Mycobacterium bovis Bacillus Calmette-Guérin Secreting Active Cathepsin S Stimulates Expression of Mature MHC Class II Molecules and Antigen Presentation in Human Macrophages[1]

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A successful Th cell response to bacterial infections is induced by mature MHC class II molecules presenting specific Ag peptides on the surface of macrophages. In recent studies, we demonstrated that infection with the conventional vaccine *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) specifically blocks the surface export of mature class II molecules in human macrophages by a mechanism dependent on inhibition of cathepsin S (Cat S) expression. The present study examined class II expression in macrophages infected with a rBCG strain engineered to express and secrete biologically active human Cat S (rBCG-hcs). Cat S activity was completely restored in cells ingesting rBCG-hcs, which secreted substantial levels of Cat S intracellularly. Thus, infection with rBCG-hcs, but not parental BCG, restored surface expression of mature MHC class II molecules in response to IFN-γ, presumably as result of MHC class II invariant chain degradation dependent on active Cat S secreted by the bacterium. These events correlated with increased class II-directed presentation of mycobacterial Ag85B to a specific $CD4^+$ T cell hybridoma by rBCG-hcs-infected macrophages. Consistent with these findings, rBCG-hcs was found to accelerate the fusion of its phagosome with lysosomes, a process that optimizes Ag processing in infected macrophages. These data demonstrated that intracellular restoration of Cat S activity improves the capacity of BCG-infected macrophages to stimulate $CD4^+$ Th cells. Given that Th cells play a major role in protection against tuberculosis, rBCG-hcs would be a valuable tuberculosis vaccine candidate. ***The Journal of Immunology,*** **2007, 179: 5137–5145.**

It is estimated that one-third of the global population is asymptomatically infected with *Mycobacterium tuberculosis* (Mtb)[4] and that 10% of infected individuals will develop active tuberculosis (TB) as a result of reinfection or reactivation of an original infection (1). This risk is increased ~10-fold in individuals coinfected with HIV (2, 3). The intracellular Mtb bacterium has developed sophisticated strategies to persist inside the host macrophage within a vacuole that escapes intracellular killing mechanisms (4, 5).

Current strategies to limit the spread of TB infection with antibiotics is facing many problems, including: 1) difficulties in accessing health services for most TB patients and 2) the logistics required for the administration of multiple drugs over periods of 6 mo or more to avoid emergence of drug resistance. Indeed, new antibiotics that could have the potential to shorten the duration of therapy and prevent resistance development are still at the experimental stage (6). Thus, improved preventive vaccination is still considered as the best cost-effective strategy to stop the spread of TB, especially in developing countries (1, 7). In this respect, the availability of an effective vaccine by the year 2015 was recently proposed as a realistic objective by The Global Plan to Stop TB (World TB Day, March 24, 2006). To reach this goal, it is estimated that at least 20 vaccine candidates should enter phase I safety trials with about half going forward phase II trials by the year 2015 (7).

The current bacille Calmette-Guérin (BCG) vaccine was introduced in 1921 and is still the only option available to prevent TB disease. It is derived from a live, weakened strain of *Mycobacterium bovis*, which causes TB in cattle. BCG has been shown to protect against meningeal TB in young children (8, 9) but is unreliable against the highly infectious adult pulmonary TB (10, 11). Therefore, designing a better TB vaccine is a major goal of many investigators in the field of TB research.

Although BCG vaccine is generally safe and rarely induces disease in humans, it appears to mimic virulent Mtb strains in their capacity to block phagosome maturation (12–14) and to inhibit Ag processing and presentation to Th cells. Indeed, earlier studies showed that BCG organisms growing in human macrophages were sequestered from recognition by immune $CD4^+$ cells although infected cells continued to express MHC class II molecules (15).

Newly synthesized class II α- and β-chains in the macrophage associate with the invariant (Ii) chain and then exit the endoplasmic reticulum subsequently localizing to an acidic endosomal/lysosomal compartment referred to as the MHC class II compartment (MIIC) (16). In the MIIC, removal of the Ii chain and peptide

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[4] Abbreviations used in this paper: Mtb, *Mycobacterium tuberculosis*; TB, tuberculosis; BCG, bacillus Calmette-Guérin; Ii chain, invariant chain; MIIC, MHC class II compartment; Cat S, cathepsin S; OADC, oleic acid, albumin, and dextrose solution; PPD, purified protein derivative; wt, wild type; MDM, monocyte-derived macrophage; MFI, mean fluorescence intensity; TR-DXT, Texas Red-conjugated dextran.

loading are believed to be critical for appropriate export of peptide-loaded class II molecule to the cell surface (17, 18). Using the human macrophage model of THP-1 cells and primary macrophages, we found a reduced surface export of class II molecules (~50% inhibition) in BCG-infected cells by a mechanism dependent on intracellular sequestration of α, β dimers despite an apparent normal signaling response to IFN-γ (19). Further studies examining the nature of class II molecules that do reach the cell surface in BCG-infected cells revealed a predominance of Ii chain-associated class II molecules and suggested that mycobacteria specifically block the surface export of peptide-loaded class II molecules (20). The same study demonstrated that cell infection with BCG inhibited both basal and IFN-γ-induced cathepsin S (Cat S) expression by a mechanism dependent on IL-10.

Cat S protease is crucial for the maturational processing of MHC class II (degradation of the Ii chain) before their export to the macrophage cell surface (21–23). Inhibition of Cat S expression in BCG-infected cells correlated with a significant reduction in the expression of mature, peptide-loaded class II molecules, and an increase in the export of an immature (associated with the Ii chain) class II population to the surface of macrophages (20). Thus, impaired surface expression of mature class II molecules in BCG-infected cells may at least partially explain the failure of the vaccine BCG to induce efficient antituberculosis immunity.

In the present study, a rBCG strain secreting active human Cat S was engineered and evaluated for its capacity to induce normal intracellular trafficking of class II molecules. The results obtained provided evidence for complete restoration of Cat S activity which resulted in surface expression of mature MHC class II molecules and the subsequent presentation of mycobacterial Ags to Th cells.

