Characterization of Human CD8+ T Cells Reactive with Mycobacterium tuberculosis-infected Antigen-presenting Cells

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Summary

Previous studies in murine models, including those using the β2 microglobulin knockout mouse, have suggested an important role for CD8+ T cells in host defense to Mycobacterium tuberculosis (Mtb). At present, little is understood about these cells in the human immune response to tuberculosis. This report demonstrates the existence of human Mtb-reactive CD8+ T cells. These cells are present preferentially in persons infected with Mtb and produce interferon-γ in response to stimulation with Mtb-infected target cells. Recognition of Mtb-infected cells by these CD8+ T cells is restricted neither by the major histocompatibility complex (MHC) class I A, B, or C alleles nor by CD1, although it is inhibited by anti-MHC class I antibody. The Mtb-specific CD8+ T cells recognize an antigen which is generated in the proteasome, but which does not require transport through the Golgi-ER. The data suggest the possible use of nonpolymorphic MHC class Ib antigen presenting structures other than CD1.

Key words: M. tuberculosis • CD8+ lymphocytes • intracellular pathogens • antigen presentation • interferon-γ

It is estimated that a third of the world’s population is infected with Mycobacterium tuberculosis (Mtb),1 the causative agent of tuberculosis. Consequently, tuberculosis is the leading cause of infectious mortality worldwide, accounting for over 8 million new cases and 2.9 million deaths annually (1). Mtb is an intracellular pathogen and thus the control of infection relies on the recognition and destruction of infected cells.

There is abundant evidence to support an important role for CD4+ T cell–mediated immunity in tuberculosis (2). However, several lines of evidence also suggest a role for CD8+ T cells in controlling Mtb infection. Mice deficient in CD8+ T cells as a consequence of disruption of the gene for β2 microglobulin are more susceptible to Mtb infection compared with their wild-type littermates (3). In addition, mice in which the gene for CD8 has been disrupted are also highly susceptible to Mtb infection (4). Recently, Silva et al. found that CD8+ T cell clones generated to the Mtb heat shock protein (hsp 65) could confer partial immunity to Mtb infection in mice (5). Immunization of mice with plasmids expressing hsp 65 (6), Ag 85a (7), or the 38-kD (8) antigen resulted in the generation of antigen-specific CD8+ CTLs that were associated with protection from subsequent challenge with Mtb. Finally, Stenger et al. demonstrated that human CD8+ CTLs restricted by CD1b molecule are able to inhibit the growth of Mtb in vitro (9).

In the host response to tuberculosis infection, CD8+ T cells may exert a protective role via several mechanisms. First, in response to antigenic stimulation, CD8+ T cells produce cytokines such as IFN-γ and TNF-α. These cytokines are potent macrophage activators and their importance has been illustrated by the increased susceptibility of mice to Mtb challenge in which the genes for IFN-γ (10) and TNF-R have been disrupted (11). Second, CD8+ T cells may play a unique role in host defense to Mtb through the release of granular constituents that promote the destruction of heavily infected macrophages and MHC class II negative cells such as endothelial cells and fibroblasts. The directed exocytosis of cytolytic granules by CD8+ T cells induces apoptosis in the target cell. In this regard, it has been suggested that apoptosis will kill intracellular mycobacteria (12). However, mice deficient in the expression of perforin, granzyme, or CD95 (Fas) are still able to con-
tains infection with Mtb (13, 14), suggesting that in the mouse model the secretion of macrophage-activating cytokines such as IFN-γ and TNF-α may be sufficient for protective immunity. Alternatively, other components of the cytotoxic granule may have a direct antimycobacterial effect (9, 15).

The role of MHC class I-restricted CD8⁺ T cells in human immunity to tuberculosis remains largely unexplored. Little is known about the mechanism by which Mtb antigens might gain access to the MHC class I antigen-processing pathway. Within the macrophage, Mtb resides primarily in the phagosome (16, 17), a site thought inaccessible to MHC class I processing. However, particulate antigens have been shown to gain access to the MHC class I pathway (18, 19), although the efficiency of these pathways remains controversial (20).

This study demonstrates the existence of human Mtb-reactive CD8⁺ T cells. These cells are present preferentially in persons infected with Mtb and produce IFN-γ in response to stimulation with Mtb-infected targets. Recognition of Mtb-infected cells by these T cells is not restricted by either the MHC class I A, B, or C, alleles or by CD1, although it is inhibited by anti-MHC class I antibody. We demonstrate that the Mtb-specific CD8⁺ T cells recognize an antigen that is generated in the proteasome, but which does not require transport through the Golgi endoplasmic reticulum (ER). The data suggest the possible use of non-polymeric MHC class Ib antigen-presenting structures other than CD1.

