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H2-M3-Restricted CD8+ T Cells Induced by Peptide-Pulsed Dendritic Cells Confer Protection against Mycobacterium tuberculosis1

Takehiko Doi,*† Hisakata Yamada,2* Toshiki Yajima,* Worawidh Wajjwalku,† Toshiro Haras,† and Yasunobu Yoshihakistar

One of the oligomeric MHC class Ib molecules, H2-M3, presents N-formylated peptides derived from bacteria. In this study, we tested the ability of an H2-M3-binding peptide, TB2, to induce protection in C57BL/6 mice against Mycobacterium tuberculosis. Immunization with bone marrow-derived dendritic cell (BMDC) pulsed with TB2 or a MHC class Ia-binding peptide, MPT64190–198, elicited an expansion of Ag-specific CD8+ T cells in the spleen and the lung. The number of TB2-specific CD8+ T cells reached a peak on day 6, contracted with kinetics similar to MPT64190–198-specific CD8+ T cells and was maintained at an appreciable level for at least 60 days. The TB2-specific CD8+ T cells produced less effector cytokines but have stronger cytotoxic activity than MPT64190–198-specific CD8+ T cells. Mice immunized with TB2-pulsed BMDC as well as those with MPT64190–198-pulsed BMDC showed significant protection against an intratracheal challenge with M. tuberculosis H37Rv. However, histopathology of the lungs in mice immunized with TB2-pulsed BMDC was different from mice immunized with MPT64190–198-pulsed BMDC. Our results suggest that immunization with BMDC pulsed with MHC class Ib-restricted peptides would be a useful vaccination strategy against M. tuberculosis. The Journal of Immunology, 2007, 178: 3806–3813.

Tuberculosis is one of the major public health problems. About one-third of the world population has been latently infected with Mycobacterium tuberculosis (1). The tuberculosis incidence is increasing in association with increased numbers of HIV/AIDS patients. Furthermore, the emergency of multidrug-resistant strains of M. tuberculosis has worsened the problems. To prevent an epidemic of tuberculosis, Mycobacterium bovis bacillus Calmette-Guérin (BCG) is the only vaccine currently available against tuberculosis. Although BCG vaccine protects children efficiently against the early manifestations of tuberculosis (2, 3), especially meningeal tuberculosis (4), it confers incomplete protection against tuberculosis in adults presumably because BCG may not be effective for inducing long-term cellular immunity sufficient for protection against pulmonary disease (5). Furthermore, BCG, a live vaccine, may not be safe for immunocompromised hosts such as AIDS and aged patients. Therefore, it is urgently required to develop prophylactic and therapeutic vaccines for tuberculosis in place of BCG (6).

Although protection against infection with intracellular bacteria such as M. tuberculosis depends mainly on CD4+ Th1 cells, there are substantial lines of evidence that CD8+ T cells also play a requisite role (7–9). β2-microglobulin-deficient mice and TAP-deficient mice, both of which lack functional CD8+ T cells, are susceptible to infection with M. tuberculosis (10, 11). Adoptive transfer of immunized CD8+ T cells conferred protection against subsequent challenge with M. tuberculosis (12). Thereafter, various vaccination strategies have settled to efficiently induce protective memory CD8+ T cells. Peptide-pulsed mature bone marrow-derived dendritic cells (BMDC) efficiently generate high numbers of effector and memory CD8+ T cells (13–15) and there have been several studies on BMDC-based vaccines against M. tuberculosis (16–18) in which a certain level of protection was observed. However, an obstacle for clinical application of these peptide-based vaccination strategies is the polymorphism of MHC molecules (19).

