Appendix

Bacterial Culture Media

Media for E. coli

Luria-Bertani (LB) medium (per litre)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
</tbody>
</table>

The medium was sterilized by autoclaving at 1 kg/cm² (15 psi), 121°C for 20 minutes and then stored at RT.

LB agar (per litre)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
</tbody>
</table>

The medium was sterilized by autoclaving at 1 kg/cm² (15 psi), 121°C for 20 minutes and then used to pour the plates aseptically.

Media for mycobacteria

Middlebrook-7H9 broth media (per liter) for H37Rv culture

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>7H9 Broth</td>
<td>4.7 g</td>
</tr>
<tr>
<td>Tween 80</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

Dissolved 4.7 g of this dehydrated base, along with 500 µl of Tween 80 in 900 ml of DDW. The medium was sterilized by autoclaving at 1 kg/cm² (15 psi), 121°C for 20
minutes and then supplemented with 100 ml of sterile ADC enrichment at RT.

Middlebrook-7H11 agar media (per liter) for H37Rv plating

7H11 Agar  20.5 g
Glycerol  5 ml

Dissolved 20.5 g of 7H11 agar powder and 5 ml of glycerol in 900 ml of DDW. The medium was sterilized by autoclaving at 1 kg/cm² (15 psi), 121°C for 20 minutes and then supplemented with 100 ml of sterile OADC enrichment when agar cools down to 40°C. It was then used to pour the plates.

Media for mammalian cell culture

RPMI 1640 (GIBCO BRL, USA) (per liter)

RPMI powder  1 Packet
HEPES  2.3 g
NaHCO₃  2.0 g
L-Glutamine  1.46 g

The contents of one packet were dissolved in 900 ml autoclaved DDW together with other reagents. The volume was made up to 1 litre and filter sterilized using 0.22 µ filter (Millipore, India). Stored in dark at 4°C.

Hanks Balanced Salt Solution (GIBCO BRL, USA) (per liter)

HBSS powder  1 Packet
NaHCO₃  0.35 g

The contents of one packet were dissolved in 900 ml autoclaved DDW together with other reagents. The volume was made up to 1 litre and filter sterilized using 0.22 µ filter (Millipore, India). Stored in dark at 4°C.
**General stock solutions and buffers**

**EDTA, 0.1 M**  
Added 0.186 g of EDTA to 8 ml of DDW. The solution was stirred vigorously on the stirrer with 1 M NaOH to adjust the pH of the solution to 8.0. The volume was made up to 10 ml with DDW. The stock solution was filter sterilized and then stored at 4°C.

**EGTA, 0.1 M**  
Added 0.38 g of EGTA to 8 ml of DDW. The solution was stirred vigorously on the stirrer with 1M NaOH to adjust the pH of the solution to 8.0. The volume was made up to 10 ml with DDW. The stock solution was filter sterilized and then stored at 4°C.

**IPTG, 1 M**  
Dissolved 2.38 g of IPTG in 10 ml of autoclaved DDW. The stock solution was sterilized by filtration through 0.45 µ filter and stored in aliquots at -20°C.

**Tris-Cl, pH 6.8/8.8, 1 M (100 ml)**  
Dissolved 12.1 g of Tris base in 80 ml of DDW and adjusted the pH to 6.8/8.8 with conc. HCl. The volume was made up to 100 ml with DDW. The buffer was sterilized by autoclaving and then stored at RT.

**Phosphate Buffer Saline (PBS, 0.1 M, pH 7.4)**  
Solution 1: 0.2 M Na₂HPO₄ prepared by adding 17.8 g of Na₂HPO₄ in 500 ml DDW.  
Solution 2: 0.2 M NaH₂PO₄ was prepared by adding 3.12 g NaH₂PO₄ in DDW.  
The stock buffer was prepared by mixing 405 ml solution 1 with 95 ml solution 2.  
Working solution was prepared by diluting 50 ml stock buffer in 950 ml of 0.15 M NaCl.

**RBC Lysis Buffer, pH 7.4**  
Tris-Cl  10 mM  
NH₄Cl  0.9%
Reagents for electrophoresis of DNA/ RNA

**Agarose gel loading dye, 6X**
Glycerol          30%
Bromophenol Blue Dye 0.25%
Xylene cyanol    0.25%
Dissolved the constituents in desired amount of autoclaved DDW and stored the dye at 4°C.

**Ethidium bromide**
Dissolved 100 mg of ethidium bromide in 10 ml of autoclaved DDW and stored at RT in dark.

**TBE, 20X (1 litre)**
Tris base          216 g
Boric acid         110 g
0.5 M EDTA         80 ml
Dissolved the constituents in 1 litre of DDW. The buffer was sterilized by autoclaving and then stored at RT.

Reagents for SDS-PAGE and immunoblotting

**Acrylamide-bisacrylamide solution, 30%**
Dissolve 29.2 g of acrylamide and 0.8 g of bis-acrylamide in DDW (100 ml final volume). The stock solution was filter sterilized through 0.45 µ filter and stored at 4°C in dark.

**APS solution, 10%**
Dissolved 100 mg APS in 1 ml of autoclaved DDW and stored the solution at 4°C.
SDS-PAGE sample solubilizing buffer, 6X
Tris-HCl, pH 6.8  300 mM
β-mercaptoethanol  7.5%
SDS  12%
Glycerol  60%
BPB  0.6%
Dissolved the constituents in desired amount of autoclaved DDW and stored at RT.

Composition of SDS-PAGE Gels
a.  Separating (resolving) gel:

<table>
<thead>
<tr>
<th>Component 10%</th>
<th>(ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved DDW</td>
<td>2.94</td>
</tr>
<tr>
<td>1 M Tris-HCl, pH 8.8</td>
<td>3.93</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>3.6</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.105</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.04</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01</td>
</tr>
</tbody>
</table>

b.  Stacking gel:

<table>
<thead>
<tr>
<th>Component 4%</th>
<th>(ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved DDW</td>
<td>3.00</td>
</tr>
<tr>
<td>1 M Tris-HCl, pH 8.8</td>
<td>1.25</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>0.66</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.037</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.02</td>
</tr>
</tbody>
</table>
SDS-PAGE Running buffer (1 litre, 10X)
Tris base 30.2 g
Glycine 188 g
SDS (10%) 100 ml
Dissolved the constituents in 1 litre of DDW and stored at RT.

Coomassie staining solution
Methanol 40%
Acetic acid 10%
DDW 50%
Coomassie Brilliant Blue R-250 0.25%

Destaining solution
Methanol 40%
Acetic acid 10%
DDW 50%

Western transfer buffer
Tris base 48 mM
Glycine 39 mM
SDS 0.037%
Methanol 20%
Dissolved the constituents in desired amount of DDW.

Blocking buffer
5% (w/v) Bovine Serum Albumin in 1X PBS-T.

Washing buffer
0.1% (v/v) Tween-20 in 1X PBS.
Primary and secondary antibody solutions were prepared in a 1% solution of BSA in wash buffer.

Buffers used in flowcytometry studies

Fixing buffer
4% paraformaldehyde in 0.1 M PBS. Stored at 4°C.

Washing buffer
0.5% BSA in 0.1 M PBS. Stored at 4°C.

Buffers used in confocal studies

Fixing buffer (2% Paraformaldehyde)
2% paraformaldehyde in 0.1 M PBS. Stored at 4°C.

Washing buffer
0.1% BSA and 0.1% Saponin in 0.1 M PBS. Stored at 4°C.

Permeabilization buffer
0.1% BSA and 0.2% Saponin in 0.1 M PBS. Stored at 4°C.

Buffers for cytoplasmic extract preparation

Cytoplasmic Lysis buffer
- HEPES (1 M, pH 7.9) 0.5 ml
- EDTA (0.5 M, pH 8.0) 0.01 M
- KCl (2 M) 0.25 ml
- EGTA (0.1 M) 0.05 M
DDW 49.19 ml

To the cytoplasmic buffer (1ml), protease inhibitors were added just prior to the use as mentioned below:

PMSF 1 mM
Aprotinin 0.2 mg/ml
Pepstatin 0.2 mg/ml
Leupeptin 0.2 mg/ml
Sodium Vanadate 1 mM

Buffers for Protein Purification

Lysis Buffer

NaH$_2$PO$_4$ 50 mM
NaCl 300 mM
Imidazole 10 mM

The pH of the solution was adjusted to 8.0 using NaOH.

Buffers for protein purification under Denaturing conditions

Buffer B

NaH$_2$PO$_4$ 100 mM
Tris.Cl 25 mM
NaCl 600 mM
Imidazole 10 mM
Urea 8 M

The pH of the solution was adjusted to 8.0 using NaOH.

Buffer C (Wash Buffer)

NaH$_2$PO$_4$ 100 mM
Tris.Cl 25 mM
NaCl 600 mM
Imidazole 20 mM
Urea 8 M
The pH of the solution was adjusted to 8.0 using NaOH.

**Buffer D (Elution buffer)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>100 mM</td>
</tr>
<tr>
<td>Tris.Cl</td>
<td>25 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>600 mM</td>
</tr>
<tr>
<td>Imidazole</td>
<td>250 mM</td>
</tr>
<tr>
<td>Urea</td>
<td>8 M</td>
</tr>
</tbody>
</table>

The pH of the solution was adjusted to 8.0 using NaOH.
Suppression of Dendritic Cell-mediated Responses by Genes in Calcium and Cysteine Protease Pathways during Mycobacterium tuberculosis Infection

Received for publication, September 5, 2011, and in revised form, February 1, 2012. Published, JBC Papers in Press, February 15, 2012, DOI 10.1074/jbc.M111.300319

Jhalak Singhal1, Neha Agrawal1, Mohit Vashishta3, N. Gayatri Priya9, Brijendra K. Tiwari9, Yogendra Singh1, Rajagopal Raman1, and Krishnamurthy Natarajan1,2

From the 1Infectious Disease Immunology Laboratory, Dr. B. R. Ambedkar Centre for Biomedical Research and 2Gut Biology Laboratory, Department of Zoology, University of Delhi, Delhi 110007, India and 3Allergy and Infectious Diseases Laboratory, Institute of Genomics and Integrative Biology, Council of Scientific and Industrial Research, Mall Road, Delhi 110007, India

Background: RNA interference (RNAi) is a useful tool to know the function of a gene in a cell under any kind of stress.

Results: RNAi-mediated knockdown of genes in dendritic cells identified unreported genes and pathways that regulate its various functions during Mycobacterium tuberculosis infection.

Conclusion: The identified genes could be potential targets in drug and vaccine designing.

Significance: Understanding the role of host factors that regulate priming of immune responses is crucial to study host-pathogen interactions.

With rising incidence of acquired drug resistance among life-threatening pathogens, alternative approaches to improve therapy and vaccination have taken center stage. To this end, genome-wide and pathway-specific siRNA libraries are being employed increasingly to identify genes that regulate immune responses against a number of pathogens. In this study using calcium and cysteine protease pathway-specific siRNA libraries, we identified genes that play critical roles in modulating diverse functions of dendritic cells (DCs) during Mycobacterium tuberculosis infection. Knockdown of many of these genes in the two pathways resulted in reduced bacterial burden within DCs. These included genes that regulated activation of transcription factors, ubiquitin-specific peptidases, and genes that are involved in autophagy and neddylation. Knockdown of certain genes increased the expression of IL-12p40 and surface densities of costimulatory molecules in an antigen- and receptor-specific manner. Increased IL-12p40 and costimulatory molecules on DCs also promoted the development of Th1 responses from a Th2 inducing antigen. Furthermore, modulation of autophagy and oxidative burst appeared to be one of the mechanisms by which these genes regulated survival of M. tuberculosis within DCs. Although some genes regulated specific responses, others regulated multiple responses that included IL-12 production, T cell priming, as well as intracellular survival of M. tuberculosis. Further dissection of the mechanisms such as neddylation, by which these genes regulate immune responses, would improve our understanding of host parameters that are modulated during M. tuberculosis infection.

Tuberculosis poses an ever-increasing risk during one’s lifetime (1–4). The specificity breadth and intensity of immune responses to infection by Mycobacterium tuberculosis is dependent on pathogen derived molecular patterns and host responses (5–7). Elucidation of factors involved in mediating immune responses during different stages of infection, namely latent/asymptomatic, active disease and during chemotherapy remains a prerequisite for the effective control of infection both in terms of vaccine development and drug discovery (8). Multiple sets of interactions between the host and the pathogen at all stages of infection are regulated at various levels culminating in differential phenotypic outcomes. Identification of genes that positively and negatively regulate these interactions would identify factors that shape effective responses (9–11).

