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Impaired Generation of Reactive Oxygen Species during Differentiation of Dendritic Cells (DCs) by *Mycobacterium tuberculosis* Secretory Antigen (MTSA) and Subsequent Activation of MTSA-DCs by Mycobacteria Results in Increased Intracellular Survival¹

Aprajita Sinha,^{2*} Anjana Singh,^{2*} Vijaya Satchidanandam,[†] and Krishnamurthy Natarajan^{3*}

We investigated the role of reactive oxygen species (ROS) in dendritic cell (DC) differentiation by 10-kDa *Mycobacterium tuberculosis* secretory Ag (MTSA) and survival of mycobacteria therein. Compared with GM-CSF, MTSA induced lower ROS production during DC differentiation from precursors. This result correlated with higher superoxide dismutase 1 expression in MTSA stimulated precursors as compared with GM-CSF stimulation. Furthermore, a negative regulation of protein kinase C (PKC) activation by ROS was observed during DC differentiation. ROS inhibited the rapid and increased phosphorylation of PKC α observed during DC differentiation by MTSA. In contrast, ROS inhibition increased the weak and delayed PKC α phosphorylation by GM-CSF. Similar to DC differentiation, upon activation with either *M. tuberculosis* cell extract (CE) or live *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), DCs differentiated with MTSA (MTSA-DCs) generated lower ROS levels when compared with DCs differentiated with GM-CSF (GM-CSF-DCs). Likewise, a negative regulation of PKC α phosphorylation by ROS was once again observed in DCs activated with either *M. tuberculosis* CE or live *M. bovis* BCG. However, a reciprocal positive regulation between ROS and calcium was observed. Compared with MTSA-DCs, stimulation of GM-CSF-DCs with *M. tuberculosis* CE induced a 2-fold higher ROS-dependent calcium influx. However, pretreatment of MTSA-DCs with H₂O₂ increased calcium mobilization. Finally, lower ROS levels in MTSA-DCs correlated with increased intracellular survival of *M. bovis* BCG when compared with survival in GM-CSF-DCs. Although inhibiting ROS in GM-CSF-DCs increased *M. bovis* BCG survival, H₂O₂ treatment of MTSA-DCs decreased survival of *M. bovis* BCG. Overall our results suggest that DCs differentiated with Ags such as MTSA may provide a niche for survival and/or growth of mycobacteria following sequestration of ROS. *The Journal of Immunology*, 2006, 177: 468–478.

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). The World Health Organization estimates that between 2002 and 2020, ~1 billion people will be newly infected, >150 million people will get sick, and 36 million will die of tuberculosis, if control is not strengthened (1). This problem is further complicated by the variable efficacy of immunizations with *Mycobacterium bovis* bacillus

Calmette-Guérin (BCG),⁴ the only available vaccine against tuberculosis (2–4). This complication underscores the need to elucidate factors that regulate protective immune responses against this pathogen (5–8).

Among the APCs of the immune system, dendritic cells (DCs) constitute the most potent APCs and act as a bridge between the innate and the acquired arm of the immune system (9). This result is largely attributed to their ability to stimulate naive quiescent T cells, thereby initiating a primary immune response. DC subsets colonize, and are recruited to specific tissues immediately following an antigenic insult, in which they initiate different immune responses (10). 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 (11). 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 that affects mycobacterial survival in the host (12).

A number of *M. tuberculosis* secretory Ags (MTSA) released from infected macrophages target DCs and mediate host immune

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⁴ Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; DC dendritic cell; CE, cell extract; MTSA, *M. tuberculosis* secretory Ag; MOI, multiplicity of infection; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; DPI, diphenyleneiodonium chloride; SOD1, superoxide dismutase 1; [Ca²⁺]_i, intracellular calcium concentration.

responses. Because the release of secretory Ags and the recruitment of DC precursors at sites of infection are early events in the infection process, interactions between them are likely to shape the nature of subsequent immune responses including mycobacterial survival and dissemination. To this end, using a 10-kDa MTSA (also known as culture filtrate protein 10) that is expressed from the RD1 region of the *M. tuberculosis* genome, we have been investigating the interactions of secretory Ags with DCs to characterize the early events that occur following infection by *M. tuberculosis*. We have shown that MTSA and many other secretory Ags activate DCs by inducing their differentiation and maturation (reviewed in Refs. 13–15). However, a challenge of these DCs with either *M. tuberculosis* cell extract (CE) (16) or live *M. bovis* BCG (our unpublished data) down-regulates primary and recall Th1 responses. This observation indicated that a putative role for Ags such as MTSA is to down-regulate protective immune responses mounted by the host against *M. tuberculosis*. On similar lines, in this study, we investigated the possibility of MTSA differentiated DCs in regulating intracellular survival of *M. bovis* BCG as a function of reactive oxygen species (ROS).

Various forms of ROS, including superoxides, are generated from different sources, including normal cellular respiration, activated PMLs, endothelial cells, and mitochondrial electron flux (17, 18). Superoxide generation by phagocytic NADPH oxidase is known to be important in bacterial killing (19). Furthermore, ROS plays a role in various cellular processes such as proliferation (20), adhesion (21), and hypertension (22). In addition, growth factors are known to stimulate ROS production through their receptors, but the mechanisms are poorly understood (23).

In this study, we first investigated the role of ROS during DC differentiation by MTSA and GM-CSF. We observed that although ROS levels are increased during DC differentiation by GM-CSF, basal ROS levels are quenched during DC differentiation by MTSA. In subsequent experiments, we investigated the role of ROS during mycobacterial activation of DCs differentiated with MTSA (MTSA-DC) and with GM-CSF (GM-CSF-DC). Similar to DC differentiation, compared with DCs differentiated conventionally with GM-CSF, MTSA-DCs showed lower levels of intracellular ROS following activation with either *M. tuberculosis* whole CE or live *M. bovis* BCG. Low levels of ROS both during MTSA-DC and following subsequent activation of fully differentiated MTSA-DCs with *M. tuberculosis* CE or *M. bovis* BCG were associated with high expression of superoxide dismutase 1 (SOD1), higher activation levels of protein kinase C (PKC), and low intracellular calcium mobilization. Furthermore, reduced levels of ROS in MTSA-DCs resulted in increased survival of *M. bovis* BCG. The functional implications of increased mycobacterial survival in MTSA-DCs are discussed.

Materials and Methods

Animals

Female BALB/c mice 4–6 wk of age kept in pathogen-free environment were used following approval from the Institutional Animal Ethics Committee.

Materials

FITC-tagged Abs against mouse CD80 (clone 1G10), CD54 (clone 3E2), H-2D^d (clone 3-25.4), PE-conjugated annexin V, and isotype controls were from BD Pharmingen. Recombinant mouse GM-CSF was from R&D Systems. DCFH-DA (dichlorofluorescein diacetate) was obtained from Molecular Probes. Ionomycin, calphostin C, PMA, *N*-acetylcysteine (NAC), diphenyleneiodonium chloride (DPI), hydrogen peroxide (H₂O₂), and fluo-3-AM (fluo-3-acetoxymethyl ester) were purchased from Sigma-Aldrich. Abs to phospho-PKC α , SOD1, phospho-ERK1/2, GAPDH and Luminol kits for chemiluminescence detection and control Abs were purchased from Santa Cruz Biotechnology. *M. tuberculosis*

CE was obtained from “Tuberculosis Research Materials and Vaccine Testing” (Colorado State University). This preparation is a sonicate of a growing culture of *M. tuberculosis* H37Rv. Additional details of its preparation and composition can be viewed at their web site (www.cvmbs.colostate.edu/microbiology/tb/top/).

Expression and purification of MTSA

Endotoxin-free MTSA was recombinantly expressed and purified as a His-tagged protein from *Escherichia coli* as described earlier (14–16). The endotoxin levels were estimated to be 0.3 EU/mg of protein.

