Phagosome-regulated mTOR Signaling during Sarcoidosis Granuloma Biogenesis

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On-line Only Supplement

Methods

Donor Recruitment.

Adult (18-70 years old) volunteers, individuals exposed to *Mycobacterium tuberculosis* (*M.tb.*) antigens [latent TB infection (LTBI)] (n = 5) and patients diagnosed with active sarcoidosis and receiving minimal treatment (no methotrexate) (n = 12), were recruited to participate in the current study. Sarcoid patients were diagnosed with active (symptomatic) pulmonary sarcoidosis, as demonstrated by chest radiography showing non-fibrotic lung involvement, and had biopsy-proven non-caseating granulomatous tissue inflammation, associated symptoms of cough and dyspnea, and the clinical decision to initiate immune suppression therapy [S1]. These patients were purified protein derivative (PPD) and/or interferon gamma (IFNγ) release assay negative using the Quantiferon test (reflecting no prior *M.tb*. exposure). Half of the sarcoidosis patients were receiving no treatment at the time of their participation, while the other half were receiving minimal treatment (5-10 mg prednisone daily). This was the same across both the high and low responder subgroups (see below). One patient (low responder) was being treated with 200 mg hydroxychloroquine daily. In all cases, we sought to recruit study subjects who were symptomatic at the time of enrollment for which treatment escalation was being considered. LTBI individuals were defined as having a positive PPD skin test and/or Quantiferon test results with no clinical signs or symptoms of active disease.

Cell Isolation, Incubation and Treatment.

Up to 120 ml of blood were withdrawn from a peripheral vein in the forearm into heparinized syringes and kept on ice until subsequent processing to isolate peripheral blood mononuclear cells (PBMCs) according to standard protocols [S2, S3]. Briefly, PBMCs were isolated from whole blood using density cushion centrifugation employing Ficoll-Paque PLUS (GE Healthcare Bio-Sciences; Pittsburgh, PA). Every 20 ml of blood were mixed with 15 ml PBS and carefully layered onto 14 ml of Ficoll and then centrifuged at 1400 rpm for 40 minutes (no brake)

at room temperature. Buffy coats were collected, diluted with RPMI 1640 media (Thermo Fisher Scientific, Inc.; Waltham, MA) and then centrifuged at 1500 rpm for 10 minutes (no brake) at 4°C. Cell pellets were pooled, re-suspended in RPMI media and then centrifuged a final time at 800 rpm for 10 minutes (no brake) at 4°C. Cells were re-suspended in RPMI media, counted and plated on 24-well tissue culture plates at 2×10^6 cells/ml (~20% monocytes/80% lymphocytes) with 10% human AB serum (Atlanta Biologicals, Inc.; Flowery Branch, GA) per well and incubated at 37°C in a 5% CO_2-humidified atmosphere. PBMCs were plated and incubated within 3 hours of blood collection.

Following a 30-minute equilibration period, cells were treated by addition of either PPD-coated beads or uncoated (PBS-washed) beads at a multiplicity of infection (MOI) of 50:1 relative to the number of representative monocytes present (~20% of all PBMCs), as detailed below. Similarly treated experiments were performed wherein equilibrated cells were pre-treated with either Rapamycin [(RAP) 100 nM, LC Laboratories; Woburn, MA], Bafilomycin A1 [(BAFA1) 500 nM, InvivoGen; San Diego, CA], Chloroquine [(CLQ) 10 µM, InvivoGen] or Ammonium chloride [(NH_4Cl) 50 µM, Sigma-Aldrich; St. Louis, MO] for 30 minutes prior to the addition of the beads. After 4 days of incubation, cells were evaluated by light microscopy for granuloma formation followed by a further addition of 50 µl human AB serum per well. At day 7, again following light microscopy evaluation (including MIPAR analysis as described below), supernatants were carefully collected (centrifuged at 1500 rpm for 10 minutes at 4°C to be free of dead cells) and stored at -80°C for later measurement of treatment-induced PBMC cytokine release by ELISA (see below). Adherent granuloma-like cell aggregates were then carefully washed twice with PBS, collected in TRIzol® (Ambion®, Life Technologies; Carlsbad, CA) and stored at -80°C for later RNA isolation and gene expression analyses (see below). In additional 6-well tissue culture plates with similarly cultured and treated PBMCs [2×10^6 cells/ml (~20% monocytes/80% lymphocytes) with 10% human AB serum], supernatants were collected (and handled as before) at 2, 24 and 48 hours post-treatment, and then the adherent granuloma-like cell aggregates were

