IL-13-regulated Macrophage Polarization during Granuloma Formation in an *In Vitro* Human Sarcoidosis Model

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Abstract

The mechanisms underlying abnormal granuloma formation in patients with sarcoidosis are complex and remain poorly understood. A novel *in vitro* human granuloma model was used to determine the molecular mechanisms of granuloma genesis in patients with sarcoidosis in response to putative disease-causing mycobacterial antigens. Peripheral blood mononuclear cells (PBMCs) from patients with active sarcoidosis and from normal, disease-free control subjects were incubated for 7 days with purified protein derivative-coated polystyrene beads. Molecular responses, as reflected by differential expression of genes, extracellular cytokine patterns, and cell surface receptor expression, were analyzed. Unbiased systems biology approaches were used to identify signaling pathways engaged during granuloma formation. Model findings were compared with human lung and mediastinal lymph node gene expression profiles. Compared with identically treated PBMCs of control subjects ($n = 5$), purified protein derivative-treated sarcoidosis PBMCs ($n = 6$) were distinguished by

the formation of cellular aggregates resembling granulomas. Ingenuity Pathway Analysis of differential expression gene patterns identified molecular pathways that are primarily regulated by IL-13, which promotes alternatively activated (M2) macrophage polarization. M2 polarization was further demonstrated by immunohistochemistry performed on the *in vitro* sarcoidosis granuloma-like structures. IL-13-regulated gene pathways were confirmed in human sarcoidosis lung and mediastinal lymph node tissues. The *in vitro* human sarcoidosis granuloma model provides novel insights into early granuloma formation, particularly IL-13 regulation of molecular networks that regulate M2 macrophage polarization. M2 macrophages are predisposed to aggregation and multinucleated giant cell formation, which are characteristic features of sarcoidosis granulomas.

Clinical trial registered with www.clinicaltrials.gov (NCT01857401).

Keywords: AmpliSeq; macrophage; peripheral blood mononuclear cells; purified protein derivative

Mechanisms of early granuloma formation during sarcoidosis are currently unknown. Existing paradigms focus on classically activated (M1) macrophages expressing

human leukocyte antigen (HLA) class II molecules that promote T-helper cell type 1 (Th1) immune cell activation in response to common antigens (e.g., *Mycobacterium*

tuberculosis [*M.tb.*]), leading to a type 2 IFN-mediated proinflammatory granulomatous response (1). However, sarcoidosis tissue macrophages express

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The uncompressed videos are accessible from this article's supplementary material page.

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Table 1. Patient Demographics

Samples	Groups	Age (Yr)	Sex (M/F)	Race (W/B/O)*
<i>In vitro</i> granuloma	Control (n = 5)	28.0 ± 2.5	3/2	3/0/2
	Sarcoidosis (n = 6)	49.8 ± 6.4	2/4	4/2/0
Lung tissue	Control (n = 6)	51.0 ± 4.1	3/3	4/2/0
	Sarcoidosis (n = 6)	43.0 ± 3.3	2/4	3/3/0
Mediastinal lymph node tissue	Control (n = 5)	43.5 ± 5.5	3/2	3/2/0
	Sarcoidosis (n = 8)	40.4 ± 4.9	3/5	5/3/0

*White/Black/Other.

markers such as CD163 (2) and matrix metalloprotease (MMP)-12 (3), indicating the presence of an alternatively activated, immune-suppressive (M2) phenotype in diseased sarcoidosis tissues. Indeed, the fusion of macrophages to form multinucleated giant cells, a histopathological feature of sarcoidosis granulomas, is mediated, at least in part, by M2 macrophages (4).

We have recently developed a human *in vitro* model of granuloma formation wherein peripheral blood mononuclear cells (PBMCs) of patients with sarcoidosis who are naive to *M.tb.* readily form cell aggregates resembling granulomas when stimulated by purified protein derivative (PPD)-coated beads (5). *In vitro* granuloma formation by sarcoidosis PBMCs was

shown to be significantly greater than that of healthy, *M.tb.*-naive controls, and proinflammatory T-cell responses were paradoxically reduced (5). These and other features of the *in vitro* model routinely recapitulate *in vivo* features of the disease (5). Using the *in vitro* human granuloma model, we sought to determine the molecular mechanisms associated with enhanced granuloma formation in patients with active pulmonary sarcoidosis using genome-wide gene expression analysis coupled with a systems biology approach.

