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Processing and Presentation of a Mycobacterial Antigen 85B Epitope by Murine Macrophages Is Dependent on the Phagosomal Acquisition of Vacuolar Proton ATPase and In Situ Activation of Cathepsin D

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Mycobacterium tuberculosis (strain H37Rv) and bacillus Calmette-Guérin (BCG) vaccine inhibit phagosome maturation in macrophages and their effect on processing, and presentation of a secreted Ag85 complex B protein, Ag85B, by mouse macrophages was analyzed. Macrophages were infected with GFP-expressing mycobacterial strains and analyzed for in situ localization of vacuolar proton ATPase (v-ATPase) and cathepsin D (Cat D) using Western blot analysis and immunofluorescence. H37Rv and BCG phagosomes excluded the v-ATPase and maintained neutral pH while the attenuated H37Ra strain acquired v-ATPase and acidified. Mycobacterial phagosomes acquired Cat D, although strains BCG and H37Rv phagosomes contained the inactive 46-kDa form, whereas H37Ra phagosomes had the active 30-kDa form. Infected macrophages were overlaid with a T cell hybridoma specific for an Ag85B epitope complexed with MHC class II. Coincident with active Cat D, H37Ra-infected macrophages presented the epitope to T cells inducing IL-2, whereas H37Rv- and BCG-infected macrophages were less efficient in IL-2 induction. Bafilomycin inhibited the induction of macrophage-induced IL-2 from T cells indicating that v-ATPase was essential for macrophage processing of Ag85B. Furthermore, the small interfering RNA interference of Cat D synthesis resulted in a marked decrease in the levels of macrophage-induced IL-2. Thus, a v-ATPase-dependent phagosomal activation of Cat D was required for the generation of an Ag85B epitope by macrophages. Reduced processing of Ag85B by H37Rv- and BCG-infected macrophages suggests that phagosome maturation arrest interferes with the efficient processing of Ags in macrophages. Because Ag85B is immunodominant, this state may lead to a decreased ability of the wild-type as well as the BCG vaccine to induce protective immunity. The Journal of Immunology, 2006, 177: 3250–3259.

Tuberculosis is the leading cause of death due to infections in humans today with at least 3 million deaths reported worldwide. The causative agent Mycobacterium tuberculosis (MTB)3 has the unique ability to survive within macrophages using diverse strategies. It can become dormant and revive later to cause reactivation. The latter is believed to be the leading cause of adult tuberculosis. The bacillus Calmette-Guérin (BCG) vaccine protects children against primary disease, but offers reduced and variable protection against the disease of young adults and reactivation (1). The search therefore continues for more effective vaccines and more effective strategies. Immunity to tuberculosis is dependent upon both CD4+ and CD8+ T cells, and macrophages and dendritic cells play a crucial role in the generation of immune response and subsequent containment of infection involving cytokines and chemokines (2). Extensive studies have therefore been performed on the paradoxical capacity of macrophages to harbor MTB leading to pathogenesis, as well as on the ability of macrophages to process mycobacterial Ags to trigger an immune response (3).

A conventional view of bacterial Ag processing is that the bacterial phagosome fuses with the lysosome, where bacteria are broken down by a variety of proteases like cathepsins in an acidiv environment (4). Peptides are then processed within the phagosomes or exported into an MHC class II-containing compartment, where other cathepsins like cathepsin S (Cat S) cleave the invariant chain and help in loading the peptides to the MHC complex (5). There is, however, continuing uncertainty on the mechanisms operative during mycobacterial Ag processing. Live, virulent MTB has been found to sequester within phagosomes of macrophages, which do not fuse with lysosomes (3, 6–8). Furthermore, several studies suggest that MTB down-regulates mycobacterial as well as nonmycobacterial Ag presentation by macrophages, decreases surface costimulation, decreases synthesis of MHC class II and CD1 molecules, reduces Ag processing, and inhibits T cell activation (9–12). Thus, it appears that the unique phagosomal sequestration of MTB enables a minimal contact with the Ag processing mechanisms of the macrophages.

Paradoxically, mycobacterial phagosomes (BCG and MTB strain H37Rv) do contain certain components of Ag processing. They have MHC class II molecules and cathepsin D (Cat D) (3,
In contrast, Cat D has the inactive 46-kDa form due to the near neutral pH of the phagosomes. Lack of acidification, in turn, appears to be due to the exclusion of vacuolar proton ATPase (v-ATPase) by virulent MTB and Mycobacterium avium phagosomes (14–16). v-ATPase has been found critical for acidification of phagosomes that leads to the pH dependent, autacatolytic generation of active forms of cathepsins (17, 18). A recent report shows that BCG can inhibit Cat S in macrophages through an IL-10-dependent mechanism (19). Thus, the biochemically identified contents of pathogenic mycobacterial phagosomes appear to be consistent with the fairly well-characterized “maturation arrest.”

Despite the perplexing nature of pathogenic mycobacterial phagosomes, an elegant study has shown that H37Ra-infected macrophages, and to some extent virulent H37Rv-infected macrophages, can process an Ag85 complex B protein, Ag85B epitope, at the phagosomal level itself and prime Ag85B-specific T cells through the MHC class II pathway (20). Thus, mycobacteria can prevent phagosome maturation, but macrophages can assemble some Ags at the phagosomal level, although the molecular mechanisms and significance of peptide assembly remain unclear.