carefully washed twice with PBS and collected (including cell scraping to thoroughly release and lyse the cells) in freshly prepared cell lysis buffer (Invitrogen, Thermo Fisher Scientific, Inc.), to which protease inhibitors [1 mM phenylmethylsulfonylfluoride (PMSF) and 10 µl/ml protease inhibitor cocktail (Sigma-Aldrich)] had been added, and stored at -80°C for later protein analyses.

Bead Preparation.

Fluoresbrite® carboxylate microspheres (Polysciences, Inc.; Warrington, PA) are fluorescently-labeled, mono-dispersed, polystyrene-based latex beads that have carboxylate groups on their surfaces which can be activated for the covalent coupling of proteins. At room temperature while under dark and sterile conditions, carboxylated microspheres (bright blue fluorescence, 1.0 µm) were either washed with and re-suspended in PBS or covalently coupled to select proteins via the carbodiimide method for later use. Briefly, beads (50-100 µl per preparation) were twice suspended in 0.1 M carbonate buffer (0.1 M Na_2CO_3 + 0.1 M $NaHCO_3$ in sterile water, pH 9.6, Sigma-Aldrich) followed each time by centrifugation at 14,000 rpm for 6 minutes. The bead pellet was then thrice suspended in 0.1 M MES buffer [2-(N-Morpholino)ethanesulfonic acid in sterile water, pH 5.6, Sigma-Aldrich] followed each time by centrifugation at 14,000 rpm for 6 minutes. To activate the carboxylate groups, the bead pellet was re-suspended in 0.1 M MES buffer, and an equal volume of 2% carbodiimide [2% 1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride dissolved in MES Buffer, Sigma-Aldrich] was added drop-wise and then allowed to gently mix for 4 hours at room temperature.

Following centrifugation at 14,000 rpm for 6 minutes, the bead pellet was thrice washed by re-suspension in 0.1 M MES buffer followed by similar centrifugation to remove unreacted carbodiimide. The bead pellet was then re-suspended in 0.2 M borate buffer (pH 8.5, Sigma-Aldrich) with 200-300 µg protein added and allowed to gently mix overnight at room temperature. The protein used for coupling to the beads was PPD (AJ Vaccines; Copenhagen, Denmark). The next day, following centrifugation at 14,000 rpm for 10 minutes, the supernatants were removed

and saved for later protein determination, and the pellet was re-suspended in 0.2 M borate buffer to which 0.25 M ethanolamine (Sigma-Aldrich) was added to quench all unreacted sites on the beads. Following gentle mixing for 30 minutes at room temperature, beads were pelleted by centrifugation at 14,000 rpm for 10 minutes and then re-suspended in 10 mg/ml human serum albumin (HSA) in 0.2 M borate buffer. Following another 30 minutes for gentile mixing to block any remaining non-specific protein-binding sites on the beads, beads were again pelleted by centrifugation at 14,000 rpm for 10 minutes. After repeating this blocking step and centrifugation, beads were re-suspended in 200-300 µl of storage buffer [0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4 (pH 7.4), 1% HSA, 0.1% sodium azide and 5% glycerol, Sigma-Aldrich] and kept at 4°C. Uncoated (PBS-washed) beads were re-suspended in the same buffer without HSA and similarly stored at 4°C. Beads were later counted and used for treatment by dilution into RPMI media and addition to the incubating PBMCs at an MOI of 50:1.