Materials and Methods

Human Subjects. Subjects were recruited from employees at Harborview Medical Center, the Fred Hutchinson Cancer Research Center, and the Corixa Corporation. Purified protein derivative of Mtb (PPD) responses were determined by the employee health service at the respective institutions. Protocols for venipuncture and apheresis were Institutional Review Board approved. HLA typing was performed on PBL by the Puget Sound Blood Center.

Monodonal Antibodies and Reagents. Culture medium consisted of RPMI-1640 supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD), 50 µg/ml gentamycin sulfate (BioWhittaker), 5 × 10⁻⁵ M 2ME (Sigma Chemical Co., St. Louis, MO), and 2 mM glutamine (GIBCO BRL, Bethesda, MD). For the generation of primary T cell lines and clones, RPMI was supplemented with 10% human serum (HS). Monoclonal antibodies were generated from hybridoma supernatants from the W6/32 and L243 cell lines obtained from American Type Culture Collection (ATCC; Rockville, MD) using the Affi-gel protein A MAPSiI kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. M. tuberculosis (H 37R v), M. bovis (ATCC 35718), M. paratuberculosis (ATCC 35726) were grown in modified Middlebrook 7H9 media. After the preparation of glycerol stocks, aliquots were frozen, and subsequently titered on Middlebrook 7H10 plates (Becton Dickinson Microbiology Systems; Cockeysville, MD). E. coli LPS was obtained from Sigma. Infectious influenza A/HK/68 virus was prepared by the University of Maryland Biotechnology Center.

Generation of Peripheral Blood Mononuclear Cells (PBMCs). PBMCs were obtained from peripheral blood of Mtb-infected donors. PBMCs were isolated by Ficoll-Hypaque density centrifugation (Sigma Chemical Co.) and washed three times with RPMI culture medium. Alternatively, PBMCs were obtained via leukapheresis. Cells were resuspended in RPMI containing 2% HS and allowed to adhere to a 12-well tissue culture flask (Corning, Cambridge, MA) at 37°C for 1 h in the presence of 10 ng/ml of GM-CSF (Immunex Corp., Seattle, WA). After gentle rocking, nonadherent cells were removed, and 30 ml of RPMI 1/10% HS containing 10 ng/ml of IL-4 (Immunex Corporation) and 30 ng/ml of GM-CSF (Immunex Corporation) was added. After 18 h, the media was removed and centrifuged, and the cell-conditioned media was plated on the adherent cells. After 5–7 d, cells were harvested with cell-disassociation media (Sigma Chemical Co.).

Flow Cytometry. Cells to be analyzed for cell surface marker expression were first incubated at 4°C in a blocking solution of PBS containing 2% normal rabbit serum (Sigma Chemical Co.), 2% normal goat serum (Sigma Chemical Co.), and 2% HS to prevent non-specific binding of mouse Ig. Cells were washed in FACS buffer (PBS containing 0.5% FBS and 0.02% sodium azide) and incubated with either FITC-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD16 (Becton Dickinson Immunocytometry Systems, San Jose, CA; 5 µg/ml) for 30 min at 4°C in a total volume of 50 µl. Cells were then washed, and flow cytometry was performed using a FACSCalibur® (Becton Dickinson) and data were collected on 10³ viable cells.

Generation of Mtb-reactive CD8⁺ T Cell Lines. 10⁶ monocyte-derived DCs were cultured overnight in the presence of Mtb (H 37R v; M OI = 100) in low adherence 12-mm wells (Costar No. 3473). After 18 h, the cells were harvested and resuspended in RPMI 1/10% HS. These cells were cultured with 3 × 10⁶ T cells depleted of CD4⁺ lymphocytes by adherence to immobilized anti-CD4 (AIS MicroCELLector; Applied Immune Sciences, Santa Clara, CA) and supplemented with IL-7 (10 ng/ml; Immunex). T cells were re-stimulated with fresh, Mtb-infected DCs on day 7. IL-2 (0.5 ng/ml; Chiron, Emeryville, CA) was added on day 8 and every other day thereafter. T cells were positively selected on day 9, and cultured in the presence of IL-2. After 2 d, cells were harvested, analyzed for the expression of CD4 and CD8 (94–98% CD8⁺), and assessed for their ability to generate IFN-γ in response to Mtb-infected cells. In this assay, 2.5 × 10⁵ DCs were co-incubated with 10⁶ T cells in 12-mm wells (Costar). After 18–24 h, supernatants were harvested and an ELISA analysis was performed to determine the concentration of IFN-γ.