Materials and Methods

Reagents and chemicals

RPMI 1640, HBSS, PMA, protease inhibitor mixture, PMSF, and trypsin-EDTA were obtained from Sigma-Aldrich. Monoclonal anti-Cat S was obtained from Calbiochem. Anti-HLA-DR mAb (clone L243), anti-Ii mAb (clone LN2), and irrelevant isotype-matched IgGs were obtained from BD Pharmingen. Mouse anti-human Lamp-1 mAb (H4A3) was obtained from Hybridoma Bank of the University of Iowa (Iowa City, IA). FITC-conjugated $F(ab')_2$ goat anti-mouse IgG and HRP-conjugated goat anti-mouse IgG were obtained from Sigma-Aldrich. Texas Red-labeled $F(ab')_2$ goat anti-mouse IgG was obtained from Caltag Laboratories. Human rIFN-γ was a gift of Genentech. Cat S inhibitor Z-Phe-Leu-COCHO and recombinant active form of human Cat S were obtained from Calbiochem. Profect P1 reagent was obtained from Targeting Systems, Cat S substrate Z-Val-Val-Arg-AMC was obtained from Bachem Bioscience, and Texas Red-conjugated dextran (40,000 molecular mass) was obtained from Molecular Probes (Invitrogen Life Technologies).

Bacteria and plasmids

Escherichia coli strain DH5α (Invitrogen Life Technologies) was grown on Luria-Bertani (Difco) medium. The shuttle vector pSMT3 (24) was provided by Dr. M. Abdallah (Vrije Universiteit Medical Centre, Amsterdam, The Netherlands). *M. bovis* BCG Pasteur (strain 1173P2) was grown in Middlebrook 7H9 broth (Difco) supplemented with 10% (v/v) oleic acid, albumin, and dextrose solution (OADC; Difco) and 0.05% (v/v) Tween 80 (Sigma-Aldrich) at 37°C on a shaker. Bacteria were harvested by centrifugation and pellets were suspended in complete medium plus 10% glycerol. Mycobacterial cultures were stored in aliquots (~5×10^8/vial) at −70°C. Before infection, BCG organisms were grown 48 h in 7H9-OADC and opsonized as follows: 10^9 mycobacteria were suspended in 1 ml of RPMI 1640 containing 20% human serum (AB^+ and purified protein derivative negative (PPD^-)) and rocked for 30 min at 37°C. Bacteria were then pelleted and suspended in 1 ml of RPMI 1640 and clumps were disrupted by multiple passages through a 25-gauge needle. To evaluate the phagocytosis of mycobacteria by THP-1 cells, bacteria (10^9/ml) were labeled by incubation with FITC (Sigma-Aldrich) at 1 μg/ml in 7H9 for 2 h at 37°C. Thereafter, FITC-labeled bacteria were washed twice with 7H9, then opsonized and used for infection as described above.

*Construction and transformation with the mycobacteria/*E. coli *shuttle expression vector pSMT3-hcs*

The sequence corresponding to the active domain of Cat S (GenBank NM_004079) was amplified by PCR from synthesized cDNA using primers catS1 (5′-ATCTAAGCTTTTGCCTGATTCTGTG-3′ containing a 5′ *Hin*dIII adaptor) and catS2 (5′-TTGGG*ATCGAT*CTAGATTTCTGGGTA-3′, containing a 5′ *Cla*I adaptor) and cloned into *Hin*dIII and *Cla*I sites leading to pSMT-CatS construct (Fig. 1*A*). The 156-bp DNA fragment spanning the ribosomal binding site and the signal sequence derived from BCG Ag85B gene (GenBank M21839) (25) was amplified by PCR using primers α forward (5′-CCAACCCTGCAGCGACGACATACAGGACAA AGGGGCA-3′) and α reverse (5′-CCGGAAAAGCTTCGCGCCCGCG GTTGCCGCTCCGCC-3′). The amplicon was then inserted upstream of Cat S between the *Pst*I and *Hin*dIII restriction sites thereby generating the shuttle vector, pSMT3-hcs (Fig. 1*B*), in which the expression of α Ag signal sequence translationally fused to Cat S is under the control of the heat shock protein 60 promoter. The signal sequence targets the expressed protein for secretion through the mycobacterial cell membrane and wall. The recombinant vector was electroporated into *M. bovis* BCG strain at 2.5 kV, 1000 Ohm, and 25 μF in 0.2-cm cuvettes (Bio-Rad) and transformants were selected on standard 7H10 agar medium in the presence of 50 μg/ml hygromycin (24).

Cell culture

The human monocytic cell line THP-1 (American Type Culture Collection) was cultured in RPMI 1640 supplemented with 5% FCS (Invitrogen Life Technologies), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) in the presence of PMA (20 ng/ml) at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Human PBMC were isolated from fresh blood obtained from PPD-negative, healthy donors by centrifugation over Histopaque as previously described (26) then $CD14^+$ cells were purified using the StemSep Human Monocyte Enrichment kit obtained from StemCell Technologies. Monocyte-derived macrophages (MDM) were prepared by culturing freshly isolated monocytes in RPMI 1640 supplemented with L-glutamine, antibiotics, and 10% autologous serum in a humidified 5% CO_2 incubator for 4–5 days. Dead monocytes or THP-1 cells were removed by several washes with HBSS and adherent monolayers were replenished with culture medium without antibiotics and infected with opsonized mycobacteria (bacteria to cell ratio of 25:1 for THP-1 and 10:1 for MDM). After a period of 2–3 h, partially attached, noningested bacteria were removed by extensive washing with HBSS. This procedure resulted in an infection rate of 70–80% with ~5–10 bacteria/cell.

Cell surface staining and flow cytometry

To measure cell surface expression of MHC class II, the culture plates were scraped with a rubber policeman and cells were collected in HBSS containing 0.1% NaN_3 and 1% FCS (staining buffer). Cells were then labeled with anti-class II mAbs or irrelevant isotype-matched IgG for 20 min. After two washes with staining buffer, samples were labeled with FITC-conjugated $F(ab')_2$ goat anti-mouse IgG for 20 min. At the end of staining, cells were washed twice and fixed in 2% paraformaldehyde in staining buffer. To control for cell viability, cells were incubated with propidium iodide (0.5 μg/ml in staining buffer) for 10 min. Cell fluorescence was analyzed using a FACSCalibur flow cytometer (BD Biosciences). Viable cells were identified by exclusion of propidium iodide. Relative fluorescence intensities of 10,000 cells were recorded as single-parameter histograms (log scale, 1024 channels, and 4 decades).

