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Secreted Immunodominant *Mycobacterium tuberculosis* Antigens Are Processed by the Cytosolic Pathway

Jeff E. Grotzke,* Anne C. Siler,† Deborah A. Lewinsohn,‡ and David M. Lewinsohn*,†

Exposure to *Mycobacterium tuberculosis* can result in lifelong but asymptomatic infection in most individuals. Although CD8+ T cells are elicited at high frequencies over the course of infection in both humans and mice, how phagosomal *M. tuberculosis* Ags are processed and presented by MHC class I molecules is poorly understood. Broadly, both cytosolic and noncytosolic pathways have been described. We have previously characterized the presentation of three HLA-I epitopes from *M. tuberculosis* and shown that these Ags are processed in the cytosol, whereas others have demonstrated noncytosolic presentation of the 19-kDa lipoprotein as well as apoptotic bodies from *M. tuberculosis*-infected cells. In this paper, we now characterize the processing pathway in an additional six *M. tuberculosis* epitopes from four proteins in human dendritic cells. Addition of the endoplasmic reticulum-Golgi trafficking inhibitor, brefeldin A, resulted in complete abrogation of Ag processing consistent with cytosolic presentation. However, although addition of the proteasome inhibitor epoxomicin blocked the presentation of two epitopes, presentation of four epitopes was enhanced. To further examine the requirement for proteasomal processing of an epoxomicin-enhanced epitope, an additional six *M. tuberculosis* epitopes from four proteins in human dendritic cells. Addition of the endoplasmic reticulum-Golgi trafficking inhibitor, brefeldin A, resulted in complete abrogation of Ag processing consistent with cytosolic presentation. How-}

Tuberculosis disease remains a global health concern because of the large global burden of *Mycobacterium tuberculosis*-infected individuals, the emergence of multidrug-and extensively drug-resistant *M. tuberculosis* strains, and the di- sastrous consequences of coinfection with *M. tuberculosis* and HIV. Control of *M. tuberculosis* infection is facilitated by the development of an adaptive CD4+ and CD8+ T cell immune response and the coordinated development of antimycobacterial effector mechanisms, such as production of proinflammatory cytokines (1, 2), antibacterial proteins (3, 4), and proapoptotic capacity (5–8). Because of the importance of cell-mediated immunity for control of *M. tuberculosis* infection, further understanding of the factors that promote both efficient T cell priming and recognition of infected cells are important for development of an effective *M. tuberculosis* vaccine.

*M. tuberculosis* is able to modulate the phagosomal environment after internalization, characterized by incomplete phagosomal acidification, incomplete maturation of phagosomes to a rab7+ late endosome, lack of phagolysosomal biogenesis, and continued access to extracellular nutrients, such as iron, through the ongoing fusion of early endosomes (9). Although *M. tuberculosis* is able to evade innate immune mechanisms within phagocytic cells, this does not mean that *M. tuberculosis* infection goes unnoticed. The phagosome is a component of the HLA-II processing and presentation pathway, which serves to alert CD4+ T cells to the presence of exogenous Ags. Indeed, CD4+ T cells are elicited at high frequencies after *M. tuberculosis* infection and are vital for host protection. Alternatively, because the phagosome is walled off from the cytosolic and endoplasmic reticulum (ER)-derived HLA-I processing and loading machinery, how *M. tuberculosis* Ags are processed and presented on HLA-I molecules is much less understood. Studies using bead phagosomes have shown that multiple pathways are functional. Ags can either escape the phagosome to the cytosol where they are degraded by the proteasome (cytosolic pathway) or remain within the endocytic pathway where they are degraded (vacuolar or noncytosolic pathway). In vivo data have suggested that the major pathway of cross-presentation and cross-priming is TAP and immunoproteasome dependent (10–16), providing evidence for the important role of the cytosolic pathway in the priming of the CD8+ T cell response. Nonetheless, cross-priming of cell-associated and viral Ags has been demonstrated to occur in the absence of TAP and, in some cases, relies on processing by cathepsin S (14).

The pathways by which *M. tuberculosis* Ags are presented on HLA-I are only beginning to be understood. Detailed analysis of presentation of three epitopes from two *M. tuberculosis* proteins has shown that *M. tuberculosis* Ags can be retrotranslocated out of the phagosome, giving them access to the cytosol (17). Once cytosolic, *M. tuberculosis* proteins require proteasomal degradation and TAP transport into either the ER or back into the phagosome, showing the use of the cytosolic processing and presentation pathway (17, 18). Conversely, there is support for endocytic processing of *M. tuberculosis*-derived Ags and apoptotic bodies. Presentation of the 19-kDa lipoprotein (lpqh) did not require TAP.

