# *Mycobacterium bovis* BCG Attenuates Surface Expression of Mature Class II Molecules through IL-10-Dependent Inhibition of Cathepsin S[1]

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**We have previously shown that macrophage infection with *Mycobacterium tuberculosis* and *M. bovis* bacillus Calmette-Guérin (BCG) partially inhibits MHC class II surface expression in response to IFN-$\gamma$. The present study examined the nature of class II molecules that do in fact reach the surface of infected cells. Immunostaining with specific Abs that discriminate between mature and immature class II populations showed a predominance of invariant chain (Ii)-associated class II molecules at the surface of BCG-infected cells suggesting that mycobacteria specifically block the surface export of peptide-loaded class II molecules. This phenotype was due to inhibition of IFN-$\gamma$-induced cathepsin S (Cat S) expression in infected cells and the subsequent intracellular accumulation of $\alpha\beta$ class II dimers associated with the Cat S substrate Ii p10 fragment. In contrast, infection with BCG was shown to induce secretion of IL-10, and addition of blocking anti-IL-10 Abs to cell cultures restored both expression of active Cat S and export of mature class II molecules to the surface of infected cells. Consistent with these findings, expression of mature class II molecules was also restored in cells infected with BCG and transfected with active recombinant Cat S. Thus, *M. bovis* BCG exploits IL-10 induction to inhibit Cat S-dependent processing of Ii in human macrophages. This effect results in inhibition of peptide loading of class II molecules and in reduced presentation of mycobacterial peptides to CD4$^+$ T cells. This ability may represent an effective mycobacterial strategy for eluding immune surveillance and persisting in the host.** ***The Journal of Immunology,* 2005, 175: 5324–5332.**

The intracellular organism *Mycobacterium tuberculosis* (Mtb)[4] resides almost exclusively within macrophages of infected individuals. The immune response mounted to Mtb is sufficient to prevent most people from developing active tuberculosis, but is insufficient to bring about sterile immunity (1, 2). Thus, breakdown of immune responses designed to contain the infection results in reactivation and replication of the bacilli, with necrosis and damage to lung tissue (3, 4). Indeed, Mtb continues to cause disease in ~8 million people each year, resulting in a death every 10 s (5). This alarming situation has resulted in a revival of multidisciplinary interest in tuberculosis research with a special focus on understanding how pathogenic mycobacteria impair macrophage defense mechanisms.

Under most conditions, macrophages ingesting bacteria and parasites elaborate a rapid innate immune response starting by phagosome acidification that paralyzes the invaders followed by a regulated phagosome transport along the endocytic and lysosomal pathway in which pathogens are exposed to degradative enzymes (6, 7). Ultimately, macrophages initiate a cell-mediated adaptive immune response by processing and presenting microbial Ags to specific T cells in the context of MHC cell surface molecules (8, 9). Activated T cells secrete IFN-$\gamma$, which activates macrophage bactericidal activity and increases expression of MHC class II molecules and costimulatory molecules on the cell surface (10–12). Contrasting with this scenario, several independent investigations suggested that macrophages infected with Mtb 1) have decreased ability to present peptide Ags to Th cells (11, 13) and 2) respond poorly to IFN-$\gamma$ in terms of MHC class II molecule expression and Ag presentation (14–17). Thus, as the ability of mycobacteria to evade the host immune response contributes largely to its success as a pathogen, the mechanisms underlying attenuation of macrophage class II expression and Ag presentation are of significant interest.

Newly synthesized class II $\alpha$- and $\beta$-chains associate with invariant chain (Ii), then exit the endoplasmic reticulum subsequently localizing to an acidic endosomal/lysosomal compartment referred to as the MHC class II compartment (MIIC) (8). In the MIIC, removal of Ii and peptide loading are believed to be critical for appropriate export of peptide-loaded class II molecule to the cell surface (18). The processing of Ii has been shown to involve a coordinated action of different proteases generating Ii intermediates p22 and p10, down to the class II-associated Ii peptide (CLIP) (8, 9). Asparagine endopeptidases generate p22, whereas

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Received for publication February 14, 2005. Accepted for publication August 2, 2005.

[1] This work was supported by operating Grant MOP-43891 from the Canadian Institutes of Health Research (CIHR) and the British Columbia Lung association and by an establishment Grant CI-SCH-26 from Michael Smith Foundation for Health Research (MSFHR). Z.H. was supported by scholar awards from the CIHR and MSFHR. K.S. and A.T. were supported by the TBVets Charitable Foundation. A.D. is a recipient of postdoctoral fellowships from CIHR and MSFHR.

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[4] Abbreviations used in this paper: Mtb, *Mycobacterium tuberculosis*; Ii, invariant chain; MIIC, MHC class II compartment; Cat S, cathepsin S; MFI, mean fluorescence intensity; BCG, bacillus Calmette-Guérin; MOI, multiplicity of infection; CLIP, class II-associated Ii peptide.

0022-1767/05/$02.00

cysteine proteases play essential role for p22 proteolysis (19, 20). Indeed, cathepsin S (Cat S) has recently been shown to be the most important, if not the only, protease responsible for the late steps in Ii processing to CLIP in human APCs (21). The CLIP fragment is subsequently exchanged, under the catalytic effect of HLA-DM, with the peptide Ag to be presented at the surface of the APC.

Our investigations have previously showed normal steady-state levels of $\alpha\beta$ dimers and intracellular sequestration of large proportion of class II molecules in cells infected with mycobacteria, indicating distal effects on class II expression likely involving maturation and transport to the cell surface (15, 22). These findings suggested the possibility that proteases involved in the maturational processing of class II molecules might be defective in cells harboring live mycobacteria.

In the present study, we examined further the basis for abnormal trafficking of class II molecules to the cell surface in cells infected with bacillus Calmette-Guérin (BCG). The results obtained provided evidences for inhibition of Cat S expression resulting in reduced expression of mature MHC class II molecules at the cell surface.