Although most of CD8+ T cells recognize peptides on highly polymorphic class Ia molecules, some CD8+ T cells recognize peptides presented by class Ib molecules which have limited polymorphism (20). H2-M3 is a member of MHC class Ib molecules showing specificity for hydrophobic peptide sequences initiating with N-formyl methionine derived from only bacteria or mitochondrial proteins (21, 22). Chun et al. (23) have identified M. tuberculosis-derived peptides which bind H2-M3 and showed an involvement of H2-M3-restricted CD8+ T cell response in murine models of M. tuberculosis infection. Thus, H2-M3-binding peptides may serve as a good candidate for universal vaccine against M. tuberculosis (24). At present, it is unclear whether immunization with H2-M3 peptide induces long-lasting protective immunity to M. tuberculosis infection.

In the present study, we examined the effects of vaccination with BMDC pulsed with a H2-M3-binding peptide, TB2 (23) or a MHC

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3 Abbreviations used in this paper: BCG, Mycobacterium bovis; DC, dendritic cell; BMDC, bone marrow-derived DC; MFI, mean fluorescent intensity.

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class Ia (H-2D\(^b\))-binding peptide, MPT64\(_{190–198}\) (25–27) on \textit{M. tuberculosis} H37Rv infection in mice. We found that immunization with TB2-pulsed BMDC elicited long-lasting Ag-specific CD8\(^+\) T cells, leading to protection against intratracheal infection with \textit{M. tuberculosis} at a level comparable to MPT64\(_{190–198}\)-pulsed BMDC.

### Materials and Methods

#### Animals

Six- to 8-wk-old female C57BL/6 mice (Charles River Laboratories) and C57BL/6 Ly5.1-congenic mice (The Jackson Laboratory) were used. They were housed in a pathogen-free environment throughout the experiment.

#### Microorganisms

\textit{M. tuberculosis} strain H37Rv was grown in Middlebrook 7H9 medium (Difco) supplemented with albumin-dextrose-catalase enrichment (Difco) and Tween 80 at 37°C. The bacteria in the culture were stored in Middlebrook 7H9 medium supplemented with 20% (v/v) glycerol at −80°C until they were used.

#### Abs and synthetic peptides

Following Abs were used: FITC-conjugated anti-IFN-γ (R-4-6A2), Cy5-conjugated anti-CD3 (53-67), PE-conjugated anti-CD44 (IM7), biotin-conjugated anti-CD45.1 (A20), and streptavidin-Cy5 (eBioscience). The H2-D\(^b\)-restricted peptide, MPT64\(_{190–198}\) (FAVTNDGVI) (25–27), Mt6234\(_{309–318}\) (GAPINSATAM) (28–29), 38 KD4\(_{129–137}\) (AQVYNNLP) (30), the H2-K\(^b\)-restricted peptide, OVA\(_{257–264}\) (SIINFEKL), and the H2-M\(^3\)-restricted peptide, TB2 (f-MLVLLV), TB4 (f-MFLIDV), TB7 (f-MILLV) (23), and LemA (f-MIGWII) (1–6) Listerial peptide.

#### Generation of peptide-pulsed BMDCs and immunization

RBC-depleted bone marrow cells were cultured at 1 x 10\(^6\) cells/ml in RPMI 1640 medium (Sigma-Aldrich) supplemented with 20 ng/ml murine IL-4 and 20 ng/ml murine GM-CSF (PeproTech) at 37°C with 5% CO\(_2\). Three days after the initial culture, two-thirds of the medium containing small nonadherent cells were removed and fresh RPMI 1640 containing GM-CSF and IL-4 was added back. At day 6, 1 μg/ml LPS (Sigma- Aldrich) was added to induce maturation. After an overnight culture, 5 μM synthetic peptides are added to the cultures 3 h before harvest. The non-adherent cells were harvested and then layered onto 15% metrizamide (Sigma-Aldrich). After a centrifugation at 600 x g for 20 min at 20°C, mononuclear cells at the interface were collected and washed twice before immunization. These cells were 80–90% CD11c\(^+\) and expressed high levels of CD80, CD86, CD40 molecules. C57BL/6 mice were injected i.v. with 1 x 10\(^5\) peptide-pulsed BMDC via the dorsal tail vein. Control mice received either PBS or none peptide-pulsed BMDC.