Methods

See the data supplement for additional methodological details.

Study Subjects

This study was approved by The Ohio State University Institutional Review Board (2014H0380) and is registered with www.clinicaltrials.gov (NCT01857401). Sarcoidosis inclusion criteria were based on

Figure 1. *In vitro* granuloma-like cell aggregation of sarcoidosis peripheral blood mononuclear cells in response to treatment with purified protein derivative (PPD)-coated beads. Representative light photomicrographs (10×) obtained 7 days after sarcoidosis or control peripheral blood mononuclear cells were incubated with either PPD-coated or uncoated beads. (A) PPD-coated beads induced prominent cellular aggregation in the sarcoidosis group (black arrows) that was comparatively minimal in the (C) similarly treated controls. Treatment with uncoated beads elicited no cellular aggregation in either (B) the sarcoidosis or (D) control group.

biopsy-proven noncaseating granulomatous tissue inflammation in the absence of other likely causes as per established criteria (6) with radiographic evidence of active (nonfibrotic) lung disease and negative screening for *M.tb.* (PPD skin test and/or IFN- γ release assay negative result using the QuantiFERON test; QIAGEN). The study subjects were not active smokers and were not actively treated with immune suppressants. Blood samples were obtained from two study groups: 1) patients with active pulmonary sarcoidosis ($n = 6$) and 2) matching disease-free, PPD skin test-negative, and/or QuantiFERON-negative control subjects ($n = 5$). In addition, granulomatous tissues (lung [$n = 6$] and mediastinal lymph node [$n = 8$]) obtained from additional unrelated groups of sarcoid patients and matching disease-free control subjects ($n = 6$ and $n = 5$, respectively) who met the criteria mentioned above were also processed and analyzed, as detailed below and in the data supplement, to determine the gene expression differences between the groups. The demographic features of all study subjects in each group and the diverse clinical characteristics of the patients with sarcoidosis in the present *in vitro* granuloma study and from whom granulomatous tissues were obtained are summarized in Table 1 and in Tables E1 and E2 in the data supplement, respectively.

PPD-coated Bead Preparation

Fluoresbrite carboxylated microspheres (bright blue fluorescence, 1.0 μm ; Polysciences) were either covalently coupled to tuberculin PPD via the carbodiimide method (further described in the data supplement) or washed and resuspended in PBS, then diluted with RPMI media and added at a concentration of 50:1 relative to the number of monocytes/macrophages present in each sample.

Human PBMC Isolation and Culture Conditions

PBMCs were isolated from whole-blood samples using Ficoll-Paque PLUS (GE Healthcare Life Sciences) (5) as detailed further in the data supplement. PBMC preparations (2×10^6 cells/ml) were then cultured in RPMI 1640 medium containing 10% human AB serum and either uncoated (PBS-washed) or PPD-coated beads for 7 days with or without leflunomide (50 μM) before treatment.

Extracellular Cytokine Analysis

Human PBMC-cleared supernatants were analyzed 7 days after treatment for IFN- γ , IL-10, and IL-13 by ELISA according to the manufacturer's recommendations.

Granuloma Gene Expression Analysis

Granuloma cells were harvested into TRIzol reagent (Thermo Fisher Scientific), and total RNA was purified of DNA contamination using the SpinSmart RNA purification kit (Thomas Scientific). RNA libraries were prepared using the Ion Torrent AmpliSeq Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific) from 10 ng of RNA. Quantitative gene expression analysis was performed using the AmpliSeq platform as previously described (5) and as specified further in the data supplement.

Gene expression data were normalized and postalignment statistical analyses performed using DESeq2 (7), and statistical comparisons of gene expression were determined from the normalized read counts based on a 10% false discovery rate (DESeq2-adjusted $P \leq 0.05$) and a fold change cutoff of 2 for each comparison.

Frozen Tissue RNA Isolation and Gene Chip Analysis

Total RNA was isolated from frozen lung or mediastinal lymph node tissues using TRIzol reagent according to the

manufacturer's protocol and as described further in the data supplement. The RNA was purified using the RNeasy Mini Kit (QIAGEN), and the integrity of the purified total RNA samples was assessed qualitatively on an Agilent 2100 Bioanalyzer (Agilent Technologies). Samples were then processed using high-density oligonucleotide Human Genome U133 Plus 2 arrays (Affymetrix), which analyze the expression levels of over 47,000 transcripts and variants, including 38,500 well-characterized human genes.

