Brief Definitive Report

Induction of M3-restricted Cytotoxic T Lymphocyte Responses by *N*-formylated Peptides Derived from *Mycobacterium tuberculosis*

By Taehoon Chun,* Natalya V. Serbina,‡ Dawn Nolt,§‡ Bin Wang,* Nancy M. Chiu,* JoAnne L. Flynn,‡ and Chyung-Ru Wang*

*From the *Gwen Knapp Center for Lupus and Immunology Research, Committee on Immunology and Department of Pathology, University of Chicago, Chicago, Illinois 60637; and the ‡Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261; and the §Department of Pediatrics, Division of Infectious Disease, Children's Hospital, Pittsburgh, Pennsylvania 15261*

Abstract

Major histocompatibility complex (MHC) class I–restricted $CD8^+$ T cells play a critical role in the protective immunity against *Mycobacterium tuberculosis* (Mtb). However, only a few Mtb peptides recognized by MHC class Ia–restricted $CD8^+$ T cells have been identified. Information on epitopes recognized by class Ib–restricted T cells is even more limited. M3 is an MHC class Ib molecule that preferentially presents *N*-formylated peptides to $CD8^+$ T cells. Because bacteria initiate protein synthesis with *N*-formyl methionine, the unique binding specificity of M3 makes it especially suitable for presenting these particular bacterial epitopes. We have scanned the full sequence of the Mtb genome for NH_2-terminal peptides that share features with other M3-binding peptides. Synthetic peptides corresponding to these sequences were tested for their ability to bind to M3 in an immunofluorescence-based peptide-binding assay. Four of the *N*-formylated Mtb peptides were able to elicit cytotoxic T lymphocytes (CTLs) from mice immunized with peptide-coated splenocytes. The Mtb peptide–specific, M3-restricted CTLs lysed the Mtb-infected macrophages effectively, suggesting that these *N*-formylated Mtb peptides are presented as the naturally processed epitopes by Mtb-infected cells. Furthermore, T cells from Mtb-infected lungs, spleen, and lymph nodes responded to *N*-formylated Mtb peptides in an M3-restricted manner. Taken together, our data suggest that M3-restricted T cells may participate in the immune response to Mtb.

Key words: infection • MHC • vaccine • *N*-formylated peptides • *Mycobacterium tuberculosis*

Introduction

Tuberculosis is a leading cause of infectious mortality in the world (1). The causative agent of tuberculosis, *Mycobacterium tuberculosis* (Mtb), is an intracellular bacterial pathogen that resides within phagosomes of infected macrophages. Despite the noncytosolic location of Mtb, endogenous Mtb-derived antigens can be presented to $CD8^+$ T cells, presumably through an alternative MHC class I antigen processing pathway (2–4). Studies of experimental tuberculosis in mice indicate that $CD8^+$ T cells contribute to immune defense against Mtb by releasing cytokines such as IFN-γ and TNF-α and directly lysing infected cells (5–10). Although the identification of Mtb antigens for T cell recognition is critical in the design of T cell–based vaccines, relatively few T cell epitopes for Mtb-specific $CD8^+$ T cells have been identified. Among them are MHC class Ia–binding peptides derived from 38-kD lipoprotein (11), 19-kD lipoprotein (12), and early secretory antigenic target 6 (13) and CD1-bound lipid antigens derived from the mycobacterial cell wall (14, 15). A recent study has shown that some human Mtb-reactive CTLs are not restricted by MHC class Ia nor by CD1 molecules, suggesting that other class Ib molecules may be involved in presenting Mtb antigens (16).

H2-M3 is an MHC class Ib molecule that has a unique specificity for *N*-formylated hydrophobic peptides derived from mitochondria and bacteria (17, 18). Given that class Ia

Address correspondence to Chyung-Ru Wang, Gwen Knapp Center for Lupus and Immunology Research, University of Chicago, 924 E. 57th St., R412, Chicago, IL 60637-5420. Phone: 773-702-4725; Fax: 773-702-1576; E-mail: cwang@midway.uchicago.edu

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molecules do not bind *N*-formylated peptides appreciably (19), M3 may have been selected in evolution for the specialized presentation of this conserved structure of bacterial peptides. Three listerial antigens presented by M3 have been defined: LemA, Fr 38, and AttM (20–22). Each antigen is recognized by CTLs from infected animals. Adoptive transfer of M3-restricted, LemA-specific CTLs can provide protective immunity against listerial infection, indicating that M3-restricted T cells may play a role in host defense against intracellular bacterial infection (23).

Recently, we have shown that M3 surface expression is undetectable in most cell types due to the lack of endogenous antigens. The majority of M3 is retained in an immature peptide-receptive form and traffics rapidly to the cell surface upon the addition of exogenous *N*-formylated peptide (24). This property allows us to search for novel pathogen-derived peptides that bind to M3 in normal APCs. Identification of bacterial peptides that are able to prime an M3-restricted CTL response will raise the prospect of using these peptides in vaccine development.

