plates (Corning Life Sciences) for 2 h, followed by removal of nonadherent cells. Adherent cells were incubated in complete RPMI 1640 medium (1% human AB serum) with 10 ng/ml IL-4 and 50 ng/ml GM-CSF (R&D Systems), with repeat addition of these cytokines on days 2 and 4. On day 5, nonadherent, immature DCs were transferred to a new 6-well plate and matured with 20 ng/ml LPS (Sigma-Aldrich) over a 48-h period before harvest and use in an allogeneic MLR.

Responder T cells

PBMCs from two different, unrelated normal control donors were frozen in aliquots for use throughout the study as responder T cells against each subject’s DCs. PBMC aliquots were thawed and plated for 4 h to allow for spontaneous cell death, with nonadherent cells passed through a 45-μm filter and recounted before use as responder T cells against subject-derived mDCs in an allogeneic MLR. Results were analyzed as T cell proliferation counts from the one single donor consistently used throughout the protocol. MLR results were also separately analyzed for each subject’s mDCs by whichever of the two unrelated donors’ T cells provided the highest proliferation counts (high responder T cells). To evaluate allogeneic responses of subject T cells, flow-through cells remaining after a subject’s pDC isolation were stored at −80°C for later use against normal control Mo-DCs.

Allogeneic MLR

Each subject’s isolated mDCs were plated in quadruplicate in 96-well round-bottom plates (Corning Life Sciences) and serially diluted against fixed concentrations of responder T cells from two unrelated donors in two separate MLRs. These allogeneic MLRs were incubated for 4 days, followed by overnight pulsing with [3H]thymidine at 1 μCi/well (PerkinElmer). Pulsed cells were harvested by lysis and aspiration to filter mat and read by a beta scintillation counter (Betaplate 1200, PerkinElmer). Results were calculated as counts per minute at each DC-T cell ratio minus the background (T cell-only wells). Wells containing DCs only for each subject showed minimal reactivity at day 5 ([200 cpm). T cell response was monitored by concurrently run positive controls stimulated by soluble mAbs to CD1 (5 μg/ml) and CD28 (10 μg/ml) (R&D Systems). Viability for all cell types used in our experiments was determined by exclusion of propidium blue during cell counting.

ELISA evaluation of soluble protein levels

Subjects’ EDTA-treated plasma was obtained by refrigerated centrifugation within 15 min of phlebotomy. Both plasma and cell-free supernatants from overnight PBMCs and MLRs were divided into multiple aliquots and stored at −80°C, with later analysis in duplicate (plasma) or triplicate (supernatants) by commercial ELISA for MCP-1, soluble IL-2 receptor, TGF-β (Pierce-Endogen), high-sensitivity IL-10, high-sensitivity IL-6, TNF-α (Innitrogen), and IFN-γ (eBioscience) according to manufacturers’ instructions. IL-12 was assessed by ELISA for either total IL-12, including both the p40 subunit and the p70 homodimer (eBioscience), or more specifically for the bioactive p70 form of the IL-12 cytokine (Pierce-Endogen). Evaluations for IL-23, a heterodimeric cytokine comprised of the p19 and p40 subunits, were performed using an ELISA without interference or cross-reactivity with the IL-12p40 monomer or IL-12p70 heterodimer (eBioscience). Absorption levels at manufacturers’ specified wavelengths were determined by spectrophotometer (VersaMax 384 Plus, Molecular Devices), with results generated against concurrently run standard curves.

Statistics

Data from this prospectively designed study are presented graphically in box plots as median, interquartile range, and range, unless otherwise indicated. All statistical comparisons of differences between normal controls and patients were performed using SPSS software (version 11.0) using the Mann-Whitney U test for independent data and the Spearman coefficient for bivalent correlation between sets of data.
FIGURE 1. Reduced skin DTH responses in sarcoidosis subjects. Sarcoidosis subjects (Sarc) have a reduced DTH skin test response to Candida compared with normal controls (NC) and disease controls with systemic sclerosis (SSc). Skin DTH responses were measured as the erythema present around the intradermal injection site at 48 h. Median, interquartile, and range are shown, with outliers represented by open circles. *, p ≤ 0.025.

