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Mast Cell Activation by Mycobacterium tuberculosis: Mediator Release and Role of CD48

Samira Muñoz,*† Rogelio Hernández-Pando, ‡ Soman N. Abraham, § and Jose Antonio Enciso2*

Mast cells (MC) are abundant in the lung and other peripheral tissue, where they participate in inflammatory processes against bacterial infections. Like other effector cells of the innate immune system, MC interact directly with a wide variety of infectious agents. This interaction results in MC activation and inflammatory mediator release. We demonstrated that MC interact with Mycobacterium tuberculosis, triggering the release of several prestored reagents, such as histamine and β-hexosaminidase, and de novo synthesized cytokines, such as TNF-α and IL-6. A number of M. tuberculosis Ags, ESAT-6, M TSA-10, and MPT-63, have been implicated in MC activation and mediator release. A MC plasmalemmal protein, CD48, was implicated in interactions with mycobacteria because CD48 appeared to aggregate in the MC membrane at sites of bacterial binding and because Abs to CD48 inhibited the MC histamine response to mycobacteria. Cumulatively, these findings suggest that MC, even in the absence of opsonins, can directly recognize M. tuberculosis and its Ags and have the potential to play an active role in mediating the host’s innate response to M. tuberculosis infection. The Journal of Immunology, 2003, 170: 5590–5596.

Infection with Mycobacterium tuberculosis continues to be one of the major global health threats, claiming ~2 million lives annually (1). Human infection with M. tuberculosis occurs mainly at the lung and is believed to involve both a carefully deployed series of adaptive bacterial virulence factors and inappropriate host immune responses (2). M. tuberculosis is naturally acquired through the respiratory route. Following penetration of the mucosal barrier, the bacteria are associated with intraepithelial leukocytes and subsequently conveyed to the draining lymph nodes (3). From human and animal models of tuberculosis infection, the primary effector cells implicated to date in the control of tuberculosis have been T lymphocytes and alveolar and interstitial macrophages (4). However, other cell types, such as dendritic cells (5), type II pneumocytes (6), endothelial cells, and fibroblasts (7), have been reported to phagocyte M. tuberculosis, suggesting that many other cell types may be involved in the immunological control of M. tuberculosis in the lung.

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3 Abbreviations used in this paper: MC, mast cells; Cal, calcium ionophore; GPI, glycosyl phosphoinositol; MOL, multiplicity of infection; PMC, peritoneal mast cells; RBL, rat basophilic leukemia cell line.
Here we investigated whether MC were capable of direct recognition of *M. tuberculosis*. We observed MC were activated by these bacteria and that the MC responses included significant release of preformed mediators and de novo synthesized mediators. We have also identified three mycobacterial Ags capable of activating MC and identified the GPI-anchored protein, CD48, as a critical mediator of activation following exposure to mycobacteria. These findings suggest that MC potentially play a critical role in modulating the early inflammatory and immune responses to *M. tuberculosis* infection.

**Materials and Methods**

**Strains and growth media**

*M. tuberculosis* strain H37Rv was grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with 10% oleic acid, albumin, dextrose, catalase enrichment medium (Difco, Detroit, MI) at 37°C. Mid-log-phase cultures were used for all the experiments. Cell suspensions were obtained by 15-s sonication in the presence of Tween 80, counted, and generally used at a multiplicity of infection (MOI) of 10 bacteria/MC.

**Recombinant Ags**

Immunodenominated secreted Ags of *M. tuberculosis*, MTSa-10 (18), MPT-63 (19), and ESAT-6 (20), were provided by Dr. M. Gennaro. These recombinant Ags were derived from genes present in *M. tuberculosis* and virulent *M. bovis*, but were missing from the genomes of all substrains of *M. bovis* bacillus Calmette-Guérin and common species of atypical mycobacteria.