We demonstrate in this study that the v-ATPase is an important initial determinant of the mycobacterial Ag85B processing by macrophages through a Cat D-dependent mechanism. Virulent MTB strain H37Rv, attenuated MTB H37Ra and BCG vaccine Ags were processed differently by murine macrophages due to the differences in the content and function of these proteins. Interestingly, Ag85B processing seems to be unrelated to the virulence of mycobacterial strains within macrophages. Ag85B is an immunodominant Ag of MTB and the DNA encoding Ag85B has been successfully used as a vaccine against tuberculosis in animal models (21–23). We therefore suggest that understanding the mechanisms of Ag85B or other similar peptide Ag presentations in macrophages may enhance our ability to prime adaptive immunity, generate immunological memory and develop successful vaccination strategies.

Materials and Methods

Macrophages

C57BL/6 mice bone marrow-derived macrophages (BMDMs) were cultured in DMEM with 10% FBS and 10 ng/ml mouse GM-CSF for 7 days, rested and used as monolayers in slide cultures for Ab stains, 24-well plates for IL-2 assays or growth curves and 15-ml flasks for phagosome preparations.

Mycobacteria and growth within macrophages

Stock cultures from American Type Culture Collection (ATCC) were grown in Dubos broth and used fresh after three washes in PBS. They were routinely >90% viable as evaluated by fluorescein diacetate stains. GFP-expressing H37Rv (ATCC 27294), H37Ra (ATCC 25177), and BCG (ATCC 35734) were prepared from ATCC stock as described previously (24). The Ag85B gene knockout strain (fhpBΔ) has been previously described by our group (25). It was cultured in Dubos broth with 25 µg/ml kanamycin and used earlier. Freshly grown unlabeled mycobacteria were also labeled with PKH-26 (Sigma-Aldrich) as per the manufacturer’s instructions to prepare red-labeled bacteria and used immediately for infections. PKH-26 red (PKR)-stained bacteria appeared somewhat shorter compared with GFP-expressing strains. Growth curves for mycobacteria in naive or IFN-γ-activated macrophages were determined by previously described methods (26).

Ag processing and presentation assays

The Ag85 epitope-specific (241-256) T cell hybridoma (BB7) was a gift from Drs. C. Harding and H. Boom (Case Western Reserve University, Cleveland, OH). It has been previously characterized to release IL-2 after specific binding to Ag85B peptide complexed with MHC class II (10). Macrophages were plated in 12-well plates and used as naive cells or were activated for 6 h with IFN-γ (400 U/ml). They were infected with mycobacteria at a multiplicity of infection (MOI) of 1:1, 1:5, or 1:10 as required.

After a 4-h infection, monolayers were washed and were either fixed with 1% paraformaldehyde for 15 min followed by a wash or were used directly to overlay T cells. Fixed macrophages were overlaid with BB7 T cells at a ratio of 1:20 and IL-2 in the supernatant was measured 4 or 24 h later using a sandwich ELISA kit (R&D Systems). Unfixed monolayers overlaid with BB7 T cells as described gave a better yield of IL-2. Thus, results with the latter assay are shown throughout this study. Viability of T cells was ascertained using alamar blue. Macrophages were added with 50 nM bafilomycin A1 during infection in both assay wells as indicated. The robustness and specificity of the IL-2 release assay was established by two criteria. First, macrophages were infected with an Ag85B knockout mutant (fhpBΔ) that was generated and characterized by our previous study (25). Macrophages are unable to process this mutant, and generate and present the Ag85B epitope because Ag85B is deleted. The fhpBΔ was thus included as an internal control in IL-2 assays. Second, the peritoneal macrophage derived cell lines PMJ2-PC (MHC class II+) and PMJ2-R (MHC class II deficient) have different levels of surface MHC class II, although both are phagocytic. Mycobacteria-infected PMJ2-R is unable to present the Ag85B peptide to T cells, indicating that MHC class II is essential for priming of the BB7 T cells (data not shown).

Phagosomal pH measurement

The pH of the phagosomes in the presence or absence of bafilomycin was measured by a ratiometric method using fluorescent dextran from Molecular Probes as described (DM-NERF dextran) (27).

Immunostains

Immunostains were performed as described elsewhere (28). Briefly, naive or IFN-γ-activated (400 U/ml for 18 h) or bafilomycin-treated (50 nM) macrophages infected at a ratio of 1:10 with mycobacteria for 4 h were washed and incubated for 24 h. Slides were washed three times with PBS, fixed in 2% fresh paraformaldehyde, permeabilized with 0.1% saponin and 0.05% Triton X-100 in PBS and 2% normal mouse serum, and stained with primary Abs for 18 h at 4°C (1500, Cat D Ab; Santa Cruz Biotechnology). Goat Ab to the A subunit of mouse v-ATPase (MTS5SQSQIQ QLAAEKRKAD-KLH) was produced by ProteinTech (data not shown). This Ab recognized a major band at 110 kDa (A unit) and at least two other bands of v-ATPase corresponding to the B and E unit and was similar in reactivity with the v-ATPase of the A subunit of the v-ATPase (29). Abs to mouse Cat D and mouse Rab7 were from Santa Cruz Biotechnology. Ab to mouse lysosome-associated membrane protein 1 (LAMP1, ID4B) was obtained from the Development Studies Hybridoma Bank. Macrophages were counterstained with Texas Red-conjugated Abs (Jackson ImmunoResearch Laboratories) for 2 h, washed again, mounted in elvanol mountant, and examined using a DeltaVision laser confocal microscope. Isotype controls were used for all experiments.

