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

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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,1 Jhalak Singhal,1 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, thereby compromising their ability to interact with Ag-specific T cells. Day-1 and -5 genes downmodulated proinflammatory cytokine production from DCs, thus impairing 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 Mycobacterium 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 Mycobacterium 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 Mycobacterium 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

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hypothesis that *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^b-, I-A, HLA-ABC, HLA-DQDR, IFN-γR, IL-12Rβ, and IL-10R were from BD Biosciences (San Jose, CA). Recombinant mouse GM-CSF was from R&D Systems (Minneapolis, MN), Dicholorofluorescin diacetate (DCFH-DH) was obtained from Molecular Probes (Eugene, OR). ELISA kits were from eBioscience (San Diego, CA) or BD Biosciences. Abs to various signaling molecules and Luminol kits for chemiluminescence detection were from eBioscience (San Diego, CA) or BD Biosciences. Abs to various signaling molecules and Luminol kits for chemiluminescence detection were from eBioscience (San Diego, CA) or BD Biosciences.

**Generation of DCs**

DCs were differentiated with GM-CSF, as described earlier (22–25). Briefly, following RBC lysis of spleen homogenates, adherent cells were removed by two rounds of panning over plastic plates. Following this, cells were washed twice with cold PBS, and lysed in buffer containing 10 mM HEPES (pH 7.9), 10 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**

*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 −85°C, and viable bacteria were enumerated by plating serial dilutions on 7H10 agar. DCs or PMA-stimulated (50 ng/ml) human THP-1 monocyte/macrophage cells were transfected with 10 µg 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 µF for 40 ms and incubated for 36 h. Following transfection, cells were infected with 1 MOI *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 co-cultured 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 Pam3CSK4 for various times. For some experiments, mouse peritoneal macrophages were transfected with day-1 or -5 Ags and stimulated for various times with Pam3CSK4 to monitor SOCS1 and cytokine profiles or stimulated with LPS to detect NO. NO was estimated in culture supernatants using the Greiss reagent. Alternatively, for some experiments, some Ags were expressed in *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.

**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 dichloro-DCA (25). The nonfluorescent DCFH-DA readily diffuses into the cells where it is hydrolyzed to the polar derivative nonfluorescent dichloro-DCA. The concentration of ROS was measured as the ratio of cellular fluorescence in the presence and absence of an antioxidant, typically N-acetyl-L-cysteine (NAC).

**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 EDTA, 0.1 M EGTA, 0.5% Nonidet P-40, and 2 µg/ml each 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 were resolved on 10% SDS-PAGE and transferred onto nitrocellulose membrane (Hybond C pure, Amersham Biosciences, Arlington Heights, IL). The blots were then probed with Abs to various molecules, followed by a labeled secondary Ab. Further, a parallel set of samples was run with buffers containing area, as per the manufacturer’s instructions (Qiagen). Excess area was removed by conventional-step dialysis, with reducing concentrations of urea in 10 mM NaH2PO4 buffer (pH 8).
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 (SirM), 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 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...
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 advancement in kinetics of expression. Likewise, in DCs transfected with Rv3416, a higher expression of SOCS1 was evident at 12 and 24 h of TLR2 stimulation, indicating advanced kinetics along with prolonged 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 oxidative burst from infected DCs**

We demonstrated earlier that CFP-10 impairs the generation of an oxidative burst from mycobacteria-infected DCs (25). This consequently leads to increased survival and multiplication of mycobacteria. Therefore, we investigated whether day-1 and -5 Ags would mimic the action of CFP-10 in preventing the generation of ROS. To that end, we stimulated Rv2463- or Rv3416-transfected DCs with *M. bovis* BCG and monitored the ROS levels by flow cytometry. As shown in Fig. 5, stimulation of MOCK-transfected DCs with BCG led to an increase in ROS levels with time (Fig. 5A, 5D, 5G). In contrast, BCG stimulation of Rv2463-transfected (Fig. 5B, 5E, 5H) or Rv3416-transfected (Fig. 5C, 5F, 5I) DCs prevented ROS generation by *M. bovis* BCG. These results indicate that day-1 and -5 Ags prevent the generation of oxidative burst in DCs that would lead to better survival of the pathogen in these cells.

### 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>
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Mouse bone marrow-differentiated DCs were transfected with day-1 (bold type) or day-5 (italic type) Ags, followed by *M. tuberculosis* H37Rv. Surface levels of indicated markers were monitored by FACS. Data 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>
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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. Surface levels of indicated markers were monitored by FACS. Data 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).

FIGURE 2. Day-1 and -5 Ags downmodulate proinflammatory cytokine secretion from DCs. DCs were transfected with indicated Ags for 36 h, followed by infection with 1 MOI M. tuberculosis H37Rv for 48 h. Culture supernatants were evaluated for the indicated cytokines. MOCK represents transfection of DCs with empty vector. Data represent the mean ± SD of three independent experiments.

FIGURE 3. Day-1 and -5 Ags suppress Th1 cell responses from DCs. DCs were transfected with indicated Ags for 36 h, followed by infection with 1 MOI M. tuberculosis H37Rv for 48 h and then were cocultured for 48 h with T cells enriched from M. tuberculosis-infected mice. Culture supernatants were screened for the levels of indicated cytokines. MOCK represents transfection of DCs with empty vector. Data represent the mean ± SD of three independent experiments.
These results indicate that day-1 and -5 Ags exhibit complementary roles toward the evasion of protective immune responses from macrophages. Because cytokines 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 Pam3Csk4 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...
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.

In the next series of experiments, we characterized the immune responses mediated by these Ags and the mechanisms used. Results indicate that although day-1 and -5 Ags mediate immune suppression, they use different strategies that are commensurate with the functions of the cells of the immune system. For example, although day-1 Ags activate DCs with respect to surface marker expression (including MHC and costimulatory molecules), the day-5 Ags prevent any such activation, indicating the suppression of DC functions at later times postinfection. However, all Ags inhibited the activation of macrophages. These results indicate that early activation of DCs could be beneficiary to the pathogen; however, at later times postinfection, inhibition of DC activation needs to be achieved to establish a safe niche. Further, all Ags curtailed proinflammatory cytokine secretion from DCs.

*M. tuberculosis* is known to downmodulate the expression of MHC class II expression on infected macrophages early in the infection process (37). We show in this article that Ags expressed early during the infection 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...
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 nitrosative 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