Among the antigen presenting cells of the immune system, dendritic cells (DCs) are the most potent and act as a bridge between the innate and the acquired arm of the immune system (12). This is attributed largely to their ability to stimulate naïve quiescent T cells, thereby initiating a primary immune response. DC subsets colonize, and are recruited to specific tissues immediately following an antigenic insult, where they initiate divergent immune responses. Depending upon the activation status, DCs initiate either inflammatory or regulatory responses that determine whether a pathogen will be cleared or retained, thus, grossly affecting the survival of the host (13, 14). Although macrophages are the preferred hosts for mycobacteria, it is being increasingly recognized that M. tuberculosis infects DCs as well and DCs are crucial to initiate protective immune responses affecting mycobacterial survival in the host (15, 16). Therefore, regulation of DC function in the context of mycobacterial infection is a key area that needs

* This work was supported by grants from the Department of Biotechnology, Ministry of Science and Technology, Government of India (to K. N.).

1 Present address: Immunology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India.

2 To whom correspondence should be addressed: Dr. B. R. Ambedkar Centre for Biomedical Research, University of Delhi, Delhi 110007, India. Tel.: 91-11-27667151 or 91-11-27666272; Fax: 91-11-27666248; E-mail: knatarajan@acbr.du.ac.in.

3 The abbreviations used are: DC, dendritic cell; DCFH-DA, dichlorofluorescein diacetate; DC-SIGNR1, DC-SIGN-related molecule 1; TLR, Toll-like receptor; manLAM, mannosylated LipoArabinoMannan, ROS, reactive oxygen species; MOI, multiplicity of infection; SNRK, SNF-related kinase; USP25, ubiquitin-specific peptidase 25; SOD1, superoxide dismutase 1.
Specific Genes Regulate Dendritic Cell Function to M. tuberculosis

detailed investigation. Mycobacteria target a number of surface receptors on DCs, e.g. the mannose receptor, CD11b (Mac-1), CD11c and DEC-205, TLR2, TLR4, and TLR9 (8, 17, 18). Some of these receptors are employed by macrophages as well.

Recently, RNAi has emerged as an important genomic tool to carry out large-scale functional studies. The use of siRNA libraries against a specific pathway is a powerful technique to study the effect of that pathway on the function of a set of related genes inside a cell (9–11). Two key pathways that are targeted by M. tuberculosis in DCs (and also macrophages) are the calcium pathway that affects the survival and proinflammatory response generation from DCs (19, 20) and the cysteine protease pathway that largely affect antigen processing and presentation to T cells, thereby modulating priming of T cells early on in the infection process (21). Our own work has also highlighted the role of calcium homeostasis in regulating the survival of M. tuberculosis both in vitro and in vivo (22).

Therefore, in the light of the above, in this study, we elucidated the role of genes of these two pathways in modulating DC function with respect to M. tuberculosis infection by employing pathway-specific siRNA libraries. Our results identify a set of yet unreported genes that are targeted by M. tuberculosis in modulating the activation and function of DCs with respect to cytokine secretion and proinflammatory T cell responses, anti-defense mechanisms such as autophagy, and reactive oxygen species generation.

EXPERIMENTAL PROCEDURES

Animals—All experiments were conducted following approval from the institutional animal ethics committee. Female BALB/c mice 4–6 weeks of age that were kept in pathogen-free environment were used.

Materials—Fluorescence-tagged antibodies against mouse CD80, CD86, CD54, and CD40 were from BD Biosciences. Recombinant mouse GM-CSF was from R&D Systems (Minneapolis, MN). Antibodies to Beclin-1, ATG5, β-actin, superoxide dismutase 1 (SOD1), siRNAs against mouse genes, and Luminol kits for chemiluminescence detection were purchased from Santa Cruz Biotechnology. Control siRNAs from Santa Cruz Biotechnology (catalog no. sc-37007) was used as a nonspecific control. Pathway-specific siRNA libraries for primary screening were from Dharmacon (Lafayette, CO). siRNAs for the secondary screen were procured from Santa Cruz Biotechnology. ELISA kits were from ebScience (San Diego, CA). Dichlorofluorescin diacetate (DCFH-DA) and FITC-tagged Alexa Fluor 488 were obtained from Molecular Probes (Eugene, OR). Recombinant M. tuberculosis antigens Rv2463 and Rv3416 were expressed and purified as described recently (23). TLR2 ligand Pam3Csk4 was purchased from Inovigen (San Diego, CA). The following reagent was obtained through BEI Resources (NIAID, National Institutes of Health; purified lipoarabinomannan (LAM) from Mycobacterium tuberculosis, strain H37Rv, NR-14848).

Generation of DCs—DCs were differentiated with GM-CSF as described previously (23, 24). Briefly, bone marrow from the tibias and femurs of BALB/c mice were flushed out, and lymphocytes and I-A<sup>+</sup> cells were depleted following magnet-assisted cell sorting. Cells were cultured in RPMI 1640 medium containing 10% FCS, 0.05 mM 2-mercaptoethanol, 1 mM sodium pyruvate plus 15 ng/ml GM-CSF.

Transfection of DCs with siRNA and Stimulation—For siRNA transfections, 4 x 10<sup>6</sup>/ml bone marrow precursors were transfected with 60 pmol of siRNA for 72 h using the Hiperfect transfection reagent (Qiagen) in OPTIMEM medium (Invitrogen). GM-CSF was added 5 h following transfection, and incubation was continued for 72 h for DC differentiation. Knock-down was verified by RT-PCR, following which, cells were stimulated either with 1 μg/ml Pam3Csk4 or 5 μg/ml mannosylated lipoarabinomannan (manLAM) and/or either with 15 μg/ml Rv2463 or Rv3416 for 24 h. For some experiments, siRNA-transfected DCs were infected with M. tuberculosis H37Rv at 2.5 MOI for indicated times. Cells were processed for monitoring colony-forming units (cfu), reactive oxygen species (ROS) measurement or Western blotting as described below.

Flow Cytometry—Cells were stained for the surface levels of CD80, CD86, CD54, and CD40 using FITC-tagged monoclonal antibodies and analyzed by flow cytometry on FACS Calibur (BD Biosciences) as described previously (23). The data were plotted and analyzed using CellQuest Pro software.

T Cell Enrichment and Processing—Mice were immunized intraperitoneally with chicken egg ovalbumin (50 μg/mouse) for 7 days. Mice were sacrificed, and splenic T cells were enriched by magnet-assisted cell sorting as described previously (25). Briefly, following RBC lysis of spleen homogenates, adherent cells were removed by two rounds of panning over plastic plates. Following this, cells were incubated with anti-CD11c, anti-CD11b, anti-I-A<sup>+</sup>, and anti-B220 microbeads to remove contaminating DCs, macrophages, MHCII<sup>+</sup> cells, and B lymphocytes, respectively. The purity of the enriched T cells was 98%, as ascertained by surface staining with CD90. The percentage of I-A<sup>+</sup> cells was 0.05%. T cells were cocultured with siRNA-transfected and ovalbumin-stimulated DCs for 48 h, and cytokines were measured in culture supernatants.

Measurement of Cytokines—Cytokines in the culture supernatants were measured by employing a sandwich ELISA as described previously (23, 24). The samples were diluted to obtain absorbance in the linear range of the standards.

Western Blotting for Signaling Molecules—At the end of incubation, cells were chilled on ice, washed once with ice-cold PBS, and lysed in buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% Nonidet P-40, and 2 mM each of aprotinin, leupeptin, and pepstatin. The suspension was centrifuged at 13,000 rpm for 2 min at 4 °C. The supernatant was designated as the cytoplasmic extract. Twenty micrograms of cytoplasmic extract was resolved on 10% SDS-PAGE and subsequently transferred onto nitrocellulose membrane (Hybond C pure, Amersham Biosciences). The blots were then probed with antibodies to various molecules, followed by HRP-labeled secondary antibodies. Furthermore, a parallel set of samples was run separately on SDS-PAGE and probed for β-actin as loading control. The blots were later developed by chemiluminescence using the luminol reagent.

Measurement of Intracellular Reactive Oxygen Species—Intracellular ROS levels were measured by flow cytometry, as described previously, using the redox-sensitive dye DCFH-DA (23, 26). The nonfluorescent DCFH-DA readily diffuses into...
the cells where it is hydrolyzed to the polar derivative nonfluorescent dichlorofluorescin, which is oxidized in the presence of H2O2 to the highly fluorescent dichlorofluorescein. Thirty minutes prior to the end of each incubation period, 1 × 10^6 cells/ml were incubated with 10 μM DCBF-DA in the dark. Cells were thoroughly and quickly washed with pulse spin and immediately acquired for analyses in FACSCalibur (BD Biosciences). The data were plotted and analyzed using CellQuest Pro software.

Confocal Microscopy—2 × 10^6/mL siRNA-transfected DCs were stimulated with manLAM along with Rv2463 for 4 h. Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin, and incubated with antibodies against Beclin-1 or ATG5 followed by anti-rabbit FITC-tagged Alexa Fluor 488. Cells were again fixed with 4% paraformaldehyde. Confocal imaging was performed with Nikon A1 laser scan confocal microscope with 60X objective magnification, numerical aperture 1.4, refractive index 1.5, Plan Apo optics equipped with an argon laser, using excitation and emission wavelength of 488 and 525, respectively. Data were analyzed using the NIS Elements AR software. Expression levels were quantified as average of sum intensity of each fluorescent field.

Statistics—A two-tailed Student’s t test was carried out to obtain p values. Values of p < 0.05 were considered as significant.

RESULTS

Genes of Calcium Calmodulin and Cysteine Protease Pathways Modulate Survival of M. tuberculosis within DCs—First, we employed pathway-specific libraries to identify genes that regulated the survival of M. tuberculosis inside DCs, in keeping with the now accepted view that in addition to macrophages, M. tuberculosis also targets DCs during primary infection (15). Knockdown of a number of genes in either the calcium calmodulin pathway (supplemental Fig. 1) or cysteine protease pathway (supplemental Fig. 2) significantly modulated the survival of M. tuberculosis in DCs. This included an increase as well a decrease in the bacterial burden within DCs. This indicated that the two pathways contain genes that play positive and negative roles in regulating the survival of mycobacteria within DCs, although knockdown of very few genes in the cysteine protease pathway significantly increased intracellular bacterial loads. To proceed further, we chose to characterize the function of genes whose knockdown inhibited the survival of M. tuberculosis inside DCs because understanding the mechanisms by which these genes inhibited host-mediated responses against M. tuberculosis would identify strategies for either vaccine or drug development. These included calcium/calmodulin-dependent protein kinase II (Camkia) that regulates activation of transcription factors NF-κB and NF-AT and the activation of the MAPK pathway (27); proviral integration site 2 (Pim2) reported as an anti-apoptotic protein (28). It also included a number of serine threonine kinases such as SNF-related kinase (Snrk) and testis-specific serine kinase 1 (Stk22a), which regulate chromatin remodeling. These also act as a novel substrate for liver kinase B1 (Lkb1) and are involved in the spermatogenesis and hormone regulation in the testis (29). In addition, knockdown of Prkaa2 (protein kinase AMP-activated, α2), which modulates the activation of K+ channels and Ulk1 (gene51-like kinase I)-mediated autophagy (30), also decreased bacterial burden. As inhibiting these genes resulted in enhanced killing of M. tuberculosis by DCs, this indicated that M. tuberculosis modulates the activity and/or function of these genes for its survival and regulation of immune responses from DCs. Although the role of calcium in mediating the survival of M. tuberculosis has been well studied, the role of these genes in M. tuberculosis infection has not yet been documented.

Similarly, genes in the cysteine protease pathway, whose knockdown resulted in reduced survival of M. tuberculosis included members of the ubiquitin-specific protease family, e.g. Usp25 (ubiquitin-specific peptidase 25) and Usp9y (ubiquitin-specific peptidase 9Y). Other genes that had a similar effect were Uchl1 (ubiquitin carboxyl-terminal hydrolase L1), which modulates free monomeric ubiquitin levels and has been identified as a novel gene for Parkinson disease (31), Lgmn (legumain), another cysteine endopeptidase that regulates apoptosis (32), and Ctsh (cathepsin H), which plays a crucial role in processing and presentation of antigen peptides to T cells (33). Interestingly, knockdown of SUMO/Senp8 (sentrinspecific peptidase 8), which plays an important role in neddylation and sumoylation (34) had a significant effect on the survival of M. tuberculosis within DCs. A recent report indicated the role of one such ubiquitin protease during M. tuberculosis infection, wherein some of the PE-polymorphic CG-proteins of M. tuberculosis were found to be resistant to these proteases (35). However, their roles in specifically regulating M. tuberculosis survival have not been reported.