Generation of Mtb-reactive CD8⁺ T Cell Clones. T cells were cloned by limiting dilution in the presence of 2 × 10⁵ irradiated (3,000 rad using a 137Cs source) heterologous PBMCs, 5 × 10⁴ irradiated (7,000 rad) heterologous lymphoblastoid cell line (LCL) cells, anti-CD3 (10 ng/ml), and recombinant IL-2 (10 ng/ml). T cells were generated in our laboratory using supernatants from the cell line 9B5-8. Cell lines were maintained by continuous passage in RPMI culture medium supplemented with 10% FBS. Clone 10D10-82, an HIV Gag-specific CD8⁺ T cell clone to the B44 restricted peptide 103 (AA 303-322; TLR AER ASO DVKNM-WMTETLL) was provided by Dr. Stanley Riddell. Clone D150M 58-16, an A2.1-restricted influenza matrix protein specific CD8⁺ CTL (AA 58-66; GILGFVFTL) was provided by Dr. Steven Fleng (Corixa Corporation).

Generation of Peripheral Blood Mononuclear Cells (PBMCs). PBMCs were isolated from heparinized blood by centrifugation over Ficoll-Hypaque (Sigma Chemical Co.) and washed three times with culture medium. Alternatively, PBMCs were obtained via leukapheresis. Cells were resuspended in RPMI containing 2% HS and allowed to adhere to a T-75 tissue culture flask (Costar, Cambridge, MA) at 37°C for 1 h in the presence of 10 ng/ml of GM-CSF (Immunex Corp., Seattle, WA). After gentle rocking, nonadherent cells were removed, and 30 ml of RPMI 1/10% HS containing 10 ng/ml of IL-4 (Immunex Corporation) and 30 ng/ml of GM-CSF (Immunex Corporation) was added. After 18 h, the media was removed and centrifuged, and the cell-conditioned media was plated on the adherent cells. After 5–7 d, cells were harvested with cell-disassociation media (Sigma Chemical Co.).

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Generation of Mtb-reactive CD8⁺ T Cell Clones. T cells were cloned by limiting dilution in the presence of 2 × 10⁵ irradiated (3,000 rad using a 137Cs source) heterologous PBMCs, 5 × 10⁴ irradiated (7,000 rad) heterologous lymphoblastoid cell line (LCL) cells, anti-CD3 (10 ng/ml), and recombinant IL-2 (10 ng/ml).
Evaluation of the specificity of T cell clones for Mtb was performed as follows: Mtb-infected DCs and control uninfected DCs were prepared as above, and seeded at 3-5 \times 10^4 cells per well in 96-well flat-bottomed plates (Costar) in 100 \mu l of RPMI/10% HS. 5 \times 10^4 T cells were added in 100 \mu l of media, and supernatants were harvested after 18-24 h for determination of IFN-\(\gamma\). Assays were performed in the presence of 1.0 ng/ml IL-2.

Metabolic Inhibition of Antigen Presentation. 1 h before the addition of Mtb to DCs, lactacystin (40 \mu M; E.J. Corey, Harvard Biolabs, Harvard, Cambridge, MA), brefeldin A (10 \mu g/ml; Sigma Chemical Co.), chloroquine (100 mM; Sigma Chemical Co.), or cytochalasin D (10 \mu g/ml; Sigma Chemical Co.) was added to the culture medium. After 18 h of coincubation with Mtb, cells were harvested and fixed in either 1% paraformaldehyde (Sigma Chemical Co.) or 0.1% glutaraldehyde (Grade I, 25% aqueous; Sigma Chemical Co.). After vigorous washing, fixed DCs were selected for the ability to generate IFN-\(\gamma\) in response to Mtb-infected DCs. All of the CD8\(^+\) T cells were able to generate IFN-\(\gamma\) after incubation with the Mtb-infected DCs. In addition to Mtb, clone 23 responded strongly to DCs infected with M. bovis and minimal to DCs infected with M. avium (Fig. 4). Taken together, these data demonstrate that the CD8\(^+\) T cell clones are specific for Mtb complex mycobacteria.