Measurement of Cat S activity

Cat S activity was measured using the fluorogenic substrate Z-Val-Val-Arg-NHMec as described (20, 27). Adherent THP-1 cells were scraped in Cat S extraction buffer (0.01% Triton X-100 in 0.1 M potassium phosphate buffer containing 1 mM EDTA (pH 7.5)) and frozen/thawed three times. Samples were sonicated for 5 s with a Sonic Dismembrator 60 (Fisher Scientific) on ice, and cell debris and membranes were removed by centrifugation at 12,000 $\times$ *g* for 30 min at 4°C. Fifty microliters of the soluble fractions (~100 μg of protein) were added to 50 μl of the reaction buffer (0.1 M potassium phosphate buffer, 5 mM EDTA (pH 7.5), and 5 mM DTT), and samples were incubated for 45 min at 40°C to inactivate cathepsin L. Thereafter, 50 μl of 12.5 μM Z-Val-Val-Arg-AMC (Cat S/L substrate) were added to the mixtures. After an additional incubation at room temperature for 10 min in the dark, fluorescence was measured at λex/em 360/460 nm in a VersaFluor instrument (Bio-Rad). Cat S activities were calculated by reference to a standard curve using a range (0–250 ng/ml) of active recombinant human Cat S. Reaction mixtures with BSA instead of cell lysates were used to control for background and nonspecific

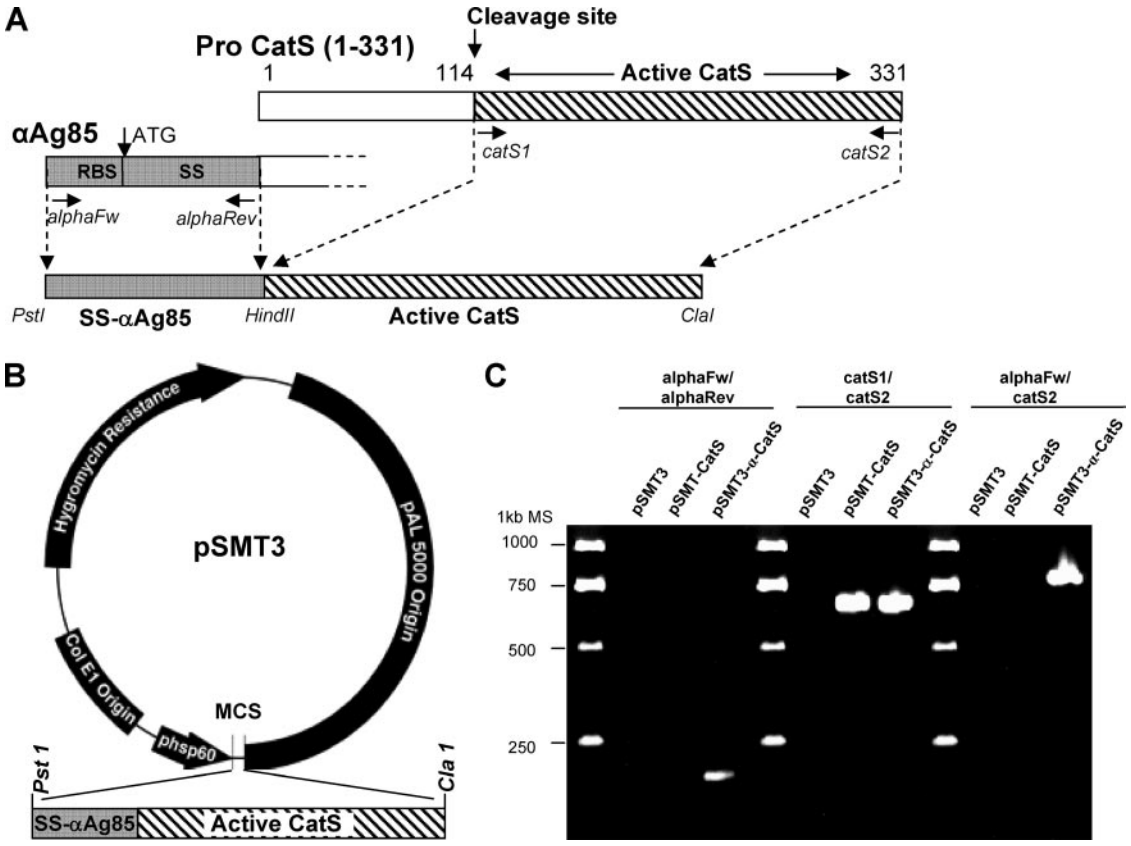

FIGURE 1. Construction of BCG strains that secrete recombinant active Cat S. *A*, The cDNA fragment encoding human active Cat S was obtained by RT-PCR on total RNA extracted from IFN-γ-stimulated THP1 cells. The amplicon of 654 bp was cloned between the *Cla*I and *Hin*dIII sites in the pSMT3 vector. *B*, The α Ag portion was amplified from BCG DNA and inserted upstream of the Cat S fragment in the pSMT3 vector to obtain the recombinant plasmid pSMT-hCat S. *C*, Positive clones were confirmed by PCR using different sets of primers.

activity and results were expressed after subtracting the fluorescence value corresponding to this control.

Fluorescence microscopy

Adherent macrophages to glass coverslips were mounted on microscope slides in FluorSave (Calbiochem-Novabiochem) to minimize photobleaching. Slides were then examined by digital confocal microscopy using an Axioplan II epifluorescence microscope (Carl Zeiss) equipped with a ×63/1.4 Plan-Apochromat objective (Carl Zeiss). Images were recorded using a CCD digital camera (Retiga EX; QImaging) coupled to the Northern Eclipse software (Empix Imaging).

Ag processing and presentation assays

Adherent THP-1 cells to 24-well plates were incubated with 200 U/ml IFN-γ with or without bacteria. Following 24 h of incubation, all medium was removed and cells were fixed in 1% paraformaldehyde, followed by incubation with DB1 T cell hybridoma, which responds specifically to a mycobacterial Ag85B peptide presented by infected THP-1 cells (28, 29). Supernatants were harvested after 24 h, and assessed for IL-2 by a specific sandwich ELISA (BD Biosciences).

Statistical analysis

All data are expressed as the mean ± the SD. Statistical analysis was performed using the Student *t* test. Values of $p < 0.05$ were considered to be significant.

Results

Analysis of rBCG-expressing Cat S

A previous study showed that *M. bovis* BCG inhibits basal and IFN-γ-induced Cat S expression in macrophages leading to reduced Ii chain degradation and a subsequent attenuation of surface expression of mature class II molecules (20). Therefore, to restore Cat S-mediated Ii chain degradation in BCG-infected cells, the sequence corresponding to active Cat S was amplified and cloned in pSMT3 in tandem with the α Ag 85B signal peptide to direct expression of active Cat S and its secretion through mycobacterial membrane and cell wall (Fig. 1, *A* and *B*). The resulting pSMT-Cat S vector construct was electroporated into *M. bovis* BCG and selected positive clones were initially screened by restriction enzymes and PCR (Fig. 1*C*) followed by sequencing. The new recombinant strain was named rBCG-hcs.

