# HLA-I PROCESSING AND PRESENTATION OF M*YCOBACTERIUM TUBERCULOSIS*-DERIVED ANTIGENS

by

Jeffrey E. Grotzke

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CERTIFICATE OF APPROVAL

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This is certify that the Ph.D. dissertation thesis of

Jeffrey E. Grotzke

has been approved

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| Thesis Advisor | David M. Lewinsohn, M.D., Ph.D. |
| Committee Member | David C. Parker, Ph.D. |
| Committee Member | Ann B. Hill, Ph.D. |
| Committee Member | David J. Hinrichs, Ph.D. |
| Committee Member | Jeffrey A. Gold, M.D. |

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List of Abbreviations

APC-Antigen presenting cell

β2m- Beta(2)-microglobulin

BCG-*Mycobacterium bovis* bacillus Calmette-Guerin

BSA-Bovine serum albumin

CFP10-10kDa culture filtrate protein

DC-Dendritic cell

DC-SIGN-Dendritic cell-specific ICAM-3 grabbing non-integrin

DOTS-Directly observed therapy, short-course

EEA-1-Early endosomal antigen-1

eGFP-Enhanced green fluorescent protein

ELISPOT-Enzyme-linked immunospot

ESAT-6-6kDa early secreted antigenic target

exoA-Exotoxin A

GM-CSF-Granulocyte monocyte colony stimulating factor

HIV-Human Immunodeficiency Virus

HLA-Human leukocyte antigen

ICS-Intracellular cytokine stain

IFN-γ-Interferon-gamma

IL-4-Interleukin-4

iNOS-Inducible nitric oxide synthase

LAM-Lipoarrabinomannan

LCL-Lymphoblastoid cell line

MHC-Major Histocompatibility Complex

Mtb-*Mycobacterium tuberculosis*

OVA-ovalbumin

PBMC-Peripheral blood mononuclear cells

PDI-Protein disulfide isomerase

PI3P-Phosphatidylinositol 3 phosphate

PIM-Phosphatidylinositol mannoside

PLC-Peptide loading complex

PLG-Poly-lactide poly-glycolide

RD1-Region of Difference 1

RNI-Reactive nitrogen intermediates

TAP-Transporter associated with antigen processing

TB-Tuberculosis disease

TfR-Transferrin receptor

TNF-α-Tumor necrosis factor-alpha

TPPII-Tripeptidyl peptidase II

TST-Tuberculin skin test

WHO-World Health Organization

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Abstract

*Mycobacterium tuberculosis* (Mtb) resides in a phagosome that resists maturation. While this compartment is accessible to the Class II processing pathway, the mechanisms by which Mtb antigens are processed and presented on Class I molecules is poorly understood. In this dissertation, I present findings which characterize the pathway by which ten Mtb epitopes, presented by both classical and nonclassical Class I molecules are processed in dendritic cells. We find that Mtb antigens access the cytosol by retrotranslocation from the phagosome. Here, Mtb proteins are degraded by the proteasome or a potentially novel cytosolic protease. Peptides derived from cytosolic proteolysis are transported by TAP, and nine of ten epitopes require ER-golgi transport to the cell surface. In contrast to these epitopes, nonclassical antigen presentation of HLA-E associated antigen does not require ER-golgi transport or new protein synthesis, suggesting that HLA-E loading does not occur in the ER. Instead, HLA-E loading occurs in the phagosome, with the aid of phagosome-localized members of the Class I peptide loading complex. Furthermore, Class I presentation of Mtb antigens does not rely on the Mtb virulence locus, Region of Difference 1, which has been hypothesized to create a pore in the phagosomal membrane. Together these data demonstrate that the cytosolic pathway of cross-presentation is the dominant pathway for Mtb antigen presentation and that the Mtb phagosome is able to participate in presentation of Class I antigens. Also, presentation by HLA-E follows a similar but distinct processing pathway compared to classical Class I molecules. Finally, an undefined cytosolic protease may be involved in generation of class I epitopes.

# Chapter 1

# Introduction

Overview

The goal of this thesis research was to determine the pathway by which *Mycobacterium tuberculosis* (Mtb) antigens are processed and presented on Human Leukocyte Antigen Class I (HLA-I) molecules, with an emphasis on the processing and presentation pathway of the non-classical HLA-I molecule, HLA-E. Our laboratory has generated numerous $CD8^+$ T cell clones using Mtb-infected dendritic cells (DC). With the use of multiple approaches, lab members have identified novel antigens and minimal epitopes presented by a diverse repertoire of HLA-I alleles. This approach has generated sensitive tools to monitor antigen processing and presentation by Mtb-infected DC of these epitopes on multiple HLA-I alleles. The research presented herein would not have been possible without the contributions of many past and present lab members as well as collaborators who have helped us define T cell epitopes. Analysis of cross-presentation of these Mtb antigens has yielded the following three main findings:

1. The Mtb-containing phagosome is able to assist in presentation of Mtb antigens on HLA-I molecules, a previously unidentified function of the Mtb phagosome.
2. Although multiple pathways are functional for cross-presentation, all Mtb antigens examined by our laboratory use the cytosolic pathway, with minor differences in details seen dependent on the antigen/epitope.
3. The Region of Difference 1 (RD1) of Mtb is not required for cross-presentation of Mtb antigens.

These findings are important for several reasons. First, this is one of the first and most in depth looks at cross-presentation using a natural model. Most prior studies have been

done using latex beads coupled with ovalbumin (OVA) or pathogens expressing OVA. Second, the epitopes we have examined are all naturally occurring and present at relatively high frequencies in latently or actively infected Tuberculosis (TB) patients, meaning that we are likely examining processes as they would occur in the human host. Finally, these results present novel findings not only to the Mtb field, but to antigen processing in general.

*Mycobacterium tuberculosis*-Biological Significance

Mtb is a facultative intracellular pathogen that is very well adapted to surviving within macrophages and the human host. Despite widespread use of the BCG (*Mycobacterium bovis* bacillus Calmette-Guerin) vaccine and increased availability of effective drug therapy, Mtb remains one of the most common causes of infectious disease morbidity worldwide. In 2005 alone, the World Health Organization (WHO) estimated that there were 8.8 million new cases, and an estimated 1.6 million TB-related deaths (1). At the forefront of the TB epidemic is the emergence of drug resistant strains of Mtb. These strains are resistant to the front-line antibiotics, isoniazid and rifampin (multidrug-resistant), and sometimes second-line drugs (extensively drug-resistant) as well. Furthermore, the interplay between Human Immunodeficiency Virus (HIV) and Mtb infection has led to Mtb being the leading cause of death in AIDS patients.

Through the WHO-sponsored DOTS (Directly Observed Therapy, Short-course) program, effective TB therapy is now available to 89% of the world's population. However, the likelihood of DOTS therapy resulting in the eradication of TB is limited by the large reservoir of latently infected individuals as well as delays in diagnosis. In fact,

the WHO reports that although the TB incidence rate has decreased over the last several years, the total number of TB cases has increased (1). Indeed, it has been estimated that very high case detection rates would be required to significantly decrease the global burden of TB. However, a vaccine of even modest efficacy when combined with existing DOTS programs offers the potential to dramatically reduce the burden of TB (2). Therefore, understanding the mechanisms by which Mtb infections are controlled is dramatically important to the development of successful vaccine candidates.

*Mycobacterium tuberculosis*-Cell Wall

The structure of the Mtb cell wall is unique to mycobacteria. Due to the presence of complex and diverse lipids, mycobacteria stain with an acid-fast procedure, and are unreactive with gram stains (3). The Mtb cell wall consists of a bilayer lipid membrane, followed by peptidoglycan and arabinogalactan layers. Attached to the arabinogalactan layer is an outer envelope consisting mostly of mycolic acid residues as well as other lipids, glycolipids, and some proteins (4, 5). Notably, the mycobacterial lipids lipoarabinomannan (LAM) and phosphatidylinositol mannoside (PIM) are loosely inserted in the inner lipid bilayer. As will be discussed below, many of the properties related to Mtb survival, persistence, and pathogenicity can be attributed to the large amounts of lipids generated by the bacterium. Alternately, the lipids present in the Mtb cell wall represent good substrates for presentation by the non-classical Class I molecues of the CD1 family.

*Mycobacterium tuberculosis*-Uptake and Subsequent Events

The phagocytosis of Mtb is achieved through the interaction of the bacterium with varying receptors. In fact, it has been difficult to show the requirement of a single receptor, due to the redundant molecules that are able to facilitate Mtb phagocytosis (6). In macrophages, complement receptors, scavenger receptors, mannose receptor, and lipid rafts have all been demonstrated to participate in Mtb entry (7). In DC, the C-type lectin dendritic cell-specific ICAM-3 grabbing non-integrin, DC-SIGN, has been shown to interact with BCG and Mtb, and more specifically LAM, and lead to internalization (8, 9). In broncho-alveolar lavage from TB patients, alveolar macrophages were shown to express DC-SIGN, an induction not seen in other lung pathologies (10). This suggests that DC-SIGN may be involved in Mtb internalization by alveolar macrophages in vivo.

Upon cellular uptake, Mtb resides in a phagosome that does not fully acidify or undergo phagolysosomal biogenesis. This is achieved through interfering with calcium signaling and phosphatidylinositol 3-phosphate (PI3P) generation (7). Normally, phagosomes mature rapidly after phagocytosis. This is achieved first through fusion of early endosomes, then late endosomes, and finally phagolysosomal biogenesis, with decreasing pH at each step. The maturation of the phagosome can be monitored by the cellular markers present. For example, recently phagocytosed particles are positive for the early endosomal markers rab5 and transferrin receptor (TfR), while more mature phagosomes will be negative for these markers but positive for late endosome markers such as rab7. Phagosomes that have fully acidified will be positive for vacuolar ATPases and active forms of lysosomal hydrolases (11).

Examination of these markers on mycobacterial phagosomes in macrophages has

revealed important characteristics of the Mtb phagosome and has been essential to determining at which step Mtb is able to block maturation. It was found that the mycobacterial phagosome is positive for the early endosomal markers rab5 and TfR, and has access to extracellularly-added transferrin (12-14). This established that mycobacterial phagosomes prevented rab conversion to rab7$^{+}$ late endosomes and, importantly, that the phagosome undergoes continued fusion with early endosomes. The latter function has been attributed to the mycobacterial lipid PIM, which is able to stimulate fusion with early endosomes (15). Another mycobacterial lipid, LAM, as well as the protein sapM, have been shown to be the mediators of maturation block (16-18). The addition of LAM to latex beads leads to a partial block of phagosomal acidification. These phagosomes are more acidified than an Mtb phagosome but less than an uncoupled latex bead phagosome (16). The mechanism by which LAM is able to block phagosomal maturation is through the prevention of calcium signaling, which thereby prevents the PI3P generation necessary for downstream fusion events. SapM, a PI3P phosphatase, acts to convert any generated PI3P back to PI3, giving mycobacteria dual mechanisms to prevent this signaling pathway (18). The consequence of inhibiting PI3P generation is the lack of recruitment of the rab5 effectors, early endosomal antigen-1 (EEA-1) and hVPS34 (a PI3 kinase) (16, 17, 19). Through these mechanisms, Mtb is able retain an early endosomal-like phenotype, evade innate immune destruction, and survive in a favorable environment hidden within a cell.

Importantly, there are mechanisms by which the Mtb maturation block can be overcome. Mtb that has been opsonized with specific antibodies and is taken up by the Fc receptor is delivered to an acidic compartment (20). Unfortunately, this mechanism

seems to pertain little to in vivo events, as there is very little to no effect of a previous antibody response on preventing reinfection (21, 22). IFN-γ treatment of macrophages also leads to phagosomal maturation (23). As will be discussed below, IFN-γ is absolutely vital to the control of bacterial replication and dissemination, a demonstration of the necessity of acquisition of an adaptive immune response.

Although Mtb is able to prevent phagosomal maturation in DC (24), the pH of the Mtb phagosome has not been evaluated in this cell type. There are important differences in phagosomal maturation between macrophages and DC that suggest that there might be a significantly different pH in these cell types. The laboratory of Sebastian Amigorena has clearly established that, through the rab27a-mediated delivery of an NADPH-oxidase (NOX2), DC phagosomes actually maintain a neutral pH for at least three hours after phagocytosis of latex beads (25, 26). Conversely, macrophage phagosomes acidify to a pH around 5 during this same time, demonstrating that this pathway is confined to DC. The outcome of increased pH is a less degradative environment and increased presentation of phagosome-associated antigen. The presence of both Mtb- and DC-specific mechanisms to prevent acidification put forward the hypothesis that the pH of an Mtb phagosome should be higher in DC than that seen in macrophages. This difference could have important implications in the presentation of Mtb antigens.

There is evidence to suggest that Mtb is able to escape the phagosome and reside in the cytosol of DC in vitro. Using electron microscopy, van der Wel, et al reported that after 48 hours of infection, Mtb was found in the cytosol without a detectable phagosomal membrane (27). This finding has been met with great controversy and requires more characterization using different methods as well as confirmation in vivo.

Cellular Immunity to Mtb Infection

Following exposure to Mtb, it has been estimated that 90% of those who develop evidence for a cellular immune response (Tuberculin skin test positive ($TST^+$)) remain healthy throughout their lifetime. This persistent infection has been termed latent TB infection (LTBI) and reflects successful immune-mediated containment of Mtb. A vigorous Th1 response, while effective in containing the growth of Mtb, cannot eradicate the bacteria. While little is known about the bacterial burden in humans latently infected with Mtb, it is clear that the organism can persist for many years (28, 29). Furthermore, those with LTBI are characterized by the presence of high-frequency Mtb-specific T cells, consistent with the hypothesis that persistent antigen is driving these responses. Hence, LTBI might be better termed persistent TB infection.

In vivo, the development of a specialized immune structure, the granuloma, plays a central role in the containment of Mtb. Granulomas consist of macrophages, macrophage-derived giant cells, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, neutrophils, and B lymphocytes in a tightly structured and hence histologically recognizable pattern. The integrated and localized response conferred by the granuloma may be central to immune containment. For example, dysregulated granuloma formation has been associated with impaired immunity to Mtb (30-32).

Available data has demonstrated that growth inhibition of Mtb requires the adaptive acquisition of an Mtb-specific, Th1-type cellular immune response. Such a response reflects the coordinated interaction with macrophages and T cells. Abundant experimental evidence supports a central role for the interaction of $CD4^+$ lymphocytes and macrophages. In this model, Mtb or Mtb-derived antigens are processed and

presented via macrophage associated MHC-II. Antigen recognition by $CD4^+$ T cells then leads to the release of pro-inflammatory cytokines, which in turn limit intracellular Mtb growth. Both human and mouse data support an essential role for $CD4^+$ T cells and MHC-II. In the mouse model, depletion of $CD4^+$ T cells prior to infection leads to increased bacterial burden and shortened survival (33-35). These results are mirrored in knockout models as both $CD4^{-/-}$ and MHC-$II^{-/-}$ mice are extremely susceptible to Mtb infection (36). Similarly in humans, patients with HIV-mediated $CD4^+$ depletion have a dramatically increased incidence of tuberculosis disease (37, 38).

Antigen specific activation of $CD4^+$ T cells leads to the release of the pro-inflammatory cytokines IFN-γ and TNF-α. Mice with a targeted disruption of the IFN-γ gene are highly susceptible to Mtb infection, fail to produce nitric oxide synthase (NOS), and develop a disseminated form of disease characterized by irregular granulomas and large areas of tissue necrosis (39, 40). Humans with mutations in the IFN-γ receptor exhibit increased susceptibility to mycobacterial infections (41), while exogenously administered IFN-γ can improve outcome (42-44). Furthermore, individuals with severe active tuberculosis have a relative deficiency of IFN-γ production (45, 46). TNF receptor knockout or mice depleted of TNF-α by monoclonal antibodies are extremely susceptible to Mtb, showing increased bacterial loads, dysregulated granuloma formation, and shortened survival (47). Additionally, humans receiving anti-TNF-α therapy have increased vulnerability to mycobacterial infection (31).

One important mechanism by which both IFN-γ and TNF-α exert their protective role is through the activation of macrophages. Macrophage activation plays a crucial role in combating Mtb infection and leads to the upregulation of Class I and Class II

molecules as well as the production of reactive nitrogen and oxygen species. In the mouse model, reactive nitrogen intermediates (RNI) have been demonstrated to inhibit Mtb growth both in vivo and in vitro. This is most dramatically illustrated by the inducible nitric oxide synthase (iNOS) knockout mouse, which exhibits increased susceptibility to infection with Mtb (48, 49). In humans, a direct role for RNI has been more difficult to demonstrate, although iNOS has been detected within the granuloma (50).

It is becoming apparent that one of the mechanisms by which IFN-γ increases control of mycobacterial growth is through the induction of autophagy (51, 52). In the process of autophagy, cytosolic proteins/organelles are enveloped by an autophagosome, which then fuses to lysosomes for degradation of the sequestered components. This pathway serves to remove old and/or damaged proteins and organelles, and is induced under conditions of starvation (52). Gutierrez et al showed that starvation or IFN-γ treatment of macrophages leads to autophagosomal localization of BCG and Mtb, with a significant decrease in bacterial viability (53). Autophagy can be induced by p47 GTPases (53, 54), which are IFN-γ inducible and are vital to controlling Mtb infection in mice (55), implying a link between the induction of autophagy by IFN-γ and the outcome of infection. Further studies directly examining the role of autophagy in vivo are needed to verify the protective nature of this process in Mtb infection.

Evidence for $CD8^{+}$ T lymphocyte involvement in Mtb containment

The anatomy of the adaptive immune response to infection with Mtb suggests that $CD8^{+}$ T cells are intimately involved in the host response to Mtb. Following infection

with Mtb, antigen-specific IFN-γ producing $CD8^+$ T cells traffic to the lung as soon as 2 weeks post infection (56, 57). Also, $CD8^+$ T cells are present within the granuloma, where they have access to and are poised to prevent dissemination of infected cells (58). Finally, Mtb-specific $CD8^+$ T cells specific for numerous antigens can be isolated at high frequencies from humans and mouse models, consistent with the hypothesis that $CD8^+$ T lymphocytes are constantly being stimulated with antigen.

Early mouse studies looking at the importance of T cell subsets in controlling Mtb infection gave conflicting results. Several studies showed little or no effect of depletion or adoptive transfer of Mtb-specific $CD8^+$ T lymphocytes (33, 34, 59). However, studies using adoptive transfer of $CD8^+$ enriched cells from Mtb infected mice or depletion of $CD8^+$ cells showed that this subset could mediate a modest decrease in bacterial load (35, 60). In most cases, the protection afforded by the $CD4^+$ subset was much greater than that seen by $CD8^+$ T cells. However, Orme found that $CD8^+$ T cells harvested 25 days post infection were uniquely able to reduce bacterial loads in the lung following a high dose aerosol infection (60). Furthermore, De Libero et al were able to demonstrate that Mtb-specific $CD8^+$ T cell lines were capable of inhibiting Mtb growth in vitro (61).

Further support for a protective role of $CD8^+$ T cells in the host response to infection with Mtb comes from experimental vaccines targeting $CD8^+$ T cell responses. These have demonstrated protective efficacy. When mice were immunized with a DNA vaccine encoding hsp65 from *Mycobacterium leprae*, Bonato et al found that adoptive transfer of $CD8^+$ T cells leads to increased protection, even over that seen by transferred $CD4^+$ T cells (62). Also, immunization with DC pulsed with a $CD8^+$ and $CD4^+$ epitope from Ag85A, but not either epitope singly, confers protection to Mtb challenge (63).

More definitive evidence has come from mice deficient in critical components of $CD8^+$ T cell antigen recognition. Beta(2)-microglobulin (β2m) functions as the light chain for MHC-I molecules, such that mice deficient in β2m are deficient in $CD8^+$ T cells. Following high dose intravenous infection, $\beta2m^{-/-}$ mice show increased bacterial loads in the liver, lung, and spleen and quickly succumb to infection (64). Although granulomas were present in $\beta2m^{-/-}$ mice, detailed pathological analysis revealed macrophages containing multiple intracellular bacteria. Hence, it appears that mice deficient in $CD8^+$ T cell responses are unable to control intracellular Mtb growth.

In addition to its role in antigen presentation, β2m is also important for recognition by Natural Killer cells and in iron metabolism. Here, Schaible et al found that the susceptibility of $\beta2m^{-/-}$ animals to infection with Mtb could be partially mitigated by iron chelation therapy (65). However, other models of CD8 deficiency have been established that more directly demonstrate the ability of $CD8^+$ T cells to participate in the protective response to Mtb infection. First, Behar and colleagues demonstrated that after high dose intravenous challenge, mice deficient in the TAP (Transporter Associated with antigen Processing) molecule succumb to infection with increased bacterial burdens in the liver, lung and spleen (66). TAP is required for the generation of MHC-I:peptide complexes. Hence, mice deficient in TAP are also deficient in MHC-I restricted T cell responses. Furthermore, Sousa et al demonstrated increased susceptibility to intravenous challenge with Mtb in mice deficient in the T cell coreceptor CD8α (67). Finally, mice that do not have cell-surface MHC-I ($K^{b-/-}/D^{b-/-}$) were examined by Rolph et al, and found to have a similar phenotype (68). When the susceptibility of $MHC\text{-}I^{-/-}$ and $CD8^{-/-}$ mice were examined using low dose aerosol infection, the consequence of $CD8^+$ T cell

deficiency was present, but less dramatic (49). Finally, Joanne Flynn and colleagues have demonstrated that depletion of $CD8^+$ T cells in cynomolgus macaques leads to disseminated TB disease (Unpublished). Overall, these data strongly support a role for $CD8^+$ T cells in the control of Mtb infection.

Kinetics of the T lymphocyte response

Studies in mice looking at the development of Mtb-specific T lymphocytes after infection show a distinct difference in kinetics compared to acute and chronic viral infections. As opposed to the peak lymphocyte response seen from day 6-10 in viral infections, the peak response in the lung of Mtb-infected mice occurs 4-6 weeks post-infection (56, 57, 69), although $CD4^+$ and $CD8^+$ T cells are detectable in the lung as early as two weeks post-infection (56, 57). After a contraction phase, there is a dynamic flux of Mtb-specific $CD8^+$ T cells characterized by phases of expansion and contraction, although the magnitude of this depends on the report (69, 70).

Surprisingly, there is very little ex vivo data from the mouse model tracking the frequency, kinetics, and tissue localization of epitope-specific $CD8^+$ T cells following infection with Mtb. Kamath and colleagues demonstrated that $CFP10_{32-39}$ and $TB10.4_{20-28}$ specific $CD8^+$ T cells were present at high frequency in the pulmonary lymph nodes, spleen, and lung (56). These cells accumulate rapidly between two and three weeks post infection, peaking at four to five weeks. Furthermore, $CFP10_{32-39}$ specific $CD8^+$ T cells could account for 30% of the $CD8^+$ T cells in the lung, demonstrating that antigen specific $CD8^+$ T cells are recruited to the major site of infection. Importantly, antigen-specific $CD8^+$ T cells produced IFN-γ and showed in vivo cytolytic activity, even at 260

days post infection. Similar results were obtained by Billeskov et al, who demonstrated that the frequency of TB10.4$_{3-11}$-specific CD8$^+$ T cells accounted for 8% of lung CD8$^+$ T cells at the peak of the response (69). While it has been reported that the frequency of Mtb-specific CD8$^+$ T cells in the lymph nodes and spleen does not represent the lung frequency (56), the frequency of TB10.4$_{3-11}$-specific CD8$^+$ T cells was similar in the blood and lung (69). We have found similar results in a non-human primate model of Mtb infection during progressive disease (71), suggesting that our analysis of frequencies in human peripheral blood is likely consistent with that in the lung.

The chronic nature of Mtb infection might influence generation of CD8$^+$ memory cells through persistent stimulation. Therefore, of interest is what memory cell populations are generated after infection. In the lung, Kamath and colleagues found that all of the TB10.4$_{20-28}$-specific CD8$^+$ T cells were effector or effector memory T cells, while a subset of lymph node and spleen tetramer positive cells had a central memory phenotype (72). After antibiotic treatment of infected mice, tetramer positive CD8$^+$ T cells in the lung, spleen, and lymph nodes reverted to central memory cells, suggesting that constant antigenic stimulation in the lung drove the high frequency of effector cells. Conflicting with this, Caccamo et al demonstrated that in children with active disease, Ag85A-specific CD8$^+$ T cells were predominately central memory cells in the blood, and this proportion decreased upon drug therapy (73). These discrepancies are probably due to differences in tissues sampled, although this doesn't completely explain why central memory cells would decrease after therapy. Regardless, these results show the generation of distinct memory populations after Mtb infection in humans and mice.

Mtb models

Much of the available data regarding the immune response to Mtb infection has come from the mouse model. Generally, the broad strokes painted by the mouse model have had strong correlates with human immunity. However, successful design of a vaccine for Mtb may require a more detailed understanding of the human correlates of protective immunity. Here, important limitations to the mouse model highlight the need to develop a more complete understanding of correlates of protective immunity in humans.

Potentially significant differences between mice and humans include both the effector function of $CD8^+$ T cells and their ability to recognize infected cells. For example, in the murine model, RNI plays an unambiguous and central role in the containment of Mtb infection in the activated macrophage. While clearly present in the human granuloma (50), it has been difficult to directly demonstrate a role for RNI in human macrophages. Hence, alternate mechanisms for the control of intracellular Mtb infection may assume greater importance in the human model. Components of the granule exocytosis pathway, either by virtue of inducing apoptosis or directly through the release of anti-mycobacterial proteins, may be of greater importance in the human paradigm. For example, the cytolytic granule component, granulysin, is present in humans but not in mice and has been demonstrated to have a direct anti-bacteriostatic effect in vitro (74). Also, mice lack expression of several non-classical Class I molecules, including group I CD1 molecules (CD1a, b, c) and HLA-E that could limit the potential repertoire of $CD8^+$ T cells that could recognized Mtb-infected cells. CD1 molecules have been shown to present mycobacterially-derived lipids, glycolipids and

lipopeptides, giving human $CD8^+$ T cells a unique mechanism of recognizing infected cells (75-80). HLA-E-restricted $CD8^+$ T cells may represent a significant portion of the human $CD8^+$ T cell response (81, 82). Because of these differences, it is important to examine the roles of $CD8^+$ T cells in humans and other animal models, including rabbits, guinea pigs, and non-human primates. These animals show a more similar pathology to human TB and may allow functional analysis of molecules not expressed in mice.

Class I antigen processing and presentation

$CD8^+$ T cells recognize transformed, virally, and bacterially infected cells through the interaction of the T cell receptor and antigenic peptides complexed onto Class I molecules. The peptides that are loaded onto Class I molecules are generally endogenously expressed, but as will be discussed below, Class I can present exogenous antigens, a processed termed cross-presentation. All eukaryotic cells contain proteasomes which serve to degrade cellular proteins, and Class I antigen presentation relies heavily on this pathway for the sampling of intracellular proteins. Cells contain specialized machinery to aid in optimal loading of cellular degradation products onto Class I molecules, namely the peptide loading complex (PLC).

Proteasomes play a pivotal role in the generation of protein fragments that are loaded onto Class I molecules (83, 84). The structure of proteasomes is cylindrical with a central open pore which unfolded proteins are threaded through and exposed to the catalytic sites. The catalytic core is the 20S proteasome, which contains six active sites, two of each with the following proteolytic activities: chymotrypsin-like, trypsin-like, and caspase-like (also called peptidyl-glutamyl peptide hydrolyzing activity). Although there

have not been any identified cleavage motifs of proteasomes, each active site shows specificity in the nature of the amino acid residue after which it cleaves. The chymotrypsin-like activity cleaves after hydrophobic residues, the trypsin-like activity cleaves after basic residues, and the caspase-like activity cleaves after acidic residues. In addition to the 20S core, 26S proteasomes consist of the 19S regulatory complex cap at each end. The regulatory subunits aids in polyubiquitin recognition and removal and protein unfolding. The 26S proteasome is the main cellular protease involved in protein turnover. The proteasome generates peptides from 2-25 amino acids with a mean length under 8 residues, meaning that 85-90% of proteasome products are too short for Class I binding (85-87). Peptide fragments that exit the proteasome are then subject to the protease rich cytosol, and it has been shown that peptides have a half-life of only a couple of seconds within cells (88). Using this pathway, cells are able to recycle the amino acids from old or misfolded proteins and prevent the buildup of these products. However, some peptides are able to escape proteolysis and enter the Class I presentation pathway.

The main function of the PLC is to promote proper assembly of Class I molecules and binding of high affinity peptides, with specific members of the PLC playing specialized and unique roles in this process. Members of the PLC include calnexin, calreticulin, ERp57, TAP, tapasin, and protein disulfide isomerase (PDI). The Class I heavy chain is cotranslationally translocated into the ER membrane, where it associates with calnexin (89, 90). Calnexin aids in folding of the heavy chain and formation of the intrachain disulfide bond (91, 92). After dissociation from calnexin, the heavy chain binds nonconvalently with β2m and then associates with the PLC. Here, Class I, TAP, tapasin, ERp57, calreticulin, and PDI can be co-immunoprecipitated (90, 93, 94). While

the overall function of the PLC is the loading of Class I molecules with optimal peptides, the role of all of the individual components has not been completely defined. The most important member of the PLC is TAP, whose function is to transport cytosolic peptides into the ER (95-97). Tapasin acts to stabilize TAP heterodimers and link the transporter to Class I (98-102), keeping the peptide supply and Class I in close proximity. ERp57 is covalently bound to tapasin and noncovalently bound to calreticulin (103-109), which interacts with Class I by association with N-linked glycans (110). The role of these interactions and the effects of ERp57 and calreticulin on peptide loading are unclear, although ERp57 has been shown to be involved in recruitment of Class I to the PLC and peptide editing (111, 112). PDI is able to oxidize the disulfide bond in the peptide binding groove of Class I and also serve as a peptide chaperone (93). Both functions appear to be involved in binding of high affinity peptides, although how PDI interacts with the PLC has not been characterized.