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Abbreviations used in this paper: BFA, brefeldin A; DC, dendritic cell; ER, endoplasmic reticulum; HS, human serum; LCL, lymphoblastoid cell line; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MHC-I, MHC class I; MOI, multiplicity of infection; Mtbc, *Mycobacterium tuberculosis*; Ox, oxidized; RD1, region of difference 1; TTPHl, tripeptidyl peptidase II.

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transport but did require trafficking outside of the mycobacterial phagosome (19). One striking feature of mycobacterially infected cells is the large amounts of vesicular trafficking of bacterial-derived proteins and lipids seen within the cell (20–22). This finding suggests that M. tuberculosis proteins may have unique access to the endocytic pathway, as well as access to bystander cells. Along those lines, Schaible et al. (8) showed that apoptotic bodies from mycobacterially-infected cells can be taken up by uninfected bystander cells and processed in an acidification-dependent but proteasome-independent manner. In all, these findings present a diverse picture of Ag processing and presentation pathways in M. tuberculosis-infected cells.

In this report, we seek to provide a more broad understanding of which pathway(s) are required for HLA-I presentation of six recently defined epitopes from secreted M. tuberculosis proteins. We screened Ag presentation in M. tuberculosis-infected cells using inhibitors that definitively distinguish cytosolic from noncytosolic pathways and find that presentation of all six of these epitopes use the cytosolic pathway.

**Materials and Methods**

**Reagents**

Inhibitors of Ag processing were obtained from EMD Biosciences (San Diego, CA; epoxomicin, bafilomycin, leupeptin, pepstatin A, and AAF-CCMK), Sigma-Aldrich (St. Louis, MO; chloroquine and cycloheximide), or Invitrogen (Carlsbad, CA; brefeldin A [BFA]). Soluble CFP10 protein was provided by GlaxoSmithKline Biologicals (Hamilton, MT). Peptides were synthesized by Genemed Synthesis (San Francisco, CA).

**Bacteria, virus, and cells**

H37Rv-eGFP has been described previously (17). Before infection, bacteria were sonicated for 20 s, passed 15 times through a tuberculin syringe, and sonicated again to obtain a single-cell suspension. Multiplicity of infection (MOI) varied depending on the experiment. Adenovirus-ICP47 (23) and adenoviral vectors were provided by Dr. D. Johnson (Oregon Health and Science University, Portland, OR).

PMBCs were obtained from human donors via leukapheresis according to Institutional Review Board-approved protocols and processed as described previously (24). Dendritic cells (DCs) were generated by culturing adherent PBMCs for 5 d in the presence of GM-CSF (10 ng/ml; Amgen, Thousand Oaks, CA) and IL-4 (10 ng/ml; R&D Systems, Minneapolis, MN) in RPMI 1640 medium (Invitrogen) supplemented with 10% pooled human serum (HS), l-glutamine (4 mM; Invitrogen), and gentamicin (50 μg/ml; Invitrogen).

**T cell expansion**

The T cell clones used in this report are detailed in Table I. T cell clones were expanded as previously described (18), except that some of the expansions were done in Stemline T cell expansion media (Sigma-Aldrich) supplemented with 1% FBS (HyClone, Logan, UT) and 4 mM l-glutamine.

**ELISPOT assay**

IFN-γ ELISPOT was performed as described previously (24).

**Inhibition of Ag presentation**

Day 5 DCs were plated in a 24-well ultra-low adherence plates (Costar, Cambridge, MA) at 5 × 10^5/well in RPMI 1640 medium/10% HS supplemented with GM-CSF and IL-4. DCs were pretreated with inhibitors (1–10 μM epoxomicin, 0.1 μg/ml BFA, 0.2 μM bafilomycin, or 10 μg/ml cycloheximide) for 1 h prior to infection with H37Rv-eGFP (MOI = 20) or addition of Ag.