To validate our results, we shortlisted some of the genes from the above two pathways that significantly reduced the survival of M. tuberculosis inside DCs and investigated their ability to modulate M. tuberculosis survival in DCs using siRNAs from a different source. As shown in Fig. 1, knockdown of all of the 13 shortlisted genes reduced the survival of M. tuberculosis by >50%, and therefore, these 13 genes were selected for subsequent experimentation and characterization. Efficiency of knockdown of select genes was ascertained by RT-PCR (supplemental Fig. 3).

DC Genes Differentially Regulate IL-12p40 Production by TLR2 and DC-SIGNR1—We next carried out a detailed characterization of the functional effects regulated by the validated genes. First, we investigated the ability of these genes to influence the production of IL-12p40 from DCs. We had shown recently that TLR2 induces higher IL-12p40 production in DCs when compared with DC-SIGN (DC-specific ICAM-3 grabbing non-integrin) R1 in the context of M. tuberculosis infection. To this end, we stimulated TLR2 with Pam3Csk4 and DC-SIGNR1 with manLAM as described previously (24). As shown in Fig. 2, knockdown of many genes significantly up-regulated IL-12p40 expression over and above that obtained following TLR2 stimulation. Maximum effects with >2-fold increases were observed upon knockdown of Camkia, Usp25, Prkaa2, Lgmn1, and Senp8. Expectedly, and consistent with our earlier report, DC-SIGNR1 stimulation did not result in any detectable induction of IL-12p40. However, following knockdown of either Pim2 or Prkaa2 or Usp25 or Snrk or Usp9y or Stk22a, a significant induction of IL-12p40, albeit to different levels, was now observed (Fig. 2). These results indicated that the genes that
negatively regulated survival of *M. tuberculosis* also negatively regulated IL-12p40 production, more significantly following stimulation of DC-SIGNR1. Furthermore, some genes such as *Usp25* and *Snrk* mediated IL-12 increase from both receptors, whereas others displayed a receptor specific effect, indicating compartmentalization of functions.

Specific Genes Regulate Dendritic Cell Function to *M. tuberculosis*

---

**FIGURE 1.** Knockdown of select genes of the two pathways in DCs results in decreased survival of *M. tuberculosis*. DCs were transfected with siRNAs against indicated genes belonging to either the calcium calmodulin pathway or cysteine protease pathway for 72 h, followed by infection with 2.5 MOI *M. tuberculosis* H37Rv for 48 h. Cell lysates were plated onto 7H11 agar plates, and cfu were monitored after 2–3 weeks. Mock represents knockdown with a control siRNA. Data represent mean ± S.D. of three independent experiments. *, *p* = 0.012 for Mock versus Camkii; **, *p* = 0.017 for MOCK versus Usp25; ***, *p* = 0.006 for Mock versus Ctsk; and ****, *p* = 0.016 for Mock versus Snrk. TTN, titin.

**FIGURE 2.** Knockdown of specific genes results in enhanced IL-12p40 expression from TLR2 and DC-SIGNR1. DCs were transfected with siRNAs against specific genes as in Fig. 1, followed by stimulation with either 1 μg/ml Pam3Csk4 (Pam) or 5 μg/ml manLAM for 24 h. Mock represents knockdown with a control siRNA. IL-12p40 levels in culture supernatants were measured by ELISA. Bars represent mean ± S.D. of three independent experiments. Upper panel, *, *p* = 0.012 for Mock versus Prkaa2; **, *p* = 0.006 for Mock versus Dcamkl1; ***, *p* = 0.013 for Mock versus Senp8; ****, *p* = 0.03 for Mock versus Usp9y. Lower panel, *, *p* = 0.016 for Mock versus Usp25; **, *p* = 0.01 for Mock versus Snrk; ***, *p* = 0.009 for Mock versus Stk22a.

Specific Genes Modulate *M. tuberculosis* Antigen-mediated IL-12p40 Production—We recently reported the enrichment and functional characterization of *M. tuberculosis* genes expressed inside macrophages as a function of infection and time. These genes were named as day1 and day5 antigens, based on their expression patterns at 24 and 120 h post-infection, respectively (23). Characterization of these antigens revealed their role in suppression of TLR2 and *M. tuberculosis* mediated IL-12p40 production, *M. tuberculosis* mediated expression of surface T cell costimulatory and MHC molecules, surface expression of cytokine receptors, generation of ROS and antigen-specific T cell responses. Furthermore, overexpression of
these day1 and day5 antigens also resulted in increased survival of *M. tuberculosis* inside DCs as well as macrophages. In light of the above results, we therefore investigated the role of the host genes identified in the present study in mediating the effects of the day1 and day5 antigens in some of the above mentioned responses. To this end, we knocked down genes in DCs followed by stimulation with either 15 μg/ml Rv2463 or Rv3416 for 24 h. Cells were later stimulated with either 1 μg/ml Pam3Csk4 (Pam) or 5 μg/ml manLAM for 24 h. Culture supernatants were screened for the levels of IL-12p40. Mock represents knockdown with a control siRNA. Data represent mean ± S.D. of three independent experiments. Upper panel, *p = 0.004 for Mock + Rv2463 + Pam3Csk4 versus Camkiia + Rv2463 + Pam3Csk4; **, p = 0.01 for Mock + Rv2463 + Pam3Csk4 versus Snrk + Rv2463 + Pam3Csk4; ***p = 0.017 for Mock + Rv3416 + Pam3Csk4 versus Usp25 + Rv3416 + Pam3Csk4; ****, p = 0.1 for Mock + Rv3416 + Pam3Csk4 versus Stk22a + Rv3416 + Pam3Csk4. Lower panel, *p = 0.16 for Mock + Rv2463 + manLAM versus Camkiia + Rv2463 + manLAM; **, p = 0.009 for Mock + Rv2463 + manLAM versus Senp8 + Rv2463 + manLAM; ***p = 0.012 for MOCK + Rv3416 + manLAM versus Usp25 + Rv3416 + manLAM; ****, p = 0.004 for MOCK + Rv3416 + manLAM versus Senp8 + Rv3416 + manLAM.

**Modulation of Costimulatory Molecule Expression by Host Genes**—A primary function of a DC is to initiate T cell responses, during which the surface densities of costimulatory molecules play a determinant role. We therefore investigated the ability of three genes (based on their ability to significantly modulate IL-12p40 responses) to modulate the surface densities of key costimulatory molecules on DCs. Our results indicate that these genes differentially influenced the surface densities of key costimulatory molecules. For example, as shown in Table 1, knockdown of *Snrk* increased the expression of CD80 following stimulation with Rv2463 (day1 antigen) in the context of TLR2 but not DC-SIGNR1. However, no significant changes were observed with Rv3416 (day5 antigen) in the context of either TLR2 or DC-SIGNR1. Similarly, knockdown of *Usp25* resulted in increased expression of CD80 and CD54 following stimulation with Rv3416 and DC-SIGNR1. Similar to *Usp25*, knockdown of *Senp8* showed a similar pattern with increased expression of all the costimulatory molecules (CD80, CD86, CD40) with maximum increase in the levels of CD54 following stimulation with Rv3416 and DC-SIGNR1.

Furthermore, the three genes showed an antigen- and receptor-specific modulation of costimulatory molecules. Typically, stimulation of DC-SIGNR1 in the context of Rv3416 a day5 antigen was more effective in increasing the expression of most
Specific Genes Regulate Dendritic Cell Function to M. tuberculosis

costimulatory molecules following knockdown of different genes when compared with similar stimulation of TLR2. This indicated that early priming of T cells by the day1 antigen (Rv2463) was regulated by these genes via modulation of IL-12

TABLE 1
Knockdown of specific genes modulates surface densities of costimulatory molecules on DCs

The indicated genes were knocked down in DCs with specific siRNAs followed by stimulation with either Pam,Csk (Pam) or manLAM along with Rv2463 or Rv3416, as indicated. The surface levels of indicated markers were monitored by flow cytometry. Data are represented as increase (+) or decrease (−) over that observed in control siRNA transfected and similarly stimulated DCs. Number of + or − indicates levels of increase or decrease, respectively. Data are representative of two independent experiments.

<table>
<thead>
<tr>
<th>Gene/stimulation</th>
<th>CD80</th>
<th>CD86</th>
<th>CD40</th>
<th>CD54</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNRK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rv2463 + Pam</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Rv2463 + manLAM</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Rv3416 + Pam</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Rv3416 + manLAM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>USP25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rv2463 + Pam</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Rv2463 + manLAM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rv3416 + Pam</td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Rv3416 + manLAM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SENP8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rv2463 + Pam</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Rv2463 + manLAM</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Rv3416 + manLAM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(As observed in Fig. 3); the late or secondary priming of T cells by the day5 antigen (Rv3416) was regulated at the level of costimulatory molecules by these genes. These results further indicate a role for these genes in influencing immune responses as a function of time and receptor triggering. These observations are consistent with the documentation that during M. tuberculosis infections, TLR2 triggering occurs early on in the infection process and is followed by stimulation of DC- sign by soluble manLAM secreted by infected macrophages as the infection proceeds with time (36).

Knockdown of Genes Induce Th1 Responses by Th2-promoting Antigen—Keeping the above results in mind, we next investigated the quality of T cell responses from ovalbumin-stimulated DCs following knockdown of the genes. Ovalbumin is an antigen that is known to induce Th2 responses and is a typical antigen widely used in mouse models of asthma (37). Because a potent Th1 response is a prerequisite and a marker for protective responses during M. tuberculosis infections, we investigated whether knockdown of the genes would skew the ovalbumin-induced Th2 response to a Th1 response. To this end, genes were knocked down in DCs followed by stimulation with ovalbumin for 24 h. The DCs were then co-cultured with T cells enriched from ovalbumin-primed mice. The levels of IFN-γ and IL-10 were scored in the supernatants 48 h later. As shown in Fig. 4, ovalbumin induced a typical Th2 response with higher

FIGURE 4. Knockdown of Prkaa2, Usp25, and Senp8 induces Th1 responses from ovalbumin-stimulated DCs. Indicated genes were knockdown in DCs followed by stimulation with 15 μg/ml ovalbumin for 24 h followed by co-culture for 48 h with T cells enriched from ovalbumin-primed mice. Culture supernatants were screened for the levels of IFN-γ and IL-10. Mock represents knockdown with a control siRNA. Data represent the mean ± S.D. of three independent experiments. Upper panel, *p = 0.013 for Mock versus Prkaa2; **p = 0.01 for Mock versus Senp8; ***p = 0.013 for Mock versus Cts; ****p = 0.014 for Mock versus Snr. Lower panel, *p = 0.012 for Mock versus Pim2; **p = 0.02 for Mock versus Prkaa2; ***p = 0.014 for Mock versus Senp8; ****p = 0.008 for Mock versus Usp9.
levels of IL-10 when compared with IFN-γ. However, knockdown of Prkaa2, Senp8, Dcamkl1, and Usp25 (and to some extent knockdown of Snrk and CtsH) significantly skewed the Th2 response to a Th1 response by increasing the ratio of IFN-γ over IL-10. However, knockdown of Camkiia, Pim2, Ttn, and Uchl1 had no significant effect, whereas knockdown of Stk22a had a marginal decrease in IFN-γ levels, indicating that not all genes have the ability to influence all responses. Nevertheless, these results were consistent with the role of these genes in influencing IL-12p40 levels from TLR2 and DC-SIGNR1. The results further indicated that these genes played a negative role in the priming of Th1 responses from infected or antigen-stimulated DCs during M. tuberculosis infections and inhibiting these genes could indeed potentiate Th1 responses.

**Genes of Calcium and Cysteine Protease Pathways Increase Autophagy in DCs**—Of late, autophagy has emerged as a key defense mechanism employed by infected cells to clear intracellular pathogens (38, 39). A recent report elucidated the role of autophagic pathway. Similar opposite effects were seen in the case of Stk22a and Senp8, wherein no significant changes were observed with respect to Beclin-1 levels, but a significant increase in the levels of ATG5 were observed. Similarly, knockdown of all genes (except Stk22a) increased the expression levels of Beclin-1 following by stimulation with DC-SIGNR1 along with the day1 antigen. On the other hand, knockdown of only four genes, namely Camkiia, Usp25, Senp8, and Stk22a, significantly increased the expression of ATG5.

