HLA-E Presents Glycopeptides from the *Mycobacterium tuberculosis* Protein MPT32 to Human CD8⁺ T cells

Melanie J. Harriff, Lisa M. Wolfe, Gwendolyn Swarbrick, Megan Null, Meghan E. Cansler, Elizabeth T. Canfield, Todd Vogt, Katelynne Gardner Toren, Wei Li, Mary Jackson, Deborah A. Lewinsohn, Karen M. Dobos & David M. Lewinsohn

Infection with *Mycobacterium tuberculosis* (MtB), the bacterium that causes tuberculosis, remains a global health concern. Both classically and non-classically restricted cytotoxic CD8⁺ T cells are important to the control of MtB infection. We and others have demonstrated that the non-classical MHC I molecule HLA-E can present pathogen-derived peptides to CD8⁺ T cells. In this manuscript, we identified the antigen recognized by an HLA-E-restricted CD8⁺ T cell clone isolated from an MtB latently infected individual as a peptide from the MtB protein, MPT32. Recognition by the CD8⁺ T cell clone required N-terminal O-linked mannosylation of MPT32 by a mannosyltransferase encoded by the Rv1002c gene. This is the first description of a post-translationally modified MtB-derived protein antigen presented in the context of an HLA-E specific CD8⁺ T cell immune response. The identification of an immune response that targets a unique mycobacterial modification is novel and may have practical impact in the development of vaccines and diagnostics.

Tuberculosis (TB), caused by infection with *Mycobacterium tuberculosis* (MtB), remains a leading cause of infectious disease morbidity and mortality worldwide, with 10.4 million new cases, including nearly 500,000 multidrug-resistant (MDR-TB) cases, and 1.8 million deaths in 2015 according to the 2016 Global Tuberculosis Report (World Health Organization). The lack of an effective vaccine, the emergence of MDR-TB, and co-infection with HIV have hampered the effort to eradicate TB. Nonetheless, most people infected with MtB successfully contain the infection. Critical to this containment is a robust, TH1-type cellular immune response. While CD4⁺ T cells are essential in containing MtB infection, CD8⁺ T cells play a unique role through their ability to preferentially recognize and eliminate heavily infected cells, as well as their ability to recognize and lyse Class II negative cells. Furthermore, CD8⁺ T cells are particularly important in mouse and non-human primate models of persistent MtB infection, highlighting the importance of eliciting a CD8⁺ T cell response during vaccination.

CD8⁺ T cell antigens are presented on classical (MHC Class Ia) or non-classical (MHC Class Ib) molecules. Classical Class I molecules, encoded by HLA-A, -B, and -C, are highly polymorphic and present a highly diverse array of specific peptide sequences. Non-classical Class I molecules, on the other hand, have limited polymorphism and present pathogen modified antigens or altered self-ligands in the context of infection. For example, CD1 molecules present glycolipids and MR1 molecules present Vitamin B metabolites. Additionally, while CD8⁺ T cells restricted by classical Class I molecules undergo clonal expansion and require time to manifest their effector responses, CD8⁺ T cells restricted by non-classical Class I molecules such as MR1 are immediately able...
to elicit effector mechanisms upon encounter with antigen. The human CD8+ T cell response to Mtb infection has been shown to involve antigen presented on both classical and non-classical Class I molecules. HLA-E is a non-classical Class I molecule that was originally described as binding ligands for NK receptors9–11. However, many have shown that HLA-E also restricts αβ T cells that respond to pathogen-derived antigens12–15.

HLA-E has been well-studied in the context of its ability to regulate NK cell activation through interaction with CD94-NKG2 receptors. In this regard, HLA-E binds leader sequence peptides derived from Classical MHC-I molecules, and the levels of HLA-E cycling to and from the surface are regulated by the availability of MHC-I leader sequences9. When MHC-I expression is disrupted, such as during a viral infection, the altered HLA-E surface expression is recognized by NK cells via the CD94-NKG2 receptor, and the cell is more susceptible to NK cell lysis. Surprisingly little is known about the pathogen-derived ligands for HLA-E, and how these ligands contribute to interaction with the T cell receptor (TCR) of HLA-E restricted αβ T cells. A limited number of studies have shown that there is recognition of pathogen-derived ligands presented on HLA-E that occurs through interaction with the TCR15–16. For example, HLA-E restricted T cells recognize peptides from the GroEL protein of Salmonella typhi and secrete granzyme B and IFN-γ in response to S. typhi-infected target cells16. Joosten et al.15 used bioinformatics analysis and subsequent functional characterization to identify potential HLA-E ligands from Mtb, and we have also recently identified HLA-E ligands presented in the context of Mtb infection (unpublished data). Both studies identified numerous peptides that were recognized by CD8+ T cells from PPD+ donors15, as well as TB patients and healthy donors (unpublished data). Interestingly, recent studies analyzing T cell responses to predicted Mtb HLA-E epitopes demonstrate that HLA-E restricted CD8+ T cells can have a Th2 phenotype17,18. These studies underscore the potential importance of HLA-E in the response to Mtb.