DCs were harvested after 15–16 h of infection, pelleted, and fixed with 1% paraformaldehyde for 15 min. The reaction was stopped with an equal volume of 0.4 M lysine or RPMI 1640 medium/10% HS, and DCs were incubated with antibodies to CD3 (UCHT1; BD Biosciences, San Jose, CA) and IFN-γ (Beckman Coulter, Fullerton, CA) in the presence of 0.2% saponin (Sigma-Aldrich). Cells were analyzed using a FACSCalibur or LSR II (BD Biosciences).

**In vitro proteasome digestion**

A synthetic peptide encompassing aa 2–21 of CFP10 (AEMKTDATLAAQAGNFER) was synthesized, purified to >99% purity (Genemed Synthesis), and resuspended in PBS. Purified 205/immunoprecipitates (5 μg; BIOMOL, Plymouth Meeting, PA) were incubated with 50 μg peptide in a total volume of 0.5 ml proteasome digestion buffer (50 mM Tris-HCl and 0.5 mM EDTA [pH 7.6]) for 20 h at 37°C in the presence or absence of epoxomicin (1 μM) or chymotrypsin (10 μg/ml; EMD Biosciences). The resulting digests were centrifuged through a Microcon tube with a 10-kDa cutoff and the flow-through frozen at −80°C.

Proteasome digests (20 pmol) were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an Agilent 1100 series capillary LC system (Agilent, Palo Alto, CA) and an LTQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Electrospray ionization was performed with an ion mass source fitted with a 34-gauge metal needle. Samples were applied at 20 μl/min to a trap cartridge (Michrom Biosources, Auburn, CA) and then switched onto a 0.5 × 250-mm Zorbax SB-C18 column (Agilent) using a mobile phase containing 0.1% formic acid, 7–30% acetonitrile gradient over 30 min, and 10 ml/min flow rate. Survey (MS) scans were used to determine the m/z values of the eluting peptides were alternated with three data-dependent MS/MS scans, where individual peptide ions were isolated and fragmented to determine their amino acid sequences. The collection of these MS/MS spectra used the dynamic exclusion feature of the instrument’s control software so that both major and minor ions in the survey MS spectra triggered data-dependent MS/MS scans. Peptide sequences were determined by comparing the observed MS/MS spectra to theoretical MS/MS spectra of peptides generated from a Uniprot protein database containing 223,100 entries and the program Sequest (version 27, revision 12; ThermoFinnigan) with no specification of potential cleavage sites and using a differential search for oxidized methionines. Summaries of identified peptides were then tabulated using BioWorks software (version 3.3; ThermoFinnigan) and were data filtered so that peptide probabilities were 1.0 × 10^-4 or better. The extent of peptide cleavage in each incubated sample was measured by the decrease in the area of the base peak for the intact peptide in each chromatogram using Xcalibur software (ThermoFinnigan) and the appearance of new base peaks in the chromatograms for the various degraded forms of the peptide identified both by mass and by Sequest analysis.

T cell clone analysis of digests were performed in Stemline T cell expansion media (Sigma-Aldrich) and the appearance of new base peaks in the chromatograms for the various degraded forms of the peptide identified both by mass and by Sequest analysis.

**Results**

Presentation of M. tuberculosis Ags requires ER-Golgi transport but not acidification

Using CD8 T cells cloned from individuals with latent or active tuberculosis, our laboratory has identified M. tuberculosis-derived HLA-I epitopes. This Ag discovery method includes cloning CD8 T cells on M. tuberculosis-infected DCs, determination of the Ag recognized using overlapping peptide pools, and subsequent determination of the minimal epitope and restricting allele (26–28).
CD8+ T cell clones used in this paper and the minimal epitopes and restricting allele are summarized in Table I. Of note, we have found preferential use of HLA-B alleles for the presentation of *M. tuberculosis* Ags, and generally, responses to these epitopes make up a substantial proportion of the *M. tuberculosis*-specific CD8+ response in the individual from which they were defined (Table I) (26).