## Materials and Methods

### *Reagents and chemicals*

RPMI 1640, HBSS, PMA, protease inhibitor mixture, PMSF, and trypsin-EDTA were obtained from Sigma-Aldrich. Anti-human HLA-DR mAb (clone TU36) and irrelevant isotype-matched IgGs were from Caltag Laboratories. Monoclonal anti-Cat S was from Calbiochem, anti-Ii mAb (clone PIN-1) was a generous gift from Dr. P. Cresswell (Yale University, New Haven, CT). Anti-human HLA-DR mAb (clone L243) was from BD Pharmingen. FITC-conjugated $F(ab')_2$ goat anti-mouse IgG and HRP-conjugated goat anti-mouse IgG were from Sigma-Aldrich. Human rIFN-$\gamma$ was a generous gift of Genentech (South San Francisco, CA). Cat S inhibitor Z-Phe-Leu-COCHO and recombinant active form of human Cat S were from Calbiochem. Cat S substrate Z-Val-Val-Arg-AMC was from Bachem Bioscience. Human IL-10 ELISA kit and blocking anti-IL-10 mAb (clone JES3-9D7) were from eBioscience.

### *Mycobacterial strains*

*M. bovis* BCG Pasteur (strain 1173P2) was provided by Dr. R. Stokes (University of British Columbia, Vancouver, Canada). BCG was grown in Middlebrook 7H9 broth (Difco) supplemented with 10% (v/v) OADC (oleic acid, albumin and dextrose solution; Difco) and 0.05% (v/v) Tween 80 (Sigma-Aldrich) at 37°C to an $OD_{600}$ of 0.5 on a rotating platform (50 rpm). Bacteria were harvested by centrifugation and pellets were suspended in complete media plus 10% glycerol. Mycobacterial cultures were stored in aliquot ($\sim 5 \times 10^8$/vial) at $-70$°C. Before infection, bacteria were grown 48 h in 7H9-OADC and opsonized as follows: $10^9$ mycobacteria were suspended in 1 ml of RPMI 1640 containing 50% human serum ($AB^+$ and $PPD^-$) 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. Killing mycobacteria was performed by 2 h incubation at 37°C in the presence of 50 $\mu$g/ml gentamicin. CFU counts and analysis of proteins secreted in culture media supplemented with [$^{35}$S]methionine showed no protein secretion or colony growth for gentamicin-treated bacteria (data not shown).

### *Differentiation and infection of THP-1 cells*

The 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 $\mu$g/ml). Cells were seeded at a density of $10^5$/cm$^2$ and allowed to adhere and differentiate in the presence of PMA (20 ng/ml) at 37°C in a humidified atmosphere of 5% $CO_2$ for 24 h. Depending on the quantity of cell material needed, 3-, 6-, or 10-cm diameter cell culture dishes (BD Biosciences) were used. Cells were then washed three times with HBSS and adherent monolayers were replenished with culture medium without antibiotics and infected with opsonized mycobacteria (bacteria to cell ratio of 25:1). After a period of 3 h, partially attached noningested bacteria were removed by 5 min treatment with trypsin-EDTA and extensive washing with HBSS. This procedure resulted in an infection rate of 80–90% with an approximate range from 5 to 10 bacteria per cell. Cell treatment with trypsin was done 24 h prior to stimulation with IFN-$\gamma$, and this treatment did not detach cells from the plates and had no apparent effect on MHC class II expression in control IFN-$\gamma$-treated noninfected cells (data not shown).

### *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. Cells were then washed twice with staining buffer and labeled with FITC-conjugated $F(ab')_2$ goat anti-mouse IgG for 20 min. To control for cell viability, cells were incubated with propidium iodide (0.5 $\mu$g/ml staining buffer) for 10 min, and cells were then washed twice and fixed in 2% paraformaldehyde in staining buffer. 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) and the mean fluorescence intensity (MFI) was calculated for each histogram. Results are expressed as MFI index, which corresponds to the ratio calculated using: [(MFI of cells + specific Ab)/(MFI of cells + irrelevant isotype-matched IgG)].

### *Measurement of Cat S activity*

Cat S activity was measured using the fluorogenic substrate Z-Val-Val-Arg-NHMec as described (23, 24). 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 in a Sonic Dismembrator 60 (Fisher Scientific) in ice and cell debris, and membranes were removed by centrifugation at 12,000 × *g* for 30 min at 4°C. Fifty microliters of the soluble fractions (~100 $\mu$g of protein) were added to 50 $\mu$l 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 $\mu$l of 12.5 $\mu$M Z-Val-Val-Arg-AMC (Cat S/L substrate) were added to the mixtures. After additional incubation at 40°C for 10 min in the dark, fluorescence was measured at $\lambda$ex/em 360/460 nm in a VersaFluor instrument (Bio-Rad). Cat S activities were calculated by reference to a standard curve using a range (20 to 20,000 U/ml) of active recombinant human Cat S. Reaction mixtures with BSA instead of cell lysate were used to control for background and nonspecific activity and results were expressed after subtracting the fluorescence value corresponding to this control.

### *RNA isolation, RT-PCR, and quantitative RT-PCR*

RNA preparation, cDNA synthesis, and PCR conditions were previously described (22, 25). The following primers were used for both RT-PCR and quantitative RT-PCR: Cat S sense TCA ACT GAA AAA TAT GGA A and antisense CCT TCT TCA CCA AAG TTG TGG CC. $\beta$-actin sense CAC CCC GTG CTG CTG ACC GAG GCC and antisense CCA CAC GGA GTA CTT GCG CTC AGG were used as endogenous control. Controls included in the RT-PCR were no RNA and RNA without reverse transcriptase. Quantitative RT-PCR was performed in a total volume of 20 $\mu$l. Each reaction sample consisted in 1:1 mixture of diluted (1/100) cDNA sample to DYNAMO SYBR Green Q-PCR Master mix (MJ Research) combined to a 1:1 mixture of gene-specific forward and reverse primers to the same SYBR Green Master mix. Quantitative RT-PCR was developed in a DNA Engine Opticon PCR cycler (MJ Research). Thermal cycler conditions were as follows: 1 × 10 min 95°C, 41 cycles of denaturation (1 min, 95°C), annealing (1 min, 55°C), and extension (1 min, 70°C). Samples were normalized using the $\Delta$Ct-based algorithm (Opticon Monitor analysis software).