The limited polymorphism of HLA-E and lack of down-regulation during HIV infection makes the continued identification of Mtb-specific HLA-E antigens an attractive goal in developing novel vaccine candidates or diagnostic targets. In fact, recent studies demonstrate that HLA-E plays an important role in the expansion of SIV-reactive T cells following vaccination of rhesus macaques19. Therefore, we used proteomics and mass spectrometry coupled with immunological assays to identify the cognate antigen for a previously described HLA-E restricted CD8+ T cell clone isolated from a latently infected individual (D160 1–23)13. We show that the antigen associated with the D160 1–23 T cell clone is the post-translationally modified glycoprotein MPT32 (also called Rv1860 or Apa), and that the epitope for the clone relies on N-terminal O-linked mannosylation of this protein. This is the first description of a glycoprotein ligand associated with HLA-E, and has implications with regard to identification of additional pathogen-relevant HLA-E ligands.

Results
Proteomic characterization of Mtb subcellular fractions recognized by a human HLA-E restricted T cell clone, D160 1-23. We previously isolated an HLA-E restricted CD8+ T cell clone from a latently infected individual (D160 1–23)13. To identify potential HLA-E specific antigens, subcellular fractions generated from Mtb whole cell lysate and the culture filtrate were tested against D160 1–23 in IFN-γ ELISPOT assays. The activity of this T cell clone was unique to the cell wall fraction and was enhanced when tested against cell wall material subjected to organic extraction to remove non-covalently cell wall associated lipids (delipidated cell wall, dCW) (Fig. 1a). Proteolytic digestion of dCW by the non-specific protease pronase resulted in further enhancement of T cell activity to a single peptide fraction. Preliminary LC-MS/MS analysis of the peptides isolated to this fraction (data not shown). Reverse phase HPLC fractionation of pronase-digested dCW successfully isolated biological activity to a single peptide fraction. Preliminary LC-MS/MS analysis of the peptides isolated to this fraction identified several proteins, with four related peptides confidently identified from the protein chaperone HspX (Table 1).

T cell reactivity is contained to RP-HPLC fractions that contain the Mtb protein MPT32 (Rv1860). Synthetic analogs of overlapping peptides from HspX were tested for their ability to activate the D160 1–23 clone; however, no T cell activity was identified for these synthetic reagents (data not shown). Interrogation of native HspX demonstrated that neither this protein, nor any of its peptides, elicited a response by D160 1–23 (data not shown). As we and others have demonstrated that biological activity is associated with the native protein as a result of its chaperone function20, an HspX knockout mutant, X4-19 ΔHspX, was used to determine whether HspX chaperoned the cognate antigen or was essential to retain/stabilize the biologically active product. From these studies we confirmed that T cell reactivity was enhanced in pronase-digested delipidated cell wall from the X4-19 ΔHspX strain of Mtb (Fig. 1a). This validated that the HspX protein does not contain the HLA-E ligand for D160 1–23 and was not required for its activity; thus the X4-19 ΔHspX strain was used for all subsequent experiments to remove this confounding protein.

RP-HPLC fractionation and testing for T cell activation was repeated with pronase-digested whole cell lysate (WCL) from the X4-19 ΔHspX strain, and biological activity was consistently reduced primarily to one fraction, F9 (Fig. 1b). LC-MS analyses of active fractions were used to generate a consensus peptide list (Table 2). Again, none of these candidate proteins, produced as synthetic peptide reagents, nor recombinant or native proteins, demonstrated biologic activity when tested (data not shown). To further resolve the source of the observed T cell activity, the active fraction was subjected to additional treatments. Boiling with 4% SDS and 1 mM DTT for 30 min to reduce the proteins did not ablate IFN-γ secretion by D160 1–23 (Fig. 1c). Additional RP-HPLC of F9 on an extended gradient of acetonitrile associated T cell activity to sub-fraction F9-7 (Fig. 1d). Peptide fragments ranging from 700 Da to 2100 Da were visualized in Fraction F9-7 using matrix-assisted laser desorption ionization, time-of-flight (MALDI-TOF) mass spectrometry. Through complementary analyses of sub-fraction F9-7 MALDI-TOF and F9 LC-MS data, peptide sequences could be assigned for several ion species identified by MALDI-TOF (Table 3). This included the identification of several peptides from MPT32 (Rv1860), a protein that was not identified in other fractions.
The amino terminus of native MPT32 contains the HLA-E epitope. MPT32, a 45 kDa alanine and proline-rich glycoprotein, is a known CD4+ and CD8+ T cell antigen that is secreted into the culture filtrate of actively growing cells. To confirm that MPT32 contains the antigen for D160 1–23, native MPT32 was isolated from the culture filtrate proteins of Mtb. Whole or pronase-digested native MPT32 and CFP were then used in an ELISPOT assay with IFN-γ release by the HLA-E restricted T cell clone, D160 1–23, as a readout. Pronase-digested MPT32 resulted in strong IFN-γ production by D160 1–23, as did pronase-digested CFP (Fig. 2a). Titration of purified pronase-digested native MPT32

Table 1. Peptides identified from LC-MS analysis of pronase-digested dCW active fraction. Proteins and peptides were accepted as potential candidates if probability scores were calculated as greater than or equal to 95% and 90%, respectively.
MPT32 protein sequence. All unique peptide sequences identified in F8 are listed in Table 4. Tandem MS analyzed a total of 10 unique peptides (normalized weighted spectra), with F8 containing approximately 20% of the fragments belonging to the N-terminus of MPT32.21 To identify glycosylated MPT32 peptides, we performed GlycanX analysis, which uses Skyline, an open-source application for building quantitative mass spectrometry assays. Here, we used Skyline to build a quantitative method for identifying glycopeptides of MPT32 that are putatively generated following digestion with Pronase. MS1 data corresponding to known glycopeptides of MPT32 that are putatively generated following digestion with Pronase was acquired from F7, 8, and 9 using this method and identified one MS1 signal uniquely abundant in F8, corresponding to the native epitope within the MPT32 protein.