The evolution of this complex structure suggests that the PLC members should be important for peptide loading, cell surface Class I expression, and generation of effective $CD8^+$ T cell responses. Lack of expression of individual PLC proteins leads to variable effects on these factors (reviewed in (113)). In brief, TAP deficiency leads to the greatest defect in cell surface Class I expression and a lack of a CD8 immune response in most models. Tapasin deficiency also impacts Class I presentation, but these effects are somewhat dependent on the Class I allele examined. The effect of calreticulin, ERp57, and PDI deficiency is much less severe, resulting in only a 2-4 fold decrease in cell surface Class I expression, as opposed to the 90 fold decrease in TAP deficient cells. The generation of $CD8^+$ T cells has yet to be addressed in mice lacking these molecules

(calreticulin knockout is embryonic lethal (114) and PDI knockout is lethal to yeast (115)).

Cross-presentation: Presentation of exogenously-derived antigens by Class I molecules

As opposed to Class I molecules, Class II molecules exit the ER with the peptide binding groove blocked by the invariant chain (116). Class II is targeted to the endosomal system, where invariant chain is degraded by cathepsins, and peptides from exogenous antigens are loaded. Class II loading has been shown to occur in early endosomes, late endosomes, phagosomes, lysosomes, and the Class II loading compartment. Because Mtb resides in a phagosome, Mtb antigens are efficiently processed and presented by Class II molecules. However, how Mtb antigens are cross-presented by Class I molecules is poorly understood. In this dissertation, cross-presentation is defined as Class I presentation of antigens that are exogenously derived and are not produced within the cytosol.

Data obtained by examining antigen processing and presentation of particulate-associated antigen, such as latex bead or bacterially associated proteins, have suggested two separate but non-mutually exclusive pathways. These have been broadly characterized as cytosolic and non-cytosolic models. In the cytosolic model, phagosomal antigens escape into the cytosol where they are processed by the conventional Class I machinery. In this model, processing and presentation of exogenous antigen by Class I is similar to that seen with endogenous proteins. In accordance with the cytosolic model, data suggests that the phagosome, following fusion with the ER, may directly participate in the processing and presentation of phagosomally derived antigens. Alternatively, the

non-cytosolic model of cross-presentation does not rely on the conventional machinery. Instead, it is thought that generation of peptide epitopes and Class I loading occurs in the endocytic pathway. How these models may apply to cross-presentation of Mtb antigens is illustrated in Figure 1.

Cytosolic Models

In support of the cytosolic model, the laboratory of Dr. Rock has shown that loading macrophages with bead-associated ovalbumin (OVA) leads to MHC-I presentation in a TAP and proteasome dependent manner (117). These experiments show that phagosomal proteins are able to escape the phagosome and localize to the cytosol. Consistent with presentation of endogenously expressed antigen, this pathway relies on ER-Golgi transport, as presentation is inhibited with brefeldin A (BFA). The ability of antigen to escape from the phagosome has been examined in Mtb infection. Mazzaccaro et al showed that loading of macrophages with soluble OVA leads to little presentation of OVA peptides on Class I molecules. However, when macrophages are loaded with soluble OVA and co-infected with either Mtb or BCG, presentation of OVA peptides on Class I increases in direct relation to mycobacterial number. Furthermore, MHC-I presentation was dependent on infection with live bacteria (118). Similarly, Teitelbaum and colleagues demonstrated that, when injected into the cytosol, fluorescent markers up to 70kDa were able to localize to phagosomes containing live but not heat-killed Mtb (119). From these results, it has been argued that infection with live mycobacteria results in a pore in Mtb-resident phagosomes, giving antigen access to the cytosol. Consistent with cytosolic antigen processing, data from our laboratory demonstrates that processing

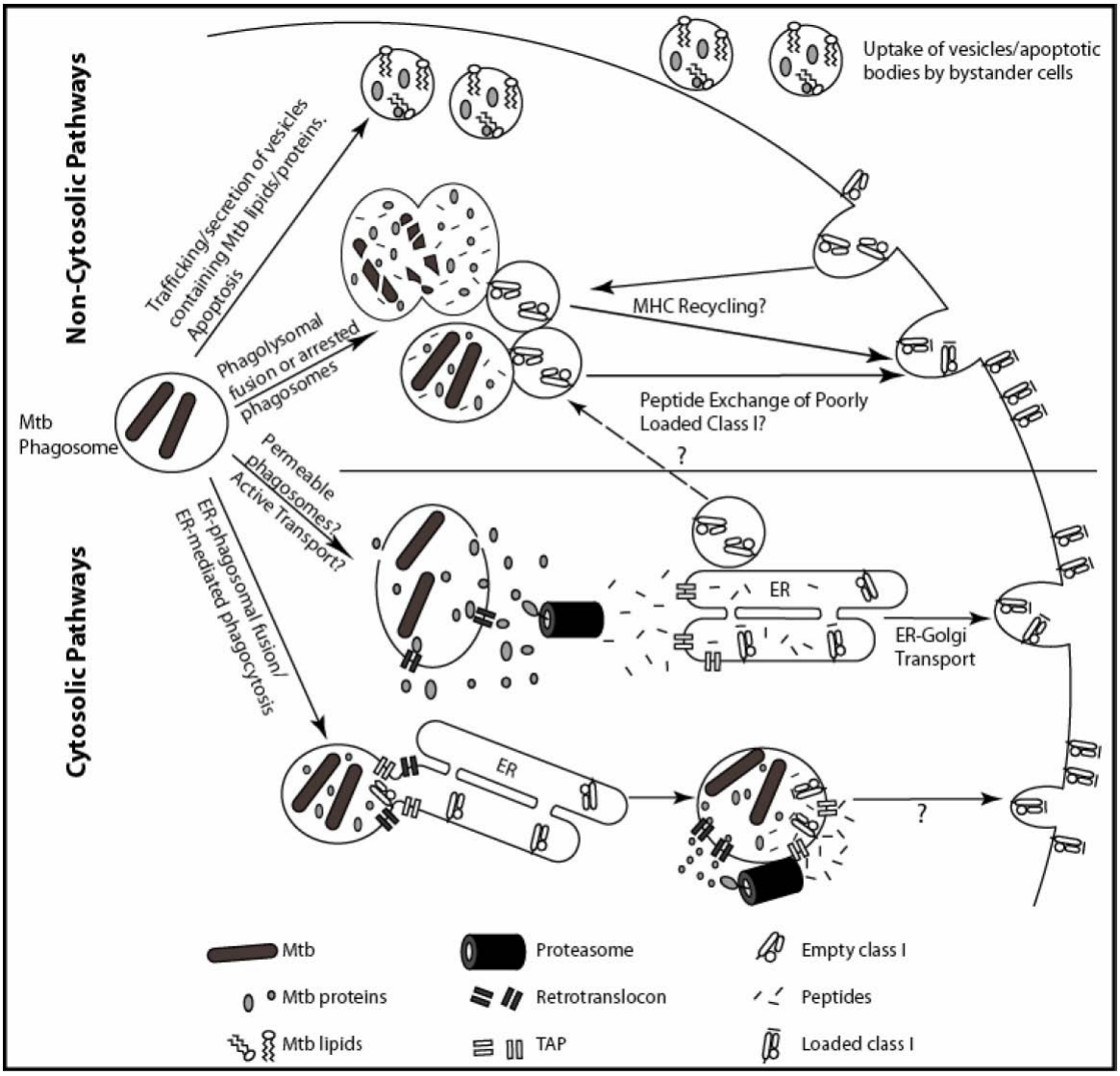


Figure 1. Possible mechanisms of cross-presentation of Mtb antigens. See text for details.

of the Mtb secreted protein, CFP10, follows the traditional Class I processing pathway, in that processing is dependent on proteasomal degradation and is inhibited with BFA (120). However, in a recent study, detection of radioactive or fluorescently labeled Mtb antigens was low within the cytoplasm, suggesting that relatively low amounts of Mtb proteins localize to the cytosol (121).

In an extension of the cytosolic model, several reports have described phagosomes as competent for cross-presentation (122-124). In these studies, phagosomes (due to ER-mediated phagocytosis (a process in which the ER assists in phagocytosis (125)) or other ER fusion events) were shown to contain the protein translocation complex Sec61 in conjunction with PLC members (TAP, calnexin, tapasin, calreticulin, Erp57, PDI, and MHC-I), associate with immunoproteasomes, and contain MHC-I:peptide complexes. In this model, antigens escape the phagosome, are degraded by the proteasome, and then transported by TAP back into the phagosomal lumen where loading occurs. Trafficking of phagosome derived MHC-I:peptide complexes to the cell surface was only partially inhibited by treatment with BFA, suggesting that ER-golgi transport is not required for cell surface presentation. More detailed characterization of this pathway has provided a mechanism for cytosolic access of phagosomal antigens. Ackerman et al demonstrated that phagosomes acquire the cellular retrotranslocation machinery (126). This normally ER-localized translocon facilitates transport of phagosomal antigens to the cytosol. Although the notion of ER-mediated phagocytosis is controversial (127), together these data characterize the role of phagosomes as autonomous organelles for cross-presentation. While further determination of the significance of this pathway in presentation of Mtb antigens is needed, it is interesting to note that our laboratory and

others have reported the existence of lactacystin-sensitive (128), brefeldin A-insensitive (128, 129) processing pathways that would be consistent with this newly described pathway.

Non-cytosolic models

There is experimental evidence supporting an alternate, non-cytosolic pathway of cross-presentation, also referred to as the vacuolar pathway (130-133). Using an OVA epitope fusion protein expressed in *Escherichia coli* or *Salmonella typhimurium* as well as OVA associated with latex beads, the laboratory of Cliff Harding has described a pathway that is TAP- and proteasome-independent as well as BFA-insensitive. These data show that presentation through this pathway does not rely on access to cytosolic or ER-derived processing machinery. In these experiments, increasing the availability of peptide-receptive (i.e. MHC-I bound with low affinity peptides) MHC-I molecules through incubation at 26 °C, addition of exogenous β2m, or addition of stabilizing peptides leads to an increased presentation of particulate-associated antigen. From these and other data, this group has proposed that exogenous antigens can be loaded onto MHC-I molecules through a vesicular recycling pathway. Here, empty or poorly stabilized MHC-I molecules intersect with the MHC-II processing pathway and are loaded through peptide exchange with endosomally-derived peptides. Recently, the molecular basis for this pathway has been elucidated as Shen et al demonstrated that Cathepsin S is required for the generation of the OVA epitope when processed endosomally (134). Therefore, particulate antigens do not necessarily need to have access to the cytosolic MHC-I processing machinery.

Mtb lipids and proteins have been demonstrated to gain access to small exosomes (135-137). Beatty et al found that Mtb or BCG-infected macrophages secrete lipid vesicles containing Mtb-derived cell wall components. These were found to be present throughout infected cells, in the extracellular milieu, and in uninfected bystander cells, perhaps making Mtb lipids and proteins accessible to multiple processing pathways. Furthermore, Neyrolles et al demonstrated that the Mtb 19kDa lipoprotein showed similar trafficking characteristics and was presented to $CD8^+$ T cells in a TAP-independent manner (138). Similarly, Schaible et al showed that Mtb-induced apoptosis led to the release of apoptotic bodies containing Mtb-derived lipids and proteins (121). Cross-presentation of these vesicles after phagocytosis by bystander cells was dependent on phagosomal acidification but not proteasomal degradation.

From these data, an increasingly complex and dynamic picture of the mechanisms by which particulate-associated antigens are cross-presented by Class I molecules has emerged. Although the relevance of any of these processing pathways in presentation of Mtb-derived antigens in vivo is unknown, it is likely that a combination of these pathways is utilized for the priming of naïve $CD8^+$ T cells and presentation of antigen by infected macrophages/DC to memory-effector $CD8^+$ T cells. Additionally, these data raise the possibility that the physical characteristics of the antigen or the nature of the particulate might govern which pathway is used.

Cross-presentation/Cross-priming in vivo

$CD8^+$ T cells can be primed by DC that are not virally infected, but instead cross-present cell-associated viral antigens (cross-priming). One of the questions raised by the

identification of distinct cross-presentation pathways is the relevance of either one in the priming of $CD8^+$ immune responses. This question has been addressed in several different systems. In one of the first characterizations of components required for cross-priming in vivo, Huang et al demonstrated that the cross-priming of $CD8^+$ T cells (using tumor cells expressing influenza nucleoprotein) requires TAP (139). In a system where only non-hematopoietic cells can be infected with poliovirus (expressing OVA), Sigal et al also found that cross-priming of OVA-specific $CD8^+$ T cells was completely TAP-dependent (140). Similar results have been established when OVA, under the control of non-APC promoters, is transfected by gene gun delivery (141), or after macropinocytosis of parvovirus-like particles that contain OVA (142). The role of immunoproteasomes in cross-priming has also been examined. LMP7 (IFN-γ inducible proteasome subunit) deficient female mice show a large deficiency in the cross-priming of HY male antigen-specific $CD8^+$ T cells (143). All of these data paint the picture that the dominant pathway for cross-priming/presentation in vivo is through the cytosolic pathway. However, using poly-lactide poly-glycolide (PLG)-associated OVA, cell associated OVA, and influenza infection, Shen et al demonstrated that although the cytosolic pathway is the most sensitive and dominant, the non-cytosolic pathway is also functional in cross-priming in vivo (134). Furthermore, Bertholet et al demonstrated that proliferation of adoptively transferred OVA-specific $CD8^+$ T cells to *Leishmania major* infection in vivo was TAP-independent , suggesting that cross-presentation of Leishmania antigens uses the vacuolar pathway in vivo (144). Even though multiple systems have been studied, most of these systems have still used OVA processing and presentation as a readout, and very few have studied cross-priming/presentation of naturally occurring immunodominant epitopes in

true infection models. Further in vitro and in vivo work is needed addressing these issues.

Non-classical antigen presentation and the HLA-E restricted response to Mtb

Generally, the $CD8^+$ immune response to infection has been studied looking at classically-restricted $CD8^+$ T cells. Here, classical MHC restriction is defined as peptide antigen presented in the context of polymorphic, MHC-Ia molecules. However, non-classically restricted $CD8^+$ T cells have also been described. Non-classical, or MHC-Ib molecules are monomorphic or show low polymorphism compared to MHC-Ia molecules. These include the mouse molecules CD1d, H2-M3, and Qa-$1^b$ and the human group 1 CD1 molecules and HLA-E, -F, and -G.

Previously, HLA-E (human) and Qa-$1^b$ (mice) molecules have been shown to bind and present leader peptides derived from classical Class I molecules (145-149). Consequently, HLA-E/Qa-$1^b$ cell surface expression is a surrogate for the expression of MHC-I molecules. As a result, downregulation of MHC-I leads to diminished cell surface expression of HLA-E/Qa-$1^b$. Because HLA-E/Qa-$1^b$ binds to the NK inhibitory heterodimer CD94/NKG2, decreased expression of HLA-E/Qa-$1^b$ leads to NK activation, providing one mechanism by which NK cells recognize reduced HLA-I expression on virally infected or transformed cells (150-152). We have described human Mtb-specific $CD8^+$ T cell clones from a LTBI donor that are restricted by HLA-E (81). Our data then suggest that pathogen-derived antigen can also be presented by HLA-E, a finding that has been confirmed for CMV (153) and *Salmonella typhi* (154).

Antigen presentation by HLA-E molecules is similar to that by classical Class I

molecules. HLA-E associates with the PLC in the ER (147, 155), where it is loaded with leader peptides. The maturation of HLA-E shows slow kinetics, with a half-life in the ER of 60-90 minutes versus 15-30 minutes for classical Class I molecules (147). The role of TAP in presentation of leader peptides is controversial, as there is data for TAP-dependent and -independent cell surface expression (147, 149, 156-158). This may be due to the location of the epitope within the leader peptide, which may enter the ER after cleavage by signal peptidase (158, 159). However, proteasome degradation has been shown to be required for presentation of leader peptides and *Salmonella Typhi* antigens (154, 159). Thus, the role of TAP in presentation of HLA-E is likely to be dependent on individual antigens.

To determine the contributions of classically versus non-classically restricted $CD8^+$ T cells in Mtb-infected patients, limiting dilution analysis from LTBI and active TB patients has demonstrated that a substantial proportion of the Mtb-specific $CD8^+$ T cell response was neither CD1 nor HLA-Ia restricted (unpublished observations, (82)). These data suggest that non-classically restricted $CD8^+$ T cells play a major role in the immune response to Mtb infection. Phenotypically, HLA-E restricted $CD8^+$ T cells express the $\alpha/\beta$ TCR, produce IFN-$\gamma$ and lyse Mtb-infected DC and macrophages (81, 128). However, the frequencies and recall capacities that this subset exhibits is not yet known.

Several characteristics of non-classical antigen presentation suggest a unique role for this response. First, the low degree of polymorphism of these molecules suggests that the array of peptides and/or non-peptide antigens presented would be limited, possibly allowing for recognition of pathogen-derived patterns. Where specific antigens for

MHC-Ib molecules have been described, patterns have become evident. For example, H2-M3 binds to N-formylated peptides (160, 161), which are specific for bacteria and mitochondria, while CD1 molecules present lipids, glycolipids, and lipopeptides (75-80). Second, it has been reported that H2-M3-restricted T cells respond with quicker kinetics to primary *Listeria monocytogenes* infection than MHC-Ia restricted T cells, but there is not an enhanced recall response for H2-M3-restricted T cells following infection with Listeria (162). These and other data have been used to suggest that non-classically restricted cells are a source for early IFN-γ. Whether non-classically restricted T cells represent a recall response to antigenic exposure or pre-armed effector cells remains controversial. $CD8^+$ T cells in $K^{b-/-}/D^{b-/-}$ mice, which represent MHC-Ib restricted $CD8^+$ T cells show a partially activated phenotype more associated with antigen experienced T cells than naïve cells (163). Moreover, Urdahl et al found that H2-M3-restricted cells show this phenotype directly after thymic selection. These data support the hypothesis that H2-M3-restricted T cells emerge from the thymus as pre-formed memory-effector cells. Whether the pre-activated phenotype can be broadly applied to other MHC-Ib restricted T cells is not known. Alternatively, there is evidence that group 1 CD1-restricted T cells, present in both humans and guinea pigs, constitute a recall response to mycobacterial infection. While CD1-restricted responses can be found in persons who have not been infected with Mtb, several studies found that the CD1-restricted T cell frequency is increased in individuals that have been immunized with BCG (164) or exposed to Mtb (77, 165), consistent with a recall response.

The role of HLA-E/Qa-$1^b$ antigen presentation and non-classically restricted $CD8^+$ T cells in Mtb infection is undefined. One study has examined the outcome of Mtb

infection in mice lacking classically restricted $CD8^+$ T cells ($K^{b-/-}/D^{b-/-}$) (166). In the absence of classical MHC-I, only $CD8^+$ T cells restricted by non-classical MHC-I molecules should be present. Although $CD8^+$ T cells trafficked to the lung and were able to produce IFN-γ after in vitro stimulation with anti-CD3, these mice showed irregular granuloma formation and decreased survival compared to wild type mice. Although these findings suggest that non-classically restricted $CD8^+$ T cells are not required for protection against Mtb infection, there are several caveats to this study. There is a large decrease in the number of $CD8^+$ T cells in these mice, and there may be a minimum threshold of CD8 recruitment for protection. More importantly, mice lacking classical Class I molecules are deficient in Class I leader peptides needed for cell surface expression of Qa-$1^b$, probably effecting the thymic selection of Qa-$1^b$-restricted T cells. Therefore, these mice might also be deficient in some non-classically restricted $CD8^+$ T cell subsets as well. It would be interesting to examine the role of Qa-$1^b$-restricted T cells in Mtb infection using the Qa-$1^b$ knockout mouse. Our finding of high frequency non-classically restricted $CD8^+$ T cells in TB patients is suggestive of a role of these cells in the Mtb response. Furthermore, the data presented herein implicate HLA-E in the presentation and surveillance of phagosomal antigens.

# Chapter 2

# The *Mycobacterium tuberculosis* phagosome is a HLA-I processing competent organelle

Jeff E. Grotzke, Anne M. Chamberlin, Dawn Nolt, and David M. Lewinsohn

***Summary:***

*Mycobacterium tuberculosis* (Mtb) resides in a long-lived phagosomal compartment that resists maturation. The manner by which Mtb antigens are processed and presented on Class I molecules is poorly understood. We examined the processing and presentation pathways for two Mtb-derived antigens, each presented by a distinct HLA-I allele (HLA-B8 versus HLA-E). Presentation of both antigens is blocked by the retrotranslocation inhibitor exotoxin A. After reaching the cytosol, these antigens require proteasomal degradation and TAP transport, but differ in the requirement for ER-golgi egress and newly synthesized HLA-I. Phenotypic analysis of the Mtb phagosome shows the presence of Class I loading accessory molecules, including TAP and PDI. Furthermore, loaded HLA-E:peptide complexes are present within the Mtb phagosome. Together, these data suggest that the phagosome plays a role in cross-presentation of Mtb-derived antigens, and that HLA-E may play a unique role in the response to phagosomal pathogens.

***Introduction:***

*Mycobacterium tuberculosis* (Mtb) remains a leading cause of morbidity and mortality worldwide, and is the leading cause of death in AIDS patients. The cellular immune response is vital for controlling Mtb infection and preventing development of active TB. Through the production of pro-inflammatory cytokines, $CD4^+$ and $CD8^+$ T cells are able to activate macrophages to upregulate anti-microbial defenses, such as reactive nitrogen intermediates and autophagy (52, 54, 167, 168). These cellular mechanisms maintain latent TB infection indefinitely in 90-95% of immunocompetent individuals. Thus, the recognition of Mtb-infected cells by T cells is central to prevention of uncontrolled replication. In this report, we seek to define the mechanisms by which Mtb antigens are cross-presented on HLA-I molecules.

Following uptake by phagocytic cells such as macrophages and dendritic cells (DC), Mtb resides in a modified phagosomal compartment with the characteristics of an early endosome (7). Mtb is able to inhibit phagosomal maturation and lysosomal fusion, and thus survive within the host cell. Because this compartment is a component of the HLA-II processing pathway, the mechanisms by which Mtb-derived antigens are processed and presented on HLA-I molecules remain incompletely understood.

Studies using model particulates have shown that phagosome-associated antigens can use several pathways for cross-presentation (169). Antigens can be degraded within a vacuolar pathway, without accessing the cytosol or endoplasmic reticulum (ER (132)). This pathway does not rely on conventional MHC Class I (MHC-I) processing molecules such as the proteasome and transporter associated with antigen processing (TAP). Rather, it has been postulated that peptides are generated in a vacuolar compartment, and are

subsequently loaded onto recycled MHC-I within this compartment (133, 170). For ovalbumin (OVA), cathepsin S plays a critical role in generating the cognate peptide (134).

Alternately, there is abundant evidence demonstrating that phagosomal antigens are able to access the cytosol, allowing processing to occur using the conventional MHC-I pathway, including proteasomal degradation, transport of peptide fragments into the ER by TAP, ER loading onto MHC-I molecules, and egress of loaded complexes through the ER-golgi (117). More recently, it has been demonstrated that certain antigens access the cytosol through the acquisition of retrotranslocation machinery from the ER, which serves to actively transport phagosomal antigens into the cytosol (126).

It has been demonstrated that loading of peptides onto MHC-I can take place within the phagosomal environment. Following export of antigen into the cytosol and proteasomal degradation, the resultant peptides are transported by TAP into the phagosomal lumen where they are loaded onto MHC-I molecules (ER-phagosomal pathway, (123, 124)). It has been suggested that ER-derived MHC-I loading machinery is delivered through ER-mediated phagocytosis and/or subsequent fusion events (125). Although the notion of ER-mediated phagocytosis, and particularly the relative contribution of ER-membrane to the phagosomal membrane remains controversial (127), multiple groups have detected members of the MHC-I peptide loading complex and functional TAP, glycosylation, and retrotranslocation machinery in latex bead phagosomes (123-126, 171).

The mechanisms by which Mtb antigens are cross-presented on Class I molecules are incompletely defined, but it appears that multiple pathways may be used (168). Initial

studies suggested that live mycobacteria may create a pore in the phagosomal membrane (118, 119). We have shown that the processing of an epitope from CFP10, an immunodominant Mtb antigen, occurs through a proteasome-dependent, TAP-dependent, and brefeldin A(BFA)-sensitive cytosolic pathway (120). Alternately, Neyrolles and colleagues demonstrated that presentation of the 19kDa lipoprotein does not rely on TAP transport (138). Schaible et. al. showed that presentation of antigens derived from apoptotic vesicles from Mtb or *Mycobacterium bovis* BCG-infected dendritic cells occurs through a vacuolar pathway that requires acidification but not proteasomal degradation (121). Finally, we have shown that presentation of an antigen presented by the non-classical HLA-I molecule, HLA-E, occurs through a proteasome-dependent, but BFA-independent pathway, suggesting that an alternate pathway may be used after proteasomal degradation (128). Of note, one other group has reported BFA-independent presentation of unidentified Mtb antigen(s), although the role of the proteasome was not examined (129). These data are consistent with that shown for the ER-phagosomal pathway (Houde et al., 2003).

In this report, we take advantage of T cells clones restricted by HLA-E, HLA-B8, and HLA-B44 to examine the processing and presentation pathway for two Mtb-derived antigens. While both antigens require cytosolic processing as reflected by dependence on the proteasome and TAP, processing of the HLA-E antigen is distinguished by the possible use of recycled HLA-E. Characterization of the Mtb phagosome reveals the presence of HLA-I, TAP, and protein disulfide isomerase (PDI), and Mtb phagosomes contain HLA-I:peptide complexes. Overall, our data argue that the Mtb phagosome itself plays a role in presentation of Mtb antigens.

***Results:***

**Mtb antigens access the cytosol but differ in their requirement for ER-golgi transport**

To define the molecular requirements for processing and presentation of two Mtb antigens, $CD8^+$ T cell clones generated in our laboratory were utilized. Clone D480 F6 recognizes the 9mer epitope EMKTDAATL from CFP10 ($CFP10_{3-11}$) in the context of HLA-B0801 (172). D160 1-23 is restricted by the non-classical HLA-I molecule, HLA-E. Although we have not yet defined the antigen or minimal epitope for this clone, it responds to the cell wall and TX-114 soluble Mtb fractions (81). A $CD4^+$ T cell clone (D454 E12) specific for CFP10 (unpublished data) was used as a control.

To determine the antigen processing pathway, the effect of inhibitors of proteasomal degradation (epoxomicin), ER-golgi transport (BFA), or vacuolar ATPase activity (bafilomycin) on processing of Mtb-derived antigens was assessed. Human monocyte-derived DC were treated with each inhibitor for one hour prior to infection with Mtb. Following overnight incubation, DC were fixed and used in an IFN-γ ELISPOT assay using Mtb-specific T cell clones as indicators.