### Quantification of Ag-specific CD8\(^+\) T cell response

The number of CD8\(^+\) T cells specific for MPT64\(_{190–198}\) or TB2 was determined by intracellular staining for IFN-γ. The cells were incubated for 5–6 h with or without 5 μM of synthetic peptides in the presence of 10 μg/ml brefeldin A at 37°C. For the surface staining, cells were first incubated with a mAb directed against the FcγIII/II receptors (2.4G2) and were incubated with PE-conjugated anti-CD44 mAb and Cy5-conjugated anti-CD8 mAb. The cells were fixed, permeabilized, and further stained with FITC-conjugated anti-IFN-γ mAb. Samples were run on a FACS Calibur flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

#### In vivo cytotoxicity assay

B6-Ly5.1\(^+\) splenocytes were divided and labeled with either a high concentration (5 μM) or a low concentration (0.5 μM) of CFSE (Invitrogen Life Technologies). CFSE\(^{low}\) cells were pulsed with 5 μM synthetic peptide for 1 h at 37°C, whereas CFSE\(^{high}\) cells left uncoated. After washing, the cells were mixed in equal proportions (2 x 10\(^5\) total cells/200 μl) and injected i.v. into mice immunized with peptide-pulsed DC 6 days previously. Splenocytes in the recipients were harvested 4 h later for flow cytometric analysis. Percent-specific lysis was calculated according to the formula (1 – (ratio primed/ratio unprimed) x 100), where the ratio unprimed = percent CFSE\(^{low}\)/percent CFSE\(^{high}\) cells remaining in nonimmunized recipients, and ratio primed = percent CFSE\(^{low}\)/percent CFSE\(^{high}\) cells remaining in immunized recipients.

#### Infection of immunized mice with \textit{M. tuberculosis}

Mice were anesthetized by i.p. injection of pentobarbital sodium and trachea were exposed. Infection was effected by intratracheal inoculation with 1 x 10\(^5\) viable CFU of \textit{M. tuberculosis} H37Rv diluted in 50 μl of PBS. The numbers of viable bacteria in organs were measured 7 or 28 days after infection by plating serial dilutions of whole organ homogenates on supplemented Middlebrook 7H10 agar (Difco) enriched with 10% oleic acid-albumin-dextrose-catalase (Difco) and 0.5% glycerol, and incubated at 37.5°C for 3 wk. Colonies were counted and total tissue CFU calculated.

#### ELISPOT assay

The numbers of MPT64\(_{190–198}\) or TB2-specific T cells in the lungs and spleen after infection with \textit{M. tuberculosis} were determined by an ELISPOT assay (Mouse IFN-γ ELISPOT Set; BD Biosciences) according to the manufacturer’s instructions. Lung mononuclear cells were prepared by collagenase digestion and were pooled from three mice. To supplement

#### Table I. Synthetic peptides used in this study\(^a\)

<table>
<thead>
<tr>
<th>Peptide Sequence and Location</th>
<th>Gene Designation and Putative Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPT64(_{190–198})</td>
<td>FAVTNDGVI (190–198)</td>
</tr>
<tr>
<td>Mt6234(_{309–318})</td>
<td>GAPINSATAM (309–318)</td>
</tr>
<tr>
<td>38 KD4(_{129–137})</td>
<td>AQVYNNLP (129–137)</td>
</tr>
<tr>
<td>OVA(_{257–264})</td>
<td>SIINFKEI (257–264)</td>
</tr>
<tr>
<td>TB2 (f-MLVLLV)</td>
<td>(1–6)</td>
</tr>
<tr>
<td>TB4 (f-MFLIDV)</td>
<td>(1–6)</td>
</tr>
<tr>
<td>TB7 (f-MILLV)</td>
<td>(1–5)</td>
</tr>
<tr>
<td>LemA (f-MIGWII)</td>
<td>(1–6)</td>
</tr>
</tbody>
</table>

\(^a\) The sequence and annotation information of \textit{M. tuberculosis} was obtained from The Institute for Genomic Research (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi) and The Institut Pasteur (http://genolist.pasteur.fr/TubercuList/).