Results

Sarcoidosis subjects have decreased DTH reactions to Candida

All subjects reacted measurably to Candida skin testing with the exception of two normal controls and two sarcoidosis patients. However, Candida DTH reactions in sarcoidosis subjects were significantly reduced compared with reactions in the normal control group as well as to a disease control group comprised of individuals with systemic sclerosis, an immune-mediated disease characterized by fibrosis of skin and internal organs (Fig. 1). Measurable reactions to Trichophyton and tuberculin purified protein derivative were very rare among all subject groups and were not included in the data analysis.

Elevation of inflammatory plasma protein levels in sarcoidosis subjects

Sarcoidosis subjects were ambulatory outpatients with low to moderately elevated levels of systemic inflammation, as reflected by the group erythrocyte sedimentation rate median of 12 mm/h (range 1–77 mm/h) compared with the normal control group’s median erythrocyte sedimentation rate of 4 mm/h (range 1–26 mm/h) (p = 0.004). Nonetheless, significant elevations of several important immune markers were present in their plasma, including the Th1-type cytokine IFN-γ, the proinflammatory cytokines IL-6 and TNF-α, and soluble IL-2 receptor, an indirect indicator of T cell activation (Table I). In the sarcoidosis group, we found significantly high plasma levels of total IL-12, comprised of both the p40 monomer and the heterodimeric p70 cytokine. However, specific assay for the bioactive IL-12p70 protein did not detect measurable levels in either subject group (data not shown), while low but comparable group levels of IL-23 were found, suggesting that the differential findings in IL-12 plasma levels between the normal controls and sarcoidosis subjects was largely due to the presence of the p40 subunit. Plasma levels of the antiinflammatory cytokines IL-10 and TGF-β were comparable between sarcoidosis and normal control groups.

Similar numbers of circulating blood pDCs and mDCs are present in sarcoidosis and normal controls

To determine circulating blood levels of mDCs and pDCs in our subjects, we used flow cytometry to analyze whole blood-stained specimens. DC subsets (mDCs, pDCs) were identified by gating on the p40+ cells (Fig. 2a) that expressed high levels of HLA-DR and either CD11c+ mDCs (b) or CD123+ pDCs (c). The DC subset of the gated live cells for subjects was multiplied by their respective total blood leukocyte count per microliter as determined by clinical laboratory on a concurrently drawn blood specimen. Results for the number of mDCs (d) and pDCs (e), and the ratio of the two subsets (f) are expressed as the median, interquartile range, and range, with open circles representing outliers.

Table I. Plasma levels of soluble proteins

<table>
<thead>
<tr>
<th>Soluble Protein</th>
<th>Normal (n = 33)</th>
<th>Sarcoidosis (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12p40/p70 (pg/ml)</td>
<td>37 (0–91)</td>
<td>88 (4–476)**</td>
</tr>
<tr>
<td>IL-23 (pg/ml)</td>
<td>0.1 (0.0–43.0)</td>
<td>1.8 (0.0–60.2)</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>3.2 (1.9–8.7)</td>
<td>4.3 (3.2–14.1)**</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>3.8 (2.6–18.5)</td>
<td>7.4 (4–33)**</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.1 (0.7–3.3)</td>
<td>2.4 (1–22)*</td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>260 (155–533)</td>
<td>313 (157–690)</td>
</tr>
<tr>
<td>Soluble IL-2 receptor (U/ml)</td>
<td>361 (82–526)</td>
<td>551 (339–4373)**</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>1.1 (0.8–1.4)</td>
<td>1.1 (0.5–5.7)</td>
</tr>
<tr>
<td>TGF-β (pg/ml)</td>
<td>107 (25–1232)</td>
<td>76 (0–413)</td>
</tr>
</tbody>
</table>

a Significance levels are indicated as: *, p ≤ 0.035; **, p < 0.001.