Presentation of $CFP10_{3-11}$ was almost completely inhibited in the presence of the epoxomicin and BFA, with no effect of bafilomycin treatment (Figure 1A). This drug inhibition profile is consistent with the cytosolic cross-presentation pathway, and are similar to those previously published by our group for the $CFP10_{2-11}$ (HLA-B44 restricted) epitope (120). HLA-E antigen presentation was also almost completely inhibited with epoxomicin, but only partially inhibited in the presence of BFA (50% inhibition, Figure 1A). These data demonstrate that although the HLA-E antigen

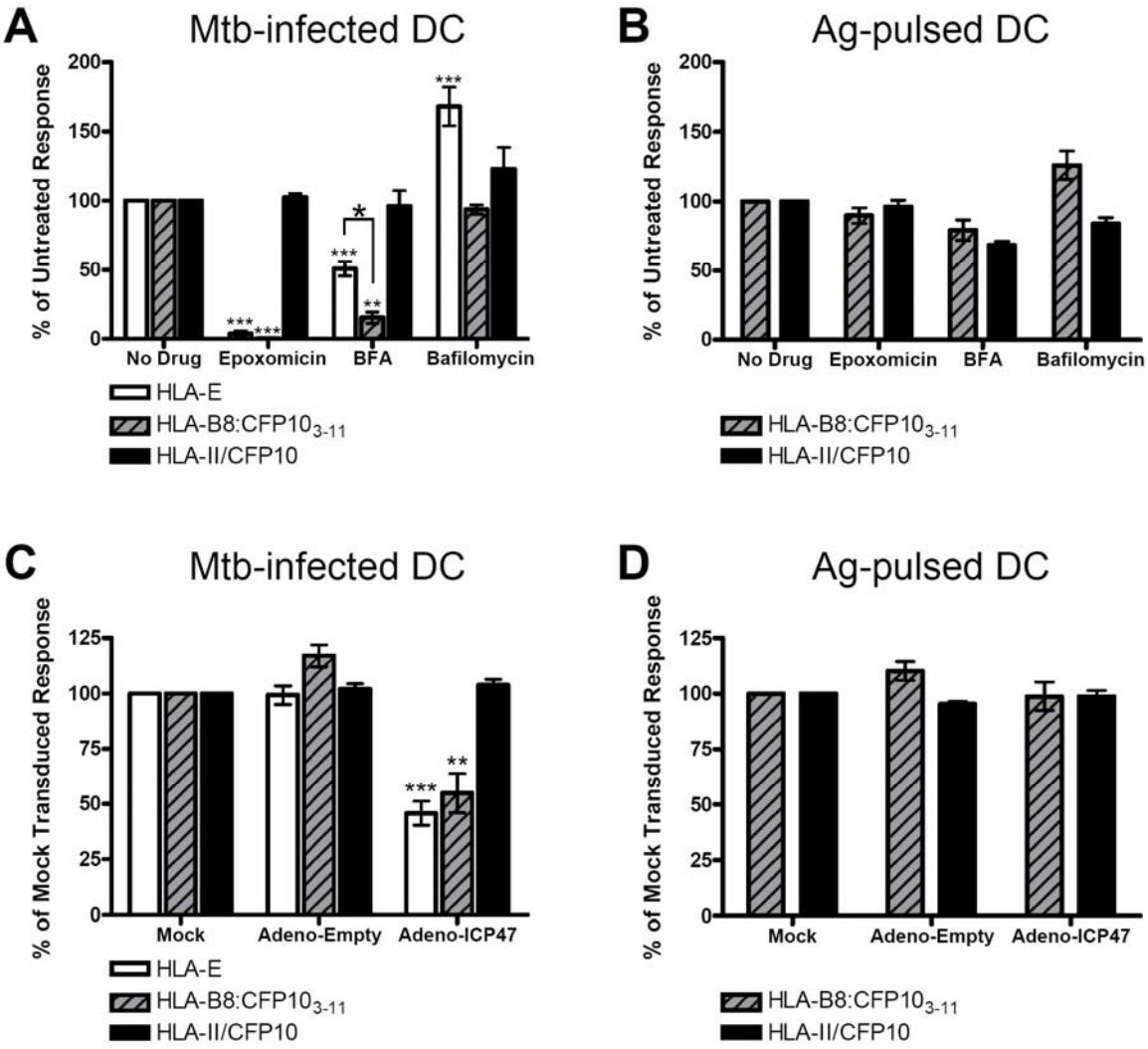


Figure 1. Mtb antigens are processed by the cytosolic pathway. (A&B) Human monocyte-derived DC were treated with media, epoxomicin (1μM), BFA (0.1μg/ml), or bafilomycin (0.2μM) for one hour before infection with H37Rv-eGFP (A) or addition of CFP10 and $CFP10_{3-11}$ (B). After 15-16 hours in the presence of the inhibitor, DC were harvested, fixed, washed extensively and used as APC in an IFN-γ ELISPOT assay where T cell clones are effectors. Data has been normalized to the untreated control, and each bar reflects the mean +/- SEM of at least three experiments per clone. Asterisks denote statistical significance compared to untreated DC, except where indicated, using Student's t test (***p<0.001, **p<0.01, *p<0.05). (C&D) DC were transduced with either empty vector or adenovirus-ICP47 using Lipofectamine 2000. After 6-26 hours, DC were washed and infected with H37Rv-eGFP (C) or antigen (D). Following overnight Mtb infection, T cell clones were added and IFN-γ production was assessed by intracellular cytokine staining. Each bar represents the mean +/- SEM of seven independent experiments. Asterisks denote statistical significance compared to mock transduced DC using Student's t test (***p<0.001, **p<0.01)

requires cytosolic proteasomal degradation, ER-golgi transport is not required for presentation. Furthermore, bafilomycin treatment actually enhanced HLA-E antigen presentation, suggesting that phagosomal acidification leads to the destruction of the epitope.

As a control for drug toxicity and specificity, neither epoxomicin nor BFA interfered with Mtb-derived CFP10 presentation on HLA-II molecules. Also, there was minimal effects of inhibitor treatment on presentation of soluble CFP10 to the $CD4^+$ clone D454 E12 or $CFP10_{3-11}$ peptide to the $CD8^+$ clone D480 F6 (Figure 1B). Overall, these data indicate that two Mtb antigens utilize different processing pathways to be ultimately presented to $CD8^+$ T cells.

**Presentation of Mtb antigens requires TAP transport**

The requirement of proteasomal processing argues for cytosolic access of CFP10 and the HLA-E antigen. To determine if TAP transport is required for presentation of these epitopes, we used an adenoviral vector expressing ICP47. ICP47 is a herpes simplex virus-I protein that binds to the cytosolic domain of TAP, thereby preventing peptide binding and subsequent peptide transport (173, 174). Transduction of DC with adenovirus-ICP47, but not with the empty vector, led to a modest reduction in HLA-I cell surface expression. Conversely, no effect of ICP47 on cell surface HLA-II was observed (data not shown). When ICP47 expressing DC were subsequently infected with Mtb, HLA-E antigen presentation was inhibited by 55% while CFP10 presentation was inhibited by 45% compared to mock transduced DC (Figure 1C). These data demonstrate that TAP is required for the presentation of these two Mtb proteins. In contrast, there was

no ICP47-mediated inhibition of presentation of either soluble or Mtb-derived CFP10 by HLA-II, nor was there any defect in ICP47-expressing cells to present the $CFP10_{3-11}$ peptide (Figure 1D).

**Mtb antigens require export out of the phagosome but do not require newly synthesized HLA-E**

The ER contains retrotranslocation machinery that functions to dislocate misfolded proteins into the cytosol where they can be degraded (175). Recent data show that the phagosome acquires this machinery and that retrotranslocation is required for cross-presentation of soluble OVA (126). To determine if Mtb antigens require transport out of the phagosome for cytosolic access, the *Pseudomonas aeruginosa* protein Exotoxin A (exoA) was employed. ExoA inhibits protein synthesis by ADP-ribosylation of elongation factor-2 (176). Through interactions with sec61 and other members of the retrotranslocation machinery, exoA is also able to inhibit retrotranslocation (126, 177). DC treated with exoA were inhibited by 80% in their ability to process and present $CFP10_{3-11}$, while presentation of the HLA-E antigen was inhibited by 45% (Figure 2A).

Because exoA has multiple functions, we sought to determine whether the effect of exoA was due to a block in retrotranslocation or inhibition of protein synthesis. Treatment of DC with exoA led to a dramatic inhibition of vaccinia virus-eGFP expression (Figure 2C), showing that exoA is a potent inhibitor of protein synthesis in primary human DC. Consistent with its effect as an inhibitor of protein synthesis, exoA blocked the presentation of vaccinia-expressed HIV p24 to a p24 specific $CD8^+$ T cell clone (Figure 2D).

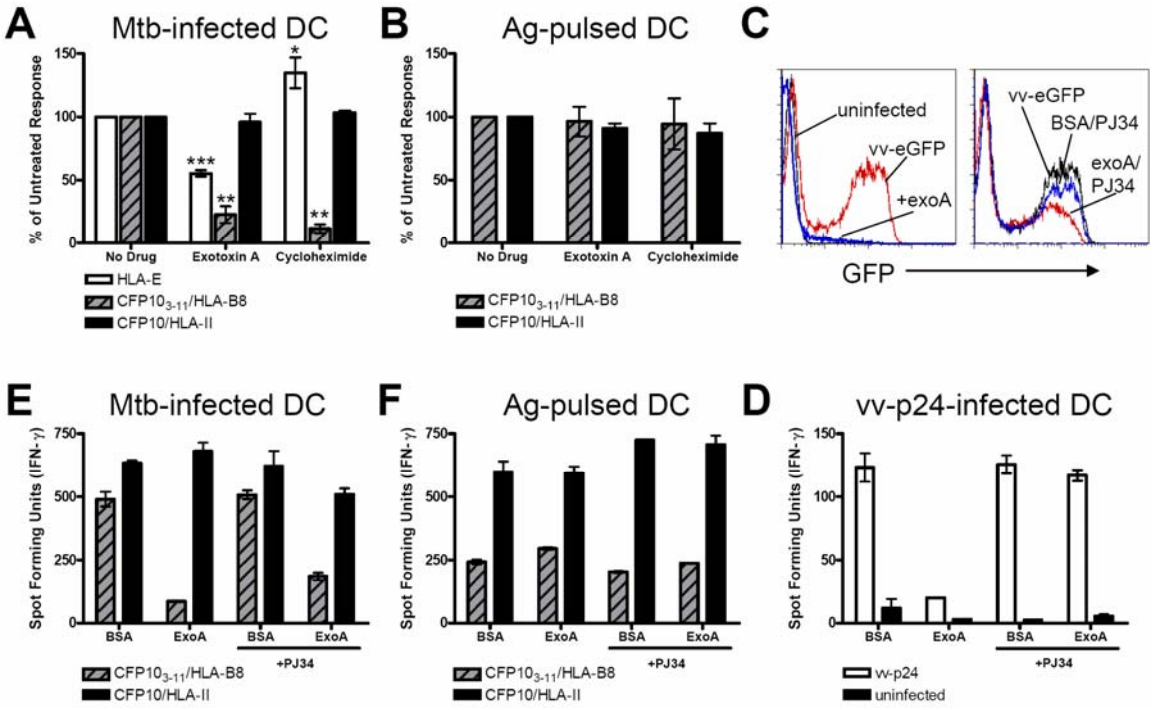


Figure 2. Mtb proteins require retrotranslocation for presentation. (A&B) DC were treated with exoA or cycloheximide for one hour before infection with H37Rv-eGFP (A) or addition of CFP10 and $CFP10_{3-11}$ (B). DC were harvested, fixed, and assessed for their ability to stimulate T cell clones as described. Each bar reflects the mean +/- SEM of at least three experiments. Asterisks denote statistical significance compared to untreated DC using Student's t test (***$p<0.001$, **$p<0.01$, *$p<0.05$). (C&D) DC were treated with exoA, exoA/PJ34, or BSA/PJ34 for one hour prior to infection with vaccinia virus expressing eGFP (C) or HIV p24 (D). After 16-18 hours, DC were harvested and GFP expression analyzed by flow cytometry (C) or fixed and used to stimulate the HIV $p24_{306-316}$-specific $CD8^+$ clone 16A7 (D). Data are representative of two experiments. (E&F) DC treated with exoA, BSA, exoA/PJ34, or BSA/PJ34 for one hour were subsequently infected with H37Rv-eGFP (E) or pulsed with antigen (F) overnight, harvested, fixed, and assessed for their ability to stimulate T cell clones. Data are representative of two experiments.

The protein synthesis inhibitor cycloheximide was used to examine the effect of protein synthesis on presentation of Mtb antigens. Treatment of DC with cycloheximide inhibited presentation of Mtb-derived $CFP10_{3-11}$ by 90%, while enhancing presentation of the HLA-E antigen (Figure 2A). These data argue two important points. First, exoA inhibition of HLA-E antigen presentation is not due to inhibition of protein synthesis and is likely an effect of retrotranslocation block. Second, newly synthesized HLA-E is not required for antigen loading. This suggests that HLA-E loading occurs through a recycling pathway that does not require nascent HLA-E, while the BFA data suggests that loading does not occur within the ER.

To determine whether retrotranslocation was involved in CFP10 processing, the exoA inhibitor PJ34 was utilized. PJ34 binds to the catalytic domain of exoA with high affinity, thereby inhibiting ADP-ribosylation and abrogating the effect of exoA on protein synthesis (178). Co-incubation of PJ34 and exoA restored vaccinia virus-eGFP expression to similar levels as seen in untreated or BSA/PJ34 cotreated DC (Figure 2C). Furthermore, presentation of endogenously-expressed antigen was indistinguishable to that from BSA or BSA/PJ34 treated controls (Figure 2D). Together, these data confirm that PJ34 blocks the protein synthesis inhibition activity of exoA, and demonstrate that PJ34 itself does not inhibit protein synthesis or endogenous antigen presentation.

While not as effective as exoA alone, exoA/PJ34 inhibited Mtb-derived $CFP10_{3-11}$ presentation by over 60%, and had little effect on presentation of Mtb-derived CFP10 by HLA-II (Figure 2E). Presentation of peptide or soluble CFP10 was not blocked by exoA, cycloheximide, or exoA/PJ34 (Figure 2B&F), further controlling for toxicity of these inhibitors. In total, these data argue that presentation of both Mtb antigens are facilitated

by retrotranslocation from the phagosome for cytosolic access.

**The Mtb phagosome contains HLA-I loading accessory molecules**

Since our data demonstrates that HLA-E antigen presentation requires TAP transport, yet is insensitive to cycloheximide and only partially blocked by BFA, we hypothesized that peptide loading may occur within the Mtb phagosome. To determine if the Mtb phagosome contains proteins associated with HLA-I peptide loading, flow organellometry was employed. Mtb phagosomes were separated from plasma and ER membranes using a 27% percoll gradient as modified from the protocol of Ramachandra et al. (179). Briefly, DC were pulsed with H37Rv-eGFP or magnetic beads for 20 minutes, washed, and then incubated for 40 minutes or overnight. Harvested cells were homogenized, intact cells and nuclei removed, and the resulting supernatant layered onto 27% percoll and spun at 36,000xg for one hour. Figure 3A demonstrates the relative enrichment of plasma membrane, ER membranes, lysosomes, and phagosomes. While there is some overlap between the phagosomal and lysosomal fractions, phagosomes are clearly separated from the ER and plasma membrane fractions. Phagosome-containing fractions were pooled and used for flow organellometry.

To analyze phagosomal maturation, Mtb phagosomes were compared to phagosomes containing polystyrene-coated magnetic beads at early (60 minutes) and late timepoints (overnight). Magnetic beads were gated on their distinct scatter profile (data not shown). Early phagosomes were either LAMP-$1^{lo/-}$/HLA-$I^{+}$, LAMP-$1^{+}$/HLA-$I^{lo/-}$, or double negative (Figure 3B). The latter population likely represents unphagocytosed beads. Analysis of the LAMP-$1^{lo/-}$/HLA-$I^{+}$ population revealed phagosomes that are

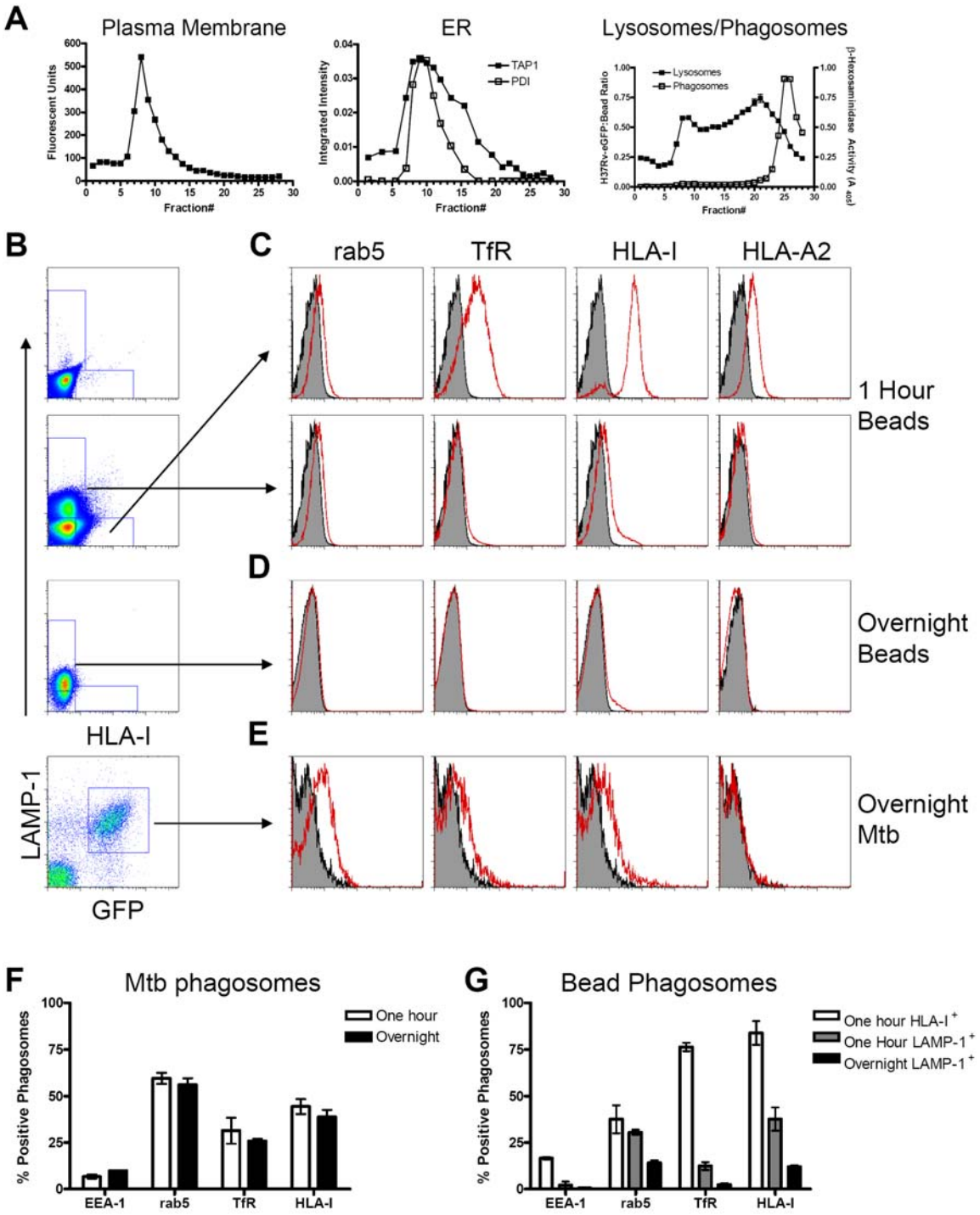


Figure 3. The Mtb phagosome retains characteristics of an early endosome. (A) Representative figure showing organelle distribution after percoll separation of homogenate from Mtb-infected DC. The plasma membrane was labeled with a PE-conjugated antibody to HLA-II prior to homogenization and fluorescence was detected by fluorometry. For detection of ER, fractions were assessed for the presence of TAP1 and PDI by western blot. An enzymatic assay for β-hexosaminidase was used for detection of lysosomes. Finally, fractions were

examined for the presence of H37Rv-eGFP by flow cytometry and quantitated using a reference latex bead population. For flow cytometric analysis of Mtb phagosomes, the final 2 ml of the gradient was pelleted, fixed, permeabilized, and stained with antibodies of interest. (B) Magnetic bead and Mtb phagosomes were gated based on FSC/SSC (not shown) and then on LAMP-1/HLA-I (beads) or LAMP-1/GFP (Mtb). The top panel represents isotype stained magnetic bead phagosomes. (C-E) Analysis of phagosome maturation on LAMP-$1^{lo/-}$/HLA-$I^{+}$ magnetic bead phagosomes (C, top panel), LAMP-$1^{+}$/HLA-$I^{lo/-}$ magnetic bead phagosomes (C, bottom panel), and LAMP-$1^{+}$ overnight magnetic bead (D) and Mtb phagosomes (E). Plots include isotype staining (shaded histograms) as well as staining with the indicated antibody (red lines). (F&G) Quantitative analysis of phagosomes over time. The percent positive number represents Overton cumulative histogram subtraction of the isotype control from the indicated stain. Each bar represents the mean +/- SEM of three experiments per timepoint.

largely positive for the early endosomal markers rab5 and transferrin receptor (TfR), and strongly positive for HLA-A2 and HLA-I (Figure 3C). This population likely represents beads that have recently been phagocytosed. Consistent with this hypothesis, this population is absent following an overnight chase. Alternately, the LAMP-$1^{+}$/HLA-$I^{lo/-}$ population had greatly reduced levels of early endosomal markers after one hour and were essentially negative after an overnight incubation (Figure 3D&F), and demonstrate normal phagosomal maturation.

In contrast, flow organellometry of Mtb phagosomes revealed a different phagosomal phenotype. After one hour of Mtb infection, the LAMP-$1^{lo/-}$/HLA-$I^{+}$ population seen in magnetic bead phagosomes was not detected (data not shown), possibly a reflection of the smaller number of events obtained for Mtb phagosomes. As a result, Mtb phagosomes could be accurately gated solely based on GFP and LAMP-1 (Figure 3B). One hour Mtb phagosomes contained similar or greater levels of early endosomal markers as seen in the LAMP-$1^{+}$/HLA-$I^{lo/-}$ magnetic bead population (Figure 3F&G). However, overnight Mtb phagosomes remained positive for rab5, TfR, and HLA-I (Figure 3E&F), consistent with the early endosomal arrest that has been previously described (7).

Having purified and phenotyped the Mtb phagosome, we asked whether HLA-I associated loading molecules were present. In addition to HLA-I (Figure 3), the Mtb phagosome contained loading accessory molecules TAP1, TAP2, PDI, as well as proteins containing the KDEL ER retrieval sequence (Figure 4A,B&D). These proteins were found at similar levels at one hour and overnight, suggesting that these molecules are stable within the Mtb phagosome. Furthermore, Mtb phagosomes were negative for ER-

resident proteins calnexin and calreticulin, suggesting that the presence of ER proteins was selective, and not the result of gross contamination during homogenization or staining. In addition, detection of ER proteins in the Mtb phagosome did not correlate with the cellular levels of these proteins in DC (Figure 4C, note TAP2 versus TAP1, calnexin, and calreticulin).

To further control for contamination, we performed a mixing experiment in which HLA-A2 could be used to discern membrane contamination. HLA-$A2^-$ DC were infected with Mtb and mixed with uninfected HLA-$A2^+$ DC. Following homogenization and percoll separation, phagosomes were stained with an antibody to HLA-A2. We did not detect any HLA-A2 in the Mtb phagosome after mixing (Figure 5A), suggesting that there is little non-specific association of membranes with Mtb phagosomes during processing. Interestingly, while Mtb phagosomes are positive for HLA-I (Figure 3), we did not detect HLA-A2 in phagosomes from HLA-$A2^+$ DC (Figure 5A). However, we were able to detect HLA-A2 staining in LAMP-$1^{lo/-}$/HLA-$I^+$ magnetic bead phagosomes (Figure 3C) and in lymphoblastoid cell lines (LCL) (Figure 5B). Because HLA-A2 is expressed at much higher levels in DC than any of the ER molecules examined, we conclude that there could only be minimal contamination of ER proteins in our phagosomal preparations.

Finally, we assessed the levels of cis- and trans-golgi markers GM130 and golgin-97, respectively. Neither protein was present in the Mtb phagosome (Figure 5C), but were detected in DC (Figure 5D). Together, these data suggest that HLA-I and associated loading accessory molecules are bona fide constituents of the Mtb phagosome.

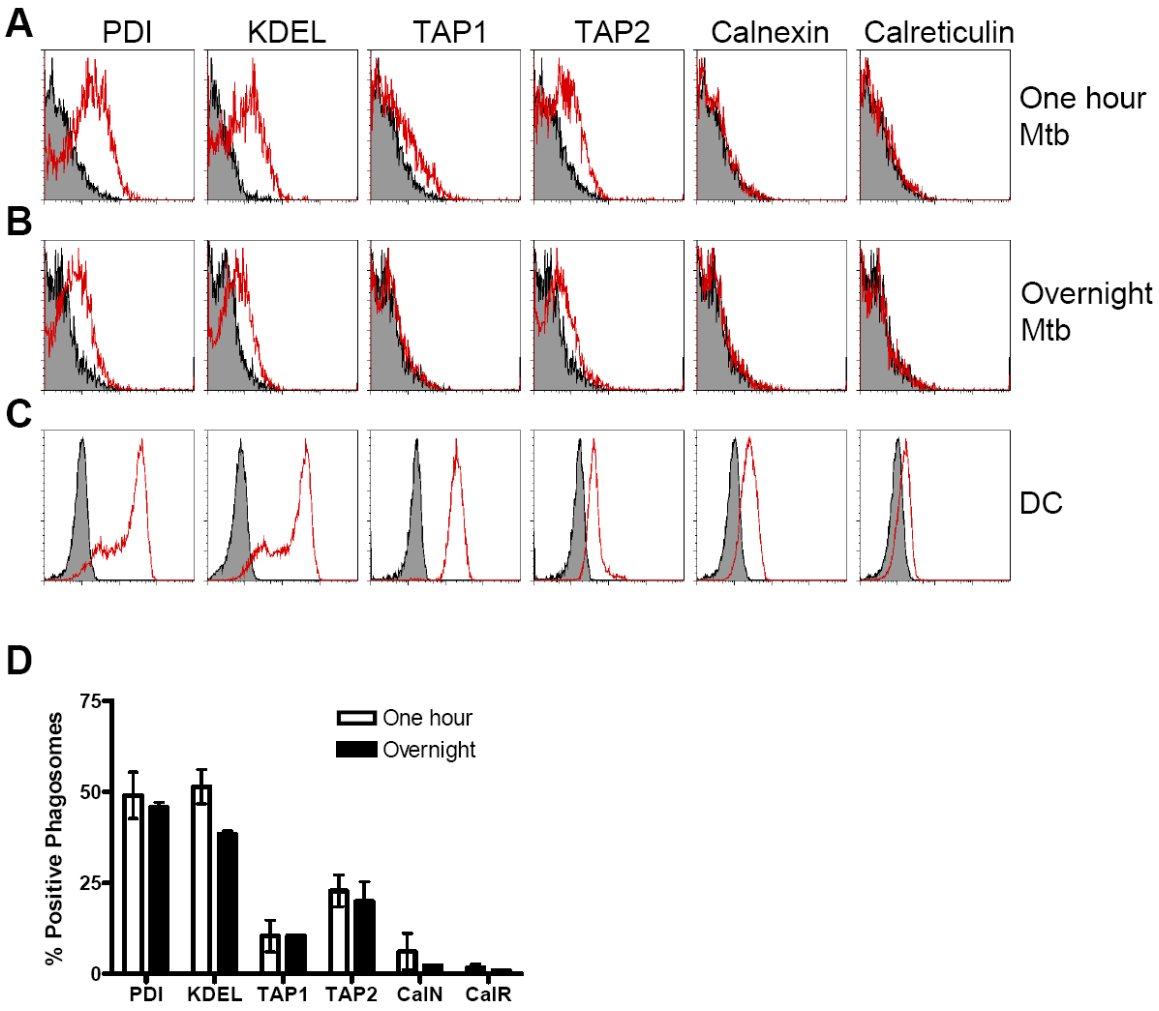


Figure 4. The Mtb phagosome contains HLA-I loading accessory molecules. (A&B) DC were pulsed with H37Rv-eGFP for 20', washed and incubated for 40' (A) or overnight (B). Phagosomal fractions were prepared as in Figure 3 and stained with the indicated antibodies. Data are representative of three experiments per timepoint. (C) Intact DC were fixed, permeabilized, and stained with the indicated antibodies. (D) Quantitative analysis of Mtb phagosomes over time. The percent positive number represents Overton cumulative histogram subtraction of the isotype control from the indicated stain. Each bar represents the mean +/- SEM from three experiments per timepoint.

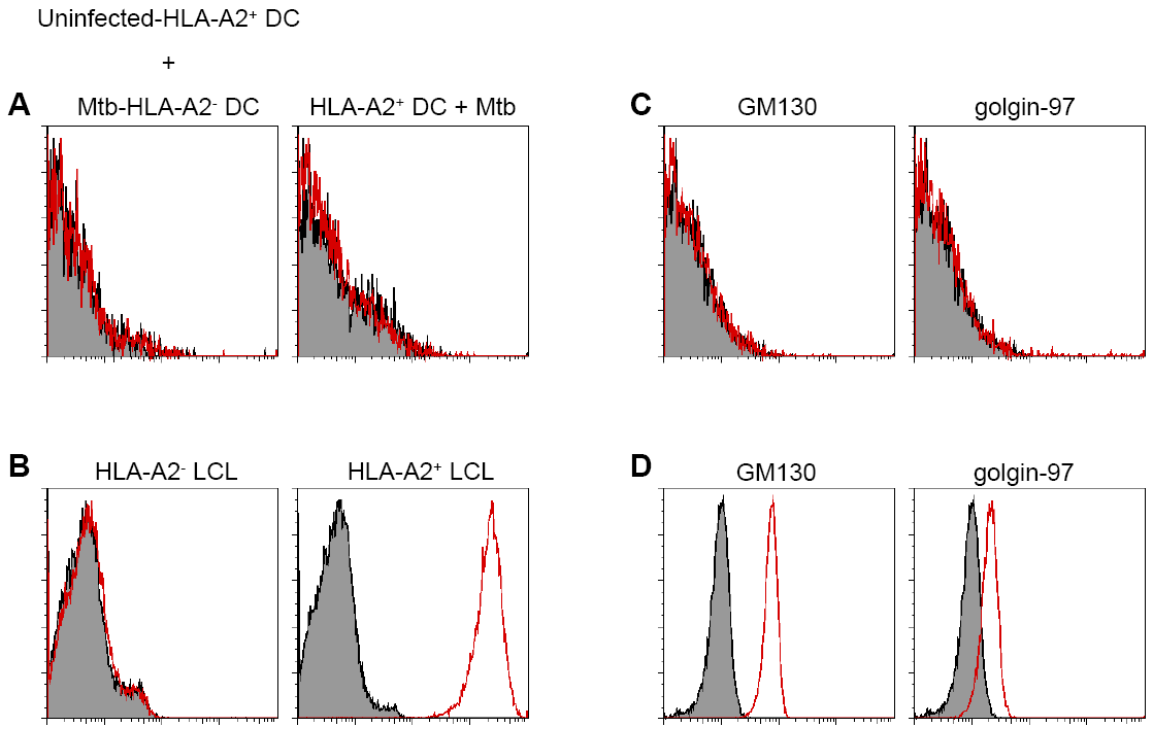


Figure 5. Analysis of contamination on Mtb phagosomes. (A) HLA-A2$^-$ or HLA-A2$^+$ DC were infected with H37Rv-eGFP for 20', washed, and incubated for an additional 40'. HLA-A2$^-$ DC were mixed with uninfected HLA-A2$^+$ DC, homogenized, and homogenate separated using 27% percoll as described. Phagosomes were stained with an antibody to HLA-A2. Shaded histograms represent isotype staining. Data is representative of three experiments. (B) HLA-A2$^-$ or HLA-A2$^+$ LCL were fixed, permeabilized, and stained with an antibody to HLA-A2. (C&D) Mtb phagosomes (C) or DC (D) were analyzed for the presence of cis- and trans-golgi markers GM130 and golgin-97, respectively. Data in C are representative of three experiments each after a 40' or overnight chase. Data in D are representative of two experiments.