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APCs in the lung cells, mitomycin C-treated syngeneic splenocytes were added at a ratio of 1:1 lung cells/APCs. Two-fold serial dilutions of the 100-μl admixture were added in triplicate to the wells precoated with anti-mouse IFN-γ mAb starting at 10^5 lung cells/well. The wells were further added with 100 μl of RPMI 1640-FCS containing no Ag, 5 μM MPT64190 –198 or 5 μM TB2. After 24 h of incubation at 37°C and 5% CO₂, unattached cells were aspirated from the wells and the remaining cells were lysed with distilled water. The wells were washed again with PBS containing 0.05% Tween 20 and incubated with a second biotinylated anti-mouse IFN-γ mAb. The wells were then washed with PBS-Tween 20, incubated for 1 h with streptavidin-HRP, washed, and developed with 3-amino-9-ethyl-carbazol as substrate. After washing and drying, the number of spots per well was counted with the aid of a digital microscope at 40. The number of cells specific for each peptide was calculated by subtracting the number of spots formed in the absence of Ag from that formed in its presence. Experiments were repeated twice.

Histopathology

Tissues were preserved in 10% buffered formalin, embedded in paraffin, sectioned, and stained with H&E. Random sections including hilar of the lung from five mice per group were examined.

FIGURE 1. Expansion of Ag-specific CD8⁺ T cells after immunization with M. tuberculosis-derived peptide-pulsed BMDCs. A, CD8⁺ T cells in the spleens of naive mice or the mice immunized with H2-M3 (upper panels) or MHC class Ia (lower panels) restricted peptide-pulsed BMDCs 6 days previously were harvested and restimulated with the indicated peptide in vitro. IFN-γ-producing CD8⁺ T cells were detected by a flow cytometer. Data are representative of three separate experiments and are expressed as means ± SD of three mice in each group. *p < 0.05, **p < 0.01, significantly different from the value of TB2-specific CD8⁺ T cells.

FIGURE 2. Kinetics of the absolute number of MPT64190–198-specific or TB2-specific, IFN-γ-producing CD8⁺ T cells in the spleens and the lungs. To calculate the number of Ag-specific CD8⁺ T cells, we subtracted the percentage of IFN-γ⁺ CD8⁺ T cells in unstimulated samples from the peptide-stimulated value. Data are representative of three separate experiments and are expressed as means ± SD of three mice in each group. *p < 0.05, significantly different from the value of MPT64190–198-specific CD8⁺ T cells.

H2-M3 restricted peptide

MHC class Ia restricted peptide

A

Immunization: DC-TB2
Stimulation: TB2 Naive TB2

Immunization: DC-TB4
Stimulation: TB4 TB7 DC-LemA LemA

Immunization: DC-Mtb32A
Stimulation: Mtb32A309–318 DC-38kDa 38kDa129–137 DC-OVA OVA35–53

B

DC-MPT64

MPT64190–198

C

TB2

MPT64

Peptide (μM)

D

MFI

Mhct class Ia H2-M3

Mtb32A MPT64 OVA TB4 LemA TB2 TB7

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CD8

MPT64190–198 gave a strong T cell response similar to the control M. tuberculosis also tested three MHC class Ia-restricted peptides derived from TB2 induced the strongest expansion of Ag-specific T cells. We found this maturation step was necessary for in-vivo expansion by peptide-pulsed BMDCs, which were treated with LPS to induce full maturation. We found this maturation step was necessary for in-vivo expansion of Ag-specific T cells in preliminary experiments (data not shown). As shown in Fig. 1A, an expansion of CD8+ T cells producing IFN-γ was observed 6 days after immunization with H2-M3-binding peptides. Among the peptides tested, TB2 induced the strongest expansion of Ag-specific T cells. We also tested three MHC class Ia-restricted peptides derived from M. tuberculosis (Table I) for their immunogenicity. Although MPT64190–198 gave a strong T cell response similar to the control OVA, the other two M. tuberculosis-derived peptides induced only marginal expansion of Ag-specific T cells. Therefore, we used TB2 and MPT64190–198 as representative of H2-M3-binding and MHC class Ia-binding peptides, respectively, in the subsequent experiments. It is of note to find that, mean fluorescent intensity (MFI) for IFN-γ staining of MPT64190–198-specific CD8+ T cells was higher than that of TB2-specific CD8+ T cells at any concentration of the peptides (Fig. 1, B and C). Furthermore, CD8+ T cells specific for MHC class Ia-restricted peptides generally showed higher MFI than those specific for H2-M3-binding peptide (average MFI 77.8 vs 55.0, respectively, p = 0.007) (Fig. 1D).