Granuloma Detection from Light Photomicrographs.

Light microscopic images of each well of incubating cells were acquired 7 days post-treatment using the EVOS™ FL Cell Imaging System (Invitrogen, Thermo Fisher Scientific, Inc.). Six random fields were imaged for each well using the 10X objective. To quantify the average percent granuloma area affected within the field of view (FOV), the images were imported into the visual analysis software, Materials Image Processing and Automated Reconstruction (MIPAR™ v2.2.5; Worthington, OH). Briefly, all images were subjected to histogram equalization, filtering with a median filter, and pixel-clustering using the built in “smart cluster” algorithm to initiate aggregate identification. By visual inspection, a size cut-off of 1200 pixels was empirically determined and implemented in order to reject cellular aggregates that were deemed to be too small to be classified as a true granuloma. Generally, the aggregates that were rejected based upon this size cut-off contained ≤4 macrophages. Each image was subjected to additional steps including clean-up and smoothing to achieve consistent granuloma boundaries without holes in

the interior. Occasionally, a final manual clean-up step was required to eliminate artifactual granulomas. From the feature identification recipe, the following data were generated and exported to examine the effects of RAP, BAFA1, CLQ or NH_4Cl pre-treatment: the percent area affected by granulomas relative to the entire FOV (area fraction).

Results were obtained from 6 independent experiments for both the high responder and low responder sarcoid groups. These two sub-groups were differentiated according to the analyzed Evos images demonstrating the induced granulomatous response to PPD treatment. The breakpoint for differentiation was pre-determined to be an area fraction of 2% based upon prior experimental experience with this model. It is well known that there is a relatively broad spectrum of phenotypes in sarcoidosis, and responsiveness in our model to PPD varies pending the patient's phenotype. In order to properly evaluate the effect of the pre-treatments, it was necessary to have a positive response to the PPD treatment. Granuloma formation with an area fraction <2% was so minimal as to fail to demonstrate any notable effect of the blocker pre-treatments. Area fraction results for the low responder sarcoid group are presented in Figure S1.

Cytokine Release Measurements.

Human PBMC supernatants, carefully collected 7 days after treatment with either uncoated beads or PPD-coated beads (alone or following a 30-minute pre-treatment with either RAP, BAFA1, CLQ or NH_4Cl), were analyzed for their TNFα, IFNγ, IL-1β and IL-10 (eBioscience, Inc., Thermo Fisher Scientific, Inc.) concentrations by ELISA according to the manufacturer's recommendations. Cytokine ELISA sensitivities were 4 pg/ml for TNFα and IFNγ and 2 pg/ml for IL-1β and IL-10. In addition, human PBMC lysates, carefully collected 24 hours after treatment with either uncoated beads or PPD-coated beads (again, alone or following a 30-minute pre-treatment with either RAP, BAFA1 or CLQ), were analyzed for their presence of *p*-mTORc1, *p*-S6 and *p*-STAT3 by ELISA (Cell Signaling Technology; Danvers, MA) in accordance with the

recommended manufacturer's kit instructions. Cytokine release results for the low responder sarcoid group are presented in Figure S2.

RNA Isolation, Amplicon Sequencing and AmpliSeq™ Performance.

RNA extraction was performed using TRIzol® Reagent (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, granuloma-like cell aggregates were collected and homogenized in TRIzol® then phase-separated with chloroform. The upper aqueous phase was collected, and the RNA present was precipitated by adding an equal volume of isopropyl alcohol. The RNA precipitate was pelleted by centrifugation and dissolved in 50 µl of RNase-free water. The RNA was then further purified using the SpinSmart™ RNA Purification kit (Denville Scientific, Inc.; Holliston, MA). Aqueous RNA was mixed with SpinSmart™ reagents and bound to a SpinSmart™ RNA Binding Column. The column membrane was washed with supplied buffers, and membrane-bound RNA was DNase-treated on column according to the manufacturer's protocol. Enzyme-treated RNA was washed to remove degraded deoxynucleic acids then eluted in RNase-free water. RNA concentration was measured using the Qubit™ RNA assay (Thermo Fisher Scientific, Inc.), and RNA integrity assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA). Samples with a RIN > 8 were used for cDNA synthesis.