**Loaded HLA-E:peptide complexes are present in the Mtb phagosome**

To assess whether peptide loading occurs in the Mtb phagosome, percoll-separated fractions were tested for their ability to stimulate $CD8^+$ T cell clones in the absence of DC. Mtb phagosomes were purified by percoll density gradient and, as was described above, were localized to fractions 22-28 and were well separated from plasma membrane and ER positive fractions. Percoll separated fractions were freeze-thawed to expose lumenal HLA-I, and incubated with $CD8^+$ T cell clones specific for HLA-E or $CFP10_{2-11}$:HLA-B44. We have previously reported that presentation of $CFP10_{2-11}$ is sensitive to BFA and lactacystin and requires TAP transport (120), following the same pathway as $CFP10_{3-11}$ presented in this paper. As expected, both clones responded to plasma membrane/ER fractions, revealing the presence of loaded HLA-I:peptide complexes (Figure 6A&B). Loaded HLA-E:peptide complexes were also detected in phagosome containing fractions at all timepoints examined, suggesting that peptide loading can in fact occur within the Mtb phagosome and that this is a rapid and ongoing process. Little to no HLA-B44:$CFP10_{2-11}$ complexes were detected within phagosome fractions, suggesting that less $CFP10_{2-11}$ loading occurs within the phagosome. These data are consistent with BFA data, showing that almost all $CFP10_{2-11}$ processing and presentation requires ER-golgi transport (120). To exclude the possibility that pre-formed peptide was simply being presented by T cells, we tested fractions from HLA-I mismatched DC for their ability to stimulate Mtb-specific $CD8^+$ clones and were unable to detect a response (data not shown).

To quantitatively assess the degree of plasma membrane contamination of the phagosome-containing fractions, plasma membrane was labeled with a PE-conjugated

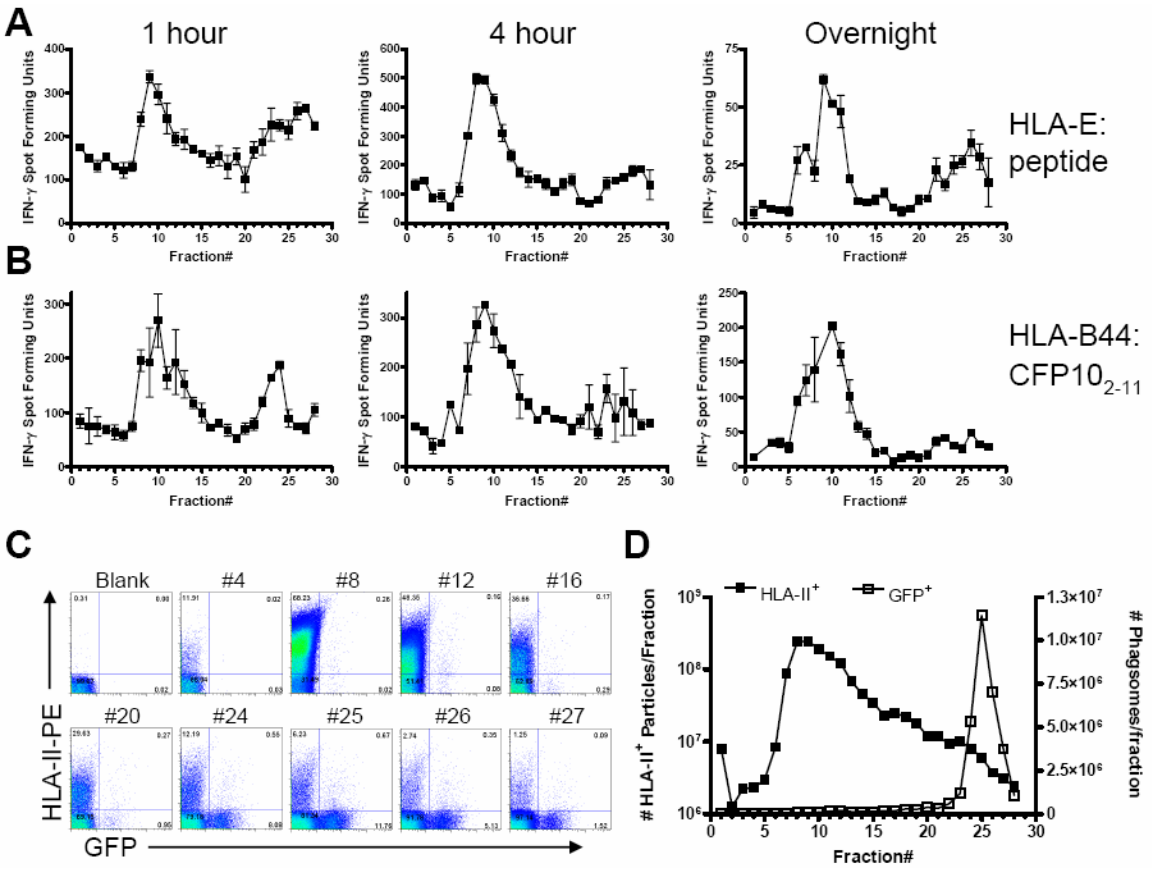


Figure 6. HLA-E:peptide complexes are present in phagosomal fractions. (A&B) DC were pulsed with H37Rv-eGFP for the indicated times, and homogenate separated using 27% percoll as described. Each fraction was freeze-thawed and tested for its ability to stimulate HLA-E restricted (A) or HLA-B44:CFP10$_{2-11}$ specific (B) CD8$^+$ T cell clones in the absence of additional APC. IFN-γ production was measured using ELISPOT. The mean +/- SEM of duplicate wells is presented. Data are representative of one experiment per timepoint. (C&D) Individual fractions were analyzed by flow cytometry to assess HLA-II-PE (plasma membrane) and H37Rv-eGFP fluorescence. Selected fractions are shown (C) including a blank (latex beads, homogenization buffer, and 27% percoll), the peak plasma membrane fraction (#8) and phagosomal fractions (#24-27). Prior to flow cytometry, fractions were mixed with a reference latex bead concentration at a known concentration. The same number of latex bead events were collected for all fractions and used to quantitate the number of plasma membrane and phagosome particles as described in experimental procedures (D).

antibody to HLA-II prior to homogenization, and presence of plasma membrane in each fraction was assessed by flow cytometry. Phagosomal fractions contained a significant amount of $PE^+$ plasma membranes, with very little associated with phagosomes (Figure 6C). The number of plasma membrane particles was quantified using a reference bead population (see materials and methods). Consistent with data obtained in Figure 3A, the number of plasma membrane particles were strongly associated with the plasma membrane peak, and decreased in every fraction thereafter (Figure 6D). These data, then, suggest that the IFN-γ response in the phagosomal fractions cannot be attributed to gross plasma membrane contamination, a conclusion strengthened by the relative enrichment of HLA-E specific activity.

**Discussion:**

Here, we find that presentation of both the HLA-E antigen and CFP10 require proteasomal degradation and TAP transport. Presentation of both antigens is blocked by the retrotranslocation inhibitor exoA. In contrast to presentation on HLA-B8, HLA-E presentation is only partially BFA-sensitive and is enhanced with cycloheximide treatment. We find the presence of HLA-I and ER-derived HLA-I loading accessory molecules PDI and TAP in the Mtb phagosome. Accordingly, we are able to detect loaded HLA-E:peptide complexes in the Mtb phagosome.

These data demonstrate two distinct cytosolic pathways for processing and presentation of Mtb antigens that in both cases are facilitated by retrotranslocation from the phagosome. After proteasomal degradation, both peptide fragments require TAP transport. $CFP10_{3\text{-}11}$ then follows a pathway that is characteristic of presentation of

endogenous proteins, being sensitive to BFA and cycloheximide. In contrast, the HLA-E antigen follows a different pathway, being incompletely blocked by BFA and enhanced by cycloheximide. This argues that newly synthesized HLA-E is not required for loading, and that once loaded, HLA-E:peptide complexes do not require transport through the ER-golgi to the cell surface.

In accordance with the inhibitor data, we detected HLA-E:peptide complexes, but few HLA-B44:$CFP10_{2-11}$ complexes within the Mtb phagosome. Presentation of latex bead delivered OVA through the ER-phagosomal pathway has been shown to be only partially BFA sensitive, consistent with our HLA-E data (124). As such, we are able to detect HLA-I and members of the HLA-I peptide loading complex within the Mtb phagosome. Ackerman et al. reported that the majority of phagosome-associated MHC-I is endoglycosidase H resistant (post-golgi (171)). This is consistent with our hypothesis that recycled HLA-E can be loaded in the phagosome.

ExoA blockade suggests that Mtb antigens require active transport out of the Mtb phagosome. The finding that exoA blocks protein synthesis and endogenous antigen processing of vaccinia-delivered antigens is conflicting with published data using the DC-like cell line, KG-1 (126). This discrepancy may be due to the use of a cell line versus ex vivo generated DC, as we saw only minor differences in vaccinia-eGFP expression and vaccinia-expressed antigen presentation in LCL treated with exoA (data not shown). The NAD analog PJ34 could be used to uncouple the protein synthesis and retrotranslocation inhibition functions of exoA. This substrate binds to the catalytic domain of exoA and prevents the ADP-ribosylation function (178). When primary DC were cotreated with exoA and PJ34, GFP expression and endogenous antigen

presentation were restored, while the inhibition of Mtb antigen presentation was unaffected. These data strongly argue that the exoA inhibition is due to retrotranslocation block, and that the retrotranslocation machinery plays a role in presentation of Mtb antigens.

While the ICP47 data demonstrates a role for TAP transport of both Mtb antigens, we cannot discern whether the blockade occurs at the ER or phagosomal membrane. Our detection of TAP and loaded HLA-E in the Mtb phagosome indirectly argues that phagosomal TAP is functional. Furthermore, as antigen presentation was only partially inhibited in the presence of ICP47 (about 50%), it is possible that some antigen is processed in a manner independent of ICP47. However, the efficiency of epoxomicin blockade demonstrates that all peptide generation occurs using cytosolic proteasomes, and suggests that the incomplete ICP47 inhibition is due to incomplete ablation of TAP function.

Our experiments have all been carried out using human DC. While DC are the likely subset to prime naïve $CD8^+$ T cells and have been shown to be infected in vivo (180), Mtb infects and resides in macrophages as well. Therefore, for infected cells in the lung to be recognized by HLA-E specific $CD8^+$ T cells, this pathway would also have to be operational within macrophages. While Mtb-infected macrophages are recognized by the HLA-E restricted clone, we have not evaluated the Mtb phagosome in these cells. However, ER-mediated phagocytosis was first described within macrophages (125). We are currently investigating whether HLA-E antigen processing follows the same pathway in macrophages.

Several reports have not detected ER-mediated phagocytosis or the presence of

ER proteins within latex bead or mycobacterial phagosomes (27, 127). While we have not assessed the role of ER-mediated phagocytosis of Mtb, we are able to detect ER-associated proteins and peptide loading in the phagosome. Thus, our data agrees with others examining cross-presentation of latex bead associated antigen (123, 124, 126).

Although it is likely that there is some contamination of phagosomal preparations, several results demonstrate that the phenotypic and functional presence of ER-resident proteins cannot be attributed to contamination. First, we do not see the presence of all ER proteins examined, suggesting that there is either selective delivery or selective retention of certain proteins. Second, we find no presence of cis- or trans-golgi proteins, which should not have access to the Mtb phagosome. Third, the maturation phenotype seen by flow cytometry is in agreement with published reports using other methods, including the use of intact cells (7). Finally, we were unable to detect contaminating HLA-A2$^{+}$ membranes on Mtb phagosomes after mixing HLA-A2$^{-}$ infected DC with uninfected, HLA-A2$^{+}$ DC. Our findings strongly indicate that the Mtb-phagosome selectively contains ER-resident proteins.

A recent report has shown that Mtb is able to escape the phagosome after 48-96 hours of infection (27), a process which requires the Region of Difference 1 (RD1, the only region that is absent from all strains of BCG, but present in all Mtb strains, (181)). This would give Mtb antigens access to the cytosol where they would be processed as endogenously expressed antigens. The authors hypothesized that this may increase antigen presentation. We have examined Mtb-infected DC at less than 18 hours post infection, a time when all Mtb should be present within the phagosome. Furthermore, we and others have shown that RD1 is not required for presentation of Mtb antigens on Class

I molecules (69, 120). This excludes phagosomal escape as a confounding factor in our interpretations.

Our findings of divergent processing pathways during Mtb infection raise the question of how these pathways may be accessed. One hypothesis is that specific HLA-I alleles influence the pathway chosen. We have examined the processing and presentation pathways for 10 different epitopes from 6 Mtb antigens presented by 8 different HLA-I alleles to date and have yet to find another antigen that is only partially inhibited by BFA (manuscript in preparation). This suggests that HLA-E may play a specific role in surveillance of phagosomal antigens. This hypothesis is currently being intensely investigated by our lab.

Besides the possibility that HLA-I alleles may influence the processing pathway of Mtb antigens, it is also likely that the antigens themselves play a role. CFP10 is a secreted protein encoded in RD1, a region which has been shown to be required for Mtb virulence (182-185). Alternately, the HLA-E antigen is detected in the Mtb cell wall fraction, with little to no detection in the Mtb culture filtrate (secreted proteins (81)). This leads to the hypothesis that nonsecreted, and perhaps cell-wall associated proteins may preferentially be processed through the ER-phagosomal pathway, while secreted proteins reach the cytosol more efficiently and at higher concentrations. All of the HLA-Ia restricted Mtb antigens we have examined are either secreted or putative secreted proteins, consistent with this hypothesis.

Once antigen is loaded onto HLA-I within the phagosome, the complex must then be transported to the cell surface. To our knowledge, no specific pathway has been identified that shows trafficking from the phagosome to the cell surface. Furthermore, it

is possible that this pathway intersects with other compartments before transport to the cell surface, making identifying a candidate pathway difficult. There is actually very little information on trafficking pathways to and from the phagosome. These pathways likely play an important role in the response to phagosomal pathogens.

In many of these experiments, we examined CFP10 processing and presentation on HLA-II molecules to control for non-specific inhibitor effects. HLA-II presentation can occur using either a nascent HLA-II or recycling pathway (186). BFA had little effect on CFP10 (both soluble and Mtb-derived) HLA-II presentation, similar to that seen in the HLA-II recycling pathway (187-189). Similar to this finding, cycloheximide treatment had no effect on CFP10 presentation. Together, these data argue that CFP10 is processed through the HLA-II recycling, rather than nascent pathway. Unexpectedly, Mtb-derived CFP10 presentation was unaffected by bafilomycin treatment. However, previous reports have demonstrated that MHC-II presented Mtb antigens do not require acidification for presentation (190). Also, there is a report of optimal epitope binding from a secreted Mtb protein on HLA-DR17 at neutral pH (191). Finally, DC have a specialized mechanism to increase the phagosomal pH and prolong antigen half-life (25, 26). These data combined with the Mtb maturation block provide an explanation for the lack of bafilomycin inhibition of HLA-II CFP10 presentation.

In conclusion, we have examined in detail the cross-presentation of two Mtb-derived antigens. We find that both antigens access the cytosolic proteasome and TAP transporter by retrotranslocation out of the phagosome. After proteasomal degradation, these two antigens take divergent pathways. CFP10 mostly follows the traditional HLA-I presentation pathway, while the HLA-E antigen follows the ER-phagosomal pathway.

These data suggest a potentially unique role for HLA-E in surveillance and presentation of phagosomal antigens.

***Experimental Procedures:***

**Reagents and antibodies**

Inhibitors of antigen processing were obtained from EMD Biosciences (epoxomicin, bafilomycin, and PJ34), Sigma (cycloheximide and *Pseudomonas aeruginosa* exotoxin A), and Invitrogen (brefeldin A).

The following antibodies were used for flow cytometry: TAP1, TAP2, EEA-1, rab5, GM130, HLA-A2, LAMP-1-PE (BD Biosciences), PDI, KDEL, calreticulin (Stressgen), HLA-I-FITC or unconjugated (B9.12.1, Coulter), TfR (Biosource), calnexin (Affinity Bioreagents), golgin-97 and goat anti-mouse IgG Alexa Fluor-647 (Invitrogen).

Soluble CFP10 protein was provided by Corixa Corp. Peptides were synthesized by Genemed Synthesis Inc.

Magnetic particles (2.8μm tosylactivated beads, Dynal) were coupled to BSA according to the manufacturer's protocol before addition to DC.

**Bacteria, virus, and cells**

H37Rv-eGFP was provided by Dr. Joel Ernst (New York University, New York, NY) and was grown in Middlebrook 7H9 broth supplemented with Middlebrook ADC (Fisher), 0.05% Tween-80, 0.5% glycerol, and kanamycin (50μg/ml). Before infection, bacteria were sonicated for 20 seconds, passaged 15 times through a tuberculin syringe, and sonicated again to obtain a single cell suspension. Multiplicity of infection varied

depending on the experiment.

Adenovirus-ICP47 (174) and other adenoviral vectors were provided by Dr. David Johnson (OHSU). Vaccinia virus expressing HIV p24 was provided by Therion Biologic Corp. and vaccinia virus expressing eGFP was provided by Corixa Corp.

PMBC were obtained from normal donors via leukapheresis and processed as previously described (81). DC were generated by culturing adherent PBMC for 5 days in the presence of GM-CSF (10ng/ml, Amgen) and IL-4 (10ng/ml, R&D systems) in RPMI (Gibco) supplemented with 10% pooled human serum (HS), L-glutamine (4mM, Gibco), and gentamicin (50μg/ml, Invitrogen).

**T Cell Expansion**

Clones D160 1-1B, D160 1-23, and D480 F6 have been previously described (81, 172, 192). D454 E12 is a HLA-II restricted clone that responds to CFP10 (unpublished). Clone 16A7 is a HLA-B44 restricted $CD8^+$ T cell clone that responds to amino acids 306-316 (AEQASQEVKNW) of HIV p24 and was provided by Dr. Stan Riddell (Fred Hutchinson Cancer Research Center, Seattle, WA). T cell clones were expanded as previously described (120), except that some of the expansions were done in Stemline T cell expansion media (Sigma) supplemented with 1% FBS (Hyclone) and 4mM L-glutamine.

**ELISPOT Assay**

96 well nitrocellulose-backed plates (Millipore) were coated with 10 μg/ml mouse anti-IFN-γ (1-D1K; Mabtech) overnight at 4°C. Plates were washed with PBS (Gibco)

and blocked with RPMI/10% HS at room temperature. DC, antigens, and T cells were added according to individual experiments and incubated 18-48 hours at 37°C. After washing with PBS/0.05% Tween-20, 1 μg/ml biotinylated anti-IFN-γ mAb (7B6-1; Mabtech) was added. After 2 hours of incubation at room temperature, plates were washed and avidin/biotinylated enzyme (HRP) complex (Vectastain ABC Elite Kit; Vector) was added, and plates were incubated for an additional hour. Plates were washed and 3-amino-9-ethylcarbazole substrate (Vectastain AEC substrate kit; Vector) was added. After 4–10 min, the colorimetric reaction was stopped by washing with distilled water. Spots were quantified using an AID ELISPOT plate reader (Cell Technology, Inc.).

**Inhibition of Antigen Presentation**

Day 5 DC were plated in a 24 well ultra low adherence (ULA) plates (Costar) at $5x10^5$/well in RPMI/10% HS supplemented with GM-CSF and IL-4. DC were pretreated with inhibitors (1μM epoxomicin, 0.1μg/ml BFA, 0.2μM bafilomycin, 10μg/ml cycloheximide, 10μg/ml exoA, or 10μg/ml exoA with 200 fold molar excess PJ34) for one hour before infection with H37Rv-eGFP (MOI=20), vaccinia virus (MOI=2), or addition of antigen (0.5-1μg/ml CFP10, 1μg/ml $CFP10_{3-11}$). In experiments using exoA/PJ34 or BSA/PJ34, these compounds were co-incubated at room temperature for 30' before addition to DC.

DC were harvested after 15-16 hours of infection, pelleted and fixed with 0.5% paraformaldehyde for 15 minutes. The reaction was stopped with an equal volume of 0.4M Lysine or RPMI/10% HS and DC were washed extensively with RPMI/10% HS.

Fixed DC were then added to an IFN-γ ELISPOT plate at varying quantities so that antigen was the limiting factor (25,000 Mtb-infected DC/well for $CD8^+$ clones, 1,000 Mtb-infected DC/well for $CD4^+$ clones, and 2,000 Ag-pulsed DC/well for both $CD4^+$ and $CD8^+$ clones). T cells clones were added at 10,000/well and plates were incubated at 37°C for 18 hours before development.

**ICP47-mediated TAP Inhibition**

DC ($1x10^5$) were added to wells of a 96 well flat bottom plate in Opti-Mem serum-free media (Gibco). Adenovirus particles or media were incubated with lipofectamine 2000 (Invitrogen) for 25 minutes at room temperature. Liposome/adenovirus was added to DC (MOI=100, 0.5μl/well lipofectamine 2000) and incubated for 2-4 hours at 37°C. RPMI/10% HS supplemented with GM-CSF and IL-4 was added to wells and incubated for another 6-26 hours. Media was removed and replaced with RPMI/10% HS containing H37Rv-eGFP (MOI=15-20 for $CD8^+$ T cell clones, 0.2-20 for $CD4^+$ clones) or antigen (0.25μg/ml $CFP10_{3-11}$, 0.005μg/ml CFP10) and incubated overnight at 37°C. After 15-16 hours, media was removed and T clones were added ($1.5x10^5$/well) in the presence of BFA (10μg/ml). After a six hour stimulation, T cell clones were harvested, fixed with 1% paraformaldehyde, and stained with antibodies to CD3 (UCHT1, BD Biosciences) and IFN-γ (Coulter) in the presence of 0.2% saponin (Sigma). Cells were analyzed using a FACSCalibur or LSR II (Becton Dickinson).

**Flow organellometry**

DC were added to a 6 well ULA plate ($2.5x10^6$/well) in RPMI/10% HS supplemented with GM-CSF and IL-4 and allowed to adhere. H37Rv-eGFP (MOI=5-7.5) or magnetic beads (1-10/cell) were added to wells and centrifuged onto DC at 22°C for 5 min. DC were then incubated for an additional 15 min at 37°C for a total pulse time of 20 min. DC were placed on ice and washed with cold RPMI and incubated for an additional 40 min or overnight at 37°C. DC were washed on ice with cold RPMI and harvested. DC were resuspended in ice cold homogenization buffer (0.25M sucrose, 10mM Hepes, pH=7.4) containing protease inhibitors (Complete Mini protease inhibitor cocktail tablet, Roche) and homogenized by passaging through a 23 gauge needle. At this point, magnetic beads were purified using a particle concentrator (Dynal). For Mtb phagosomes, intact cells and nuclei were removed by two spins at 200xg, 4°C. Postnuclear supernatant was layered onto 27% percoll (Amersham) and centrifuged for one hour at 4°C, 36,000xg in a 70.1Ti rotor (Beckman).

The phagosome-containing fractions (final 2ml of gradient) were fixed with 1% paraformaldehyde on ice for 15 minutes, washed, and stained with primary antibodies (5μg/ml) for 40 min on ice in the presence of 0.2% saponin, 2% HS, 2% goat serum, and 0.5% FBS. After washing, goat anti-mouse-IgG-Alexa Fluor-647 was added (1:1000) and incubated for 40 min. Phagosomes were then washed twice and stained with anti-LAMP-1-PE and anti-HLA-I-FITC for 40 min. Phagosomes were washed and analyzed using a FACSCalibur or LSR II.

We analyzed the percentage of positive phagosomes using several different methods. The Overton histogram subtraction method in Flowjo software (Treestar) was used to compare the isotype- and experimentally-stained populations. The values

obtained from this analysis are shown in Figures 3 and 4. We also analyzed each experiment by setting the isotype gate so that 5% of the events are positive, and then applied this gate to stained samples. After subtracting the isotype value, these numbers gave almost identical results as that seen using Overton analysis (data not shown).

**Subcellular Fractionation**

DC were plated in 6 well ULA plates as above. H37Rv-eGFP (MOI=7.5) was added to wells and centrifuged onto DC for 5 min at 22°C. DC were incubated for an additional hour or overnight, without washing. After infection, DC were placed on ice, washed with cold RPMI, and harvested. Before homogenization, DC were labeled with a PE-conjugated antibody to HLA-II (Coulter) to track plasma membrane. Excess antibody was removed by centrifugation, cells were resuspended in homogenization buffer, homogenized and separated on a percoll gradient as above. The percoll gradient was manually fractionated into 28 fractions (332μl each).

Lysosomes were detected using an enzymatic assay to detect β-hexosaminidase activity as previously described (179). ER containing fractions were identified using western blot for PDI and TAP1 (Stressgen). For plasma membrane and phagosome detection, fractions were fixed with paraformaldehyde. Plasma membrane was detected using a fluorometer with an excitation filter of 530+/-12.5nm and emission filter of 590+/-17.5nm. Phagosomes were not detectable using a fluorometer due to the high autofluorescence of percoll. Phagosomes were detected using a flow-based assay, where fixed fractions were mixed with a reference population of 2μm latex beads (Polysciences) at a known concentration and analyzed using a FACSCalibur or LSR II. Phagosomes

were detected based on GFP fluorescence and quantified using the ratio of $GFP^+$ events to latex beads. Plasma membrane was also quantified using this assay and gave similar results to fluorometer data. Quantitation of organelles was done using the following equation:

ratio of $PE^+$ or $GFP^+$ to latex beads * #beads/ml * dilution factor * fraction volume

For detection of loaded HLA-I:peptide complexes, fractions were frozen overnight at -80°C and thawed. 20-45µl fractions were added to duplicate wells of an ELISPOT plate containing 20,000 T cell clones and incubated for 18-48 hours at 37°C before development.

***Acknowledgements:***

I would like to acknowledge Anne Chamberlin (OHSU) for her role in this project. Anne performed many of the flow organellometry experiments in figures 3-5. I would also like to thank Dr. Clifford Harding and Dr. Lakschmi Ramachandra (Case Western Reserve University, Cleveland, OH) for their technical assistance with flow organellometry and subcellular fractionation. Dawn Nolt (OHSU) has tirelessly attempted to examine the Mtb phagosome in DC using immunofluorescence. Finally, I would like to thank Joel Ernst (New York University, New York, NY) for providing eGFP expressing H37Rv, David Johnson (OHSU) for the provision of adenoviral vectors, and Stan Riddell (Fred Hutchinson Cancer Research Center, Seattle, WA) for providing the HIV p24-specific $CD8^+$ T cell clone.

# Chapter 3

# *Mycobacterium tuberculosis* antigens preferentially utilize the cytosolic pathway for cross-presentation

Jeff E. Grotzke, Anne M. Chamberlin, and David M. Lewinsohn

***Summary:***

We have previously characterized the presentation of two HLA-I epitopes from *Mycobacterium tuberculosis* (Mtb) and shown that these antigens are processed using the cytosolic pathway. To discern the dominant pathway of Mtb antigen cross-presentation, we analyzed the presentation of an additional seven epitopes from five Mtb proteins presented by 6 different HLA-I alleles. Presentation of all epitopes was blocked in the presence of the ER-golgi trafficking inhibitor, brefeldin A. However, presentation of four of these epitopes was enhanced by blocking proteasomal degradation. To resolve this apparent discrepancy, we examined the role of the transporter associated with antigen processing (TAP), and found that TAP transport is required for presentation. Furthermore, presentation was not inhibited in the presence of cathepsin or acidification blockers. Together, these data show that Mtb antigens are processed and presented using a cytosolic pathway and suggest that a potentially novel cytosolic protease or proteases may be involved in epitope generation.

***Introduction:***

Tuberculosis (TB) disease remains a global health concern due to the large global burden of Mtb-infected individuals, the emergence of multidrug- and extensively drug-resistant Mtb strains, and the impact of Mtb infection on HIV infection. Control of Mtb infection requires the development of an adaptive $CD4^+$ and $CD8^+$ T cell immune response and the subsequent production of pro-inflammatory cytokines. Because of the importance of cell-mediated immunity for control of Mtb infection, further understanding of the factors required for the generation of this response are important for development of an effective Mtb vaccine.

Mtb is able to modulate the phagosomal environment after internalization, characterized by incomplete phagosomal acidification, blockade of rab conversion to $rab7^+$ late endosomes, lack of phagolysosomal biogenesis, and continued access to extracellular nutrients (such as iron) through the continued fusion of early endosomes. Although Mtb is able to evade innate immune mechanisms within phagocytic cells, this does not mean that Mtb 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 antigens.

On the other hand, because the phagosome is walled off from the cytosolic and ER-derived HLA-I processing and loading machinery, how Mtb antigens are processed and presented on HLA-I molecules is much less understood. Studies using bead phagosomes have shown that multiple pathways are functional for antigen cross-presentation. In these pathways, antigens can either escape the phagosome to the cytosol where they are degraded by the proteasome (cytosolic pathway), or they can remain

within the endocytic pathway where they are degraded (vacuolar or non-cytosolic pathway). In vivo data has suggested that the major pathway of cross-presentation and cross-priming is TAP- and immunoproteasome-dependent (134, 139-143, 193), 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 antigens have been demonstrated to occur in the absence of TAP, and rely on processing by cathepsin S (134). Furthermore, OVA expressed by *Leishmania major* has been shown to cross-presented using the vacuolar pathway in vivo (144).