**Abs and immunoreagents**

Mouse anti-rat CD48 (mlgG1, clone OX-45) were obtained from Serotec (Toronto, Canada), and mouse mAb to rat CD117 was purchased from BD PharMingen. Mouse anti-rat CD48 (mIgG1, clone OX-45) were obtained from Serotec (Dorset, UK) on exposed autoradiograms and normalized using housekeeping strategies. The multiprobe template set rCK-1 (BD PharMingen) was used to detect specific mRNAs. PCRs were contrasted with uranium salts and analyzed with the electron microscope.

**Histamine release assay**

PMC were plated in 96-well tissue culture microplates (1 × 10^5 cells/well) and exposed to mycobacteria (MOI of 10) or to different concentrations (5, 10, and 15 ng/ml) of recombinant mycobacterial Ags for 30 min at 37°C in a 5% CO_2_ atmosphere. The supernatants were separated, and the monolayers were treated with Triton X-100 (0.1%). β-Hexosaminidase release was measured in both supernatants and cell fractions using the β-hexosaminidase substrate (p-nitrophenyl-N-acetyl-β-D-glucosaminide). The OD was measured at 405 nm with a Multiskan Ascent microplate reader (Labsystems). β-Hexosaminidase release was expressed as percentage of the total β-hexosaminidase content and calculated by the formula:

\[
\text{β-hexosaminidase release} = \frac{\text{OD supernatant}}{\text{OD supernatant} + \text{OD pellet}} \times 100
\]

As described above, the hexosaminidase spontaneous release was subtracted from stimulated release.

**RNA isolation and RNase protection assay**

RBL-2H3 cells were exposed to mycobacteria or to mycobacterial recombinant Ags, MTSa-10, MPT-63 (5 ng/ml in each case), and ESAT-6 (15 ng/ml), for 6 h, and total RNA was isolated using TRIzol (Life Technologies). The multiprobe template set rCK-1 (BD PharMingen) was used to synthesize the [γ-32P]UTP-labeled riboprobes with transcription kit (BD PharMingen). RNase protection assay was performed with 10 μg of each target RNA using the RNase protection assay kit according to the manufacturer’s instructions (BD PharMingen). The samples were loaded onto an acrylamide-urea sequencing gel next to the labeled control probes and run at 30 W for 1.5 h. The gel was absorbed to filter paper, dried under vacuum, and exposed on Kodak X-AR film (Rochester, NY) with intensifying screens at ~80°C.

The relative amounts of poinflammatory cytokine gene expression were measured by scanning densitometry (GeneGenius; Syngene, Cambridge, U.K.) on exposed autoradiograms and normalized using housekeeping gene values.

**Cytokine release**

PMCs were incubated with recombinant Ags, MTSa-10, MPT-63 (5 ng/ml in each case), and ESAT-6 (15 ng/ml), or were exposed to mycobacteria (MOI of 10) under 5% CO_2_ at 37°C. Cell-free supernatants were collected after 6 h of exposure for the measurement of TNF-α release and after 24 h for IL-6 measurement. The concentrations were determined using rat TNF-α and IL-6 ELISA kits (Endogen, Woburn, MA).

**Immunofluorescence assay**

PMCs (5 × 10^5) grown on glass coverslips were incubated with previously labeled mycobacteria (MOI of 10) and allowed to bind and interact for 2 h at 37°C. Briefly, after incubation with mycobacteria, MC were washed with PBS and fixed with 4% paraformaldehyde; non-specific binding was blocked with 5% BSA, and cells were sequentially incubated with rat CD48 Ab (1/100). For ultrastructural studies the cells were pelleted by centrifugation, fixed again with 4% paraformaldehyde dissolved in Sörensen buffer, pH 7.4, for 2 h at 4°C, and embedded in LR-White hydrophilic resin. Thin sections from 70–90 nm were mounted on nickel grids and incubated for 2 h at room temperature with goat anti-mouse IgG conjugated to 5-nm gold particles (Sigma-Aldrich) diluted 1/20. The grids were contrasted with uranium salts and analyzed with the electron microscope.
Statistical analysis
Values were expressed as the mean ± SD, and the significance between values was tested by Student’s t test.