A nitogen Presentation Is Not Restricted to a Specific MHC Class I Allele. In an attempt to define a restricting MHC class I allele for the Mtb-reactive CD8\(^+\) T cell clones, a panel of DCs was generated that matched the T cells at one MHC class I allele for the Mtb-reactive CD8\(^+\) T cell clones, a panel of DCs was generated that matched the T cells at one MHC class I allele for the Mtb-reactive CD8\(^+\) T cell clones, a panel of DCs was generated that matched the T cells at one MHC class I allele for the Mtb-reactive CD8\(^+\) T cell clones. A panel of DCs was generated that matched the T cells at one MHC class I allele for the Mtb-reactive CD8\(^+\) T cell clones. A panel of DCs was generated that matched the T cells at one MHC class I allele for the Mtb-reactive CD8\(^+\) T cell clones.
Human CD8⁺ T Cells to Mycobacterium tuberculosis

Antigen Presentation Requires Phagocytosis and Proteasomal Degradation, but Does Not Require Golgi-ER Transport. The failure to demonstrate HLA-A-, B-, or C restriction of these T cell clones suggested that antigen presentation could be occurring through a nonpolymorphic MHC Class Ib molecule such as CD1 or HLA-E, -F, or -H. To derive insights into the cellular processes required to present Mtb antigens to CD8⁺ T cells, inhibitors known to interfere with discrete stages of antigen processing were used. DCs were preincubated in the presence of inhibitor for 1 h, pulsed overnight with Mtb in the presence of inhibitor, and fixed in 0.1% glutaraldehyde. As shown in Fig. 7 A, neither chloroquine (an inhibitor of phago-lysosomal acidification and thus MHC class II-dependent peptide presentation) nor brefeldin A (which inhibits Golgi-ER transport, and thus MHC class I-dependent peptide presentation) affected the Mtb-induced generation of IFN-γ. In contrast, the addition of either the phagocytosis inhibitor cytochalasin D or the potent proteasomal inhibitor lactacystin resulted in complete inhibition. Lactacystin did not appear to...
be nonspecifically toxic to the DCs, as it did not inhibit antigen presentation to an Mtb-reactive CD4\(^+\) T cell clone using the same DCs (Fig. 7B). Moreover, both brefeldin A and lactacystin were effective in blocking the presentation of the influenza matrix peptide to an HLA-A2-restricted CTL clone by influenza virus-infected DCs (Fig. 7B). Taken together, these data indicate that the processing and presentation of Mtb antigen to these CD8\(^+\) T cells requires phagocytosis of the bacteria, with antigen gaining entry to the cytoplasm where proteasomal degradation occurs. However, the absence of inhibition by brefeldin A demonstrates that the antigen must bypass the Golgi-ER, possibly being processed and released into the extracellular milieu, and then presented.

**Discussion**

Studies in mouse models have demonstrated the importance of CD8\(^+\) T lymphocytes in protective immunity to tuberculosis. In this paper, we provide definitive evidence that human CD8\(^+\) T cells recognize protein antigens presented by Mtb-infected DCs and macrophages. The antigenic specificity of this response was clearly established using a reciprocal specificity analysis with an HIV p24-reactive CTL. Moreover, all of the healthy Mtb-infected donors that have been tested to date have strong CD8\(^+\) T cell re-
sponses to Mtb-infected DCs, whereas only two out of six of those who are not Mtb infected have shown responses, indicating the recall nature of these responses and suggesting the importance of CD8+ T cells in protective immunity. Furthermore, activation of T cell clones is dependent on the presence of Mtb and APCs. Finally, the T cell clones respond to Mtb and the closely related pathogenic M. bovis, but not to M. avium.

The presentation of Mtb antigen to CD8+ T cells was inhibited by the W6/32 antibody, suggesting that the T cell recognizes antigen in the context of MHC class I. However, attempts to identify a specific HLA-A-, -B-, or -C-restricting allele were unsuccessful. Because the W6/32 antibody inhibits both classical and nonclassical MHC class I molecules such as HLA-E, -F, or -H, it is possible that the antigen is presented by such a nonpolymorphic MHC class Ib molecule (23, 24). Our data suggest that CD1a, -b, or -c are not required based on the ability of cells lacking these markers to present Mtb-derived antigen. The expression and function of CD1d and -e remain unclear, leaving restriction by this molecule a possibility. Unfortunately, a variety of cell lines were not able to process and present antigen derived from live Mtb, precluding the use of this approach to further define the restricting allele.