Gene Network Analysis

Functional and network analyses of gene expression data derived from the AmpliSeq results and the Affymetrix Human Genome U133 Plus 2 chips were performed independently using Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems Inc.; www.ingenuity.com).

Results

In Vitro Cell Aggregation Resembling Granulomas

Incubation of PBMCs from patients with sarcoidosis ($n = 6$) or control subjects ($n = 5$) with uncoated beads for 7 days yielded minimal cell aggregation. By contrast, treatment of sarcoidosis PBMCs with PPD-coated beads elicited the formation of

Video 1. Macrophages and lymphocytes comprise confocal immunofluorescence imaging of granuloma-like structures in three-dimensional (3D) animation. Confocal immunostaining shows the participation of CD11b⁺ macrophages and CD3⁺ lymphocytes in a granuloma-like structure of cells obtained from a patient with sarcoidosis in response to purified protein derivative-coated beads 7 days after treatment. Confocal z-stacks were reconstructed into a 3D animation for video viewing. CD11b and CD3 are shown in red and green, respectively; DAPI is shown in blue. Scale bar: 30 μm .

granuloma-like multicellular complexes compared with nominal cell aggregation under these conditions in normal healthy control PBMCs (Figure 1). Immunofluorescence analysis of granuloma-like structures demonstrated central clustering of macrophages with peripherally situated lymphocytes as exhibited in a three-dimensional (3D) video view of the granuloma shown in Video 1.

Gene Expression Profile of *In Vitro* Sarcoidosis Granulomas

Comparison of RNA expression measured by AmpliSeq in sarcoidosis PBMCs incubated for 7 days with PPD-coated beads with identically treated control PBMCs yielded 1,274 significantly differentially expressed genes (unpaired group comparison; >2 -fold; $P < 0.05$ by DESeq2 [7]). In contrast, no differentially expressed genes were detectable when we compared sarcoidosis with controls after treatment with uncoated beads for 7 days, indicating that the observed gene expression changes were related specifically to PPD. Figure 2 presents mean normalized counts plotted against average fold change (MvA plot) and volcano plots of the gene expression data, demonstrating distinct gene expression characteristics under these conditions. Using a gene network analysis that is based on known molecular interactions (IPA), it is feasible to identify molecular patterns that provide insight into disease mechanisms. The results of this upstream analysis designed to identify primary regulators, based on statistical probability, responsible for the gene expression changes observed in the data set (8) identified IL-13 as the cytokine most strongly regulating the differential expression of genes in the sarcoidosis group (Figure 3). At a higher level of analysis based on the IPA tool, which shows the strongest interactions among differentially expressed genes, an interactive network of molecules coregulated by IL-13 and IL-1B was identified (Figure 4A). Using the same IPA network analysis, we also identified IL-13 coregulated networks in independently examined human sarcoidosis lung and lymph node tissues (Figures 4B and 4C, respectively).

Alternatively Activated (M2) Macrophage Polarization in Sarcoidosis Granulomas

The macrophage gene expression profile of PBMCs from patients with sarcoidosis 7 days after PPD treatment was compared