Enrichment of DC precursors from bone marrow and generation of DCs

DCs were differentiated with either GM-CSF or MTSA as described before (12–14). Briefly, bone marrow from the tibia and femurs of BALB/c mice were flushed out and lymphocytes and I-A⁺ 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 either 15 ng/ml GM-CSF or 20 μ g/ml MTSA for 3 days. DCs differentiated with MTSA are referred to as MTSA-DCs, whereas DCs differentiated with GM-CSF are referred to as GM-CSF-DCs. We have shown that this method gives a homogenous population that is 99% DCs with negligible contaminating monocytes or macrophages (14). For some experiments, bone marrow cells were incubated with different concentrations of NAC or DPI for 1 h before stimulation of cells with either MTSA or GM-CSF. Cells were stained for surface molecules by FITC-tagged Abs (BD Biosciences) and analyzed by flow cytometry using FACSCalibur (BD Biosciences). The data were plotted using CellQuest software (BD Biosciences).

Measurement of intracellular ROS

Intracellular ROS levels were measured by flow cytometry in cells cultured in serum-free medium and loaded with the redox-sensitive dye DCFH-DA (24). The nonfluorescent DCFH-DA readily diffuses into the cells where it is hydrolyzed to the polar derivative DCFH, which is oxidized in the presence of H₂O₂ to the highly fluorescent DCF. Thirty minutes before the end of each incubation period, $\sim 1 \times 10^6$ cells were incubated with 10 μ M DCFH-DA in 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 software.

Analyses of phospho-PKC α , phospho-ERK, and SOD1 levels

Phospho-PKC α , phospho-ERK, and SOD1 levels were monitored by Western blotting. At the end of incubation, cells were chilled on ice and 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 M EGTA, 0.5% Nonidet P-40, and 2 μ g/ml 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. The 20 μ g of cytoplasmic extract were then resolved on 10% SDS-PAGE and subsequently transferred onto nitrocellulose membrane (Hybond C pure; Amersham Biosciences). The blots were then probed with Abs to phospho-PKC α or SOD1 or phospho-ERK followed by HRP-labeled secondary Abs. Furthermore, a parallel set of samples were run separately on SDS-PAGE and probed for classical PKC α or ERK or GAPDH as loading control. The blots were later developed by chemiluminescence using the Luminol reagent.

Transfection of bone marrow precursors with Cu/Zn SOD

Bone marrow precursors were transfected with either an irrelevant plasmid or Cu/Zn SOD (SOD1) by electroporation using Gene Pulser II (Bio-Rad) at 0.180 kV, 975 μ F for 40 msec. Following standardization for transfection efficiency, transfected bone marrow precursors were stimulated 14 h later with either MTSA or GM-CSF for analyses of intracellular ROS or monitoring PKC α phosphorylation as described.

Estimation of intracellular calcium levels

Intracellular calcium levels were monitored essentially as previously described (25). Briefly, either 2×10^7 GM-CSF-DCs or MTSA-DCs were loaded with 1 μ M fluo-3-AM for 45 min at 37°C in culture medium. The cells were thoroughly washed with HBSS and suspended in fresh culture medium. An aliquot of cells was diluted in culture medium, and when required, stimulated with *M. tuberculosis* CE, and real-time increase in intracellular calcium concentration ([Ca²⁺]_i) was monitored immediately over a period of 5 min by FACS using FACSCalibur (BD Biosciences), and the data were analyzed using the CellQuest software. For some groups,

GM-CSF-DCs were treated with NAc or DPI and MTSA-DCs were treated with 50 μM H_2O_2 for 30 min. Cells were later loaded with fluo-3-AM and stimulated with *M. tuberculosis* CE to monitor intracellular calcium levels over a period of 5 min.

Infection of DCs with *M. bovis* BCG

M. bovis BCG strain SSI-1331 from Statens Serum Institute was grown in Middlebrook 7H9 liquid medium supplemented with ADS (albumin/dextrose/saline) at a final concentration of 5, 2, and 0.8 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. Fully differentiated MTSA-DCs or GM-CSF-DCs were infected with *M. bovis* BCG at a multiplicity of infection (MOI) of 1 for 5 h. Extracellular bacteria were washed off, and DCs were processed for estimation of ROS, phospho-PKC α , or SOD1 levels as mentioned.

Intracellular survival of *M. bovis* BCG in DCs

Fully differentiated MTSA-DCs or GM-CSF-DCs were infected with MOI of 1 for *M. bovis* BCG as described. Following washes, extracellular bacteria were killed off by treatment with gentamicin. Infected DCs were lysed 72 h postinfection, serially diluted, and plated onto 7H10 agar. Two to three weeks later, plates were scored for CFU.

Results

MTSA generates low levels of ROS during DC differentiation

Toward characterization of the interactions of DCs with MTSA, we have previously shown that Ags such as MTSA induce differentiation of mouse bone marrow precursors into immature DCs (14). These DCs are phenotypically (based on surface marker expression) and morphologically similar to DCs differentiated conventionally with GM-CSF. However, functional characterization of MTSA-DCs showed that, unlike GM-CSF-DCs that induce Th1 responses to a challenge with *M. tuberculosis* whole CE, MTSA-DCs induced Th0 responses to *M. tuberculosis* whole CE in an IL-10 and TGF- β -dependent mechanism (16). This response indicated that Ags such as MTSA are secreted to down-regulate Th1 responses to subsequently released mycobacterial components or mycobacteria via initial activation of DCs. In this study, to begin, we first examined the role of ROS during DC differentiation by MTSA. In subsequent experiments, we looked at modulation of ROS levels in MTSA-DCs following activation with *M. tuberculosis* whole CE or infection with live *M. bovis* BCG and the consequent effect thereof on the survival of intracellular mycobacteria. As MTSA-DCs and GM-CSF-DCs share similar phenotype and maturation status (both are immature DCs; Ref. 14) for comparative purposes, we did parallel experiments with GM-CSF. This approach identifies the differences and similarities between the two

differentiated DCs and highlights the physiological significance of DC differentiation by MTSA.

We first investigated whether ROS are generated and required for DC differentiation by MTSA and GM-CSF. We monitored intracellular ROS levels by flow cytometry as a function of reduction of the redox sensitive dye DCFH-DA (see *Materials and Methods*). As shown in Fig. 1 stimulation of bone marrow precursors with GM-CSF increased ROS levels within 30 min that were maintained at a higher level than unstimulated controls up to 2 h. In contrast, a decrease in ROS levels with respect to unstimulated control was noticed upon stimulation with MTSA. This decrease was maintained up to 1 h of stimulation, and at 2 h ROS levels were at par with the unstimulated control. Furthermore, this difference in ROS levels was maintained until these cells fully differentiated into DCs as later shown (see Fig. 5). We have shown earlier that DCs differentiated with either GM-CSF or MTSA were homogenous and do not contain any monocytes or macrophages (14). Nevertheless, to confirm that the observed ROS levels (especially at 72 h) were from DCs and not from any contaminating monocytes or macrophages in our DC populations, we conducted immunophenotypic analyses for the presence of various monocytes or macrophages in MTSA-DCs and GM-CSF-DCs by monitoring the expression of CD43 that is present on most macrophages and monocytes. Concurrent with our earlier studies (14), no CD43 $^+$ cells were observed in our DC populations. The majority of the cells were positive for CD11c and CD11b that are expressed on most myeloid DCs. These results indicated that ROS levels observed in the cells were in DCs only. Furthermore, we stimulated freshly isolated CD43 $^+$ CD11b $^+$ CD11c $^-$ peritoneal monocytes/macrophages isolated from naive mice with either MTSA or GM-CSF and monitored changes in ROS levels. Similar to DCs a reduction in ROS levels was observed in peritoneal macrophage/monocytes upon stimulation with MTSA. However, no effect on ROS levels was observed following stimulation of peritoneal monocytes/macrophages with GM-CSF (data not shown). These results further reiterated that the observed increase in ROS levels were in GM-CSF-DCs only and not in any contaminating monocytes/macrophages in the population. The decrease in ROS levels in MTSA-stimulated peritoneal macrophages/monocytes indicated that similar to DCs, MTSA might down-regulate ROS levels in monocytes/macrophages also. However, because no CD43 $^+$ cells were present in MTSA-DCs, this rules out the possibility that the observed decrease in ROS levels in DCs is due to contaminating monocytes/macrophages.