Expression and secretion of Cat S by rBCG-hcs was examined by Western blotting (Fig. 2*A*). Although no signal was detected in the supernatant of parental BCG and pMST3-transformed BCG strains, anti-Cat S mAb recognized a single band in the culture supernatant of rBCG-hcs (molecular mass ~28 kDa) that matches the size of recombinant active human Cat S (rHuCat S) used as control. However, the same Ab detected two bands in total lysates of rBCG-hcs: a low molecular mass band, presumably corresponding to mature Cat S, that matched the position of rHuCat S, and a second band (higher molecular mass), which likely corresponds to Cat S fused to signal peptide. As expected, no band was detected in lysates from parental BCG or BCG transformed with empty pSMT3 vector.

To verify whether Cat S expression within the bacterium affects its viability, we measured the growth of the recombinant strain in standard medium (Middlebrook 7H9 broth plus OADC) at different time points over ~12 days. Results in Fig. 2*B* showed that at different stages of bacterial growth, the rate of rBCG-hcs multiplication was similar to that of BCG expressing vector alone (BCG-pSMT3) and wild-type BCG. Therefore, expression of Cat S had no apparent toxic side effect on the bacterium. Moreover,

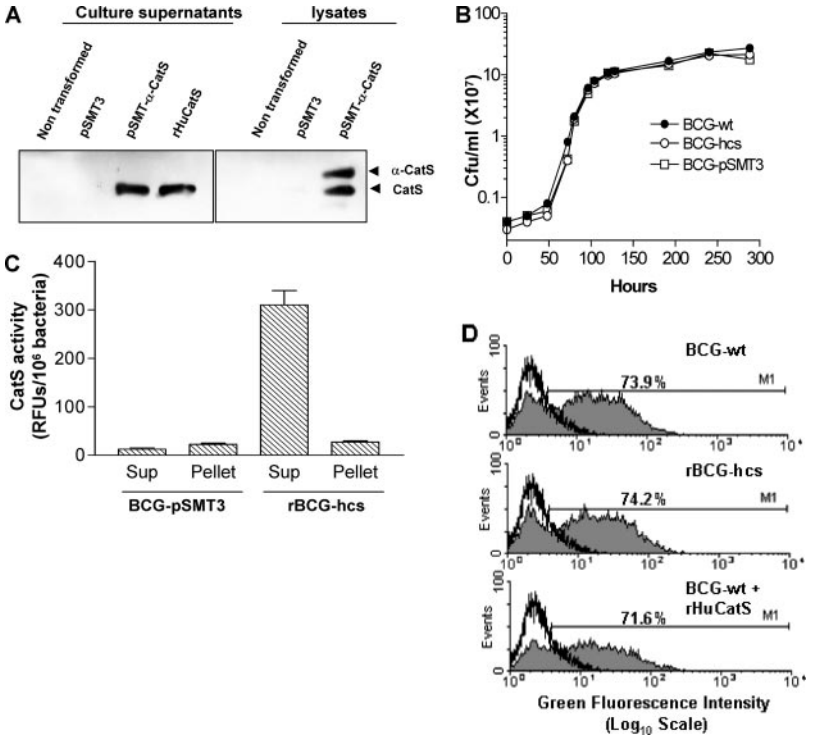

FIGURE 2. Characterization of rBCG clones. *A*, Culture filtrates (50 μl) of late log-phase culture (10^8 CFU/ml) along with total lysates of 5×10^8 bacteria were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 3% BSA and probed with anti-Cat S mAb (1 μg/ml). Membranes were then incubated with HRP-conjugated goat anti-mouse IgG and developed by ECL. *B*, Aliquots from exponential cultures of BCG-wt, BCG transformed with empty pSMT3 vector (BCG-pSMT3), or pSMT3 expressing human Cat S (rBCG-hcs) were grown at 37°C in Middlebrook 7H9 broth supplemented with 10% OADC. Absorbance (600 nm) was measured at the indicated times and values were converted to CFU as described (64). *C*, Cat S activity was measured in culture supernatants and in lysate of bacterial pellets using the fluorogenic substrate Z-Val-Val-Arg-AMC as described in *Materials and Methods.* Values are represented as relative fluorescence units per 10^6 CFU. *D*, PMA-differentiated THP-1 cells in 6-well culture plates were incubated with serum-opsonized FITC-labeled BCG-wt or rBCG-hcs (multiplicity of infection (MOI), 25:1) in the absence or presence of rHuCat S (1 μg/ml). After 2-h incubation at 37°C, partially attached but noninternalized bacteria were removed by trypsin treatment and HBSS washes as described (19). Samples were then analyzed in a BD FACSCalibur flow cytometer. Open histograms, Autofluorescence of noninfected cells; solid histograms, cells exposed to fluorescent bacteria. In each panel, the percent value indicates the proportion of cells containing BCG as calculated by WinMDI 2.8 software. Data shown are from one of two independent experiments that yielded similar results.

Cat S activity was undetectable in total bacterial lysates, while significant activity was observed in rBCG-hcs supernatants (Fig. 2*C*). This result indicated that Cat S is inactive until it is cleaved from the α Ag85 signal peptide and secreted.

Before developing detailed studies of macrophage responses to rBCG-hcs, we examined whether Cat S secretion could affect the uptake of the bacterium. Adherent THP-1 cells were infected with either rBCG-hcs (or parental BCG) freshly labeled with FITC, and bacterial uptake was measured by flow cytometry. Fluorescence histograms presented in Fig. 2*D* showed similar rates of infection with rBCG-hcs and BCG-wild type (wt), suggesting that secretion of active Cat S during adherence of rBCG-hcs to the host cell surface does not alter the function of receptors involved in the ingestion of the bacterium. Similarly, treatment with various concentrations (up to 1 μg/ml) of recombinant human Cat S for 2 h did not reduce macrophage capacity to ingest FITC-labeled BCG.