Kinetic analysis revealed that the number of TB2-specific CD8+ T cells in the spleen and the lung peaked on day 6 after immunization and contracted until day 20, and then was maintained an appreciable level at least 60 days (Fig. 2). The absolute number of TB2-specific CD8+ T cells tended to be higher than that of MPT64190–198-specific CD8+ T cells at the peak, but there were no significant differences. Although the number of TB2-specific CD8+ T cells was lower than that of MPT64190–198-specific CD8+ T cells at day 60, it was still above background. These data showed that not only MHC class Ia-restricted MPT64190–198 but also H2-M3-restricted TB2-pulsed mature BMDC induced long-lasting Ag-specific CD8+ T cells.

**Statistical analysis**

The statistical significance of the bacteria number was determined by one-way ANOVA. Other data were determined by the Student t test. Differences with a p value of <0.05 were considered significant. Analyses were completed using SPSS software.

**Results**

**Induction of MHC class Ia- and H2-M3-restricted CD8+ T cell expansion by peptide-pulsed BMDCs**

Chun et al. (23) have identified several M. tuberculosis-derived peptides binding to a MHC class Ia molecule, H2-M3. We first compared immunogenicity of three of these peptides named TB2, TB4, and TB7 (Table I), all of which were shown to be immunogenic in M. tuberculosis-infected C57BL/6 mice (23). We examined expansion of Ag-specific T cells after immunization with peptide-pulsed BMDC, which were treated with LPS to induce full maturation. We found this maturation step was necessary for inducing clear expansion of Ag-specific T cells in preliminary experiments (data not shown). As shown in Fig. 1A, an expansion of CD8+ T cells producing IFN-γ was observed 6 days after immunization with H2-M3-binding peptides. Among the peptides tested, TB2 induced the strongest expansion of Ag-specific T cells. We also tested three MHC class Ia-restricted peptides derived from M. tuberculosis (Table I) for their immunogenicity. Although MPT64190–198 gave a strong T cell response similar to the control

**Effector functions of TB2- and MPT64-specific CD8+ T cells**

As potential of IFN-γ production seemed different between MHC class Ia- and H2-M3-restricted CD8+ T cells by intracellular flow
cytometric analysis (Fig. 1, B–D), we further compared the functions of TB2-specific CD8+ T cells and MPT64190–198-specific CD8+ T cells 6 days after immunization. IFN-γ and TNF-α production by spleen CD8+ T cells were measured by ELISA. Although there was no significant difference between the number of TB2-specific CD8+ T cells and that of MPT64190–198-specific CD8+ T cells in the spleen as shown in Fig. 2, TB2-specific CD8+ T cells produced less IFN-γ than MPT64190–198-specific CD8+ T cells at any concentration of the peptide (Fig. 3A). The level of TNF-α production was also lower in TB2-specific CD8+ T cells than MPT64190–198-specific CD8+ T cells (Fig. 3B).