Cat D active site labeling

The active sites of Cat D can be probed using a fluorescent tracer, bodipy-peptatin-F1 (BFP; Molecular Probes) (30). Macrophages were infected with PKH-26-labeled red mycobacteria for 4 h (MOI, 1:1) incubated for 24 h, and then fixed and permeabilized. Macrophages were then labeled with 1 µg/ml BFP for 30 min, washed three times with permeabilization buffer, mounted and examined as described earlier. Control PKR-H37Ra infected macrophages were stained with 1 µg/ml unlabeled peptatin A (Sigma-Aldrich) as described.

Detection of phagosomal proteins by immunoblot analysis

Macrophages were grown as monolayers in 75-ml flasks and infected at a ratio of 1:2 with mycobacteria for 4 h, incubated for another 24 h. Phagosomes were fractionated as per the procedures described by Ulrich et al. (13, 31). Briefly, macrophages were scraped, washed three times in a fractionation buffer (PFB) with 10 mM HEPES, 5 mM EDTA, 5 mM EGTA (pH 7.0), and suspended in PFB with anti-protease mix consisting of 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM PMSF. Pellets were then homogenized in a glass tissue homogenizer 10 times and passed 10 times through a 28-gauge needle. Lysates were centrifuged at 500 × g for 5 min to sediment nuclei and the postnuclear supernatant was layered on a step gradient of 50 and 12% sucrose in PFB. After centrifugation at 10000 × g for 60 min, the interphase of phagosome fraction was collected and further purified by passing through two successive cushions of 70- and 400-kDa Ficoll in PFB as described before. The final purified phagosomal pellet was collected by centrifugation at 10,000 × g for 15 min and suspended in SDS sample buffer. Samples heated at 98°C for 5 min were then electrophoresed using either 7–15% or
10–15% gradient SDS gel electroblotted, and membranes were probed with Cat D Ab (C-20, SC6486; Santa Cruz Biotechnology). Bands were visualized using a chemiluminescence kit from Amersham Biosciences. Macrophages were infected with latex beads at a ratio of 1:5 for 1 h and phagosomes prepared after 4 h were analyzed as positive controls. Latex beads mature rapidly and hence were purified earlier at 4 h compared with mycobacteria. To detect mycobacterial Ag85 complex proteins, phagosomes were probed with TD17 mAb, a gift from Dr. K. Huygen (Pasteur Institute, Brussels, Belgium). TD17 specifically recognizes the three proteins of the Ag85 complex (32). The mAb HYT27 also has a similar activity against Ag85 complex (data not shown).

**Titration and intracellular effects of bafilomycin**

Cytotoxicity was tested as follows. Macrophages were incubated with varying doses of bafilomycin (10-fold increase from 0.1 to 100 nM), and BMDMs were tested for viability over 72 h using alamar blue. A dose of 50 nM was found nontoxic for over 24 h. To determine whether bafilomycin affected the delivery of Cat D to phagosomes, rhodamine signals of BMDMs were infected with mycobacteria at an MOI of 1:1, and incubated for 24 h in the presence or absence of 50 nM bafilomycin. Phagosomes were then fractionated and Western blot analysis was performed using an Ab to Cat D as described. Latex beads were internalized as controls and their phagosomes fractionated at 4 h after phagocytosis.

**Small interfering RNA (siRNA) inhibition of Cat D and IL-2 response**

The siRNA kit from Santa Cruz Biotechnology was used as per the manufacturer’s instructions to inhibit Cat D in macrophages. The siRNAs contain a mix of three sequences tailored for inhibition of murine Cat D: CUGCCGCAAGUCUCAUCUAU, CUCACUCAUUGCAAAGAUAtt, and CUAACCCUAAUCUCUG-UUUtt. The control siRNA contained CUGCGCAAGUUCACAUCUAtt, CUCCAUUCAUUGCAAGAUAtt, and CUACCCUAAUCUCUG-UUUtt. The control siRNA contained three matched sequences, each of which had a missense. Macrophages plated in 12-well plates were transfected with siRNA or control siRNA for 24 h. Macrophages were then washed and infected with the GFP strains of mycobacteria for 4 h and washed. They were overlaid with BB7 T cells as positive controls. Latex phagosomes prepared after 4 h were analyzed as positive controls. Latex beads were internalized as controls and their phagosomes fractionated at 4 h after phagocytosis.