Results
MC mediator release upon activation by M. tuberculosis.
MC release inflammatory mediators when they are activated. Many of the mediators are presynthesized and stored in granules, whereas others are de novo synthesized and released upon stimulation. To study MC activation by M. tuberculosis, we examined the release of β-hexosaminidase and histamine from rat PMC following 30-min exposure to the bacteria. Both these MC products are typically prestored in cytoplasmic granules. As shown in Fig. 1, appreciable PMC release of histamine (A) and β-hexosaminidase (B) was observed. This response was comparable to that induced by 5 μM calcium ionophore (Caf), a well-known and powerful MC activator, and was significantly higher than the response elicited by 3% BSA (control). Indeed, the amount of MC β-hexosaminidase induced by 3% BSA was comparable to the spontaneous release observed in unstimulated MC.

We also determined whether MC released TNF-α and IL-6, two early response cytokines, upon exposure to M. tuberculosis. Although MC also prestore TNF-α in their granules (23), both cytokines are also de novo synthesized and released upon activation. We investigated the profile of mRNA encoding the two cytokines in the rat MC line, RBL-2H3, after 6 h of exposure to mycobacteria H37Rv. Because of the requirement for large numbers of cells, we resorted to using a MC line for this assay. As shown in Fig. 2, there was an increase in mRNA levels for TNF-α and IL-6 in MC after exposure to bacteria. These increases were significantly higher than the levels obtained in control cells exposed to BSA. Densitometric values and the percent increment in mRNA for each cytokine were derived following normalization with values obtained with two housekeeping genes L-32 and GAPDH.

To confirm that the increment in mRNA levels correlates with a corresponding increase in protein expression, we investigated whether primary PMC were able to release TNF-α and IL-6 following in vitro exposure to M. tuberculosis H37Rv. As shown in Fig. 3, PMC evoked significant release of TNF-α (A) and IL-6 (B) compared with the medium controls. Taken together, MC activation by mycobacteria involves the release of prestored mediators, such as histamine and hexosaminidase, and recently synthesized mediators, such as TNF-α and IL-6.

MC activation by recombinant mycobacterial Ags
Many bacterial components can potentially serve to activate MC. Recently, three recombinant mycobacterial Ags (ESAT-6, MTS-A-10, and MPT-63) were shown to evoke a powerful immunological

![Figure 1](image1.png)  
**FIGURE 1.** Histamine and β-hexosaminidase release by MC following exposure to M. tuberculosis. Freshly isolated PMC were incubated with 3% BSA (Control), H37Rv strain at a MOI of 10, or 5 μM calcium ionophore A23187 (Caf) for 30 min. The percentage of histamine (A) and β-hexosaminidase (B) released by the MC was determined in each case. Spontaneous release was subtracted in all cases. Results shown represent mean values (±SD) from three independent experiments. The significance of the values was determined using Student’s t test: \( p \leq 0.05 \).

![Figure 2](image2.png)  
**FIGURE 2.** Increase in proinflammatory cytokine mRNA levels in MC following exposure to M. tuberculosis. A. Autoradiograms of RNAse protection assays. Ten micrograms of total RNA from RBL-2H3 cells stimulated for 6 h with H37Rv strain (MOI of 10) were analyzed by RNAse protection assay. B. Densitometric analysis showing the percent increase for all experiments described in A. The relative amounts of IL-6 and TNF-α mRNA were evaluated by scanning densitometry (Syngene) of test autoradiograms and were normalized using housekeeping gene values. These results are representative of three independent experiments.