In this study, we identified M3-binding peptides derived from Mtb. We showed that CTLs with specificity for *N*-formylated Mtb peptides can be generated in mice. These T cells are capable of lysing Mtb-infected macrophages. In addition, M3-restricted *N*-formylated peptide–specific T cells are present in Mtb-infected mice, suggesting that the M3-restricted T cells may contribute to the defense against mycobacterial infection. These *N*-formylated M3-binding peptides identified from Mtb, a prominent human pathogen, may prove useful in the search for functional homologues of M3 in humans.

Materials and Methods

Abs and Peptides. Abs used in this study include: FITC-conjugated mAbs specific for hamster IgG; CD4 (RM4-5); TCR-β (H57-597); Vβ5 (MR9-4); Vβ6 (RR4-7); Vβ7 (TR310); Vβ8 (MR5-2); Vβ9 (MR10-2); Vβ12 (MR11-1); Vβ14 (14-2); and Vα3 (RR3-16); and PE-conjugated mAbs specific for CD8α (53-6.7); Vβ2 (B20.6); Vβ3 (KJ25); Vβ4 (KT4); Vβ10 (B21.5); Vβ11 (RR3-15); Vβ13 (RR12-3); Vα2 (B20.1); Vα8 (B21.14); and Vα11 (RR8-1) (BD PharMingen). The hamster mAb130 was purified as described previously (24). Synthetic peptides were purchased from Research Genetics. Peptide sequences are described in Table I. All peptides were >90% pure as determined by mass spectrometry. Peptides were dissolved in DMSO at concentrations of 10 mM.

Bacteria. Mtb (Erdman strain; Trudeau Institute, Saranac Lake, NY) was passed through mice, grown in culture once, and frozen in aliquots (25). Frozen aliquots were used to start cultures at a concentration of 2.5×10^6 cells/ml in liquid medium (7H9 Middlebrook; Difco Laboratories, Inc.), and bacteria were grown in 5% CO_2 at 37°C. 6–7-d-old cultures were used for infections. Bacteria were washed and resuspended in DMEM (Life Technologies) before infection of cell cultures.

Cell Lines and Cell Cultures. Fibroblast cell lines were maintained in RPMI 1640 (Mediatech) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 20 mM Hepes, 50 μM 2-ME, penicillin, and streptomycin (RPMI 10), and the macrophage cell line P388 was cultured in DMEM (Mediatech) containing 10% FBS.

Table I. *Synthetic Peptides Used in This Study*

Peptide	Sequence	Gene designation and annotation
TB1	fMIVVLV	Rv1021 (conserved hypothetical protein)
TB2	fMLVLLV	Rv0476 (hydrophobic protein; contains β-ketoacyl synthase active site)
TB3	fMWYYLF	Rv2182C (conserved hypothetical protein; contains ATP/GTP binding site)
TB4	fMFLIDV	Rv0277C (conserved hypothetical protein)
TB5	fMLFAAL	Rv0072 (ABC-transporter transmembrane subunit)
TB6	fMFFLDA	Rv0749(conserved hypothetical protein)
TB7	fMILLV	Rv1686C (probable transmembrane protein)
Fr38	fMIVIL	Listerial peptide
LemA	fMIGWII	Listerial peptide
ND1	fMFFINIL	Mitochondrial peptide
COI	fMFINRW	Mitochondrial peptide

The sequence and annotation information of Mtb was obtained from The Sanger Center (GenBank/EMBL/DDBJ accession no. AL123456).

For CTL assays, cells were harvested with 0.2% collagenase (type IV) and EDTA-saline (140 mM NaCl, 5 mM KCl, 12.5 mM Na_2HPO_4, 5.8 mM NaH_2PO_4, 0.2% glucose, and Na_2EDTA, pH 7.2). TR8.4a is a derivative of the B10.CAS2 fibroblast cell line that has been transfected with genomic DNA encoding $M3^{wt}$ (26). P388-M3 is a macrophage cell line that has been transfected with *M3* cDNA under the control of CMV promoter.

Computer-aided Search of M3-binding Peptide and Computer Modeling. The Mtb sequences in The Sanger Center database were searched with the Findpattern program (Genetic Computer Group) allowing no mismatches with the following sequence: fM(Y,F,W,I,L)(Y,F,I,L,V)ZXX (Z, amino acid with nonpolar side chain; X, any amino acid; reference 27). 41 peptides were identified that bear this motif. We modeled 20 such peptides into the binding groove of the M3/MTF crystal structure with program O on a Silicon Graphics machine (28, 29). The backbone conformation of the peptide was left unchanged, and rotameric states of the new side chains were assigned to optimize van der Waals interactions and avoid steric hindrance.