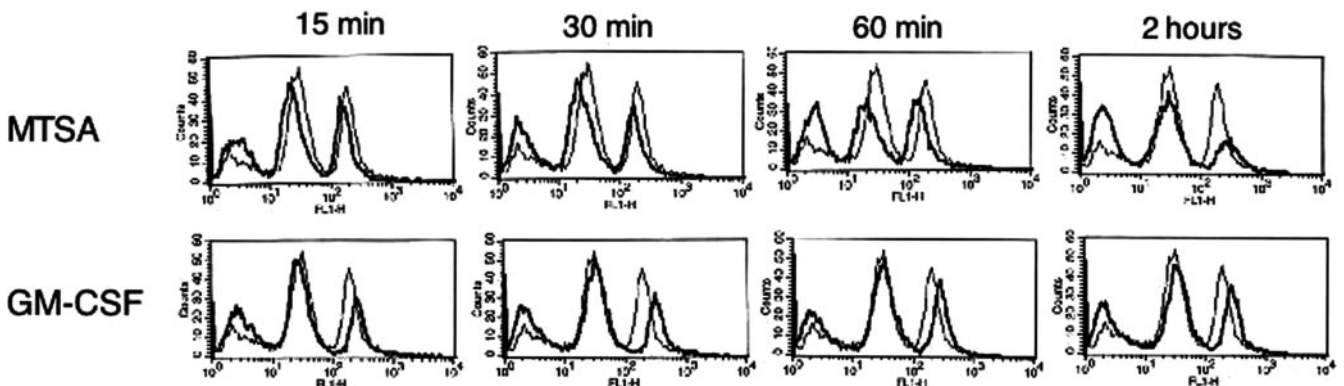


FIGURE 1. MTSA generates negligible levels of ROS during DC differentiation. Bone marrow precursors were cultured in serum-free medium for indicated times. Thirty minutes before the end of incubation period, cells were loaded with 10 μM DCFH-DA. Cells were quickly and thoroughly washed and immediately analyzed for ROS levels by FACS. *Top*, Stimulation with MTSA. *Bottom*, Stimulation with GM-CSF. Stimulated cells (thick line histogram) and unstimulated cells (thin line histogram) are represented. Data from one of five independent experiments are shown.

We next investigated whether ROS requirement is essential for DC differentiation. To this end, we incubated bone marrow precursors with two well-characterized ROS inhibitors, NAc and DPI (24). Although NAc is known to block ROS generation by inhibiting the action of the NADPH oxidase (24), DPI is an inhibitor of flavoproteins and thus inhibits both phagocytic and nonphagocytic NADPH oxidases, NO synthase, NADPH quinone:oxidoreductase, and mitochondrial NADH dehydrogenases (26–29). For monitoring DC differentiation, we looked at the surface densities of CD80, CD54, and H-2D (MHC class I), as representative of costimulatory, adhesion and MHC molecules. Furthermore, these markers are expressed at high levels on both MTSA-DCs and GM-CSF-DCs (14). As shown in Fig. 2, incubation of precursors with either NAc or DPI completely blocked the up-regulation of all the three markers in a dose-dependent manner, by both MTSA (Fig. 2, *left*) and GM-CSF (Fig. 2, *right*) during DC differentiation. These results indicated that ROS play a crucial role in the differentiation of precursors into DCs, such that inhibiting ROS does not allow the precursors to develop into DCs. This finding was despite the fact that MTSA stimulation failed to appreciably increase ROS to detectable levels. In addition, the levels of MHC class II and CD86 were also completely inhibited by both the inhibitors (data not shown). We ascertained that incubation with either DPI or NAc had no effect on cell viability. To this end, cells were incubated with 10 μ M DPI or 50 mM NAc along with either GM-CSF or MTSA for 72 h. Additionally, cells were incubated with 100 μ M DPI along with GM-CSF. At the end of the incubation period, cells were stained with Annexin V. In the presence of either 10 μ M DPI or 50 mM NAc, the cell viability was maintained over 95% indicating that neither NAc or DPI used at their respective concentrations had any effect on cell viability, whereas cells with 100 μ M DPI along with GM-CSF showed considerable loss in viability (data not shown). Concurrent with the fact that GM-CSF stimulation produced more ROS than stimulation with MTSA, a higher dose of DPI or NAc was required for near complete inhibition of surface marker up-regulation (to levels observed in unstimulated cultures) as compared with MTSA stimulated cultures (Fig. 2). Furthermore, of the three markers analyzed H-2D (MHC class I) showed the greatest sensitivity to ROS inhibition by both NAc and DPI, whereas CD80 was relatively more resistant. This was true for both MTSA- and GM-CSF-mediated DC differentiation, indicating differential regulation of surface marker up-regulation by ROS. TNF- α is secreted during DC differentiation of precursors and TNF- α has been postulated to aid DC differentiation (30). Furthermore, ROS is known to induce TNF- α secretion in human DCs (31). Therefore, as an indicator of DC differentiation, we also looked at TNF- α levels in the presence of ROS inhibitors during DC differentiation by MTSA and GM-CSF. NAc or DPI treatment of precursors before stimulation with MTSA or GM-CSF also sig-

nificantly inhibited TNF- α (data not shown). These results indicated that ROS are required for TNF- α secretion during DC differentiation.

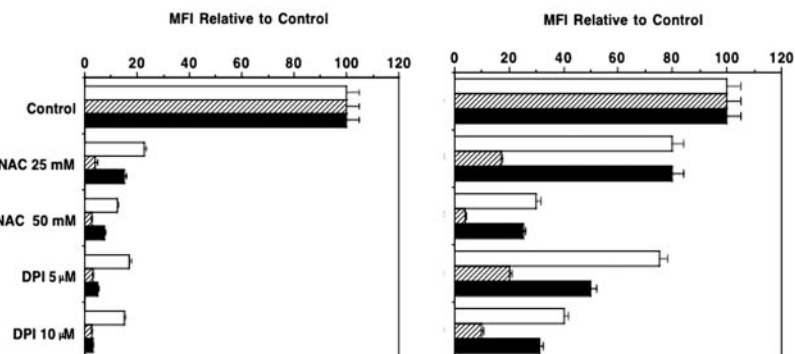
Keeping these identified dose thresholds in mind, and for a realistic comparison of the effects of ROS on DCs differentiated with MTSA or with GM-CSF, all subsequent experiments were conducted by treatment of cells with either 50 mM NAc or 10 μ M DPI before stimulation.

SOD1 quenches ROS generated during DC differentiation by MTSA

Because MTSA stimulation generated low levels of ROS we investigated whether this could be a result of high levels of an intracellular ROS scavenger. Of the different intracellular ROS scavengers, the various isoforms of SOD are well characterized (32). SOD converts superoxide into H₂O₂ and oxygen (32). As shown in Fig. 3A, MTSA stimulation indeed induced a 24-fold increase in SOD1 levels within 30 min that was evident up to 6 h poststimulation. In contrast, a weak increase in SOD1 expression (3-fold) was observed post-GM-CSF stimulation that was lost by 6 h. Importantly, overall levels of SOD1 were higher at all time points during DC differentiation by MTSA as compared with GM-CSF. This indicated that higher levels of SOD1 might scavenge the superoxides generated following stimulation with MTSA, resulting in low levels of detectable ROS. Furthermore, no changes in SOD1 levels were observed in peritoneal monocytes/macrophages stimulated with MTSA or GM-CSF, indicating that the observed effects were specific to DCs (data not shown). This result also indicated that the decrease in ROS levels observed in DCs and monocytes/macrophages following MTSA stimulation could be regulated at different levels. This further indicated that the interaction of DCs and monocytes/macrophages with *M. tuberculosis* and/or *M. tuberculosis* Ags could be differentially regulated as discussed later.