The Ion Torrent AmpliSeq™ Transcriptome Human Gene Expression kit (Thermo Fisher Scientific, Inc.) uses targeted Next Generation sequencing to simultaneously measure the expression levels of over 20,000 human RefSeq genes in a single assay. The small (~110 base pair average) amplicon size and optimized primer design provides more uniform gene expression results over a broad range of RNA quality [S4]. Reverse transcription was performed on 10 ng of the total granuloma RNA, using the AmpliSeq™ Whole Transcriptome primers with the included SuperScript® VILO™ cDNA Synthesis kit. The resulting cDNA was amplified for 12 cycles, by means of Ion AmpliSeq™ primers and technology to accurately maintain expression levels of all

genes. The AmpliSeq™ primer sequences were partially digested in the same reaction tube using proprietary AmpliSeq™ reagents. Barcoded adapters were added and ligated to individual reactions, and the resulting libraries were purified using the included Agencourt AMPure XP reagents (Beckman Coulter, Inc.; Brea, CA) without any additional amplification. The limited number of PCR cycles reduces biased amplification of highly abundant molecules and also reduces dropout of low abundance transcripts, as well as minimizes the confounding effect of undesirable PCR duplicates produced by higher cycle numbers. This facilitates a precise and sensitive linear range of measurement of gene expression over 6 orders of magnitude [S4]. The purified library concentrations were determined by quantitative real-time PCR with Ion Torrent™ adapter primers and SYBR® Green detection. The quantified barcoded libraries were diluted to 100 pM. Libraries were combined and pooled in equal amounts for emulsion PCR on the Ion OneTouch™2 instrument. The OneTouch™2 system uses emulsion PCR to produce templated Ion Sphere Particles used for Ion Torrent™ semiconductor sequencing. Templated libraries were sequenced with the Ion Proton™ Sequencer, using Ion PI™ Sequencing 200 Kit v2 reagents on Ion PI™ Chips.

Raw sequencing data was processed on the Ion Proton™ Sequencer and transferred to the Ion Proton™ Torrent Server for primary data analysis. Gene level transcript quantification from sequence read data was performed using the associated Torrent Suite™ analysis plugin, “AmpliSeqRNA”. Unlike other RNA-Seq analyses that require normalization approaches such as RPKM (reads per kilobase of transcript per million reads mapped), the small amplicon size and targeted AmpliSeq™ Transcriptome amplicons make it unnecessary to normalize data to account for transcript length bias [S4]. The analyzed granuloma libraries were sequenced to an average depth of 11.6 million reads (± 3.2 million) with 92% (± 5%) of the reads mapping on target. Typically 56% (± 3%) of the 20,800 AmpliSeq™ target genes were detected in the granuloma RNA. AmpliSeq™ has been reported to yield RNA profiles with good precision and reproducibility

[S4]. In our hands, analyses yielded correlation values of r = 0.96-0.99 for technical and biological replicates.

AmpliSeq™ gene expression data were normalized and post-alignment statistical analyses performed using *DESeq2* [S5], and statistical comparisons of gene expression were determined from the normalized read counts based upon a 10% false discovery rate (*DESeq2*-adjusted $p \leq 0.05$) and a fold-change cut-off of 2 for each comparison.

Gene Network Analysis.