CD8+ T cell clones were used to monitor the processing and presentation requirements of the specific epitopes recognized by each clone, using inhibitors that distinguish cytosolic and non-cytosolic processing pathways. Presentation of *M. tuberculosis* Ags in human DCs was first screened in the presence of ER-Golgi transport (BFA), acidification (bafilomycin), as well as proteasome (epoxomicin) blockade. In brief, DCs were treated with inhibitors for 1 h prior to infection with an H37Rv strain that expresses eGFP. After 15–16 h of infection in the presence of inhibitors, DCs were harvested, fixed, washed extensively, and used as stimulators in an overnight IFN-γ ELISPOT assay. Using eGFP fluorescence as a marker for intracellular infection, we were unable to detect significant differences in the percentage of cells infected or the degree of infection after treatment with these inhibitors (data not shown), demonstrating that inhibitor treatment did not affect uptake of bacteria. As shown in Fig. 1, treatment with the ER-Golgi trafficking inhibitor BFA resulted in a marked reduction in presentation of all of the epitopes examined. These data are consistent with the cytosolic presentation pathway (29). Conversely, vacuolar processing of phagosomal Ags for class I presentation generally requires acidification (8, 30). As shown in Fig. 1, treatment of DCs with the vacuolar ATPase inhibitor, bafilomycin, did not significantly inhibit presentation of any of the epitopes tested. To ensure that bafilomycin was effective at the concentration used, DCs were pretreated with bafilomycin or DMSO as a control and then incubated with beads labeled with pH-dependent (fluorescein) and pH-independent (Alexa Fluor 647) dyes. When cells that had internalized a single bead were compared, bafilomycin-treated cells showed a 2-fold increase in fluorescein fluorescence, with no increase seen in Alexa Fluor 647 fluorescence (data not shown), indicating that bafilomycin did in fact raise the phagosomal pH. Presentation of synthetic peptides was not inhibited in the presence of BFA or bafilomycin, arguing against nonspecific effects of drug treatment. Taken together, these data confirm that the cytosolic presentation pathway is primarily used for *M. tuberculosis* Ags, and this pathway is not significantly affected by the inhibitors used in this study.

**Table I. Summary of clones used**

<table>
<thead>
<tr>
<th>Clone Name</th>
<th>Aga</th>
<th>Amino Acid Sequence</th>
<th>Restricting Allele</th>
<th>Ex Vivo Frequencyb</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D160 1–23</td>
<td>ND</td>
<td>ND</td>
<td>HLA-E</td>
<td>ND</td>
<td>24, 27</td>
</tr>
<tr>
<td>D480 F6</td>
<td>CFPI0109-11</td>
<td>EMKTDAAATL</td>
<td>HLA-B0801</td>
<td>1/646</td>
<td>26</td>
</tr>
<tr>
<td>D466 H4</td>
<td>CFPI0109-9</td>
<td>AEMKTDAA</td>
<td>HLA-B4501</td>
<td>1/102</td>
<td>26</td>
</tr>
<tr>
<td>D466 D6</td>
<td>CFPI0109-12</td>
<td>AEMKTDAAATLA</td>
<td>HLA-B4501</td>
<td>1/125</td>
<td>26</td>
</tr>
<tr>
<td>D481 C10</td>
<td>CFPI0109-83</td>
<td>NIRQAGQVVV</td>
<td>HLA-B1502</td>
<td>1/146</td>
<td>26</td>
</tr>
<tr>
<td>D504 B10</td>
<td>Ess1234-34</td>
<td>QTVEDEARRM6</td>
<td>HLA-B570I</td>
<td>1/55</td>
<td>Unpublishedc</td>
</tr>
<tr>
<td>D454 H1-2</td>
<td>DPV33-43</td>
<td>AIVINTCHNYQG</td>
<td>HLA-B1501</td>
<td>1/10,417</td>
<td>26</td>
</tr>
<tr>
<td>D443 H9</td>
<td>Ag85B144-153</td>
<td>ELPOQLSARNR</td>
<td>HLA-B2410</td>
<td>&lt;1/25,000</td>
<td>26</td>
</tr>
<tr>
<td>D454 E12</td>
<td>CFP10</td>
<td>ND</td>
<td>HLA-II</td>
<td>ND</td>
<td>17</td>
</tr>
</tbody>
</table>

*a* Numbers denote amino acid position within the protein.

*b* Frequency of CD8+ T cells present in PBMCs specific for the indicated epitope.