**Results**

**Growth curves of MTB strains in macrophages and effects of IFN-γ**

One measure of the virulence of MTB strains is their ability to grow progressively in macrophages. Fig. 1 shows that the virulent H37Rv strain of MTB was able to grow at least by 1 log_{10} over 7 days, whereas the avirulent H37Ra strain as well as the attenuated BCG vaccine showed a modest decline. However, when macrophages were activated with IFN-γ, all three strains showed a further decline in viability.

**Phagosomes of virulent and avirulent mycobacterial strains show a differential acquisition of v-ATPase in macrophages**

Although lysosomes are extensively acidified by v-ATPase, bacterial phagosomes also acquire v-ATPase independent of lysosomal fusion (27, 33, 34). To examine whether mycobacterial phagosomes acquired v-ATPase, freshly grown, highly viable (> 90%) GFP-expressing strains of mycobacteria were phagocytosed into naïve as well as IFN-γ-activated macrophages, and stained for mouse v-ATPase 24 h after infection followed by Texas Red-labeled anti-Ig conjugates. Laser confocal microscopy showed that avirulent H37Ra acquired v-ATPase even in naïve macrophages (Fig. 2A, top). H37Rv and BCG phagosomes effectively excluded v-ATPase. Prior activation of macrophages with IFN-γ enhanced the colocalization of v-ATPase on to all three mycobacterial phagosomes (Fig. 2B). Quantitative scoring of v-ATPase acquisition has been shown in Fig. 2C. The exclusion of v-ATPase by virulent H37Rv and BCG is consistent with previous reports that MTB excludes v-ATPase (16). Because IFN-γ is known to enhance phagosome maturation (35, 36), its enhancing effects were anticipated. Acquisition of v-ATPase by H37Ra phagosomes is different from the report of the Russell group (16), who found that H37Ra excluded v-ATPase in macrophages. Although we have no clear explanation for the discrepancy, we used a different preparation of polyclonal monospecific Ab to the A subunit of v-ATPase that was nonetheless similar in specificity to the mAb used in the latter study (data not shown). To verify that the mycobacterial strains behaved within the macrophages as reported in literature, macrophages were also stained with Abs to Rab7, which is known to be excluded by BCG and H37Rv, and to LAMP1, which is acquired by all mycobacterial phagosomes to varying levels (3, 37). These studies showed that all three mycobacterial phagosomes acquired LAMP1 to varying degrees but uniformly excluded Rab7 (Fig. 3). This result confirmed that our mycobacterial strains trafficked as anticipated with regard to two well-established phagosomal markers.

These studies together indicate that v-ATPase is excluded by H37Rv and BCG phagosomes. IFN-γ enhances the phagosome maturation process in macrophages and a part of this process is evidently the delivery of v-ATPase to phagosomes. v-ATPase can be delivered to avirulent H37Ra phagosomes even without IFN-γ. Thus, v-ATPase delivery to mycobacterial phagosomes appears to be modulated by an intrinsic property of mycobacteria. This property does not appear to be related to their virulence because both BCG and H37Ra are attenuated, but only H37Ra phagosomes acquire v-ATPase.

**Differential acidification of mycobacterial phagosomes is due to v-ATPase**

Because mycobacterial phagosomes differentially acquired v-ATPase, the pH of the phagosomes was determined using the ratiometric method with fluorescent-labeled dextrans (27). Macrophages were incubated in the presence or absence of bafilomycin, a v-ATPase-specific inhibitor. BCG as well as H37Rv phagosomes maintained their pH above 6.0 over 3 days. This result is consistent with the previous reports that H37Rv and BCG phagosomes have near neutral pH (Fig. 4A). H37Ra phagosomes showed a striking decline in the pH over 3 days of incubation. Bafilomycin inhibited the colocalization of v-ATPase with H37Ra phagosomes (Fig. 4B) that appears to have effectively raised the pH to near 6.0. Thus,
v-ATPase localization is the major mechanism of acidification in mycobacterial phagosomes because blockade of this enzyme prevented their acidification.

Differential generation of active forms of Cat D within mycobacterial phagosomes

Cat D is synthesized by naive as well as mycobacteria-infected macrophages (data not shown). Cat D is an aspartic protease that is targeted to the phagosomes early during phagosome maturation (38, 39). Cat D is synthesized in the rough endoplasmic reticulum of the trans Golgi network in a 51- or 53-kDa form and it is broken down into a 46- to 47-kDa intermediate form while in transit to phagosome (13, 31). Active forms of Cat D, 30–31 kDa and 18–20 kDa, are produced by the active ATP-driven acidification of the phagosome, most likely due to v-ATPase (40). Previous reports show that despite the ability of MTB strains to inhibit maturation, their phagosomes contain abundant Cat D, but in its immature 46-kDa form (13, 16, 31). It has also been shown that Cat D does not break down to its active forms (30 and 18 kDa) due to the near neutral pH of mycobacterial phagosomes (13). To detect phagosomal Cat D, macrophages were infected with mycobacterial strains and incubated in the presence or absence of bafilomycin.
Phagosomes of mycobacteria were then fractionated at 6 and 24 h postinfection using sucrose gradients and purified pellets analyzed by Western blot using Abs to Cat D. Latex beads were internalized and analyzed as positive controls. As anticipated, H37Ra phagosomes showed a progressive drop in the pH that is restored by prior incubation with 50 nM bafilomycin (*, p < 0.01, using t test). BCG and H37Rv phagosomes maintain their pH (± SEM) above 6.0. Data represent three independent experiments.