### *Metabolic labeling and detection of Ii fragments*

Control and BCG-infected cells were stimulated with IFN-$\gamma$ for 24 h. Z-Phe-Leu-COCHO (Cat S inhibitor) was added at 50 mM 6 h before radiolabeling and maintained during the pulse-chase period. Infected cells and cells treated with Z-Phe-Leu-COCHO were washed with HBSS and incubated for 1 h in methionine-free RPMI 1640. Cells were then pulsed with 200 $\mu$Ci/ml [$^{35}$S]methionine for 1 h in methionine-free RPMI 1640 supplemented with 5% dialyzed FCS. Cells were washed three times with HBSS and chased for 5 h in complete media. At the end of the radiolabeling period, cells were extensively rinsed with HBSS and lysed by scraping in Tris-buffered saline containing 1% Nonidet P-40, 1 mM PMSF, and protease inhibitors mixture. Cell debris was removed by centrifugation in a microfuge for 20 min at 4°C and immunoprecipitations using the anti-Ii mAb (clone PIN-1) were performed 2 h at 4°C using equal amounts of

TCA precipitable radioactivity from each treatment group. Immune complexes were collected by adsorption to protein A and released by boiling agarose beads in 2× SDS sample buffer. Samples were separated by 7–20% gradient SDS-PAGE, dried, and exposed to x-ray films.

### *Western blotting of Cat S*

Control and infected cells were treated with IFN-$\gamma$ for 24 h then washed with HBSS and whole cell lysates were prepared in Nonidet P-40 lysis buffer (50 mM sodium acetate, 5 mM $MgCl_2$, 0.5% Nonidet P-40, pH 7.4) in the presence of protease inhibitors mixture and PMSF. Proteins (50 $\mu$g/lane) were separated by 12% SDS-PAGE then transferred to nitrocellulose membrane and probed with anti-Cat S mAb. To assess the amount of individual proteins in each sample, after detection of bound anti-Cat S mAb, membranes were stripped and reprobed with anti-actin Abs and developed by ECL as described (15, 25).

### *Statistical analysis*

All data are expressed as the mean ± SD. Statistical analysis was performed using Student's *t* test. Values of $p < 0.05$ were considered to be significant.

## Results

### Mycobacterium bovis *BCG increases surface expression of immature class II molecules in macrophages*

Initial experiments examined the effect of BCG on surface expression of MHC class II molecules by flow cytometry analysis of cell stained with anti-class II mAb clone Tu36. Consistent with previously published data (15, 22), the fluorescence histograms in Fig. 1*A* showed that infection with live but not killed BCG partially inhibited (~55% reduction) IFN-$\gamma$-induced surface expression of class II molecules in differentiated THP-1 cells. Tu36 mAb recognizes all class II subpopulations including $\alpha\beta$ dimers associated with either Ii or Ii degradation products as well as peptide-loaded molecules (26). To specifically determine the nature of class II molecules that reach the surface of infected cells, staining with mAb PIN-1 was done. PIN-1 recognizes immature class II molecules associated with intact or partially degraded Ii (27). In parallel, comparison was made with cells stained with mAb L243, which recognizes peptide-loaded class II molecules as well as those still loaded with CLIP fragment (26, 28). The data in Fig. 1*B* showed that IFN-$\gamma$ stimulation of BCG-infected cells brought about an increase in expression of immature class II molecules only. In contrast, the increase in class II expression in response to IFN-$\gamma$ by control cells was accounted for nearly exclusively by mature $\alpha\beta$ dimers. Of interest, the phenotype of the IFN-$\gamma$ response observed for infected cells was recapitulated in cells where Cat S, a cysteine protease that plays a major role in Ii processing (21, 29, 30), was inhibited by pretreatment with the Cat S inhibitor Z-FL-COCHO (31).

### *Cells infected with BCG show reduced Cat S activity*

Cat S has an established role in the processing of Ii in human APCs (21). This finding, together with the similarity in the qualitative distributions of cell surface class II subpopulations between BCG-infected cells and those exposed to the Cat S inhibitor Z-Phe-Leu-COCHO (Fig. 1*B*), suggested that infected cells may have reduced Cat S activity. To examine this hypothesis, BCG-infected cells were incubated with IFN-$\gamma$, and endogenous Cat S activity was measured in cell lysates. As shown in Fig. 2*A*, although IFN-$\gamma$ treatment of control cells resulted in a doubling of Cat S activity, enzyme activity was in fact dramatically reduced in BCG-infected cells to a level below the basal activity observed in cells not infected and not treated with IFN-$\gamma$. Consistent with this observation, RT-PCR and quantitative RT-PCR experiments on total RNA showed that infection with BCG was associated with reduced Cat S mRNA expression in IFN-$\gamma$-treated cells (Fig. 2, *B* and *C*), suggesting that reduced Cat S activity was related to bacterial effects either on transcription of the Cat S gene or on mRNA stability.