Myeloid DC function is decreased in individuals with sarcoidosis

To study potential differences in DC function between normal healthy controls and individuals with sarcoidosis, we isolated immature DCs ex vivo directly from the subjects’ blood. This method allowed study of subject DCs without introduction of exogenous
from a single normal donor. Normal healthy controls were plated at a concentration of 200,000/well and analyzed (not shown). When proliferation rates of the high-responder T cells were analyzed over the final 18 h is shown (a). Similar group differences were seen when proliferation rates of the high-responder T cells were analyzed (not shown). b, Subject T cells from either sarcoidosis patients or normal healthy controls were plated at a concentration of 200,000/well against a constant number of T cells from an unrelated donor in an allogeneic MLR and incubated for 5 days. Logarithmic plotting of group means and SEM of [3H]thymidine uptake by a single donor’s proliferating T cells over the final 18 h is shown (a). 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Similar group differences were seen when proliferation rates of the high-responder T cells were analyzed (not shown).

Functional testing of T cells and DC subsets from study subjects and correlations with skin DTH reactions. Ex vivo-isolated mDCs from sarcoidosis subjects (Sarcoid), normal healthy controls (Normal), and systemic sclerosis subjects (SSc) were serially diluted in 96-well round-bottom plates against a constant number of T cells from an unrelated donor in an allogeneic MLR and incubated for 5 days. Logarithmic plotting of group means and SEM of [3H]thymidine uptake by a single donor’s proliferating T cells over the final 18 h is shown (Fig. 3a). Similar group differences were seen when proliferation rates of the high-responder T cells were analyzed (not shown). b, Subject T cells from either sarcoidosis patients or normal healthy controls were plated at a concentration of 200,000/well against a constant number of T cells from an unrelated donor in an allogeneic MLR and incubated for 5 days. Logarithmic plotting of group means and SEM of [3H]thymidine uptake by a single donor’s proliferating T cells over the final 18 h is shown (a). Similar group differences were seen when proliferation rates of the high-responder T cells were analyzed (not shown). b, Subject T cells from either sarcoidosis patients or normal healthy controls were plated at a concentration of 200,000/well against a constant number of T cells from an unrelated donor in an allogeneic MLR and incubated for 5 days. Logarithmic plotting of group means and SEM of [3H]thymidine uptake by a single donor’s proliferating T cells over the final 18 h is shown (a). Similar group differences were seen when proliferation rates of the high-responder T cells were analyzed (not shown). b, Subject T cells from either sarcoidosis patients or normal healthy controls were plated at a concentration of 200,000/well against a constant number of T cells from an unrelated donor in an allogeneic MLR and incubated for 5 days. Logarithmic plotting of group means and SEM of [3H]thymidine uptake by a single donor’s proliferating T cells over the final 18 h is shown (a). Similar group differences were seen when proliferation rates of the high-responder T cells were analyzed (not shown). b, Subject T cells from either sarcoidosis patients or normal healthy controls were plated at a concentration of 200,000/well against a constant number of T cells from an unrelated donor in an allogeneic MLR and incubated for 5 days. Logarithmic plotting of group means and SEM of [3H]thymidine uptake by a single donor’s proliferating T cells over the final 18 h is shown (a). Similar group differences were seen when proliferation rates of the high-responder T cells were analyzed (not shown). b, Subject T cells from either sarcoidosis patients or normal healthy controls were plated at a concentration of 200,000/well against a constant number of T cells from an unrelated donor in an allogeneic MLR and incubated for 5 days. Logarithmic plotting of group means and SEM of [3H]thymidine uptake by a single donor’s proliferating T cells over the final 18 h is shown (a). Similar group differences were seen when proliferation rates of the high-responder T cells were analyzed (not shown). b, Subject T cells from either sarcoidosis patients or normal healthy controls were plated at a concentration of 200,000/well against a constant number of T cells from an unrelated donor in an allogeneic MLR and incubated for 5 days. Logarithmic plotting of group means and SEM of [3H]thymidine uptake by a single donor’s proliferating T cells over the final 18 h is shown (a). Similar group differences were seen when proliferation rates of the high-responder T cells were analyzed (not shown). b, Subject T cells from either sarcoidosis patients or normal healthy controls were plated at a concentration of 200,000/well against a constant number of T cells from an unrelated donor in an allogeneic MLR and incubated for 5 days. Logarithmic plotting of group means and SEM of [3H]thymidine uptake by a single donor’s proliferating T cells over the final 18 h is shown (a). Similar group differences were seen when proliferation rates of the high-responder T cells were analyzed (not shown). b, Subject T cells from either sarcoidosis patients or normal healthy controls were plated at a concentration of 200,000/well against a constant number of T cells from an unrelated donor in an allogeneic MLR and incubated for 5 days. Logarithmic plotting of group means and SEM of [3H]thymidine uptake by a single donor’s proliferating T cells over the final 18 h is shown (a). Similar group differences were seen when proliferation rates of the high-responder T cells were analyzed (not shown).