Figure 2. Differential gene expression after PPD-coated bead treatment of peripheral blood mononuclear cells (PBMCs) derived from patients with sarcoidosis compared with similarly treated PBMCs from normal healthy control subjects. (A) The output type “MvA” plots of normalized gene expression data wherein each point represents a gene, with the y-axis representing a log(base 2) fold change in expression after 7-day treatment with PPD-coated beads of PBMCs derived from patients with sarcoidosis compared with identically treated PBMCs from normal, healthy control subjects. The x-axis is the log average of the gene expression level. All genes with an adjusted P value of 0.05 (representing a 5% false discovery rate) and at least a twofold change (highlighted by the two horizontal gray lines) in the magnitude of gene expression between patients with sarcoidosis and control subjects are shaded red. The blue line represents smoothed local mean expression (fitted using a generalized additive model) with the surrounding 95% confidence interval shaded light blue. The dashed gray contour lines represent a two-dimensional density estimation. Genes that are most differentially expressed in response to PPD-coated beads in patients with sarcoidosis compared with control subjects are highlighted in the top part of the MvA plot, whereas those genes most differentially expressed in control subjects compared with patients with sarcoidosis are at the bottom. (The top five most differentially expressed genes in each case are labeled.) (B) Volcano plots of the same data wherein the y-axis corresponds to transcripts with high statistical significance ($-\log_{10}$ of P value) and the x-axis corresponds to fold change of gene expression (log base 2) were also generated from the same data set. Labeled transcripts on the upper left side of the plot have strong statistical significance with relatively low expression after treatment of PBMCs with PPD-coated beads of sarcoidosis versus controls. Transcripts on the upper right are more highly expressed with strong statistical significance between sarcoidosis and controls after PPD treatment. The dotted line corresponds to a P value cutoff of 0.05, and genes failing to meet this cutoff are shaded gray. Data were generated from all 11 experiments (six sarcoidosis and five controls).

Figure 3. Pathway analysis predicts IL-13 as an important upstream regulator of the observed gene expression changes. Predictions are based on two statistical measures reflecting data set genes known to be regulated by each specific cytokine or transcription factor (overlap *P* value) and activation (*z*-score) based on the known direction by which the upstream regulator is known to influence the gene target (inhibition vs. activation). Details of the statistical methods are provided by Ingenuity Systems (8). Applying this statistical approach to the differential gene expression pattern of PBMCs of patients with sarcoidosis compared with healthy control subjects determined 7 days after treatment with PPD-coated beads yielded the above IL-13-regulated network. Data were generated from all 11 experiments (six sarcoidosis and five controls).

with that of identically treated PBMCs of disease-free control subjects, reflecting a strong polarization toward putative M2 molecules, as reflected by patterns of highly expressed transcripts (Table 2) and reduced expression of genes conforming to the M1 phenotype (*SLC7A5*, *IL2RA*, *GZMA*, *FAS*) (not shown). Among these putative M2 molecules, *CD163* gene expression (and its receptor) was particularly elevated in sarcoidosis granulomas after PPD-coated bead treatment relative to treatment-matched controls, and *CD163* is regulated by M2

cytokines IL-13 (9) and IL-10 (10, 11). Higher relative *CD163* protein expression was confirmed in sarcoidosis granulomas by immunohistochemistry compared with matching controls after PPD-coated bead treatment (Figure 5 and in a 3D video view of the granuloma shown in Video 2).

Macrophage M2 polarization via IL-13 activation of STAT6 (signal transducer and activator of transcription 6) was further demonstrated by significantly diminished granuloma formation

when PPD-stimulated sarcoid PBMCs were pretreated with the STAT6 inhibitor leflunomide (Figure 6). Insight into the mechanisms by which M2 macrophages aggregate is further provided by the observed increase in *CD11* and *CD18* expression in PPD-treated PBMCs from sarcoidosis (12). *CD11b* and *CD18* combine to form the heterodimeric protein Mac-1 (macrophage-1 antigen), which has implications for macrophage aggregation, as discussed below.

Figure 4. Pathway analysis of gene expression identifies IL-13 to be a mediator of the sarcoidosis granulomatous response. (A) The gene network most highly overrepresented (see the METHODS section regarding AmpliSeq analyses) in PBMCs from patients with sarcoidosis ($n = 6$) relative to control subjects ($n = 5$) 7 days after PPD treatment was predicted to be strongly regulated by IL-13 and IL-1 β . Genes and gene products are represented by their common abbreviated names according to established direct (solid lines) or indirect (dashed lines) interactions and the specific nature of the interaction. These include binding (straight line), activation (arrow), inhibition (truncated line), or either activation or inhibition (truncated line with arrow). Genes that are significantly overexpressed in the sarcoidosis group relative to controls are highlighted in shades of red, wherein darker shades conform to higher differential expression values. The shape of each gene “node” denotes the function of the gene product, as indicated in the key. (B) Pathway analysis of gene expression results (from Affymetrix GeneChip arrays) obtained from human sarcoidosis lymph nodes ($n = 5$) compared with disease-free control lymph nodes ($n = 8$) using a cutoff of greater than or equal to twofold change ($P < 0.05$) identified IL-13 and IL-2 as putative mediators of the granulomatous response, including induction of STAT1 (signal transducer and activator of transcription 1)-mediated inflammatory signaling. (C) Pathway analysis of gene expression results (from Affymetrix GeneChip arrays) obtained from human sarcoidosis lung tissues ($n = 6$) compared with disease-free control lung tissues ($n = 6$) using a cutoff of greater than or equal to twofold change ($P < 0.05$) identified IL-13 as a putative mediator of the granulomatous response, including regulation of metalloproteases MMP-12 and ADAMDEC1, the expression of which correlated significantly with pulmonary sarcoidosis disease severity (3).