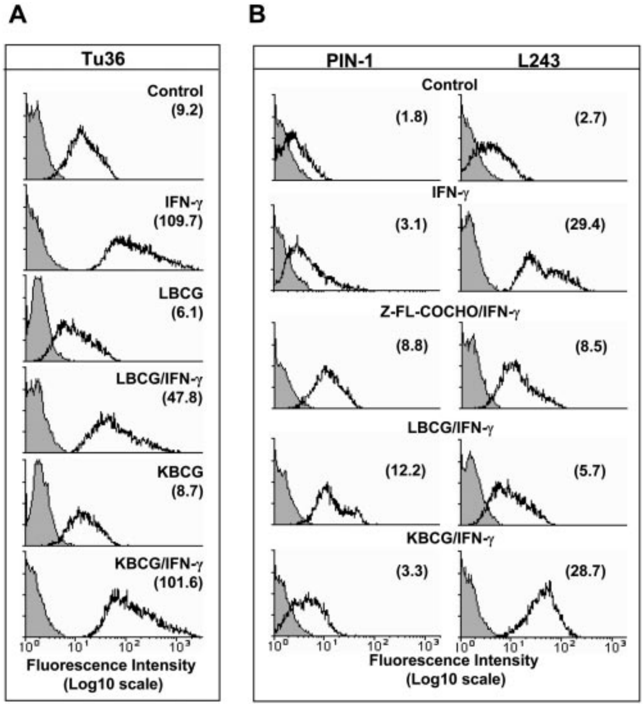

**FIGURE 1.** Surface expression of mature class II molecules is reduced in cells infected with BCG. *A*, PMA-differentiated THP-1 cells were incubated with opsonized live (LBCG) or killed (KBCG) *M. bovis* BCG at a bacterium to cell ratio of 25:1 for 24 h, and then IFN-$\gamma$ (200 U/ml) was added for 24 h. Cells were then incubated for 20 min with anti-HLA-DR mAb (clone Tu36) or irrelevant mAb. 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. *B*, Cells were infected with live (or killed) BCG or exposed to 50 nM Z-FL-COCHO (Cat S inhibitor) before IFN-$\gamma$ stimulation. Cells were then washed and scraped, and aliquots were stained with L243 mAb, PIN-1 mAb, or irrelevant isotype-matched IgG. Results are expressed as histograms of fluorescence intensity. Gray histograms represent cells stained with irrelevant isotype-matched IgG and open histograms represent cells stained with specific mAb. Values in parentheses (*inset*) 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. Data shown are from one of four independent experiments that yielded similar results.

### *Ii p10 fragments accumulate in BCG-infected cells*

In human APCs, Cat S represents the main cysteine protease involved in generating CLIP from the Ii intermediate fragment p10 (21, 29, 30). Given the evidence we found for reduced expression of mature Cat S protein and activity in BCG-infected cells, we sought to determine whether this had any impact on Ii processing. Cells were either untreated, infected with BCG, or treated with Cat S inhibitor, stimulated with IFN-$\gamma$, and pulse-chased with [$^{35}$S]methionine. Ii and its intermediates were immunoprecipitated with PIN-1 mAb, which also recognizes the Cat S product p10 (32). Samples were then analyzed by SDS-PAGE and autoradiography. The results shown in Fig. 3 indicate that BCG-infected cells accumulated significant amounts of p10 fragments and this was comparable to that observed in Z-Phe-Leu-COCHO-treated cells. In contrast, there were almost no Ii intermediates in IFN-$\gamma$-stimulated control cells. These results suggest that abortive processing of Ii accounts for increased surface expression of immature class II molecules by BCG-infected cells.

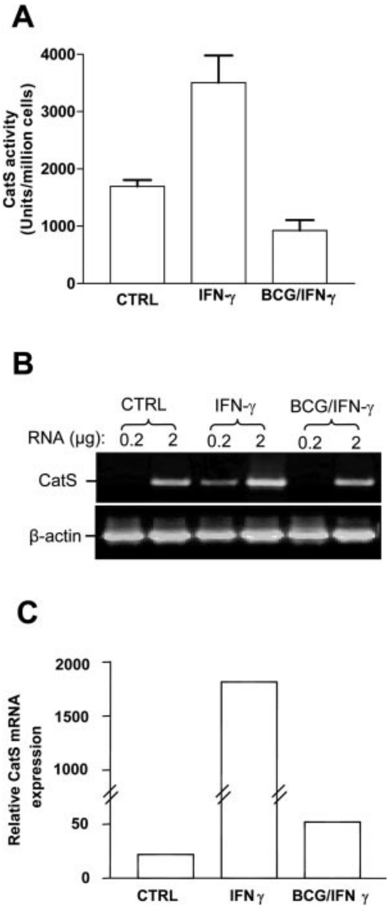

**FIGURE 2.** Cat S activity and gene expression are reduced in BCG-infected cells. *A*, PMA-differentiated THP-1 cells were left untreated (CTRL) or infected with opsonized live BCG at a bacteria to cell ratio of 25:1 for 24 h, and then IFN-γ (200 U/ml) was added for an additional 24 h. Cell lysates were prepared and assayed for Cat S activity using the fluorogenic substrate Z-Val-Val-Arg-AMC as described in *Materials and Methods*. *B*, Total RNA was extracted from control (CTRL), IFN-γ-treated, and BCG-infected/IFN-γ-treated cells. RNA samples were quantified by a UV spectrophotometer and equal amounts of RNA (0.2 and 2 μg) were analyzed by RT-PCR using Cat S and β-actin specific primers as previously described (22, 25). *C*, Total RNA were prepared from control and infected cells and submitted to quantitative RT-PCR as described in *Materials and Methods*. Mean ± SD of three independent experiments (*A*) and from one of two independent experiments (*B*) that yielded similar results are shown, and *C* represents the average of two experiments.

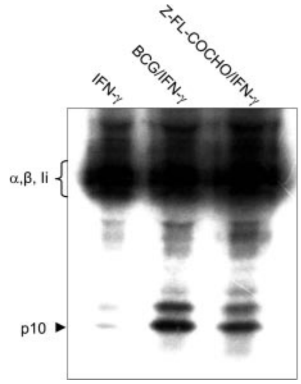

**FIGURE 3.** BCG-infected THP-1 cells accumulate Ii p10 fragment. PMA differentiated THP-1 cells were infected or not (control) with BCG then stimulated with 200 U/ml IFN-γ for 24 h. Cells were then pulse-chased with [$^{35}$S]methionine as described in *Materials and Methods*. Cat S inhibitor was added 6 h before and during radiolabeling. Cells were then lysed, immunoprecipitated with PIN-1 mAb, and the protein complexes were analyzed by 7–20% gradient SDS-PAGE and autoradiography. The data shown are from one of three independent experiments that yielded similar results.

### *Cat S inhibition in BCG-infected cells is dependent on IL-10 secretion*

Previous studies have shown that IL-10 is a major cytokine that antagonizes macrophage antimicrobial effector function (30, 33, 34) including export of mature class II molecules to the plasma membrane (35). Thus, we examined whether inhibition of mature class II expression in BCG-infected cells is also dependent on IL-10 induction. First, we verified whether BCG induces IL-10 production in our cell system model. Supernatants were collected from cells coincubated for 24 h with BCG at various multiplicities of infection (MOI) and assayed for IL-10 by a human IL-10 ELISA kit. As has been observed previously for Mtb (36, 37), BCG was found capable of inducing the secretion of substantial amounts of IL-10 (320 pg/ml at MOI of 25:1) by THP-1 cells (Fig. 4*A*). Of particular interest, immunostaining with clones L243 and PIN-1 mAbs showed that the addition of blocking anti-IL-10 mAb to BCG-infected cells significantly restored surface expression of mature class II molecules (70% of the response to IFN-γ) (Fig. 4*B*), and this was dependent on reduced expression of immature dimers. Consistent with these findings anti-IL-10 mAb also blocked the inhibitory effect of BCG on the catalytic activity of Cat S (Fig. 4*C*).