**FIGURE 4.** Effect of bafilomycin on v-ATPase-dependent regulation of pH in mycobacterial phagosomes. BMDMs were infected with *gfp* strains of mycobacteria, incubated untreated or treated with 50 nM bafilomycin. The pH of phagosomes was measured using a ratiometric method with fluorescent dextrans (A) or the macrophages stained for v-ATPase 24 h later (B). A, H37Ra phagosomes show a progressive drop in the pH that is restored by prior incubation with 50 nM bafilomycin (*, p < 0.01, using t test). BCG and H37Rv phagosomes maintain their pH (± SEM) above 6.0. Data represent three independent experiments. B, Laser confocal microscopy illustration showing bafilomycin inhibits v-ATPase colocalization with *gfp*-H37Ra phagosomes. Bafilomycin effects on BCG and H37Rv are not apparent because they exclude v-ATPase on their own (data not shown).

Phagosomes of mycobacteria were then fractionated at 6 and 24 h postinfection using sucrose gradients and purified pellets analyzed by Western blot using Abs to Cat D. Latex beads were internalized and analyzed as positive controls. As anticipated, H37Ra phagosomes contained a strong 46-kDa Cat D band (Fig. 5). H37Ra, conversely, contained the 30-kDa band, and treatment with bafilomycin prevented the 46-kDa band from substantially degrading into the 30-kDa form. This finding suggested that bafilomycin prevented Cat D breakdown by blockade of v-ATPase that, in turn, maintained the pH near 6.0.

**FIGURE 5.** Cat D activity in purified mycobacterial phagosomes. BMDMs were infected with mycobacteria for 4 h and incubated for 6 or 24 h in the presence or absence of bafilomycin. Phagosomes were purified on sucrose gradients and tested for Cat D in Western blot analysis. Macrophages infected with latex beads, which mature rapidly and were purified earlier compared with mycobacteria, were analyzed as positive controls (Mr, molecular weight marker). H37Rv and BCG phagosomes show the 46-kDa form of Cat D, whereas H37Ra shows a significant 30-kDa form. Bafilomycin treatment of H37Ra prevents substantial breakdown of the 46-kDa form of Cat D. One of three similar experiments is illustrated.

*Specificity of bafilomycin and active site labeling of Cat D*

Even though bafilomycin is a specific inhibitor of v-ATPase, it has been reported to affect endosomal sorting events between the plasma membrane and early endosomes (41, 42). Although Cat D is transported from trans Golgi network to phagosomes and was not likely to be affected, we sought to rule out potential interference by bafilomycin. Phagosomes of BCG, H37Ra, and H37Rv were therefore harvested from bafilomycin-treated macrophages along with postnuclear supernatants and were probed for Cat D. Fig. 6A shows that bafilomycin treatment of macrophages did not affect the presence of Cat D forms (46 kDa plus traces of 30 kDa) in the postnuclear supernatants representing the cytosol or in the purified phagosome pellets containing the immature 46-kDa form. To further confirm that Cat D in H37Ra phagosomes was enzymatically active, the fluorescent active site specific probe BPF was used (30). BPF colocalized with red-labeled H37Ra phagosomes, but not with H37Rv or BCG phagosomes (Fig. 6, B and C). Finally, to verify that phagosomes contained mycobacterial Ags relevant to this study, they were probed with TD17 mAb specific for Ag85 complex (32). Two major bands were observed in all three strains (Fig. 6D). The bands corresponded to protein A (32 kDa) and protein B (31 kDa) proteins (C protein was at the same size as A protein) (25). The fbpBΔ mutant that lacks Ag85B, as anticipated, gave only a thick band around 32 kDa. It should be noted that as much as 5 μg of protein per lane of phagosomal protein had to be loaded to detect the Ag85 complex proteins. In summary, these studies indicate that bafilomycin inhibits v-ATPase delivery that prevents the breakdown of immature Cat D to active forms within the H37Rv and BCG phagosomes. H37Ra phagosomes acidify and allow autocatalytic production of the 30-kDa Cat D active form.