Leukocyte Immunoglobulin-Like Receptor Family B Molecule expression

All five members of the LILRB (leukocyte immunoglobulin-like receptor family B)

family (*LILRB1-5*) were overexpressed 7 days after PPD treatment in sarcoidosis PBMCs relative to controls (Table 3). Likewise, members of the LILRB family were overexpressed in established

granulomas derived from diseased human sarcoidosis tissues. LILRB molecules have important implications for regulating M2/Th2 immune responses (discussed below).

Table 2. *In Vitro* Granuloma Gene Expression Reflecting M1/M2 Polarization

M2 Macrophage Genes*		M1 Macrophage Genes	
Gene	Fold Change	Gene	Fold Change
CD163	11.1	CXCL9	4.0
MMP2	10.0	HLA-DMA	4.0
HNMT	7.9	CXCL10	3.3
HS3ST2	7.2	IL18	3.2
STAB1	6.7	CD68	3.1
CHIT1	6.4		
MS4A4A	6.3		
CCL18	6.2		
MMP12	5.8		
MAF	5.1		
CCL13	4.7		
IL10	4.4		
CD163L	4.2		
MRC1	4.0		
LTA4H	3.4		

*Representative of a total of 30 M2 macrophage genes (based on PubMed and Google searches of terms *gene expression* and *M2 macrophages* and including References 2, 7, and 11) using cutoff values of greater than or equal to twofold change in expression ($P < 0.02$).

Figure 5. Immunofluorescence imaging of granuloma-like structures demonstrates persistent macrophage CD163 expression on sarcoidosis PBMCs after PPD stimulation. Representative photomicrographs of immunostained PBMCs in granuloma-like structures 7 days after bead treatment using confocal microscopy. DAPI and CD163 staining are shown in blue and red, respectively (see data supplement for methods and antibodies used). (A) PBS-washed (uncoated) and (B) PPD-coated, bead-stimulated PBMCs obtained from a PPD-naïve, healthy control subject showing high CD163 expression on macrophages that was markedly decreased after PPD stimulation. In contrast, images of granuloma-associated macrophages obtained from a patient with sarcoidosis demonstrated high CD163 expression after (C) uncoated bead stimulation that persisted after (D) stimulation with PPD-coated beads. (E) Staining of PPD-stimulated PBMCs from a donor with sarcoidosis using a concentration-matched isotype control (to CD163) antibody. In each panel, a matching differential interference contrast image is shown next to each composite image. All images are representative of the results from four independent experiments. Scale bars: 10 μ m.

M1/M2 Cytokine Ratios during *In Vitro* Human Granuloma Formation

IFN- γ promotes M1 polarization, whereas IL-13 and IL-10 promote M2 polarization (9–11, 13). IFN- γ /IL-10 and IFN- γ /IL-13 differential expression ratios were lower in sarcoidosis compared with controls, consistent with a relative M2 cytokine balance (Table 4).

Discussion

Mechanisms of early granuloma formation in sarcoidosis are enigmatic and complex, evolving from the dynamic interaction of multiple immune cell types, the features of which are changing over time. Complex genetic factors that predispose to sarcoidosis in humans, not readily modeled in animals, are yet another variable that needs to be considered. Human tissue samples are of limited value for understanding early granuloma genesis in that they provide a static image of well-formed, established granulomas of unknown age and presumably representing a chronic maintenance phase of granuloma. Nonetheless, certain molecular features, such as increased *MMP12* (an IL-13-regulated molecule [14]) expression, are observed at all stages of experimental *in vivo* granuloma formation (15), implying common core mechanisms throughout all stages of granuloma formation that are regulated by IL-13. Thus, the *in vitro* model of granuloma formation employed in the present study, coupled with an unsupervised genomic systems biology approach, allowed us to consider novel mechanisms contributing to early granuloma formation in sarcoidosis. In contrast to currently held paradigms implicating Th1 and M1 (proinflammatory) mechanisms (16), our findings demonstrate strong M2 polarization during early granuloma formation in patients with a chronically active pulmonary sarcoidosis phenotype, and they further incriminate IL-13 as a proximal regulator of these events.