Myeloid DC function in the allogeneic MLR is directly related to subjects’ skin DTH responses to Candida

We next asked whether there was any relationship in our subjects between their mDC function in vitro and their in vivo skin DTH responses to Candida recall Ags. We found that skin DTH responses to Candida were directly related to mDC function in vitro, as measured by allogeneic responses of T cells from a single donor (Fig. 3c) or from the high-responder T cells (data not shown). Within both normal control and sarcoid subject groups, greater in vivo skin immunity to recall Ags was significantly associated with greater in vitro activity of mDCs in the MLR.

Attenuated mDC function is not explained by reduced expression of activation markers

Insufficient costimulatory molecule expression during Ag presentation by DCs can result in T cell anergy. To further examine the decreased MLR function of mDCs from sarcoidosis subjects, we measured expression of costimulatory molecules by subject mDCs immediately before the isolation and plating of these cells in the allogeneic MLR (Fig. 4a–e). Our initial studies revealed that, while expression of the costimulatory and maturation makers CD40, CD80, CD83, and class II (HLA-DR) were relatively low on immature mDCs isolated from freshly drawn blood, these makers spontaneously up-regulated on mDCs studied after incubation overnight in PBMC culture (data not shown). Contrary to expectations, sarcoidosis mDCs examined just before MLR plating showed significantly increased expression of CD40 compared with normal control mDCs (Fig. 4g), while CD83 and the other costimulatory molecules were comparably up-regulated (Fig. 4, f and h–j). No significant correlations were observed between mDC expression of these markers and their ability to drive allogeneic T cell proliferation in the MLR. Also, we did not find any group differences in levels of HLA-DR expression on immature blood mDCs at the time of phlebotomy (data not shown). Thus, attenuated function of sarcoid-derived mDCs in the allogeneic MLR was not associated with lack of phenotypic maturation.
Attenuated mDC function does not reflect differences in inflammatory cytokine exposure during PBMC overnight culture

Because exposure to soluble secreted factors such as cytokines during the transition from the immature to mature state may also affect subsequent mDC Ag presentation function, we next determined cytokine levels in supernatants obtained from the overnight PBMC cultures from which mDCs were isolated. Significantly increased levels of MCP-1, IL-6, and IL-12p40/p70 were found in supernatants from the sarcoid subjects’ PBMCs compared with levels from normal control PBMC cultures, while IL-23 and IL-10 levels were comparable between the two groups (Fig. 5a–e). No detectable levels of IFN-γ, IL-12p70, or TGF-β were found in either group. The supernatant findings reflected the relative plasma level differences of these cytokines for the two subject groups (Table I). However, neither supernatant nor plasma levels of these cytokines correlated with allogeneic MLR responses induced by subject mDCs.

Minimal differences in MLR cytokine levels at 24 h

We also questioned whether there were group differences in cytokines released into culture supernatants during the allogeneic MLR. Because of limited availability of ex vivo isolated mDCs for our assays, we selected a single time point of 24 h to measure soluble protein levels in the MLR supernatants (Fig. 5f–l). Unlike findings in PBMC culture supernatants, no group differences were...
seen in levels of MCP-1, IL-6, and IL-12p40/p70 from MLR supernatants (Fig. 5f–h). In contrast, IL-23 remained present in the normal control MLR supernatants but was largely undetectable in the sarcoidosis group (Fig. 5i). A wide range of IL-10 levels were found in the sarcoidosis mDC-driven MLR, resulting in no statistically significant differences between the two groups (Fig. 5j). Importantly, we did not find reduced levels of the key Th1-type cytokine IFN-γ levels in MLRs containing sarcoid-derived mDCs (Fig. 5k) nor correlations of these levels with IL-12 production in plasma, overnight PBMC supernatants, or MLR supernatants. We also measured levels of the antiinflammatory cytokine TGF-β in the MLR supernatants (Fig. 5l), but again found no statistically significant difference between the two groups. Thus, the increased levels of inflammatory factors noted in overnight cultures of sarcoid PBMCs and/or plasma from sarcoid subjects did not persist in the MLRs containing mDCs isolated from these individuals. Additionally, no significant correlations were noted between cytokines in the 24 h MLR supernatants and mDC function in the allogeneic MLR.

