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CD8+ T Cell Protective Immunity against Chlamydia pneumoniae Includes an H2-M3-Restricted Response That Is Largely CD4+ T Cell-Independent1

Amy Tvinnereim and Benjamin Wizel2

CD8+ T cells are important for immunity to the intracellular bacterial pathogen Chlamydia pneumoniae (Cpn). Recently, we reported that type 1 CD8+ (Tc1) from Cpn-infected B6 mice recognize peptides from multiple Cpn Ags in a classical MHC class Ia-restricted fashion. In this study, we show that Cpn infection also induces nonclassical MHC class Ib-(H2-M3)-restricted CD8+ T cell responses. H2-M3-binding peptides representing the N-terminal formylated sequences from five Cpn Ags sensitized target cells for lysis by cytolytic effectors from the spleens of infected B6 mice. Of these, only peptides fMFFAPL (P1) and fMLYWFL (P4) stimulated IFN-γ production by infection-primed splenic and pulmonary CD8+ T cells. Studies with Cpn-infected Km−/−/D b−/− mice confirmed the Tc1 cytokine profile of P1- and P4-specific CD8+ T cells and revealed the capacity of these effectors to exert in vitro H2-M3-restricted lysis of Cpn-infected macrophages and in vivo pulmonary killing of P1- and P4-coated splenocytes. Furthermore, adoptive transfer of P1- and P4-specific CD8+ T cells into naive Km−/−/D b−/− mice reduced lung Cpn loads following challenge. Finally, we show that in the absence of MHC class Ia-restricted CD8+ T cell responses, CD4+ T cells are largely expendable for the control of Cpn growth, and for the generation, memory maintenance, and secondary expansion of P1- and P4-specific CD8+ T cells. These results suggest that H2-M3-restricted CD8+ T cells contribute to protective immunity against Cpn, and that chlamydial Ags presented by MHC class Ib molecules may represent novel targets for inclusion in anti-Cpn vaccines. The Journal of Immunology, 2007, 179: 3947–3957.

Infection with Chlamydia pneumoniae (Cpn), a Gram-negative obligate intracellular bacterial pathogen that causes sinusitis, bronchitis, and pneumonia, occurs in nearly every human being (1, 2). Despite their high prevalence, most Cpn infections are mild or subclinical and, thus, do not prompt antibiotic treatment. However, left untreated or inadequately treated, Cpn can persist in the host and cause chronic infections, which have been implicated in a number of respiratory and systemic inflammatory conditions that include asthma, chronic obstructive pulmonary disease, multiple sclerosis, and atherosclerosis (2–5). Thus, the development of a vaccine to prevent or ameliorate acute and chronic Cpn infection could provide considerable public health benefit. Although our knowledge of chlamydial Ags and defense mechanisms that lead to protective immunity against Cpn has increased substantially in recent years (6–13), developing vaccines or immunotherapies against Cpn will require an improved and comprehensive understanding of all the arms of the immune system that act in concert to control Cpn growth and facilitate pathogen clearance.

Several studies using mice competent or deficient in elements known to contribute to host defense against bacterial pathogens indicate that innate and adaptive immune effector mechanisms can decrease, promote, or have no role in the control of Cpn growth (6–12, 14). For instance, IFN-γ, a type 1 cytokine critical for T cell-mediated protection against Cpn (6, 7), is also produced by macrophages at levels that are sufficient to reduce pulmonary Cpn loads (8). In contrast, NK cells, which are an important early supply of IFN-γ and cytotoxic activity, are apparently not needed for innate resistance to Cpn (7). TNF also appears to be dispensable for the initial control of Cpn growth in vivo, although it does display anti-Cpn growth activity in vitro when combined with IFN-γ (6, 15). Likewise, Abs play no major role in the control of Cpn in vivo, but they do neutralize pathogen infectivity in vitro (6, 9, 16). Despite their ability to ingest and kill bacteria, neutrophils and macrophages can serve as host cells for Cpn (17, 18), and these infected phagocytes have been reported to enhance Cpn replication in epithelial cells and disseminate the infection from the respiratory tract (14, 18). Finally, evidence indicates that T cells are essential in immunity to Cpn. Although both CD4+ and CD8+ T cells contribute to protection, mainly through the secretion of IFN-γ, CD8+ T cells play the predominant role (6, 10). Thus, a full characterization of the CD8+ T cell response against Cpn will be required to develop an effective vaccine against this pathogen.