In contrast, stimulation of DC-SIGNR1 and the day5 antigen either had no effect on Beclin-1 expression following knockdown of most genes, whereas its levels were reduced below basal levels following knockdown of Snrk and Stk22a. Interestingly, the knockdown of only Snrk increased ATG5 levels, with no effects upon knockdown of the other genes. These results reiterate the receptor- and antigen-specific effects in the regulation of autophagy by these genes. For example, a reverse effect of Snrk was observed in the case of stimulation of DC-SIGNR1 with the day5 antigen, wherein a polar effect was evident with respect to regulation of the expression of two genes in the autophagic pathway. Similar opposite effects were seen in the case of Stk22a with respect to ATG5 expression in the context of TLR2 stimulation along with the day1 and the day5 antigen.
Taken together, the results in Fig. 5, display an interesting pattern, wherein specific genes in the calcium and cysteine protease pathway regulate the expression levels of different genes in the autophagic pathway, depending on the type of receptor that is stimulated by diverse antigens. Overall, the knockdown of these genes had a positive effect on autophagy, albeit at different levels, indicating a net negative role during mounting of autophagic responses during *M. tuberculosis* infection. This could reflect the redundancy that is observed for most processes in biological systems following a stress response in the form of pathogenic insult or abiotic pressures.

We also monitored the expression of Beclin-1 and ATG5 in select groups by confocal microscopy. To this end, we selected *Camkiia, Usp25*, and *Senp8* because knockdown of these genes significantly increased the expression of both Beclin-1 and ATG5 following stimulation of DC-SIGNR1 with the day1 antigen. As shown in Fig. 6, and consistent with the results in Fig. 5, knockdown of either *Camkiia* or *Senp8* or *Usp25* maximally increased the expression of Beclin-1 upon costimulation of day1 antigen (Rv2463) with DC-SIGNR1.

**Key Genes in Two Pathways Modulate Oxidative Burst in DCs**—We had earlier reported the role of ROS in mediating the survival of mycobacteria inside DCs (26). Furthermore, we had established a positive correlation between intracellular calcium influx and ROS generation in DCs. Increased intracellular calcium and ROS levels reduced the survival of mycobacteria in DCs (26). Therefore, we monitored ROS levels following knockdown of a few genes followed by specific antigen and receptor stimulation. As shown in Fig. 7, an interesting pattern was observed with respect to modulation of ROS by antigens and receptors. In the case of day1 antigen (Rv2463), knockdown of the three genes increased ROS levels by TLR2, whereas a reverse effect was observed upon stimulation of DC-SIGNR1 (Fig. 7A). In contrast, in the case of the day5 antigen (Rv3416), knockdown of the three genes increased ROS levels by DC-SIGNR1, whereas a reverse effect was observed upon stimulation of TLR2 (Fig. 7B). These results indicate that with the early antigen (Rv2463), these genes played a negative role in ROS induction from TLR2, whereas a positive role was observed following DC-SIGNR1 stimulation. Conversely, with the late antigen (Rv3416), these genes played a positive role in ROS induction from TLR2 and a negative role upon DC-SIGNR1 stimulation. A similar pattern was obtained following knockdown of *Snrk* and *Stk22a* and to an extent with *Prkaa2* (supplemental Fig. 4). These results also point to a differential activation status of these genes during a time-dependent antigenic stimulation of different receptors. Furthermore, these results add support to our earlier report wherein we proposed a complementary role for these antigens as a function of infection and time that constantly work toward keeping immune responses suppressed (23). These results indicated that modulating the oxidative burst could be one of the mechanisms by which these genes regulate survival of *M. tuberculosis* in DCs.

**Key Genes Regulate Autophagy in *M. tuberculosis*-infected DCs**—We extended the above observations with infection of DCs with live virulent *M. tuberculosis*. Specific genes were knocked down with specific siRNAs followed by infection with *M. tuberculosis* H37Rv for 24 h. First, we monitored the effect of knockdown of five genes in modulating autophagic responses in *M. tuberculosis*-infected DCs. As shown in Fig. 8,
knockdown of Camkia, Prkaa2, Usp25, Senp8, and Snrk increased the levels of Beclin-1, whereas knockdown of Stk22a had a marginal effect. On the other hand, knockdown of Camkia, Prkaa2, Usp25, and Senp8 (to an extent Stk22a) significantly increased ATG5 expression in *M. tuberculosis* infected DCs, whereas knockdown of SNRK had no effect. The fact that the increase in the levels of Beclin-1 was not as dramatic as observed following stimulation with day1 or day5 antigens, and TLR2 or DC-SIGNR1 could be attributed to the fact that during *M. tuberculosis* infection multiple receptors would be stimulated with multiple surface antigens, in contrast to stimulation of specific receptors (TLR2 or DC-SIGNR1) with specific antigens (Rv2463 or Rv3416). The cross-talk between signals emanating from multiple receptors would indeed have bearings on the levels of gene expression of the autophagic pathway. Nevertheless, the fact that there was an increase in the levels of both Beclin-1 and ATG5 by one gene or the other indicates that these genes played a net negative role in regulating autophagy during *M. tuberculosis* infection.

**Key Genes Regulate ROS Generation and SOD1 Levels in M. tuberculosis-infected DCs**—We next monitored the levels of ROS in *M. tuberculosis* DCs following knockdown of the above genes. As shown in Fig. 9, individual knockdown of all of the genes increased ROS levels in infected cells albeit with different kinetics and to different extents. Although the data in Fig. 7 indicated that an increase in ROS levels upon stimulation of either TLR2 or DC-SIGNR1 with a day1 or the day5 antigen was observed within 30 min, the kinetics of increase in ROS levels...
were different with respect to \textit{M. tuberculosis} infection. Although knockdown of \textit{Senp8} or \textit{Camkiia} or \textit{Usp25} increased ROS levels 1-h post infection, knockdown of \textit{Prkaa2} or \textit{Stk22a} increased ROS levels within 30 min. On the other hand, knockdown of \textit{Snrk} had no significant effect. These results once again reiterate that these genes played a negative role in regulating oxidative burst during \textit{M. tuberculosis} infection, and the delayed kinetics observed in some genes could be attributed to stimulation of multiple receptors as against individual receptors as argued above for autophagy.

Because generation of ROS is often associated with modulations in the levels of the ROS quencher SOD1, we monitored SOD1 levels in \textit{M. tuberculosis}-infected DCs following knockdown of the genes. As shown in Fig. 10, infection with \textit{M. tuberculosis} increased SOD1 levels with time, indicating down-modulation of oxidative burst. However, knockdown of most genes resulted in a significant decrease in SOD1 levels. With respect to the MOCK control, knockdown of \textit{Camkiia} or \textit{Usp25} resulted in a decrease of SOD1 levels by 56 and 50%, respectively. Likewise, knockdown of \textit{Prkaa2} and \textit{Stk22a} brought down SOD1 levels by 40 and 44%, respectively. On the other hand, knockdown of \textit{SENP8} and \textit{SNRK} resulted in a modest decrease of SOD1 expression levels by 22 and 13%, respectively, with respect to the MOCK control. These results indicate that although all the genes analyzed in effect decreased SOD1 levels, they varied in their kinetics in down-modulating SOD1 levels.

For example, although knockdown of genes such as \textit{Camkiia}, \textit{Usp25}, and \textit{Stk22a} down-modulated SOD1 levels at later times post-infection (2 h), knockdown of genes such as \textit{Prkaa2} and \textit{Senp8} had an early effect with respect to down-regulation of oxidative burst during \textit{M. tuberculosis} infection, and the delayed kinetics observed in some genes could be attributed to stimulation of multiple receptors as against individual receptors as argued above for autophagy.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig8.png}
\caption{Knockdown of select genes induces autophagy in \textit{M. tuberculosis}-infected DCs. DCs were transfected with siRNAs against indicated genes, followed by infection with 2.5 MOI \textit{M. tuberculosis} (\textit{M. tb}) H37Rv for 24 h. Cytoplasmic extracts were Western blotted for Beclin-1 and ATG5 expression. MOCK represents knockdown with a control siRNA. Numbers below the Beclin-1 or ATG5 blots indicate relative intensities of the bands. Data from one of two experiments are shown.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig9.png}
\caption{Knockdown of select genes enhances ROS generation in \textit{M. tuberculosis}-infected DCs. DCs were transfected with siRNAs against indicated genes and later infected with 2.5 MOI \textit{M. tuberculosis} H37Rv for indicated times. 30 min prior to the incubation period, cells were loaded with 10 \muM DCFH-DA. At the end of the incubation period, cells were quickly and thoroughly washed with culture medium and fixed with 4% paraformaldehyde for 2 h. Cells were washed once again with culture medium and immediately analyzed for ROS levels by flow cytometry. Mock represents transfection with control siRNAs. Black lines depict ROS levels in DCs transfected with control siRNAs. Green lines depict DCs transfected with gene-specific siRNAs. Data from one of two experiments are shown.}
\end{figure}
Specific Genes Regulate Dendritic Cell Function to M. tuberculosis

With the emergence of drug resistance in pathogenic strains, including M. tuberculosis, and the increasing risk and failures associated with multi-drug-resistant and extensively drug-resistant TB, together with the protracted development of new and effective drugs against these strains, research priorities have begun to focus on the abilities of the host machinery to combat long lasting and resurgent infections. To this end, several attempts have been made to understand the role of host genes in mediating protection as also the regulation of immune responses against these pathogens (9–11). The survival of M. tuberculosis in the host cell is facilitated through a dynamic interplay of cellular and intracellular immune responses. These include down-regulation and inhibition of antigen presentation, cellular activation pathways, and the activation of bacterial responses (24, 41). Furthermore, several reports point toward a role of M. tuberculosis antigens in mediating suppressive responses on host cells. For example, the 19-kDa lipoprotein inhibits IFN-γ-mediated responses in macrophages via TLR2 (42). Over the years, we have also highlighted the role of M. tuberculosis antigens, specifically CFP-10, in modulating DC functions (22–24, 43, 44).

Several studies have demonstrated the utility of RNAi-based screens, which use siRNA libraries to identify genes that play crucial roles that shape functional and phenotypic outcomes (45) and to identify potential drug targets (46). For example, siRNA libraries against the apoptotic pathway have been used to identify several genes regulating cancer (47). Furthermore, siRNA libraries have been used in systematic and cost-effective genome-wide loss-of-function screens with the aim of assessing the role of specific genes in neoplastic phenotypes and the rapid identification of novel drug targets (46). The apoptotic pathway library has been used to identify genes active during apoptosis, proliferation, and cell cycle (48). MAPK pathway-specific siRNA libraries have been used to study cell cycle regulation (49). Using similar approaches, the role of phosphatases that regulate NF-κB activation has been elucidated (50). Recently, genes regulating the level of ploidy in HeLa cells were profiled using genome-scale RNAi profiling and >2000 genes were identified to regulate ploidy in cancerous cells (51). A recent report has also identified genes in human macrophages that regulate the survival of M. tuberculosis wherein inhibiting xenophagic pathways and the formation of autophagosomes as an increase in the levels of LC3 II upon the silencing of some target genes was observed (52).

In light of the above reports, we investigated the roles of genes that mediate immune responses to M. tuberculosis via DCs. To that end, we restricted our studies to investigating the roles of genes involved in specific pathways, namely the calcium calmodulin pathway and cysteine protease pathway. These pathways were chosen based on reports, including ours, on the role of calcium in modulating immune responses to M. tuberculosis from DCs and macrophages (22) and the important role of antigen presentation in stimulating T cell responses (12–14). In addition, calcium plays a determinant role in the generation of proinflammatory responses (19, 20) and also regulates the survival of M. tuberculosis in macrophages and DCs (22).

Using siRNA libraries to these pathways, we initially investigated the role of these genes in mediating survival of M. tuberculosis inside DCs. This was carried out to shortlist crucial genes that could be pursued for further characterization. A set of 13 genes was identified whose knockdown resulted in a significant reduction in cfus. Interestingly, many of the genes have not been reported to regulate immune responses to M. tuberculosis, although these genes have been reported to influence various immune related responses. For example, TLR stimulation of DCs result in the activation of Camkiiia and it is also involved in the regulation and distribution of MHCII levels in DCs (53).
PIM-2, an anti-apoptotic protein (28, 54), is expressed at high levels in acute promyelocytic leukemia patients (55). Usp25, a ubiquitin-specific protease enzyme, is involved in deubiquitination process that has an essential role in regulating various cellular pathways, cell cycle regulation, transcription etc. Dcamkl1 (doublecortin and CaMK kinase-like-1) is expressed in post-mitotic neurons (56) and is known as a putative stem cell marker. Senp8 is a NEDD8-specific protease that has an important role in deneddylation of cullin, a substrate for neddylation (34). In addition to playing a role during apoptosis, Lgmn, an asparaginyl endopeptidase, is overexpressed in many human solid tumors, breast, colon, and prostate cancer and sparsely expressed in normal tissues (32). Snrk is reported as a novel substrate for liver kinase B1 (29). It also controls angioblast populations in the lateral plate mesoderm in zebrafish (57). Usp9y is one of the major Y-linked spermatogenesis genes, which acts as a fine tuner to improve efficiency of human spermatogenesis (58).