To further investigate whether increased SOD1 levels were indeed responsible for low ROS levels during DC differentiation by MTSA, we transfected bone marrow precursors with a plasmid expressing SOD1 (Cu/Zn SOD) and measured ROS levels at various times following stimulation with GM-CSF. As shown in Fig. 3B, GM-CSF-stimulated bone marrow cells transfected with an irrelevant plasmid (MOCK) showed higher ROS levels than MTSA-stimulated bone marrow cells (Fig. 3B, compare thick line histogram with thin in b–e). In contrast, ROS levels in GM-CSF-stimulated bone marrow cells transfected with plasmid expressing SOD1, were now reduced to the extent that the profiles were superimposable on MTSA-stimulated bone marrow cells at all time points (Fig. 3B, compare thick line histogram with thin in g–j). In fact, basal levels of ROS in unstimulated SOD1-transfected cells

FIGURE 2. ROS is obligatory for surface marker up-regulation during DC differentiation. Bone marrow precursors were stimulated with MTSA (*left*) or GM-CSF (*right*) in the presence of indicated concentrations of ROS inhibitors DPI or NAc. “Control” represents DCs differentiated in the absence of any inhibitor. Cells were stained for surface levels of CD80 (□), H-2D (▨), and CD54 (■) with FITC-conjugated Abs, and analyzed by FACS. Data are expressed as levels of mean fluorescence intensity (MFI) relative to Control. Data from one of three independent experiments are shown.



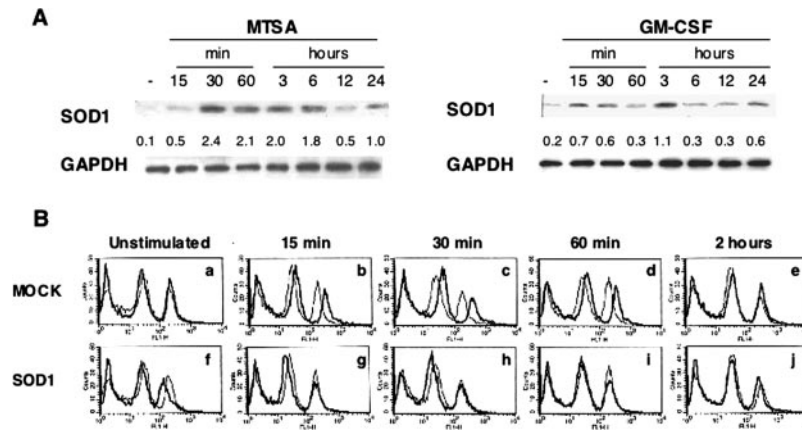


FIGURE 3. Enhanced SOD1 expression during DC differentiation by MTSA quenches ROS. *A*, Bone marrow precursors were stimulated with MTSA (*left*) or GM-CSF (*right*) for indicated times. SOD1 level in the cytoplasmic extracts were monitored by Western blotting. Values below SOD blot depict relative intensity of the bands. GAPDH levels are shown as loading control. One of three independent experiments is shown. *B*, Bone marrow cells were transfected with an irrelevant plasmid (MOCK, *a–e*) or plasmid DNA expressing SOD1 (SOD1, *f–j*). Thick line histograms in *b–e* depict ROS levels at indicated times poststimulation in GM-CSF-stimulated bone marrow precursors transfected with MOCK plasmid. Thin line histograms in *b–e* and *g–j* depict ROS levels in MTSA stimulated bone marrow cells. The thick line histograms (*a* and *f*) depict ROS levels in MOCK-transfected and SOD1-transfected, respectively, unstimulated bone marrow cells. The thin line histograms (*a* and *f*) depict unstimulated bone marrow cells.

were also quenched as compared with unstimulated MOCK-transfected cells (Fig. 3*B*, compare *a* and *f*). These results then strongly indicated that increased levels of SOD1 during DC differentiation by MTSA contribute toward quenching of ROS.

Negative regulation of PKC activation by ROS during DC differentiation

We next investigated whether ROS generated during MTSA-DC or GM-CSF-DC modulates signaling intermediates involved in the differentiation process. In investigating the intracellular mechanisms contributing to down-regulation of Th1 responses from MTSA-DCs, we have observed that MTSA stimulation induces early and increased phosphorylation of PKC α as compared with GM-CSF during DC differentiation. This increased and early activation of PKC plays a determinant role in the down-regulation of subsequent Th1 responses (A. Sinha, A. Singh, and K. Natarajan, submitted for publication). To begin with we looked at the effects of ROS inhibition on PKC α phosphorylation. As shown in Fig. 4*A*, *left*, MTSA stimulation increased the phosphorylation of PKC α within 15 min. This induced phosphorylation was further enhanced by both NAc and DPI treatment indicating a negative role of ROS in PKC α phosphorylation by MTSA. In contrast, GM-CSF stimulation resulted in a weak phosphorylation of PKC α . However, this weak phosphorylation of PKC α by GM-CSF was significantly enhanced upon pretreatment with both NAc and DPI (Fig. 4*A*, *right*), again indicating a negative regulation of ROS in PKC activation. In a mirror experiment, treatment of DC precursors with H₂O₂ completely inhibited PKC α phosphorylation following MTSA stimulation (Fig. 4*B*), thus suggesting that ROS has a regulatory effect on PKC α activation. We ensured that H₂O₂ treatment did not result in any cell death as >95% cells were viable in the presence of H₂O₂ (data not shown). Furthermore, similar to treatment with NAc or DPI, PKC α phosphorylation was now significantly increased with advanced kinetics in GM-CSF stimulated bone marrow cells transfected with SOD1 expressing plasmid (see Fig. 4*C*), thus indicating that higher SOD1 levels quench ROS generated following stimulation and result in higher PKC activation. Similar to SOD1 levels no effect of MTSA or GM-CSF stimulation of peritoneal monocytes/macrophages on phospho-PKC α phosphorylation was observed (data not shown); once again indi-

cating that the effects seen were DC-specific and PKC α phosphorylation by ROS is regulated differently in DCs and monocytes/macrophages following stimulation with MTSA. We also looked at the activation status of MAPK that are known to play vital roles in DC differentiation by ensuring the survival of the differentiating precursors as a function of ROS (33). We have observed that MTSA and GM-CSF induce ERK1/2 phosphorylation with different kinetics. Although GM-CSF induces ERK1/2 phosphorylation within 15 min, MTSA induces ERK phosphorylation between 3 and 12 h (our unpublished results). We, therefore, analyzed the effects of ROS inhibition on ERK1/2 phosphorylation by MTSA and GM-CSF at their optimal time of activation. Phosphorylation of ERK1/2 was inhibited by both NAC and DPI in GM-CSF and MTSA stimulated precursors. A greater degree of inhibition was observed at early time points in MTSA stimulated cultures (Fig. 4*D*, *left*), while inhibition of ERK1/2 phosphorylation was more evident at later time points in GM-CSF stimulated cultures (Fig. 4*D*, *right*). This indicates a differential regulation of kinetics of ERK phosphorylation by ROS in the two DCs. These results also indicated that ROS regulates the activation of the MAPK family involved in DC survival.

ROS generation following stimulation with live mycobacteria

Having observed that ROS is differentially generated during DC differentiation by MTSA and GM-CSF and that ROS modulates activation of signaling intermediates, next it was important to investigate the role of ROS in DC activation by mycobacteria. Furthermore, such a scenario would be reminiscent of infection because secretion of Ags such as MTSA is likely to be followed by the release of mycobacterial components and/or live mycobacteria from infected macrophages. We therefore conducted investigations toward ROS generation and regulation in DCs. To this end, we stimulated fully differentiated MTSA-DCs and GM-CSF-DCs with *M. tuberculosis* CE, as representative of *M. tuberculosis* components on one hand and *M. bovis* BCG as representative of live mycobacteria in contrast. As shown in Fig. 5*A*, compared with MTSA-DCs (thin line histogram), stimulation of GM-CSF-DCs (thick line histogram) with either *M. tuberculosis* CE (*top*) or *M. bovis* BCG (*bottom*) showed a greater increase in ROS levels over unstimulated controls. Furthermore, stimulation of GM-CSF-DCs