Induction of M3 on the Cell Surface by Mtb Peptides. Splenocyte suspensions were prepared from C57BL/6 (B6) mouse spleens by mechanical disruption in RPMI 10. RBCs were removed by hypotonic lysis. One million cells were incubated in RPMI 10 with or without peptides overnight at 37°C. Cells were harvested and washed three times with PBS before cell surface staining experiments. M3 staining was detected by adding 100 μl of hybridoma supernatants (mAb130) followed by mouse anti–hamster IgG FITC. Staining with each reagent was performed for 30 min on ice in HBSS (Life Technologies) containing 2% FBS and 0.1% sodium azide (Sigma Aldrich), followed by washing with the same buffer. The stained cells were analyzed by flow cytometry using a FACSCaliber™ (Becton Dickinson) with CELLQuest™ software.

Generation and Maintenance of Mtb-specific CTL Lines. 5×10^6 splenocytes from B6 mice were cultured in 5 ml of RPMI 10 in the presence of 10 μM Mtb peptide at 37°C. After overnight incubation, cells were harvested, washed three times with PBS, and

inactivated by gamma irradiation (1,500 rads). B6 mice were primed with peptide-coated splenocytes through intraperitoneal injection and footpad injection. After 1 wk, the mice were killed, and their spleen cells were incubated with 10 μM peptides in RPMI 10 at the concentration of 10^7 cells per milliliter. Cultures were restimulated weekly with peptide-pulsed syngenic splenocytes ($2–5 \times 10^6$ cells per milliliter) and maintained in supplemented Mishell Dutton medium with IL-2 supplement (20 U/ml). IL-2 for restimulations was partially purified from the supernatant of EL4.IL2 cells (American Type Culture Collection), assayed, and used as described in reference 30.

Culture and Infection of Macrophages. Macrophages were grown from bone marrow precursors of B6 mice. In brief, cells were eluted from the femur and tibia bones of mice in DMEM 10. Cells were washed twice in DMEM, and $1–3 \times 10^6$ cells were plated in LabTek PS petri dishes (Fisher Scientific) in 25 ml DMEM 10, 1 mM sodium pyruvate (Sigma Aldrich), 2 mM L-glutamine (Life Technologies, Inc.), and 33% supernatant from L cell fibroblasts cultured for 5–7 d. All reagents were LPS-free, and no antibiotics were used. Medium was changed once after 2–3 d of culture. On day 5, medium was replaced with DMEM 10, 1 mM sodium pyruvate, and 2 mM L-glutamine. Cells were then infected with Mtb at a multiplicity of infection of 2–5. The percentage of infection was estimated in each experiment by staining aliquots of cells by the Kinyoun method for acid-fast bacteria (Difco Laboratories, Inc.). After 15–48 h of infection (depending on the assay), adherent cells were prepared for use as targets in CTL assays.

CTL Assays. One million target cells were incubated in RPMI 10 with or without 10 μM peptide overnight. After incubation with peptide, cells were washed free of excess peptide and labeled with 100 μCi [^{51}Cr]–sodium chromate for 1 h at 37°C. For infected macrophages, cells were washed twice with ice-cold PBS, incubated for 20 min on ice, and harvested by forceful pipetting before labeling in Teflon jars (Savillex). Target cells (10^4 cells) were added to round-bottomed microtiter wells containing effector cells. After 4 h, 100 μl of supernatant from each well was collected and assayed for ^{51}Cr release. The percentage of specific lysis = (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100.

IFN-γ Assay. CTLs (10^5 cells/well) were stimulated with gamma-irradiated B6 splenocytes (10^5 cells/well) in a final volume of 200 μl of RPMI 10 in the presence or absence of 10 μM Mtb peptide. After 48 h, the culture supernatants were harvested, and the levels of IFN-γ were quantitated by sandwich ELISA using commercial mAb pairs (BD PharMingen) according to the manufacturer's instructions.

In Vitro IFN-γ Production by Lymphocytes from Mtb-infected Mice. B6 and K^bD^b knockout (KO) mice (in B6 background; reference 31) were infected intravenously via tail vein with 10^5 live bacilli. At 10 and 21 d after infection, lungs, pulmonary lymph nodes, and spleens were harvested. Single-cell suspensions were obtained by crushing the organs in cell strainders (Becton Dickinson). RBCs were removed by hypotonic lysis, and the cells were washed extensively. Cells were resuspended in T cell medium (DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM Hepes, and 1% nonessential amino acids) and plated onto culture dishes for 1 h at 37°C to deplete macrophages. Lung and lymph node cells (8×10^4 and 2×10^5 cells per well, respectively) were stimulated with P388–M3 (1:1 ratio), pulsed with either a mixture of Mtb peptides (TB1-7, 2 μM each) or LemA in the presence or absence of an anti-M3 Ab (mAb130). Each condition was plated in triplicate wells in 96-well plates; each well contained a total volume of 200 μl. Spleen cells (2×10^5 cells/well) were stimulated as above, except P388–M3 pulsed with 10 μM of each TB peptide were also included as stimulators. After a 4-d incubation at 37°C, supernatants from each well were filtered with 0.45-μm filter to remove Mtb before testing for IFN-γ.