Additional experiments examined the effect of anti-IL-10 mAb on the level of active Cat S in infected cells. Cat S is first translated as inactive precursor (pro-Cat S), which is subsequently activated by autocleavage to generate mature and active Cat S (38). Cells were infected and stimulated with IFN-γ in the presence of blocking anti-IL-10 mAb. Thereafter, lysates were prepared and examined along with recombinant active Cat S by Western blotting with anti-human Cat S mAb that recognizes both pro-Cat S (36 kDa) and mature Cat S (28 kDa). Fig. 4*D* shows that IFN-γ induced strong expression of Cat S and that the band corresponding to active Cat S was almost completely absent in lysates prepared from BCG-infected cells. However, the inhibitory effect of BCG was reversed significantly when blocking anti-IL-10 was added to infected cells.

Experiments using primary macrophages derived from normal human monocytes showed that IFN-γ stimulation resulted in a class II phenotype dominated by surface expression of mature $\alpha\beta$ dimers. In contrast, as was the case for THP-1 cells, BCG-infected human monocyte-derived macrophages and monocyte-derived macrophages treated with the Cat S inhibitor Z-FL-COCHO showed reduced surface expression of mature class II molecules in favor of abnormal surface export of unprocessed $\alpha\beta$ Ii complexes (Fig. 5*A*). Blocking IL-10 with specific mAb restored expression of mature class II molecules in infected cells (Fig. 5*B*) consistent with findings with the THP-1 macrophage system model. Taken together, these observations suggested that class II molecules expressed in BCG-infected macrophages are predominantly if not exclusively immature and thus are unlikely to contribute to presentation of mycobacterial Ags to CD4$^+$ T cells.

### *Introduction of active Cat S into BCG-infected macrophages restored the expression of mature class II molecules*

To investigate more directly the role of Cat S activity on surface expression of mature class II molecules we attempted to deliver recombinant active Cat S into class II compartment in infected cells. Initial experiments examined the capacity of Profect P1 reagent (Targeting Systems) to deliver proteins into the endosomal

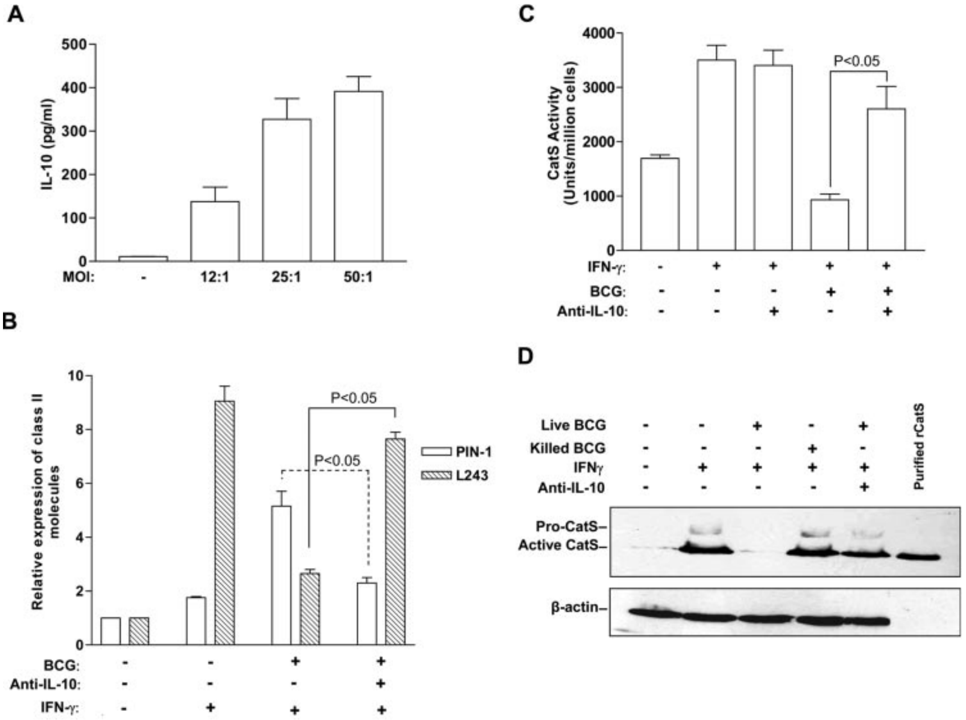

**FIGURE 4.** Cat S inhibition in BCG-infected cells is dependent on IL-10. *A*, PMA differentiated THP-1 cells were infected for 24 h with opsonized BCG at the indicated MOI. Thereafter, supernatants were collected and cleared by high-speed centrifugation. Serial dilutions of supernatants were assayed in duplicate for IL-10 by ELISA. *B*, PMA-differentiated THP-1 cells were infected with BCG (MOI of 25:1) for 6 h then blocking anti-IL-10 mAb was added at a final concentration of 10 μg/ml, and cells were incubated for a further 18 h. Cells were then washed and stimulated with IFN-γ for 24 h in the continuous presence of anti-IL-10 Abs. Cells were then recovered and aliquots were stained with specific mAb to class II subpopulations as described in Fig. 1. MFI indices were obtained from fluorescence histograms and results are expressed as the fold increase of specific fluorescence signal relative to the control untreated cells. Values are the mean ± SD of three independent experiments. *C*, THP-1 cells were infected and treated with IFN-γ and anti-IL-10 Abs as described in *B*. Cells were then recovered and lysates were prepared for Cat S assay as described in *Materials and Methods*. *D*, THP-1 cells were treated as described in *B* and *C* and lysates were prepared and analyzed by Western blotting using an anti-human Cat S mAb. Membranes were stripped and reprobed with anti-actin to assess the amount of protein in each lane. The position of translated Cat S as inactive precursor (pro-Cat S) and mature active Cat S are indicated. *A–C*, Data are mean ± SD of three independent experiments, whereas *D* are one of two independent experiments that yielded similar results.