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**FIGURE 1.** Presentation of *M. tuberculosis* Ags requires ER-Golgi transport but not acidification. DCs were pretreated with BFA (0.1 μg/ml) or bafilomycin (0.2 μM) for 1 h prior to infection with H37Rv-eGFP (MOI = 20) or addition of peptide Ag (1 μg/ml). After 15–16 h in the presence of inhibitor, DCs were fixed, washed, and used as stimulators in an IFN-γ ELISPOT assay (25,000 *M. tuberculosis*-infected DCs/well, 2,000 peptide-pulsed DCs/well) where CD8+ T cell clones are effectors (10,000 cells/well). A, Representative experiment for three clones. Each bar represents the mean ± SEM of triplicate wells. B, Data have been normalized to the untreated control, and each bar reflects the mean ± SEM of at least three experiments per clone (two for D443 H9). *p* < 0.05; **p** < 0.01 using two-tailed Student t test compared with untreated controls. Mtb, *Mycobacterium tuberculosis.*
together, these data demonstrate that presentation of all six epitopes require ER-Golgi transport, with little to no requirement for phagosomal acidification.

**Epoxomicin can either inhibit or enhance Ag presentation**

Proteasomal processing of Ags is a central feature of the cytosolic Ag-processing pathway. We have previously reported that three *M. tuberculosis* epitopes require proteasomal degradation [CFP102–12 (18), CFP103–11 (17), and an undefined Ag presented by HLA-E (17, 27)]. Unexpectedly, treatment of DCs with the specific proteasomal blocker epoxomicin showed that this inhibitor could either inhibit or enhance presentation of the examined peptides. Reportedly, presentation of CFP102–9 and Ag85B144–153 epitopes was ablated in the presence of epoxomicin, presentation of CFP102–12, CFP1075–83, EssJ24–34, and DPV33–43 epitopes was markedly enhanced. Schwarz et al. (31) reported that low-dose epoxomicin and lactacystin can paradoxically enhance Ag presentation of some epitopes, although higher doses inhibit it. However, presentation of neither CFP102–12 nor CFP1075–83 epitope was inhibited by epoxomicin treatment at concentrations up to 10 μM (Fig. 2B). This is the highest dose of epoxomicin that was nontoxic to DCs and is >20-fold higher than the dose generally required for maximal inhibition of Ag presentation in DCs (data not shown). Taken together, these data suggest that the proteasome can either generate or destroy the correct *M. tuberculosis*-derived epitope.

**Generation of epoxomicin-enhanced epitopes does not require tripeptidyl peptidase II or endocytic processing**

Given these data, we reasoned that the epoxomicin-enhanced epitopes were either generated using a lysosomal or cytosolic protease or were generated by the proteasome in a manner refractory to epoxomicin blockade. To date, tripeptidyl peptidase II (TPPII) is the only other cytosolic protease described that is able to generate the correct C terminus of epitopes for MHC class I (MHC-I) presentation. TPPII is necessary for the generation of an HIV nef (32) and an influenza virus nucleoprotein epitope (33). To establish a role for TPPII in the generation of the epoxomicin-enhanced epitopes, the TPPII inhibitor AAF-CMK was tested for its ability to block presentation of the EssJ24–34 or CFP1075–83 epitope. However, no effect of this inhibitor was observed at the highest possible nontoxic dose (data not shown). Alternately, the lysosomal proteases cathepsins D and S have been shown to participate in presentation of phagocytosed Ags (14, 34). Treatment with the cathepsin inhibitors leupeptin or pepstatin A did not block presentation of the EssJ24–34 or CFP1075–83 epitope (data not shown). Furthermore, presentation of neither of these epitopes was inhibited in the presence of chloroquine (data not shown) or baflomycin (Fig. 1), further arguing against a role of endocytic processing of these epitopes, because cathepsins are only optimally active at acidic pH. Taken together, these data suggest that proteolysis of the four proteasome-independent epitopes is not dependent on endosomal proteases or TPPII.

An epoxomicin-enhanced epitope is generated by purified proteasomes

To directly test the possibility that the proteasome is able to generate an epoxomicin-enhanced epitope, an in vitro proteasome model of Ag processing was established. The N terminus of CFP10 was chosen for analysis because it contains both epoxomicin-inhibited [CFP102–9 (Fig. 2), CFP103–11 (17)] and epoxomicin-enhanced [CFP102–12 (Fig. 2)] epitopes. Purified immunoproteasomes were incubated with CFP102–21 in the presence or absence of epoxomicin or chymotrypsin for 20 h. Because there is a predicted chymotrypsin cleavage site between aa 11 and 12, we included chymotrypsin as a control for enzymatic activity that will destroy the 2–12 epitope. The resultant peptides were assessed by LC-MS/MS and T cell-dependent IFN-γ ELISPOT. For T cell assays, the proteasomal digests were pulsed onto LCLs to increase the sensitivity of the assay. Alternately, digests were added directly onto CD8+ T cells to ensure that there was no further processing of peptides.