CD8+ T cells recognize pathogen-derived Ags presented by MHC class I molecules on the surface of infected cells. Based on structural and functional properties, MHC class I molecules can be classified within the highly polymorphic classical class Ia or the more conserved nonclassical class Ib families of proteins. We have shown that multiple Cpn Ags are targets of a CD8+ T cell response in infected mice, and that pathogen-specific Tc1 cells exhibit potent antichlamydial growth activity upon MHC class Ia-restricted

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2 This work was supported by Grant RO1 HL70641 from the National Institutes of Health.
3 Abbreviations used in this paper: Cpn, Chlamydia pneumoniae; NfM, N-terminal formylated methionine; Tc1, type 1 CD8+ T cell; IFU, inclusion-forming unit; TCM, T cell medium; i.n., intranasal; MFI, mean fluorescence intensity; LMNC, lung mononuclear cell; SC, spleen cell; mM, murine alveolar macrophage; SFC, spot-forming cell; Snplh, small protein B.
recognition of infected cells (11). However, it is now evident that nonclassical MHC class Ib molecules also function as Ag-presenting structures for CD8+ T cells during infection with intracellular bacteria (19–23).

In mice, the MHC class Ib molecule H-2-M3 binds 5- to 6-mer hydrophobic peptides with an N-terminal formylated methionine (N-FM), a residue only found in bacterial, and a set of mitochondrial, proteins (24–26). To date, three Ags from Listeria monocytogenes, four from Mycobacterium tuberculosis, and an unknown molecule from Salmonella typhimurium have been reported to prime H-2-M3-restricted CD8+ T cells following infection with these pathogens (19–23). Another murine MHC class Ib molecule that is involved in the anti-L. monocytogenes CD8+ T cell response and that acts as the dominant restricting element for S. typhimurium-specific CD8+ T cells is Qa-1b (27, 28). Although information on the protective capacity of nonclassically restricted CD8+ T cells is sparse, depletion and adoptive transfer studies in the L. monocytogenes infection model strongly suggest that these T cell effectors play an active role in antilisterial immunity (29–32). That this subset of CD8+ T cells participate in the human host response to intracellular bacteria is suggested by the fact that most M. tuberculosis-specific CD8+ T cells in the peripheral blood of subjects with latent tuberculosis infection are restricted by the human class Ib molecule HLA-E (33, 34). Moreover, recent studies in Chlamydia trachomatis-exposed individuals indicate that the majority of Chlamydia-reactive CD8+ T cells recognize infected cells in a nonclassically restricted manner (35, 36). Nevertheless, it is uncertain whether MHC class Ib-restricted CD8+ T cells are induced during Cpn infection and whether these T cells contribute to protection. Furthermore, no chlamydial Ag has yet been identified as a target of MHC class Ib-restricted CD8+ T cell responses.

Using the mouse model of Cpn infection, we provide here evidence that primed CD8+ T cells include specificities for H-2-M3-binding Cpn Ag-derived N-FM peptides, and that these T cells can lyse Cpn-infected cells and produce type 1 cytokines in an H-2-M3-restricted fashion. We also show that adoptive transfer of Cpn H-2-M3 epitope-specific T cells (Te1) reduces the bacterial burden in the lungs of Cpn-challenged mice and that the same T cell specificities are induced and maintained in the absence of CD4+ T cells.

Materials and Methods

Bacteria

Cpn isolate K6 (obtained from M. Puolakkainen, University of Helsinki, Helsinki, Finland) was grown within HL cells in Chlamydia medium as described (11). Elementary bodies were purified by density gradient centrifugation, aliquoted in a sucrose-phosphate-glutamate buffer, and stored at −70°C. The infectivity, as measured by inclusion-forming units (IFU) of purified organisms, was titrated in cycloheximide-treated HL cell monolayers.

Mice and infections

Six- to 10-wk-old female C57BL/6 (B6; The Jackson Laboratory) and B6.K−/− D2−/− (K−/− D2−/−; Taconic Farms) (37) mice were maintained in a specific pathogen-free facility and housed in microisolator cages. Animals received primary and challenge infections by intranasal (i.n.) inoculation with 105 IFU of Cpn in 40 μl of PBS under halothane anesthesia. The institutional animal care and use committee approved all experimental procedures used in this study.