**Functional response to CpG-A stimulation of sarcoidosis pDCs in vitro remains intact**

In addition to our studies of mDCs from our subjects, we also directly isolated their blood pDCs for in vitro functional testing. Plasmacytoid DCs secrete large amounts of IFN-α in response to ligation of TLR9, a pathogen-associated molecular pattern receptor specific for bacterial and viral DNA. Oligonucleotide sequences such as CpG-A are unmethylated and mimic microbial DNA, thus providing strong stimuli for pDC production of IFN-α (18). We found that pDCs directly isolated ex vivo from the blood of sarcoidosis subjects and normal healthy volunteers released similar amounts of IFN-α in response to CpG-A stimulation over an 18-h period (Fig. 6).

**Myeloid DC in vitro function correlates with in vivo measure of pulmonary disease severity**

Our in vitro functional measures for mDCs, pDCs, and responder T cells in the sarcoidosis subjects revealed that only their mDCs were functionally attenuated. To provide potential clinical relevance for our laboratory findings, we analyzed the demographic and clinical disease measures for any relationships with our subjects’ DC functional outcomes. We identified an association between mDC function in the MLR and the chest x-ray score, a radiological measurement of disease activity in the patients’ lungs (Fig. 7a). Increased severity of pulmonary disease correlated with lower mDC-driven proliferation of T cells. This same inverse relationship existed between chest x-ray scores and Candida DTH skin reactions (Fig. 7b). Interestingly, the chest x-ray score also correlated with mDC expression of the costimulatory molecule CD40 ($r = 0.029$, $p = 0.455$). We did not find any other correlations between DC outcomes and clinical measures, including disease duration, extrapulmonary disease involvement, serum angiotensin-converting enzyme level, or erythrocyte sedimentation rate, or the Short Form-36 cumulative physical or mental scores.

**Discussion**

In this study, we found significant correlations between the in vitro allogeneic immune responses by subjects’ mDCs and their in vivo skin DTH responses to the common recall Ag Candida. Importantly, this correlation existed within both normal control and sarcoid groups, even with the overall reduced DTH responses occurring in the latter group. Although anergic subjects with poorly functioning mDCs were often of the sarcoid group, there were a number of apparently healthy normal controls with these attributes. A positive response to skin testing with Candida in the general population ranges between 60 and 80%, and up to 5% of healthy adults can have complete anergy to testing with multiple different Ags, a condition of unknown clinical significance (19–21). Our results indicate that anergy to at least one common recall Ag in either normal healthy individuals or those with sarcoidosis is associated with reduced functioning of mDCs in vitro. It is possible that identification of individuals in either group with anergy to multiple skin test Ags would provide a much stronger link to poor mDC function, but the currently limited selection of commercially available reagents for skin testing precluded using this approach in our study.

Any potential relevance of in vitro mDC function to sarcoidosis disease susceptibility or its progression remains speculative at this time. These findings do suggest that individuals suffering from sarcoidosis exist within the lower range of the general population in terms of these two attributes, although whether
their immune function was compromised before disease onset remains unknown. Nonetheless, increases in anergy and mDC dysfunction in our sarcoid subjects are each separately correlated with increased severity of their pulmonary disease as measured by the chest x-ray score. The radiologic score has been in use for many years by clinical researchers of sarcoidosis to follow disease activity and provide prognostic information (17). Enlarged lymph nodes and the absence or presence of disease in the surrounding lung tissues indicates a chest x-ray score of 1 or 2, respectively, while lung tissue involvement in the absence of any lymph node enlargement rates a 3 and has the worst prognosis. Viewed in the context of our results, the use of lymph node size in this progressive radiologic scoring system might be interpreted as indicating the strength of an individual’s immune response to contain the disease.