Results

Identification of M3-binding Peptides from the Predicted Protein Sequences of Mtb. We searched the predicted protein coding sequences from Mtb for NH_2-terminal peptides that share features with other M3-binding peptides. These peptides were then modeled into the M3 structure with Program O on a Silicon Graphics machine. Seven of these *N*-formylated peptides that fit well to the M3-binding groove were synthesized and tested for M3 binding by analysis of their capacity to induce M3 surface expression (Table I). P388-M3 cells, an M3-transfected macrophage cell line, were incubated overnight with various concentrations of each Mtb peptide. Surface expression of M3 was determined by immunofluorescence assay with an anti-M3 mAb (mAb130). Fig. 1 shows that six of these Mtb peptides can significantly induce M3 surface expression. TB1 and TB7 have the highest affinity for M3, followed by TB2 and TB4, and then by TB3 and TB6. TB5 enhances M3 expression only slightly. The affinity of TB1 and TB7 to M3 is comparable to two other M3-binding peptides derived from *Listeria*, namely LemA and Fr38.

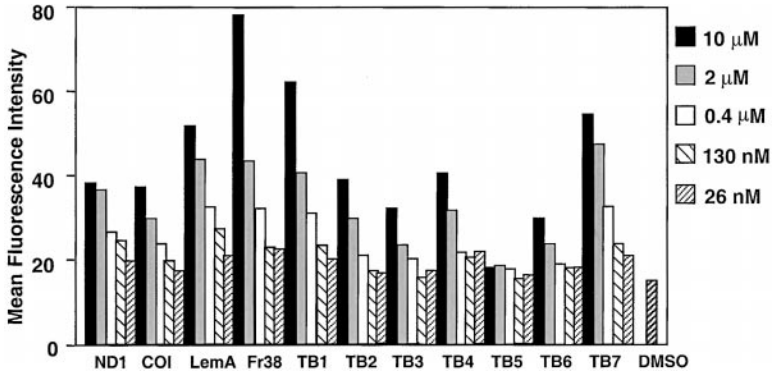

Figure 1. *N*-formylated Mtb peptides increase surface expression of M3. P388-M3 transfectants were incubated overnight with varying concentrations of *N*-formylated peptides and stained for M3 expression. The range of concentrations and the corresponding hatchmarks are shown. The bars represent mean fluorescence intensity after staining with mAb130 as described. The results are representative of two experiments.

Generation of M3-restricted Mtb-specific CTLs. Four Mtb peptides, TB2, TB4, TB6, and TB7, were chosen to represent groups that increase M3 surface levels to different extents. To access the immunogenecity, we analyzed their ability to elicit peptide-specific CTLs after in vivo priming. Eight long-term CTL lines were established from eight B6 mice (*H-2*^{b}, *M3*^{wt}) immunized with TB peptide-coated splenocytes and maintained by in vitro restimulation with irradiated splenocytes pulsed with the immunizing peptide. Cytotoxicity assays were performed to determine the antigen specificity of these CTLs. To address the question of whether these CTLs are restricted by M3, we used a B10.CAS2 fibroblast cell line that expresses an *M3* allele (*M3*^{cas}) that does not present *N*-formylated peptide efficiently and an M3^{wt}-transfected B10.CAS2 (TR8.4a) as targets. All eight CTL lines lysed TR8.4a cells sensitized with the stimulating peptide but failed to recognize B10.CAS2 fibroblasts sensitized with the same peptide (Fig. 2 A). Lysis of TR8.4a was peptide dependent, as TR8.4a cells alone were not lysed. To determine whether these TB peptides are presented exclusively by M3, we tested the ability of anti-M3 Ab to block the killing by CTLs of the peptide-sensitized P388 (*H-2*^{d}, *M3*^{wt}) cells. As shown in Fig. 2 B, mAb130 completely blocked the killing of all Mtb-specific CTL lines tested. Together, these results suggest that M3 presents *N*-formylated Mtb peptides to the CTLs.