Functional and network analyses of gene expression data derived from the AmpliSeq™ results was performed independently using Ingenuity Systems' Ingenuity Pathways Analysis (IPA) software (Ingenuity® Systems Inc., www.ingenuity.com). Briefly, gene expression changes are considered in the context of physical, transcriptional or enzymatic interactions of the gene/gene products and are grouped according to interacting gene networks through the use of IPA. The score assigned to any given gene network takes into account the total number of molecules in the data set, the size of the network, and the number of "network eligible" genes/molecules in the data set. The network score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's exact test. The network score is the negative log of this *p*-value.

Network analyses tools, such as employed in these investigations, provide an unbiased interpretation of gene array data based upon established interactions between genes/gene products, thereby reducing user bias. Gene network analyses also provide a template for interpreting large datasets generated through genome-wide gene array analyses to identify relevant biological processes.

The relatively small size of the patient population included in the gene array analysis could be considered to be a limitation of this study; however, this concern is obviated by several factors. First, the IPA analysis approach, which assigns differentially expressed transcripts to the most biologically relevant networks based upon known molecular interactions and functions, serves to

increase the statistical power of the study. For example, the odds of a single gene transcript (being identified by chance is reflected by a false discovery rate of <3%. However, the probability that multiple differentially expressed transcripts within our data set would associate with a given gene network is proportionate to the total number of differentially expressed genes, the size of the gene network, and the number of genes that are associated with the gene network. This approach allows for the identification of biologically relevant genes in relatively small experimental groups [S6, S7]. Thus, despite the fact that the pathway identified by the most highly over-represented gene network in PBMCs from patients with sarcoidosis relative to LTBI 7 days after PPD treatment, as depicted in Figure 2 (corresponding manuscript), was derived from only 12 sarcoidosis patients, the statistical probability that this particular cogent gene network would be found merely by chance is extremely low.

Lymph Node Tissue.

Patients who provided diseased mediastinal lymph node tissue samples met the operational diagnosis of sarcoidosis based upon the accepted pathological criteria. Specifically, samples displayed well-formed, non-necrotizing epithelioid granulomas in the absence of identifiable infection or foreign bodies, in accordance with diagnostic criteria described in the American Thoracic Society's joint statement on sarcoidosis [S8]. Samples exhibiting atypical pathological features, such as necrosis or fibrosis, were excluded from analysis. Tissue samples were obtained from organ donors (age-matched controls) or sarcoidosis patients undergoing surgical biopsies and provided by the Midwestern Division of the Cooperative Human Tissue Network.

Total RNA was isolated from frozen lymph node tissue using TRIzol reagent according to the manufacturer's protocol and as described previously [S9, S10]. Briefly, lymph node tissue samples were intermittently homogenized in 1 ml of TRIzol while on ice using a mini-homogenizer with a sterile RNase-free pestle (Kontes® Glass Company; Vineland, NJ) for up to one minute.

Phase separation of the RNA was carried out following the addition of 200 µl of chloroform, vigorous mixing for 15 seconds and centrifugation at 12,000 x *g* for 15 minutes at 4°C. The top RNA-containing phase was carefully transferred to a new tube, mixed with 500 µl of isopropanol, vigorously mixed for 15 seconds and centrifuged again at 12,000 x *g* for 10 minutes at 4°C. Following careful removal of the supernatant, 75% ethanol was added to the RNA pellet which was vortexed and centrifuged at 7500 x *g* for 5 minutes at 4°C. The ethanol was removed, and the pellet was allowed to air dry. The RNA pellet was then dissolved in RNase-free water by gentle pipetting and incubating at 55-60°C for 5 minutes. The RNA was purified using the Qiagen RNeasy® Mini Kit (Qiagen Inc.; Valencia, CA). The integrity of purified total RNA samples was assessed qualitatively on an Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA) and verified by the presence of two discrete electropherogram peaks corresponding to the 28S and 18S rRNA at a ratio approaching 2:1. Samples were excluded from gene array analysis if the ratio between the 3' and 5' signals exceeded 4, with ideal values being between 1 and 2.