We next evaluated the cytotoxic activity of MPT64190–198- or TB2-specific CD8+ T cells by measuring in vivo cytotoxic activity against syngeneic peptide-pulsed splenocytes 6 days after immunization. Both MPT64190–198-specific CD8+ T cells and TB2-specific CD8+ T cells lysed peptide-pulsed syngeneic splenocytes (Fig. 4). As opposed to the case of IFN-γ or TNF-α production, the cytotoxic activity of TB2-specific CD8+ T cells was significantly higher than that of MPT64190–198-specific CD8+ T cells. Taken together, these data indicated that, although H2-M3-restricted TB2-specific CD8+ T cells and MHC class Ia-restricted MPT64190–198-specific CD8+ T cells expand in similar extent with similar time kinetics after immunization with BMDC, they have somewhat different activities of function.

Response of MPT64190–198 or TB2-specific CD8+ T cells during infection with M. tuberculosis

To examine whether CD8+ T cell response to MPT64190–198 or TB2 was elicited during infection with M. tuberculosis, the number of MPT64190–198- or TB2-specific T cells in the lungs or spleen from 2 × 10^5 CFU M. tuberculosis H37Rv-infected mice was measured by an ELISPOT assay (Fig. 5). Two weeks after infection, a small number of peptide-specific IFN-γ spots was detected in the lungs and spleen. The frequency of MPT64190–198- and TB2-specific CD8+ T cells both rapidly increased from 3 wk after infection and then reached a peak at 4 wk after infection. These results clearly indicate that MPT64190–198 and TB2 are presented during infection. There was no clear difference in kinetics of the response between MPT64190–198 and TB2-specific CD8+ T cells.

H2-M3-restricted TB2-specific CD8+ T cells protect mice from intratracheal M. tuberculosis infection

To examine whether these CD8+ T cells are both protective against M. tuberculosis, we challenged the mice intratracheally with M. tuberculosis H37Rv 6 days after immunization with MPT64190–198-pulsed BMDC (DC-MPT64), TB2-pulsed BMDC (DC-TB2), or no peptide (DC-NONE). Control mice were given PBS alone. At 6 days (A) or 60 days (B) postimmunization, the mice were challenged intratracheally with 1 × 10^3 CFU of live M. tuberculosis H37Rv. Data are representative of two separate experiments and are expressed as means ± SD of four mice of each group. * p < 0.05, ** p < 0.01 significantly different from the values of PBS and DC-NONE-immunized mice.
difference of the CFU in the lung was ~1 log10 order. Thus, both MPT64<sub>190–198</sub>-specific CD8<sup>+</sup> T cells and TB2-specific CD8<sup>+</sup> T cells were protective against respiratory <i>M. tuberculosis</i> infection.

**Vaccination with TB2-pulsed BMDC conferred long-lasting protective immunity against <i>M. tuberculosis</i>**

As shown in Fig. 2, MPT64<sub>190–198</sub>-specific CD8<sup>+</sup> T cells and TB2-specific CD8<sup>+</sup> T cells were maintained for 60 days after immunization. To evaluate whether BMDC immunization can induce long-lasting protective immunity against <i>M. tuberculosis</i>, we challenged intratracheally with <i>M. tuberculosis</i> 60 days later after immunization. At 4 wk, the CFU in the lungs of DC-MPT64- or DC-TB2-immunized mice was significantly lower than those of DC-NONE-immunized mice or naive mice (Fig. 6B). The difference of the CFU in the lungs was ~1 log<sub>10</sub> order. Although the CFU in the spleens was considerably lower than that of DC-NONE-immunized mice or naive mice, there was no statistical difference. These data suggested that both MHC class Ia-restricted CD8<sup>+</sup> T cells and H2-M3-restricted CD8<sup>+</sup> T cells induced by peptide-pulsed mature BMDC elicited long-lasting protection against respiratory <i>M. tuberculosis</i> infection.