![Figure 3](image3.png)  
**FIGURE 3.** TNF-α and IL-6 released by MC following exposure to M. tuberculosis. PMC were incubated with mycobacteria H37Rv (MOI of 10). Levels of TNF-α (A) and IL-6 (B) were measured in supernatants after 6 and 24 h of exposure, respectively, using the rat TNF-α and IL-6 ELISA kits (Endogen). Values are given as the amount of cytokines produced by \( 1 \times 10^6 \) MC. Results are expressed as the mean values (±SD) and are representative of two experiments. Significance was determined using Student’s t test: \( p \leq 0.05 \).
response in the host (19–21). We compared the ability of each of these recombinant Ags to activate MC.

As shown in Fig. 4, after 30 min of PMC exposure to different concentrations of mycobacterial Ags, significant amounts of histamine (A) and β-hexosaminidase (B) were released. Interestingly, the amounts of MC hexosaminidase and histamine released were comparable to those evoked by the whole mycobacteria. We next investigated the ability of each of these mycobacterial Ags to trigger MC release of TNF-α and IL-6. As before, we examined MC for the increment in mRNA levels of TNF-α and IL-6 and for the release of these two cytokines in the extracellular medium. RBL-2H3 cells were exposed to 5 ng/ml MTSA-10 and MPT-63 and 15 ng/ml ESAT-6, and increments in TNF-α and IL-6 mRNA were determined. The concentration of Ag used in each assay was based on the concentration that evoked the best histamine release from MC (Fig. 4). As shown in Fig. 5, each of the recombinant mycobacterial Ags induced a significant increase in mRNA levels of TNF-α and IL-6 in MC compared with controls. Recombinant MTSA Ag induced a 150% increase in IL-6 mRNA expression, and a 50% increase in TNF-α mRNA expression compared with controls. To confirm that these changes in mRNA levels correlated with a corresponding increase in cytokine release, we investigated whether PMC released TNF-α and IL-6 following exposure to each of the Ags. As shown in Fig. 6, PMC released significant amounts of TNF-α (A), comparable to the amounts observed following PMC exposure to whole bacteria. In contrast, the level of IL-6 (Fig. 6B) was similar to that observed in PMC incubated with medium alone (Fig. 6). Interestingly, the limited release of IL-6 was independent of the incubation period (6 or 24 h). At this time it is unclear why there was no corresponding IL-6 release in the Ag-activated MC. Nevertheless, these data show that for the most

![FIGURE 4. Histamine and β-hexosaminidase released by MC following exposure to recombinant mycobacterial Ags. PMC were exposed to 3% BSA (C, negative control); to various concentrations of MTSA-10, ESAT-6, and MP-T63 Ags; or to 5 μM Cal, and the percent release of histamine (A) and β-hexosaminidase (B) was calculated. In each case exposure was for 30 min, and spontaneous MC release was subtracted from total release. The results shown represent the mean values (±SD) from three independent experiments. Significance was determined using Student’s t test: *, p ≤ 0.05.](image)

![FIGURE 5. Increase in mRNA levels of proinflammatory cytokines in MC following exposure to recombinant mycobacterial Ags. A, Autoradiograms of RNase protection assay. Ten micrograms of total RNA from RBL-2H3 cells stimulated for 6 h with the mycobacterial Ags, MTSA-10, MPT-63 (5 ng/ml in each case), and ESAT-6, (15 ng/ml), were analyzed by RNase protection assay. B, Densitometric analysis showing the percent increase for all experiments described in A. The relative amounts of IL-6 and TNF-α mRNA were evaluated by scanning densitometry (Syngene) of test autoradiograms and normalized using housekeeping gene values. These results are representative of three independent experiments.](image)

![FIGURE 6. TNF-α and IL-6 released by MC following exposure to recombinant mycobacteria Ags. PMC were incubated with 5 ng/ml MTSA-10 or MPT-63 or 15 ng/ml ESAT-6. The levels of TNF-α (A) and IL-6 (B) in the culture supernatants were assayed after 6 and 24 h of exposure, respectively, using rat TNF-α and IL-6 ELISA kits (Endogen). Values are represented as the amount of cytokines produced by 1 × 10⁶ MC. Results are expressed as the mean values (±SD) and are representative of two separate experiments. *, p ≤ 0.05.](image)
part, each of the mycobacterial Ags was capable of triggering MC release of presynthesized and de novo synthesized mediators.