Samples were then processed using high density oligonucleotide HG-U133 Plus 2 arrays (Affymetrix, Inc.; Santa Clara, CA), which analyze the expression levels of over 47,000 transcripts and variants including 38,500 well-characterized human genes [S9, S10]. Gene expression levels from probe intensities were estimated using a robust multi-array analysis (RMA) method with quantile normalization and background correction [11]. Functional and network analyses of gene expression data derived from the Affymetrix HG-U133 Plus 2 chips was performed using Ingenuity Systems' IPA software [S9, S10]. Briefly, gene expression changes are considered in the context of physical, transcriptional or enzymatic interactions of the gene/gene products and are grouped according to interacting gene networks through the use of Ingenuity Pathways Analysis. The score assigned to any given gene network takes into account the total number of molecules in the data set, the size of the network, and the number of "network eligible" genes/molecules in the data set. The network score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's exact test. The network score is the negative log of this *p*-value.

Statistics.

The data were derived from independent experiments and were expressed as means ± SEM, and statistical significance was based upon a value of $p < 0.05$. SigmaPlot 14.0 and SYSTAT 13.0 software were used to plot the data and carry out the analyses, respectively. The Mann-Whitney U-test (Wilcoxon Rank Sum) was employed to statistically compare hypothesis-driven differences in MIPAR-analyzed granuloma formation parameters following PPD stimulation in the presence and absence of inhibitor (RAP, BAFA1, CLQ, NH_4Cl) pre-treatments. In addition, the U-test was used to determine statistical differences in ELISA measurements of group cytokine release and *p*-mTORc1, *p*-S6 and *p*-STAT3 signaling following PPD treatment in the presence and absence of inhibitor pre-treatment based upon hypothesis-designed comparisons.

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Table S1: Genes associated with the canonical phagosome-mediated antimicrobial pathway that are differentially expressed and significantly increased 7 days after PPD-coated treatment of PBMCs obtained from Sarcoid patients compared to those of LTBI subjects

Gene	**Gene Product (Name)**	**Microbial Response Function**	**Fold Δ**	***p*-Value**
Antimicrobial Function – Antigen Recognition				
C1qA	Complement C1q subcomponent A	Serum complement component, opsonization	9.6	0.00056
C1qB	Complement C1q subcomponent B	Serum complement component, opsonization	15.0	0.00005
C1qC	Complement C1q subcomponent C	Serum complement component, opsonization	15.0	0.00005
CD163	Cluster of Differentiation 163	Macrophage scavenging receptor, antigen recognition	116.7	>0.00001
CLEC1A, CLEC4A, CLEC4D	C-lectin family member	Antigen recognition/presentation	5.0-16.9	0.00003-0.01224
CLEC7A	Dectin-1	Antigen recognition/presentation	7.8	0.00011
MRC1	Mannose receptor C, type 1	Recognized bacterial polysaccharides and promotes phagocytosis	18.7	>0.00001
TREM2	Triggering receptor expressed on myeloid cells 2	Complexes with TYROBP to bind various bacterial antigens to trigger immune responses	16.1	0.00001
TYROBP	TYRO protein tyrosine kinase binding protein	Complexes with TREM2 to bind various bacterial antigens to trigger immune responses	5.6	>0.00001
Antimicrobial Function – Antigen Presentation				
CD1A	CD1 family member	Lipid/glycolipid antigen presentation	17.2	0.01901
CD1B	CD1 family member	Lipid/glycolipid antigen presentation	28.5	0.01440
CD1C	CD1 family member	Lipid/glycolipid antigen presentation	12.1	0.02638
CD1D	CD1 family member	Lipid/glycolipid antigen presentation	13.2	0.00047