Native MPT32 glycosylated peptides are antigenic. We employed several methods to define the minimal epitope within the MPT32 protein. Testing of overlapping synthetic peptides representing the regions of MPT32 identified in F8 by ELISPOT assay with D160 1–23 did not result in any biological activity (Fig. 3a). Because some amino acids of the MPT32 sequences identified in the fraction are glycosylated, and overlapping synthetic peptides did not induce T cell responses, we used several methods to evaluate whether MPT32 glycosylation was required for T cell response. First, we enriched for glycopeptides present in the native MPT32 pronase digest using a ConA lectin column. T cell activity was predominantly retained in the ConA column eluate containing mannosylated proteins (Fig. 3b), supporting the requirement for glycosylation. Next, we generated constructs to express recombinant MPT32 with point mutations at three mannosylated N-terminal threonines (T10V, T18V, and T27V) in a ΔMptX Mtb strain. Culture filtrate protein from the recombinant MPT32 strains was pronase digested and tested for T cell activity. Substitution of valine for threonine at position 10 (T10V) had no effect on T cell response, substitution of valine for threonine at position 18 (T18V) resulted in an increased T cell response, while substitution of valine for threonine at position 27 (T27V) completely abrogated the response (Fig. 3c). Finally, we tested Mtb with a complete deletion of the Mtb protein, ΔRv1002c, which encodes a mannosyltransferase that modifies proteins at serine and threonine residues with mannose26, including MPT32.27 Analysis of the antigenic activity of pronase digested whole cell lysates and cell wall preparations from the ΔRv1002c mutant indicated that O-mannosylation is required for eliciting T cell responses (Fig. 3d). To validate these results, we tested for the ability of the D160 1–23 clone to recognize infection with Mycobacterium smegmatis, a non-pathogenic relative of Mtb that expresses a homolog of MPT32 (30% sequence homology at the N-terminus) that does not contain a threonine at positions 10, 18, or 27 (Fig. 3e). These results strongly support the hypothesis that it is highly likely that these responses are HLA-E restricted.

To identify the MPT32 antigen, the complexity of the source of biological activity in the native MPT32 protein was further reduced by performing RP-HPLC fractionation of pronase-digested native MPT32. The biological activity of MPT32 was primarily isolated to a single fraction, F8 (Fig. 2d). LC-MS analysis of fraction 8 identified a total of 10 unique peptides (normalized weighted spectra), with F8 containing approximately 20% of the fragments belonging to the N-terminus of MPT32.21 To identify glycosylated MTP32 peptides, we performed GlycanX analysis, which uses Skyline, an open-source application for building quantitative mass spectrometry assays. Here, we used Skyline to build a quantitative method for identifying glycopeptides of MPT32 that are putatively generated following digestion with Pronase. MS1 data corresponding to known glycopeptides of MPT32 that are putatively generated following digestion with Pronase was acquired from F7, 8, and 9 using this method and identified one MS1 signal uniquely abundant in F8, corresponding to the native epitope within the MPT32 protein.

Table 2. Peptides identified from LC-MS analysis of fraction 9 of pronase-digested WCL from the X4-19 ΔHspX Mtb strain.

| Protein accession number | Protein name | Observed mass (M + H) | Peptide sequence |
|--------------------------|--------------|----------------------|------------------|
| Rv00129c, Rv1886, Rv3804 | Ag85 complex | 1017.15 (F)SRPGLPVEY(E) |
| Rv3418c | GroES | 1171.27 (W)DGEDEKRIPL(D) |
| Rv2101 | HeZ | 1394.53 (T)LEEKIDEM(ox)IEE(K) |
| Rv3417 | GroEL | 1135.24 (A)KEKLEDIP(E) |
| Rv3763c | LpqH | 1375.58 (T)GVDMANPM(ox)SPVYNK(S) |

Table 3. Peptides identified from MALDI-TOF analysis of fraction 9–7 of pronase-digested WCL from the X4-19 ΔHspX Mtb strain.

| Protein accession number | Protein name | Observed mass (M + H) | Peptide sequence |
|--------------------------|--------------|----------------------|------------------|
| Rv1880 | Mpt32 | 933.07 | APPAPAPAPAGEVAP |
| Rv2560 | Hypothetical protein Rv2560 | 1203.39 | GPPGPQPPGYPP |
| Rv0014c | PknB | 2162.47 | NPPANQTSNVTNVIIIWGS |
| Rv3448 | EccD4 | 2164.69 | PWIAALTMLAAVMLGFA |

...
the conclusion that the epitope for the HLA-E restricted D160 1–23 clones includes the N-terminal T27 and that Mtb-specific O-linked mannosylation is required for HLA-E T cell activity.