compartment of differentiated THP-1 cells. Various concentrations of recombinant Cat S were mixed separately with Profect P1 and added to adherent THP-1 cells, and cells were incubated for 6 h as recommended by the manufacturer. Thereafter cell lysates were prepared and analyzed by Western blotting with anti-Cat S mAb. Results in Fig. 6*A* indicated that Profect P1 is able to deliver substantial amount of Cat S into THP-1 cells. Additional control experiments based on confocal microscopy analysis of IFN-γ-treated cells (i.e., expressing class II molecules) showed that profection with GST protein could transfect over 60% cells and that the delivered protein is able to join class II compartment (Fig. 6*B*). In this control experiment, transfection was done with GST (29 kDa) instead of active Cat S (28 kDa) because it is not possible to distinguish by intracellular staining between IFN-γ-induced Cat S and exogenously added Cat S. After validating the endosomal targeting with the Profect P1 technique, an equivalent amount of purified active recombinant Cat S or heat-inactivated enzyme were mixed separately with Profect P1 and delivered to macrophages infected with BCG and treated with IFN-γ for 18 h. Cells were collected 6 h after profection and stained with L243 and PIN-1 mAbs then analyzed by flow cytometry. As shown in Fig. 6*C*, transfection with active Cat S resulted in the return of mature class II molecules to the surface of infected cells. In contrast, expression of mature class II molecules was not observed when heat-inactivated Cat S was used as control proteins.

Taken together, these findings provide the basis for a model in which reduced expression of mature class II molecules by BCG-infected cells involves inhibition of Cat S expression and activity resulting in abortive processing of Ii.

## Discussion

Microbial proteins in their native conformation are processed by macrophages and dendritic cells and presented as short peptide Ags to Th cells in an MHC class II-restricted fashion (9). Early studies showed that macrophages can be induced to process Ag and to express class II molecules loaded with antigenic peptide by soluble factors, in particular IFN-γ (39). More recently, expression of class II molecules in response to IFN-γ has been shown to be regulated primarily at the level of transcription. This effect requires induction of the CIITA (40), and expression of the *CIITA* gene itself is dependent on STAT-1 signaling (41–43).

Defective expression of IFN-γ-induced MHC class II molecules in macrophages infected with pathogenic mycobacteria has been studied extensively and has been shown to involve multiple mechanisms (44). For example, using the RAW 264.7 mouse macrophage cell line infected with *M. avium* as a model, reduced surface expression of class II proteins was shown to be associated with inhibition of IFN-γ signaling and STAT-1 phosphorylation and MHC class II gene expression (45). Recently, inhibition of class II gene expression in macrophages infected with virulent Mtb was

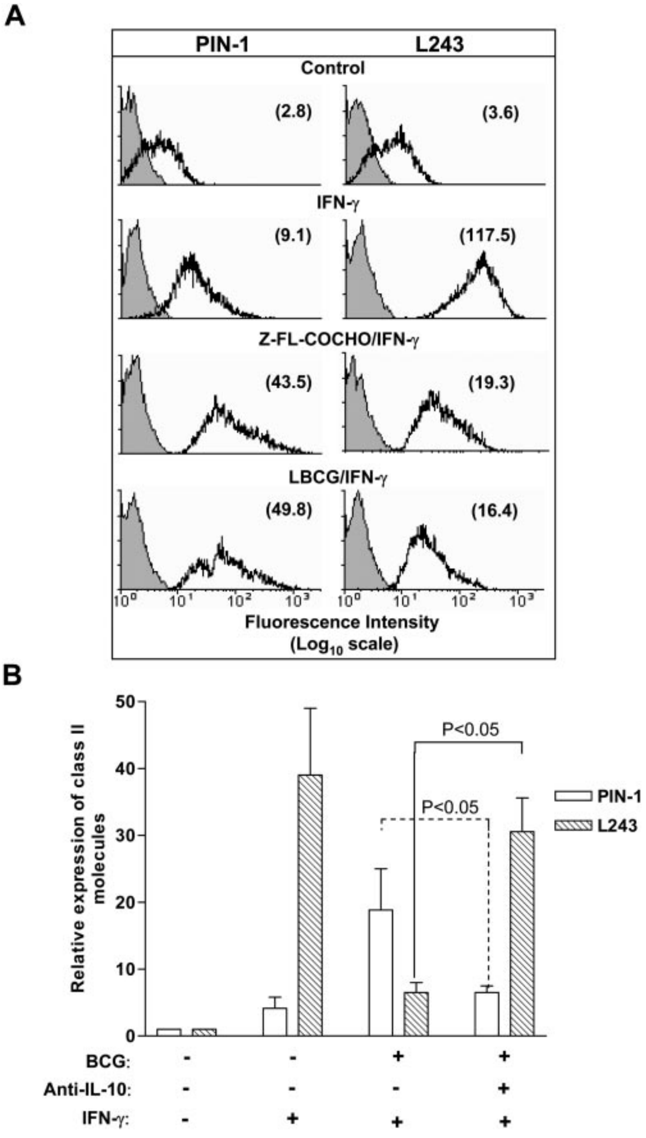

**FIGURE 5.** Surface expression of class II molecules in primary macrophages. Human PBMC were isolated from fresh blood obtained from $PPD^-$ healthy donors by centrifugation over Histopaque as previously described (70) then $CD14^+$ cells were purified using the StemSep Human Monocyte Enrichment kit obtained from StemCell Technologies. *A*, Monocytes were differentiated to macrophage by 24-h adherence to culture plates then used for infection with live (or killed) BCG or treatment with the Cat S inhibitor Z-FL-COCHO before stimulation with IFN-γ as described in Fig. 1. *B*, Macrophages were infected with live BCG then blocking anti-IL-10 mAb was added at a final concentration of 10 μg/ml and cells were incubated for a further 18 h. Cells were then washed and stimulated with IFN-γ for 24 h in the continuous presence of anti-IL-10 Abs. Cells were then washed and scraped, and aliquots were stained with L243 mAb, PIN-1 mAb, or irrelevant isotype-matched IgG as described in Fig. 1. Results are expressed as histograms of fluorescence intensity (*A*). Gray histograms represent cells stained with irrelevant isotype-matched IgG and open histograms represent cells stained with specific mAb. Values in parentheses (*inset*) indicate MFI indices calculated as described in Fig. 1. The data shown in *A* are from one of two independent experiments that yielded similar results. Data in *B* are the mean ± SD of three independent experiments.