A previous study showed that the majority of M3-restricted *Listeria*-specific CTL clones responded to multiple *N*-formylated peptides, indicating that M3-restricted T cells might be highly cross-reactive (32). To gain insight into the peptide specificity of these Mtb-specific CTLs, we examined the responses of four representative CTL lines to a panel of *N*-formylated peptides, including TB2, TB4, TB6, TB7, two mitochondrial peptides (ND1 and COI), and listerial LemA peptide (Fig. 2 C). All four CTL lines preferentially lysed the target cells pulsed with *N*-formylated peptide used for in vitro stimulation, although low levels of cross-reactivity with one or two peptides from this

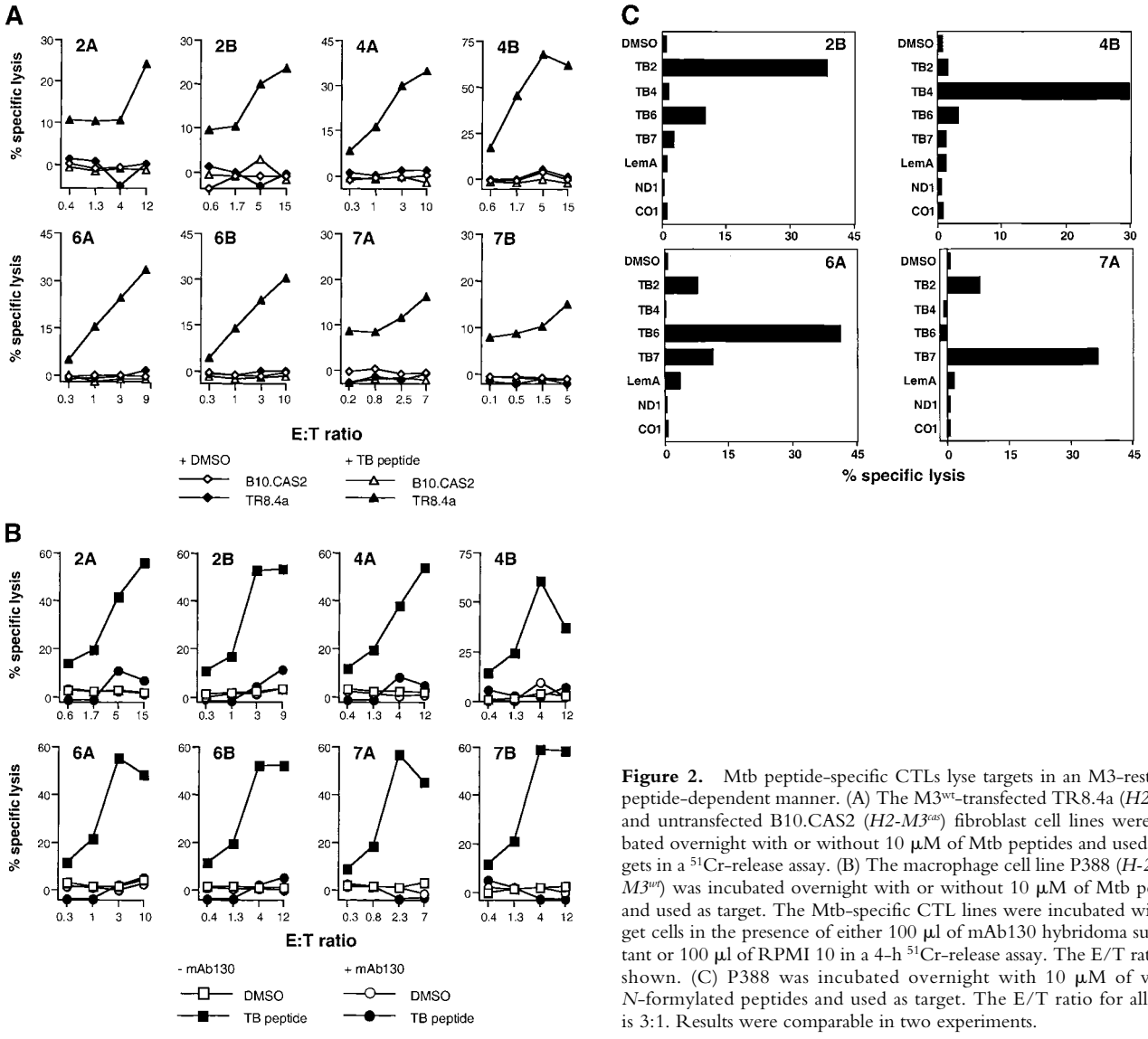

Figure 2. Mtb peptide-specific CTLs lyse targets in an M3-restricted, peptide-dependent manner. (A) The M3^{wt}-transfected TR8.4a (*H2-M3*^{wt}) and untransfected B10.CAS2 (*H2-M3*^{cas}) fibroblast cell lines were incubated overnight with or without 10 μM of Mtb peptides and used as targets in a ^{51}Cr-release assay. (B) The macrophage cell line P388 (*H-2*^{d}, *H2-M3*^{wt}) was incubated overnight with or without 10 μM of Mtb peptides and used as target. The Mtb-specific CTL lines were incubated with target cells in the presence of either 100 μl of mAb130 hybridoma supernatant or 100 μl of RPMI 10 in a 4-h ^{51}Cr-release assay. The E/T ratios are shown. (C) P388 was incubated overnight with 10 μM of various *N*-formylated peptides and used as target. The E/T ratio for all CTLs is 3:1. Results were comparable in two experiments.

panel could be detected. Thus, the recognition of these Mtb-specific CTLs are peptide specific.