The pathways by which Mtb antigens are cross-presented are only beginning to be understood. We have shown that Mtb antigens can be retrotranslocated out of the phagosome, giving them access to the cytosol (Chapter 2). Furthermore, we have found that Mtb proteins that require retrotranslocation for antigen presentation also require proteasomal degradation and TAP transport, showing the use of the cytosolic processing and presentation pathway (120). Conversely, there is support for endocytic processing of Mtb-derived antigens and apoptotic bodies. Presentation of the 19kDa lipoprotein did not require TAP transport, but did require trafficking outside of the mycobacterial phagosome (138). One striking feature of mycobacterial-infected cells is the large amounts of vesicular trafficking of bacterial-derived proteins and lipids seen within the cell (135-137). This finding suggests that Mtb proteins may have unique access to the endocytic pathway, as well as access to bystander cells. Along those lines, Schaible, et. al. showed that apoptotic bodies from mycobacterial-infected cells can be taken up by uninfected bystander cells, and process antigen in an acidification-dependent, but proteasome-independent manner (121). In all, these findings present a diverse picture of

antigen processing and presentation pathways in Mtb-infected cells.

In this report, we seek to provide a more broad understanding of which pathway(s) are required for HLA-I presentation of seven newly defined epitopes. We screened antigen presentation in Mtb-infected cells using inhibitors which distinguish cytosolic from non-cytosolic pathways. We find that while presentation of all seven of these epitopes use the cytosolic pathway, four epitopes were processed in a proteasome-independent manner raising the possibility of an alternate cytosolic proteolytic activity involved in HLA-I epitope generation.

***Results:***

**Cross-presentation of Mtb antigens requires ER-golgi transport but not acidification**

Using $CD8^+$ T cells cloned from individuals with latent or active TB, our lab has identified novel Mtb-derived HLA-I epitopes. This method includes cloning $CD8^+$ T cells on Mtb-infected DC, determination of the antigen recognized using overlapping peptide pools, and subsequent determination of the minimal epitope and restricting allele (128, 172, 192). Most of the $CD8^+$ T cell clones used in this paper have been generated using this method 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 Mtb antigens, and generally, responses to these epitopes comprise a large proportion of the Mtb-specific $CD8^+$ response (Table I (172)).

We utilized these $CD8^+$ T cell clones to monitor the requirements of processing and presentation of the specific epitopes recognized by each clone using inhibitors that

Table I: Summary of $CD8^+$ T cell clones used in this report

| Clone | Antigen Recognized | Minimal Epitope | Restricting Allele | Ex Vivo Frequency[a] | $CD8^+$ Mtb Frequency[b] | Ref. |
|---|---|---|---|---|---|---|
| D160 1-23 | ND[c] | ND | HLA-E | ND | 1/4,098 | (81, 128) |
| D160 1-1B | CFP10 | 2-11 AEMKTDAATL | HLA-B44 | 1/694 | 1/4,098 | (192) |
| D480 F6 | CFP10 | 3-11 EMKTDAATL | HLA-B0801 | 1/646 | 1/418 | (172) |
| D466 H4 | CFP10 | 2-9 AEMKTDAA | HLA-B4501 | 1/102 | 1/307 | (172) |
| D466 D6 | CFP10 | 2-12 AEMKTDAATLA | HLA-B4501 | 1/125[d] | 1/307 | (172) |
| D481 C10 | CFP10 | 75-83 NIRQAGVQY | HLA-B1502 | 1/146 | 1/617 | (172) |
| D504 B10 | EsxJ | 24-34 QTVEDEARRMW | HLA-B5701 | 1/55 | 1/1,984 | UN[e] |
| D454 H1-2 | DPV | 33-43 AVINTTCNYGQ | HLA-B1501 | 1/10,417 | 1/1,811 | (172) |
| D443 H9 | Ag85B | 144-153 ELPQWLSANR | HLA-B4102 | <1/25,000 | 1/4,716 | (172) |
| D131 10-E12 | TbH9 | 144-153 MWAQDAAAMF | HLA-B44 | ND | ND | (194) |
| D454 E12[f] | CFP10 | ND | HLA-II | ND | | UN |

[a]Donor $CD8^+$ T cell frequency using the minimal epitope
[b]Donor $CD8^+$ T cell frequency using Mtb-infected DC
[c]Not determined
[d]This frequency may be overestimated as this epitope is also weakly recognized by D466 H4
[e]Unpublished
[f]$CD4^+$ T cell clone used as a control

distinguish cytosolic and non-cytosolic processing pathways. To determine which cross-presentation pathway is used for the processing and presentation of Mtb antigens, we first screened antigen presentation in the presence of proteasome (epoxomicin), ER-golgi transport (BFA), and acidification (bafilomycin) blockers. In brief, DC were treated with inhibitors for one hour prior to infection with an H37Rv strain that expresses enhanced green fluorescent protein (eGFP). After 15-16 hours of infection in the presence of inhibitors, DC were harvested, fixed, washed extensively, and used as stimulators in an overnight IFN-γ ELISPOT assay. Using eGFP fluorescence, we were unable to detect significant differences in the percentage of cells infected or the level of infection after treatment with these inhibitors (data not shown).

As shown in figure 1, presentation of all of the epitopes examined was markedly reduced in the presence of BFA. This suggests a requirement for ER-golgi trafficking, a finding consistent with the cytosolic cross-presentation pathway (117). In agreement, treatment of DC with the vacuolar ATPase inhibitor, bafilomycin, did not significantly inhibit presentation of any of the epitopes tested. As vacuolar processing of Class I presented antigens generally requires acidification (121, 131), the lack of inhibition by bafilomycin further supports the notion that these antigens are processed using the cytosolic pathway.

To control for drug specificity and toxicity, we performed several controls. First, BFA or bafilomycin did not inhibit the presentation of peptides by DC (Figure 1B), showing that the HLA-I presentation pathway is still intact. Second, there was no inhibition of Mtb-derived CFP10 presentation on HLA-II by BFA (Chapter 2, Figure 1). HLA-II presentation can occur using either a nascent or recycling pathway. A lack of

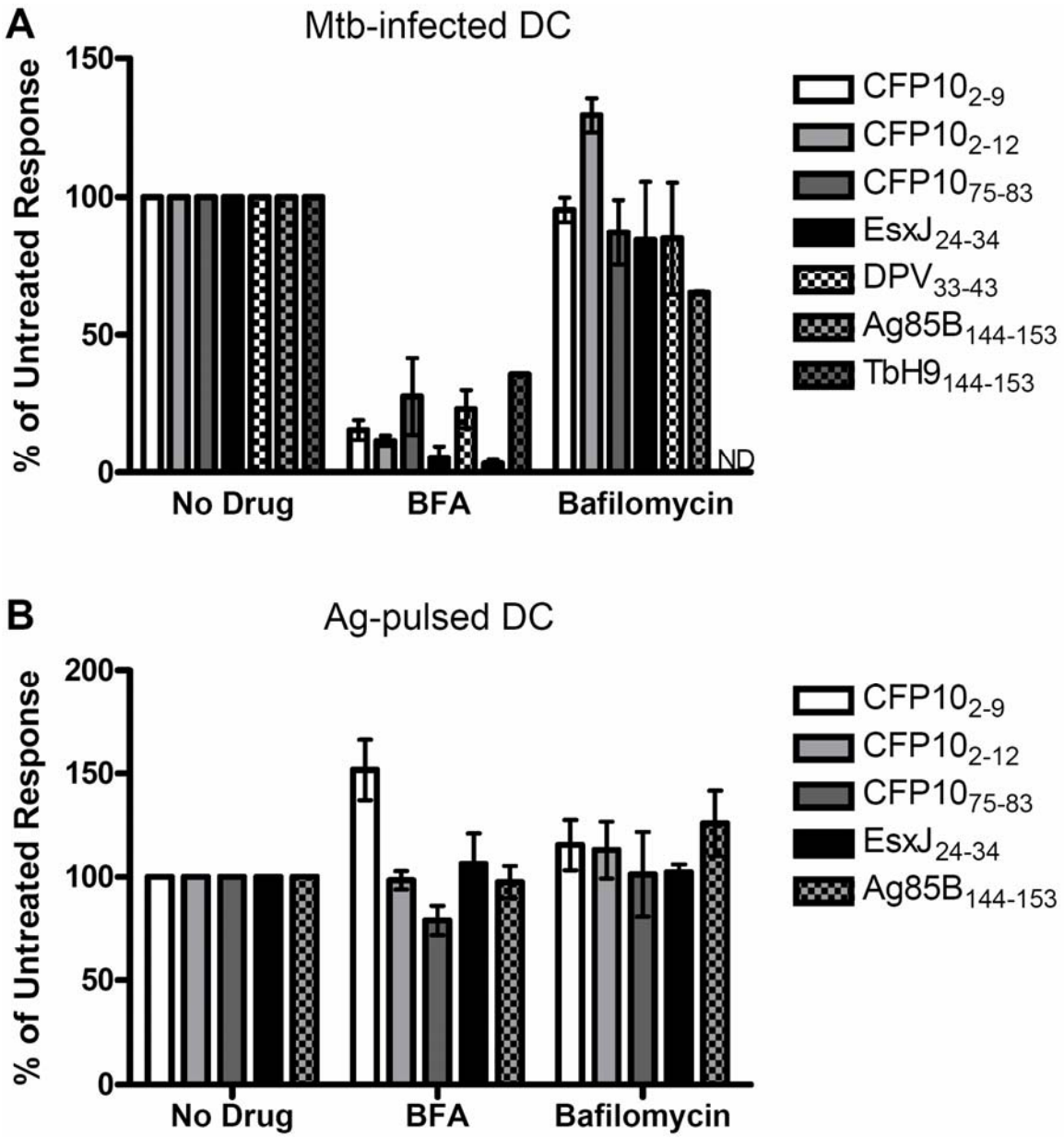


Figure 1. Cross-presentation of Mtb antigens requires ER-golgi transport, but not acidification. DC were treated with BFA (0.1μg/ml) or bafilomycin (0.2μM) for one hour before infection with H37Rv-eGFP (MOI=20, A) or addition of indicated peptides (1μg/ml). After 15-16 hours of infection, DC were harvested, fixed, washed, and added as stimulators in an IFN-γ ELISPOT assay. T cells were added in excess (10,000/well) while limiting numbers of DC were added so that antigen is the limiting factor (25,000 Mtb-infected DC/well, 2,000 Ag-pulsed DC/well). Data has been normalized to the untreated control and each bar represents the mean +/-SEM from 2-4 experiments.

BFA effect on presentation of CFP10 is consistent to that seen with the recycling pathway (187-189).

**Disparate proteasome requirement of Mtb antigens**

We next examined the role of the proteasome in the generation of Mtb-derived HLA-I epitopes. We have previously reported that three Mtb epitopes require proteasomal degradation ($CFP10_{2-11}$ (120), $CFP10_{3-11}$(Chapter 2) and the HLA-E antigen (Chapter 2, (128)). Unexpectedly, treatment of DC with the specific proteasomal blocker epoxomicin showed that this inhibitor could either inhibit or enhance presentation of the seven examined epitopes (Figure 2). While presentation of $CFP10_{2-9}$, $Ag85B_{144-153}$, and $TbH9_{144-153}$ was dramatically reduced in the presence of epoxomicin, presentation of $CFP10_{2-12}$, $CFP10_{75-83}$, $EsxJ_{24-34}$, and $DPV_{33-43}$ was markedly enhanced. Epoxomicin did not inhibit peptide presentation to $CD8^+$ T cell clones (Figure 2B), nor did it inhibit presentation of Mtb-derived or soluble CFP10 to the $CD4^+$ T cell clone, excluding any toxic effects of proteasome inhibition (Chapter 2).

It has been previously shown that proteasome inhibitor concentrations can have opposite effects on epitope generation. Schwarz, et. al. reported that low dose epoxomicin and lactacystin can enhance antigen presentation while higher doses inhibit it (195). To determine if the enhancement seen with epoxomicin treatment was due to incomplete proteasome block, we treated DC with increasing concentrations of epoxomicin. Presentation of $CFP10_{2-12}$ was not inhibited by epoxomicin treatment at concentrations up to 10μM (Figure 2C). This argues against a role of proteasomal degradation for presentation of these epitopes.

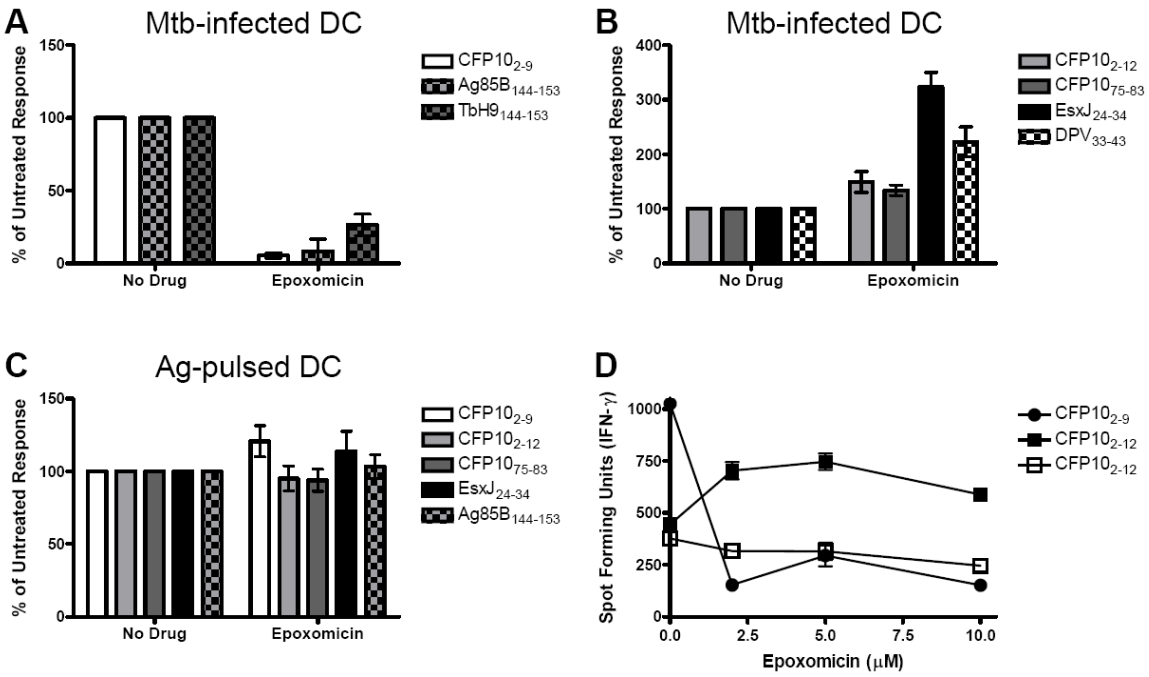


Figure 2. Differential effect of proteasome inhibition on presentation of Mtb antigens. (A-C) DC were treated with epoxomicin (1μM) for one hour prior to infection with H37Rv-eGFP (MOI=20, A-B) or addition of indicated peptides (1μg/ml, C). (D) DC were pretreated with 2-10μM epoxomicin before addition of H37Rv-eGFP (closed symbols) or 1μg/ml $CFP10_{2-12}$ peptide (open symbols). After 15-16 hours of infection, DC were added as stimulators in an IFN-γ ELISPOT assay as described. Numbers in A-C (mean +/- SEM) indicate pooled data from 2-4 experiments. Numbers in D are the mean +/- SEM of triplicate wells from one experiment.

**Presentation of proteasome-independent epitopes does not require cathepsins or Tripeptidyl Peptidase II**

Data presented to this point are inconclusive as to whether presentation of proteasome-independent epitopes are processed using the cytosolic pathway. Because BFA could potentially be interfering with a process other than ER-golgi egress of loaded HLA-I:peptide complexes to the cell surface, we tested whether other components involved in the vacuolar pathway participate in generation of these epitopes.

Shen et. al. reported that cathepsin S plays a large role in the generation of SIINFEKL when associated with PLG microspheres (134). In fact, incubation of OVA with purified cathepsin S led to the generation of the SIINFEKL epitope. In a human model of cross-presentation, Fonteneau, et. al., showed that preprocessing of cell-associated antigen required acidification and cathepsin D proteolysis before cytosolic access (196). Therefore, we tested the roles of cathepsins using the inhibitors leupeptin and pepstatin A, and retested the role of acidification using an alternate inhibitor of acidification, chloroquine.

Leupeptin is a fairly broad cysteine- and serine- protease inhibitor that acts upon cathepsins B, H, L, and S. Pepstatin A inhibits aspartic proteases such as cathepsin D (197, 198). As shown in figure 3, these inhibitors did not block presentation of $EsxJ_{24-34}$ or $CFP10_{75-83}$, and had little effect on peptide presentation. Furthermore presentation of neither of these epitopes was inhibited in the presence of chloroquine, further arguing against a role of endocytic processing of these epitopes.

We next tried to identify the cytosolic protease responsible for epitope generation. Generally, the carboxy-terminus of epitopes is generated by the proteasome (83).

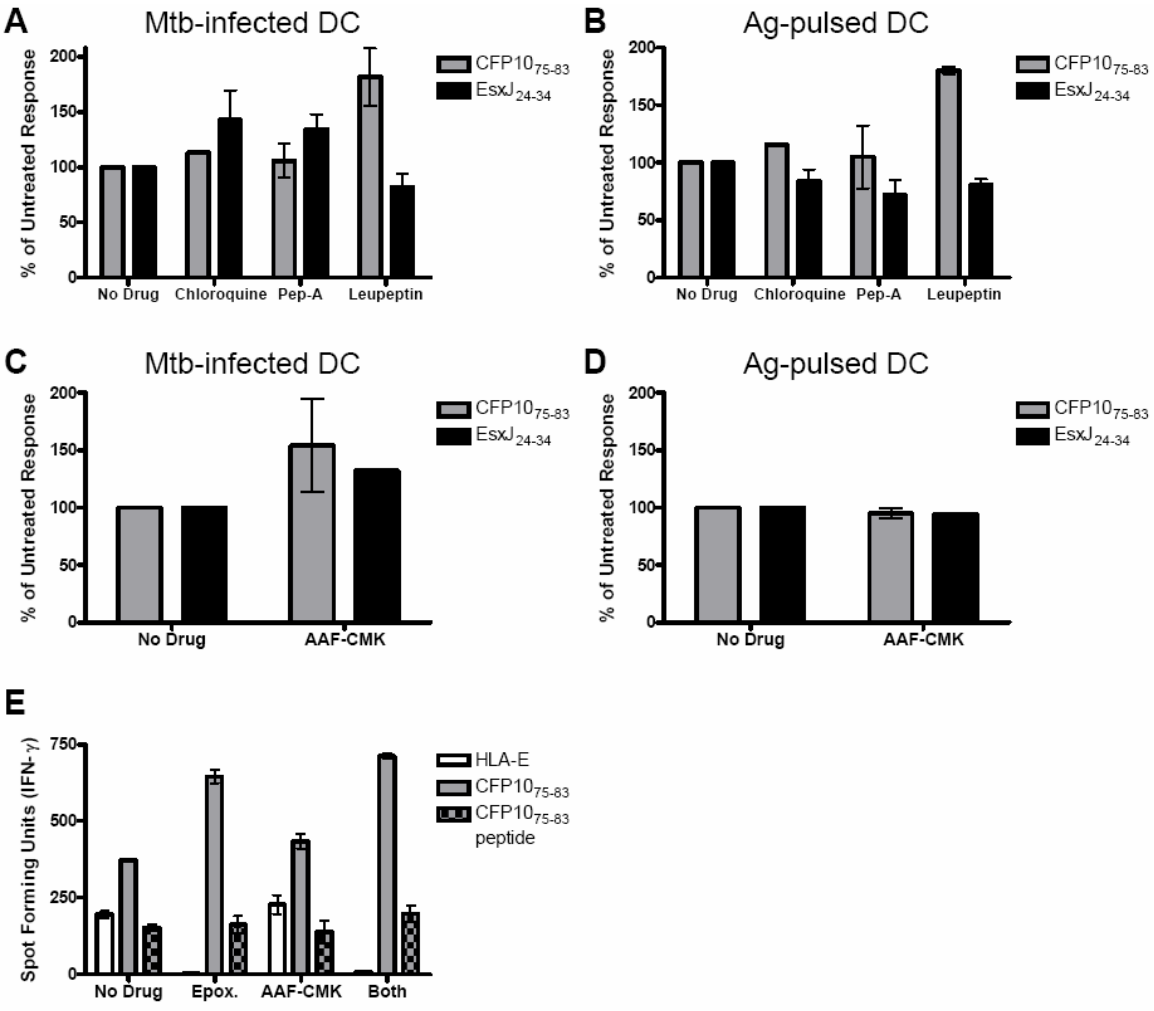


Figure 3. Proteasome-independent epitopes are not generated by cathepsins or tripeptidyl peptidase II. DC were treated with chloroquine (100μM), pepstatin A (150μM), leupeptin (100μg/ml), AAF-CMK (2μM) or 1μM epoxomicin/2μM AAF-CMK for one hour prior to infection with H37Rv-eGFP (MOI=20, A, C, & E) or addition of indicated peptides (1μg/ml, C, D, & E). After 15-16 hours of infection, DC were added as stimulators in an IFN-γ ELISPOT assay as described. Numbers (mean +/- SEM) indicate pooled data from 2-3 experiments for A&B, 1-2 experiments for C&D, or triplicate wells from 1 experiment in E.

Amino-terminal extensions can then be trimmed in the cytosol or ER by aminopeptidases (86). Other than the proteasome, the only other cytosolic protease described to date that is able to provide epitopes for MHC-I presentation is tripeptidyl peptidase II (TPPII). This protease is a huge, 4MDa complex that has both exo- and endo-peptidase activities (199). Mainly, TPPII removes three amino acid residues from the amino terminus at a time, and is involved in cellular protein turnover. However, Seifert, et. al. showed that presentation of an HIV nef epitope was enhanced by blocking proteasome degradation and instead requires TPPII endoprotease activity (200).

We tested the role of TPPII processing by using the inhibitor AAF-CMK. This blocker had no effect on the presentation of $EsxJ_{24-34}$ or $CFP10_{75-83}$, demonstrating that this protease does not play a significant role in the generation of these epitopes (Figure 3C). We hypothesized that there may be a dual requirement of the proteasome and TPPII for epitope generation. For example, although proteasome inhibition enhances antigen presentation, partial proteasome proteolysis may be required to generate the substrate for TPPII. Therefore, we cotreated cells with epoxomicin and AAF-CMK to block both activities. Presentation of $CFP10_{75-83}$ was unaffected by the presence of both inhibitors (Figure 3E), strongly arguing against a role of TPPII in epitope generation. Together, these data suggest that proteolysis of the four proteasome-independent epitopes is not dependent on cathepsins or TPPII, and may be achieved by a novel protease.

**Presentation of proteasome-independent epitopes requires TAP transport and newly synthesized HLA-I**

To demonstrate cytosolic processing of epitopes that do not require proteasomal

degradation, we examined the role of TAP transport. We have previously shown that TAP is required for the presentation of $CFP10_{3-11}$ and the HLA-E antigen using the Herpes Simplex Virus-1 encoded protein, ICP47. ICP47 binds to the cytosolic face of TAP and prevents peptide transport by preventing peptide binding to the TAP transporter. As reported in Chapter 2, when DC were transduced with an adenovirus vector expressing ICP47, the presentation of $CFP10_{3-11}$ was inhibited by approximately 50% (Figure 4A). A similar inhibition was seen for the presentation of $CFP10_{75-83}$, while presentation of $DPV_{33-43}$ showed an even greater inhibition. There was no effect of ICP47 expression on the presentation of peptide to $CD8^+$ T cell clones or Mtb-derived or soluble CFP10 to D454 E12 (Figure 4B). These data demonstrate that proteasome-independent peptides generated by the unidentified protease require transport from the cytosol for peptide loading.

In Chapter 2, we reported that the presentation of cytosolically processed CFP10 requires newly synthesized HLA-I, as presentation is blocked in the presence of cycloheximide. Alternately, we showed that presentation of the HLA-E antigen, which is loaded within the phagosome, does not require newly synthesized HLA-E. Also, the vacuolar pathway has been reported to be insensitive to cycloheximide treatment (132). Although not absolute, cycloheximide treatment represents an additional way of distinguishing between cytosolic or non-cytosolic antigen processing. Perhaps more technically correct is that it is able to distinguish the need for newly synthesized HLA-I, and that phagosome or endosomal loading does not require nascent HLA-I while the classical pathway and ER loading does.

We addressed the role of protein synthesis in the presentation of $CFP10_{75-83}$.

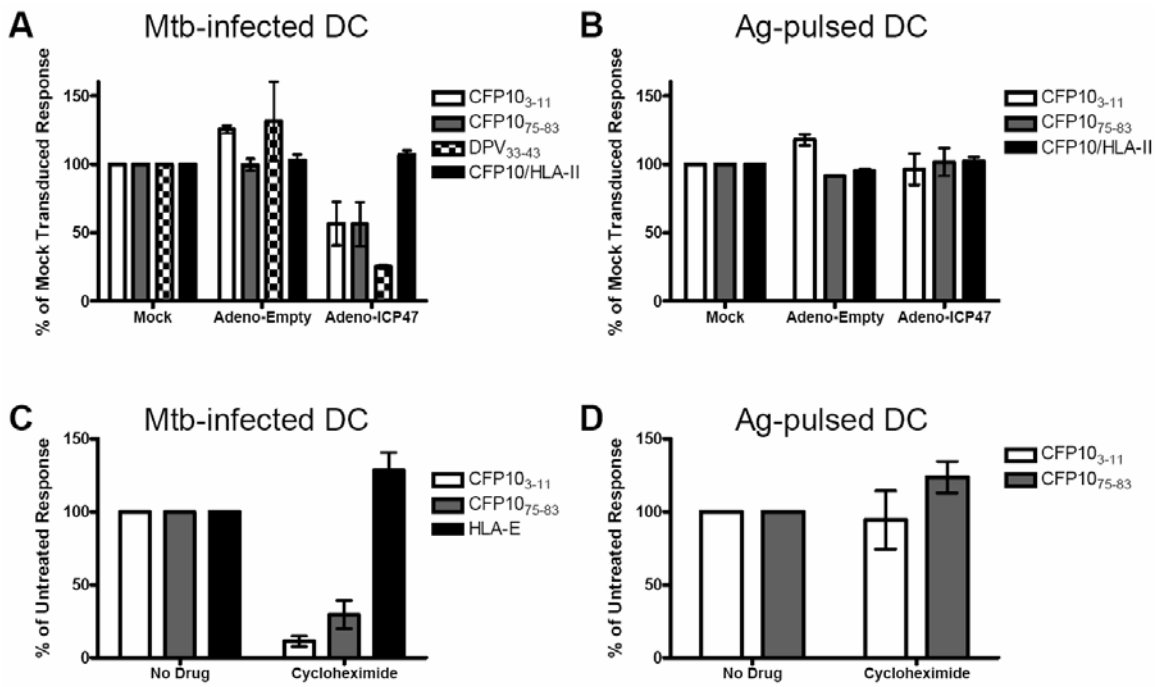


Figure 4. Proteasome-independent epitopes require TAP transport and newly synthesized HLA-I. (A&B) DC were transduced with Lipofectamine 2000 (mock) or Lipofectamine 2000 and adenovirus vectors as described. Media was removed and replaced with media containing H37Rv-eGFP (MOI=15-20 for $CD8^+$ T cell clones, 0.2-20 for $CD4^+$ clone, A) or antigen (0.25 μg/ml peptides or 0.005 μg/ml CFP10, B). IFN-γ production was assessed after a six hour stimulation. Pooled data (mean +/- SEM) represent 2-4 independent experiments. (C&D) DC were treated with cycloheximide (10μg/ml) for one hour prior to infection with H37Rv-eGFP (MOI=20, C) or addition of indicated peptides (1μg/ml, D). After 15-16 hours of infection, DC were added as stimulators in an IFN-γ ELISPOT assay as described. Numbers (mean +/- SEM) indicate pooled data from 3-6 experiments.

Cycloheximide treatment of DC led to an enhanced presentation of the HLA-E antigen (Figure 4C), consistent with that shown above. Although not as complete as the block seen with $CFP10_{3-11}$ presentation (90%), $CFP10_{75-83}$ presentation was inhibited by 70%. Peptide presentation was not markedly affected by cycloheximide treatment (Figure 4D). These data suggest that newly synthesized HLA-B1502 is required for presentation of this epitope.

**Presentation of proteasome-independent epitopes requires retrotranslocation to the cytosol**

To further examine the requirements for presentation of proteasome-independent epitopes, we examined the requirement for retrotranslocation out of the phagosome. In addition to inhibiting protein synthesis, the exotoxin A (exoA) protein from *Pseudomonas aeruginosa* has been shown to block retrotranslocation of proteins from the ER (177). Ackerman, et. al. then showed that the normally ER-localized retrotranslocation machinery was present within the phagosome, and that cross-presentation of OVA required retrotranslocation. We have expanded on these findings in Chapter 2, showing that presentation of the HLA-E antigen and $CFP10_{3-11}$ require retrotranslocation out of the Mtb phagosome for cytosolic processing.