Cell lines and culture media

The RMA-S lymphoma (H-2b; provided by H. G. Ljundgren, Karolinska Institute, Stockholm, Sweden), murine alveolar macrophage (mAM) (H-2b; obtained from Z. Chrones, University of Texas Health Center, Tyler, TX), and HL human epithelial (University of Washington, Seattle, WA) cell lines were maintained in complete medium consisting of RPMI 1640 with 10% FCS, 20 mM HEPEs, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 20 μg/ml gentamicin (all obtained from Invitrogen). The H-2-M3-transfected macrophage cell line P388-M3 (supplied by C.-R. Wang, University of Chicago, Chicago, IL) (38) was maintained in complete medium with 0.4 mM l-glutamine (Invitrogen). T cell medium (TCM) was prepared by supplementing complete medium with 50 μM 2-ME (In vitro). Chlamydia medium consisted of complete IMDM (Invitrogen) with 0.5 μg/ml glucose and 0.26 mg/ml sodium bicarbonate at 5% CO2.

Peptide-induced up-regulation of cell surface H2-M3 expression

Ligand binding to H-2-M3 was measured by the ability of exogenously added peptides to increase the expression of this class Iib molecule on the surface of P388-M3 cells (40). At 24 h after culture at 37°C for 18 h in the presence of 0.1–20 μM or absence of peptide, P388-M3 cells were harvested, washed in cold FACS buffer (PBS/1% BSA/0.05% NaN3), preincubated with Fc block (BD Biosciences) for 15 min, and then stained for 30 min on ice with a FITC-F(ab′)2 goat anti-hamster IgG (Southern Biotechnology Associates). Cells were washed two times after each staining step and then analyzed on a FACSCalibur flow cytometer using CellQuest Pro software (BD Biosciences). Results were expressed as the mean fluorescence intensity (MFI) ratio: MFI of cells incubated with peptide/MFI of cells incubated without peptide.

Preparation of target cells and CTL assay

Pepptide-pulsed RMA-S target cells were prepared in 24-well plates (107/well) by overnight incubation at 37°C in the presence of peptide (2 μM) and 10 μg/ml Con A-stimulated rat splenocytes (T25 flasks; Corning). Lung mononuclear cells (LMNC) were prepared as described (11). CD8+ T cells were purified from LMNC by positive selection and tested ex vivo for cytolytic activity.

Peptides

Deduced amino acid sequences of all the open reading frames found in the Cpn AR39 genome (39) were analyzed at their N termini for the presence of a nonamer segment bearing the H-2-M3-binding motif FM(F/Y)FIL(W/Y)FI (38), and the sequences determined by reverse-phase HPLC and mass spectrometry. All peptides were dissolved in DMSO (Sigma-Aldrich) at 0.5 μg/ml and stored at −20°C.

Generation of CTL effectors

Spleens and lungs from Cpn-infected mice were removed 7 days to 4 wk after the first or second infection. Spleen cells (SC; 4 × 106) were incubated with each Cpn peptide (2 μM) at 37°C, 6% CO2, in 10 ml of TCM using T25 flasks (Corning). After 2 days of incubation, cultures were supplemented with 5% of a supernatant from Con A-stimulated rat splenocytes (T-STIM, without Con A; BD Biosciences) and incubated for 4 additional days. To generate short-term T cell lines, effectors were restimulated with peptide (2 μM) for a second 6-day cycle as described (41). Lung mononuclear cells (LMNC) were prepared as described (11). CD8+ T cells were purified from LMNC by positive selection and tested ex vivo for cytolytic activity.
**Table I. Cpn synthetic peptides: protein sources and H2-M3-binding affinities**