Incubation of the 20-mer peptide with proteasomes resulted in a modest 15–25% reduction of the parent peptide as estimated by LC-MS/MS (data not shown), whereas chymotrypsin digestion resulted in nearly complete digestion (Fig. 3A). As predicted, chymotrypsin digestion resulted in generation of the CFP102–11 epitope but destroyed the CFP102–12 epitope (Fig. 3A, 3B). These data are consistent with proteolysis generating one epitope while destroying another.

We next asked whether the proteasome could generate the epoxomicin-enhanced epitope CFP102–12. We hypothesized that Ag presentation is increased in epoxomicin-treated DCs because of proteasome cleavage within the epitope. After digestion with purified proteasomes, CFP102–11 was readily detectable, as estimated by both IFN-γ ELISPOT and LC-MS (Fig. 3A, 3B). Alternately, incubation of CFP102–21 with proteasomes did not result in detectible levels of CFP102–12. When epoxomicin was included, the amount of CFP102–11 was drastically reduced, similar to data from *M. tuberculosis*-infected DCs. Surprisingly, epoxomicin enhanced the generation of CFP102–12.

![FIGURE 2. Presentation of *M. tuberculosis* epitopes is either inhibited or enhanced by epoxomicin. DCs were treated with epoxomicin (1–10 μM), infected with H137Rv-eGFP or pulsed with peptide, and used as APCs in an IFN-γ ELISPOT assay as described in Fig. 1. A, Data have been normalized to the untreated control, and each bar represents the mean ± SEM of at least three experiments (two for D443 H9). *p < 0.05; **p < 0.01 using two-tailed Student t test compared with untreated controls. B, DCs were treated with increasing concentrations of epoxomicin, infected with *M. tuberculosis* (M. tuberculosis-DCs) or pulsed with cognate peptides (Ag-DCs), and used as stimulators as described. One representative experiment of three is shown. Mtb, *Mycobacterium tuberculosis*.

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making it now detectible by T cell assay but not LC-MS. As demonstrated in Fig. 3C, clone D466 D6 only responds to the CFP102–12 minimal epitope in the absence of LCLs to process the extended peptide, showing that the measured response is not to a shorter peptide. When samples were analyzed by MS-MS, CFP102–12 was detected in both proteasome samples (with and without epoxomicin) but not in the undigested or chymotrypsin digested samples, further confirming the presence of this peptide. These data argue that the proteasome is better able to generate CFP102–12 in the presence of epoxomicin and rule out the requirement for an alternate cytosolic protease for generation of CFP102–12. These data also suggest that the proteasome is sufficient for generation of the remaining epoxomicin-enhanced epitopes. Furthermore, the enhancement seen with epoxomicin suggests that competition exists between different proteolytic activities of the proteasome for generation of T cell epitopes.

Presentation of epoxomicin-enhanced epitopes requires TAP transport

Having demonstrated a role for ER-Golgi transport and the proteasome in the generation of the epoxomicin-enhanced epitopes, the role of TAP was next examined. We have previously shown that TAP is required for the presentation of the CFP102–11 (18), CFP103–11, and the HLA-E epitopes (17). The HSV-1–encoded protein ICP47 is a potent inhibitor of MHC-I Ag processing via its ability to prevent peptide binding to the cytosolic face of TAP (23, 35). Infection of DCs with an adenovirus vector expressing ICP47 resulted in inhibition of both epoxomicin-inhibited (CFP102–11) and epoxomicin-enhanced (CFP102–12, CFP105–83, EsxJ24–34, and DPV33–43) epitopes (Fig. 4A). ICP47 had no effect on presentation of cognate peptide to these CD8+ T cell clones (Fig. 4B). In addition, presentation of M. tuberculosis–derived or soluble CFP10 to a CD4+ T cell clone (D454 E12) was not affected (Fig. 4A,4B). These data demonstrate a requirement for cytosolic transport of all Ags presented to CD8+ T cells tested to date.