Cat S activity in macrophages infected with rBCG-hcs

We have shown that infection with wild-type BCG inhibited Cat S expression within the host cell (20). The finding that rBCG-hcs secretes active Cat S in culture medium suggested that this recombinant strain would compensate for the inhibitory effect of the parental strains. This possibility was first confirmed by Western blot analyses that showed substantial levels of Cat S in whole cell lysates from THP-1 and MDM infected with rBCG-hcs, which were comparable to the levels induced by IFN-γ (Fig. 3*A*). Control experiments, in which cells were infected with wild-type BCG or BCG-pMST3 alone, showed no change of Cat S levels relative to nontreated cells (data not shown). The synthesis and release of Cat S from intracellular rBCG-hcs was then confirmed by confocal microscopy (Fig. 3, *B* and *C*). As expected, and relative to the control untreated cells, IFN-γ induced substantial expression of Cat S in a large majority of cells ($\sim$80% displaying abundant intracellular Cat S). In BCG-infected cells, phagosomes containing rBCG-hcs, but not BCG-wt, displayed a strong staining for Cat S and image magnification showed that Cat S secreted from rBCG-hcs was exported to different vesicles throughout the cell. More importantly, measurement of endogenous Cat S activity in THP-1 and MDM showed that infection with rBCG-hcs recapitulated IFN-γ stimulation and resulted in $\sim$2-fold increase of Cat S activity (Fig. 3*D*). In contrast, enzyme activity was reduced in cells infected with BCG-pMST3 to a level below the basal activity observed in control nontreated cells. Taken together, these data demonstrated that rBCG-hcs is ingested normally by macrophages and this results in the restoration of Cat S activity within its host cell.

MHC class II expression and Ag presentation in rBCG-hcs-infected macrophages

To examine the nature of class II molecules that reach the surface of rBCG-hcs-infected cells, immunostaining with LN2 and L243

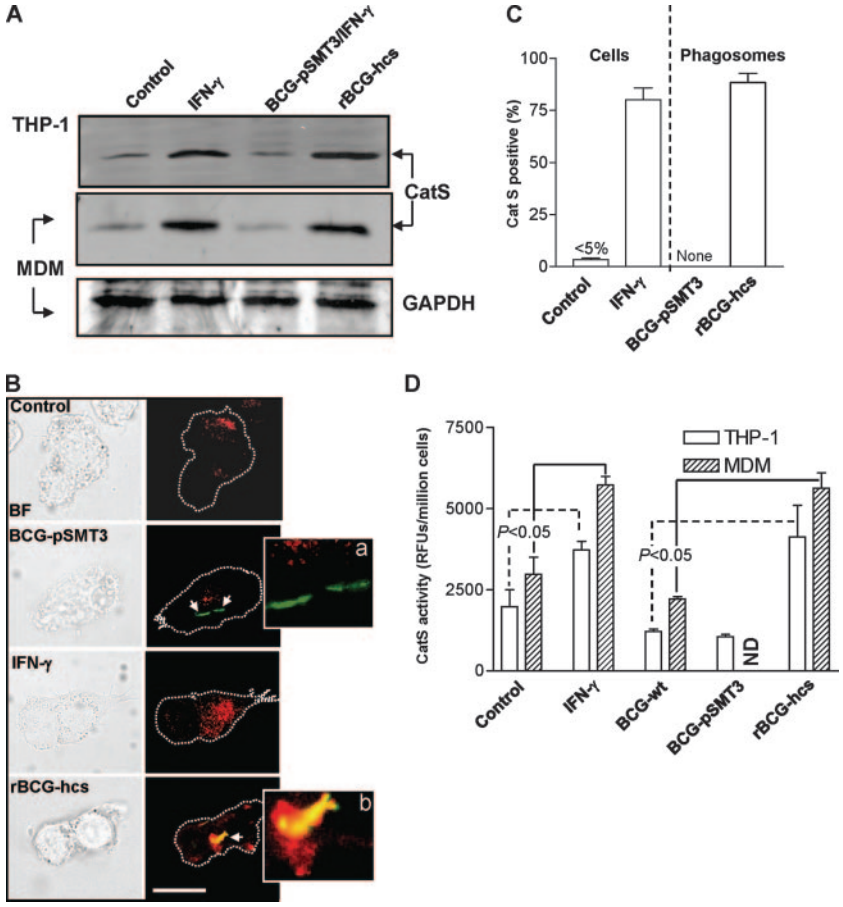

FIGURE 3. rBCG-hcs secretes active Cat S within the macrophage. *A*, Macrophages were untreated or infected overnight with BCG-pSMT3 or rBCG-hcs (multiplicity of infection (MOI), 25:1 for THP-1 and 10:1 for MDM). Where indicated, IFN-γ (200 U/ml) was added and cells were incubated for additional 24 h. Cells were washed and lysed with radioimmuno precipitation assay buffer and total soluble proteins (50 μg from THP-1 and 30 μg from MDM) were analyzed by SDS-PAGE and Western blot with anti-Cat S mAb as described in Fig. 2*A*. To ensure equal loading of protein from different sample treatments, membranes were striped and reprobed with anti-GAPDH Abs as shown in the *lower panel* of the MDM experiment reported in this figure. *B*, THP-1 cells adherent to cover slips were stimulated with IFN-γ (200 U/ml, 24 h) or infected with FITC-conjugated rBCG-hcs or BCG-pSMT3 (MOI, 25:1, overnight) or left untreated (control). Cells were then washed, fixed, permeabilized, and stained with anti-Cat S mAb and TR-labeled secondary Ab. Labeled cells were analyzed with digital confocal microscopy, and optical sections (0.2 μm) were scanned for green and red fluorescence. The image from infected cells is displayed as merge of green (rBCG-hcs) and red (Cat S) signals. The yellow signal depicts colocalization of green with red and dotted lines depict cell boundaries. Ba and Bb represent ×4 magnifications of the cell compartment containing bacteria. Bar, 10 μm. *C*, Quantification of the results shown in *B*. *D*, Macrophages were untreated or infected with BCG-pSMT3 or rBCG-hcs and stimulated with IFN-γ as indicated in *A*, then cell lysates were prepared and proteins (50 μg from THP-1 and 30 μg from MDM) were assayed for Cat S activity using the fluorogenic substrate Z-Val-Val-Arg-AMC as described in *Materials and Methods*. The data in *A* and *B* are from one of two independent experiments that yielded similar results and values in *C* are the mean ± SD of three independent experiments.