Metabolic inhibition was used to further define the mode of antigen processing, in particular with regard to the requirements for proteasomal processing (lactacystin), Golgi-ER transport (brefeldin A), phagocytosis (cytochalasin D), and endosomal acidification (chloroquine). Both cytochalasin D and lactacystin proved potent inhibitors of antigen presentation, while neither chloroquine nor brefeldin A were inhibitory. These data suggest that Mtb is phagocytosed, with Mtb-derived proteins gaining access to the cytosol where proteasomal degradation occurs. The failure of brefeldin A to inhibit antigen presentation suggests that proteasomally derived peptide does not require Golgi-ER transport and thus that the antigen presenting structure is not transported to the cell surface by the same pathway as conventional MHC class I. The precise mechanism by which such presentation occurs is unclear. Perhaps dying cells release peptides that are presented by adjacent live cells (paracrine processing).

The results presented here describe a processing pathway that is distinct from conventional MHC class I or II pathways. Exogenous particulate antigens have been reported to gain access to the class I processing pathways (18). However, in this model, processing and presentation requires both phagocytosis and Golgi-ER transport. In contrast, Pfeiffer et al. demonstrated that ova-peptide expressed in either E. coli or Salmonella as a fusion protein was presented to MHC class I-restricted T cells in a manner that was inhibited by chloroquine. Of particular interest, those studies demonstrated the presence of peptide in the extracellular milieu (19).

The simplest explanation of our data is that the processed peptide binds a nonpolymorphic antigen-presenting structure on the cell surface, perhaps on the MHC class Ib molecules such as HLA-E, -F, or -H. In bacterial infection, precedent for MHC class Ib-restricted antigen presentation exists in two model systems. CD4/CD8 double negative cytolytic T cells that are restricted by monomorphic, β2 microglobulin-associated CD1b and CD1c molecules (25, 26) have been described. These cells recognize both mycolic acid (27) and glycolipid antigens (25). Those antigens are processed by a novel chloroquine-sensitive, but HLA-DM-independent mechanism (26). The CD8+ CTLs described in this paper are not CD1 restricted, in that they recognize a proteasome-dependent antigen presented by CD1-negative Mtb-infected macrophages.

In the mouse, the monomorphic, β2 microglobulin-associated H2-M3 molecule has been demonstrated to present short formylated peptides derived from Listeria monocytogenes (28–30). Although there is no known human homologue for H2-M3, monomorphic members of the HLA family such as HLA-E, -F, or -H may be capable of presentation of mycobacterially derived peptide(s).

An alternative interpretation of these data would be the presentation of antigen by HLA class I or II structures in an unconventional manner. For example, the T cells could recognize peptide that binds to multiple HLA class I or II alleles. Such promiscuous presentation has been described for HLA-DR (31), and recently within members of the HLA-A3 superfamily (32). However, the antibody-blocking data does not suggest a direct role for HLA class II molecules. Moreover, although the inhibition observed with the anti-class I antibody W6/32 would be consistent with restriction by MHC-1α, the APCs tested do not fall within a known HLA class Iα superfamily. However, it is possible that an as yet undescribed super-family might exist. Similarly, it is conceivable that antigen presentation is occurring in a manner analogous to that of a superantigen. Although this has been described in Mtb (33), there is no precedent for a superantigen that requires proteasomal processing.

In short term cytolytic assays, we observed ∼20% specific lysis of Mtb-pulsed cells (data not shown). The relatively modest cytolyis may reflect a small subset of DCs that are sufficiently infected to present antigen, or a low abundance of antigen. In this regard, there is a paucity of published data regarding the ability of mouse MHC class Iα-restricted CTLs against defined antigens to lyse Mtb-infected cells. Recently, Zhu et al. described murine CD8+ CTL to the 38-kD antigen (8). Epitope mapping revealed that CTLs derived from DNA vaccination recognized entirely distinct peptides than did those derived from natural infection.

In summary, we have defined a novel antigen-processing pathway by which human CD8+ T cells recognize Mtb-infected DCs and macrophages. The antigen is presented in a manner that is not restricted by a specific HLA-A, -B, or -C allele. To date, all of the CD8+ T cell clones we have generated (seven clones from two donors) have the same characteristic lack of HLA-A, -B, or -C restriction. Our data indicate that Mtb-derived antigen is processed by the proteasome and thus must gain access to the cytosol. Consequently, further definition of the restricting element and the antigen it presents may yield valuable insights into the mechanism by which CD8+ CTLs contribute to host defense against Mtb.
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1640 Human CD8+ T Cells to Mycobacterium tuberculosis

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