Newly synthesized MHC-I is required for Ag presentation

Conventional MHC class I presentation requires new protein synthesis for loading onto nascent MHC molecules in the ER, although the vacuolar presentation pathway is insensitive to cycloheximide treatment (36). Presentation of cytosolically processed CFP103–11 requires newly synthesized HLA-I, because presentation is blocked in the presence of cycloheximide (17). Alternately, presentation of an HLA-E–presented Ag, which is processed cytosolically but loaded within the phagosome, does not require new protein synthesis. We examined the role of protein synthesis in the presentation of three of the epoxomicin-enhanced epitopes. As shown previously, cycloheximide treatment led to an enhanced presentation of the HLA-E–presented epitope (Fig. 5, Table II). Presentation of the epoxomicin-enhanced epitopes CFP102–12, CFP105–83, and EsxJ24–34 were inhibited by >70%, demonstrating a requirement
for nascent HLA-I. Interestingly, presentation of the epoxomicin-inhibited epitope CFP102–9 was only slightly inhibited in the presence of cycloheximide. Presentation of synthetic peptides was not significantly affected by cycloheximide treatment (Fig. 5). Taken together, these data demonstrate that all M. tuberculosis Ags characterized to date are processed cytosolically.

Discussion

There has been considerable interest in the mechanisms by which particulate-associated Ags are processed and presented in the context of the MHC-I pathway. However, much of what is known has been derived using OVA conjugated to a variety of beads or expressed by bacteria, leading to a paucity of data regarding processing of naturally occurring epitopes in biological infections. To define those epitopes generated during the natural course of infection with M. tuberculosis, we have used M. tuberculosis-infected DCs to generate CD8+ T cell clones. In this study, these clones, specific for six recently defined epitopes from four secreted proteins, were used to examine the requirements for MHC-I processing and presentation. Although the effects of inhibitors vary for different epitopes, these data demonstrate that all epitopes examined ultimately require proteasomal degradation, TAP transport, loading onto nascent HLA-I, and ER-Golgi egress. Taken together, these data demonstrate that all of the epitopes use the cytosolic pathway.

Although these and other data corroborate robust use of the cytosolic pathway for presentation of M. tuberculosis Ags on HLA-I (Table II) (17, 18), we have primarily examined secreted proteins. Similarly, secretion of OVA or the endogenous protein, GRA6, into the parasitophorous vacuole by Toxoplasma gondii leads to cytosolic MHC-I presentation (37–40). Conversely,

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FIGURE 4. TAP transport is required for presentation of all epitopes. DCs were infected with either adenoviral ICP47 or empty vector using Lipofectamine 2000. After 6–26 h, DCs were washed and infected with H37Rv-eGFP (A) or pulsed with Ag (B). Following overnight incubation, T cell clones were added, and IFN-γ production was assessed by intracellular cytokine staining. For each clone, the mean response to mock-treated, M. tuberculosis-infected DCs was at least 4-fold higher than the response to uninfected DCs. Data have been normalized to the untreated controls, and each bar represents the mean ± SEM of at least three independent experiments. *p < 0.05; **p < 0.01 using two-tailed Student t test compared with untreated controls. Mtb, Mycobacterium tuberculosis.

FIGURE 5. Protein synthesis is required for presentation of epoxomicin-enhanced epitopes. DCs were treated with cycloheximide (10 μg/ml), infected with H37Rv-eGFP or pulsed with peptide, and used as APCs in an IFN-γ ELISPOT assay as described in Fig. 1. Data have been normalized to the untreated control, and each bar represents the mean ± SEM of at least three experiments. *p < 0.05; **p < 0.01 using two-tailed Student t test compared with untreated controls. Mtb, Mycobacterium tuberculosis.

Table II. Effect of inhibitor treatment on presentation of the indicated epitope

<table>
<thead>
<tr>
<th>Epitope</th>
<th>ICP47</th>
<th>Epoxomicin</th>
<th>Brefeldin A</th>
<th>Bafilomycin</th>
<th>Cycloheximide</th>
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<tbody>
<tr>
<td>CFP102–9</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>CFP102–11,a</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>CFP102–12</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>CFP102–13</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>DPV1–13</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>EsxJ2–34</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ag85B144–153</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>HLA-E Ag6</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>ND</td>
</tr>
</tbody>
</table>

Inhibition is denoted by (−), 100–75% (−−−−), 74–50% (−−−−), 49–25% (−−−−), 25–15% (−−), 15–0% (−). Enhancement is denoted by (+).