Mtb Peptide–specific CTLs Can Effectively Kill Mtb-infected Macrophages. To assess whether Mtb peptide–induced CTLs can recognize naturally processed mycobacterial antigens, we performed CTL assays using Mtb-infected macrophages as target cells. M3 surface expression was not detectable on uninfected macrophages. Upon infection, expression increased to low but detectable levels (data not shown). The infected macrophages were specifically lysed by all the Mtb peptide-specific CTL lines (Fig. 3) but were insensitive to the lysis by a *Listeria*-specific M3-restricted CTL clone, D7 (33). The lysis of infected macrophages by M3-restricted Mtb peptide–specific CTLs indicates that these *N*-formylated peptides are naturally processed and presented by M3 in Mtb-infected macrophages. Macrophages infected for 12–18 h were already recognized by M3-restricted CTL, although in some experiments lysis was improved if the infection of macrophages was allowed to progress for 48 h before use in the CTL assay.

Characterization of M3-restricted Mtb-specific CTLs. Surface phenotypes and TCR usage of these Mtb-specific CTLs were determined by flow cytometric analysis. The results are summarized in Table II. All of the CTL lines express αβ TCRs, and most of the lines use different combinations of the Vα and the Vβ segments, suggesting that the T cell repertoire for M3-restricted responses is not limited. Four of the Mtb-specific CTL lines express the CD8 coreceptor, while the other four CTLs do not express CD4 or CD8 coreceptors. As both $CD8^+$ and dominant negative (DN) CTLs can lyse Fas-negative target cells such as peptide-pulsed fibroblasts, it is likely that both types of M3-restricted CTLs can lyse the targets via the granule exocytosis pathway. However, in the case of the CD1-restricted, Mtb-specific CTLs, the $CD8^+$ CD1-restricted CTLs lysed infected cells by a granule-dependent mechanism, whereas the DN CD1-restricted CTLs lysed targets through Fas–FasL interaction (34).

Production of IFN-γ by T cells is one of the protective mechanisms against bacterial pathogens and is particularly crucial in controlling tuberculosis (35, 36). To test the ability of Mtb-specific CTLs to secrete IFN-γ, we measured IFN-γ secreted in response to peptide-pulsed B6 splenocytes. As shown in Table II, all of the CTLs produced significant amounts of IFN-γ in response to stimulation with Mtb peptide–pulsed splenocytes. Thus, these M3-restricted, Mtb-specific CTLs could directly lyse the infected cells and release IFN-γ to modulate the cell-mediated immune response against Mtb.

M3-restricted T Cells Participate in Immune Response to Mtb In Vivo. Although the M3-restricted CTL lines were capable of recognizing antigen presented by infected mac-