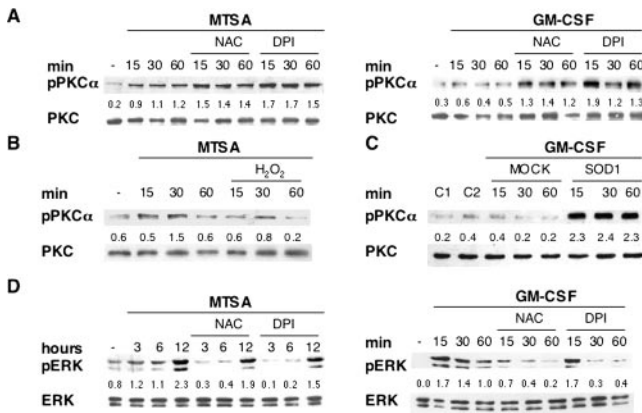


FIGURE 4. ROS negatively regulates PKC α phosphorylation during DC differentiation. Phosphorylation of PKC α (pPKC α) in A–C or of ERK1/2 (pERK) in D of MTSA- or GM-CSF-stimulated bone marrow precursors under different culture conditions is shown. A, Bone marrow cells were incubated with 50 mM NAc or 10 μ M DPI for 1 h and later stimulated with MTSA (*left*) or GM-CSF (*right*) for indicated times. B, Cells were incubated with 50 μ M H₂O₂ for 1 h before stimulation with MTSA. C, Bone marrow cells were transfected with either an irrelevant plasmid (MOCK) or plasmid-expressing SOD1. Following transfection, cells were stimulated with GM-CSF for indicated times and monitored for phospho-PKC α levels. C1 and C2 represent unstimulated bone marrow cells transfected with MOCK and SOD1, respectively. D, Bone marrow cells were incubated with 50 mM NAc or 10 μ M DPI for 1 h and later stimulated with MTSA (*left*) or GM-CSF (*right*) for indicated times. Values between blots depict relative intensity of the band. One of three experiments is shown.

resulted in higher overall levels of ROS as compared with stimulation of MTSA-DCs at all time points. Maximum ROS levels were seen at 30 min following stimulation of GM-CSF-DCs with either *M. tuberculosis* CE or *M. bovis* BCG. At this time point MTSA-DCs stimulated with *M. tuberculosis* CE showed ROS levels lower than their unstimulated controls, but the levels came back to baseline at 2 h poststimulation. This indicated a tight regulation of ROS in MTSA-DCs following activation with *M. tuberculosis* components. Furthermore, at 2 h poststimulation with *M. bovis* BCG, whereas ROS levels in GM-CSF-DCs were higher compared with its unstimulated control, the corresponding ROS levels in MTSA-DCs were lower than its unstimulated control. These results indicated that MTSA-DCs are incapable of mounting high levels of ROS following their interaction with mycobacteria or parts thereof. Furthermore, in contrast to GM-CSF-DCs, ROS levels in peritoneal macrophages/monocytes marginally decreased upon *M. tuberculosis* CE stimulation at 60 min when compared with unstimulated controls. However, no effect on ROS levels was observed following stimulation with *M. bovis* BCG at any time point of stimulation (data not shown) indicating that the interaction of mycobacteria with DCs and monocytes/macrophages is different.

Similar to DC differentiation, SOD1 levels were significantly enhanced in MTSA-DCs within 15 min following stimulation with *M. tuberculosis* CE (Fig. 5B, *top left*), whereas no significant enhancement was observed in GM-CSF-DCs even at a higher exposure of the film (Fig. 5B, *top right*). These results further reiterate the role of ROS scavenger SOD1 in preventing high levels of superoxide accumulation in MTSA-DCs. SOD1 levels were marginally enhanced in MTSA-DCs following *M. bovis* BCG stimulation (Fig. 5B, *bottom left*), whereas SOD1 levels showed a 2-fold increase in GM-CSF-DCs following *M. bovis* BCG stimulation (Fig. 5B, *bottom right*). Nevertheless, a side-by-side comparison of

absolute levels of SOD1 revealed that they were higher in stimulated MTSA-DCs as compared with stimulated GM-CSF-DCs; again indicating that higher expression of SOD1 could prevent ROS accumulation and function in MTSA-DCs. SOD1 levels showed no significant changes in peritoneal monocytes/macrophages following stimulation with either *M. tuberculosis* CE or *M. bovis* BCG (data not shown), again indicating differential regulation of ROS in DCs and monocytes/macrophages in response to mycobacteria.

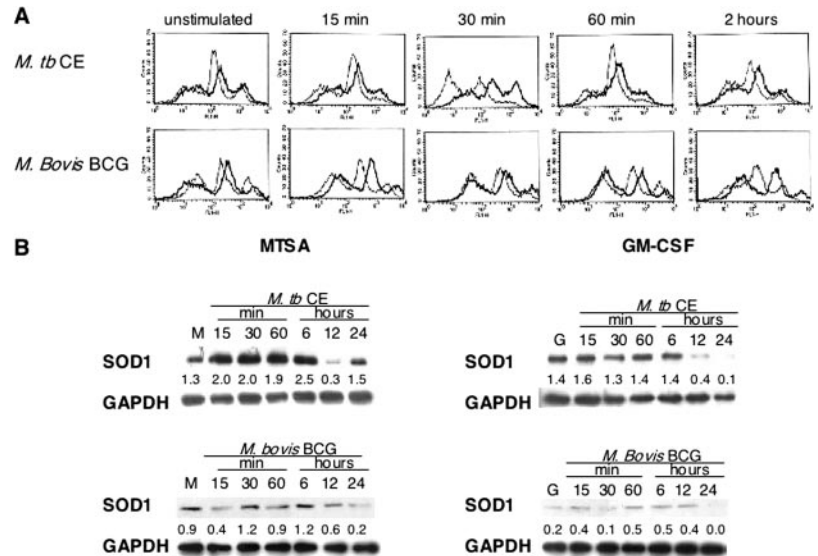
ROS negatively regulates PKC activation during DC activation by live mycobacteria

We next investigated whether the negative regulation of PKC α activation by ROS observed during DC differentiation was also prevalent in subsequent activation of DCs with live mycobacteria. To observe the effects of ROS regulation we treated MTSA-DCs with H₂O₂ and GM-CSF-DCs with NAc or DPI before stimulation with *M. tuberculosis* CE or *M. bovis* BCG. As shown in Fig. 6, *top left*, *M. tuberculosis* CE stimulation of MTSA-DCs induced rapid increase in phosphorylation of PKC α within 15 min of stimulation. This increase was completely inhibited upon pretreatment of DCs with H₂O₂. Likewise, pretreatment of GM-CSF-DCs with ROS inhibitors NAc or DPI now showed increased levels of PKC α phosphorylation and with advanced kinetics (within 15 min) as compared with the weak and delayed activation observed (at 60 min) in the absence of ROS inhibition (Fig. 6, *top right*). Similar results were obtained following stimulation of MTSA-DCs with *M. bovis* BCG, wherein H₂O₂ pretreatment abolished the induced phosphorylation of PKC α (Fig. 6, *bottom left*). Treatment of GM-CSF-DCs with DPI and NAc enhanced the degree and kinetics of PKC α phosphorylation upon *M. bovis* BCG stimulation (Fig. 6, *bottom right*). These results indicated that the negative regulation of PKC activation by ROS observed during DC differentiation continued into DC activation by mycobacteria. Similar to SOD1, no modulation of PKC α levels were observed in peritoneal monocytes/macrophages following stimulation with *M. tuberculosis* CE or *M. bovis* BCG (data not shown) indicating that PKC activation is differentially regulated in DCs and monocytes/macrophages, at least at the level of ROS.