We monitored presentation of $DPV_{33-43}$ in the presence of PJ34 and exoA. PJ34 binds to the catalytic site of exoA with high affinity and blocks the ADP-ribosylation function of exoA, while retaining the retrotranslocation inhibition. Above, we show that cotreatment of DC with these compounds does not inhibit presentation of vaccinia-expressed antigen, which should not require retrotranslocation. Presentation of $DPV_{33-43}$

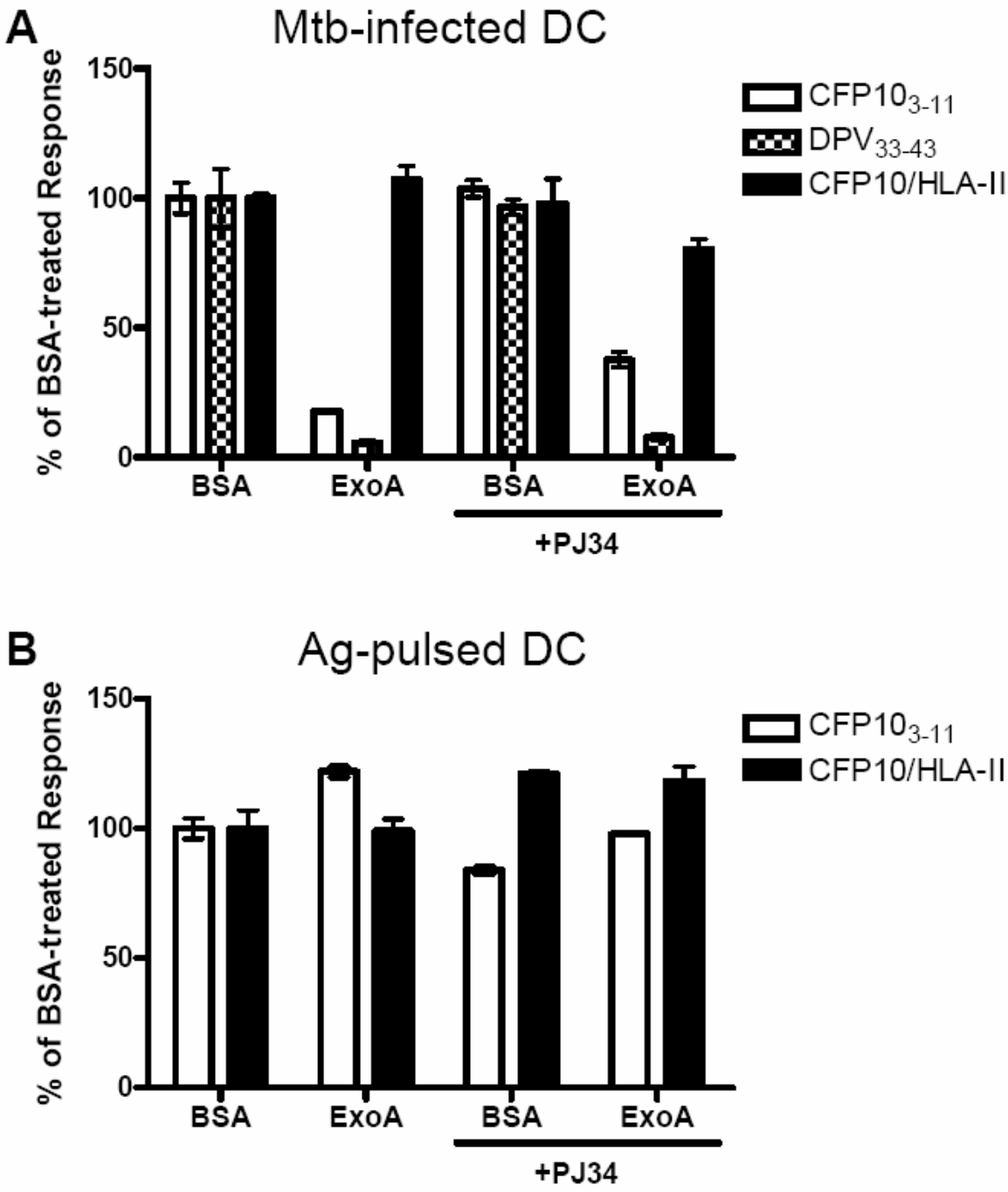


Figure 5. Presentation of $DPV_{33-43}$ requires retrotranslocation from the phagosome. DC were treated with exoA or BSA (10μg/ml) alone or with 200-fold molar excess PJ34 for one hour before infection with H37Rv-eGFP (MOI=20, A) or addition of indicated peptides (1μg/ml) or CFP10 protein (0.5μg/ml, B). After 15-16 hours of infection, DC were added as stimulators in an IFN-γ ELISPOT assay as described. Numbers are the mean +/- SEM of triplicate wells and are representative of two independent experiments.

was markedly inhibited in the presence of exoA or exoA/PJ34 (Figure 5A), demonstrating that DPV does in fact require cytosolic access for antigen presentation. There was no effect of BSA/PJ34 treatment, demonstrating that this effect is specific for exoA and not PJ34. Furthermore, there was no effect on presentation of $CFP10_{3-11}$ peptide to D480 F6 or Mtb-derived or soluble CFP10 presentation on HLA-II, further supporting the specificity of exoA inhibition (Figure 5B). Taken together, the ICP47, exoA, and cycloheximide data demonstrate that proteasome-enhanced antigens are processed in the cytosol.

**Discussion:**

In this chapter, we examined the requirements for cross-presentation of seven newly defined epitopes derived from five Mtb proteins presented by six different HLA-B alleles. Presentation of all seven epitopes was blocked by BFA while none were blocked by bafilomycin. The generation of three epitopes was inhibited by proteasomal inhibition while presentation of the remaining four was enhanced. Cathepsin inhibitors as well as a TPPII inhibitor did not affect presentation of proteasome-enhanced epitopes. Presentation of proteasome-enhanced epitopes requires TAP transport, new protein synthesis, and retrotranslocation from the phagosome.

Consistent with cytosolic antigen presentation, we found that presentation of all seven epitopes was blocked by BFA and thus require ER-golgi transport. Unexpectedly, we found that presentation of four epitopes was enhanced by blocking proteasome function, suggesting that the proteasome actually destroys the antigenic epitope and that it is not required for epitope generation. Although BFA sensitivity excludes the vacuolar

pathway, the lack of proteasome requirement for presentation is inconsistent with cytosolic processing. Therefore, the processing pathway for these four epitopes was ambiguous. Further evidence against the vacuolar pathway is the finding that presentation did not require acidification or cathepsin proteolysis. However, we were unable to identify the cytosolic protease responsible for epitope generation. To verify that these epitopes use the cytosolic pathway, we demonstrate that presentation of the proteasome-independent epitopes requires TAP transport, new protein synthesis, and retrotranslocation from the phagosome. From these data, we conclude that all seven epitopes are processed using the cytosolic pathway, suggesting a preferential use of the cytosolic pathway for Mtb antigen cross-presentation.

There are several potential biases to this conclusion. First, all of the proteins studied in this chapter except TbH9 (201) are secreted proteins. CFP10, Ag85B, and DPV are major constituents of the Mtb culture filtrate and CFP10 and Ag85B have been shown to be immunodominant $CD4^+$ antigens (202-204). We and others have found CFP10 to be an immunodominant $CD8^+$ antigen as well (56, 172). EsxJ is a CFP10 homologue which is secreted through the same mechanism as CFP10 (205). Because of their immunogenicity, Mtb culture filtrate proteins have been tested as vaccine candidates (202). Our findings lead to the hypothesis that secreted proteins may preferentially access the cytosol. In chapter 2, we demonstrated a distinct pathway for the non-secreted HLA-E antigen, although presentation still occurs using the cytosolic pathway. Second, we have defined these epitopes using peptide pools, which excludes the identification of modified proteins (e.g. lipoproteins). Non-cytosolic processing of an Mtb-derived lipoprotein has been described (138), suggesting that modified proteins may be processed

using non-cytosolic pathways. We have made an Mtb expression library in *Mycobacterium smegmatis* (Marisa Frieder, unpublished) that should allow us to identify modified antigens recognized by $CD8^+$ T cells, and subsequently examine the processing and presentation requirements.

Initially, we hypothesized that screening Mtb antigen presentation in the presence of epoxomicin, BFA, and bafilomycin would distinguish between the cytosolic and vacuolar pathways. Presentation using the cytosolic cross-presentation pathway requires proteasomal degradation (117), but the requirement for ER-golgi transport is dependent on whether antigen trafficking follows the traditional HLA-I or ER-phagosomal pathway (Chapter 2 (117, 124)). Because the vacuolar pathway has neither of these requirements, but instead requires acidification (131-133), bafilomycin was included in the initial screens. While this approach worked for defining the processing pathway for three of the epitopes, the results for the remaining epitopes were inconclusive. This suggests that until there is a better understanding of the intricacies of cross-presentation, processing and presentation pathways should be characterized on a case by case basis. Furthermore, conclusions drawn by examining only one aspect of a particular pathway (i.e. proteasome inhibition) may lead to inaccurate conclusions, as we shown that cytosolic processing can occur without the proteasome and that cytosolic derived peptides can be loaded within the phagosome (Chapter 2). Also, at least two reports have shown a requirement for both cytosolic and endocytic protease activity (142, 196).

The observation that proteasome blockade resulted in enhanced presentation of four epitopes was surprising. Inhibition of proteasome function could cause either direct (i.e. lack of protein degradation and proper generation of epitope C-termini) or indirect

effects (i.e. lack of HLA-I trafficking due to peptide depletion) on antigen presentation. It is hard to imagine a scenario where the indirect effects of proteasome blockade would enhance antigen presentation. For example, if proteasome inhibition led to a decrease in HLA-I trafficking, this should also affect the levels of HLA-I available for loading in the endocytic pathway, which has been hypothesized to use recycled HLA-I (133, 170). Therefore, we conclude that the enhancement observed is due to destruction of the epitope by the proteasome. Furthermore, this argues that although peptide generation may not occur within the cytosol, antigen must at least traffic to the cytosol for this effect to occur.

Interestingly, three out of the four proteasome-independent epitopes are 11mers, while $CFP10_{75-83}$ is a 9mer. This suggests that longer peptides may be preferentially degraded by the proteasome. Consistent with this hypothesis, the proteasome-independent epitope from HIV nef is a 10mer (amino acids 73-82 (200)). Detailed study of proteasomal degradation products has shown that size can range from 2-25 amino acids, with a mean size of less than 8 amino acids (85-87). This demonstrates that although the proteasome predominantly creates short peptides, it does not have an inherent inability to produce epitopes of this size.

There are several publicly available programs that predict proteasome cleavage sites, based on characterization of actual proteasome degradation products. Predictions of cleavage sites for the proteins containing the enhanced epitopes suggest strong cleavage sites within these epitopes, explaining why blocking proteasomal degradation may enhance presentation. However, cleavage sites are also predicted within epitopes that are generated by the proteasome. This is not surprising as data has suggested that

proteasome can both create and destroy epitopes, and that the correct C-termini of epitopes actually represents a minority of proteasome cleavage products (85, 195). As more real life proteasome products are characterized, proteasome prediction algorithms should improve.

One caveat of the cathepsin and acidification blocker experiments is that we do not have a positive control for inhibition of activity. Inhibitors were used at the highest non-toxic concentration possible and were at or above published concentrations shown to inhibit the relevant function. Suggesting that bafilomycin treatment affected acidification was the finding that presentation of the HLA-E antigen was enhanced by treatment (Chapter 2). A similar effect was seen with presentation of $CFP10_{2-12}$, one of the proteasome-enhanced epitopes. Together these data suggest that acidification and downstream effects lead to destruction of these epitopes. Furthermore, the preponderance of evidence suggests that proteasome-independent epitopes are processed in the cytosol, incongruent with a role for acidification or cathepsin processing.

Similarly, we do not have a positive control for blocking TPPII activity. Sequence analysis of the proteasome-independent epitopes suggests that they would be poor substrates for TPPII. Exoprotease activity of TPPII removes three amino acid residues at a time, but is unable to cleave proline-X bonds (206). Because this activity would destroy most epitopes, and especially $CFP10_{2-12}$, this excludes exoprotease function in generating the correct C-termini. Although not absolute, the TPPII endoprotease activity predominantly cleaves after lysine and arginine activities, present at none of the proteasome-independent epitopes.

Together, these data demonstrate that presentation of all seven epitopes occurs via

the cytosolic pathway. Thus, the predominant pathway for cross-presentation of Mtb antigens relies on cytosolic access. The proteasome does not appear to be involved in the generation of four of these epitopes. Although we can not formally exclude a role for proteasomal or TPPII proteolysis, our data strongly suggest that a novel cytosolic protease plays a major role in presentation of HLA-I antigens.

***Experimental Procedures:***

**Reagents**

Inhibitors of antigen processing were obtained from EMD Biosciences (epoxomicin, bafilomycin, leupeptin, pepstatin A, AAF-CMK and PJ34), Sigma (chloroquine, cycloheximide, and *Pseudomonas aeruginosa* exotoxin A), or Invitrogen (brefeldin A). Soluble CFP10 protein was provided by Corixa Corp. Peptides were synthesized by Genemed Synthesis Inc.

**Bacteria, virus, and cells**

H37Rv-eGFP was provided by Dr. Joel Ernst (New York University, New York, NY) and was grown in Middlebrook 7H9 broth supplemented with Middlebrook ADC (Fisher), 0.05% Tween-80, 0.5% glycerol, and kanamycin (50μg/ml). Before infection, bacteria were sonicated for 20 seconds, passaged 15 times through a tuberculin syringe, and sonicated again to obtain a single cell suspension. Multiplicity of infection varied depending on the experiment.

Adenovirus-ICP47 (174) and other adenoviral vectors were provided by Dr. David Johnson (OHSU).

PMBC were obtained from normal donors via leukapheresis and processed as previously described (81). DC were generated by culturing adherent PBMC for 5 days in the presence of GM-CSF (10ng/ml, Amgen) and IL-4 (10ng/ml, R&D systems) in RPMI (Gibco) supplemented with 10% pooled human serum (HS), L-glutamine (4mM, Gibco), and gentamicin (50μg/ml, Invitrogen).

**T Cell Expansion**

The T cell clones used in this report are detailed in Table 1. T cell clones were expanded as previously described (120), except that some of the expansions were done in Stemline T cell expansion media (Sigma) supplemented with 1% FBS (Hyclone) and 4mM L-glutamine.

**ELISPOT Assay**

96 well nitrocellulose-backed plates (Millipore) were coated with 10 μg/ml mouse anti-IFN-γ (1-D1K; Mabtech) overnight at 4°C. Plates were washed with PBS (Gibco) and blocked with RPMI/10% HS at room temperature. DC, antigens, and T cells were added according to individual experiments and incubated 18-48 hours at 37°C. After washing with PBS/0.05% Tween-20, 1 μg/ml biotinylated anti-IFN-γ mAb (7B6-1; Mabtech) was added. After 2 hours of incubation at room temperature, plates were washed and avidin/biotinylated enzyme (HRP) complex (Vectastain ABC Elite Kit; Vector) was added, and plates were incubated for an additional hour. Plates were washed and 3-amino-9-ethylcarbazole substrate (Vectastain AEC substrate kit; Vector) was added. After 4–10 min, the colorimetric reaction was stopped by washing with distilled

water. Spots were quantified using an AID ELISPOT plate reader (Cell Technology, Inc.).

**Inhibition of Antigen Presentation**

Day 5 DC were plated in a 24 well ultra low adherence (ULA) plates (Costar) at 500,000 cells/well in RPMI/10% HS supplemented with GM-CSF and IL-4. DC were pretreated with inhibitors (0.1μg/ml BFA, 0.2μM bafilomycin, 1-10μM epoxomicin, 100μM chloroquine, 150μM pepstatin A, 100μg/ml leupeptin, 2μM AAF-CMK, 10μg/ml cycloheximide, 10μg/ml exoA, or 10μg/ml exoA with 200 fold molar excess PJ34) for one hour before infection with H37Rv-eGFP (MOI=20) or addition of antigen. In experiments using exoA/PJ34 or BSA/PJ34, these compounds were co-incubated at room temperature for 30' prior to addition to DC.

DC were harvested after 15-16 hours of infection, pelleted and fixed with 0.5% paraformaldehyde for 15 minutes. The reaction was stopped with 135ul of 0.4M Lysine or RPMI/10% HS and DC were washed extensively with RPMI/10% HS. Fixed DC were then added to an IFN-γ ELISPOT plate at varying quantities so that antigen was the limiting factor (25,000 Mtb-infected cells/well for $CD8^+$ clones, 1,000 Mtb-infected cells/well for $CD4^+$ clones, and 2,000 Ag-pulsed cells/well for both $CD4^+$ and $CD8^+$ clones). T cells clones were added at 10,000 cells/well and plates were incubated at 37°C for 18 hours before development.

**ICP47-mediated TAP Inhibition**

DC (100,000) were added to wells of a 96 well flat bottom plate in 20μl Opti-

Mem serum-free media (Gibco). Adenovirus particles or media were incubated with lipofectamine 2000 (Invitrogen) for 25 minutes at room temperature. Liposome/adenovirus was added to DC (20μl/well, MOI=100, lipofectamine 2000 at 0.5μl/well) and incubated for two to four hours at 37°C. RPMI/10% HS supplemented with GM-CSF and IL-4 was added to wells and incubated for another 6-26 hours. Media was removed and replaced with RPMI/10% HS containing H37Rv-eGFP, antigen, or nothing and incubated overnight at 37°C. After 15-16 hours, media was removed and T clones were added (150,000/well) in the presence of BFA (10μg/ml). After a six hour stimulation, T cell clones were harvested, fixed with 1% paraformaldehyde, and stained with antibodies to CD3 (UCHT1, BD Biosciences) and IFN-γ (Coulter) in the presence of 0.2% saponin (Sigma). Cells were analyzed using a FACSCalibur or LSR II (Becton Dickinson).

***Acknowledgements:***

I would like to strongly acknowledge Anne Chamberlin for her role in these experiments. She performed many of the experiments in Figures 1-3 and 4B. Without her assistance, many of these experiments would still be ongoing. Many past and current members of the lab were involved in $CD8^+$ T cell cloning, epitope mapping, and HLA restriction studies. Therefore, I would like to thank Amy Heinzel, Katie Tanner, Matt Cook, Ervina Winata, Meghan Cansler, and Megan Null for generation and characterization of the reagents used in this chapter that are summarized in Table I.

# CHAPTER 4

# The role of Region of Difference 1 in presentation of *Mycobacterium tuberculosis*-derived antigens

Jeff E. Grotzke and David M. Lewinsohn

**Summary:**

The Region of Difference 1 (RD1) is a locus that is present in all strains of Mtb, but absent in all strains of the attenuated vaccine strain *Mycobacterium bovis* bacillus Calmette-Guerin (BCG). Therefore, much emphasis has been placed on the role of this region in Mtb virulence. Deletion of RD1 attenuates Mtb while introduction of RD1 into BCG leads to an increase in virulence. Our collaborators and others have demonstrated that RD1 encodes a secretion system that plays a large role in secretion of Mtb proteins. Furthermore, there is experimental evidence suggesting that one of the RD1 encoded proteins, 6kDa early secreted antigenic target (ESAT-6), is able to create a pore in lipid bilayers. These data have been used to argue that RD1 may play a specialized role in access of antigens to the cytosolic MHC-I processing machinery, by either creating a pore in the phagosomal membrane or mediating escape of the bacterium from the phagosome. We have analyzed the role of RD1 in presentation of Mtb antigens using strains that are mutated or deleted for individual RD1 genes. We show that processing and presentation of $CFP10_{2-11}$ is processed by the cytosolic pathway. We analyzed the role of individual RD1 genes on secretion and presentation of CFP10, showing that CFP10 presentation only occurs in when antigen is secreted. Finally, we show that HLA-I presentation occurs in DC infected with RD1 mutants, arguing against a requirement for this secretion pathway in cross-presentation of Mtb antigens.

***Introduction:***

The factors that promote virulence of Mtb are only beginning to be elucidated. Comparative genomic analysis of strains of mycobacterial species has yielded important insights into genes and loci that may play a role in Mtb virulence. Work done by multiple groups has identified genetic regions that differ between virulent Mtb, avirulent Mtb, and BCG (181, 207-210). Notably, Region of Difference 1 (RD1) is the only genetic region that is present in all virulent strains of Mtb, but absent in BCG strains. Moreover, the avirulent Mtb strain, H37Ra, shows up to a 12-fold decrease in expression of genes encoded in this region (211). This finding has provoked a large amount of research examining the function of RD1, individual RD1 genes, and the role they play in Mtb virulence.

The RD1 locus encodes nine genes (Rv3871-Rv3879), including the immunodominant CD4 and CD8 antigens, CFP10 (Rv3874) and ESAT-6 (Rv3875). We and others have demonstrated that those with latent or active Mtb infection have strong T cell responses to these antigens. As evidenced by the large number of shared and unique epitopes we have identified in CFP10 and the high ex vivo $CD8^+$ T cell frequency to these epitopes (172), the response to these antigens represents a significant component of Mtb immunity. As such, much effort has been placed on Mtb diagnostic tests using responses to these antigens. Because ESAT-6 and CFP10 are absent in most environmental mycobacteria, antigen-specific responses likely represent Mtb infection, and these tests are vital for detecting true Mtb infection in BCG-vaccinated individuals.

Although there has been a lot of effort focused on studying the immune response to RD1 antigens, the function of RD1 genes is still not clear. It was originally

hypothesized that RD1 may encode a novel secretion system due to sequence analysis and the fact that, when expressed in BCG, secretion of ESAT-6 required a secretory sequence (212). This proved to be the case as three groups reported that individual RD1 genes are required for secretion of ESAT-6 and CFP10 (182, 183, 185). Moreover, mutants that did not secrete these molecules were attenuated in vitro and in vivo, suggesting a role for secreted proteins in pathogenicity. RD1 mutants were unable to lyse macrophages, leading to large numbers of intracellular bacteria. Furthermore, two groups have reported that ESAT-6 is able to disrupt lipid bilayers (183, 213). These data have led to the hypothesis that ESAT-6/CFP10 secretion by the RD1 encoded system creates a pore in the phagosomal membrane that might create a pathway for cytosolic access. Although disputed, a recent report demonstrates that CFP10 is required for phagosomal escape of Mtb (27). Together, these data have led to the hypothesis that ESAT-6 and CFP10 may play a vital role in presentation of Mtb antigens on Class I molecules.

Here, we tested the role RD1 genes in cross-presentation of Mtb antigens. The data reported here suggest that the RD1 encoded secretion system is not required for global cross-presentation of Mtb antigens, although it is required for presentation of CFP10.

***Results:***

**RD1 genes are required for secretion of CFP10 and ESAT-6**

To determine the role of RD1 in cross-presentation of Mtb antigens, we made use of Mtb strains mutated in individual RD1 genes as well as deleted for the entire RD1 region in collaboration with Dr. David Sherman (Seattle Biomedical Research Institute,

Seattle, WA). Culture supernatant from log phase bacteria was filtered and concentrated to examine secreted proteins. LCL were pulsed with culture filtrate or bacterial pellet proteins and the presence of ESAT-6 and CFP10 was detected using $CD4^+$ T cell clones as a readout. Using an ELISPOT assay for IFN-γ production, we were able to detect CFP10 with high sensitivity, as low as 2ng/ml (Figure 1). Detection of ESAT-6 was less sensitive, with a limit of detection of 100ng/ml. As expected, we did not detect CFP10 (Rv3874) or ESAT-6 (Rv3875) in the culture filtrate or bacterial pellet of ΔRD1 or the corresponding mutant strains, demonstrating the lack of expression in the mutant strains. There was a large decrease or lack of secretion of both proteins in strains with transposon insertions in Rv3870, Rv3871, and Rv3876, but both proteins were detectable within the bacterial pellet. Complementation with plasmids encoding the mutated genes led to recovery of secretion, showing that individual RD1 genes are required for secretion of ESAT-6 and CFP10.

Unexpectedly, we did not detect ESAT-6 or CFP10 in the pellets of strains mutated in CFP10 and ESAT-6, respectively. It has been shown that these proteins form a tight 1:1 heterodimer before secretion (214). Because RNA analysis of H37Rv:tn3874 showed wild type expression levels of Rv3875 (182), the lack of detection of these proteins suggests that these proteins are unstable in the absence of the binding partner.

**Presentation of $CFP10_{2-11}$ requires TAP transport**

We next examined the role of ESAT-6/CFP10 secretion in presentation of Mtb antigens. Because secretion of these proteins has been postulated to affect cytosolic access of Mtb antigens, we first examined whether $CFP10_{2-11}$ is processed using the

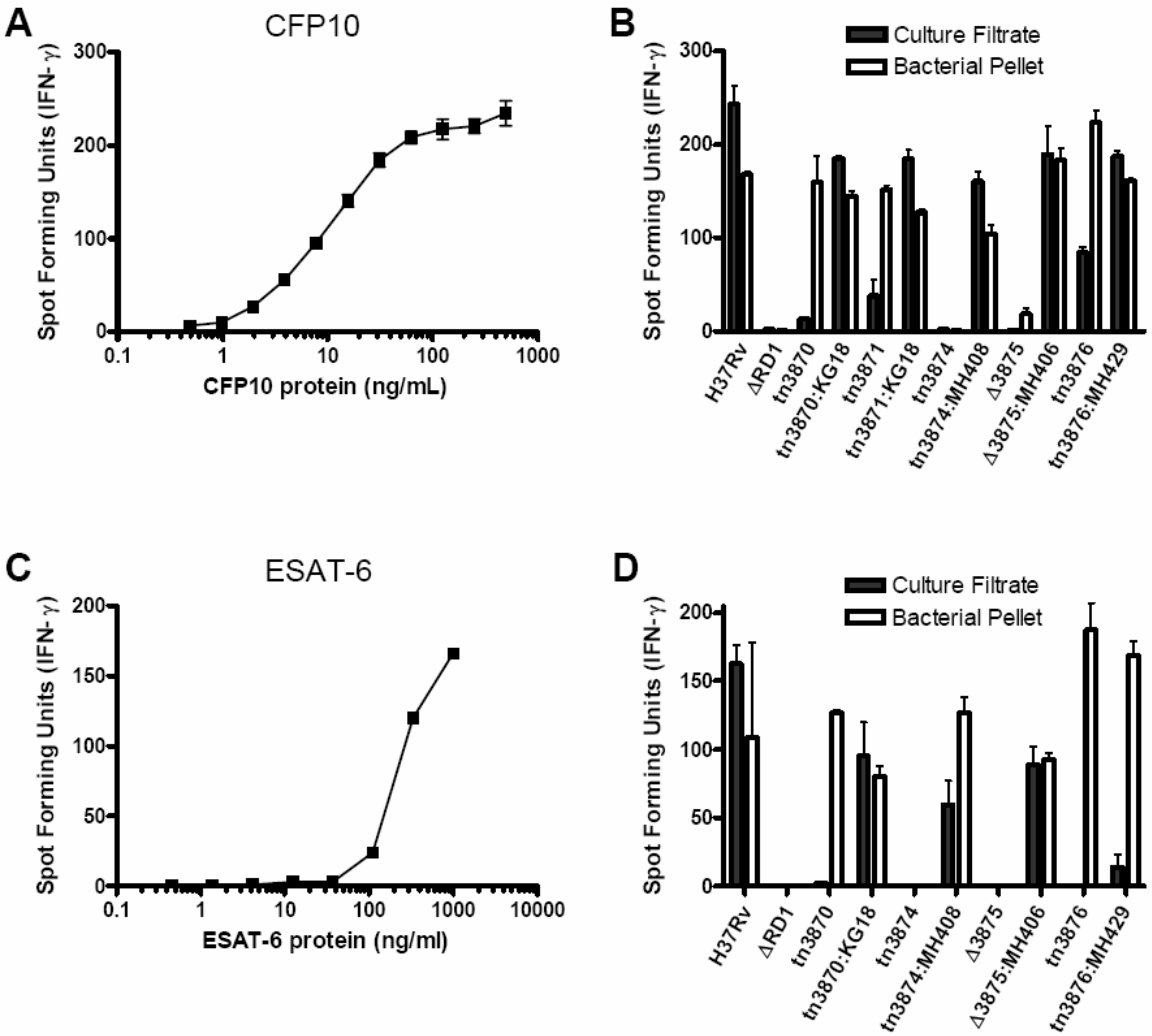


Figure 1. Lack of ESAT-6/CFP10 secretion in the absence of RD1 genes. LCL were pulsed with recombinant protein (A & C), secreted (culture filtrate) or intracellular (bacterial pellet) proteins (B & D) for 1-2 hours. CD4+ T cell clones were added and IFN-γ assessed by ELISPOT after an overnight infection. Data represent the mean +/- SEM of duplicate wells and results are representative of two separate experiments. Plasmids used for complementation encode the genes shown in parentheses, KG18 (Rv3870, Rv3871, Rv3872), MH408 (Rv3874, Rv3875), MH406 (Rv3874, Rv3875), MH429 (Rv3876).

cytosolic pathway. Therefore, we assessed the role of TAP transport in presentation of $CFP10_{2-11}$. DC were transduced with an adenoviral vector expressing the TAP inhibitor ICP47 or the empty parental adenoviral vector. After 30 hours of adenoviral infection, we detected a modest downregulation of cell surface HLA-I, with no effect on cell surface HLA-II (data not shown). When ICP47-expressing DC were infected with Mtb, there was a large reduction of $CFP10_{2-11}$ presentation, while presentation of CFP10 on HLA-II was unaffected (Figure 2A). In addition, there was no defect in the ability of DC to present $CFP10_{2-11}$ peptide or CFP10 recombinant protein, excluding toxicity of ICP47 expression or a defect in HLA-I presentation (Figure 2B). Finally, we assessed Mtb infection by using an H37Rv strain expressing eGFP and detected no difference in adenovirus-ICP47, empty-adenovirus, and mock transduced DC (data not shown).

The responses seen using this protocol were relatively low compared to untransduced DC, especially for HLA-II presentation (data not shown). To further demonstrate the role of TAP in presentation of $CFP10_{2-11}$, we used an alternate protocol and assessed IFN-γ production by intracellular cytokine staining. As demonstrated in Figure 2C, we again saw inhibition of Mtb-derived $CFP10_{2-11}$ presentation with ICP47 expression, with no effect on CFP10 HLA-II presentation. These results demonstrate that TAP is required for the presentation of $CFP10_{2-11}$, and that this protein is cross-presented using the cytosolic pathway.