mAbs was done as described (20). LN2 recognizes immature class II molecules associated with intact or partially degraded Ii (30) while L243 recognizes peptide-loaded class II molecules as well as those still loaded with CLIP fragment (31, 32). FACS analysis data (fluorescence histograms and mean fluorescence intensity (MFI) indices) shown in Fig. 4*A* indicated that BCG-pMST3 infection of THP-1 and MDM before IFN-γ stimulation brought about expression of immature class II molecules only. In contrast, the increase in class II expression in response to rBCG-hcs-infected cells was accounted for nearly exclusively by mature α, β dimers. Of interest, the phenotype observed for rBCG-hcs-infected cells was abolished in cells where Cat S activity was blocked by pretreatment with the inhibitor Z-FL-COCHO (33). Alternatively, complementation of BCG-wt-infected cells with recombinant active Cat S restored the expression of mature class II molecules as shown in our previous work (20). Thereafter, the DB1 T cell hybridoma was used to examine the ability of rBCG-hcs-infected cells to present Ag. DB1 is specific for the mycobacterial Ag 85B (epitope 97–112) presented by HLA-DR1 (28, 34). THP-1 cells (HLA-DR1/DR2) were infected with rBCG-hcs or BCG-pSMT3 and stimulated with IFN-γ before coculture with DB1 cells. The IL-2 levels measured in culture supernatants indicated that DB1 cells recognized the Ag85B epitope processed from whole rBCG-hcs (Fig. 4*B*). Additional assays in the absence of IFN-γ showed that rBCG-hcs, but not BCG-wt, is capable of inducing macrophage presentation of Ag85B to DB1. These observation correlated with the finding that rBCG-hcs, in contrast to BCG-wt, is capable of inducing expression of detectable levels of mature class II molecules at the surface of macrophages (Fig. 4*C*). The role of Cat S was confirmed further with experiments showing that complementation of BCG-wt-infected cell with recombinant active Cat S restored IFN-γ-driven macrophage stimulation of DB1 (Fig. 4*B*). Thus, Cat S secreted by rBCG-hcs within the host macrophage generated conditions for efficient delivery of mycobacterial Ags by MHC class II molecules.

Persistence of rBCG-hcs within the macrophage

Maturation of microbial phagosomes to phagolysosomes contributes significantly to the normal processing and presentation of Ags

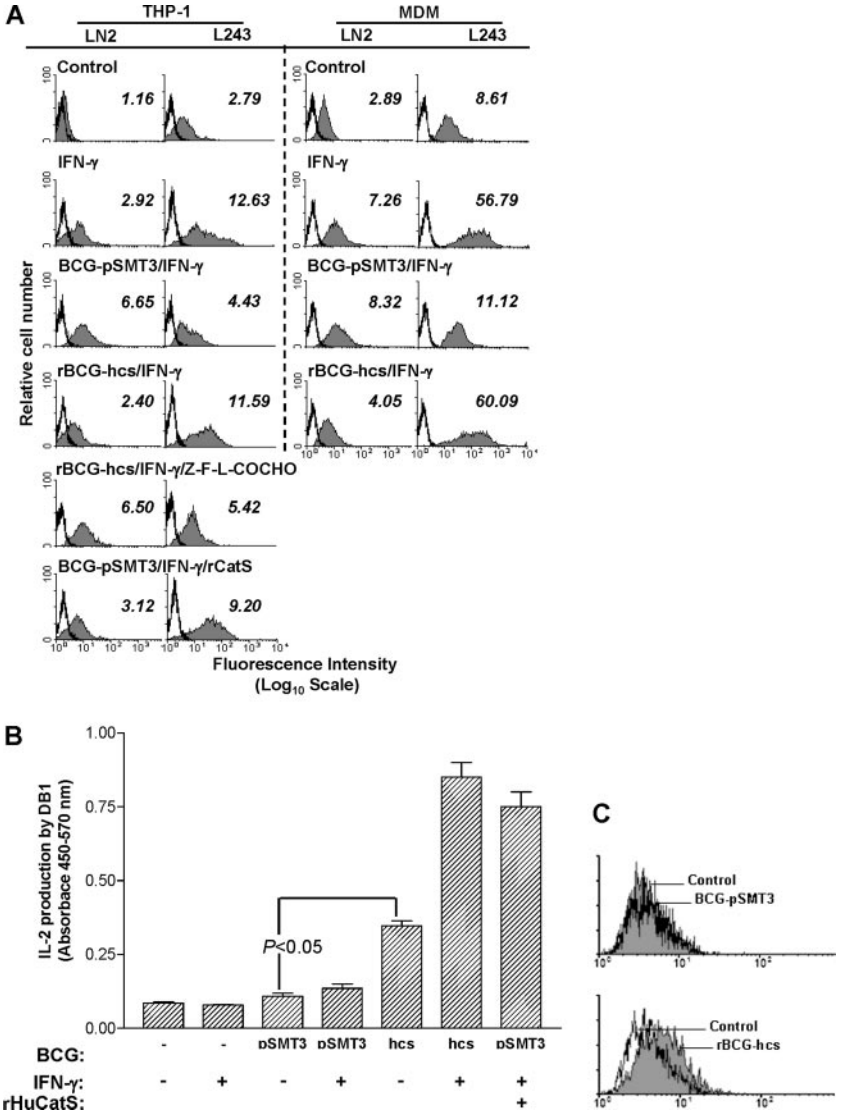

FIGURE 4. Cell infection with rBCG-hcs restores expression of mature class II molecules and Ag presentation. *A*, THP-1 cells and MDM were incubated with the indicated bacteria (MOI, 25:1 for THP-1 and 10:1 for MDM) for 24 h, and then IFN-γ (200 U/ml) was added for 24 h. Cat S inhibitor (Z-FL-COCHO, 50 nM) and exogenous recombinant active human Cat S (rHuCatS, 500 ng/ml) were added 6 h before culture termination. Cell complementation with rHuCatS was performed with Profect P1 reagent in serum-free medium as described previously (20). Macrophages were then washed, scraped, and aliquots were stained with L243 mAb, LN2 mAb, or irrelevant isotype-matched IgG for 20 min at room temperature. After two washes, cells were labeled with FITC-conjugated $F(ab')_2$ goat anti-mouse IgG for 20 min, washed, fixed, and analyzed by flow cytometry. Results are expressed as histograms of fluorescence intensity. The MFI was calculated for each histogram with the WinMDI 2.8 software. Open histograms, Cells stained with irrelevant isotype-matched IgG; solid histograms, cells stained with specific mAb. Values in the *top right* of each panel indicate MFI indices, which correspond to the ratio the MFI of cells incubated with specific Ab to the MFI of cells stained with irrelevant isotype-matched IgG. *B*, THP-1-derived macrophages adherent in 24-well culture plates (5×10^5/well) were untreated or infected with rBCG-hcs or BCG-wt for 24 h as described in *A*. Cells were then washed and left untreated or stimulated with 200 U/ml IFN-γ for additional 24 h. Cells were then fixed in 1% paraformaldehyde and incubated with DB1 T hybridoma cells (specific for Ag85) (5×10^5/well) for 20 h. Supernatants were harvested and assessed for IL-2 by sandwich ELISA. Where indicated, macrophages were complemented with rHuCatS 6 h before fixation as described in *A*. *C*, Adherent macrophages were infected with BCG-wt or rBCG-hcs for 24 then stained with L243 mAb and analyzed by FACS as described in *A*. The data in *A* are from one of three independent experiments that yielded similar results and values in *B* are the mean ± SD of three independent experiments. The data in *C* are from one of two independent experiments that yielded similar.