As reported by others, several responses observed with live infections can be reproduced by stimulating immune cells such as DCs or macrophages with M. tuberculosis antigens (22, 42–44). To this end, we also recently reported the role of antigens expressed inside of M. tuberculosis-infected macrophages as a function of infection and time in modulating M. tuberculosis-mediated responses to DCs and macrophages (23).

Therefore, in the next set of experiments, we investigated the ability of these genes to modulate M. tuberculosis antigen-specific responses from DCs. To that end, again based on our earlier report (24), we focused on the roles of two receptors, TLR2 and DC-SIGNR1, which induce contrasting responses with respect to M. tuberculosis (59). Taken together, the results indicate that knockdown of many of these genes resulted in enhanced proinflammatory cytokine responses, as observed by increased levels of IL-12p40 from both TLR2 and to some extent DC-SIGNR1. This was further corroborated with increases in the surface densities of costimulatory molecules, as well as induction of Th1 responses from an antigen known to favor Th2 responses. These results emphasize an as yet unknown function of these genes in regulating immune responses to M. tuberculosis.

Following this, we next investigated the mechanism involved in regulation of M. tuberculosis survival inside DCs as mediated by the identified genes. To that end, our results indicated that they regulate autophagy and the generation of oxidative bursts. The results indicate that knockdown of most of the genes are able to increase the expression of Beclin-1 and ATG5, autophagic markers upon TLR2 and DC-SIGNR1 stimulation with day1 and day5 M. tuberculosis antigens. Likewise, the generation levels of ROS also showed an enhanced effect following knockdown of genes upon stimulation of TLR2 and DC-SIGNR1 with day1 and day5 M. tuberculosis antigens. Interestingly, ROS was regulated by these genes in a manner that was reflective of time-based responses. This indicated differential activation of these genes at different times post-infection when antigens secreted from infected macrophages would counter DCs that are recruited to the sites of infection and modulate their function as proposed in our earlier report (23).

Collectively, our results indicate the identification of genes that play a significant role in modulating immune responses to M. tuberculosis from DCs. Genes such as Usp25, Snrk, and Senp8 displayed broad negative regulation with respect to proinflammatory responses from DCs as well as regulation of M. tuberculosis survival. Furthermore, many of the genes identified in this report acted in a specific manner that are indicative of fine tuning of immune responses during infection and the ability of M. tuberculosis to modulate the activation status of these genes for immune evasion. Interestingly, Senp8 is involved in neddylation of various intracellular molecules (60). The role of neddylation during M. tuberculosis infection has not been reported. Some of the above results were extended to experimentation with live M. tuberculosis infections and similar results were obtained. Most genes down-regulated autophagy and ROS during live infections thereby adding support to the above observations. This indicated that these genes played a negative role during specific antigenic stimulation as well as whole bacterial infection. Therefore, delineating the mechanisms employed by these genes in regulating some of these responses could increase our understanding on the roles played by these genes in the pathogenesis of M. tuberculosis.

REFERENCES

Specific Genes Regulate Dendritic Cell Function to M. tuberculosis


Suppression of TLR2-Induced IL-12, Reactive Oxygen Species, and Inducible Nitric Oxide Synthase Expression by Mycobacterium tuberculosis Antigens Expressed inside Macrophages during the Course of Infection

Deepti Gupta, Sachin Sharma, Jhalak Singhal, Akash T. Satsangi, Cecil Antony and Krishnamurthy Natarajan

*J Immunol* 2010;184;5444-5455; Prepublished online 12 April 2010;
doi:10.4049/jimmunol.0903283
http://www.jimmunol.org/content/184/10/5444

References

This article cites 42 articles, 25 of which can be accessed free at:
http://www.jimmunol.org/content/184/10/5444.full.html#ref-list-1

Subscriptions

Information about subscribing to *The Journal of Immunology* is online at
http://www.jimmunol.org/subscriptions

Permissions

Submit copyright permission requests at
http://www.aai.org/ji/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at
http://www.jimmunol.org/etoc/subscriptions.shtml/
Suppression of TLR2-Induced IL-12, Reactive Oxygen Species, and Inducible Nitric Oxide Synthase Expression by Mycobacterium tuberculosis Antigens Expressed inside Macrophages during the Course of Infection

Deepti Gupta, Sachin Sharma, Jhalak Singhal, Akash T. Satsangi, Cecil Antony, and Krishnamurthy Natarajan

We report the enrichment of and immune responses mediated by genes expressed by Mycobacterium tuberculosis inside macrophages as a function of time. Results indicate that M. tuberculosis expresses different genes at different times postinfection. Genes expressed early (day 1) following infection enhance M. tuberculosis-mediated activation of dendritic cells (DCs), whereas genes expressed later (day 5) in the infection prevent DC activation. However, all genes downmodulated MHC class I and II expression on infected macrophages, thus compromising their ability to interact with Ag-specific T cells. Day-1 and -5 genes downmodulated proinflammatory cytokine production from DCs, thus impairing cytokine signal 3 during DC–T cell cognate interactions. Consequently, T cells activated by Ag-experienced DCs secreted low levels of IFN-γ and IL-17 but maintained high IL-10 secretion, thus inducing suppressor responses. Further characterization revealed that day-1 and -5 genes increased TLR2-induced expression of suppressors of cytokine signaling 1 from DCs and downmodulated IL-12 expression. In addition, day-1 and -5 genes prevented the generation of reactive oxygen species in DCs. In contrast, although day-5 genes increased TLR2-mediated suppressors of cytokine signaling 1 expression in macrophages, day-1 genes downmodulated the expression of inducible NO synthase 2. Similar downregulation of immune responses was observed upon exogenous stimulation with day-1 or -5 Ags. Finally, day-1 and -5 genes promoted enhanced survival of M. tuberculosis inside DCs and macrophages. These results indicate that M. tuberculosis genes, expressed inside infected macrophages as a function of time, collectively suppress protective immune responses by using multiple and complementary mechanisms. The Journal of Immunology, 2010, 184: 5444–5455.

The global burden of mortality and morbidity due to tuberculosis caused by the intracellular pathogen Mycobacterium tuberculosis shows a steady increase, with the emergence of antibiotic resistance and coinfection with HIV (1–3). According to the World Health Organization, >9 million cases were recorded in 2007. This problem is further complicated by the variable efficacy of immunizations with Mycobacterium bovis bacillus Calmette-Guérin (BCG), the only available vaccine against tuberculosis (4, 5). This underscores the need to elucidate factors that regulate protective immune responses against this pathogen (6–8).

Dendritic cells (DCs) and macrophages constitute major cell types of the immune system and contribute toward generating immune responses following M. tuberculosis infection (9–11). Although DCs are the most potent APCs and are largely instrumental in priming T cells (12), macrophages serve as the long-term hosts for mycobacteria. A recent study showed that following aerosol infection, M. bovis BCG infects 50–60% of macrophages and 30–40% of DCs in the lungs (13). This indicates that, in addition to macrophages, DCs are primarily infected by mycobacteria at comparable frequencies. Thus, the nature of the immediate and long-term immune responses generated depends on the quality and quantum of responses initiated by M. tuberculosis and its Ags.

M. tuberculosis secretes a number of Ags in axenic cultures (14). Many of these Ags are potential candidates in various vaccine formulations and as diagnostic markers (15–20). However, the physiological roles for many of these Ags at sites of infection have not been completely deciphered. Over the last few years, we have been working toward elucidating the above aspect using M. tuberculosis culture filtrate protein of 10 kDa (CFP-10) as a model Ag (reviewed in Ref. 21). Briefly, we demonstrated that CFP-10 induces the differentiation and activation of DCs. However, CFP-10 DCs induce suppressor responses to M. tuberculosis (22–24). Further, by modulating the redox potential, these DCs also serve as depots for the multiplication and survival of mycobacteria (25). These results indicated that the expression of Ags, such as CFP-10, as the infection progresses in time could be a strategy used by M. tuberculosis toward immune evasion.

To test the above hypothesis, we enriched M. tuberculosis genes from infected macrophages at different times postinfection and characterized the immune responses mediated by these genes. These include modulation of DC and macrophage activation and function and their ability to alter T cell priming. The results validated our
hypothesis that \textit{M. tuberculosis} expresses different genes as the infection progresses in time. Using different but complementary mechanisms, these Ags inhibit protective immune responses, especially those mediated by TLR2. These involve increased expression of suppressors of cytokine signaling 1 (SOCS1), resulting in the inhibition of IL-12 secretion and decreased induction of oxidative and nitrosative bursts. These responses collectively help the pathogen to evade protective immunity.

Materials and Methods

Animals

All experiments were conducted following approval from the institutional animal ethics committee. Female BALB/c mice, 4–6 wk of age and kept in a pathogen-free environment, were used.

Materials

Fluorescence-tagged Abs against mouse or human CD80, CD86, CD54, H-2D\textsuperscript{b}, I-\textalpha, HLA-ABC, HLA-DQDR, IFN-\gammaR, IL-12\textbetaR, and IL-10\textbetaR were from BD Biosciences (San Jose, CA). Recombinant mouse GM-CSF was from R&D Systems (Minneapolis, MN), Diphtheria toxin from Bioreagents (Eugene, OR), ELISA kits were from eBioscience (San Diego, CA) or BD Biosciences. Abs to various molecules and Luminol kits for chemiluminescence detection were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TLR2 ligand Pam\textsubscript{3}Csk4 was purchased from Invivogen (San Diego, CA). Kits for the isolation of \textit{M. tuberculosis} RNA were from Qiagen (Valencia, CA). MicroBEnrich kit and poly(A) tailing kit were procured from Ambion (Austin, TX). Amplification of cDNA from poly(A)\textsuperscript{+} tailing M. tuberculosis RNA was carried out using the CLONTECH SMART kit.

Infection of TPH-1 cells and enrichment of \textit{M. tuberculosis} genes

Human TPH-1 monocyte cells were stimulated with 50 ng/ml PMA for 16 h to differentiate them into macrophages, followed by extensive washes. The cells were then infected with 10 multiplicity of infection (MOI) \textit{M. tuberculosis} H37Rv for 24 h and 5 d (120 h). At the end of the incubation period, macrophages were lysed with a nonionic detergent, and bacteria were pelleted. To avoid contamination, the bacterial pellet was treated with DNase and RNase to remove all mammalian (macrophage) RNA and DNA. This was followed by bacterial lysis and isolation of \textit{M. tuberculosis} total RNA. From this preparation, \textit{M. tuberculosis} RNA was further enriched using the MicroBEnrich kit that selectively depletes all mammalian RNA. At each step, we confirmed the presence of \textit{M. tuberculosis} RNA by PCR-based amplification of housekeeping gene \textit{Rv2244} or \textit{Rv3601c} and the absence of macrophage RNA (\beta-actin) (data not shown). The RNA preparation was then poly(A)\textsuperscript{+} tailing and amplified using the CLONTECH SMART kit. The amplified products were ligated into pGEMT Easy cloning vector (Promega, Madison, WI), followed by transformation of competent Escherichia coli cells. Colonies were partially sequenced to identify the amplified genes. Following this, fresh primers were designed to amplify genes from \textit{M. tuberculosis} genomic DNA, and amplicons were cloned in pFLAG-CMV-6a (Sigma-Aldrich, St. Louis, MO).

Infection of mice and enrichment of \textit{M. tuberculosis} Ags

Groups of mice (\textit{n} = 5/group) were infected with 4 \times 10\textsuperscript{6} \textit{M. tuberculosis} mouse i.v. via the tail vein for 3 or 15 d. Total RNA from the lungs was enriched and subjected to RT-PCR for the detection of day-1 and -5 Ags at various times postinfection.

Generation of DCs

DCs were differentiated with GM-CSF, as described earlier (22–25). Briefly, bone marrow from the tibias and femurs of BALB/c mice were flushed out, and lymphocytes and I-\textalpha\textsuperscript{+} cells were depleted following MACS. Cells were cultured in RPMI 1640 medium containing 10% FCS, 0.05 M 2-ME, 1 mM sodium pyruvate plus 15 ng/ml GM-CSF for 3 d. We showed that this method results in a homogenous population that is 99% DCs, with negligible contaminating monocytes or macrophages (25).