**Cross-presentation of $CFP10_{2-11}$ requires CFP10 secretion**

To address the role of ESAT-6/CFP10 secretion in presentation of Mtb antigens, we monitored antigen presentation in RD1 mutants. Infection of DC with RD1 mutant

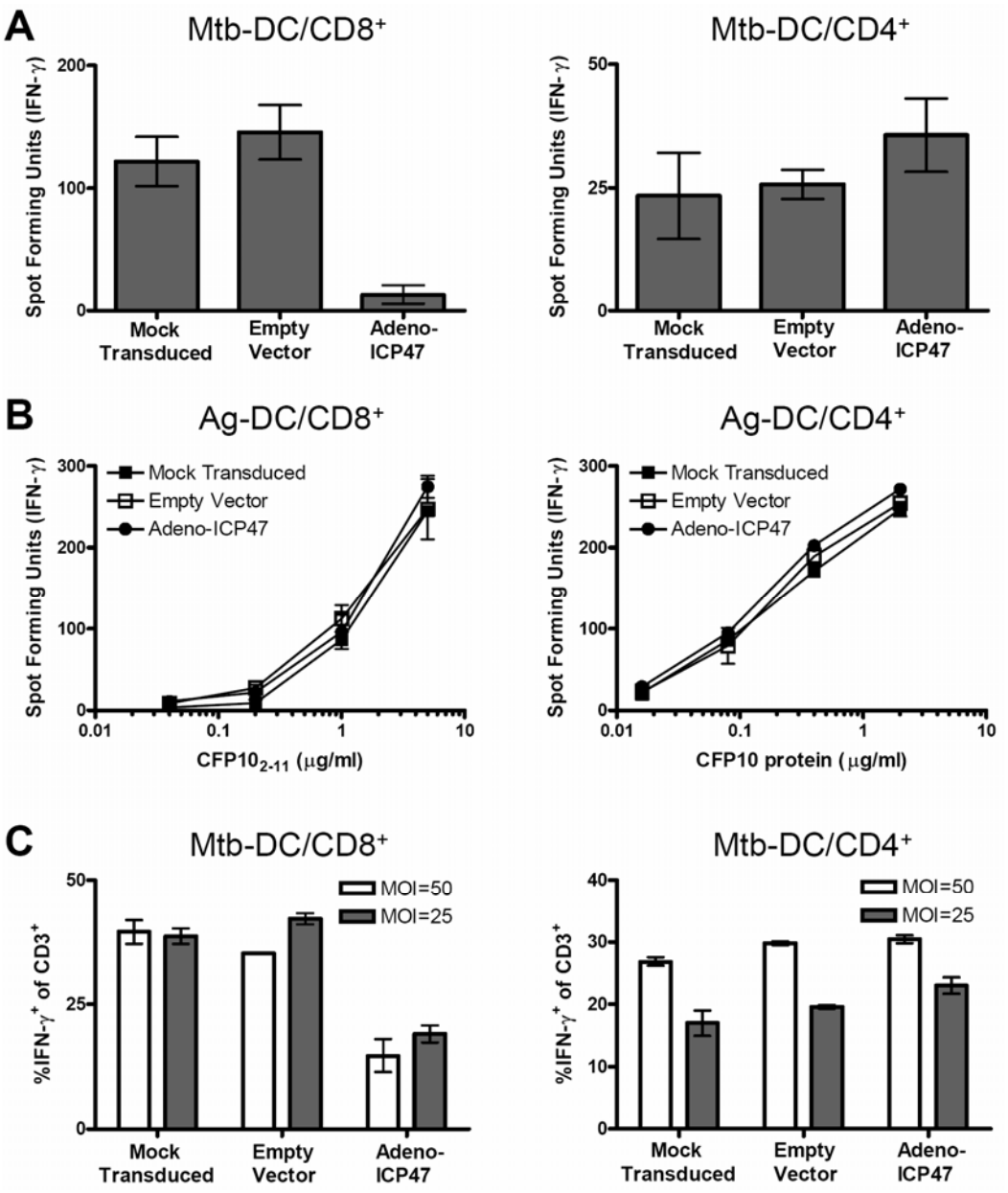


Figure 2. Cross-presentation of $CFP10_{2-11}$ requires TAP. DC were mock infected, infected with adenovirus-expressing ICP47 or with control adenovirus (MOI=100). After 30 hours, DC were infected overnight with H37Rv (MOI=50, A) or loaded overnight with $CFP10_{2–11}$ peptide or CFP10 protein (B). The T cells were then added to DC, and IFN-γ assessed by ELISPOT. Each bar represents the mean of triplicate wells, and the data are representative of three separate experiments. (C) DC were mock infected or infected with adenovirus as described and then infected with H37Rv at the indicated MOI. T cell clones were added for 6 h, with BFA present for the last 2 hours of the stimulation. After staining, CD3 cells were analyzed for IFN-γ expression. Each bar represents the mean of duplicate wells, and the data are representative of three separate experiments.

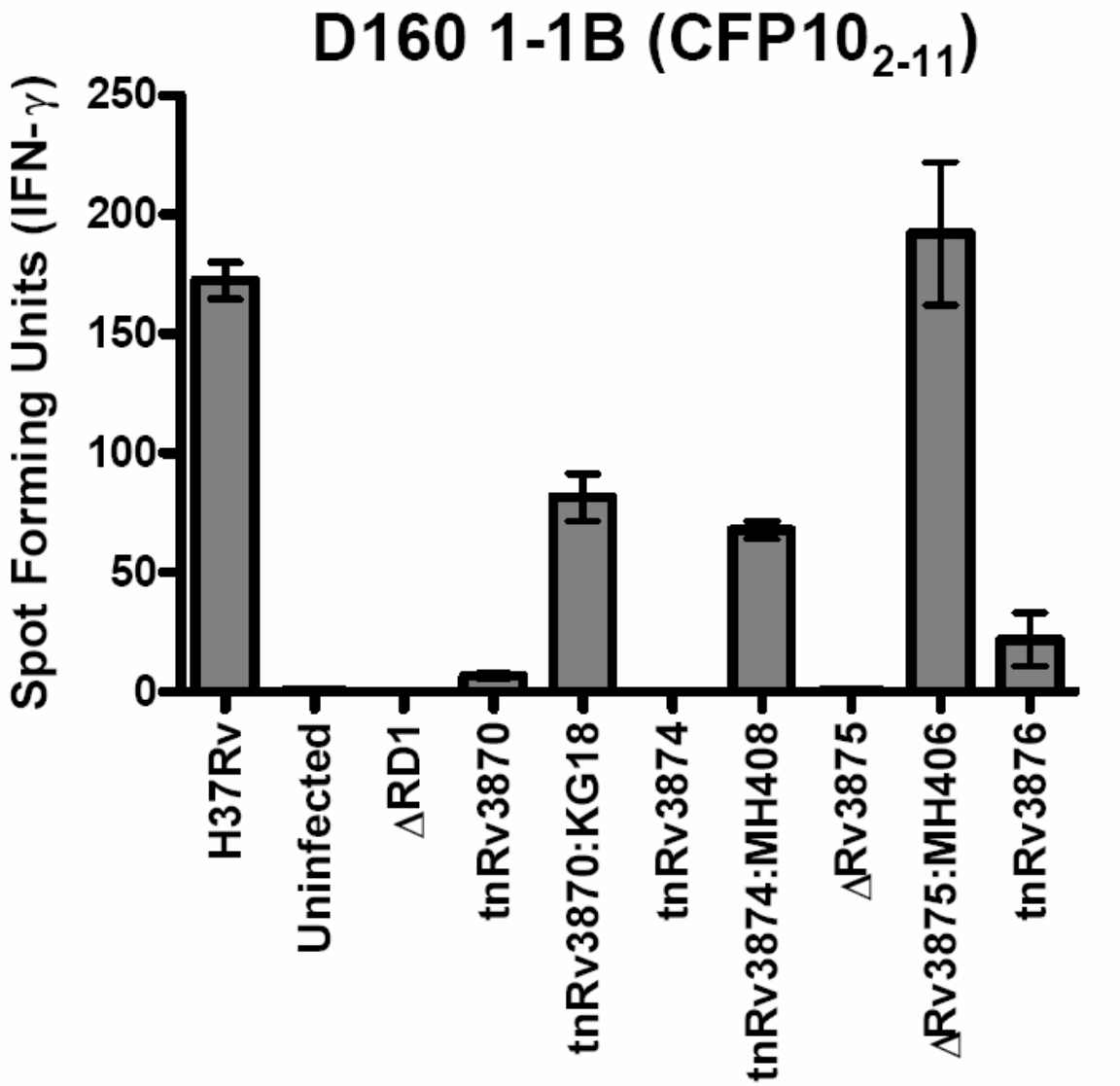


Figure 3. ESAT-6/CFP10 secretion is required for presentation of CFP10$_{2-11}$. DC were infected overnight with the indicated Mtb strains. Harvested DC were incubated with D160 1-1B CD8$^+$ T cells overnight and IFN-γ assessed by ELISPOT. Numbers indicate the mean +/- SEM of triplicate wells and are representative of two separate experiments.

strains showed that antigen presentation was reduced not only in strains lacking CFP10, ESAT-6, or both, but also in strains that express CFP10 but show lack of or decreased secretion (H37Rv:tn3870 and H37Rv:tn3876, respectively, Figure 3). Presentation of $CFP10_{2-11}$ correlated with secretion as we saw more presentation in H37Rv:tn3876 than H37Rv:tn3870 (compare CFP10 secretion by these strains in Figure 1). Complementation of mutants with the associated genes led to a recovery of antigen presentation, showing that the effect was due to transposon insertion in the RD1 gene, and not alternate transposon insertion sites.

As stated above, the avirulent Mtb strain, H37Ra, shows a dramatic decrease in expression of RD1 genes (211). We were unable to detect presentation of $CFP10_{2-11}$ in H37Ra-infected DC (data not shown). However, we were able to detect CFP10 presentation on HLA-II, showing that at least some CFP10 is expressed within this strain. Although we cannot exclude other differences between H37Ra and H37Rv, the lack of recognition of H37Ra by $CD8^+$ T cells is consistent with the effects seen with lack of CFP10 secretion. Taken together, these data argue that CFP10 requires secretion for presentation on HLA-I.

**RD1 is not required for cross-presentation of Mtb antigens**

Thus far, we have established that individual RD1 genes are required for secretion of ESAT-6 and CFP10, and that secretion of these proteins is required for the presentation of CFP10 on HLA-I. To address the role of ESAT-6/CFP10 secretion on presentation of non-RD1 antigens, we used a $CD8^+$ T cell clone that responds to the nine amino acid sequence, ASPVAQSYL, from the Mtb protein DPV in the context of HLA-

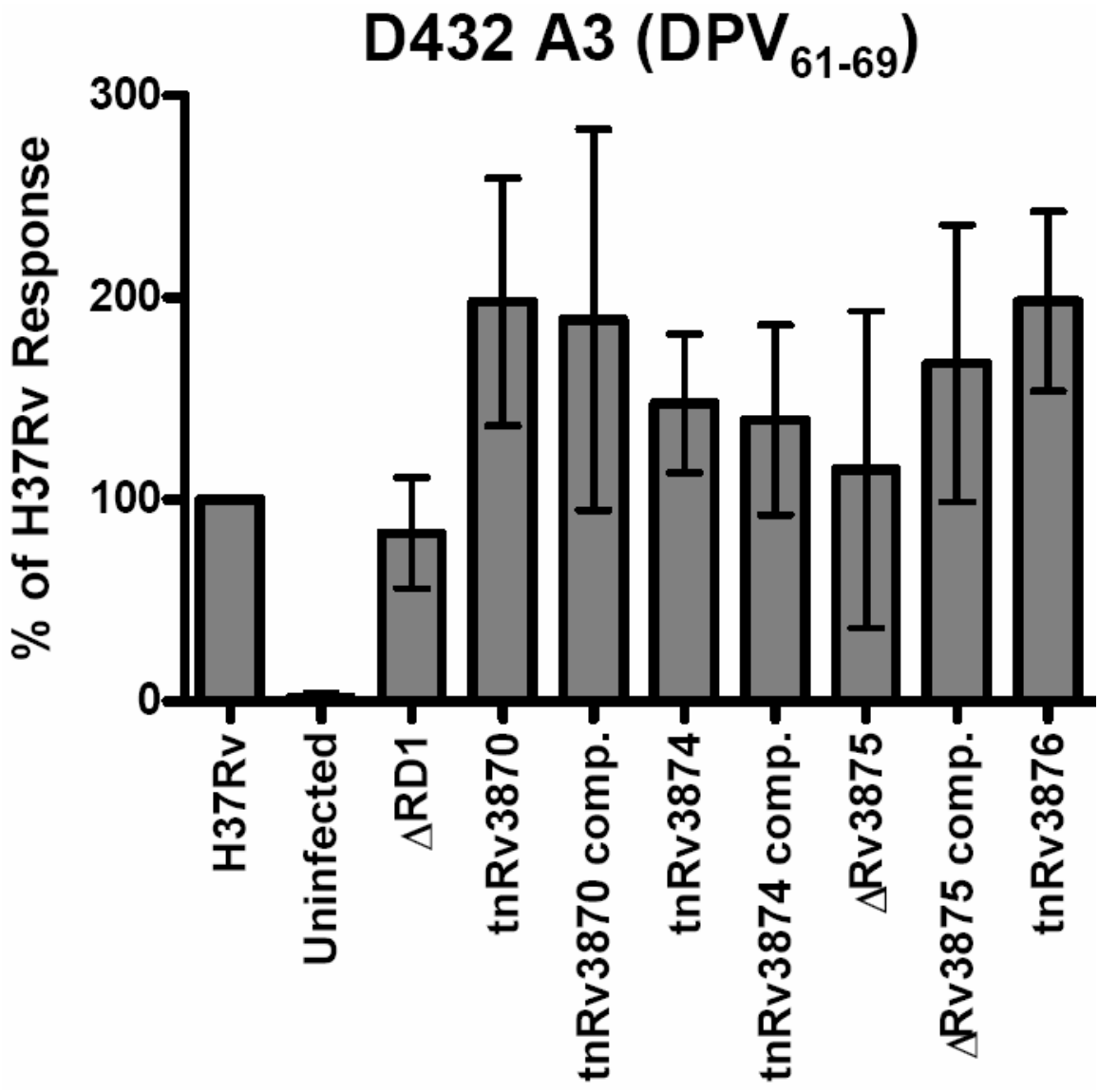


Figure 4. ESAT-6/CFP10 secretion is not required for presentation of $DPV_{61-69}$. DC were infected overnight with the indicated Mtb strains. Harvested DC were incubated with D432 A3 $CD8^+$ T cells overnight and IFN-$\gamma$ assessed by ELISPOT. Numbers (mean +/- SEM) are pooled data from three experiments. No significant differences were found between the response to H37Rv and any of the RD1 mutants using one-way ANOVA.

B3514. DC infected with RD1 mutant strains were able to process and present $DPV_{61-69}$ as well or better that wild type H37Rv or the complemented mutant strains (Figure 4). A similar result was seen for presentation of the HLA-E antigen (data not shown). These findings argue against a global role of RD1 in cross-presentation of Mtb antigens.

***Discussion:***

In this chapter we examined the role of RD1 in the cross-presentation of Mtb antigens. Deletion of or transposon insertion in RD1 genes led to a decrease or lack of secretion of CFP10 and ESAT-6. These and other data have been used to show that RD1 functions as a novel Mtb secretion system (182, 183, 185). Presentation of the cytosolically processed epitope, $CFP10_{2-11}$, required secretion of these RD1 encoded proteins. However, the RD1 region or secretion of RD1-encoded proteins did not affect presentation of the non-RD1 antigen, DPV.

Because RD1 is missing from all strains of BCG and downregulated in H37Ra, understanding the role of these genes has been an area of intense investigation. Results from studying RD1 mutants or CFP10 and ESAT-6 proteins have led to multiple hypotheses on what role this secretion system plays in pathogenicity and antigen presentation. Initial studies looking at antigen presentation within infected macrophages suggested that mycobacterial phagosomes were permeable. Coincubation of macrophages with mycobacteria and soluble OVA led to cross-presentation of OVA that was not seen with OVA alone or with heat-killed mycobacteria (118). Furthermore, microinjection of fluorescent dextrans into mycobacterially-infected cells showed that substrates up to 70kDa could localize within phagosomes, a finding again dependent on

live bacteria (119). However, neither of these papers addressed whether this was an active or passive process. Because "permeability" required live infection, it could be argued that mycobacterial phagosomes express factors that either promote cytosolic access or recruit host cell components that aid in cross-membrane transport. The finding that ESAT-6 could alter membrane permeability (183, 213, 215) made for an attractive hypothesis on the role of RD1 secreted proteins in cytosolic access of phagosomal antigens. We demonstrate here that lack of ESAT-6/CFP10 secretion abrogates antigen presentation of the $CFP10_{2-11}$ epitope on HLA-B44. This is not surprising as CFP10 should be retained within the bacterium in the absence of an intact secretion system. Retention of CFP10 should hypothetically prevent CFP10 from accessing both phagosomal and cytosolic proteases, avoiding both the vacuolar and cytosolic cross-presentation pathways. We have previously demonstrated that CFP10 is processed using the vacuolar pathway (BFA-independent) when complexed with PLG microspheres (120). This suggests that the pathway by which CFP10 is processed is dependent on the nature of the associated particulate, and that there is little vacuolar processing of CFP10 in the absence of secretion.

In the absence of RD1 or ESAT-6/CFP10 secretion, we did not find any significant differences in the presentation of $DPV_{61-69}$. This finding demonstrates that RD1 is not required for global cross-presentation of Mtb antigens. However, we have not demonstrated that this epitope requires cytosolic processing. We have shown that presentation of another epitope from DPV ($DPV_{33-43}$) requires TAP transport (Chapter 3), suggesting that DPV does access the cytosol. Also, the overwhelming majority of HLA-I presented Mtb antigens use the cytosolic pathway (Chapter 2). Furthermore, we also saw

presentation of the cytosolically processed HLA-E antigen in the absence of RD1 (data not shown). Finally, in the mouse model, Billeskov, et al have confirmed that cross-presentation of the Mtb protein TB10.4 does not require RD1 (69). Together, these data exclude a requirement of RD1 for Mtb antigen cross-presentation.

A recent report has suggested that Mtb is able to escape the DC phagosome and reside in the cytosol, a process that is dependent on CFP10 (27). These authors reported that Mtb remains within the phagosome for the first 48 hours of infection, and is detectable in the cytosol increasingly thereafter. The authors hypothesized that phagosomal escape may be a major pathway of HLA-I presentation. While this finding is refuted by many researchers who have failed to see cytosolic bacteria, we can exclude a role of phagosomal escape in the experiments described here. We were able to detect antigen presentation when all Mtb should be within the phagosome, either in the absence of CFP10 or in infections no longer than 18 hours. Furthermore, we generally see relatively efficient presentation of Mtb antigens after 15-16 hours of infection (multiple experiments in Chapters 2 and 3), excluding the need for phagosomal escape for HLA-I presentation.

In conclusion, our and others' data have been used to demonstrate that the proteins encoded in RD1 function as a specialized secretion system for ESAT-6 and CFP10. Secretion of these proteins is not required for cross-presentation of Mtb antigens. These findings suggest that the membrane lysing function of ESAT-6 is not necessary for cytosolic access of Mtb antigens. It is more likely that this activity is required for host cell lysis and/or phagosomal escape after long-term infection.

***Experimental Procedures:***

**Bacteria and Virus**

RD1 mutants, complements, and H37Rv strains used were all characterized and provided by David Sherman (Seattle Biomedical Research Institute, Seattle, WA). Adenoviral vectors were provided by David Johnson (OHSU, Portland, OR).

**PBMC and T cell clones**

PMBC were obtained from normal donors via leukapheresis and processed as previously described (120). DC were generated by culturing adherent PBMC for 5 days in the presence of GM-CSF (10ng/ml, Amgen) and IL-4 (10ng/ml, R&D systems) in RPMI (Gibco) supplemented with 10% pooled human serum (HS), L-glutamine (4mM, Gibco), and gentamicin (50μg/ml, Invitrogen).

Clone D160 1-1B is a HLA-B44-restricted $CD8^+$ T cell clone specific for $CFP10_{2–11}$ (AEMKTDAATL (192)). Clone 38.1-1 is a $CD4^+$ T cell clone specific for $CFP10_{73–87}$ (STNIRQAGVQYSRAD (182)). Clone D481B D2 is a $CD4^+$ T cell clone specific for ESAT-6 (unidentified epitope, Unpublished). Clone D432 A3 is an HLA-B3514-restricted $CD8^+$ T cell clone specific for $DPV_{61-69}$ (ASPVAQSYL (172)). T cell clones were expanded as described above.

**Cell pellet and culture filtrate preparation**

Proteins from culture filtrate and bacterial cell pellets were processed by Mark Hickey as described (182, 216, 217).

**ELISPOT**

LCL ($2x10^4$/well) were pulsed with CFP10 or ESAT-6 recombinant protein (Corixa Corp.), culture filtrate, or bacterial pellet proteins for 1-2 hours at 37°C. $CD4^+$ T cells (750/well) were added and incubated overnight at 37°C. IFN-γ release was detected by ELISPOT as described above.

DC ($5x10^5$/well) were infected with H37Rv or RD1 mutant strains (MOI=50) in 24 well ultra-low adherence plates (Costar). After 18 hours at 37°C, DC were harvested and added to triplicate wells of the ELISPOT plate ($2x10^4$/well). $CD8^+$ T cell clones ($1-2x10^3$/well) were added and incubated overnight at 37°C.

In experiments using adenovirus, 125µl Opti-Mem (Gibco) containing $5x10^5$ DC was added to wells of a 24 well ultra-low adherence plate. Adenovirus was mixed with Lipofectamine 2000 (Invitrogen) for 20 minutes at room temperature and added to DC (MOI=100). After 2 hours, RPMI/10% HS supplemented with GM-CSF and IL-4 was added and incubated for an additional 30 hours. DC were harvested, counted, and added to the ELISPOT plate ($2x10^4$/well) in addition to Mtb (MOI=50), peptide, or protein antigens for 18 hours. T cell clones ($1x10^3$/well) were added and incubated overnight at 37°C.

**Intracellular cytokine staining**

A total of $10^5$ monocyte-derived DC in 20µl of Opti-Mem medium was seeded in a 96-well flat-bottom plate. After a 20 minute incubation of adenovirus with Lipofectamine 2000 (Invitrogen Life Technologies), 20µl of adenovirus liposomes was added to the DC and incubated for 2 hours at 37°C. Medium containing GM-CSF and

IL-4 was added, and DC were incubated for an additional 28-30 hours. DC were then infected with H37Rv at varying MOIs or pulsed with peptide or protein antigen for 18 h. After washing, 1–1.5x$10^5$ T cells were added and incubated for 6 hours, with the addition of BFA (10µg/ml) for the final 2 hours. After harvesting, cells were fixed, permeabilized, stained with anti-CD3 (UCHT1; BD Pharmingen) and anti-IFN-γ (4S.B3; eBioscience), and analyzed on a FACSCalibur.

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***Notes:***

Data in figures 1A, 2, and 4 have been previously published (120, 182).

# CHAPTER 5

# DISCUSSION

Because of the low number of active TB cases each year in the US (around 10,000 cases), little attention is given to TB disease by the general population. However, Mtb is one of the leading contributors to infectious disease mortality worldwide, and greatly impacts the outcome of immunosuppression. This is most easily seen in the HIV infected population. It has been estimated that in contrast to infected healthy individuals, which have a lifetime 10% risk of developing active disease, HIV-infected patients have a yearly 10% risk of reactivation (218). Mtb infection has now become the leading cause of death of AIDS patients. Because of these staggering numbers, there has recently been more emphasis placed on understanding the immune response to Mtb infection. Furthermore, groups that have traditionally solely supported HIV research have begun to funnel some of that money towards Mtb research.

LTBI patients, although a potential source of new infections, represent individuals who have successfully controlled Mtb infection. Numerous reports have demonstrated that a strong Th1 response is required for the control of infection in these individuals and in mice. Production of pro-inflammatory cytokines by T lymphocytes plays a vital role in the activation of infected cells to upregulate innate and adaptive immune mechanisms. While the $CD4^+$ T lymphocyte subset clearly plays a crucial role in the Mtb adaptive immune response, $CD8^+$ T lymphocytes have also been shown to affect the outcome of infection. One possible strategy in vaccine development would be to decrease the percentage of LTBI patients who develop active disease, which would rapidly decrease new infections. To do this, however, a detailed understanding of the quality and quantity of T lymphocyte generation and maintenance is required. Central to this is how T cells interact with and recognize infected cells. This includes recognition of priming APC,

likely DC, and recognition of infected targets (macrophages, DC, and epithelial cells) in the lung. Because effective T lymphocyte responses are able to contain Mtb growth, understanding the factors that contribute to recognition of infected cells is fundamental for comprehension of the relevant components of Mtb immunology.

In this regard, we hypothesize two distinct roles that $CD8^+$ T cells may play during Mtb infection (168). First, $CD8^+$ T cells may uniquely recognize Mtb-infected cells, by recognition of non-classical HLA-I molecules or HLA-II$^-$ epithelial cells. For example, CD1 molecules are able to present mycobacterial-derived lipids, glycolipids, and lipopeptides; increasing the repertoire of mycobacterial products that can potentially be recognized by effector cells beyond peptide antigens (75-79). Similarly, because $CD8^+$ T cells specialize in recognizing intracellular infection, they are able to discriminate the level of Mtb infection within DC and preferentially recognize heavily infected cells (219). Second, $CD8^+$ T cells may preferentially utilize the granule exocytosis pathway versus production of pro-inflammatory cytokines (219). Thus, $CD8^+$ T cells may play an essential immune-surveillance role by recognizing heavily infected cells that may be unresponsive to IFN-γ, inducing apoptosis by granule exocytosis, depriving Mtb of a favored environment, and allowing fresh APC to bear the burden of Mtb containment.

With this in mind, our lab has focused on the role of $CD8^+$ T cells in human Mtb infection. We have found that Mtb-infected DC are able to present antigen using the non-classical Class I molecule, HLA-E (81). Strikingly, we are able to detect a large population of non-classically restricted $CD8^+$ cells that respond to Mtb-infected DC in LTBI and active TB patients. In normal donors, almost all $CD8^+$ T cells that respond to

Mtb-infected DC are non-classically restricted. While we do not presume that all of these responses are HLA-E restricted, these results suggest that non-classical antigen presentation may be an important component of the $CD8^+$ T cell response, by providing unique mechanisms of recognition.

We have not defined the Mtb antigen presented by HLA-E. Crude preparations of Mtb cell wall and TX-114 soluble cell wall proteins are recognized by HLA-E restricted $CD8^+$ T cell clones when processed by DC (81). The antigen is proteinaceous in nature, as antigenic activity of cell wall proteins is destroyed by treatment with trypsin (unpublished) and presentation requires proteasomal degradation (Chapter 2 (128)). Furthermore, all other described HLA-E binders have been peptides (145-148, 154, 158, 220-222). We cannot rule out that the HLA-E presented antigen is modified. In fact, certain data actually suggest that it is. Treatment of the TX-114 Mtb fraction with pronase or chymotrypsin actually enhances the antigenic activity of the fraction, implying that the epitope is protected from digestion. HPLC-fractionated, pronase-digested TX-114 fraction has consistently yielded sequence from the Mtb heat shock protein, HspX, in the antigenically-active fractions. However, DC pulsed with HspX overlapping peptides do not stimulate HLA-E restricted clones. Finally, HspX is antigenic when native protein is purified from Mtb, but not recombinant protein expressed in *E. coli*. Digestion of native HspX with pronase again leads to enhanced antigenicity. To this end, we can not conclude whether the HLA-E antigen is HspX or an HspX-chaperoned protein that is protected from digestion by association with HspX.

In other models of non-classical restriction, $CD8^+$ T cells have distinct characteristics from classically restricted T cells. Proliferation and function of H2-M3

restricted $CD8^+$ T cells peaks earlier than that seen for MHC-Ia restricted clones, but they proliferate only weakly upon secondary infection (162). These data have led to the hypothesis that H2-M3 restricted $CD8^+$ T cells are an innate subset that provides an early source of IFN-γ. Whether HLA-E restricted $CD8^+$ T cells act as innate or adaptive T cells remain to be determined, as this is a hard question to address in humans. However, we have demonstrated the presence of Mtb-reactive, non-classically restricted $CD8^+$ T cells in the thymus and cord blood that produce IFN-γ ex vivo, suggesting that a population of Mtb-reactive innate $CD8^+$ T cells exist (Marielle Gold, unpublished). Once the HLA-E antigen has been identified, tetramer analysis will allow enumeration of the frequency of HLA-E restricted $CD8^+$ T cells and comparison between normal donors, Mtb-infected adults and neonates should address this question.

In experiments presented here, we have characterized the processing and presentation pathway of HLA-E presented antigen in comparison with presentation of CFP10 on the classical HLA-I molecule, HLA-B8 (Chapter 2). Similar to presentation of $CFP10_{3\text{-}11}$, presentation of the HLA-E antigen requires retrotranslocation from the phagosome, proteasomal degradation, and TAP transport. These requirements are characteristic of cytosolic cross-presentation. However, several features of HLA-E antigen presentation are distinct from $CFP10_{3\text{-}11}$. Presentation of the Mtb-derived HLA-E antigen is only partially blocked in the presence of BFA, and does not require new protein synthesis. Both of these findings are inconsistent with presentation of endogenous antigens along the traditional HLA-I pathway, and thus are inconsistent with HLA-E loading within the ER and subsequent ER-golgi egress. We then tested the hypothesis that HLA-E loading may occur at a site other than the ER. Stimulation of

D160 1-23 with subcellular fractions showed the presence of loaded HLA-E:peptide complexes in fractions containing phagosomes, implying that HLA-E loading can occur within the Mtb phagosome. Loaded HLA-E:peptide complexes were present in phagosomal fractions at one to eighteen hours post-infection, suggesting that loading within the phagosome occurs rapidly and is ongoing. Furthermore, we detected less HLA-B44:$CFP10_{2-11}$ complexes in phagosomal fractions. Taken together, these data show that although HLA-E presentation of Mtb-derived antigen shows similarities to presentation by classical HLA-I molecules, the overall pathway is distinct.