to elicit adaptive immunity (4, 35–37). However, Mtb and BCG strains often block this process to ensure persistence and replication within the macrophage (12, 38). Therefore, we examined whether the restoration of Ag presentation in rBCG-hcs-infected macrophages was directly linked to phagolysosome fusion. Adherent THP-1 cells were preloaded with Texas Red-dextran (TR-DXT) and infected with FITC-labeled mycobacteria. Alternatively, infected macrophages were fixed/permeabilized and stained with anti-Lamp-1 and TR-conjugated secondary Abs as described (39). TR-DXT is a nondegradable, cell-permeable probe that accumulates in lysosomes (40, 41) and Lamp-1 is widely used as an endogenous marker of late endosomes and lysosomes (42). The fluorescence images obtained showed that in rBCG-hcs-infected cells, a large majority of phagosomes displayed extensive colocalization with TR-DXT (84.5 ± 10.6%) and Lamp-1 (85.0 ± 9.8%) positive organelles suggestive of phagolysosome fusion (Fig. 5*A*). As expected, only a minor fraction of phagosome containing parental BCG (BCG-wt, 8.5 ± 3.5%) or BCG transformed with

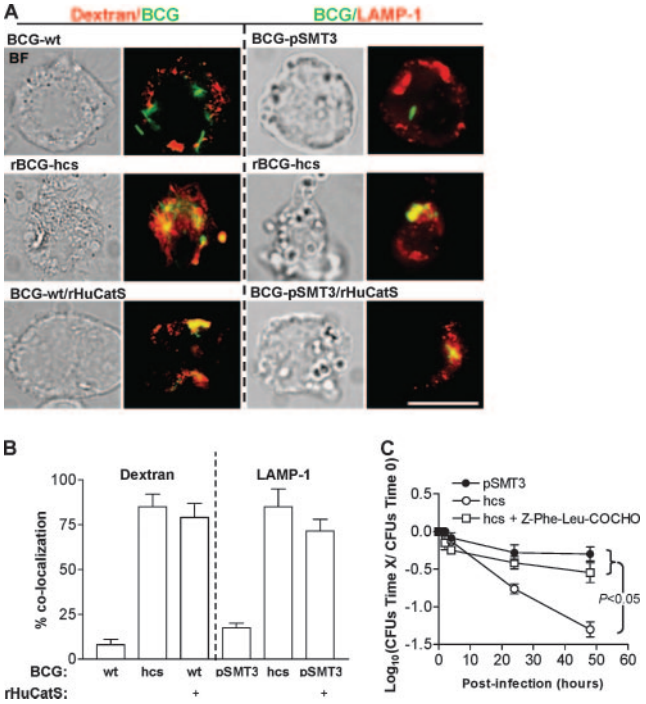

FIGURE 5. rBCG-hcs phagosome maturation and survival within the macrophage. *A*, *Left panel*, THP-1 cells were differentiated with PMA in the presence of TR-DXT (500 μg/ml) and chased for 3–4 h. TR-DXT-loaded cells were left untreated or complemented with rHuCatS (500 ng/ml) 30 min before exposure to FITC-labeled mycobacteria. Cells were allowed to ingest fluorescent bacteria and chased for 2 h. *Right panel*, Infected macrophages were permeabilized and stained for endogenous Lamp-1 with H4A3 mAb and revealed with TR-labeled secondary Ab as described previously (39). Cover slips were then mounted on microscope slides and examined by digital confocal microscopy as described in Fig. 3*B*. In the merge panels, the yellow signal emanating from colocalization of red and green fluorescence signals is indicative of substantial fusion between phagosomes containing FITC-labeled BCG organisms and TR-DXT (*left panel*) or Lamp-1 (*right panel*) positive lysosomes. *B*, Quantification of the results shown in *A*. *C*, THP-1-derived macrophages were infected with BCG-wt or rBCG-hcs for 3 h at 37°C in culture medium. Partially attached, noningested bacteria were removed by 5-min treatment with trypsin-EDTA and extensive washing with HBSS. Culture plates were filled with culture medium and reincubated at 37°C in the absence or in the presence of Cat S inhibitor (Z-FL-COCHO, 50 nM). At the time points indicated, bacteria were released by treatment with 0.1% Triton X-100 in PBS and serial dilutions were plated on 7H11 plates in triplicate. The results are expressed as described (19, 65). The data in *A* are from one of three independent experiments that yielded similar results; values in *B* are the mean ± SD of colocalization of 50–70 cells from three individual experiments and values in *C* are the mean ± SD of three independent experiments.

empty vector (BCG-pSMT3, 12.5 ± 2.5%) showed colocalization with the lysosomal markers, indicating a significant inhibition of phagolysosome fusion consistent with previous observations (12, 43). However, complementation with recombinant active Cat S restored phagolysosome fusion in a large majority of BCG-wt (79.0 ± 8.0%) or BCG-pSMT3 (71.0 ± 6.5%) phagosomes. These additional experiments confirmed the direct role of Cat S in phagosome maturation.

To establish a link between Cat S-mediated phagolysosome fusion and bacterial survival, infected macrophages were lysed, and the lysates were diluted and spread on 7H11 Middlebrook plates to determine the number of mycobacterial CFU remaining. Interestingly, survival of rBCG-hcs was ~10-fold lower than control BCG at 48 h postinfection (Fig. 5*C*). CFU counts were then repeated in macrophage treated with Z-Phe-Leu-COCHO and the results obtained showed that blocking Cat S secreted intracellularly restored substantially the survival of rBCG-hcs. Taken together, these data demonstrate that increased Ag presentation by macrophages infected with rBCG-hcs is associated with accelerated mycobacterial phagosome maturation and intracellular killing dependent on secretion of active Cat S.