[a] See Refs. 17 and 18.
[b] Lactacystin was used instead of epoxomicin.
presentation of secreted OVA expressed by Leishmania major is TAP and proteasome independent (40), demonstrating that cytosolic processing of secreted proteins is not absolute. As non-cytosolic processing of an M. tuberculosis-derived lipoprotein has been previously described (19), our findings then lead to the hypothesis that M. tuberculosis-secreted proteins may preferentially access the cytosol. In this regard, it is interesting to note the dependence on bacterial secretion of CFP10 for T cell priming (41).

The mechanism(s) by which M. tuberculosis proteins gain access to the cytosol remain to be elucidated. It has been reported that the whole M. tuberculosis bacterium is able to escape the phagosome and reside in the cytosol (42). However, the DCs used in this study were fixed at a time prior to bacterial escape (18 h postinfection), excluding this possibility. As M. tuberculosis expresses several effectors that potentially function in the cytosol (e.g., SgpM, PknG, and CFP10 (43–45)), the cytosolic delivery of proteins may be beneficial to the bacterium. However, this comes at the cost of potential detection by CD8+ T cells. Whether M. tuberculosis facilitates translocation of proteins to the cytosol or uses host cell machinery has yet to be determined. The normally ER-localized cellular retrotranslocation machinery can be localized to phagosomes and function in the cytosolic translocation and MHC-I presentation of phagosomal Ags (46). We have demonstrated that the retrotranslocation inhibitor Pseudomonas aeruginosa exotoxin A inhibits presentation of two M. tuberculosis epitopes, including one from CFP10 (17), suggesting that host cell machinery plays a role in this process. Furthermore, we and others have demonstrated that an intact region of difference 1 (RD1) is not required for MHC-I presentation of a non-RD1 Ag or priming of M. tuberculosis-specific CD8+ T cells (18, 47), suggesting that any potential membrane disruptions (42, 48, 49) initiated by the RD1-encoded Ess-1 secretion system are not strictly required for cytosolic access. Although these data implicate the host cell for translocation of M. tuberculosis proteins to the cytosol for Ag presentation, they do not exclude a role for M. tuberculosis-induced translocation. Further analysis is necessary to identify the specific components required for cytosolic access of M. tuberculosis proteins.

The observation that proteasome blockade by epoxomicin resulted in enhanced presentation of four epitopes was surprising. Although inhibition of proteasome function could cause either direct (i.e., lack of protein degradation and proper generation of epitope C terminus) or indirect effects (i.e., lack of HLA-I trafficking because of peptide depletion) on Ag presentation, it is hard to imagine a scenario where the indirect effects of proteasome blockade would enhance Ag presentation. Furthermore, we were able to confirm these findings by incubating purified proteasomes with a synthetic extended peptide from CFP10, showing that the proteasome is in fact better at generating CFP102–12 in the presence of epoxomicin. Although there are many possible explanations for this phenomenon, we hypothesize several different scenarios that would lead to the observed enhancement. First, the epoxomicin-enhanced epitopes may not be generated by the chymotrypsin-like activity of the proteasome. Most proteasome inhibitors, including epoxomicin, more potently inhibit the chymotrypsin-like activity of the proteasome. Blocking the chymotrypsin-like activity may allow the other proteolytic activities to generate the correct C terminus. This scenario would suggest competition between different proteasome activities for generation of MHC-I–presented peptides, possibly evidenced by the more efficient generation of CFP102–11 versus CFP102–12 in the absence of epoxomicin (Fig. 3). A second possibility is an altered peptide repertoire generated in the presence of proteasome inhibitors. Interestingly, three of the four epoxomicin-enhanced epitopes are 11-mers. This suggests that longer peptide epitopes are inefficiently generated by the fully active proteasome, consistent with previous reports demonstrating that the mean size of proteasome products is <8 aa (50–52). Decreased proteolytic activity in the presence of inhibitors may result in more efficient generation of longer epitopes. Similarly, epitopes may be both generated and then immediately destroyed when the proteasome is fully active. The addition of a proteasome inhibitor may decrease excessive degradation of peptides allowing for more efficient epitope generation. Nonetheless, CFP102–12 (Fig. 3) and the other epoxomicin-enhanced epitopes can be generated by the proteasome in the absence of inhibitors, as evidenced by the high-frequency CD8+ T cell response to these epitopes.

Taken together, these data demonstrate that presentation of all six epitopes occurs via the cytosolic pathway. Thus, the predominant pathway for MHC-I presentation of immunodominant M. tuberculosis Ags relies on cytosolic access and processing.

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Disclosures

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References