<table>
<thead>
<tr>
<th>ID</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gene Designation(s)&lt;sup&gt;b&lt;/sup&gt; (Annotated Source)</th>
<th>Ratio MFI&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>fMFAPFL</td>
<td>CP0021/Cpn0725 (Conserved hypothetical protein)</td>
<td>3.12 ± 0.15</td>
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<tr>
<td>2</td>
<td>fMLLGAY</td>
<td>CP0323/Cpn0430 (NADH ubiquinone oxidoreductase subunit E)</td>
<td>1.34 ± 0.14</td>
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<tr>
<td>3</td>
<td>fMLINLS</td>
<td>CP0413/Cpn0347 (ABC transporter, putative permease protein)</td>
<td>1.97 ± 0.24</td>
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<tr>
<td>4</td>
<td>fMLHYFL</td>
<td>CP0421 (SsrA-binding protein, SmpB)</td>
<td>2.90 ± 0.17</td>
</tr>
<tr>
<td>5</td>
<td>fMLPEGK</td>
<td>CP0531/Cpn0233 (Hypothetical protein)</td>
<td>1.28 ± 0.14</td>
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<tr>
<td>6</td>
<td>fMLLLLE</td>
<td>CP0797, CP0728, CP0733, CP0764 (Hypothetical proteins)</td>
<td>1.73 ± 0.04</td>
</tr>
<tr>
<td>7</td>
<td>fMLFPPFR</td>
<td>CP0739 (Conserved hypothetical protein)</td>
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<tr>
<td>8</td>
<td>fMLLLTL</td>
<td>CP0766 (Hypothetical protein)</td>
<td>2.86 ± 0.12</td>
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<td>fMLFLIS</td>
<td>CP0795/Cpn0155 (Hypothetical protein)</td>
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<tr>
<td>10</td>
<td>fMFFIAV</td>
<td>CP0826/Cpn1026 (Late transcription unit A protein, LtuA)</td>
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<tr>
<td>11</td>
<td>fMVTLSK</td>
<td>CP0845 (Conserved hypothetical protein)</td>
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<tr>
<td>12</td>
<td>fMILITQ</td>
<td>CP0928/Cpn0933 (Predicted disulfide bond isomerase)</td>
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<tr>
<td>13</td>
<td>fMLVLLIL</td>
<td>CP1029/Cpn0840 (Conserved hypothetical protein)</td>
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<td>14</td>
<td>fMLMVTAT</td>
<td>CP0866/Cpn0989 (N utilization substance protein B, NusB)</td>
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<td>fMILIRKS</td>
<td>CP1039/Cpn0831 (Hypothetical protein)</td>
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<td>16</td>
<td>fMLYRVF</td>
<td>CP0835 (Hypothetical protein)</td>
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<td>18</td>
<td>fMHYKSL</td>
<td>CPn1007 (Hypothetical protein)</td>
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<tr>
<td>Fr38</td>
<td>fMLVIL</td>
<td>Lmo1602 (L. monocytogenes protein, unknown function)</td>
<td>5.15 ± 0.13</td>
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<tr>
<td>COI</td>
<td>fMFINIK</td>
<td>Cytochrome c oxidase subunit I (mitochondrial protein)</td>
<td>2.06 ± 0.38</td>
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<sup>a</sup> Sequence in single letter amino acid code. Sequences conform to H2-M3-binding motif: IM at position 1, YFWL at position 2, YFILV at position 3, any amino acid residue with a nonpolar side chain at position 4, and any amino acid residue at positions 5 and 6.

<sup>b</sup> Gene names designations as annotated from the sequenced genomes of Cpn strains AR39 and CWL029 (www.tigr.org/igs-scripts/CMR2/GenomePage3.spl?database=bcp, reported in Ref. 39; www.tigr.org/igs-scripts/CMR2/GenomePage3.spl?database=ntcp01, reported in Ref. 52).

<sup>c</sup> Binding affinity as assessed by the ability of peptides (10 μM) to increase M3 surface expression on P388-M3 cells. Ratio MFI is mean fluorescence intensity in presence of test peptide over the fluorescence intensity in the absence of peptide (high, ≥2.5; intermediate, 2.0–2.4; low, ≤1.9). Values represent the mean ratio MFI ± SEM obtained from three independent experiments.