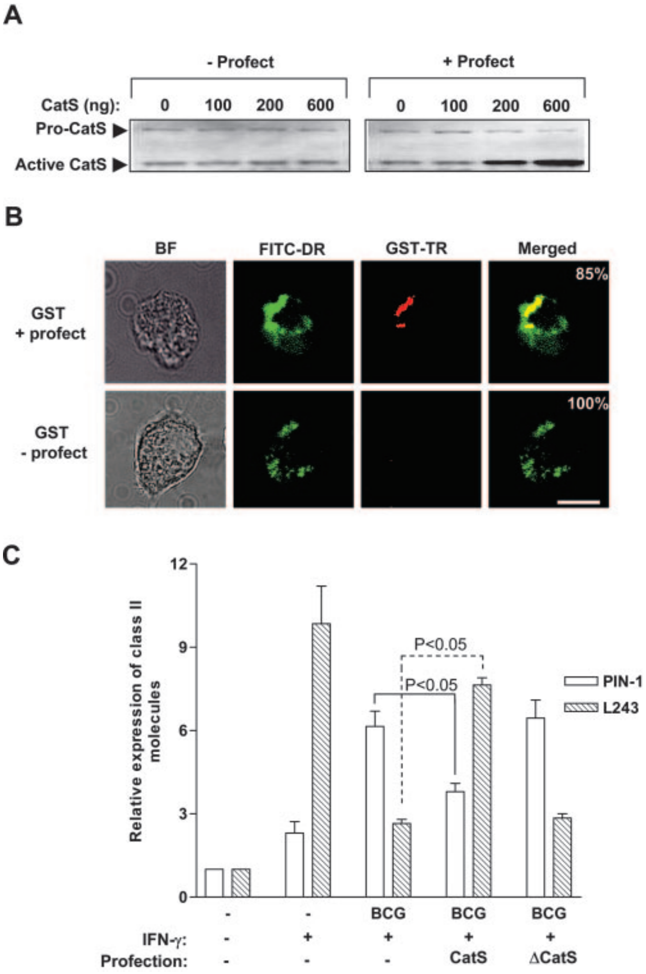

**FIGURE 6.** Introduction of active Cat S into BCG-infected cells restored surface expression of mature class II molecules. *A*, PMA differentiated THP-1 cells were incubated for 6 h at 37°C with various concentrations of free Cat S or in complex with Profect P1 reagent in serum-free media according to the manufacturer's instructions. Cells were washed and lysed with RIPA buffer and analyzed by SDS-PAGE and Western blot with anti-Cat S mAb. *B*, PMA differentiated THP-1 cells were stimulated with IFN-γ for 18 h then incubated for 6 h with free GST or GST in complex with Profect P1. Cells were washed, fixed, permeabilized, and stained with anti-GST mAb and Texas Red (TR)-labeled secondary Ab. Cells were then stained with FITC-labeled anti-DR mAb. Labeled cells were analyzed with digital confocal microscopy, and optical sections (0.2 μm) were scanned for green and red fluorescence. The images are displayed with green (HLA-DR), red (GST), and yellow signals, with the latter depicting colocalization of green with red. Bar, 10 μm. *C*, PMA differentiated THP-1 cells (~5 × $10^6$ in 10-cm diameter culture plate) were infected for 24 h with opsonized BCG (MOI of 25:1) then stimulated with 200 U/ml IFN-γ for 18 h. Cells were then transfected with active Cat S as follow: purified active recombinant Cat S (600 ng) or an equivalent amount of heat-inactivated enzyme (ΔCatS) were incubated separately with Profect P1 reagent in serum-free media. Thereafter, the protein-Profect P1 complexes were added to the plates and cells were reincubated at 37°C for a period of 6 h. Cells were then stained with L243 mAb and PIN-1 mAb and examined by flow cytometry as described in Fig. 1. The data in *A* and *B* are from one of three independent experiments that yielded similar results and data in *C* are the mean ± SD of three independent experiments.

recently shown to involve histone deacetylase complex formation at the HLA-DR promoter, resulting in histone deacetylation and gene silencing (46).

In other studies, such as the human macrophage model of THP-1 cells infected with Mtb Erdman and BCG Pasteur, it was found that IFN-γ-induced STAT-1 activation and expression of *CIITA* and MHC class II genes occurred normally (15, 22). However, despite apparently normal signaling responses to IFN-γ, in the latter study infected cells showed marked intracellular sequestration of immature class II molecules and reduced surface expression, in particular in cells infected with BCG (22). Albeit at a reduced level, infected cells continued to deliver substantial amounts of class II molecules to the cell surface, but the nature of these molecules was not determined.

Maturation and transport of class II molecules to the cell surface is associated with important changes in their biochemical properties in particular the enzymatic processing of Ii and peptide loading in the MIIC (18). In the present study, experiments using PIN-1 mAb, which recognizes the N-terminal portion of Ii (27), and L243 mAb, which recognizes mature peptide-loaded class II molecules as well as those still loaded with the CLIP (26, 28), demonstrated that macrophage infection with BCG was associated with the nearly exclusive export of immature class II molecules associated with either intact or partially degraded Ii to the cell surface. These findings suggested the possibility of a defect in Ii processing in infected cells.

Cat S is the principal protease involved in late steps of Ii processing in which p22 is converted to p10 and then to CLIP (21, 29, 30). This conclusion is based upon experiments that used Cat $S^{-/-}$ cells and cells exposed to Cat S inhibitor Z-Phe-Leu-COCHO, which showed abnormal intracellular accumulation of the Ii fragment p10 (28). Thus, the apparent arrest of class II processing in BCG-infected cells that we observed could have been explained by a defect at the level of Cat S. In fact, the finding that Cat S activity was reduced in cells infected with BCG and the observation of p10 fragment accumulation in both infected cells and cells treated with Z-Phe-Leu-COCHO suggested that Cat S is a major target of mycobacteria in infected macrophages. Indeed, mRNA analysis and Western blot data showed substantial attenuation of IFN-$\gamma$-induced Cat S gene expression and correspondingly Cat S protein levels in infected cells.