Although not ideal, a normal skin DTH response to a common recall Ag provides in vivo evidence for an intact cellular immune memory response. Macrophages and DCs within the challenged skin take up the Ags, become activated, and then induce memory T cells to clonally expand and respond in an Ag-specific manner. In individuals with sarcoidosis, high prevalence of partial or complete clinical anergy to recall Ags and even synthetic skin sensitizers suggests that dysfunction exists somewhere within this immunological circuit. However, unlike primary immune deficiency diseases associated with anergy or acquired types such as HIV infection, any impairment that exists in the cellular immunity of sarcoidosis patients appears relatively mild, for these patients do not develop recurrent infections typical of an immune-compromised host. Additionally, the reported resolution of anergy upon remission of sarcoidosis (22) suggests that a relationship exists between the immune abnormality and the underlying pathogenesis.

Numerous attempts have been made over the years to explain the paradoxical immunity underlying sarcoidosis. Early studies proposed that soluble factors such as PGE₂ released by monocytes from patients with active sarcoidosis were responsible for inhibiting in vitro lymphocytic responses to mitogen and Ags (9). Others suggested that decreased ratios of CD4⁺ Th cells to CD8⁺ suppressor T cells in the circulating blood of patients was responsible for their reduced peripheral immunity (8). More recently it was reported that sarcoidosis patients have marked increases in circulating CD4⁺ regulatory T cells that have suppressive in vitro effects on T cell proliferation (23). However, specific linking of in vivo DTH responses with the immune function of a particular cell type has not been described in sarcoidosis or in the general human population.

We previously demonstrated augmentation of DC activation markers and increased T cell infiltration of granulomas in cutaneous sarcoidosis patients during their early clinical response to treatment with thalidomide (24), a drug with known immune-stimulatory effects on mDCs and T cells (25, 26). During early thalidomide treatment, the sarcoid granulomas showed increased infiltration by T lymphocytes, multinucleated giant cells, and DCs, followed by later breakup and regression of these structures. This unexpected finding suggested to us a potential link between attenuated cellular immunity in an individual and susceptibility to onset or persistence of the granulomatous inflammation characteristic of sarcoidosis. To further explore this observation, we have focused our attention on the DC, a critical early element during the initiation of an effective immune response against antigenic stimuli.

Because changes in the composition of circulating DC subsets and their response to maturation stimuli can affect an individual’s cellular immunity, we determined levels of circulating DC subsets in sarcoidosis subjects and normal controls at the time of skin DTH testing as well as examined the phenotypic and functional characteristics of their DCs after ex vivo isolation from peripheral blood. Our method was specifically designed to provide DCs that were unmanipulated by exogenously added cytokines to detect potential immune function abnormalities that were present or acquired during in vivo development. Our results showed that sarcoidosis patients had normal circulating numbers of both DC subsets in the blood and normal in vitro responses by pDCs to TLR9 ligation. However, mDCs isolated from peripheral blood of sarcoidosis subjects generated reduced allogeneic T cell responses in vitro, despite up-regulated expression of MHC class II and costimulatory molecules. In contrast, T cells from these sarcoidosis subjects proliferated vigorously to normal control Mo-DCs at levels comparable to T cells from the healthy volunteers. Importantly, our evaluation method did not show reduced in vitro allogeneic immune responses with mDCs isolated from patients with scleroderma, a Th2-type immunity-associated, skin fibrosing disorder that is not associated with clinical anergy. Furthermore, we identified an association between the in vitro capacity of mDCs from our study participants to induce allogeneic T cell responses and in vivo measured outcomes in these subjects, including the skin DTH response and a radiological score of lung disease activity, thus providing potential clinical relevance for our results.

Because DCs were initially reported in sarcoidosis as being rarely present in noncutaneous sites (27), there have been limited studies of DCs in this disease. In an early report involving anergic sarcoidosis patients, reduced numbers of epidermal Langerhans cells were found in both lesional and noninvolved skin, with the most marked reductions seen in patients with multisystemic disease (28). Later investigators have detected DCs in bronchoalveolar washings from sarcoidosis patients (29). More recently, it was shown that airway mDCs from sarcoidosis patients had increased expression of CD80 and decreased expression of CD86 compared with normal healthy volunteers, although overall DC numbers were comparable between the two groups (30).