HLA-DMA, HLA-DOA, HLA-DOB, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB2, HLA-DRA, HLA-DRB1	Major histocompatibility complex class II	Binds exogenous antigens for endocytic processing enabling lysosomal/phagosomal degradation	4.4-16.9	>0.00001-0.04748
LGMN	Legumain	Processing of bacterial peptides for MHC class II presentation	22.5	>0.00001
Antimicrobial Function – Phagosome Formation, Maturation and Phagolysosomal Fusion				
LAMP1	Lysosomal-associated membrane protein 1	Fusion of lysosomes with phagosomes	5.6	0.00005
LAMP2	Lysosomal-associated membrane protein 2	Fusion of lysosomes with phagosomes	2.5	0.00001
LAMP5	Lysosomal-associated membrane protein 5	Fusion of lysosomes with phagosomes	23.0	0.00598
RAB5C	RAS oncogene family member RAB5C	Associates with phagosomes and regulates membrane trafficking	2.5	0.00008
RAB7A	RAS oncogene family member RAB7A	Fusion of phagosomes with lysosomes	3.5	>0.00001
RAB20	RAS oncogene family member RAB20	Regulates phagosome maturation	4.9	0.00389
RAB31	RAS oncogene family member RAB31	Intracellular membrane trafficking and phagosomal maturation	12.4	>0.00001
RAB32	RAS oncogene family member RAB32	Phagosome maturation	7.3	0.00014
RAB34	RAS oncogene family member RAB34	Phagosome maturation, lysosomal/phagosomal fusion	5.4	0.00086
RAB42	RAS oncogene family member RAB42	Phagosome maturation, lysosomal/phagosomal fusion	9.1	0.00196
RILP	RAB-interacting lysosomal protein	Fusion of phagosomes with lysosomes	3.2	0.00261
TUBB6	Tubulin subunit-Beta class 6	Microtubule formation enabling intracellular trafficking	9.8	>0.00001
Antimicrobial Function – Intracellular Phagosomal Antibacterial Molecule				
ATP1B1	ATPase Na^+/K^+ transporting subunit beta 1	Phagosomal/lysosomal acidification	10.9	0.00003

ATP1B4	ATPase Na^+/K^+ transporting subunit beta 4	Phagosomal/lysosomal acidification	59.9	0.00374
ATP6AP1	ATPase H^+ transporting accessory protein 1	Phagosomal/lysosomal acidification	6.0	0.00001
ATP6V0A1, ATP6V0B, ATP6V0C, ATP6V0D1, ATP6V0D2, ATP6V1A	V-type ATPases	Phagosomal/lysosomal acidification	3.9-6.2	0.00002-0.00087
CALR	Calreticulin	Ca^{+2} chaperone, ER Ca^{+2} signal transduction	2.7	0.00422
CTSA, CTSB, CTSC, CTSG, CTSH, CTSK, CTSS, CTSZ	Cathepsin	Lysosomal proteolysis	2.7-31.8	>0.00001-0.02547
LIPA	Lysosomal lipase A	Phagosome-mediated bacterial lipolysis	11.5	0.00001
LYZ	Lysozyme	Mediates oxidant-dependent bacteriolysis in phagosomes	10.4	0.00014
MPEG1	Macrophage-expressed protein 1 (Perforin-2)	Phagosome-mediated bacterial lysis	63.4	>0.00001
MPO	Myeloperoxidase	Mediates oxidant-dependent bacteriolysis in phagosomes	22.2	0.00002

Table S2: Genes associated with the canonical phagosome-mediated antimicrobial pathway that are differentially expressed and significantly increased 7 days after PPD-coated treatment of PBMCs obtained Sarcoid patients compared to those of Control subjects