Positive regulation of calcium by ROS during DC activation by mycobacteria

A key second messenger that governs various aspects of cellular activation is [Ca²⁺]_i (30). Furthermore, calcium signals are generated and modulated upon challenge of DCs with mycobacterial Ags (34). It was thus of interest to see the regulation of calcium by ROS during DC activation. We have earlier observed that stimulation of GM-CSF-DCs with *M. tuberculosis* CE induces a rapid influx of calcium from the medium to a greater extent than the influx seen in MTSA-DCs. Stimulation of GM-CSF-DCs with *M. tuberculosis* CE induced 200 nM calcium (Fig. 7A, *profile a* and *right panel*), whereas only 89 nM was observed in MTSA-DCs (Fig. 7A, *profile b*), indicating that calcium-mediated responses are suppressed in MTSA-DCs. Pretreatment of GM-CSF-DCs with either NAc or DPI completely inhibited any increase in intracellular calcium levels (Fig. 7A, *profiles c* and *d*). In contrast, pretreatment of MTSA-DCs with H₂O₂ now induced intracellular calcium influx to levels comparable to those observed in *M. tuberculosis* CE-stimulated GM-CSF-DCs (Fig. 7A, *profile e*). These results indicated that although ROS negatively regulates PKC activation, it has a positive effect on intracellular calcium influx. We extended the observations of positive regulation of calcium by ROS by transfecting GM-CSF-DCs with plasmid expressing SOD1 (to

FIGURE 5. Activation of MTSA-DCs with live mycobacteria induces low ROS levels. **A**, ROS levels in fully differentiated MTSA-DCs (thin line histogram) or GM-CSF-DCs (thick line histogram) stimulated with either 20 μ g of *M. tuberculosis* CE (top) or *M. bovis* BCG at 1 MOI (bottom) for indicated times. “Unstimulated” represents ROS levels before stimulation. **B**, SOD1 levels in MTSA-DCs (left) or GM-CSF DCs (right) stimulated either with 20 μ g of *M. tuberculosis* CE (top) or *M. bovis* BCG at 1 MOI (bottom) for the indicated times. GAPDH levels are shown as loading control. M and G represent SOD1 levels in unstimulated MTSA-DCs and GM-CSF DCs, respectively. Data from one of five independent experiments are shown.



quench ROS) before stimulation with *M. tuberculosis* CE. As expected, no influx of calcium was observed in GM-CSF-DCs transfected with SOD1 following stimulation with *M. tuberculosis* CE, whereas MOCK-transfected DCs resulted in increased calcium influx (data not shown). These results further support that the calcium influx observed in DCs following stimulation with mycobacteria is regulated by ROS. Singh et al. (35) have recently reported a cross-regulation of ROS and calcium during the activation of B lymphocyte receptor that effects subsequent expression of a number of genes. To see whether such reciprocal regulation of ROS and calcium exist during activation of DCs by mycobacteria, we measured ROS levels in MTSA-DCs and GM-CSF-DCs in the presence of ionomycin, a calcium ionophore. As shown in Fig. 7B, pretreatment of MTSA-DCs with ionomycin resulted in a 2- to 3-fold increase in ROS levels following stimulation with either *M. tuberculosis* CE or *M. bovis* BCG. However, no appreciable increase in ROS levels was observed in corresponding GM-CSF-DCs. In fact a marginal decrease in ROS levels was evident upon stimulation with *M. tuberculosis* CE. Because ROS levels in *M. tuberculosis* CE stimulated GM-CSF-DCs are already high (Fig. 5A), this could probably reflect upper threshold limits and a feedback regulation that prevents further increase in ROS levels so as to avoid deleterious effects on the cell itself. That this was true was confirmed in experiments when GM-CSF-DCs were stimulated with a low dose of *M. tuberculosis* CE along with ionomycin. A weak increase in ROS levels was observed with a low dose of *M. tuberculosis* CE that was significantly enhanced by cotreatment with ionomycin (data not shown). These data indicate that at low concentrations of ROS, there exists a reciprocal regulation of ROS by calcium that might play a role in subsequent DC functions.

Increased ROS correlates with decreased intracellular survival of mycobacteria in DCs

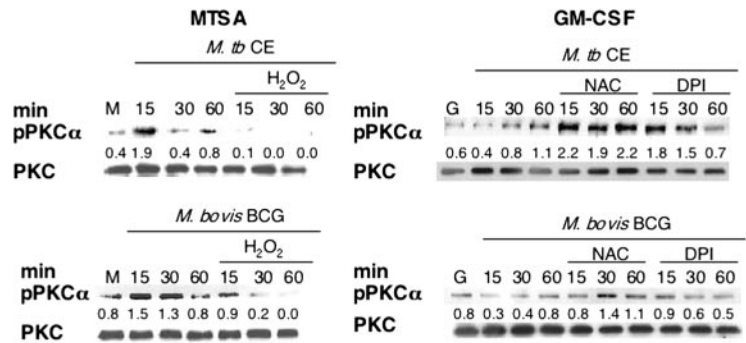
To investigate functional significance of the above observations, we investigated whether differential ROS levels in fully differentiated GM-CSF-DCs vs MTSA-DCs would affect intracellular survival of mycobacteria. To this end, we infected fully differentiated MTSA-DCs and GM-CSF-DCs with *M. bovis* BCG and 72 h postinfection cells were lysed and scored for intracellular bacteria by CFU method. As shown in Fig. 8, the number of CFU from MTSA-DCs was 3-fold higher as compared with GM-CSF-DCs. Treatment of fully differentiated MTSA-DCs with H₂O₂ reduced the number of CFU by 2-fold, whereas treatment of fully differentiated

GM-CSF-DCs with NAc increased the number of CFU by 3-fold. Surprisingly, DPI treatment of fully differentiated GM-CSF-DCs did not result in any CFU on 7H10 plates. This probably could be a result of toxic effects of DPI on the growth of mycobacteria on culture medium. This was confirmed when *M. bovis* BCG was cultured in the presence of DPI. Compared with control 7H10 agar plates that supported good growth of the bacilli, no CFUs were seen in the culture plates containing 5 μ M DPI (data not shown). These results suggest that DPI acted directly on mycobacteria and prevented their growth by possibly killing them. Furthermore, inhibiting classical PKCs with calphostin C resulted in a 2-fold decrease in survival of *M. bovis* BCG in MTSA-DCs. Concurrent with the limited roles of PKC in DC functions (A. Sinha, A. Singh, V. Satchidanandam, and K. Natarajan, submitted for publication), treating GM-CSF-DCs with PMA to activate PKCs had no significant effect on the CFU counts. Furthermore, pretreatment of MTSA-DCs with ionomycin to increase intracellular calcium levels (and thereby increase ROS levels), resulted in a 30% decrease in the number of CFU. Concurrent with the data in Fig. 7B, no appreciable decrease in the CFU from GM-CSF-DCs treated with ionomycin was observed. Overall, these results indicated that higher levels of ROS in GM-CSF-DCs contributed toward reduced survival or growth of mycobacteria. Conversely, reduced levels of ROS in MTSA-DCs results in increased survival or growth of mycobacteria, indicating that MTSA-DCs (and possibly other MTSA-DCs) may provide a suitable niche for mycobacteria to reside and evade immune responses.

Discussion

Toward understanding the early events that ensue following infection by *M. tuberculosis*, we have been elucidating the interactions of MTSA with DCs and their outcome on host-mediated immune responses. Many MTSA are promising vaccine and diagnostics candidates (36–42). However, despite the large volume of data available on these Ags their physiological role(s) at sites of infection have received scant attention. In view of this, using MTSA (also known as culture filtrate protein 10) as a model Ag, we have thus far shown that these Ags activate DCs by inducing their differentiation from bone marrow precursors (14). However, MTSA-DCs induced Th0 responses to a challenge with *M. tuberculosis* CE in a TGF- β - and IL-10-dependent mechanism (16) indicating that Ags such as MTSA down-regulate proinflammatory responses to mycobacteria following initial activation of DCs.

FIGURE 6. ROS negatively regulates PKC α phosphorylation in mycobacteria activated DCs. Phosphorylation of PKC α (pPKC α) in fully differentiated MTSA-DCs (*left*) or GM-CSF DCs (*right*) stimulated with 20 μ g of *M. tuberculosis* CE (*top*) or *M. bovis* BCG at 1 MOI (*bottom*) for indicated times is shown. MTSA-DCs were incubated with 50 μ M H₂O₂ for 1 h before stimulation. GM-CSF-DCs were incubated with 50 mM NAc or 10 μ M DPI for 1 h before stimulation. M and G represent phospho-PKC α levels in unstimulated MTSA-DCs and GM-CSF-DCs, respectively. Values between plots indicate relative intensity of the band.



Various forms of ROS are highly reactive O₂ metabolites that include superoxide radical O₂⁻, H₂O₂, hydroxyl radical (OH⁻), and others that are generated from various sources where they mediate host defense responses against invading pathogens (26, 43, 44) and cell signaling mediated effects (45, 46).

DCs are known to produce ROS, albeit at low levels, with both signaling and antipathogenic functions. In addition, it has been reported that oxidative stress induced following exposure to H₂O₂ influenced DC maturation and function by regulating surface expression of MHC molecules, chemokine and cytokine expression including TNF- α (47). Recently, bovine DCs have been shown to produce ROS in response to TLR agonists (48, 49). However, data on the role of ROS in regulating T cell responses is controversial (50, 51).