Two opposing hypotheses have been suggested concerning the way in which nascent $\alpha\beta$ Ii complexes reach the Golgi apparatus in normal cells; they may be directly targeted to endosomes by means of a pathway dependent on the AP-1 clathrin adaptor complex bypassing the cell surface, or they may first be directed to the plasma membrane and then rapidly internalized via an AP-2-dependent pathway (47, 48). In this context, a recent report from the laboratory of Benaroch and colleagues (49) showed that AP-2 depletion led to a large increase in surface levels of $\alpha\beta$ Ii complexes, inhibited their rapid internalization, and strongly delayed the appearance of mature MHC II in intracellular compartments. Therefore, we can speculate that accumulation of unprocessed $\alpha\beta$ Ii complexes at the surface of infected cells might result from a saturation of the AP-2 recycling pathway. Such hypothesis does not rule out the possibility that BCG infection might also directly alter the AP-2 recycling system.

It has been well established in humans, that IL-10 is able to induce a state of Ag-specific unresponsiveness (50, 51). Effects of IL-10 on human monocytes include, but are not limited to down-regulation of plasma membrane expression of major MHC class II molecules (53, 50) as well as inhibition of the expression of co-stimulatory molecules such as ICAM-1, CD80, and CD86 (52, 53). Moreover, infection of macrophages with *M. tuberculosis* has been shown to induce IL-10 production (54), but its modulatory role in BCG-infected cells has not been clearly defined. Indeed, in the present study, infection of macrophages with BCG was found to induce IL-10 production and incubation of cells in the presence of neutralizing Abs to IL-10 restored both Cat S expression and surface expression of mature class II molecules. Taken together, these findings establish a role for IL-10 in the antagonistic effect of BCG on IFN-$\gamma$-induced Cat S activity and therefore on the surface expression of mature class II molecules. This conclusion is consistent with the previously published finding that IL-10 attenuated Cat S activity and the export to the cell surface (but not synthesis) of class II molecules in dendritic cells activated with the proinflammatory cytokines IL-1 and TNF-$\alpha$ (55). It is also consistent with the observation of an improved protective immune response to BCG in IL-$10^{-/-}$ mice (56).

A series of elegant biochemical and molecular biological studies have revealed that members of the SOCS (suppressor of cytokine signaling) family of proteins act in a negative feedback loop, inhibiting the cytokine-activated JAK/STAT signaling pathway to modulate cellular responses (reviewed in Ref. 57). Based on the finding that SOCS-3 is a negative feedback regulator of IFN-$\gamma$ signaling (58) and that IL-10 is a specific inducer of SOCS-3 (59), one can suggest that BCG attenuation of IFN-$\gamma$ responses would be mediated by a mechanism dependent on IL-10-induced SOCS-3 inhibition of IFN-$\gamma$-activated JAK1/STAT-1 or JAK2/STAT-1 signaling.

It is of interest to view our results in the context of those of Pancholi et al. (13), who observed that BCG organisms growing in human monocyte-derived macrophages were apparently sequestered from recognition by immune CD4$^{+}$ cells. These investigators considered this finding to be independent of effects on class II expression because they found that infected cells continued to express class II molecules. However, the nature of these class II proteins was not explored. Based upon the results in this report, we conclude that sequestration of BCG from effective Ag presentation is likely related to maturational arrest of class II molecules processing. This technique appears to be a previously unrecognized strategy used by mycobacteria to evade immune recognition.

The efficacy of BCG as a vaccine varies from 0 to 80% in different populations, with a consistently low efficacy in many parts of the world where anti-tuberculosis protection is the most needed (60, 61). Although BCG vaccine is generally safe and rarely induces disease in human, it appears to mimic virulent Mtb strains in their capacity to inhibit Ag processing and presentation to T cells. Thus, impaired surface expression of mature class II molecules in BCG-infected cells as demonstrated in the present study may at least partially explain the failure of the vaccine BCG to induce efficient anti-tuberculosis immunity.

The mechanisms used by mycobacteria to inhibit MHC class II expression are complex and still not completely understood. Pathogenic mycobacteria produce numerous protein and other molecules within host cells (62–65), with the potential to interfere with innate or adaptive immune responses. In this context, it has been shown that macrophage exposure to purified mycobacterial 19 kDa Ag (p19), a pathogen associated molecule secreted within the host cells, partially inhibited IFN-$\gamma$-induced surface expression of MHC class II molecules (66, 67). Moreover, this appeared to be related to attenuation of MHC gene transcription by p19 (68). At the same time, recent studies showing that BCG p19$^{-}$ bacilli were equally effective in inhibiting class II-directed Ag presentation by macrophages when compared with wild-type BCG (69), which suggests that mycobacteria probably use multiple virulence factors to interfere with MHC class II expression and function.

In conclusion, the results presented show that macrophages infected with the vaccine strain BCG express immature MHC class II populations, molecules that are known to be ineffective for Ag presentation to T cells. The data show that Cat S inhibition via mycobacterial-induced IL-10 plays an important role in the maturational arrest of class II processing. Further study of the mechanisms that contribute to inhibition of Ag presentation by macrophages infected with mycobacteria in general and BCG in particular may advance the development of improved BCG-based immunization strategies for inducing host resistance to tuberculosis. For example, engineering of BCG so as to avoid IL-10 induction or to constitutively express Cat S may lead to the development of more effective recombinant vaccines.

## Acknowledgments

We thank Neil Reiner for critically reviewing the manuscript. We also thank R. Stokes for providing *M. bovis* BCG cultures, P. Cresswell for the gift of the PIN-1 mAb, M. Lopez for assistance with cell cultures, and Genentech Inc. for the gift of human rIFN-$\gamma$.

## Disclosures

The authors have no financial conflict of interest.

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