CD48 involvement in MC activation by mycobacteria

The plasma membrane of MC comprises glycolipid-rich entities called lipid rafts in which several GPI-anchored proteins are located. Several of these GPI-anchored proteins have been implicated in bacterial binding and/or bacteria-mediated cell activation, including mycobacteria species (14). CD48 is one such GPI-anchored moiety that has been specifically implicated in the MC TNF-α response to FimH-expressing E. coli (16). We examined the role of CD48 in MC activation following exposure to mycobacteria. We incubated PMC with increasing concentrations of CD48-specific Ab and then exposed the mixture to H37Rv bacteria (MOI of 10). Pretreatment of PMC with CD48-specific Ab markedly inhibited histamine release in a dose-dependent fashion, whereas Abs directed to rat CD117, a well-known MC membrane marker, did not (Fig. 7). To further demonstrate the specificity of the CD48 Ab, we show that PMC histamine release following calcium ionophore treatment was not affected by CD48-Ab (Fig. 7). Thus, CD48 appears to be involved in MC activation by mycobacteria.

Enrichment of CD48 in MC plasmalemmal regions making direct contact with mycobacteria

If CD48 is involved in MC responses to mycobacteria, it is expected that this protein will be enriched at cellular regions found in direct contact with the bacteria. Using CD48-specific Ab, we sought to determine whether this was the case in experiments involving confocal microscopy and immune electron microscopy. Monolayers of PMC were exposed to a suspension of PKH26-labeled mycobacteria H37Rv for 5, 30, 60, and 120 min. After this the MC were fixed and stained with FITC-labeled anti-CD48 Ab and then subjected to confocal microscopy. Distinct patches of CD48 staining were observed in cellular regions found in direct contact with bacteria (Fig. 8). This is consistent with the recruitment and aggregation of CD48 in these cellular sites. It is noteworthy that most of these interactions between bacteria and MC seemed to occur only by 120 min. Electron microscopy (Fig. 9), confirmed this finding when the interactions between mycobacteria and RBL-2H3 cells were examined after 120 min of incubation. As shown in Fig. 9A, strong CD48 labeling (depicted by gold particles) was observed in MC regions in immediate contact with the bacteria. It is noteworthy that in several cases, bacteria appeared to be partially internalized by the MC (Fig. 9B), while in some cells the microorganisms were totally engulfed and encircled by abundant CD48 immunolabeling (Fig. 9C), raising the possibility that CD48 may be involved in MC uptake of mycobacteria.
Discussion
The results of this study provide the first evidence of a direct interaction between *M. tuberculosis* and MC. We have shown that MC are capable of recognizing mycobacteria and respond by releasing the proinflammatory mediators histamine, TNF-α, and IL-6 and by internalizing bound bacteria. We have also shown that the GPI-anchored protein, CD48, is involved in MC activation and uptake of *M. tuberculosis*. The mediators released by MC are critical for initiating the innate as well as the adaptive immune responses to bacteria.

Histamine is a very well-known MC product that can influence the balance of Th1/Th2 responses. A number of studies have reported that histamine preferentially enhances Th1 responses by triggering histamine type 1 receptor, whereas Th2 responses are negatively regulated (24, 25). On the other hand, there are also reports showing that histamine suppresses IL-12 and stimulates IL-10 secretion via histamine type 2 receptors, changes that may result in a shift of the Th1/Th2 balance toward Th2 dominance (26). Cumulatively, these findings together with our results suggest that MC-derived histamine in addition to its well-known roles in inducing bronchoconstriction, mucus secretion, increment of vascular permeability, and edema production (27), have the potential to play an important regulatory role in the immune response to *M. tuberculosis*.