**Discussion**

CD8+ T cells play an important role in protective immunity against Mtb. CD8+ T cells recognize a highly diverse repertoire of peptides associated with classical class I molecules (HLA-Ia). Non-classical HLA-Ib molecules also recognize a variety of mycobacterial antigens, including glycolipids presented on CD1 molecules and vitamin B metabolites presented on MR1, and protein-based ligands presented on HLA-E. Although a role for non-classical CD8+ T cells during Mtb infection is not yet clear, non-classically restricted CD8+ T cells make up a majority of Mtb-specific CD8+ T cell response, suggesting they may be important to the immune response to Mtb infection. HLA-E has limited polymorphism, and is likely to present a limited repertoire of antigens. Additionally, in contrast to classical class I molecules, HLA-E is

**Table 4.** Peptides identified from RP-HPLC analysis of fraction 8 of pronase digested native MPT32.
not downregulated during HIV infection. Together, these factors make targeting Mtb-specific HLA-E restricted CD8+ T cell responses a novel and attractive approach for vaccine development.

We previously isolated an Mtb-specific HLA-E restricted CD8+ T cell clone (D160 1–23) from a latently infected individual, and sought to use this clone to identify Mtb ligands for HLA-E. Prior definition of D160 1–23 allowed for a biochemical and proteomic approach to identify the cognate ligand. Using these methods, we demonstrate that the N-terminal O-mannosylated glycopeptide of MPT32 contains the epitope for D160 1–23. O-mannosylation of serine or threonine residues of mycobacterial proteins has been described for T cell antigens including the 19 kDa lipoprotein and the B cell antigen SodC, however it is not known whether glycosylation of these proteins contributes to cognate interactions between the class I molecule and the T cell receptor. MHC-I molecules are capable of binding and presenting glycosylated peptides, and presentation of aberrant glycosylated peptides is thought to be important to T cell recognition of tumor antigens. This is the first description of a pathogen-derived glycosylated antigen recognized by HLA-E restricted CD8+ T cells. These findings may be important to vaccine and drug development as the enzymes responsible for glycosylation of MPT32 are specific to mycobacteria. In this regard, even between Mtb and the related non-pathogen M. smegmatis, there are differences in the degree of glycosylation of MPT32. The potential importance of glycosylation to T cell recognition during Mtb infection is underscored by the inability of D160 1–23 to recognize M. smegmatis-infected target cells.

MPT32 is a relatively well-characterized secreted Mtb protein. MPT32 was first described by Nagai et al. in 1991 as a component of the Mtb secretome. Sites of MPT32 glycosylation were then thoroughly characterized by Dobos et al. and Romain et al. In addition to stimulating antibody production, MPT32 also strongly induces the production of IFN-γ by both CD4+ and CD8+ T cells. Additionally, MPT32 provides protection in guinea pig challenge models when administered as DNA or protein subunit vaccine. Although MPT32 is not glycosylated when administered as a component of subunit vaccines, a number of groups have looked at the impact of glycosylation on antigenicity, immunogenicity, and protection. Romain et al. demonstrated that deglycosylation of MPT32 resulted in reduced DTH reaction in BCG immunized guinea pigs as well as reduced responses of CD4+ T cells in ex vivo stimulation assays. Similarly, Horn et al. demonstrated that deglycosylation of MPT32 resulted in decreased stimulation of T cells isolated from the lymph nodes of BCG vaccinated

---

**Figure 3.** Native MPT32 glycosylated peptides are antigenic. (a–d) DC were pulsed with the following samples and used as antigen presenting cells in an ELISPOT assay with IFN-γ release (spot forming units (SFU)) by the HLA-E restricted T cell clone, D160 1–23, as a readout. (a) Synthetic overlapping peptides representing Mpt32. (b) Flow-through, wash, and eluate following concavalin-A column enrichment of pronase-digested native Mpt32. (c) Pronase-digested CFP from the Mpt32 T10V, T18V, and T27V recombinant Mtb strains. (d) Pronase digested whole cell lysates and culture filtrate proteins from wild-type Mtb or the ΔRv1002c Mtb mutant. (e) DC were infected with M. smegmatis (Msm) or Mtb as a positive control or left uninfected (UI) and used as antigen presenting cells in an ELISPOT assay with IFN-γ release (spot forming units (SFU)) by the HLA-E restricted T cell clone, D160 1–23, as a readout. Blocking with the W6/32 antibody is shown for Mtb infected cells. Results are the mean and standard deviation from four independent experiments.
glycolipids. Lyophilized cell wall was subjected to two extractions of 2 h each followed by one 18 h extraction via BCA (Thermo Pierce). 4 °C for 4 h to recover cell membrane. Cell wall, membrane and cytosol were all dialyzed and protein quantified water (10:10:3, v/v/v) each at 22 °C. The fully delipidated cell wall was dried under N2 and resuspended in PBS, was resuspended in phenyl sepharose buffer A (10 mM KH2PO4 (pH 7.2), 1 mM EDTA, 1 mM DTT (1 L) and 0.1% ASB-14 pH 7.4 to maximize solubility. Cell wall protein was quantified by bicinchoninic acid (BCA) assay via filtration through a 0.22 μm membrane and concentrated using a stirred cell apparatus under N2, followed by dialysis into 0.01 M NH4HCO3, and centrifuged at 16,000 × g at 4 °C for 1 h to generate cell wall. The supernatant was subsequently centrifuged at 100,000 × g for 30 minutes and supernatant removed for a second salt cut at 70%. The 40% pellet was dried under N2 and further extracted twice for 2 h and one 18 h extraction with chloroform/methanol (2:1, v/v) at a ratio of 30 mL/g of cell wall. Extractions were performed at 22 °C with agitation. Centrifugation at 27,000 g for 30 min was performed to collect cell wall material. The 2.1 extracted cell wall was dried under N2 and further extracted twice for 2 h and one 18 h extraction with chloroform/methanol/ water (10:10:3, v/v/v) each at 22 °C. The fully delipidated cell wall was dried under N2 and resuspended in PBS, 0.1% ASB-14 pH 7.4 to maximize solubility. Cell wall protein was quantified by bicinchoninic acid (BCA) assay (Thermo Pierce).