Keeping the information presented in mind, to further our understanding on the physiological roles played by these secretory Ags, we investigated whether DCs differentiated by some of these Ags (such as MTSA) could provide a safe and conducive environment for mycobacteria to survive (and possibly multiply) in addition to offering protection from cell-mediated elimination. Because MTSA-DCs and GM-CSF-DCs shared phenotypic and maturation status but differed in the functional outcome with respect to the quality of Th responses, we envisaged that the differences could be manifested during the DC differentiation process. To this end, we investigated whether MTSA would modulate ROS levels first during DC differentiation from precursors and also following subsequent activation of fully differentiated DCs with mycobacteria. We show that, compared with GM-CSF, MTSA generated low levels of ROS during DC differentiation. Nevertheless, ROS was found to be obligatory for DC differentiation by both stimuli. We also ensured that the observed levels in ROS and the differences in ROS levels in cells stimulated with either MTSA or GM-CSF were in DCs and not in any contaminating cells such as monocytes or macrophages.

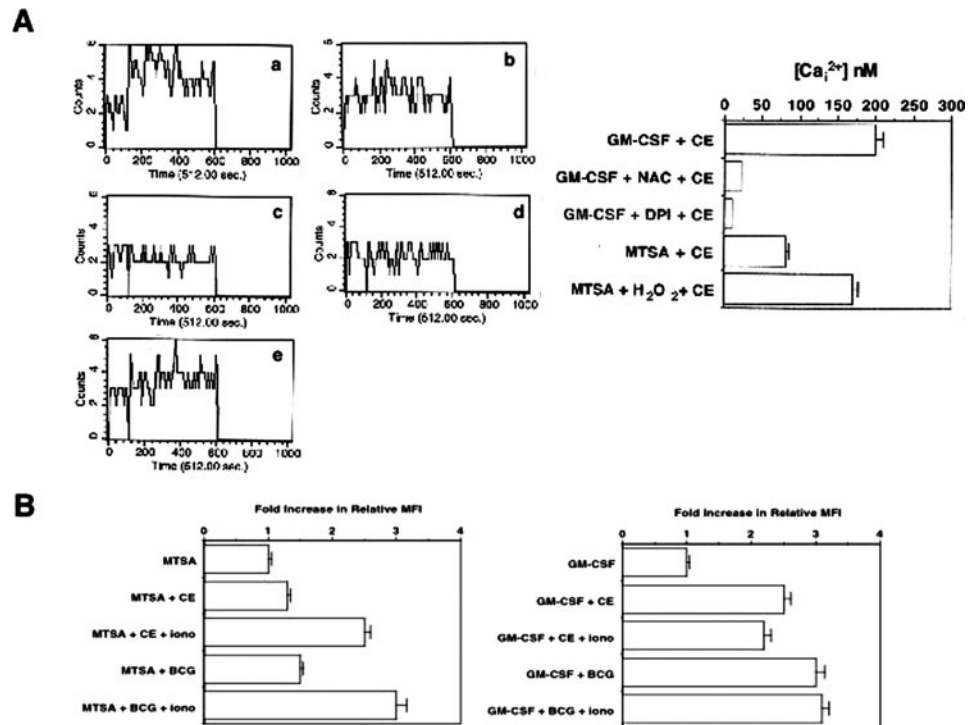
Low levels of ROS could be attributed to high levels of Cu/Zn SOD or SOD1 a known ROS scavenger (52, 53) that showed increased expression in MTSA-stimulated cells as compared with GM-CSF-stimulated cells. This was further confirmed in experiments where overexpression of SOD1 led to decreased levels of ROS in GM-CSF-stimulated bone marrow cells to levels observed in MTSA stimulated cells. We next investigated the role of decreased ROS levels in modulating the activation status of signaling intermediates, involved in DC differentiation by MTSA. We have observed that PKC plays a central role both during DC differentiation by MTSA and also in down-regulating Th1 responses from MTSA-DCs. Results showed that ROS negatively regulated PKC α phosphorylation such that inhibiting ROS in GM-CSF-DCs either with pharmacological inhibitors or via high expression of SOD1 enhanced the kinetics and degree of PKC α phosphorylation, while supplementing ROS in MTSA-DCs completely inhibited

PKC α phosphorylation. ROS was also required for activation of the MAPK pathway as NAc and DPI treatment inhibited ERK1/2 phosphorylation. In keeping with the role of ROS in modulating cellular functions (20–23), taken together these results implicated the role of ROS in DC differentiation by modulating the activations status of signaling intermediates involved therein.

In the next set of experiments, we investigated the roles played by ROS upon DC activation by mycobacteria. In the context of *M. tuberculosis* infection, ROS have been implicated in many aspects (54). *M. tuberculosis* infection of diverse cell types generates an oxidative burst that affects intracellular survival of mycobacteria and other cellular functions. For example, *M. tuberculosis* infection of neutrophils releases ROS in a protein tyrosine kinase and PLC- γ dependent manner (55). Furthermore, infection of monocytes with *M. tuberculosis* and an endogenous oxidative burst resulted in killing of intracellular mycobacteria (56). Infection of macrophages induces IFN- γ dependent generation of ROS and reactive nitrogen intermediates (57). *M. tuberculosis* infection of macrophages further regulates TNF- α secretion and *M. tuberculosis* Ag 85 expression (58). Furthermore, the Cu/Zn superoxide dismutase of *M. tuberculosis* has been demonstrated to play a role in enhancing its survival such that deletion of the same results in increased killing of intracellular mycobacteria in infected macrophages (53). This implicated a direct role of ROS in macrophage-mediated control of intracellular *M. tuberculosis*. Conversely, it has been also argued that intracellular survival of mycobacteria within macrophages is better regulated by reactive nitrogen intermediates as compared with ROS (54, 59).

Importantly, *M. tuberculosis* has been shown to interact differently with DCs as compared with macrophages. For example, infection of DCs with *M. tuberculosis* induces their activation by up-regulating costimulatory and MHC molecules (60). This also results in secretion of IL-12 and IFN- γ from infected DCs. On the other hand, infection of macrophages with *M. tuberculosis* results in down-regulation of MHC class II (8), MHC class I (61), IFN- γ responsiveness and IL-12 production, resulting in reduced inducible NOS and *phox* expression (57). Similarly, macrophage infection with *M. bovis* BCG also results in down-regulation of peptide-loaded MHC class II to the cell surface (62), whereas infection of DCs with BCG cell wall skeleton leads to their activation (63). Our results also suggest that *M. tuberculosis* Ags such as MTSA interact differently with DCs and monocytes/macrophages. Although stimulation of both DCs and monocytes/macrophages resulted in down-regulation of basal ROS levels, this down-regulation was regulated at different levels. Although it is governed by increased SOD1 levels in DCs, this was not so in the case of monocytes/macrophages. These results exemplify the role of diverse cell types in regulating the immune responses generated against mycobacteria in the back-drop of ROS by various cells of the immune system.

FIGURE 7. Reciprocal regulation of ROS and calcium following activation of DCs with *M. tuberculosis* CE. **A**, Real-time increase in intracellular calcium in fully differentiated GM-CSF-DCs (profiles *a*, *c*, and *d*) or MTSA-DCs (profiles *b* and *e*) following stimulation with 20 $\mu\text{g/ml}$ *M. tuberculosis* CE over a period of 5 min. For profiles *c* and *d*, GM-CSF-DCs were incubated for 1 h with 50 mM NAC and 10 μM DPI, respectively, before stimulation. For profile *e*, MTSA-DCs were incubated with 50 μM H_2O_2 for 30 min before stimulation. The $[\text{Ca}^{2+}]_i$ in nanomoles (*right*) at the end of 5 min of stimulation. Data from one of three experiments are shown. **B**, ROS levels in MTSA-DCs (*left*) or GM-CSF-DCs (*right*) following stimulation for 30 min with either *M. tuberculosis* CE or *M. bovis* BCG in the presence or absence of 1 μM ionomycin. Data are presented as fold increase in the relative mean fluorescence intensity (MFI) to the respective unstimulated control.