Processing of cells

\textit{M. tuberculosis} H37Rv was grown in Middlebrook 7H9 liquid medium supplemented with albumin/dextrose/catalase at a final concentration of 5, 2, and 0.003 g/l, respectively, along with 0.05% Tween 80. Aliquots were frozen at \textasciitilde 85\degree C, and viable bacteria were enumerated by plating serial dilutions on 7H10 agar. DCs or PMN-stimulated (50 ng/ml) human THP-1 monocyte/macrophage cells were transfected with 10 ng pFLAG-CMV-6a (vector control) or pFLAG-CMV-6a expressing individual genes by electroporation using Gene Pulser II (Bio-Rad, Hercules, CA) at 0.25 kV, 960 \mu F for 40 ms and incubated for 36 h. Following transfection, cells were infected with 1 MOI \textit{M. tuberculosis} for 48 h. Flow cytometry was carried out using FACS-Calibur (BD Biosciences). The data were plotted using CellQuest Pro software. For some experiments, DCs were cocultured with T cells for 48 h, and supernatants were monitored for various cytokines. For some experiments, gene-transfected DCs or THP-1 cells were stimulated with the TLR2 ligand Pam\textsubscript{3}Csk4 for various times. For some experiments, mouse peritoneal macrophages were transfected with day-1 or -5 Ags and stimulated for various times with Pam\textsubscript{3}Csk4 to monitor SOCS1 and cytokine profiles or activated with LPS to detect NO. NO was estimated in culture supernatants using the Greiss reagent. Alternatively, for some experiments, some Ags were expressed in \textit{E. coli} as recombinant proteins and modulations in the activation of surface markers on DCs and macrophages, cytokine profiles in DCs, and NO levels in macrophages were monitored.

Recombinant expression of proteins in \textit{E. coli}

\textit{Rv1483}, \textit{Rv3416}, and \textit{Rv0353} were cloned in pQE31 (Qiagen) vector, whereas \textit{Rv2463} was cloned in pET28b (Novagen, Madison, WI) and expressed as His-tagged recombinant proteins in \textit{E. coli} following standard procedures. The expression of \textit{Rv1483} was observed in the soluble fraction, whereas the expression of the other three proteins was observed as inclusion bodies. Proteins expressed as inclusion bodies were purified by batch method with Nickel affinity columns. Further, a parallel set of samples was run with buffers containing urea, as per the manufacturer’s instructions (Qiagen). Excess urea was removed by conventional-step dialysis, with reducing concentrations of urea in 10 mM NaH\textsubscript{2}PO\textsubscript{4} buffer (pH 8).

T cell enrichment and processing

Mice were infected i.v. with 4 \times 10\textsuperscript{6} \textit{M. tuberculosis} per mouse for 2 wk. Splenic T cells were enriched, as described earlier, by MACS (22–24). Briefly, following RBC lysis of spleen homogenates, adherent cells were removed by two rounds of panning over plastic plates. Following this, cells were incubated with anti-CD11c, anti-CD11b, anti-I-\textalpha, and anti-B220 microbeads to remove contaminating DCs, macrophages, MHC-II\textsuperscript{+} cells, and B lymphocytes, respectively. The purity of the enriched T cells was 98%, as ascertained by surface staining with CD90. The percentage of I-\textalpha\textsuperscript{+} cells was <0.5%. T cells were cocultured with gene-transfected and \textit{M. tuberculosis}-infected DCs for 48 h, and cytokines were measured in culture supernatants.

Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) levels were measured by flow cytometry, as described previously, using the redox-sensitive dye DCFH-DA (25). The nonfluorescent DCFH-DA readily diffuses into the cells where it is hydrolyzed to the polar derivative nonfluorescent dichlorofluorescin, which is oxidized in the presence of \textit{H}_{2}O\textsubscript{2} to the highly fluorescent dichlorofluorescin. Thirty minutes prior to the end of each incubation period, \textasciitilde 1 \times 10\textsuperscript{6} cells/ml were incubated with 10 \mu M DCFH-DA in the dark. Cells were thoroughly and quickly washed with pulse spin and immediately acquired for analyses in FACS-Calibur (BD Biosciences). The data were plotted and analyzed using CellQuest software.

Western blotting for signaling molecules

At the end of incubation, cells were chilled on ice, washed once with ice-cold PBS, and lysed in buffer containing 10 mM HEPES (pH 7.9), 10 mM Tris. Then, the cytoplasmic membrane was solubilized with buffer containing 1% Triton X-100, 0.5% Nonidet P-40, and 2 \mu g/ml each aprotinin, leupeptin, and pepstatin. The suspension was centrifuged at 13,000 rpm for 2 min at 4\degree C. The supernatant was designated as the cytoplasmic extract. Twenty micrograms of cytoplasmic extract were resolved on 10% SDS-PAGE and subsequently transferred onto nitrocellulose membrane (Hybond C pure, Amersham Biosciences, Arlington Heights, IL). The blots were then probed with Abs to various molecules, followed by horseradish peroxidase-conjugated secondary Abs. Further, a parallel set of samples was run separately on SDS-PAGE and probed for GAPDH as loading control. The blots were later developed by chemiluminescence using the Luminol reagent.
Statistics

The Student t test was carried out for all experiments; $p < 0.05$ for different groups was considered significant.

Results

**M. tuberculosis expresses different genes inside macrophages as a function of infection time**

Our earlier work showed that *M. tuberculosis* CFP-10 activates DCs that induce suppressor responses (24). Likewise, DCs activated with total culture filtrate proteins enriched from axenically growing *M. tuberculosis* reproduced the results obtained with CFP-10 (23). Based on these results, we hypothesized that *M. tuberculosis* would express similar genes inside infected macrophages as a function of time during the course of infection that would play a role in the suppression of protective immune responses. Therefore, to test this hypothesis, we infected PMA-stimulated THP-1 human macrophages for different times and enriched *M. tuberculosis* genes. Using a set of procedures we could enrich 10 genes: 5 from 24 h and 5 from 120 h postinfection. Although at this stage one cannot be sure of the antigenic nature of these genes, we prefer to address them as Ags because, as presented below, they influenced the quality of T cell responses and modulated cytokine profiles from APCs. Therefore, genes enriched at day 1 (24 h) postinfection are hereafter collectively called day-1 Ags, and genes enriched at day 5 (120 h) postinfection are collectively called day-5 Ags. These two time points were chosen based on the observation that, in our culture conditions, maximum THP-1 cells remain infected and contain viable bacilli at these time points (data not shown). Beyond day 5, most macrophages were nonviable and contained very little culturable bacilli. Table I lists the 10 Ags enriched from infected macrophages and their putative functions. Interestingly, all day-1 Ags matched with the Rubin list of genes required for survival inside macrophages (26). This indicated that *M. tuberculosis* expresses these Ags early following an infection. In contrast, none of the Ags enriched at day 5 of infection belonged to the Rubin list. However, all of the day-5 Ags were previously shown to play a major role in promoting latent infection under in vitro culture conditions (27–29). Significant among these were Rv3416 (WhiB3), Rv3911 (SigM), and Rv2391 (SirA).

These results indicated that *M. tuberculosis* expressed different Ags at different times postinfection that play specific roles. Although the day-1 Ags ensure survival of *M. tuberculosis* inside macrophages, day-5 Ags could create conditions to induce persistence/latency. However, a PubMed search indicated that the quality and quantity of immune responses mediated by any of the Ags have not been characterized.

**Day-1 and -5 Ags display differential expression in *M. tuberculosis*-infected mice**

We next investigated whether the differential expression of genes inside *M. tuberculosis*-infected macrophages also follows similar kinetics of expression in vivo in *M. tuberculosis*-infected mice. To that end, mice were infected with *M. tuberculosis* for 3 and 15 d, and the expression of the genes was monitored in the lungs. As shown in Fig. 1, the expression of three of five day-1 Ags was detected within 3 d of infection, and their expression was below detectable levels at day 15 postinfection. We could not detect the expression of the remaining two day-1 Ags at day 3, possibly because of different kinetics of expression. In contrast, the expression of all day-5 Ags was detectable only at day 15 of infection, whereas it was below detectable levels at day 3 of infection. These results indicate that the expression kinetics of day-1 and -5 Ags were essentially similar in vitro and in vivo. Furthermore, this also indicated that *M. tuberculosis* regulates the secretion of different Ags at different times postinfection in vitro (inside macrophages) and in vivo (in mice).

**Day-1 and -5 Ags differentially activate DCs and macrophages**

Next, to characterize the roles played by these Ags in modulating *M. tuberculosis*-induced immune responses, we expressed these Ags inside DCs and macrophages and followed up with *M. tuberculosis* infection. Modulations in the surface densities of key activation markers, such as costimulatory molecules, MHC molecules, and cytokine receptors, were investigated. The results are represented in Tables II and III. As shown in Table II, all day-1 Ags enhanced *M. tuberculosis*-mediated activation of DCs. This was evident with significant increases in the surface levels of costimulatory molecules, MHC class I and II, and receptors for IFN-γ and IL-12 and a weak increase in IL-10R levels. Among the day-1 Ags, Rv2463 was the most potent in enhancing *M. tuberculosis*-induced activation of DCs. In contrast, day-5 Ags had no effect or downmodulated the expression of *M. tuberculosis*-induced surface markers. In particular, the downmodulation was more severe on MHC class I and II and CD54 molecules: three of five Ags downmodulated their expression levels. These results indicated that Ags expressed at different times postinfection differentially activate DCs.

We next carried out similar experiments with macrophages. As shown in Table III, and in contrast to the effects on DCs, day-1 and -5 Ags severely downmodulated MHC class I and II expression on macrophages. This indicated that infected macrophages would not be responsive to Ag-specific T cells, thereby leading to the downregulation of effector T cell responses. The expression of CD40 alone was upregulated by most Ags, whereas the expression of CD80 and CD86 was minimally modulated, indicating that costimulatory molecules were mostly unaffected by these Ags. With respect to cytokine receptors, all day-1 Ags upregulated the expression of all three receptors, whereas all day-5 Ags downmodulated the expression of all receptors. Further, the above results indicated that day-1 Ags promoted cytokine responses early during the infection; however, at later stages, with the expression of day-5 Ags these cytokine responses would be suppressed. These results indicated that Ags expressed by *M. tuberculosis* at different times postinfection differentially modulate the activation of DCs and macrophages.

**Day-1 and -5 Ags downmodulate proinflammatory cytokine responses from *M. tuberculosis*-infected DCs**

We next investigated the cytokine patterns from DCs mediated by the Ags. As shown in Fig. 2, day-1 and -5 Ags significantly downmodulated the expression of proinflammatory cytokines IL-12p40, -6, and -17. In fact, Ags such as Rv2463, Rv0082, Rv0981, and Rv3416 completely abrogated the ability of DCs to secrete all three cytokines in response to *M. tuberculosis* infection. In contrast, Ags such as Rv3723, Rv1483, Rv2391, Rv3911, and Rv0353 differentially downmodulated cytokine secretion. These results indicated that *M. tuberculosis* expresses Ags with different functional effects at different stages of infection. This also indicated that each Ag would downmodulate proinflammatory T cell responses using different mechanisms. For example, although day-1 Ags upregulated costimulatory molecules and MHC molecules, thereby increasing signal 1 and 2 for productive T cell responses, by abrogating the secretion of IL-12, a key cytokine that drives proinflammatory responses via signal 3, they ensure the generation of suppressor responses to *M. tuberculosis* from these DCs. In
contrast, by downmodulating DC activation and cytokine secretion, day-5 Ags paralyze T cell responses that favor the pathogen at later times postinfection. Additionally, because day-1 Ags downmodulated surface levels of MHC class I and II on macrophages, this further ensures that Ag-activated T cells would be ineffective on infected macrophages, thereby contributing to defective clearance of the pathogen.