Purification of Native MPT32. Ammonium sulfate (NH4)2SO4 was added to the culture filtrate proteins (CFP) to a final concentration of 40% and proteins allowed to separate for 1 hour at 4 °C. The CFP was then centrifuged at 16,000 × g for 30 minutes and supernatant removed for a second salt cut at 70%. The 40% pellet was resuspended in phenyl sepharose buffer A (10 mM KH2PO4 (pH 7.2), 1 mM EDTA, 1 mM DTT (1 L) and a gradient of buffer B (10 mM Tris-base (pH 8.9), 1 mM EDTA, 1 mM DTT (1 L), then C (10 mM Tris-Base (pH

Materials and Methods  Human Subjects and ethics statement. This study was conducted according to the principles expressed in the Declaration of Helsinki. All study participants were adults. Study participants, protocols, and consent forms were approved by the Oregon Health & Science University Institutional Review Board (OHSU IRB0000186). Written informed consent was obtained from all participants and all samples were anonymized using a random three digit code by the study coordinator.

Peripheral blood mononuclear cells (PBMC) were obtained by apheresis from healthy, LTBI, and Mtb-infected adult donors recruited from OHSU as previously described45, or via IRB-approved advertisement at local TB clinics. Uninfected individuals were defined as healthy individuals with a negative tuberculin skin test and no known risk factors for infection with Mtb. Individuals with LTBI were defined as healthy individuals with a positive tuberculin skin test and no symptoms and signs of active TB. Individuals with active TB were diagnosed by the TB Controller for Multnomah or Washington Counties in Oregon, United States, and confirmed by positive sputum culture for Mtb.

Bacteria and Cell Lines. Mycobacterium tuberculosis strain H3Rv was originally provided as strain TMC102 from the Trudeau Mycobacterial Collection and is currently available from the Biodefense and Emerging Infections Resources Repository (BEI Resources, Manassas, VA). Similarly, CDC1551 was originally provided from the Centers for Disease Control and Prevention and is available from BEI Resources. X4-19 (∆HspX) was kindly provided from Russel Karls and Fred Quinn (University of Athens, GA).

A549 cells were obtained from the American Type Culture Collection (ATCC, CCL-185) and cultured in F12K with 10% heat inactivated FBS. Human monocyte-derived dendritic cells (DC) were prepared as described46. Briefly, PBMC obtained as described above were resuspended in RPMI with 2% heat-inactivated human serum (HuS) and allowed to adhere to a T-75 (Costar) flask at 37°C for 1 h. After gentle rocking, non-adherent cells were removed and 10% heat-inactivated HuS in RPMI containing 10 ng/ml IL-4 (R&D Systems) and 30 ng/ml GM-CSF (Sanofi) was added to the adherent cells. After 5 days, cells were harvested with cell-dissociation medium (Sigma-Aldrich) and used as indicated in assays. The D160 1–23 human HLA-E restricted CD8+ T cell clone was expanded and maintained as previously described13.

Isolation of Subcellular Fractions and Delipidation of Cell Wall. M. tuberculosis, H3Rv was cultured in glycerol-alanine-salts (GAS) media for 14 days at 37°C, followed by harvest of supernatant and cells, lysis and subcellular fractionation as described previously47. Briefly, culture filtrate proteins were separated from cells by filtration through a 0.22 μm membrane and concentrated using a stirred cell apparatus under N2, followed by dialysis into 0.01 M NH4HCO3. Whole cells were inactivated by γ-irradiation and resuspended in breaking buffer (PBS, 1 mM EDTA) supplemented with protease inhibitors, DNase and RNase for serial passage through a French pressure cell (Thermo Scientific). Whole cell lysate was dialedyzed into 0.01 M NH4HCO3, and centrifuged at 27,000 × g at 4°C for 1 h to generate cell wall. The supernatant was subsequently centrifuged at 100,000 × g for 4 h to recover cell membrane. Cell wall, membrane and cytosol were all dialyzed and protein quantified via BCA (Thermo Pierce).