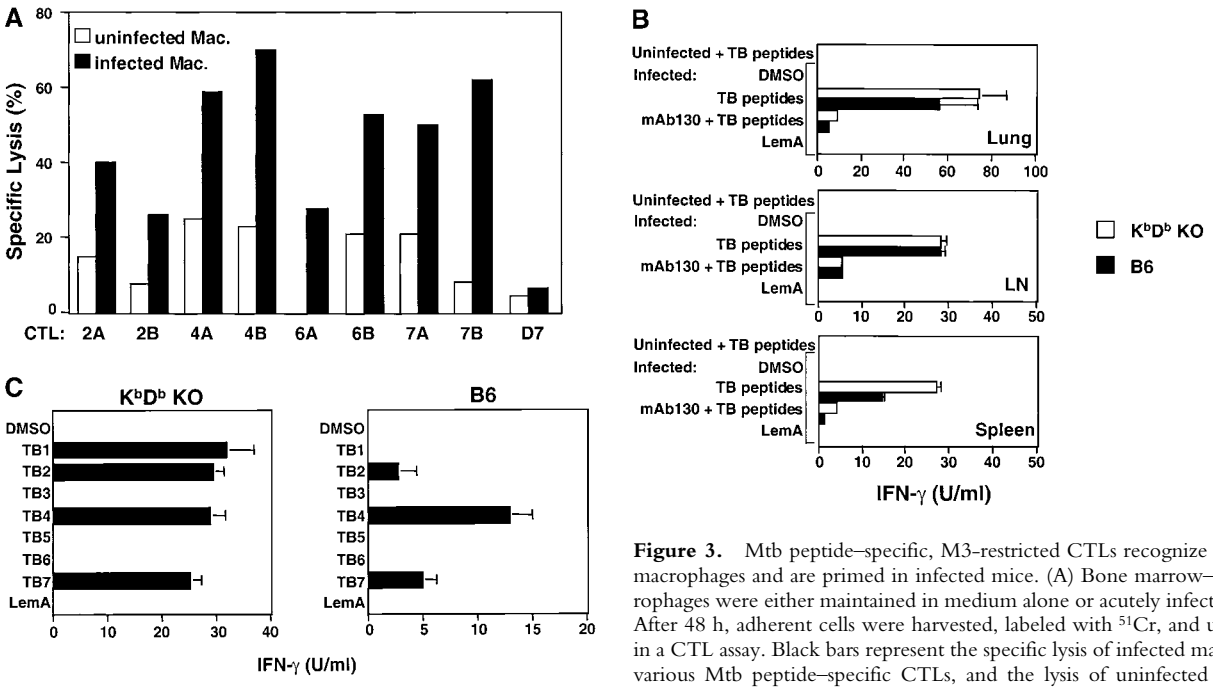

Figure 3. Mtb peptide–specific, M3-restricted CTLs recognize Mtb-infected macrophages and are primed in infected mice. (A) Bone marrow–derived macrophages were either maintained in medium alone or acutely infected with Mtb. After 48 h, adherent cells were harvested, labeled with ^{51}Cr, and used as targets in a CTL assay. Black bars represent the specific lysis of infected macrophages by various Mtb peptide–specific CTLs, and the lysis of uninfected macrophages (white bars) was included for comparison. The E/T ratio was 30:1 for CTL4B and 10:1 for the rest of the CTL lines. Results shown are the means from triplicate wells and the standard errors were <5%. Results were comparable in three experiments. (B) IFN-γ production by lymphocytes from Mtb-infected mice in response to Mtb peptide stimulation. Lymphocytes from lung and lymph nodes (LN) and spleen of K^bD^b KO or B6 mice were harvested at 21 d after infection and stimulated with a mixture of TB peptides (TB1–TB7, 2 μM each). After 4 d, the amounts of IFN-γ in the culture supernatants were measured by ELISA. Results shown are the means from triplicate wells, and the standard errors are shown. (C) Reactivity of splenocytes from Mtb-infected mice to various TB peptides. Splenocytes from mice at 21 d after infection were stimulated with each TB peptide (10 μM) in the presence or absence of an anti-M3 Ab (mAb130). After 4 d, the levels of IFN-γ in the culture supernatants were quantitated by ELISA. Splenocytes from uninfected mice did not secrete detectable amounts of IFN-γ in response to TB peptide stimulation. For B and C, three K^bD^b KO and two B6 mice were used for each experiment, and the results were comparable in two experiments. Cells from mice infected for 10 d gave similar results but with lower IFN-γ production.

Table II. *Surface Phenotypes and IFN-γ Secretion of Mtb-specific CTLs*

CTL	Peptide specificity	Surface phenotype*	IFN-γ secretion‡
			U/ml
2A	TB2	DN, TCR-αβ$^+$ (Vα3$^+$Vβ8$^+$)	26 ± 5
2B	TB2	DN, TCR-αβ$^+$ (Vα11$^+$Vβ6$^+$)	18 ± 1
4A	TB4	CD8$^+$, TCR-αβ$^+$ (Vα2$^+$Vβ14$^+$)	73 ± 15
4B	TB4	CD8$^+$, TCR-αβ$^+$ (Vα:UNKVβ8$^+$)	49 ± 10
6A	TB6	DN, TCR-αβ$^+$ (Vα2$^+$Vβ10$^+$)	34 ± 8
6B	TB6	CD8$^+$, TCR-αβ$^+$ (Vα2$^+$Vβ:UNK)	24 ± 2
7A	TB7	DN, TCR-αβ$^+$ (Vα:UNKVβ5$^+$)	116 ± 6
7B	TB7	CD8$^+$, TCR-αβ$^+$ (Vα3$^+$Vβ5$^+$)	115 ± 5

*CTL lines were stained with various Abs and analyzed by FACS®. DN, CD4$^-$CD8$^-$; UNK, unknown, i.e., no positive staining with the Abs specific to the following V segments: Vα2, 3, 8, and 11 and Vβ2–14 and 17.
‡Antigen-dependent IFN-γ production by Mtb-specific CTLs was assessed in response to various Mtb peptide–pulsed splenocytes. CTLs cultured with splenocytes alone did not secrete detectable amounts of IFN-γ (<1 U/ml).