*Ag85B presentation by macrophages*

Whereas v-ATPase acidifies the phagosomes, active forms of Cat D can generate peptides that can be subsequently presented to T cells through the MHC class II complex (5, 43). Cat D-generated peptide fragments of mycobacteria are known to be T cell reactive (44). We thus hypothesized that v-ATPase, Cat D, and Ag85 complex Ags within the mycobacterial phagosomes could lead to peptide presentation. A T cell hybridoma specific to Ag85B epitope has been described and has been extensively used to measure Ag85B processing in macrophages (10, 45). This cell line was used in this study to determine the ability of macrophages infected with mycobacteria to process and present the epitope (aa 241–256) from Ag85B. Naive or IFN-γ-activated macrophages were infected and incubated in the presence or absence of bafilomycin. In preliminary validation studies, macrophages infected with wild-type or *gfp*-expressing mycobacteria showed nearly identical ability to induce IL-2 from T cells and, thus the profiles for macrophages with unlabeled strains are shown. Fig. 7A illustrates that H37Ra-infected macrophages were able to prime a significant IL-2 release, whereas BCG-infected macrophages were modest inducers of IL-2. H37Rv-infected macrophages were unable to induce significant IL-2 on their own. It should be noted that IL-2 levels were measured at two time intervals (Fig. 6A at 4 h and 6B at 18 h, post overlay). Macrophages infected with the fbpBΔ mutant that lacks the Ag85B were unable to prime for IL-2, thus confirming the specificity of the assay. Fig. 7 also illustrates the effects of IFN-γ and bafilomycin on macrophage-mediated priming. Prior activation of macrophages with IFN-γ enhanced the IL-2 levels induced by all three types of macrophages. IFN-γ-activated macrophages increased T cell-derived IL-2 production most likely
through enhancing v-ATPase colocalization. In contrast, bafilomycin treatment of macrophages inhibited IL-2 release, suggesting that acidification of phagosomes via v-ATPase was necessary. Together, these observations suggested that bafilomycin sensitive proteases were involved within the macrophages for the production of peptide epitopes necessary to prime IL-2.

Cat D plays a major role in the processing of Ag85B

Although Cat D, Cat B, Cat H, Cat F, and others have been reported to be capable of generating peptides during lysosomal degradation of Ags (17, 18), Cat D was the most prominent protease we found in mycobacterial phagosomes (Fig. 5). We therefore sought to determine whether Cat D was involved in the generation of Ag85B peptide within macrophages that could then be loaded into the MHC class II complex before presentation. Macrophages were therefore transfected with siRNA specific for mouse Cat D and incubated for 18 h. Control macrophages were transfected with missense siRNA. Fig. 8A shows the dose response titration of the siRNA and a 50-nM dose was used in all subsequent experiments. In another specificity control assay, siRNA-treated macrophages were tested for Cat D as well as other cathepsins. Fig. 8B shows that siRNA caused a specific inhibition of Cat D, but not of three other cathepsins.

siRNA treated and washed macrophages were infected with GFP strains of mycobacteria and tested for Ag presentation using BB7 T cells. In addition, macrophages were stained for Cat D using immunostains. Macrophage-T cell coulture supernatants were tested for IL-2 at 24 h after overlay because H37Rv produced very little IL-2 after a 4-h overlay. The results with IL-2 were dramatic. There was a marked reduction of IL-2 when macrophages were pretreated with siRNA for Cat D, indicating that Cat D was involved in the peptide generation process within H37Ra- and BCG-infected macrophages (Fig. 8C). Thus, processing and generation of Ag85B epitope appears to involve the enzymatic action of Cat D within macrophages.

Intracellular sites of processing for Ag85B

Fig. 5 showed a paradoxical observation. BCG and H37Rv phagosomes did not contain active forms of Cat D as assessed by Western blot analysis. Yet, they induced modest levels of IL-2 that was inhibited almost completely by siRNA to Cat D (Fig. 8C). This result suggested a possibility that trace amounts of active Cat D forms present in their phagosomes, but not detectable by Western blots, could have generated the Ag85B epitope. The other possibility is speculative but more intriguing. Being a secreted Ag, Ag85B is known to be exported to other compartments in the macrophages from the phagosomes. Previously, immunoelectron microscopic studies showed the occurrence of Ag85 complex proteins near the vicinity of phagosomes in human monocytes (46). We hypothesized that Ag85B could localize to certain compartments in the macrophages like MHC class II-containing compartments, where they can encounter mature forms of Cat D. This process could, in turn, generate an Ag85B epitope required for triggering IL-2 from T cells. We thought that this mechanism could explain why both BCG and H37Rv did not contain active forms of Cat D but still induced some IL-2 that was inhibitable by bafilomycin as well as siRNA for Cat D. It should be noted in this model that BCG and H37Rv prevent acidification of their phagosomes through intrinsic factors thus preventing Cat D breakdown. However, if soluble Ags escape from phagosomes and sequester within other compartments, mycobacteria may not be able to modulate the pH regulation of such compartments. Indeed, laser confocal microscopy analysis revealed that in mycobacteria-infected macrophages stained for v-ATPase as well as for Cat D, as many as 10% of macrophages showed an atypical staining pattern. On occasion, mycobacteria colocalized with Cat D, but spherical and
tubular cytoplasmic sites staining positively for Cat D or v-ATPase and green fluorescent bacterial material were also evident (Fig. 9). We suggest that such sites could potentially harbor soluble mycobacterial Ags, Cat D and v-ATPase. Efforts are underway to demonstrate colocalization of these molecules using corresponding Abs (e.g., TD17) and triple color imaging.