The cell wall fraction was subjected to organic extraction to remove non-covalently associated lipids and glycolipids48. Lyophilized cell wall was subjected to two extractions of 2 h each followed by one 18 h extraction with chloroform/methanol (2:1, v/v) at a ratio of 30 mL/g of cell wall. Extractions were performed at 22°C with agitation. Centrifugation at 27,000 g for 30 min was performed to collect cell wall material. The 2.1 extracted cell wall was dried under N2 and further extracted twice for 2 h and one 18 h extraction with chloroform/methanol/water (10:10:3, v/v/v) each at 22°C. The fully delipidated cell wall was dried under N2 and resuspended in PBS, 0.1% ASB-14 pH 7.4 to maximize solubility. Cell wall protein was quantified by bicinchoninic acid (BCA) assay (Thermo Pierce).

β2-microglobulin (β2M) was expressed and confirmed by Western blot analysis and isotype specific ELISA (Invitrogen). Tetramers were generated with the peptides and HLA-E restricted T cells recognizing the peptides were detected among circulating T cells. When isolated from human PBMC and stimulated with peptide, these HLA-E restricted T cells produce Th2 cytokines. These results reveal a novel Th2 T cell population in the human immune response to Mtb. The role of glycosylated epitopes, such as the MPT32 peptide identified here, in production of Th1 or Th2 cytokines by HLA-E restricted CD8+ T cells remains to be explored, but these results demonstrate that HLA-E restricted CD8+ T cells have the potential to play a unique and important role in the human immune response to infection with Mtb.
the phen-seph column (flow through) was exchanged into concavalin-A (ConA) binding buffer (50 mM KH2PO4, approximate concentration of 0.1 ug/ul in Buffer A. Sample were kept frozen at −80 °C prior to ELISPOT testing.

**Pronase Digestion.** Pronase (250 ku, EMD Millipore) was resuspended in digestion buffer - PBS, 0.1% ASB-14 for subcellular fractions or 0.2 M NH4HCO3 for purified proteins, at a concentration of 5.0 mg/ml. Digestion was performed at 37 °C, 16 h at an enzyme to substrate ratio of 1:20 for all samples. Enzymatic reactivity was quenched with rapid freeze thaw cycles at −80 °C.

**IFN-γ Elispot assay.** IFN-γ ELISPOT analysis was performed as previously described11. Briefly, 96-well nitrocellulose backed plates were coated as recommended by the manufacturer with capture mouse anti-IFN-γ overnight at 4 °C. Plates were then washed three times with PBS (Invitrogen) and blocked with RPMI + 10% HS for 1 h at room temperature. Antigen presenting cells (20,000) were added, then pulsed with antigen for 1 hr. T cell clones (20,000) were then added, and the plate incubated overnight at 37 °C. Plates were extensively washed and anti-IFN-γ secondary antibody conjugated to HRP was added. AEC developer substrate was added and the reaction stopped by washing with distilled water.

**Reverse Phase HPLC.** Whole protein digests (1 mg to 5 mg) were separated on Waters Alliance 2695 HPLC (Waters Inc, Millford) using a Grace Vydc Everest 300 Å monomeric C18 column (4.6 × 150 mm, 238EV5115). Fractions were collected every 2 minutes over a gradient of 0–50% B over 30 minutes, 0.5 ml/min. Buffer A − 0.1% TFA in water, Buffer B − 0.1% TFA, 90% acetonitrile. For fraction 9 A, the gradient was modified to begin at 15% B and increased to 30% B over 30 minutes. Fractions were collected every 3 minutes at a flow rate of 0.5 ml/ min. For all peptide separations fractions were concentrated using vacuum centrifugation and resuspended at an approximate concentration of 0.1 ug/ul in Buffer A. Sample were kept frozen at −80°C prior to ELISPOT testing.

**Concavalin-A lectin chromatography of peptides.** 3 mg of purified MPT32 was subjected to Pronase digestion as described above (concentration of 1 mg/ml). Peptides were incubated with in ConA buffer as described above with rocking, in the presence of 2 ml of Con-A conjugated sepharose slurry (i.e. 1 ml settled resin) (GE Life Sciences) at 4 °C, for 16 h in a 15 ml reaction tube. Resin-peptide mix was poured into an open column and allowed to settle. Flow through was collected. Resin was washed with 10 column volumes of binding buffer and collected. The peptides were eluted off with 10 CV of elution buffer. Collected fractions were exchanged back into 0.1% TFA after processing through solid-phase sep-pak columns (Waters) following manufacturer's instructions.

**Mass spectrometry.** Liquid Chromatography Mass Spectrometry (LC-MS): Peptides were separated on a nanospray column (Zorbax C18, 5 μm, 75 μm ID 6 150 mm column) and samples were eluted into a LTQ linear ion trap mass spectrometer (Thermo) as described previously46. MALDI-TOF: 1 ml of peptide sample is mixed with 1 ml of a mixed matrix solution (10 mg/ml α-Cyano-4-hydroxycinnamic acid (CHCA) and 10 mg/ml 2,5-Dihydroxybenzoic acid (DHB); 1:1 v/v) in 50% ACN, 0.1% TFA. The mixture was spotted on the MALDI target and allowed to air dry. The sample was analyzed by an Ultraflex-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica) in positive ion, reflector mode using a 25 kV accelerating voltage. External calibration was performed using a peptide calibration mixture (4 to 6 peptides) on a spot adjacent to the sample. The raw data was processed in the FlexAnalysis software (version 3.3, Bruker Daltonics).