Day-1 and -5 Ags downmodulate proinflammatory T cell responses to M. tuberculosis

Keeping the above results in mind, we next investigated the quality of T cell responses from Ag-stimulated DCs. To that end, Ag-transfected DCs were infected with *M. tuberculosis* and cocultured with T cells enriched from *M. tuberculosis*-infected mice. As shown in Fig. 3, day-1 and -5 Ags induced suppressor responses with very low to undetectable expression of IFN-γ and IL-17. In contrast, IL-10 levels were expressed at very high levels and largely were not modulated. We also observed low levels of IL-12p40 during cognate DC–T cell interactions. These results clearly indicate that by modulating the activity of DCs these Ags ensured that subsequently elicited proinflammatory T cell responses were also abrogated. Table IV summarizes the extent and profile of the downmodulation of Ag-influenced cytokine secretions from *M. tuberculosis*-infected DCs and the corresponding effect on T cell responses from these DCs. As can be seen from Table IV, for most Ags, the extent of downmodulation of IL-12, for instance, correlated well with the extent of downmodulation of IFN-γ, such that a 100% downmodulation of IL-12, -6, or -17 in DCs effectively resulted in a 100% downmodulation of IFN-γ in T cells that interacted with the corresponding Ag-stimulated DCs.

**Rv2463 and Rv3416 induce higher expression of SOCS1 from TLR2**

We recently showed that stimulation of mouse and human DC-specific intercellular adhesion molecule-3-grabbing non-integrin (SIGN) homologs induces a higher expression of SOCS1, whereas the stimulation of TLR2 results in lower SOCS1 expression (30). High SOCS1 expression results in lower expression of IL-12 in the

---

### Table I. *M. tuberculosis* Ags enriched from THP-1 macrophages infected for 24 h (day 1) or 120 h (day 5)

<table>
<thead>
<tr>
<th>Ag</th>
<th>Alternate Name</th>
<th>Putative Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv1238</td>
<td>sugC</td>
<td>Probable sugar-transport ATP-binding protein ABC transporter</td>
</tr>
<tr>
<td>Rv2463</td>
<td>lipP</td>
<td>Probable esterase/lipase</td>
</tr>
<tr>
<td>Rv3723</td>
<td>NA</td>
<td>Probable conserved transmembrane protein</td>
</tr>
<tr>
<td>Rv0082</td>
<td>NA</td>
<td>Probable oxido-reductase</td>
</tr>
<tr>
<td>Rv1483</td>
<td>fabG1</td>
<td>3-Ketoacyl-acyl carrier protein reductase (mycolic acid biosynthesis protein)</td>
</tr>
<tr>
<td>Rv2391</td>
<td>sirA</td>
<td>Probable ferredoxin-dependent sulphite reductase</td>
</tr>
<tr>
<td>Rv3911</td>
<td>sigM</td>
<td>Possible alternate RNA polymerase σ factor</td>
</tr>
<tr>
<td>Rv0981</td>
<td>mprA</td>
<td>Mycobacterial persistence regulator (two-component response transcriptional regulatory protein)</td>
</tr>
<tr>
<td>Rv3416</td>
<td>whiB3</td>
<td>Transcriptional regulatory protein WhiB-like</td>
</tr>
<tr>
<td>Rv0353</td>
<td>hspR</td>
<td>Probable heat shock protein transcriptional repressor</td>
</tr>
</tbody>
</table>

PMA-stimulated THP-1 macrophages were infected with 10 MOI *M. tuberculosis* H37Rv for 24 h (day 1) and 120 h (day 5). Ags expressed at these time points were enriched as detailed in Materials and Methods. Ags in bold type represent those enriched after 24 h of infection, whereas Ags in italic type represent those enriched after 120 h of infection.

---

**FIGURE 1.** Day-1 and -5 Ags show different kinetics of expression in vivo. Mice were infected i.v. with $4 \times 10^6$ *M. tuberculosis*/mouse for 3 or 15 d. Total RNA was enriched from lungs. An aliquot of the RNA was used in RT-PCR to screen for the expression of day 1 (A) or day 5 (B) Ags at both times of infection. Arrows indicate specific bands of the right size. Data from one of two experiments are shown.
context of *M. tuberculosis* infection. Inhibiting SOCS1 by RNA interference enhanced IL-12 expression and reduced intracellular bacterial loads. Therefore, we investigated whether day-1 and -5 Ags also influenced TLR2-mediated SOCS1 expression. Because Rv2463 (a day-1 Ag) and Rv3416 (a day-5 Ag) were the most potent in downmodulating DC activation (Table IV) and were also detected in vivo in infected mice, we chose to carry out subsequent experiments with these two Ags.

To investigate modulations in SOCS1 expression from TLR2, DCs transfected with Rv2463 or Rv3416 were stimulated with Pam3Csk4, a characterized TLR2 ligand, and SOCS1 levels were monitored. As shown in Fig. 4A, TLR2 induced SOCS1 expression at 24 h in mock-transfected DCs. However, in DCs transfected with Rv2463, TLR2 induced a strong expression of SOCS1 within 12 h, indicating advanced kinetics along with increased expression. These results indicate that day-1 and -5 Ags quantitatively and kinetically increased SOCS1 expression in DCs. These results also suggest that the downmodulatory effects of SOCS1 could be initiated early in the course of infection.

Day-1 and -5 Ag(s) downmodulate TLR2-mediated IL-12 expression from DCs

We next investigated whether higher expression of SOCS1 would translate into subversion of IL-12 production, thereby influencing the induction of suppressor responses. It has been argued that ligation of TLR2 by *M. tuberculosis* activates DCs, leading to greater expression of IL-12 and protective immune responses (31). Therefore, we stimulated Rv2463- or Rv3416-transfected DCs with Pam3Csk4. As shown in Fig. 4B and 4C, both Ags downmodulated TLR2-induced IL-12 and -6 production. Rv2463, a day-1 Ag, was more potent than Rv3416, a day-5 Ag, in downmodulating IL-12, indicating that subversion of signal 3 via expression of SOCS1 early during immune priming could result in the inhibition of subsequent Th1 responses from these DCs.

**Table II. Modulations in surface densities of indicated markers upon *M. tuberculosis* H37Rv infection in mouse DCs transfected with the indicated Ag**

<table>
<thead>
<tr>
<th>Ag</th>
<th>CD80</th>
<th>CD86</th>
<th>CD54</th>
<th>CD40</th>
<th>HLA-ABC</th>
<th>HLA-DQDR</th>
<th>IFN-γR</th>
<th>IL-12R</th>
<th>IL-10R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv1238</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Rv2463</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Rv3723</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Rv0082</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Rv1483</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Rv1431</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Rv1353</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Mouse bone marrow-differentiated DCs were transfected with day-1 (bold type) or day-5 (italic type) Ags, followed by *M. tuberculosis* H37Rv infection with 1 MOI. Values are presented as increase (+) or decrease (−) in the levels of indicated markers in *M. tuberculosis*-infected Ag-transfected DCs over *M. tuberculosis*-infected empty vector-transfected DCs. The number of symbols (+ or −) indicates the extent of the increase or decrease, respectively.

+/−: No significant change.

**Table III. Modulations in surface densities of indicated markers upon *M. tuberculosis* H37Rv infection in human macrophages transfected with the indicated Ag**

<table>
<thead>
<tr>
<th>Ag</th>
<th>CD80</th>
<th>CD86</th>
<th>CD54</th>
<th>CD40</th>
<th>HLA-ABC</th>
<th>HLA-DQDR</th>
<th>IFN-γR</th>
<th>IL-12R</th>
<th>IL-10R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv1238</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Rv2463</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Rv3723</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Rv0082</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Rv1483</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Rv1431</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Rv1353</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

PMA-stimulated THP-1 macrophages were transfected with day-1 (bold type) or day-5 (italic type) Ags, followed by infection with 1 MOI *M. tuberculosis* H37Rv. Values are presented as increase (+) or decrease (−) in the levels of indicated markers in *M. tuberculosis*-infected Ag-transfected cells over *M. tuberculosis*-infected empty vector-transfected cells. The number of symbols (+ or −) indicates the extent of the increase or decrease, respectively.

+/−: No significant change.
Day-1 and -5 Ag(s) downmodulate key macrophage functions

In parallel, we similarly investigated the ability of these two Ags to modulate critical functions of macrophages. As with DCs, we investigated modulations in TLR2-mediated SOCS1 expression. As shown in Fig. 6A, TLR2 stimulation alone did not result in any significant increase in SOCS1 levels. However, the day-5 Ag Rv3416 increased SOCS1 expression in macrophages, whereas the day 1 Ag Rv2463 had minimal effect. This indicates that in macrophages, TLR2 responses are downmodulated at later times post-infection by increased SOCS1 expression, whereas early responses may not contribute toward SOCS1 regulation. We were unable to detect IL-12 expression from TLR2-stimulated THP-1 cells; therefore, modulations of this cytokine could not be investigated. However, we carried out similar experiments with mouse peritoneal macrophages. As shown in Fig. 6B and 6C, Rv2463 and Rv3416 significantly downmodulated TLR2-induced IL-12 and -6 expression in mouse macrophages. These results indicate that similar to DCs, these Ags downmodulated proinflammatory cytokine secretion from macrophages.

We next investigated the modulation of the reactive nitrogen pathway by monitoring the levels of inducible NO synthase (iNOS) 2, a key enzyme involved in the generation of reactive nitrogen species, immediately following infection (8). Interestingly, in human THP-1 macrophages, the day-1 Ag Rv2463 downmodulated iNOS2 expression, whereas the day-5 Ag Rv3416 had no significant effect (Fig. 7A). We also monitored the ability of these two Ags to modulate NO production. As shown in Fig. 7B, in human THP-1 macrophages, Rv2463 downmodulated LPS-induced nitrite production, whereas Rv3416 had minimal effects. In mouse peritoneal macrophages, both Ags downmodulated iNOS2 expression, although the downmodulation was better with the day-5 Ag Rv3416 (Fig. 7C). Similarly, Rv2463 downmodulated NO levels in peritoneal macrophages, whereas Rv3416 had minimal effects (Fig. 7D).
These results indicate that day-1 and -5 Ags exhibit complementary roles toward the evasion of protective immune responses from macrophages. Because macrophages rely more on the reactive nitrosative pathways for mounting protective responses immediately following infection (8), subversion of these reactive species via downmodulation of iNOS by day-1 Ags would be strategically useful for M. tuberculosis for survival inside macrophages. This was further confirmed when Rv2463- and Rv3416-mediated modulations in ROS levels were investigated. Similar to DCs, day-1 and -5 Ags inhibited the generation of ROS from macrophages, although the extent of ROS generation by infected macrophages was lower compared with infected DCs (data not shown). This once again indicated that macrophages are poor generators of ROS, compared with DCs, and that they rely more on the reactive nitrogen species for pathogen clearance. Additionally, although with slight variation in kinetics, the downmodulation of iNOS and NO levels was obtained with human and mouse macrophages.

**Exogenous addition of day-1 and -5 Ags downmodulates key DC and macrophage functions**

To extend the above observations, we recombinantly expressed two day-1 Ags (Rv2463 and Rv1483) and two day-5 Ags (Rv3416 and Rv0353) in E. coli and exogenously stimulated DCs and macrophages. Modulations in the levels of surface markers, cytokines, SOCS1, iNOS2 levels, and T cell responses in the context of M. tuberculosis infection were monitored. Fig. 8A shows the purity of the rAgs on a silver-stained SDS-PAGE. To start, we monitored the modulations in surface markers required for a productive T cell response. As shown in Fig. 8B, Rv2463 and Rv0353 downmodulated the M. tuberculosis–induced expression of most markers in DCs and macrophages. Rv1483 and Rv3416 marginally increased the levels of some markers or had no significant effect.

We next investigated the ability of the two Ags to modulate SOCS1 levels in DCs and macrophages. As shown in Fig. 8C, Rv2463 increased TLR2-mediated SOCS1 expression in DCs, whereas the day-5 Ag Rv3416 had no significant effect. These results are in agreement with the data obtained in Fig. 4, when these Ags were expressed inside DCs, indicating a similar modulation of DC functions, irrespective of intracellular versus extracellular stimulation. However, Rv1483 and Rv0353 had no appreciable effect on TLR2-induced SOCS1 in DCs; in fact, they downmodulated its levels at later times poststimulation. However, an interesting pattern was observed when modulations in iNOS2 levels in human macrophages were monitored (Fig. 8D). Although Rv2463 and Rv3416 downmodulated iNOS2 expression in macrophages, the effects with Rv3416 were more potent. This was complementary to the modulations in SOCS1 profiles observed in DCs by the two Ags; Rv2463 increased SOCS1 levels, whereas Rv3416 had no appreciable effect. Similarly, although Rv1483 and Rv0353 did not increase TLR2-mediated SOCS1 expression, both Ags significantly downmodulated TLR2-induced iNOS2 expression in macrophages.