Discussion

Many Ags of MTB are immunogenic in humans and animals. Mice immunized with BCG are protected against subsequent challenge against virulent MTB. MTB and BCG both induce strong T cell as well as Ab responses. These observations confirm that mycobacterial Ags are processed within macrophages and dendritic cells and are presented to T cells. However, the mechanisms of protein Ag processing in mycobacteria remain poorly understood with the exception of Ag85B. Extensive studies by the Harding and Boom groups (10–12, 20, 45) have shown that Ag85B is processed at the phagosomal level in macrophages infected with virulent and avirulent MTB strains, and one of its epitopes (aa 241–256) is rapidly presented to T cells. Initial studies showed that virulent MTB are processed less efficiently compared with avirulent strains. Subsequent studies showed that live virulent, live avirulent and virulent MTB killed by different means were all processed at different rates and thus processing of Ag85B was independent of the viability of mycobacteria. Based on these studies, Ramachandra et al. (20) suggested that mechanisms of phagosome maturation inhibition by MTB are likely to be different from those modulating Ag processing.

However, based on our observations in this study, we suggest that phagosome maturation events are interlinked with Ag processing and that MTB-mediated maturation arrest can affect its immunogenicity. A brief discussion of mycobacterial maturation phenomenon is relevant to clarify this issue. Lack of phagosome-lysosome fusion is the hallmark of live virulent MTB and many recent studies have examined the molecular basis. MTB phagosomes strongly acquire markers of early endosome fusion (e.g., transferrin receptor and Rab5) but the markers of late endosomes and lysosomes such as CD63 and LAMP1 are weakly acquired (3, 47). Rab7, a marker of lysosomes, has been proposed by some to be excluded by MTB, although in other studies, MTB stained positively for Rab7 but still avoided lysosomal fusion (8, 48). In murine macrophages, a coat protein TACO on mycobacterial phagosomes has been shown to prevent maturation (49). More recent

FIGURE 7. Macrophages infected with mycobacterial strains differ in processing and presenting an Ag85B epitope to the T cells. Naive, bafilomycin-treated or IFN-γ-activated BMDMs were infected with mycobacteria and the fbpBΔ control strain for 4 h, washed, and overlaid with BB7 T cells and supernatants tested 4 h (A) or 18 h (B) later for IL-2 by sandwich ELISA. BMDMs infected with H37Ra induce copious IL-2 that is inhibited by bafilomycin. BCG-infected BMDMs also induce IL-2 inhibitable by bafilomycin, whereas H37Rv-infected BMDMs induce very little IL-2. IFN-γ activation of BMDMs induces more IL-2 by BMDMs infected with all three strains. Data represent four independent experiments (± SEM), and significance was tested using the t test.

FIGURE 8. Interference of Cat D activity in macrophages using siRNA inhibits Ag85B presentation. A, BMDMs were transfected with siRNA for Cat D or missense control siRNA, washed, and incubated for 24 before lysates were probed for Cat D using Western blot analysis. Dose titration shows inhibition of Cat D synthesis in BMDM lysates. B, siRNA Cat D-transfected BMDMs (50 nM) were infected at 24 h, and lysates harvested another 24 h later were tested for Cat D as well as other cathepsins that are constitutively synthesized by BMDMs. Latex beads were internalized in macrophages as controls (Mr, molecular weight marker). siRNA for Cat D affects only Cat D but not Cat B, Cat H, or Cat F. C, Inhibition of Cat D using siRNA interferes with the presentation of Ag85B by BMDMs. BMDMs were transfected as described and overlaid with BB7 T cells and IL-2 measured after 24 h. siRNA mediated interference with Cat D inhibits Ag85B processing by BMDMs (*, p < 0.006; **, p < 0.009, using t test). Missense siRNA has no effect on IL-2 levels. Data represent three independent experiments.
studies have shown that mycobacterial lipids such as lipoarabinomannan interfere with the endosomal sorting events, whereas other lipids like phosphatidylinositol mannoside promote early endosomal fusion (50, 51). Although differences are likely to be exist between human and mouse macrophages in terms of phagosomal trafficking regulating fate of mycobacteria, it appears certain that MTB affects endosomal sorting events. As a consequence of a selective ability to fuse with early endosomes and exclusion of late endosomes, MTB phagosomes have a distinct character in terms of Ag-generating machinery. They acquire Cat D, but due to the exclusion of v-ATPase, they are inactive. MHC class II has been demonstrated in MTB-infected murine phagosomes but not Cat S, which is required for loading peptides to MHC class II (3). Although many cathepsins appear to take part in microbial degradation within lysosomes, only Cat D has been consistently found in mycobacterial phagosomes. However, it is interesting to note that both in murine macrophages as well as in human macrophages, mycobacteria like BCG acquire immature forms of Cat D that suggests that their phagosomes are near neutral in pH despite differences in the origin of macrophages (13, 52).

Data presented in this study link phagosome maturation arrest events to Ag processing in two ways. First, we showed that BCG, H37Ra, and H37Rv phagosomes differentially acquired v-ATPase within macrophages that led to a differential acidification. IFN-γ enhanced the v-ATPase delivery to all three mycobacterial phagosomes, although it should be noted that in murine macrophages, mycobacteria like BCG acquire immature forms of Cat D that suggests that their phagosomes are near neutral in pH despite differences in the origin of macrophages (13, 52).