**Peptide and Protein Identification.** LC-MS Database Searching: Tandem mass spectra were extracted, charge state deconvoluted and desisotoped by Xcalibur version 1.7 SP2. All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, v.27, rev. 11) and MASCOT (Matrix Science, v.2.3). Sequest and MASCOT were set up to search the MtbV3_Reverse database (7992 entries) assuming non-specific cleavage. Parameters for both search engines were set as variable modification on threonine. +162 da and mannobiose (+324) were set as variable modification on threonine. All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository50, 51 with the dataset identifier PXD005765 and 10.6019/PXD005765.

MALDI-TOF spectra were collected and select ions were chosen for fragmentation. Peptide sequences were elucidated via de novo calculation of amino acid fragments. M + H single charged ion masses were matched if identified using LC-MS protein identification. Scaffold (version Scaffold_3.5.1, Proteome Software Inc.) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Sequest identifications required at least deltaCn scores of greater than 0.2 and XCorr scores of greater than 1.8, 2.0, 3.0 and 4.0 for singly, doubly, triply and quadruply charged peptides. Protein identifications assigned within each peptide fraction were accepted if they contained at least 2 identified peptides. All peptide spectra were manually inspected for sequence coverage and signal to noise. MS1 peak detection of tandem LC-MS data was performed in Skyline (MacCoss Lab Software, v.3.6). Non-specific cleavage products resulting from pronase digestion of MPT32 were predicted and listed as Precursor Mass targets (+2 and +3 charge states). Putative target peptides were considered only if the M, M +1, and M +2 isotope peak signal was 3X above background and isotope dot products (idotp) ratios of 0.9 or greater.
**Generation of T10V, T18V, and T27V MPT32 mutants.** Each of the 3 N-terminal threonine (Thr) sites of glycosylation were subjected to site-directed mutagenesis (Thr → Val) (QuickChange II XL, Agilent). Briefly, the plasmid pMV261 containing the MPT32 gene was amplified with mutagenesis primers: T10V (5′-cgcgcggtaacggcgcctcg-3′), T18V (5′-cgcgcggtaacggcgcctcg-3′), and T27V (5′-acccgacgaccgctctgcgcgc-3′). The mutants (T10V, T18V, T27V) were transformed into an Mpt32 knock-out strain of Mtb, CDC1551 (kindly provided by Dr. Gyanu Lamichane at Johns Hopkins University). Transformed cells were grown for two weeks in glycerol, alanine, salts medium and cells harvested from the culture filtrate (CFP). Purification of wild type Mpt32 from Mtb CDC1551 was conducted similarly as above with minor modifications. Briefly, Mtb CFP was concentrated using a Pellicon system with a 10 kDa membrane (Millipore) and proteins precipitated with 40% ammonium sulfate. The purified protein was enriched for glycoproteins and glycoconjugates over a concavalin-A lectin chromatography column (as above), and final purification of Mpt32 was achieved by resolving the ConA column eluate using C4 RP-HPLC (using the same conditions as above for C18 polishing).

**Disruption of Rv1002c in M. tuberculosis by allelic replacement.** The avirulent auxotrophic M. tuberculosis H37Rv strain mc²606 (∆panCD ∆lebCD) (kindly provided by Dr. W. R. Jacobs, Albert Einstein College of Medicine) was grown at 37 °C in Middlebrook 7H9-OADC-0.05% tyloxapol supplemented with 0.2% casamino acids, 48 μg/ml pantothenate and 30 μg/ml L-leucine or on similarly supplemented Middlebrook 7H11-OADC agar medium. When required, kanamycin (Kan) and hygromycin (Hyg) were used at concentrations of 25 and 50 mg/ml, respectively.

The Rv1002c locus of M. tuberculosis mc²606 was disrupted by recombineering using plasmid pJV53 to transiently express the highly active mycobacteriophage-encoded recombinases, gp60 and gp61, as described. The linear DNA substrate used to achieve allelic replacement at the Rv1002c locus was PCR-amplified from M. tuberculosis H37Rv genomic DNA using primers Rv1002c-KOF1 (5′-CGGTTAAGACCGTCACCGA-3′) and Rv1002c-KOR1 (5′-ACCATGCGAACCAGATAC-3′), and 1,390-bp of the Rv1002c sequence flanked by two AgeI sites was replaced by a hygromycin resistance gene. M. tuberculosis mc²606 electrocompetent cells expressing the recombinases from pJV53 were transformed with the linear DNA substrate and double cross-over mutants selected on 7H11-OADC-Hyg plates at 37 °C. Allelic replacement at the Rv1002c locus was confirmed by PCR and sequencing.

**Data Availability.** All data generated or analyzed during this study (except raw mass spectrometry data, see below) are included in the published article. All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD005765 and 10.6019/PXD005765.

**References**

1. Lewinsohn, D. A. et al. Mycobacterium tuberculosis-specific CD8+ T cells preferentially recognize heavily infected cells. *Am J Respir Crit Care Med* **168**, 1346–1352, doi:10.1164/rccm.200306-837OC (2003).