Discussion

The nature of the cells responding to microbial infections and their relative contribution to control the infection is an area of obvious importance for rational vaccine development. In the case of mycobacterial infections, experimental TB in mouse models has shown that a $CD8^+$ T cell response is important for efficient anti-TB defense, notably in later stages of the infection process (1, 44). However, MHC class II-restricted $CD4^+$ T cells are the most important aspect of the initial protective response (45, 46). Our studies of BCG interaction with MHC class II-directed Ag presentation revealed to us that macrophage infection with BCG reduced surface export of class II molecules by a mechanism dependent on intracellular sequestration of α, β dimers (19). Indeed, the same study showed that BCG infection has no effects of infection on IFN-γ signaling (Jak/STAT pathway) or class II genes transcription (19). Further investigation showed that inhibition of Cat S expression in BCG-infected cells was responsible for the block of the maturation of class II molecules (20). Unlike MHC class II, IFN-γ-induced *Cat S* gene expression is not controlled by CIITA (47).

Therefore, convinced that efficient class II-directed presentation of mycobacterial Ags to Th cells would contribute significantly to induction of effective TB immune response, we upgraded the classical BCG vaccine with a vector expressing active Cat S—to compensate for the inhibition of Cat S expression engendered by the bacterium—and examined its capacity to restore the defects in class II expression and Ag presentation.

We focused on Cat S because 1) this enzyme is the principal protease involved in late steps of Ii chain processing (21–23) and 2) removal of the Ii chain is critical for appropriate export of peptide-loaded class II molecule to the cell surface (17, 18). Before being shuttled to the phagolysosome, Cat S is first synthesized as a latent precursor of a higher molecular mass protein (proCat S, 331 aa, 36 kDa) with a polypeptide extension of 8 kDa at the N terminus of the mature enzyme domain, which acts as a potent inhibitor to the cognate enzyme (48–50). Cat S is converted to the mature form (active Cat S, amino acids 115–331, 28 kDa) by limited proteolysis by other proteases, or by autocatalytic processing upon exposure to acidic environments (48). Given that BCG blocks phagosome acidification (51, 52), rBCG-hcs was engineered to express only the peptide segment corresponding to amino acids 115–331. This approach resulted in the secretion of active Cat S within the macrophage that bypasses the acidity-dependent activation. Cat S secretion by BCG was made possible by the use of the peptide signal of secreted mycobacterial α Ag85 as a carrier. Such a strategy was used successfully earlier by Matsuo et al. (53) and adopted later on by other investigators (54–56) to induce secretion of foreign proteins by mycobacteria.

Of particular note, phenotype analysis showed that BCG transformation with the active domain of human Cat S did not affect the survival and growth of the bacterium as a result of proteolytic activity within the bacterium consistent with a lack of activity in bacterial lysates as opposed to culture supernatants. It is possible that mycobacteria express an endogenous factor that binds to Cat S and blocks its activity intracellularly and, in this case, such an

inhibitor would have to detach from Cat S before (or immediately after) its secretion. Another possibility to consider is that within the bacterium, the peptide signal (SS-αAg85) linked to the active fragment of Cat S would act as a propeptide to block the enzyme until its cleavage before the release of Cat S from the bacterium.

The first evidence supporting the "Cat S approach" in enhancing the efficacy of the BCG vaccine came from experiments demonstrating that Cat S exported by rBCG-hcs within the macrophage displayed a strong activity in vitro against the classical substrate Z-Val-Val-Arg-AMC. These findings correlated with the restoration of surface expression of mature class II molecules capable of Ag presentation presumably via intracellular degradation of Ii chain by bacterial Cat S. Indeed, complementation of macrophages infected with parental BCG with exogenous active Cat S largely reproduced the phenotype observed with rBCG-hcs.

It is generally accepted that the maturation status of the microbial phagosome dictates its ability to generate peptide-MHC class II complexes in human macrophages (36, 37) and this concept would apply also to infection with the vaccine strain BCG. In fact, given the innate tendency of BCG to prevent phagosome maturation (12, 57) and acidification (37) one might expect that this vaccine may only present a part of its Ag repertoire in vivo, depending on its ability to block maturation. The in vitro data presented in this study link mycobacterial phagosome maturation to Ag processing in two ways. First, we showed that r-BCG-hcs accelerated fusion of its phagosome with lysosomes and this led to intracellular processing of mycobacterial Ag85B (a mycolyltransferase involved in cell wall biosynthesis (58), and presentation of the immunodominant $Ag85B_{97-112}$ epitope into the MHC class II presentation pathway. Second, complementation of macrophages harboring BCG with recombinant active Cat S restored both phagolysosome fusion and presentation of Ag85B. These findings provided the first evidence for a direct role of active Cat S in nullifying mycobacterial control of phagosome-lysosome fusion consistent with a previous demonstration of its major contribution to protein degradation in lysosomes (59). This work is highly relevant to vaccine development because Ag 85B is a major target of human T cell responses to Mtb and a leading vaccine candidate (60–62).

The mechanism of phagosome maturation dependent on Cat S is presently unclear but ongoing investigations of the global macrophage transcriptome by microarray indicated to us that a cluster of genes related to phagosome maturation, including autophagy 5-like, ATPase, H^+ transporting (V0 and V1) and their accessory proteins, are up-regulated in cells infected with rBCG-hcs (data not shown). These findings are in agreement with recent studies showing that phagosomal acquisition of vacuolar proton ATPase is critical for the processing and presentation of Ag85B in macrophages infected with BCG (37).

In conclusion, the finding of improved stimulation of MHC class II-restricted CD4 T cells by BCG expressing active Cat S is a step toward novel vaccine development. The central role of CD4 T cells in protection against TB has been established (63) and early studies showed that CD4 T cells are poorly stimulated by BCG (15). This discrepancy between the requirement of CD4 T cells for protection and inadequate stimulation of CD4 T cells by BCG should be considered in the development of efficient TB vaccines. Our finding that Cat S-secreting rBCG strain promotes MHC class II processing and presentation of the mycobacterial Ag 85B demonstrates a new strategy for overcoming this drawback. This work also sets the stage for future experiments to clarify whether rBCG-hcs vaccination increases protection against virulent mycobacteria.

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Disclosures

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