Alternatively Activated (M2) Macrophages and Sarcoidosis

The observed M2 polarization of macrophages in patients with sarcoidosis provides new perspective on previously documented features of macrophages in patients with active sarcoidosis. A distinguishing feature of alveolar

Video 2. Persistent macrophage CD163 expression after purified protein derivative stimulation of sarcoidosis peripheral blood mononuclear cells is demonstrated via confocal immunofluorescence imaging of granuloma-like structures in 3D animation. Confocal immunostaining shows the participation of CD163⁺ macrophages in a granuloma-like structure of cells obtained from a patient with sarcoidosis in response to purified protein derivative-coated beads 7 days after treatment. Confocal z-stacks were reconstructed into a 3D animation for video viewing. CD163 is shown in red; DAPI is shown in blue. Scale bar: 20 μ m.

macrophages from patients with sarcoidosis is enhanced antigen presentation (17), which has been interpreted to represent M1 macrophage polarization, such as is reflected by increased *HLA-DMA* (Table 2). However, M2 signatures, such as CD163 (2) and MMP-12 (5), were previously observed in human sarcoidosis tissue granulomas, and their expression correlated with pulmonary disease severity (3, 18). Moreover, M2 macrophage polarization, as reflected by increased CD163 expression, is associated with enhanced antigen presentation within a select subset of regulatory T cells, leading to lower concentrations of IFN- γ and higher concentrations of IL-10 upon stimulation (19) (lower IFN- γ /IL-10 ratio [Table 4]), as demonstrated in this study. Another interesting property of CD163-expressing cells is a paradoxical high-level release of both IL-4 (promoting Th2 immune responses) and TNF- α (an M1 marker) (20), and high concentrations of TNF- α were previously reported in this model (5). The simultaneous expression of M2 and M1 features speaks to the plasticity of macrophages that is commonly observed in the context of human disease (21). Nonetheless, the prevailing molecular signature of the *in vitro* human granuloma model is toward M2 macrophage polarization (Table 2).

The molecular mechanisms regulating M2 macrophage polarization are well established, including IL-4- and IL-13-mediated activation of STAT6, with subsequent induction of numerous downstream mediators. The present study provides novel insights into M2 polarization mechanisms during the early phases of granuloma formation in the context of sarcoidosis. Figures 3 and 4A implicate a central role for IL-13 as a proximal upstream regulator of molecular events during the early evolution of sarcoidosis granulomas. This finding is supported by increased IL-13 expression in the BAL fluid cells and PBMCs of humans with active pulmonary sarcoidosis (22), and as reflected by the central role of IL-13 in molecular networks derived from genomic analyses of human sarcoidosis tissues (Figures 4B and 4C). Indeed, in sarcoidosis lung tissues, IL-13 is predicted to regulate the expression of metalloproteases MMP-12 and ADAMDEC1 (ADAM-like decysin 1) (Figure 4C), the expression of which significantly correlates with pulmonary sarcoidosis disease severity (3). The central role of the IL-13/STAT6 signaling pathway in the genesis of sarcoidosis granulomas was supported by the inhibitory effects of leflunomide, a potent STAT6 inhibitor (23), which is effective for the treatment of sarcoidosis (Figure 6) (24, 25).