Flow cytometric analysis of cell surface activation phenotype

SC from naive and 7-day Cpn-infected mice were prepared in TCM, seeded in 24-well plates (5 × 10⁵/ml; Costar), and incubated with CTL peptides (2 μM) for 24 h at 37°C, 6% CO₂. After harvesting, peptide-stimulated effector cells were washed in cold FACS buffer, dispensed into aliquots (5 × 10⁵–1 × 10⁶), and stained for 45 min at 4°C with the following mAbs (10 μg/ml): anti-CD8α PE and PE-Cy5 (53-6.7), anti-CD44 PE (IM7), anti-CD69 FITC (H1.2F3), anti-CD62L PE (MEL-14), anti-CD11a FITC (M17/4) (all obtained from eBioscience), and fluorescently conjugated rat IgG2a, IgG2b and hamster isotype control Abs (all obtained from BD Biosciences). Cells were then washed twice and analyzed by flow cytometry (at least 10,000 events/sample) as described above. Dead cells and monocytes were excluded using forward and side scatter gating.

Cytokine determinations

SC and LMNC from 7-day Cpn-infected mice were suspended in TCM and plated onto culture dishes (Corning) for 1 h at 37°C. Nonadherent SC and LMNC were then respectively adjusted to 2.5 × 10⁵ cells/ml and 8 × 10⁶ cells/ml, and seeded in triplicate at 100 μl/well in flat-bottom 96-well plates (Costar). Each well also received 100 μl of peptide-pulsed (5 μM) irradiated (16 krad) RMA-S cells adjusted to 1 × 10⁵ cells/ml. After 4 days of incubation at 37°C, culture supernatants were harvested, and levels of IFN-γ, TNF-α, and IL-4 were determined by sandwich ELISA. Capture and detection Ab pairs were: R4-6A2/XMG1.2 (BD Biosciences) for IFN-γ, Ag affinity purified goat polyclonal Ab/Mp6-XT3 (R&D Systems; BD Biosciences) for TNF-α, and BVDV-1D11/BVDV-24G2 (BD Biosciences) for IL-4. The lower detection limits for IFN-γ, TNF-α, and IL-4 were 40, 40, and 8 pg/ml, respectively.

IFN-γ ELISPOT assay

Enumeration of Cpn peptide-specific, IFN-γ-producing T cells was assessed by ELISPOT (11), using 2-fold serial dilutions of LMNC and SC (100 μl/well) from Cpn-infected mice as effectors, and peptide-pulsed (2 μM) irradiated (16 krad) RMA-S cells (10⁴/100 μl/well) as stimulators. Effector cells also included LMNC that were depleted of CD8+ and CD4+ T cells by positive selection using anti-Ly2 and anti-L3T4 Ab-coupled magnetic beads (Miltenyi Biotec). Each effector cell population was >90% depleted of the respective T cell subset. Immune cells stimulated with irradiated unpulsed RMA-S cells in the presence or absence of PMA (50 ng/ml) and ionomycin (500 ng/ml; Sigma-Aldrich) were used as negative and positive controls, respectively.

**FIGURE 1. Cpn infection-primed lung CD8+ T cells exhibit MHC class Ia- and non-MHC class Ia-restricted CTL activity.** A. Immunofluorescent staining of Cpn-infected and uninhibited mAM for CD1a (blue), Cpn MOMP (green), and IgG3 isotype control mAbs. Rate of infection was measured by flow cytometry (at least 10,000 events/sample) as described above. Dead cells and monocytes were excluded using forward and side scatter gating.
FIGURE 2. Cpn-specific CD8+ Tc1 response includes specificities to H2-M3-binding Cpn peptides. A, SC from 10-day Cpn-infected B6 mice were cultured for 6 days with each of the six Cpn H2-M3-binding peptides and a control non-H2-M3-binding peptide (2 μM). Lytic activity of effectors was measured in a CTL assay against RMA-S cells pulsed with homologous peptide or with negative control H2-M3-binding COI peptide (2 μM). Similar results were obtained with immune SC obtained 7–30 days postinfec-
tion. B, Spleen- and lung-derived immune cells pooled from five 14-day Cpn-infected B6 mice were stimulated with irradiated RMA-S cells pulsed with N-FM Cpn peptides or control COI peptide (5 μM). Levels of IFN-γ were measured by ELISA in 4-day culture supernatants. Peptide-stimu-
lated cells from mock-infected mice did not secrete de-
tectable amounts of IFN-γ. C, LMNC pooled from five 7-day Cpn-infected B6 mice were cocultured for 24 h with peptide pulsed (5 μM) or unpulsed (DMSO) irradiated RMA-S cells, and the number of IFN-γ SFC were determined by ELISPOT. Tested effector cells also included LMNC depleted of CD4+ or CD8+ T cells. Values represent mean ± SEM of four (A) or three in-
dependent experiments (B and C). *, Differences vs lysi-
sis of respective COI peptide-pulsed targets (A, p ≤ 0.05), IFN-γ secretion from COI peptide-stimulated cells (B, p ≤ 0.01), and IFN-γ SFC for Fr38-stimulated LMNC (C, p ≤ 0.001) are significant (Student’s t test).