2. Gold, M. C. et al. Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biol* **8**, doi:10.1371/journal.phb.1000407 (2010).

3. Harriff, M. I. et al. Human lung epithelial cells contain Mycobacterium tuberculosis in a late endosomal vacuole and are efficiently recognized by CD8(+) T cells. *PLoS One* **9**, doi:10.1371/journal.pone.0097515 (2014).

4. van Pinxteren, L. A., Cassidy, J. P., Smedegaard, B. H., Agger, E. M. & Andersen, P. Control of latent *Mycobacterium tuberculosis* infection is dependent on CD8 T cells. *Eur J Immunol* **30**, 3689–3698, doi:10.1002/1521-4141(200012)30:12<3689::AID-IMMU3689>3.0.CO;2-4 (2000).

5. Chen, C. Y. et al. A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. *PLoS Pathog* **5**, doi:10.1371/journal.ppat.1000392 (2009).

6. Bjorkman, P. J. & Parham, P. Structure, function, and diversity of class I major histocompatibility complex molecules. *Annual review of biochemistry* **59**, 253–288, doi:10.1146/annurev.biochem.59.070198.001345 (1990).

7. Moody, D. B. et al. CD1c-mediated T-cell recognition of isoprenoid glycolipids in *Mycobacterium tuberculosis* infection. *Nature* **404**, 884–888, doi:10.1038/35099119 (2000).

8. Kjer-Nielsen, L. et al. MRI presents microbial vitamin B metabolites to MALT cells. *Nature* **491**, 717–723, doi:10.1038/nature11605 (2012).

9. Braud, V. M. et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* **391**, 795–799, doi:10.1038/358689 (1998).

10. Lee, N. et al. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci USA* **95**, 5199–5204 (1998).

11. Brooks, A. G. et al. Specific recognition of HLA-E, but not classical, HLA class I molecules by soluble CD94/NKG2A and NK cells. *J Immunol* **162**, 305–313 (1999).

12. Garcia, P. et al. Human T cell receptor-mediated recognition of HLA-E. *Eur J Immunol* **32**, 936–944, doi:10.1002/1521-4141(200204) (2002).

13. Heinzl, A. S. et al. HLA-E-dependent presentation of Mtb-derived antigen to human CD8+ T cells. *J Exp Med* **196**, 1473–1481 (2002).

14. Scotland, K. et al. The analysis of the natural killer-like activity of human cytolytic T lymphocytes revealed HLA-E as a novel target for TCR alpha/beta-mediated recognition. *Eur J Immunol* **31**, 3687–3693, doi:10.1002/1521-4141(200112) (2001).

15. Joosten, S. A. et al. Mycobacterium tuberculosis peptides presented by HLA-E are targets for human CD8 T-cells with cytotoxic as well as regulatory activity. *PLoS Pathog* **6**, e1000782, doi:10.1371/journal.ppat.1000782 (2010).

16. Vazquez, S. & Esteban, E. Identification of a human HLA-E-restricted CD8+ T cell subset in volunteers immunized with *Salmonella enterica* serovar Typhi strain Ty21a typhoid vaccine. *J Immunol* **173**, 5852–5862, doi:10.4049/jimmunol.173/5852 (2004).

17. van Maegaarden, K. E. et al. Human CD8+ T-cells recognizing peptides from *Mycobacterium tuberculosis* (MtB) presented by HLA-E have an unorthodox Th2-like, multifunctional, MtB inhibitory phenotype and represent a novel human T-cell subset. *PLoS Pathog* **11**, doi:10.1371/journal.ppat.1004671 (2015).

18. van Maegaarden, K. E. et al. Human CD8+ T-cells recognizing peptides from *Mycobacterium tuberculosis* (MtB) presented by HLA-E have an unorthodox Th2-like, multifunctional, MtB inhibitory phenotype and represent a novel human T-cell subset.
We would like to thank Gyanu Lamichhane (Johns Hopkins University) for providing us with the ΔMPT32 knockout Mtb strain, Katie strain (Colorado State University) for technical assistance with the MALDI-TOF analyses. This work was supported in part by Veterans Affairs Biomedical Laboratory Research and Development Program (D.M.L.), AI048090 (NIH, D.M.L.), Merit Award #I01 BX000533 from the U.S. Department of Veterans Affairs Clinical Sciences Research and Development Program (M.J.H.), Merit Award #I01 BX000533 from the U.S. Department of Veterans Affairs Biomedical Laboratory Research and Development Program (D.M.L.), AL048090 (NIH, D.M.L.), HHSN266200400081C (NIH, D.M.L.), and HHSN272200900053C (NIH, D.M.L.).
Author Contributions
M.H. and L.W. wrote the main manuscript text and prepared the figures and tables. M.H., L.W., G.W., M.N., M.C., E.C., T.V., K.G.T., W.L., and K.D. performed experiments. M.H., L.W., G.S., M.J., D.A.L., K.D., and D.M.L. contributed intellectually to project conception, experimental design, reagent generation, and/or data analysis. All authors reviewed the manuscript.

Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-04894-0

Competing Interests: The authors declare that they have no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017