In the blood, circulating levels of both DC subsets were recently reported to decrease in sarcoidosis patients, in contrast to our findings (31). However, this study determined DC numbers by first depleting PBMCs of monocytes, B cells, and T cells before quantifying DCs by flow cytometry, using the presence or absence of CD11c on HLA-DR⁺ cells to differentiate between mDCs and pDCs. In our study, we directly measured whole blood for lin⁻, HLA-DR⁺ cells expressing either CD11c or CD123 for the two major DC types, respectively. Furthermore, our recovery rates of both DC subsets directly from PBMCs were similar between sarcoidosis subjects and normal healthy controls, corroborating our flow cytometry results on the whole blood specimens.

In contrast to the limited information available on DCs in sarcoidosis, numerous reports have described how DC maturation can be altered or blocked by microbial pathogens, providing for advantage over host defenses. For example, mycobacterial infection of immature DCs can down-regulate expression of CD1 and costimulatory molecules, reduce IL-12 production, and inhibit activation of T cells (13, 32, 33). DC infection by either Mycobacterium tuberculosis or the HIV virus can cause increased secretion of the anti-inflammatory cytokine IL-10, driving T cell differentiation toward Th2-type responses and anergy (12, 13). In these microbial-driven diseases, altered DC maturation contributes to an ineffective, chronic inflammatory
cular endothelial growth factor, and PGE2, have been shown to attenuate responses that fail to clear the inciting intracellular pathogen. In sarcoidosis, however, the putative Ags responsible for driving granuloma formation remain unknown, making similar investigations difficult.

Noninfectious disease processes have also been associated with attenuated DC function. For example, in cancer patients, numerous tumor-derived soluble factors, including IL-6, IL-10, TGF-β, vascular endothelial growth factor, and PGE2, have been shown to inhibit mDC differentiation, maturation, and function (11, 14, 34–36). In contrast, the spontaneously matured mDCs from our sarcoidosis subjects did not have decreased expression of MHC class II and costimulatory molecules but instead showed elevated CD40 expression, suggesting an increased activation state possibly reflective of their exposure to higher proinflammatory cytokine levels in the PBMCs overnight. An exposure to a more inflammatory milieu has been found to influence the transition of DCs from immature to a mature state and their subsequent capacity to induce T cell responses in the MLR. Termined “exhaustion”, production of IL-12 by mDCs is limited to a narrow period of 10–18 h after initial exposure to LPS or other maturation stimuli (37). Additionally, phenotypically mature DCs with impaired IL-12 production and attenuated function have been reported in malignant diseases, including hepatocellular carcinoma (38) and breast cancer (39). However, we did not detect any deficiency in production of IL-12 by sarcoid mDCs in the early stages of the allogeneic MLR, but our investigations in this regard were limited and did not involve additional time points or other means of actively stimulating IL-12 release.

The cytokine IL-12 is a 70-kDa heterodimeric molecule composed of a constitutively transcribed 35-kDa (p35) subunit and an inducible 40-kDa (p40) subunit, with only the p70 heterodimer having full IL-12 bioactivity (40–42). In our study, we detected high levels of IL-12p40/p70 in the blood of sarcoidosis subjects or spontaneously released by their cultured PBMCs. The absence of detectable IL-12p70 in these specimens and the low group levels of the heterodimeric, p40-containing cytokine IL-23 suggests that the elevated sarcoidosis IL-12 levels are due to the free p40 subunit, a finding supported by others in sarcoidosis blood and bronchoalveolar lavage fluid (43–45). The physiological role of free p40 is not well characterized. However, in addition to its presence within the IL-12 and IL-23 heterodimers, p40 can also associate with itself to form homodimers that bind to the same receptors as the IL-12p70 molecule, suggesting a possible antagonist role that is supported by mouse models but remains controversial in human studies (46, 47).

The sarcoidosis subjects in this study had increased plasma levels of IFN-γ, a Th1-type cytokine produced upon IL-12 stimulation that is critical for granuloma formation. However, in the 24 h MLRs we found that mDCs from sarcoid and normal control groups induced similar levels of IFN-γ by responder lymphocytes. Due to limited availability of subject DCs, we were unable to collect supernatants at later and possibly more optimal time points for differentiating IFN-γ production between the two groups. Also, we did not measure the maximally stimulated capacity for IFN-γ production by these mDC-expanded lymphocyte populations. Others have reported increased IFN-γ release in stimulated and unstimulated cells from sarcoid bronchoalveolar lavage specimens, while similar studies of sarcoid peripheral blood lymphocytes have shown mixed results (48–51). Thus, further study will be necessary to evaluate the Th1 profile of lymphocytes expanded by ex vivo-isolated sarcoid mDCs.