**Lung histopathology of the mice immunized with TB2 or MPT64<sub>190–198</sub> after intratracheal <i>M. tuberculosis</i> infection**

As we observed some differences in the activity of function between MPT64<sub>190–198</sub> and TB2-specific CD8<sup>+</sup> T cells in vivo as well as in vitro (Figs. 3 and 4), it is of interest to compare histopathological changes in the lungs of the mice immunized with the different peptides. DC-MPT64-immunized mice had larger pulmonary infiltrates composed of formed macrophages (Fig. 7, B and E) compared with control (Fig. 7, A and D) or DC-TB2-immunized mice (Fig. 7, C and F). Areas of bronchopneumonia were clearly evident and frankly necrotic areas were observed in part (Fig. 7B). In contrast, although large infiltrates were also observed in the lungs of DC-TB2-immunized mice, there were few necroses and the structure of the walls of the alveoli comparatively avoided destruction (Fig. 7C). There tended to be greater numbers of lymphocytes in the inflammatory infiltrate compared with DC-MPT64-immunized mice. Both perivascular and interstitial lymphoid infiltrates were observed (Fig. 7F). These histopathological features suggest that the protection mechanism of TB2-specific CD8<sup>+</sup> T cells against <i>M. tuberculosis</i> infection is different from that of MPT64<sub>190–198</sub>-specific CD8<sup>+</sup> T cells.

**Discussion**

Because CD8<sup>+</sup> T cells play a requisite role in the resistance to mycobacterial infection, Ag-specific CD8<sup>+</sup> T cells are major target for vaccine design for tuberculosis. We here showed the first evidence that immunization with mature BMDC pulsed with either MHC class Ia (H-2D<sup>B</sup>) binding MPT64<sub>190–198</sub> or class Ib (H2-M3) binding TB2 peptide induced long-lasting Ag-specific CD8<sup>+</sup> T cells and conferred protection against an intratracheal challenge with <i>M. tuberculosis</i>.

There have been several studies on BMDC-based vaccination against <i>M. tuberculosis</i> infection models in mice (16–18). McShane et al. (18) reported that mice immunized with immature BMDC pulsed with either MHC class I- or MHC class II-restricted Ag85A peptide was not protective against <i>M. tuberculosis</i> challenge. Nakano et al. (16) reported that retroviral Ag85A gene-transduced, incompletely matured BMDC immunization was not effective enough in terms of clearance of <i>M. tuberculosis</i> from the tissues. In contrast, Malowany et al. (17) reported that adenoviral Ag85A gene-transduced mature BMDC induced much longer immune response compared with immature peptide-pulsed BMDC. These findings clearly indicated the importance of maturational stage of BMDC for vaccination. We also observed a clear difference in inducing T cell response between immature and mature BMDC (data not shown). Thereafter, we used LPS-stimulated BMDC for immunization and successfully induced protective CD8<sup>+</sup> T cell responses against <i>M. tuberculosis</i> infection.

There have been some reports showing an involvement of H2-M3-restricted CD8<sup>+</sup> T cells in <i>M. tuberculosis</i> infection. Chun et al. (23) identified several H2-M3-binding peptides by scanning the full sequence of the <i>M. tuberculosis</i> genome. Although they showed CD8<sup>+</sup> T cell responses to some of these peptides including TB2 after infection with <i>M. tuberculosis</i>, it has been unknown whether the H2-M3-restricted CD8<sup>+</sup> T cells are protective against <i>M. tuberculosis</i> infection. Dow et al. (31) also identified several H2-M3-binding peptides derived from <i>M. tuberculosis</i> and showed CTL response to these peptides. They also examined protection against <i>M. tuberculosis</i> challenged 10 days after immunization with some of these peptides. However, these peptides were longer...
than the predicted length of H2-M3-binding peptides revealed by the crystallography (32) and also by bioassays (33), and none of these peptides were identical with the peptides identified by Chun et al. (23). In this study, we found TB2 elicited strongest T cell response after immunization with peptide-pulsed BMDC. Thereafter, we used TB2 and found that immunization with TB2 confers significant protection as vaccine against M. tuberculosis challenged even 60 days after immunization.