Macrophages are generally recognized as the main source of TNF-α during *M. tuberculosis* infection (28). Our studies have implicated MC as an important source of TNF-α following exposure to *M. tuberculosis*. Although we have demonstrated that de novo synthesis of TNF-α occurs in MC, it is conceivable that some of the TNF-α detected in the extracellular medium were prestored in MC granules. It is noteworthy that MC are the only cell type in the body that can store this cytokine in its preformed state, before release after cell activation (23). Given that MC are resident cells in the lung, and TNF-α is considered a crucial mediator of both protection and pathology during tuberculosis (29), these results suggest that MC-derived TNF-α are likely to play a key modulatory role in *M. tuberculosis* infection. During the early stages of infection, MC TNF-α may be critical for the activation of macrophages, for the differentiation and activation of immature monocytes recruited to the site of infection, as well as for granuloma formation (30, 31). During the more chronic stages of the infection, TNF-α appears especially important for maintaining the integrity of the granulomas and for the control of bacteria, because its depletion results in desegregation of the granulomas and exacerbation of the infection (32).

We also observed that IL-6, another proinflammatory cytokine, is produced by MC after activation by *M. tuberculosis*. Elevated concentrations of IL-6 have been detected in bronchoalveolar lavage from patients with active pulmonary tuberculosis (33). This cytokine contributes to host defense by activating neutrophils and stimulating the growth and function of T cells (34). Experimental animal models of tuberculosis have shown that, like TNF-α, IL-6 is produced by activated macrophages at the beginning of the infection and during the early phases of granuloma formation (35). Studies in vitro have reported that appreciable amounts of IL-6 are produced by cultured spleen cells 2 wk postinfection with *M. tuberculosis*, and that this concentration was maintained throughout the 5-wk infection period (36). Our results suggest that MC may be an important source of IL-6 during mycobacterium infection.

We observed that *M. tuberculosis* recombinant Ags MTSA-10, MPT-63, and ESAT-6, could induce the release of inflammatory mediators from MC in vitro at comparable levels to those induced by the whole organism. These Ags were chosen from a group of proteins that are actively produced by *M. tuberculosis* during its growth. They have previously been shown to be strongly immunogenic, generating protective immune responses in both patients and experimental animals (18–20). Thus, it is plausible that these secreted Ags participate in MC activation in vivo and cause the release of inflammatory mediators, including several proinflammatory cytokines, particularly during the early phase of the infection.

Although *M. tuberculosis* can be internalized by several host cell types (5–7), it is generally assumed that macrophages are the primary host cells in which pathogenic mycobacterium reside in. Paradoxically, these macrophages are also implicated in host defense against mycobacteria. Our in vitro findings reveal that MC respond vigorously to mycobacteria through the release of a range of inflammatory mediators and by internalizing adherent bacteria. It is difficult to predict the contribution of MC to the overall immune response to *M. tuberculosis*. However, by comparing the progress of mycobacterial infections in MC-deficient and wild-type mice, it will be possible to accurately assess how much of these MC responses contribute to bacterial clearance. That the GPI-anchored moiety, CD48, has been implicated in *M. tuberculosis* activation of MC is consistent with other studies implicating GPI-anchored moieties as coreceptors on immune cells for *M. kansasi* (17). Since GPI-anchored moieties are typically found in lipid rafts, it also implicates these plasmamembrane microdomains in MC activation by *M. tuberculosis*. Although lipid rafts have been involved in the entry of *M. bovis* into macrophages (37), the contribution of lipid rafts in the uptake of *M. tuberculosis* remains to be established.

In summary, this report represents the first description of the interactions of MC with *M. tuberculosis*. We have identified several Ags on the bacteria capable of activating MC, and conversely, we have identified CD48 as a critical MC determinant involved in these interactions. Whether the mycobacterial Ags, MTSA-10, MPT-63, and ESAT-6, directly bind CD48 is the focus of our current studies.

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