rophages, it remained to be determined whether T cells specific for these *N*-formylated peptides were elicited in response to Mtb infection in mice. Lymphocytes were isolated from Mtb-infected mice at 10 and 21 d after infection and examined for IFN-γ production by restimulating in vitro with either a mixture of TB peptides (TB1-7) or individual TB peptide. Both K^bD^b KO and B6 mice were examined to determine whether M3-restricted responses are augmented in the absence of class Ia molecules. At 21 d after infection, lymphocytes harvested from lungs, lymph nodes, and spleens of infected K^bD^b KO and B6 mice secreted IFN-γ upon in vitro stimulation with TB1-7, but not with LemA peptide (Fig. 3 B). The response was blocked by the addition of anti-M3 Ab (mAb130), suggesting that TB peptide–specific, M3-restricted T cells are present in Mtb-infected mice. Similar results were obtained with lymphocytes isolated from infected mice at 10 d after infection, but the magnitude of the response was lower (data not shown). Three out of the seven TB peptides, TB2, TB4, and TB7, readily induced IFN-γ from the spleen cells of infected B6 and K^bD^b KO mice (Fig. 3 C), but not from uninfected mice (data not shown). The response to TB1 was only detected from infected K^bD^b KO mice. In addition, lymphocytes from infected K^bD^b KO mice secreted substantially larger amounts of IFN-γ in response to TB peptides, indicating that M3-restricted T cells are enriched in K^bD^b KO mice. Taken together, these results suggest that *N*-formylated M3-binding peptides are effectively presented during Mtb infection, and the resulting M3-restricted T cells may contribute to the immunity against Mtb.

Discussion

The Mtb genome sequence was published recently (27). This allowed us to search for *N*-formylated mycobacterial peptides with sequence homology to other M3-binding peptides. More than 40 candidate peptides were identified from 3,924 predicted protein coding sequences of Mtb. Our analysis of the four candidate peptides demonstrates the feasibility of using this approach to identify functionally relevant epitopes for M3-restricted T cells in Mtb infection. The fact that all of the CTLs elicited by these four *N*-formylated Mtb peptides can recognize and lyse Mtb-infected macrophages suggests that the *N*-formylated peptides might be prevalent antigens during bacterial infections. In addition, three *N*-formylated peptides used in this study can be effectively presented by M3 in Mtb-infected mice, suggesting that *N*-formylated peptide–specific, M3-restricted T cells may play a role in the immune response to Mtb.

The unique ligand specificity of M3 may contribute to its efficacy in antigen presentation by allowing *N*-formylated antigenic peptides access to a pool of empty M3 in the endoplasmic reticulum without competition from many self-peptides (24). During infection, presentation of *N*-formylated bacterial peptides by M3 is rapid and undiluted, which might provide a potent activation signal to M3-restricted T cells. Consistent with this notion, Kerksiek et al. (37) have shown that M3-restricted responses appear to be earlier and more prominent than class Ia–restricted responses in the primary infection of mice with *Listeria monocytogenes*. In our studies, the response to the *N*-formylated TB peptides was stronger at 21 d after infection compared with 10 d after infection, suggesting that the kinetics of the response may vary with different pathogens. *N*-formyl modification is a feature common to bacterial proteins. Thus, it is possible that M3 might be able to present antigens derived from a variety of intracellular bacteria, and M3-restricted responses might contribute to the defense against infection by a wide range of intracellular pathogens.

Limited polymorphism of M3 suggests that M3 may have evolved to present conserved and unique ligands to T cells. Focusing the immune response on common microbial entities that are rare or absent from host cells can broaden the host specificity against infectious agents without increasing the risk of autoimmunity. The CD1 molecules exemplify this strategy by presenting bacterial lipids to T cells (14, 15). *N*-formylated peptides are extremely rare in mammalian cells but serve as a widely used signal sequence for protein secretion in bacteria. Therefore, it would be advantageous for the host to preserve such antigen presentation through evolution. Although Southern blot analysis failed to detect M3 orthologs in humans (26), it remains to be determined whether functional homologues of M3 exist in humans. HLA-E and Qa-1 do not share striking sequence similarity to each other, although each appear to have evolved to bind leader peptides of class Ia molecules in humans and mice, respectively (38, 39).

MHC-unrestricted, Mtb-specific CTLs have been identified in humans (16). However, the antigen(s) and the restriction element(s) for these CTLs have not been determined. Our results suggest that if a human homologue of M3 exists, it would be possible to elicit CTL responses from infected individuals by in vitro stimulation with Mtb-

derived *N*-formylated peptides. Currently, we are attempting to use the M3-binding peptides described in our study as candidate peptides for developing human CTLs. If this approach succeeds, *N*-formylated peptides presented by a nonpolymorphic MHC molecule could potentially be used as novel vaccines against Mtb infection in a broad range of recipients.

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