The importance of H2-M3-restricted CD8+ T cells has been more clearly shown in Listeria monocytogenes infection. It was recently reported that H2-M3-deficient mice were impaired in early bacterial clearance during primary L. monocytogenes infection (34). H2-M3-restricted CD8+ T cells play a role in early protection against a primary L. monocytogenes infection by expanding quicker than class Ia-restricted CD8+ T cells (35, 36). In the present study, however, we did not find difference in the kinetics of expansion between H2-M3-restricted CD8+ T cells and MHC class Ia-restricted CD8+ T cells either after immunization with BMDC or during M. tuberculosis infection. In the former case, the discrepancy between the previous observations and our findings may be explained by different efficacy in Ag processing between MHC class Ia-binding peptides and H2-M3-binding peptides. For the presentation by MHC class Ia molecules, antigenic peptides in the cytosol are translocated to the lumen of the endoplasmic reticulum by TAP and loaded onto peptide-receptive MHC class Ia complexes. Stably conformed and peptide-filled class Ia complexes then egress from the endoplasmic reticulum to the cell surface. In contrast, TAP did not appear to be absolutely necessary for the presentation of N-formylated peptides by H2-M3 molecules (37). In addition, the supply of endogenous N-formylated mitochondrial peptides is limited and a significant pool of H2-M3 exists intracellularly, which can be rapidly mobilized to the surface when provided with appropriate exogenous N-formylated peptides (38). Therefore, it is suggested that MHC class Iib-binding Ag peptides are more rapidly presented by APCs. Immunization with peptide-pulsed mature BMDC may circumvent these time-dependent factors. In the case of in vivo infection, one of the major differences between L. monocytogenes and M. tuberculosis is their growth rate. M. tuberculosis divide slowly and their Ags are presented gradually with time, which may conceal the lag of response of MHC class Ia-restricted CD8+ T cells behind that of H2-M3-restricted CD8+ T cells. Additionally, there seems to be a difference in Ag processing between L. monocytogenes and M. tuberculosis. In contrast to L. monocytogenes, which actively escapes phagosomes and enters the cytosol, M. tuberculosis resides within phagosomes which has features similar to those of an early endosome (39, 40). Nevertheless, M. tuberculosis-derived peptides are cross-presented by MHC class I pathway, which is supposed to be far less efficient than the case of L. monocytogenes. These differences in Ag processing and presentation of different microbes may also be involved in the discrepancy.

There were some differences in the activities of effector functions between H2-M3-restricted TB2-specific CD8+ T cells and MHC class Ia-restricted MPT64°190–198°-specific CD8+ T cells, although both were protective against M. tuberculosis infection. Cytotoxic activity of TB2-specific CD8+ T cells was higher than that of MPT64°190–198°-specific CD8+ T cells, whereas the ability to produce IFN-γ or TNF-α was the opposite. Such differences are usually observed after immunization with different peptides, even restricted by the same MHC molecule and could be related to the stability of the MHC complexes. In this regard, it is notable to find that the expression levels of IFN-γ were generally lower in H2-M3-restricted CD8+ T cells than in MHC class Ia-restricted CD8+ T cells by intracellular flow cytometric analysis (Fig. 1D), suggesting that the difference in the activities of function between TB2- and MPT64°190–198°-specific CD8+ T cells may be generalized to difference between MHC class Ia- and MHC class Iib-restricted CD8+ T cells. Further detailed analysis is needed to test this possibility. These differences in activities of function of CD8+ T cells might result in the different histopathology of the lung between MPT64°190–198° and TB2-immunized mice. The lungs of MPT64°190–198°-pulsed BMDC-immunized mice following M. tuberculosis infection had large pulmonary infiltrates composed of formed macrophages. In contrast, the lungs of TB2-pulsed BMDC-immunized mice following M. tuberculosis infection had less necrosis and reduced pulmonary injury.

In conclusion, our results clearly indicated that vaccination with mature BMDC pulsed with a H2-M3-binding peptide significantly confers protection against M. tuberculosis. Because MHC class Iib molecules including H2-M3 have an advantage of limited polymorphism, immunization with MHC class Iib-restricted peptides would be a novel vaccination strategy against M. tuberculosis infection for a broad range of recipients.

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Disclosures

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