Because TAP is required for presentation of the HLA-E antigen, and loading occurs within the phagosome, we reasoned that the Mtb phagosome must contain components of the HLA-I peptide loading complex, as put forth by multiple groups using latex bead phagosomes (122-125). To address this question we developed a method to phenotype phagosomes from Mtb-infected DC by flow cytometry. This technique is sensitive and allows the analysis of thousands of events per experiment. We found that, in contrast to magnetic bead phagosomes, Mtb phagosomes retained an early endosomal phenotype (TfR and rab5 positive) after overnight infection. These data are consistent with that shown by other groups using alternate methods (7). Furthermore, we detected the stable localization of HLA-I, PDI, TAP1, and TAP2 in the Mtb phagosome, giving mechanistic plausibility to phagosomal loading.

There has been a lot of controversy regarding the role of the ER in phagocytosis and the localization of ER proteins in the phagosome (27, 127, 223). Some of this objection is theoretical (223), while one group has quantitated the contribution of ER and plasma membrane to phagosomal membrane formation (127). These authors concluded

that the ER could only minimally contribute to the phagosomal membrane, as the membrane is initially derived from the plasma membrane and then from endosomes. We conclude that these findings are not disparate with our results, as we make no claims as to how ER proteins access the Mtb phagosome, nor have we tracked phagosomes early enough to define when these proteins first appear.

Although many groups have been able to detect ER proteins in latex bead phagosomes (122-125), others have been unable to detect ER proteins in latex bead and mycobacterial phagosomes (27, 127). We have several lines of evidence to suggest that our detection of these ER localized proteins is not due to contamination. First, we do not see the presence of all ER proteins in the Mtb phagosome, which is consistently negative for calnexin and calreticulin. Second, the level of staining of ER proteins in the Mtb phagosome does not correlate with the intracellular detection of these proteins in intact DC. Third, phagosomes are negative for the cis- and trans-golgi markers GM130 and golgin-97, respectively. All of these data are inconsistent with gross contamination of Mtb phagosomes with organellar membranes during homogenization, staining, and analysis. Finally, we used an allele specific marker to show that HLA-A2$^{+}$ membranes from uninfected cells do not contaminate Mtb phagosomes. Because of the high levels of HLA-A2 staining in intact DC, it is likely that we would detect the presence of HLA-A2 if there was significant contamination. From these data, we conclude that there could only be minimal contamination of Mtb phagosome preparations.

Interestingly, we found that HLA-A2 was not present in the Mtb phagosome at any timepoint examined, and we obtained similar results for HLA-B7 (data not shown). However, we detected relatively high levels of HLA-I at all timepoints using a pan-HLA-

I antibody that recognizes both classical and non-classical HLA-I molecules. These findings coupled with the observed phagosomal loading of HLA-E suggest that one mechanism by which HLA-E could uniquely function in antigen presentation is preferentially trafficking or enhanced recycling to the phagosome. Indeed, we are able to detect HLA-E in phagosome fractions by western blot (data not shown). We are currently assessing whether HLA-E is enriched in phagosomes relative to classical HLA-I molecules by comparing levels of HLA-E and non-HLA-E Class I in the phagosome and intact DC.

There is precedent for sequences that affect trafficking of MHC-I molecules. Lizee, et al demonstrated that a tyrosine residue in the cytoplasmic tail of MHC-I molecules was required for trafficking to the endosomal compartment and cross-presentation (224). While this finding has not been substantiated (225), further analysis of HLA-I allele specific trafficking patterns is needed. In the CD1 family, different isoforms traffic to different endosomal compartments, at least partially due to differences within the cytoplasmic tail (226-232). The HLA-E cytoplasmic tail is not drastically distinct from the HLA-I tail consensus sequence. It is four amino acids shorter than the HLA-A2 tail, but only one amino acid is missing compared to HLA-B alleles. In comparison to HLA-A2, the HLA-E tail has identical amino acids at 19/29 positions, and similar amino acids at another 2 positions. Nonetheless, several reports have described differences in binding of viral proteins between HLA-E and HLA-A2. Human Cytomegalovirus encoded US11 and HIV nef cause downregulation of cell surface HLA-A2, but not HLA-E (233-235). When the cytoplasmic tails were swapped, HLA-E became sensitive to the viral genes while HLA-A2 became resistant. Similar studies will

help us determine whether the cytoplasmic tail of HLA-E plays a role in phagosomal trafficking.

The lack of requirement of new protein synthesis for HLA-E presentation of Mtb antigen suggests that nascent HLA-E is unnecessary. This is similar to that seen in the vacuolar pathway (cycloheximide insensitive (132)), and presentation using this pathway has been hypothesized to use recycled MHC-I molecules (133, 170). To date, this result has been the most obvious difference between presentation of the HLA-E antigen and other Mtb-derived antigens that are processed using the cytosolic pathway, and our most provocative data suggesting that loading does not occur within the ER. This finding suggests several characteristics of the ER-phagosomal pathway. First, although the Mtb phagosome has access to ER proteins, it is unlikely that the majority of HLA-I is delivered from the ER. Second, the species of HLA-I that is loaded in this pathway is likely recycled molecules. Consistent with these assessments, Ackerman et al demonstrated that the majority of latex bead associated Class I was endoglycosidase H resistant (had trafficked through the golgi (236)). Furthermore, there is some intriguing data showing that HLA-E has a higher turnover rate than classical Class I molecules (147). We are currently performing studies to determine the relative amount of nascent and recycled HLA-I and HLA-E in Mtb phagosomes by examining endoglycosidase H sensitivity and the presence of cell surface-derived HLA-I in the phagosome.

The finding of members of the peptide loading complex in the Mtb phagosome is similar to what others have shown in latex bead phagosomes (122-125), however, we did not detect caltreticulin or calnexin. It is possible that these proteins are present at levels below our level of detection. Alternately, these proteins may be not delivered to Mtb

phagosomes or not retained as other ER molecules are. Gagnon et al demonstrated that calnexin is rapidly degraded in macrophage phagosomes, while the cytosolic tail was still detectable (125). These two members of the peptide loading complex are not likely to be required for peptide loading in the Mtb phagosome. Calnexin assists in the formation of the peptide loading complex but does not associate with Class I heavy chain:β2m heterodimers (89, 90). While calreticulin is a component of the peptide loading complex, calreticulin only associates with monoglucosylated Class I heavy chain (110), which is unlikely to be present on the recycling pool of HLA-I within the phagosome.

The finding of such high levels of PDI in the phagosome is intriguing. Until recently, PDI was not a known component of the peptide loading complex. Park et al demonstrated that PDI regulates the oxidation state of the disulfide bond in the Class I peptide binding groove, and decreasing PDI expression with siRNA decreases Class I maturation and thermostability (93). Furthermore, they showed that the peptide chaperone domain of PDI is important for optimal peptide loading. Both of these PDI roles may be important for peptide loading in the phagosome. In the proteolytic environment of the phagosome, the peptide chaperone function may protect TAP transported peptides from degradation. PDI may also be required to keep the disulfide bond in the peptide binding groove oxidized. Further study of the role of PDI will yield insights on whether there is a functional role in presentation of the HLA-E antigen.

In chapter 3, our goal was to determine the dominant pathway by which Mtb antigens are cross-presented. Our laboratory has had a long standing interest in the identification of immunodominant Mtb epitopes that are recognized by $CD8^+$ T cells. Using a T cell based approach, whereby $CD8^+$ T cells cloned on Mtb-infected DC are

used to identify the recognized antigen, minimal epitope, and restricting allele, we have identified numerous epitopes that for the most part comprise a significant proportion of the donor Mtb-specific $CD8^+$ T cell response. Interestingly, we have found that only a minority of the identified epitopes are 8mers and 9mers, with the majority being 10- and 11mers (172). Furthermore, all but one of the identified epitopes was presented by HLA-B alleles (172). We then used these clones as a readout for antigen presentation in the presence of inhibitors of individual components of antigen processing and presentation pathways.

We first attempted to distinguish cytosolic and non-cytosolic antigen presentation. Previous studies have shown that the cytosolic pathway requires proteasomal degradation and ER-golgi transport (although not for the ER-phagosomal pathway), but not acidification, while the inverse is true for non-cytosolic antigen presentation. Therefore, we initially screened Mtb antigen presentation using inhibitors of proteasomal degradation, ER-golgi trafficking, and acidification. This approach was successful for three of the seven epitopes studied. Presentation of $CFP10_{2-9}$, $Ag85B_{144-153}$, and $TbH9_{144-153}$ was blocked in the presence of epoxomicin and BFA, but not bafilomycin. These data suggest that these three epitopes are processed using the cytosol, and these epitopes were not further characterized. However, the remaining four epitopes ($CFP10_{2-12}$, $CFP10_{75-83}$, $DPV_{33-43}$, and $EsxJ_{24-34}$) gave conflicting results being sensitive to BFA, unaffected by bafilomycin, and enhanced by epoxomicin. BFA sensitivity and bafilomycin insensitivity suggests cytosolic processing, while the lack of a requirement for the proteasome is indicative of non-cytosolic processing. To resolve these discrepancies, we used several approaches. First, we show that presentation of $CFP10_{75-83}$ and $EsxJ_{24-34}$

does not require cathepsins which would likely be involved in non-cytosolic processing. Then we tested the requirement for cytosolic factors in presentation of these epitopes. Presentation of $CFP10_{75-83}$ and $DPV_{33-43}$ require TAP transport, a definitive requirement of cytosolic cross-presentation. Furthermore, presentation of $CFP10_{75-83}$ requires new protein synthesis, again characteristic of the conventional Class I pathway. Finally, DPV requires retrotranslocation from the phagosome for presentation of $DPV_{33-43}$, showing that this protein requires cytosolic access for presentation. Together, these data strongly support that presentation of the proteasome-independent epitopes occurs using the cytosolic pathway.

We have now looked at the processing requirements of ten epitopes from six Mtb proteins that are presented on eight different HLA-I alleles. Presentation of all of these epitopes occurs using the cytosolic pathway, suggesting that this is the main pathway of antigen presentation in Mtb-infected DC. There are several potential biases to this conclusion. First, although we have studied ten different epitopes, five of them are from CFP10, four of which are present in the amino terminal 12 amino acids. However, there are high frequency responses to these epitopes in the donor from which the epitope was defined, suggesting that presentation of these epitopes occurs efficiently in vivo. Furthermore, we have shown that CFP10 can be processed through different pathways depending on the delivery method (PLG microspheres versus Mtb (120)), meaning that presentation may not occur through the same pathway for every epitope. Intriguingly, we find that this is the case, as some CFP10 epitopes require proteasomal degradation and others do not. A second caveat is that there may be a bias in our $CD8^+$ T cell cloning procedure which would affect the epitopes that we identify. Although we find this

unlikely, we have discovered an unproportionately high number of HLA-B restricted epitopes (172). Again, however, the high frequency of responses to these epitopes suggests that if we are biased toward anything, it is towards detecting immunodominant epitopes. Another bias is that all of the proteins studied, except for TbH9 and the HLA-E antigen, are secreted proteins. These proteins may have easier access to the retrotranslocation machinery and cytosol than cell wall or intracellular proteins. Finally, we have not identified any epitopes from lipoproteins, which have been shown to have distinct vesicular trafficking patterns and can be processed in a TAP-independent manner (136-138). While we believe that Mtb antigens can be processed using alternate pathways, our findings suggest that immunodominant Mtb antigens preferentially use the cytosolic pathway.

Interestingly, in our analysis of these epitopes we have only identified one epitope that appears to preferentially use the ER-phagosomal pathway. This is surprising as our data indicates that a high proportion of phagosomes have detectable levels of TAP and PDI. Therefore, unless there are other factors at play, such as HLA-I allele-specific phagosomal trafficking discussed above, there should not be an inherent bias of loading within the ER or phagosome for a specific epitope. There does seem preferential loading in the ER, as presentation of all epitopes besides the HLA-E antigen is blocked by BFA. This does not exclude some portion of phagosomal loading for these epitopes, as very few are completely blocked. Unfortunately, we do not have an unequivocal way to distinguish between the traditional cytosolic and the ER-phagosomal pathway. We and others have noted that partial BFA sensitivity is characteristic of the ER-phagosomal pathway (Chapter 2, (124)), but it is hard to distinguish these pathways based on partial

inhibition as these results are not always clear cut. We have found that cycloheximide distinguishes between presentation of the HLA-E antigen and $CFP10_{3-11}$/$CFP10_{75-83}$, although more characterization is needed before we can conclude that this inhibitor is truly able to distinguish between the pathways. In fact, if we were to repeat the experiments in Chapter 3, we would likely screen epitope presentation in the presence of cycloheximide instead of bafilomycin. This would give us a potential marker for vacuolar/ER-phagosomal processing, and when combined with epoxomicin and BFA data, should more clearly define the presentation pathway. Until we define a more definitive indicator of the ER-phagosomal pathway and/or determine the HLA-I loading site for each epitope, we can not confidently discern the amount of loading that occurs within the ER or phagosome.

One potential way to discriminate the traditional cytosolic and ER-phagosomal pathway would be the requirement of recycled HLA-I for presentation. We have addressed this to an extent by the use of cycloheximide, which shows that newly synthesized HLA-E is not required for presentation. Analysis of the effect of cycloheximide on the remaining epitopes would determine the relative contribution of newly synthesized HLA-I required for presentation and further confirm the unique presentation pathway of HLA-E. To show the use of recycled HLA-I, we could deplete the HLA-I recycling pool by treating cells with acid and allowing internalization and subsequent degradation of cell surface HLA-I. Alternately, we could block endocytosis, and thereby recycling of HLA-I.

We hypothesized that cytosolic processing would be blocked by the proteasome inhibitor epoxomicin. To date, this is the most specific proteasome inhibitor, with no

known inhibition of other proteases (237, 238). However, presentation of four epitopes was enhanced by blocking proteasome function. Generally, generation of Class I presented peptides has been attributed to the chymotrypsin-like activity of the proteasome, which preferentially cleaves after large hydrophobic residues (83). Because epoxomicin inhibits mainly the chymotrypsin activity, it is possible that one of the other activities is required for epitope generation. However, the carboxy-terminus of the proteasome-independent epitopes does not fit with this. The trypsin-like activity of the proteasome cleaves after basic residues and the caspase-like activity cleaves after acidic residues (83). Analysis of the epitope sequence of the inhibitor enhanced epitopes shows none of these amino acids are present at the carboxy-terminus ($CFP10_{2-12}$:alanine, $CFP10_{75-83}$:tyrosine, $EsxJ_{24-34}$:tryptophan, $DPV_{33-43}$:glutamine). Furthermore, the proteasome-dependent $CFP10_{2-9}$ epitope contains an alanine at the carboxy-terminus, similar to the proteasome-independent $CFP10_{2-12}$ epitope. There are previous reports of enhanced antigen presentation in the presence of proteasome inhibition (195, 200, 239-242). In one case, this has been resolved by increasing inhibitor concentration (195). We have tried epoxomicin at concentrations up to 10μM, much higher than is generally required to block proteasome function, with no effect on presentation of $CFP10_{2-12}$. At this concentration, the trypsin-like activity of the proteasome should be mostly inhibited, while the caspase-like activity is minimally inhibited (243). It is unlikely that the caspase-like activity is involved in generation of any of these epitopes, but the use of site-specific inhibitors could be used to further rule this out. While we can not completely rule out a role of the proteasome in generation of these inhibitor-enhanced epitopes, it is likely that another cytosolic protease is required for proteolysis.

In other cases of proteasome inhibitor-enhanced antigen presentation, two groups have shown a role for tripeptidyl peptidase II. Seifert, et al demonstrated that TPPII generated the carboxy-terminus of an HIV nef epitope (200), while Guil et al showed a requirement of TPPII in presentation of an influenza nucleoprotein epitope (241). Furthermore, inhibition of TPPII activity leads to a decrease in cell surface expression of HLA-I, similar to that seen with proteasomal blockade, suggesting a large role of TPPII in peptide processing for Class I presentation (88). However, we saw no effect of TPPII inhibition on presentation of $CFP10_{75-83}$ or $EsxJ_{24-34}$. Unfortunately, we do not have a positive control for blocking TPPII function. Once again, sequence analysis of the proteasome-independent epitopes suggests that TPPII is not the relevant protease. TPPII preferentially cleaves after lysine and arginine residues, but has also been reported to cleave after proline and glutamic acid (200, 206). There is a valine at the carboxy-terminus of the TPPII-dependent influenza epitope, although the authors did not directly show that TPPII generated the correct cleavage (241). Nonetheless, none of these amino acids are present at the carboxy-terminus of the proteasome-independent epitopes. As TPPII is the only other cytosolic protease that has been demonstrated to generate the correct carboxy-terminus of Class I presented epitopes, these findings suggest that a novel cytosolic protease or proteases may play a major role in Mtb cross-presentation and in antigen presentation in general.

One unique situation of phagosomal antigens that reach the cytosol is the access to both vacuolar and cytosolic proteases. While it has been shown that the C-termini of epitopes are created by proteasomal or TPPII degradation (83, 200), this may not necessarily be true for proteins that have access to another repertoire of proteases (i.e.

cathepsins). In fact, antigens that are processed by the ER-phagosomal or cytosolic pathway may uniquely require both proteasomal and cathepsin degradation, although we have found no effect on presentation of the HLA-E antigen, $CFP10_{75-83}$, or $EsxJ_{24-34}$ by the cathepsin S and D inhibitors leupeptin and pepstatin A, respectively (data not shown and Chapter 3, Figure 3). However, this does not exclude a role for proteolysis in multiple compartments. Fontenaeu et al demonstrated that cross-presentation of cell associated antigen was enhanced with the addition of exogenous cathepsin D and inhibited in the presence of pepstatin A (196). Presentation of this antigen uses the cytosolic pathway, as ICP47, BFA, and lactacystin all blocked presentation. Together, these data suggest that protease activity is required both before and after cytosolic access. In this system, the proteasome generates the correct carboxy-terminus of the epitope. In our system, it is possible that the carboxy-terminus is generated within the phagosome, and cytosolic aminopeptidases are required for amino-terminal trimming. This possibility requires further experimental evidence.

Our goal is to define the protease required for generation of these four epitopes. First, we need to rule out a role of the proteasome by testing alternate proteasome inhibitors (including trypsin- and caspase-like site specific inhibitors) and/or examining the peptides generated from recombinant Mtb antigens by purified proteasomes. These experiments will either confirm or exclude a role of the proteasome in epitope generation. If the proteasome is not the relevant protease, we plan to examine cytosolic fractions or purified cytosolic and endosomal proteases to determine their cleavage of recombinant Mtb antigens. These experiments will help define the protease involved in presentation of these epitopes.

In chapter 4, we examined the role of RD1 in the presentation of Mtb antigens. First, we showed that transposon insertion in RD1 genes resulted in decreased or lack of secretion of CFP10 and ESAT-6. Mtb strains that lacked secretion were not recognized by a $CD8^+$ T cell clone specific for $CFP10_{2-11}$, even though CFP10 was expressed by the bacteria. However, RD1 mutants were recognized by a clone specific for $DPV_{61-69}$.

There have been many functions speculated for RD1 due to the presence of this region in Mtb strains and the absence in the vaccine strain, BCG (181, 207-210). Certainly, deletion of RD1 from Mtb decreases virulence in vitro and in vivo, giving a phenotype similar to BCG infection (184). Conversely, addition of RD1 to BCG leads to a partial increase in virulence, although not to the same level as Mtb (244). BCG expressing ESAT-6 and CFP10 serve as a better vaccine for Mtb challenge in both mice and guinea pigs (245). Interestingly, when ESAT-6 is introduced into BCG, secretion requires either a secretory sequence or adjacent RD1 genes (212, 245). This and the observation that ESAT-6 and CFP10 lack an obvious secretory signal led to the hypothesis that RD1 encodes a novel secretion system. In collaboration with David Sherman, we analyzed the secretion of CFP10 and ESAT-6 in H37Rv strains missing the entire RD1 region or mutated in individual RD1 genes. We saw decreased or absent secretion of both proteins in strains with transposon insertions in Rv3870, Rv3871, and Rv3876, while protein was detected within the bacterial cell (Chapter 4, Figure 1 (182)). Complementation of these strains with plasmids encoding the missing genes led to a recovery of secretion. We used these and other data to argue that RD1 does in fact encode a secretion system that serves to secrete ESAT-6 and CFP10 and potentially other Mtb proteins (182). At the same time, two other groups reported similar findings (183,

185).

Interestingly, ESAT-6/CFP10 secretion mutants showed a distinct defect in cell to cell spread. The mutants were able to infect macrophages, but accumulated intracellularly (182, 185). Furthermore, Hsu et al demonstrated that ESAT-6 was able to disrupt lipid bilayers (183). Initial studies looking at Mtb cross-presentation suggested that live Mtb is able to form a pore in the phagosomal membrane (118, 119). We then hypothesized that RD1 might play a pivotal role in access of Mtb antigens to the cytosol. Analysis of CFP10 presentation in DC infected with RD1 mutants showed that presentation of the cytosolically processed $CFP10_{2-11}$ epitope requires ESAT-6/CFP10 secretion. However, presentation of $DPV_{61-69}$ was not inhibited in RD1 mutants, suggesting that RD1 is not required for the cross-presentation of all Mtb antigens, and likely not involved in cytosolic access of Mtb antigens. Unfortunately, we have not demonstrated that the $DPV_{61-69}$ epitope is processed cytosolically, but we have shown this to be true for $DPV_{33-43}$ (Chapter 3). Also, we found similar results with the cytosolically processed HLA-E antigen (data not shown). Our finding that every epitope examined is processed cytosolically suggests that it is unlikely that $DPV_{61-69}$ is processed by the vacuolar pathway. Furthermore, Billeskov et al similarly found that RD1 is not required for priming of $TB10.4_{3-11}$ $CD8^+$ T cells in mice (69).

It is not surprising that RD1 does not function for cytosolic access of Mtb antigens. Since the initial observations in the laboratory of Dr. Barry Bloom suggesting that Mtb phagosomes are permeable (118, 119), great strides have been made in understanding cross-presentation. The access of bead associated OVA to the cytosol suggest that cellular factors are able facilitate transport of antigens across the phagosomal

membrane. Ackerman, et al demonstrated that latex bead phagosomes acquire the cellular retrotranslocation machinery which performs the above mentioned function (126). We have shown that the retrotranslocation machinery is required for presentation of the HLA-E antigen, $CFP10_{3-11}$, and $DPV_{33-43}$, further refuting a requirement of RD1 in cytosolic access. The use of a cellular factor for cytosolic access does not explain the differences seen in the "permeability" of phagosomes containing live and dead Mtb. Because the retrotranslocation machinery can be recruited to latex bead phagosomes, it is likely that it should be recruited to phagosomes containing heat-killed Mtb. However, this machinery may be recruited more efficiently or may be longer lived in live Mtb phagosomes. Alternately, Mtb may encode other factors that aid in cytosolic access of Mtb proteins, as there is at least some benefit to the bacteria in targeting proteins to the cytosol (18, 246). The ESAT-6 membrane lysis activity is therefore is likely to be more important for lysing host cells (182, 185) or bacterial escape from the phagosome into the cytosol (27). In summary, these results exclude a significant role of RD1 in cross-presentation of Mtb antigens.

***Summary:***

The data presented in Chapter 2 show that the Mtb phagosome is able to participate in the cross-presentation of Mtb antigens. The phagosome acquires the cellular retrotranslocation machinery which serves to transport Mtb antigens into the cytosol where proteasome degradation occurs. Although we have not formally shown that functional TAP is present in the phagosome, its presence there and the TAP requirement for HLA-E antigen presentation suggests that the phagosome is also able to

participate in peptide transport. Finally, peptide loading can occur within the phagosome, potentially using recycled HLA-I molecules. These findings represent a significant step forward in the understanding of cross-presentation of Mtb antigens and put forth the attractive hypothesis that HLA-E may play a unique and specialized role in the detection of phagosomal pathogens.

The results presented in chapter 3 demonstrate that immunodominant Mtb antigens are primarily processed and presented using the cytosolic pathway. While the cytosolic pathway has been thought by many to be the dominant pathway of cross-presentation (134, 140), these results are almost all based on cross-presentation of OVA using various models and delivery systems. Also, one group has shown that the cross-presentation of parasite-derived OVA uses the non-cytosolic pathway (144). We show that multiple native Mtb antigens, which elicit high frequency $CD8^+$ responses and are therefore presented in vivo during the course of Mtb infection, preferentially use the cytosolic pathway. The number of epitopes examined is the broadest study of cross-presentation to date, and these findings verify what has been shown in somewhat contrived systems, and greatly add to the characterization of Mtb antigen presentation. Furthermore, although we need to formally exclude a role of the proteasome and TPPII in presentation of the proteasome inhibitor enhanced epitopes, these findings suggest that a novel protease or proteases may be involved in presentation of Mtb antigens.

Finally, in chapter 4 we examine the role of RD1 in presentation of Mtb antigens. We report that RD1 encodes a secretion system that is required for the secretion of two immunodominant Mtb proteins, ESAT-6 and CFP10. Cross-presentation of CFP10 requires secretion of these two proteins, as evidenced by lack of presentation in RD1

mutants which express CFP10 but do not secrete it. However, presentation of DPV did not require ESAT-6/CFP10 secretion, suggesting that RD1 is not required for the cross-presentation of Mtb antigens. In conjunction with the retrotranslocation data presented in chapter 2, these findings exclude that effectors secreted by RD1 are required for cytosolic access of Mtb antigens.

These data argue for the following cross-presentation pathway of Mtb-derived antigens (Figure 1). After phagocytosis, a proportion of Mtb resides in a compartment that contains members of the PLC. Mtb antigens are then translocated out of the phagosome into the cytosol. Subsequently, antigens are degraded by the proteasome or a novel cytosolic protease. Peptide fragments are then transported by TAP into the ER or phagosomal lumen, where they are loaded onto nascent HLA-I or potentially recycled HLA-E, respectively. The factors that determine which pathway a specific antigen follows remain to be elucidated, but may depend on HLA-I trafficking/recycling or properties of the specific antigen.

The finding that the Mtb phagosome participates in antigen presentation has important implications for pathogen recognition. Cells have specific mechanisms to alert the immune system of intracellular infection using HLA-I molecules or exogenous antigens using HLA-II. Thus, it is not surprising that a pathway is in place that allows HLA-I to sample phagosomal antigens and relay this information to $CD8^+$ T cells. This ensures loading of phagosome-derived pathogen-specific epitopes on HLA-I molecules and provides a novel mechanism for $CD8^+$ T cells to identify cells infected with non-cytosolic pathogens.

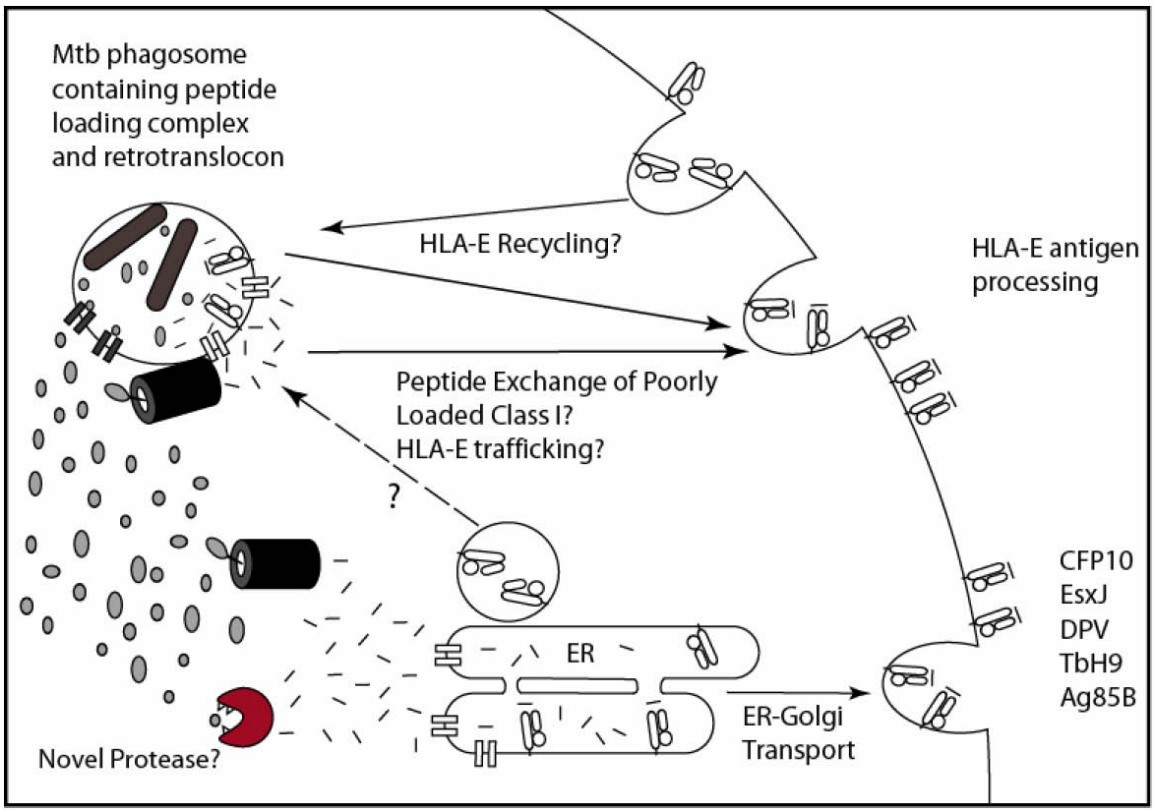


Figure 1. Cross-presentation pathways of antigens studied in this report. See text for details.

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