Molecular Mechanisms of Macrophage Aggregation in Sarcoidosis

M2 macrophage polarization favors macrophage aggregation and multinucleated giant cell formation, which are histological hallmarks of sarcoidosis granulomas. In this regard, circulating leukocytes of patients with sarcoidosis express higher concentrations of CD11b and CD18 (12), and the CD11b/CD18 heterodimer (Mac-1) promotes macrophage aggregation (26) and is incriminated in the context of granulomatous infections (27). Mac-1 is further shown to promote IL-13-mediated M2 macrophage polarization (28). Thus, we posit that CD11b and CD18, which are highly expressed in the *in vitro* sarcoidosis granuloma model (3.1- and 2.5-fold in sarcoidosis compared with controls [$P < 0.05$], respectively) and in diseased sarcoidosis lymph nodes (3.1- and 3.9-fold in sarcoidosis relative to controls [$P < 0.05$], respectively), contribute to early M2 polarization and to immune cell aggregation in the context of sarcoidosis.

Considering the Role of LILRB Family Molecules in Early Granuloma Formation

LILRB molecules promote M2 and Th2 polarization (29). The LILRB family members are expressed on various immune cells, including dendritic cells, monocytes, and lymphocytes (T cells, natural killer cells, and B cells), generally inhibiting the proinflammatory interactions among these cells and with proinflammatory S100 proteins (30). LILRB molecules are important regulators of granulomatous responses, such as leprosy (31), by reducing M1/Th1 (IFN- γ -induced) immunity (30). In the present study, all five LILRB family member genes (i.e., *LILRB1–5*) were highly expressed during early sarcoidosis granulomas (Table 3). The implications of the LILRB family in the context of sarcoidosis will require further investigation; however, we posit that LILRB molecules contribute to M2 polarization as well as T-cell anergy, as reflected by a regulatory T-cell cytokine profile (i.e., comparatively lower IFN- γ /IL-13 [Table 4]). Thus, *LILRB* expression could explain suppression of inflammatory T-cell responses (relative T-cell anergy) compared with disease-free controls observed in the *in vitro* model and as documented *in vivo* in the context of active pulmonary

Figure 6. Leflunomide (Lef) pretreatment suppresses PPD-induced granuloma formation in sarcoidosis PBMCs. (A) Representative light photomicrograph of granuloma-like structures 7 days after sarcoidosis PBMCs were treated with PPD-coated beads. (B) The same image shown in A wherein granulomas were identified and color-coded on the basis of size, employing the EVOS FL Cell Imaging System (Thermo Fisher Scientific) and MIPAR analysis software as described in the data supplement. (C) Representative light photomicrograph of granuloma formation in PBMCs from the same patient with sarcoidosis 7 days after PPD stimulation after pretreatment with leflunomide for 30 minutes. (D) The same image shown in C after analysis with the EVOS FL Cell Imaging System and MIPAR analysis software. (E) Significant reduction in the area comprised by granulomas as a percentage of the field of view in sarcoidosis PBMCs 7 days after treatment with PPD-coated beads after initial pretreatment with leflunomide. All images are representative of the results from five independent experiments. Scale bars: 200 μ m.

Table 3. *In Vitro* Granuloma Leukocyte Immunoglobulin-Like Receptor B Genes

Gene	Known Cell Targets	Fold Change	Ligands
LILRB1*	T, B, NK	3.7	HLA class I S100A8/9
LILRB2	M	4.2	HLA class I β-Amyloid
LILRB3	M, DC	2.6	IgE LILRA2
LILRB4*	M, T	3.4	HLA class I HLA-DR
LILRB5	M, T, NK	6.3	HLA class I

Definition of abbreviations: B = B cell; DC = dendritic cell; HLA = human leukocyte antigen; LILR = leukocyte immunoglobulin-like receptor; M = macrophage; NK = natural killer T cell; T = T cell.

*Also differentially expressed in human sarcoidosis tissues (lymph node).

sarcoidosis (32). The possible link between IL-13 and LILRB is provided by Shakhawat and colleagues, who demonstrated that LILRB molecules through major histocompatibility complex class I molecule engagement promote IL-13/STAT6-mediated release of IL-10 and transforming growth factor-β by monocytes/macrophages (29). As such, LILRB molecules may function upstream of IL-13/STAT6 and in concert with other regulatory elements (e.g., SHP1 [Src homology region 2 domain-containing phosphatase 1]/SHP2) (33) to promote M2 macrophage polarization and related anergy.