Keeping the above in mind, we challenged fully differentiated MTSA-DCs and GM-CSF-DCs with *M. tuberculosis* CE or *M. bovis* BCG. Our rationale for this relied on the likelihood that the release of secretory Ags such as MTSA would be followed by the release of mycobacterial components or live mycobacteria either as a result of host immune responses and/or as a result of apoptosis of infected macrophages as proposed by Kaufmann and colleagues (64). We first looked at the ability of the two DCs to generate ROS following stimulation with *M. tuberculosis* CE (representing mycobacterial components) and live *M. bovis* BCG (representing live mycobacteria). Similar to DC differentiation, ROS levels were much lower in MTSA-DCs when compared with conventional GM-CSF-DCs upon a challenge with either *M. tuberculosis* CE or *M. bovis* BCG. These results indicated that the factors governing low production of ROS during DC differentiation by MTSA continued to exert a dominant effect in DC activation. One of these factors could be the high level of SOD1 observed following stimulation of MTSA-DCs with *M. tuberculosis* CE (and to a lesser extent following stimulation with *M. bovis* BCG). The fact that SOD1 expression levels were raised in MTSA-DCs upon activation with mycobacteria, suggested an important role for the Cu/Zn form of SOD in quenching ROS in these DCs. Furthermore, the negative regulation of PKC activation as measured by phosphorylation of PKC α was also observed in both MTSA-DCs and GM-CSF-DCs upon stimulation with *M. tuberculosis* CE and *M. bovis* BCG again indicating that activation of intracellular intermediates is modulated during DC activation in a ROS regulated manner. This negative regulation of PKC was once again mediated by ROS as reiterated by overexpression of SOD1 resulting in greater activation of PKC. The fact that stimulation of freshly isolated peritoneal monocytes/macrophages with *M. tuberculosis* CE or *M. bovis* BCG did not appreciably modulate levels of either ROS or SOD1 or extent of PKC α phosphorylation again indicates that compared with monocytes/macrophages, DCs respond differently to mycobacterial Ags and/or mycobacteria. These differences could be attributed to the fact that macrophages need to be acti-

vated with IFN- γ , for any effects mediated by ROS and/or reactive nitrogen species as also emphasized by others (57, 59).

An important feature of DC activation by pathogens is intracellular calcium mobilization. We therefore investigated roles played by ROS in regulating calcium influx. Results showed that blocking ROS completely inhibited calcium influx in GM-CSF-DCs indicating that ROS was essential and positively regulated calcium mobilization in conventional DCs following a microbial insult. Conversely, treatment of MTSA-DCs with H_2O_2 now induced a rapid increase in intracellular calcium that was earlier induced rather weakly upon *M. tuberculosis* CE stimulation. These results suggested that ROS negatively regulated PKC activation while positively regulated calcium influx in professional APCs such as DCs. Cross-regulation of ROS and calcium with effects on multiple gene expression has recently been shown (35). We show the existence of similar reciprocal regulation in DC activation by mycobacterial components. This reciprocal regulation of ROS by calcium, interestingly however, is active at low ROS levels, such as in MTSA-DCs. At higher ROS levels, calcium had minimal role to play in further increasing ROS. On the contrary, a feedback regulation of ROS was observed when attempts were made to increase ROS by boosting intracellular calcium; while a positive regulation was observed when GM-CSF-DCs were stimulated with low doses of *M. tuberculosis* CE along with ionomycin. This indicates that ROS and calcium tightly regulated each other so as to have optimal effects on DC functions. In contrast, suppressing ROS and calcium responses in DCs, could be a strategy used by mycobacteria in modulating DC functions to their advantage as discussed below.

Quenching and sequestration of ROS and reactive nitrogen intermediates are some of the many strategies used by various pathogens to evade host-mediated killing and elimination (8). This is more true with respect to the "danger hypothesis" in the context of peripheral activation of DCs. ROS forms an integral component of defense with respect to invading pathogens (17–19). Therefore, reduced ROS levels in DCs activated by MTSA at peripheral sites of infection might modulate danger signals seen by the host, which

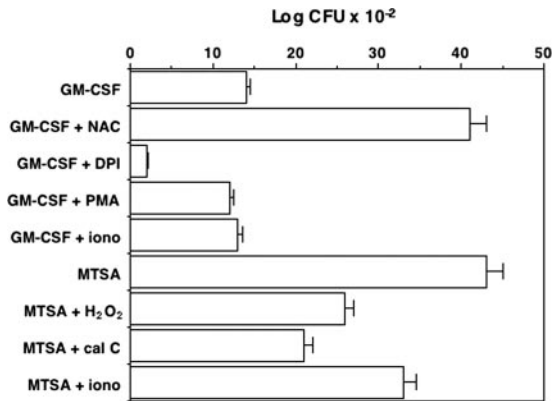


FIGURE 8. *M. bovis* BCG shows increased survival in MTSA-DCs. Either fully differentiated MTSA-DCs (MTSA) or GM-CSF DCs (GM-CSF) were infected with *M. bovis* BCG at 1 MOI for 5 h. Following elimination of extracellular bacteria by gentamicin treatment, cells were cultured for 72 h. At the end of the incubation period, cells were lysed, and cell lysate was serially diluted and plated for CFU monitoring on 7H10 agar plates. In some groups, GM-CSF DCs were treated with either 50 mM NAc or 10 μ M DPI or 100 ng/ml PMA, or 1 μ M ionomycin (iono), whereas MTSA-DCs were treated with either 50 μ M H₂O₂ or 0.1 μ M calphostin C (cal C) or 1 μ M ionomycin (iono) for 1 h before infection. Data from one of three independent experiments are shown.

again would favor the pathogen. Therefore, to give a functional meaning to the relative levels of ROS in the two DCs we looked at the ability of ROS to modulate intracellular survival of mycobacteria. Results indicated that consistent with the low production of ROS, *M. bovis* BCG showed increased survival in MTSA-DCs when compared with GM-CSF-DCs. That this was a direct effect of differential ROS levels was evident in groups where inhibition of ROS led to increased survival in GM-CSF-DCs, whereas supplementing ROS (via H₂O₂) in MTSA-DCs resulted in decreased intracellular survival to levels comparable to GM-CSF-DCs. These results are consistent with the ability of H₂O₂ to restrict survival of mycobacteria in DCs (65). In fact, as outlined above, the Cu/Zn form of SOD1 of *M. tuberculosis* has been shown to prevent killing by macrophages (53), thus adding support to our data regarding high levels of SOD1 expression in MTSA-DCs mediating increased survival of intracellular mycobacteria. The ability of mycobacteria to survive in DCs is controversial, with reports both in favor and against. Although a constrained survival of mycobacteria in DCs was reported (65), others report increased replication of mycobacteria in DCs as compared with regulated replication in macrophages (67–69). Nevertheless, our results compared two different kinds of DCs, one that is differentiated by secretory Ags, like MTSA, and the other by conventional GM-CSF-DC that have been used by most researchers.

Further, the negative regulation of PKC by ROS directly affected intracellular survival of *M. bovis* BCG in MTSA-DCs, suggesting functional implications of ROS mediated modulation of signaling machinery by Ags such as MTSA during DC differentiation and activation. Likewise, the threshold-controlled reciprocal-regulation of ROS and calcium by one another also had its effects on intracellular mycobacterial survival. Although boosting intracellular calcium in MTSA-DCs with low ROS levels decreased mycobacterial survival, it had no significant effect on survival rates under conditions with high ROS such as in GM-CSF-DCs. The role of calcium in contributing to ATP mediated killing of intracellular *M. bovis* BCG in macrophages has been reported (70). We report the existence of a reciprocal regulation of ROS and calcium in mediating survival of *M. bovis* BCG in DCs.

Collectively, our results then point toward a unique survival strategy used by mycobacteria toward immune evasion and increased intracellular survival in DCs. This includes initial secretion of Ags such as MTSA that induce the differentiation of DC from precursors. The secretory Ag differentiated DCs then not only down-regulate Th1 responses to subsequently released mycobacteria (14, 16), but also serve as depots for the survival of engulfed mycobacteria by modulating the generation and downstream functions of ROS. We are currently elucidating molecular mechanisms that are likely to regulate increased survival of mycobacteria in MTSA-DCs.

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Disclosures

The authors have no financial conflict of interest.

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