Gene	**Gene Product (Name)**	**Microbial Response Function**	**Fold Δ**	***p*-Value**
Antimicrobial Function – Antigen Recognition				
C1qA	Complement C1q subcomponent A	Serum complement component, opsonization	6.6	0.00096
C1qB	Complement C1q subcomponent B	Serum complement component, opsonization	6.4	0.00121
C1qC	Complement C1q subcomponent C	Serum complement component, opsonization	6.4	0.00080
CD163	Cluster of Differentiation 163	Macrophage scavenging receptor, antigen recognition	11.1	0.00002
CLEC1A, CLEC4A, CLEC4D	C-lectin family member	Antigen recognition/presentation	3.9-4.5	0.00017-0.04783
CLEC7A	Dectin-1	Antigen recognition/presentation	4.3	0.00078
MRC1	Mannose receptor C, type 1	Recognized bacterial polysaccharides and promotes phagocytosis	4.0	0.01194
TREM2	Triggering receptor expressed on myeloid cells 2	Complexes with TYROBP to bind various bacterial antigens to trigger immune responses	8.6	0.00001
TYROBP	TYRO protein tyrosine kinase binding protein	Complexes with TREM2 to bind various bacterial antigens to trigger immune responses	3.4	0.00085
Antimicrobial Function – Antigen Presentation				
HLA-DMA, HLA-DOA, HLA-DOB, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB2, HLA-DRA, HLA-DRB1	Major histocompatibility complex class II	Binds exogenous antigens for endocytic processing enabling lysosomal/phagosomal degradation	2.9-4.5	0.00277-0.04555
LGMN	Legumain	Processing of bacterial peptides for MHC class II presentation	4.7	0.00290

Antimicrobial Function – Phagosome Formation, Maturation and Phagolysosomal Fusion				
LAMP1	Lysosomal-associated membrane protein 1	Fusion of lysosomes with phagosomes	2.6	0.03278
LAMP2	Lysosomal-associated membrane protein 2	Fusion of lysosomes with phagosomes	2.3	0.00023
RAB5C	RAS oncogene family member RAB5C	Associates with phagosomes and regulates membrane trafficking	2.2	0.00056
RAB7A	RAS oncogene family member RAB7A	Fusion of phagosomes with lysosomes	1.9	0.00201
RAB20	RAS oncogene family member RAB20	Regulates phagosome maturation	2.9	0.01840
RAB31	RAS oncogene family member RAB31	Intracellular membrane trafficking and phagosomal maturation	3.9	0.00039
RAB32	RAS oncogene family member RAB32	Phagosome maturation	3.3	0.00243
RAB34	RAS oncogene family member RAB34	Phagosome maturation, lysosomal/phagosomal fusion	2.5	0.01432
RAB42	RAS oncogene family member RAB42	Phagosome maturation, lysosomal/phagosomal fusion	6.1	0.00066
RILP	RAB-interacting lysosomal protein	Fusion of phagosomes with lysosomes	2.4	0.02781
TUBB6	Tubulin subunit-Beta class 6	Microtubule formation enabling intracellular trafficking	2.6	0.00870
Antimicrobial Function – Intracellular Phagosomal Antibacterial Molecule				
ATP1B1	ATPase Na^+/K^+ transporting subunit beta 1	Phagosomal/lysosomal acidification	4.3	0.00139
ATP6AP1	ATPase H^+ transporting accessory protein 1	Phagosomal/lysosomal acidification	3.1	0.00262
ATP6V0A1, ATP6V0B, ATP6V0C, ATP6V0D1, ATP6V0D2, ATP6V1A	V-type ATPases	Phagosomal/lysosomal acidification	2.7-3.5	0.00017-0.04733
CALR	Calreticulin	Ca^{+2} chaperone, ER Ca^{+2} signal transduction	2.2	0.00441
CTSA, CTSB, CTSC, CTSG, CTSH, CTSK, CTSS, CTSZ	Cathepsin	Lysosomal proteolysis	2.4-7.2	0.00002-0.04598

LIPA	Lysosomal lipase A	Phagosome-mediated bacterial lipolysis	4.3	0.00268
LYZ	Lysozyme	Mediates oxidant-dependent bacteriolysis in phagosomes	5.9	0.00042
MPEG1	Macrophage-expressed protein 1 (Perforin-2)	Phagosome-mediated bacterial lysis	12.5	0.00002
MPO	Myeloperoxidase	Mediates oxidant-dependent bacteriolysis in phagosomes	5.6	0.00822