Clinical Implications of This Study

The insights provided in this paper have important clinical implications, including therapeutic ones. Doxycycline, a potent inhibitor of M2 macrophage polarization (34), and leflunomide, an inhibitor of STAT6 and related M2 gene expression (23), are effective treatments for sarcoidosis (25, 35). Given the diverse functional properties of M2 macrophages, including the “paradoxical” promotion of granuloma

formation and suppression of Th1-mediated inflammation, a nuanced targeting of these pathways may be effective in selective suppression of granuloma formation without unleashing proinflammatory T-cell responses.

Unanswered Questions and Study Limitations

The factors influencing the balance between M1 and M2 macrophage polarization are complex and dynamic. For instance, during the evolution of *M.tb.*-induced granuloma formation, a predominant M1 immune response is observed in the early stages of infection, giving way to an M2-predominant response in association with granuloma formation within 9 days (36). Moreover, human tissue samples show M2-dominant activation to be localized within tuberculous granulomas, with M1 features in adjoining nongranulomatous tissues (36). In the context of sarcoidosis, it is unclear whether there is indeed an early Th1/M1 phase preceding the M2/Th2 profile observed by Day 7 or if M1 stromal macrophages act in concert with M2

granuloma-forming macrophages. The factors specifically favoring M1 or M2 polarization in the context of chronic granulomatous inflammation are unclear; however, on the basis of striking differences between healthy control subjects and patients with sarcoidosis demonstrated in the present study in response to identical PPD-coated bead treatment, we postulate that genetic factors play important roles. Further investigations are warranted to identify the mechanisms contributing to M2 polarization in the context of sarcoidosis and to determine the clinical implications thereof.

As with any model of human disease, this study has limitations. For instance, differences in PBMC immune cell subpopulations could contribute to differential gene expression. However, gene expression in unstimulated PBMCs (treated with uncoated beads for 7 d) did not differ between sarcoidosis and controls, suggesting that baseline differences in cell population profiles do not contribute significantly to the observed gene expression changes after PPD treatment. Rather, differences appear to be contingent on stimulation with antigen. Furthermore, although the model relies on PBMCs, which could be considered a limitation, tissue inflammation is contingent on recruitment of circulating immune cells and subsequent antigen-mediated responses. Recent studies show that circulating monocytes traverse the lungs and other tissues in search of immunogenic danger signals (environmental antigens) and become activated tissue residents only if activated during transit (37). Additional immune cells are recruited while others (e.g., T cells) proliferate to further amplify the inflammatory response. Moreover, direct activation of circulating immune cells is sufficient for recapitulating important features of diseased tissue (e.g., Figures 4B and 4C). Most importantly, PBMCs of patients with active sarcoidosis harbor the complex genetic features that predispose to the disease. Thus, the model appears to be a reliable surrogate for human disease.

Conclusions

The *in vitro* human granuloma model provides novel insights into early granuloma formation in the context of sarcoidosis. Using an unbiased analysis of the molecular and genetic features of sarcoidosis granulomas, we show that

Table 4. Ratios of Cytokines Released 7 Days after PPD-coated or Uncoated Bead Treatment of PBMCs Obtained from Control Subjects and Patients with Sarcoidosis

Cytokine Ratio	Control		Sarcoid	
	PPD Beads	UNC Beads	PPD Beads	UNC Beads
IFN-γ/IL-10	60.8 ± 18.8	0.3 ± 0.2*	7.1 ± 2.9†	0.2 ± 0.1*
IFN-γ/IL-13	812.4 ± 227.0	24.1 ± 10.2*	231.2 ± 128.5‡	8.2 ± 1.4*

Definition of abbreviations: PBMC = peripheral blood mononuclear cell; PPD = purified protein derivative (from *Mycobacterium tuberculosis*) coated; UNC = uncoated, no protein.

**P* < 0.05 compared with matching PPD-coated bead treatment.

†*P* = 0.015 compared with matching treatment in the control group.

‡*P* = 0.056 compared with matching treatment in the control group.

macrophages are strongly polarized toward an M2 phenotype, which favors macrophage aggregation. Furthermore, the same mechanisms promoting M2 polarization can promote inhibitory T-cell expression, possibly involving the LILRB family, thus explaining the “immune paradox” of sarcoidosis wherein exuberant granuloma formation occurs in association with T-cell anergy (31, 38). This study further implicates inhibitors of M2

polarization as a potential approach to mitigate disease progression in patients with sarcoidosis with progressive disease. ■

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