**FIGURE 4.** Day-1 and -5 Ags suppress IL-12 from TLR2 via increased SOCS1 expression in DCs. DCs were transfected with indicated Ags and stimulated with 1 μg/ml PamCsk4 for the indicated times. Cytoplasmic extracts were analyzed for SOCS1 levels (A), whereas the culture supernatants were evaluated for IL-12p40 (B) and IL-6 (C). MOCK represents the transfection of DCs with empty vector. One of three independent experiments is shown in A, whereas for B and C, data are the mean ± SD of three independent experiments.
We next investigated the cytokine production from Ag-activated DCs in the context of *M. tuberculosis* infection, as well as in the modulation in T cell responses. As shown in Fig. 9A and 9B, all four Ags downregulated *M. tuberculosis*-induced secretion of IL-12p40 and -6 from DCs. All four Ags also downmodulated proinflammatory T cell responses from *M. tuberculosis*-infected DCs by downregulating the levels of IFN-γ, with Rv2463 and Rv0353 being the most potent (Fig. 9C, 9D). Together, the results in Figs. 8 and 9 exemplify our argument that these Ags, whether expressed intracellularly or added exogenously, complement the downmodulating functions of each other, resulting in a net downmodulation of protective responses mounted by different cells of the immune system against *M. tuberculosis*.

Rv2463 and Rv3416 promote better survival of *M. tuberculosis* inside DCs and macrophages

To test proof of principle of the above findings, we tested the ability of Rv2463 and Rv3416 to modulate the survival of *M. tuberculosis* inside DCs and macrophages. To that end, Ag-transfected cells were infected with *M. tuberculosis* H37Rv, and survival of the pathogen was monitored. Day-1 and -5 Ags differentially modulated the survival of *M. tuberculosis* inside DCs (Fig. 10A) and macrophages (Fig. 10B). Although Rv2463 (a day-1 Ag) promoted increased survival of *M. tuberculosis* inside DCs and macrophages, Rv3416 (a day-5 Ag) promoted *M. tuberculosis* survival only inside macrophages, with minimal effects on DCs. These results further validate our hypothesis that Ags expressed by *M. tuberculosis* as...
a function of time, work toward the suppression of protective immune responses, leading to increased survival of the pathogen in infected cells. Further, because day-1 Ags are essential for *M. tuberculosis* survival (26), the results obtained with Rv2463 are in agreement with their role in mediating enhanced survival of *M. tuberculosis* inside macrophages and DCs. Because Rv3416, a day-5 Ag, is known to play a role in mediating latency (27–29), albeit under in vitro conditions, its effects on the promotion of *M. tuberculosis* survival inside macrophages and not DCs also fit with its putative role, because macrophages serve as the long-term hosts for *M. tuberculosis* during persistent/latent infection.

**Discussion**

In an effort to characterize the events that mediate priming and the long-term survival of *M. tuberculosis* by modulating the activation and consequent functioning of cells of the immune system, over the years we have been elucidating the interactions of *M. tuberculosis* Ags with DCs and their outcome on host-mediated immune responses. Many *M. tuberculosis* Ags are promising vaccine and diagnostics candidates (14–20). However, despite the large volume of data available on these Ags, their physiological role(s) at sites of infection are not fully understood. Many *M. tuberculosis* protein and nonprotein Ags have been demonstrated to play roles in immune evasion. For example, mannosylated lipoarabinomannan (manLAM) expressed on the surface and later secreted by virulent *Mycobacterium* species, such as *M. tuberculosis* and *M. avium* (32, 33), was demonstrated to bind DC-SIGN homologs, such as DC-SIGNR1 (CD209b) and lymph node-SIGN, on mouse and human cells (32). Mice lacking DC-SIGNR1 induce stronger T cell responses to *M. tuberculosis* (33), indicating an inhibitory role for manLAM in T cell priming. We also recently demonstrated that one of the potent mechanisms of the inhibitory action of manLAM via DC-SIGN homologs is its ability to inhibit IL-12 secretion from DCs via increased expression and recruitment of SOCS1 to DC-SIGN homologs (30).

In addition, many *M. tuberculosis* Ags modulate TLR signaling. These include the 19-kDa lipoprotein and peptidoglycan that act on TLR2 (34). In particular, the 19-kDa Ag has been well characterized to compromise many antibacterial functions of macrophages in a TLR2-dependent manner (35). Over the years, our own work demonstrated the role of *M. tuberculosis* Ags, such as CFP-10, in modulating DC function. CFP-10 induces the differentiation of DCs (22). However, CFP-10 DCs induce suppressor responses that

**FIGURE 7.** Day-1 and -5 Ags differentially modulate iNOS2 and NO production in macrophages. A, PMA-stimulated THP-1 macrophages were transfected for 36 h with the indicated Ags. Transfected cells were stimulated with 1 µg/ml Pam3CSK4 for the indicated times. Cytoplasmic extracts were probed for iNOS2 and NO levels in mouse peritoneal macrophages that were processed as in A and B, respectively. A and C. One of two experiments. Bars in B and D represent mean ± SD of two independent experiments.

**FIGURE 8.** Exogenous addition of day-1 and -5 Ags suppresses key DC and macrophage functions. A, Silver-stained gel showing the purity of Ags recombinantly expressed in *E. coli*. B, DCs or THP-1 macrophages were stimulated with 15 µg/ml of indicated Ags for 36 h, followed by infection with *M. tuberculosis* H37Rv for 24 h. Cells were stained for the surface levels of indicated markers. Data are represented in a tabulated form: + or − represents an increase or decrease, respectively, in the levels of indicated markers in *M. tuberculosis*-infected Ag-stimulated DCs over *M. tuberculosis*-infected DCs. The number of symbols (+ or −) indicates the extent of the increase and decrease; +/− indicates no significant change. C, DCs were stimulated with 15 µg/ml of indicated Ags for 36 h, followed by stimulation with 1 µg/ml Pam3CSK4 for the indicated times; cytoplasmic extracts were Western blotted for SOCS1 expression. D, THP-1 macrophages were stimulated with 15 µg/ml of indicated Ags for 36 h, followed by stimulation with 1 µg/ml Pam3CSK4 for the indicated times; cytoplasmic extracts were Western blotted for iNOS2 expression.
include the inhibition of IFN-γ and the production of IL-10 from T cells (23, 24). In addition, CFP-10 DCs modulate ROS levels, leading to increased survival of mycobacteria within DCs (25). Conditioning DCs with appropriate cytokines and chemokines results in the mounting of protective responses and the clearance of an established M. tuberculosis infection in mice (36).

In light of the above reports, we argued that M. tuberculosis would express many more Ags, like CFP-10 and the 19-kDa Ag, as a function of time inside macrophages that would result in the continued suppression of macrophage and DC activation, creating a niche for the long-term survival of M. tuberculosis. To that end, we enriched Ags expressed inside infected macrophages as a function of time. Using a series of procedures we could enrich 10 Ags: 5 from day 1 (24 h) and 5 from day 5 (120 h) of infection. Results indicate that M. tuberculosis expresses different Ags at different times postinfection. Although Ags expressed immediately following infection are required for survival of the pathogen in infected cells, as revealed by their presence in the Rubin list, Ags expressed at later times postinfection contribute toward this downmodulation and, for the first time, present data that this modulation continues later in the infection with the expression of day-5 Ags. These results have important bearings on the extent of early and late T cell responses. For example, although day-1 Ags activate DCs, thereby indicating the possibility of a productive T cell response, the time DCs prime T cells, the infected macrophages would have expressed day-1 and, later, day-5 Ags that would result in the downmodulation of MHC class I and II molecules on the cell surface. Therefore, in effect, the T cells primed by DCs would be blind to the infected macrophage, and effector functions (e.g., CD4+ T cell-mediated activation or CD8+ T cell-mediated cytotoxicity) would be impaired. In addition, should a possible T cell recognition by infected macrophages be mediated, the low level of

FIGURE 9. Exogenous stimulation of DCs with Ags downmodulates proinflammatory responses. A and B, DCs were stimulated with 15 µg/ml of indicated Ags, followed by infection with 1 MOI M. tuberculosis H37Rv for 24 h. Culture supernatants were evaluated for the indicated cytokines. C and D, Ag-stimulated and M. tuberculosis-infected DCs were cocultured for 48 h with T cells enriched from M. tuberculosis-infected mice, and culture supernatants were evaluated for the indicated cytokines.

FIGURE 10. Rv2463 and Rv3416 promote survival of M. tuberculosis inside DCs and macrophages. DCs (A) and PMA-stimulated THP-1 macrophages (B) were transfected with Rv2463 or Rv3416 for 36 h, followed by infection with 1 MOI M. tuberculosis H37Rv for 48 h. Cell lysates were plated onto 7H11 agar plates, and CFU were monitored after 2–3 wk. MOCK represents transfection with empty vector. Data are the mean ± SD of three independent experiments.
IL-12, -6, and -17 secreted by Ag-activated DCs would ensure that these T cells mediate suppressor responses. That this was true was demonstrated in the next experiment, wherein the profile of cytokines secreted during a cognate DC–T cell interaction displayed a phenotype indicative of suppressor responses, with high levels of IL-10 and low levels of IL-17 and IFN-γ.

In the next set of experiments, we investigated the mechanisms used by these Ags to modulate immune responses from DCs and macrophages. To that end, we restricted our experiments initially to a single day-1 Ag and a single day-5 Ag, because essentially similar responses were observed when comparisons were made between all day-1 Ags and all day-5 Ags, but later we used rAgs to extend the study to four Ags. Further, these two Ags (Rv2463 and Rv3416) were the most potent in downmodulating the proinflammatory responses (Table IV). Using these Ags as models, we analyzed their ability to modulate key functions of DCs and macrophages that are indicative of protective responses (i.e., oxidative and nitrative bursts, SOCS1, and IL-12 induction). The results indicated that all of the above functions were downmodulated by one Ag or the other but with different kinetics. Although day-1 and -5 Ags subvert DC functions by similar mechanisms (involving SOCS1 and IL-12), thereby ensuring that the priming of T cell is constantly subverted as the infection progresses, their roles in modulating macrophage functions match with the kinetics of different protective responses mounted by the infected macrophage. This is evident from the results obtained for iNOS2 and IL-12; depending on the time of induction of the effector molecules, the Ags adjust and complement the functions of each other to thwart protective responses. We also demonstrated that irrespective of whether the Ags are expressed inside cells or are provided as an exogenous stimulation, they downmodulated most DC and macrophage functions like, proinflammatory cytokine secretion, downmodulation of Th1 responses, increased expression of TLR2 induced SOCS1 expression in DCs and downmodulation of iNOS2 levels in macrophages.

It has been argued that a balance between activation of TLR2 and DC-specific ICAM-3 grabbing nonintegrin or DC-SIGN during M. tuberculosis infection governs the generation of protective versus suppressor responses (38). TLR2-mediated activation results in greater IL-12 expression via increased activation of NF-κB, whereas stimulation of DC-SIGN blocks NF-κB activation, resulting in low IL-12 secretion. Based on this, it has been proposed that at initial stages of infection when the pathogen load is low, TLR2 triggering induces protective immunity and prevents the development of active tuberculosis disease. Following increased bacterial burden and the development of active disease, as a result of HIV infection or other factors (8), soluble manLAM secreted from infected macrophages at later times postinfection triggers DC-SIGN to induce suppressor responses that favor the pathogen. Therefore, in the above context, an increase in TLR2-mediated SOCS1 expression by day-1 and -5 Ags in DCs would inhibit Th1 cell priming and an increase in TLR2-mediated SOCS1 expression by day-5 Ags in macrophages at later times postinfection would inhibit TLR2 signals and possibly amplify signals by DC-SIGN. This once again emphasizes the complementary roles played by Ags expressed at different times postinfection toward immune suppression.

Importantly, M. tuberculosis was shown to interact differently with DCs compared with macrophages. For example, infection of DCs with M. tuberculosis induces their activation by upregulating costimulatory and MHC molecules (39, 40). This also results in secretion of IL-12 from infected DCs. In contrast, infection of macrophages with M. tuberculosis results in downregulation of MHC class I and II, IFN-γ responsiveness, and IL-12 production (8, 41–44). Our results also suggest that M. tuberculosis Ags expressed in infected cells at different times postinfection interact differently with DCs and macrophages, but the overall outcomes of these effects compromise the ability of the infected cell to mediate effector responses to the pathogen. Nevertheless, the use of either mechanism leads to a better survival of the pathogen in infected cells.

Together, these results exemplify the role of diverse Ags in regulating the immune responses by various cells of the immune system. They further indicate that M. tuberculosis strategically expresses different Ags at different times postinfection. These Ags differentially modulate key functions of DCs and macrophages as the infection progresses, resulting in the generation of suppressor responses.

Disclosures
The authors have no financial conflicts of interest.

References