In support of our suggestion, it is interesting to note that the BCG-overexpressing Ag85 complex was found more effective than native BCG vaccine in the mouse model (56, 60). In view of our data that cytosolic vesicles of macrophages stained for bacterial products as well as for Cat D and v-ATPase, we further propose that copious amounts of overexpressed Ag85B was probably exported from BCG to vesicular compartments in the cytosol to be processed and presented, thus accounting for these mice being immunized more effectively with various Ags of BCG including Ag85B. In other words, BCG may only present a part of its Ag repertoire, depending on its ability to interfere with maturation.

FIGURE 9. Mycobacteria-infected BMDMs show evidence of cytosolic mycobacterial products. BMDMs were infected with gfpH37Ra or gfpH37Rv for 4 h, washed, and incubated for 24 h, fixed and stained for Cat D. The ~10% of BMDMs show tubular and vesicular staining. Two profiles of H37Ra and H37Rv phagosomes are shown. gfpH37Ra-infected macrophages (left) show scattered cytosolic spherical and tubular vesicles (arrow) along with colocalization (arrowhead). gfpH37Rv-infected macrophages (right) show fewer numbers of such vesicles.

surprisingly, we found that the BCG vaccine produced less IL-2 than H37Ra, even though in mouse macrophages both showed an attenuated growth. That these strains induced different levels of IL-2 was, however, related to their ability to acquire v-ATPase that led to the differential accumulation of active Cat D isoforms. It is pertinent to note that BCG down-regulates mechanisms of Ag processing in murine macrophages through a 19-kDa lipoprotein-dependent mechanism (12). It is also possible that the lipids of BCG may interfere with maturation (50). We suggest that attenuation in mycobacterial growth within macrophages may not necessarily mean better Ag processing.

The latter observation has an interesting implication for BCG vaccine efficacy in humans. Though protection rates for BCG range from 0 to 80%, the reasons for variable efficacy remain unclear (55–57). Vaccinated individuals may respond differently to BCG, but it is also known that BCG lacks some immunodominant Ags of MTB (e.g., ESAT-6, CFP-10) or expresses them in less than required levels in its sub-strains (e.g., MPT64) (58). Furthermore, BCG has less than optimum efficacy in mice. For example, in the well-established mouse model of BCG vaccine evaluation, aerosol-challenged organisms grow nearly similar to the unvaccinated control for ~2 wk despite the fact that mice have been vaccinated 4 wk earlier with BCG (59). Given the propensity of BCG to prevent phagosome maturation (8, 37), the inability to acquire v-ATPase (our study), generate active forms of Cat D (52), and trigger inefficient IL-2 (our study), we propose that mice do not receive adequate priming by various Ags of BCG including Ag85B. In other words, BCG may only present a part of its Ag repertoire, depending on its ability to interfere with maturation.

In support of our suggestion, it is interesting to note that the BCG-overexpressing Ag85 complex was found more effective than native BCG vaccine in the mouse model (56, 60). In view of our data that cytosolic vesicles of macrophages stained for bacterial products as well as for Cat D and v-ATPase, we further propose that copious amounts of overexpressed Ag85B was probably exported from BCG to vesicular compartments in the cytosol to be processed and presented, thus accounting for these mice being immunized more effectively with Ag85B. This line of thinking suggests that Ag85 complex proteins are important for priming protective immunity in mice. Indeed, Ag85 complex is the most abundant protein complex secreted by MTB and Ag85B is one of the most frequently recognized Ags by the mouse and human immune system (61). DNA vaccines encoding for Ag85A and Ag85B have been found vaccinogenic in mice, whereas recombinant BCG overexpressing the Ag85 complex is a more effective vaccine than BCG (21, 23, 56). Due to the lack of appropriate T cells, it was not possible to measure processing of Ag85A in our study. However, it is obvious that MTB secretes Ag85B to prime T cells to induce a powerful immune response and this study shows that both v-ATPase and Cat D play a major role in its processing.

Because both H37Rv and BCG prevent acquisition of v-ATPase, a key factor in the acidification of phagosomes (16), we suggest that Ag processing in macrophages infected with these organisms is very likely to remain incomplete. Thus, virulent...
H37Rv can interfere with Ag processing, prevent an efficient immune response and survive to cause pathogenesis. In contrast, BCG may become much less effective by presenting only some of its Ags. In conclusion, this study demonstrates a key role for v-ATPase and Cat D in the macrophages during Ag85B processing. Yet this study raises additional questions. We analyzed presentation of one immunodominant epitope in this study and mycobacterial Ags are probably very diverse and are presented through multiple mechanisms. Although Cat D and related cathepsins generate peptides, it is perhaps Cat S that helps in loading the peptides to the MHC class II complex (5). It is possible that even though Cat D or similar cathepsins generate peptides for processing, Cat S or other cathepsins are essential for assembly mechanisms and the effect of MTB on the function of latter cathepsins remains unclear. Differences in the processing of structural vs secreted mycobacterial Ags are probably very diverse and are presented through MHC class II molecules through IL-10-dependent inhibition of cathepsin S (J. Immunol. 157: 5324–5332.


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Disclosures
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