We did not find group differences in supernatant levels of IL-10 or TGF-β, two cytokines reported by others to be responsible for tumor-induced immune suppression (35, 52). However, significant elevations of IL-6 were found in sarcoid plasma specimens and culture supernatants from the sarcoid-derived PBMCs. Long considered an inflammatory factor, IL-6 has more recently been noted for its immune-suppressive effects on DC differentiation. Tumor-associated overproduction of IL-6 has been shown to decrease DC numbers and impair DC maturation and function in patients with multiple myeloma (14) and chronic lymphocytic leukemia (53). Although elevated IL-6 levels could potentially be a factor in the altered mDC function observed in this study, our sarcoid subjects did not have reductions in circulating blood mDC numbers or in phenotypic maturation of DCs that were reported to occur with tumor-associated IL-6 exposure.

In the absence of a secondary, costimulatory signal, engagement of the TCR alone by a DC can lead to T cell anergy (54). The sarcoidosis-derived mDCs in our study were phenotypically mature with costimulatory molecule expression equal to or greater than normal control mDCs. More recently, secondary signaling molecules have been described on activated DCs and other immune cells that deliver inhibitory signals (55). In particular, B7-H1, signaling through its receptor PD1 on T cells, has been shown to be expressed on activated “tolerogenic” DCs and can be up-regulated in chronic viral infections (56) and malignant states (57). As we did not evaluate the presence of B7-H1 on mDCs in this study, it is possible that there was up-regulated expression of this molecule or other coinhibitory molecules on the sarcoidosis mDCs, leading to a shift toward Th2-type immune responses, anergy, or the formation of regulatory T cells.

Our study included evaluation of pDCs, which can also promote Th2-type immune responses and induce formation of regulatory T cells (58). We did not observe differences in circulating blood pDC numbers, either absolute or relative to mDCs, between our sarcoid and normal control groups. Furthermore, there were no group differences in stimulated pDC production of IFN-α, a cytokine that has been linked to the onset of sarcoidosis in patients receiving it for the treatment of melanoma or chronic viral hepatitis (59). The lack of any correlations between our pDC data and other measured outcomes in this study suggests that this DC subtype is not a major factor in the decreased cellular immunity observed in our sarcoidosis patients.

T cells from sarcoidosis subjects in this study showed strong allogeneic responses to a normal control Mo-DC. Others have found that the T cell populations from sarcoid subjects proliferate normally in response to stimulation with mitogens or Ags (8, 9). In these earlier studies, proliferation rates by T cells of either sarcoid or normal control origin were suppressed by an unidentified cell type in the sarcoid adherent cell fraction, presumably containing both macrophages and DCs. Furthermore, our study did not show a relationship between the allogeneic responses of subjects’ T cells and their DTH skin reactivity or other measured outcomes, in contrast to the significant clinical correlations found for mDC in vitro activity.

In summary, our study provides evidence that mDC function in vitro is correlated with in vivo DTH responses to _Candida_, and that both of these measured outcomes are reduced in individuals with active, untreated sarcoidosis. In this single-sampling study designed to evaluate both DC subsets, we were not able to explore whether differences in mDC function are innate to the precursor cells or acquired during their development, or whether mDC function improves with clinical changes in sarcoid disease activity. However, our observed correlations between clinical disease activity in individuals with sarcoidosis and measures of their cellular immune response in vitro and in vivo suggest that reduced, rather
than overactive, cellular immunity is associated with more severe disease.

Sarcoidosis remains for many a chronic and debilitating disease without a safe and effective treatment. Identifying abnormalities in DC function may reveal novel targets for therapeutic manipulation, providing the rationale for use in this patient population of DC-targeted agents currently being tested in the oncology and infectious disease fields.

Acknowledgments
We thank Ralph Steinman for helpful input during study initiation and manuscript preparation.

Disclosures
The authors have no financial conflicts of interest.

References