In vivo cytotoxicity assay
SC from naive K+b−/−D−/− mice were adjusted to 1 × 10^7 cells/ml in TCM and incubated with either 5 μM of Cpn CTL peptide or with DMSO-containing TCM for 1 h at 37°C. After two washes with PBS, peptide- and mock-pulsed SC were adjusted to 2 × 10^7 cells/ml in HBSS and labeled, respectively, with a high concentration (2.5 μM) and a low concentra-
tion (0.5 μM) of CFSE for 3 min at 37°C. CFSE labeling was then stopped by addition of FBS, and after extensive washing, equal numbers of CFSEbright and CFSElow cells were combined and transferred i.v. (1 × 10^7/100 μl/mouse) to naive and 10-day Cpn-reinfected K+b−/−D−/− mice. The lungs of recipient mice were harvested 18 h later, and single-cell suspensions were analyzed by flow cytometry. The percent-specific lysis of CFSE+ donor SC in each mouse was determined as follows: 1 − [(% CFSElow naive/% CFSEbright naive)/(% CFSElow infected/% CFSEbright infected)] × 100%.

Adaptive transfer of Cpn epitope-specific H2-M3-restricted CD8+ T cells

CD8+ T cells were immunomagnetically purified (>95% CD8+) by nega-
tive selection (Miltenyi Biotec) from Cpn CTL peptide P1- and P4-spe-
cific short-term T cell lines (85–87% CD8+) 6 days after Ag stimulation, and from freshly isolated naive SC. After two washes in PBS, donor cells were injected i.v. into recipient K+b−/−D−/− mice (5 × 10^9/200 μl/mouse), which were challenged 2 h later by i.n. infection with 10^6 IFU of Cpn.

Quantitation of Cpn pulmonary loads

Pulmonary Cpn infectious titers were assessed at the indicated times postinfection as described (42). Briefly, IFU titers were determined by counting the number of chlamydial inclusions in HL cell monolayers in-
culated with serial dilutions of lung homogenates and processed 72 h later for immunofluorescent staining using mAb RR402. The limit of detection is ∼30 IFU per lung.

In vivo depletion of CD4+ T cells

In vivo depletion of CD4+ T cells was performed as described previously with the following modifications (43). Briefly, K+b−/−D−/− mice were de-
pleted of CD4+ T cells by i.p. injection of the GK1.5 (anti-CD4) mAb (250 μg/100 μl) on days −4, −2, 0, +2, and +5 relative to the day of Cpn infection or pathogen re-exposure. For in vivo CD4+ T cell depletion dur-
ing the memory phase of the primary infection, K+b−/−D−/− mice received GK1.5 injections twice per week for 3 wk starting 10 days postinfection. Depletion of CD4+ T cells was 92–97% effective, as determined by stain-
ing and flow cytometric analysis of SC from PBS- or GK1.5-treated mice with the FITC-conjugated RM4-4 mAb (BD Biosciences).

Results

Cpn-specific CTL activity of lung CD8+ T cells includes a component that is non-MHC class Ia restricted

We previously noted that mononuclear cell infiltrates in the lungs of Cpn-infected B6 mice contain pathogen-specific CD8+ T cells with the capacity to produce IFN-γ (11). To investigate whether these T cells also display Cpn-specific MHC class Ia-restricted CTL activity, the lytic potential of Cpn infection-primed pulmonary CD8+ T cells was examined ex vivo using Cpn-infected
Cpn infection primes CD8+ Tc1 cells that recognize H-2-M3-binding Cpn peptides

Because H-2-M3 is the most widely studied Ag-presenting structure for nonclassically restricted murine CD8+ T cells elicited following infection with several intracellular bacterial pathogens (19–23), we investigated whether CD8+ T cells with specificity for N-fM chlamydial peptides are generated in Cpn-infected B6 mice. We searched the protein coding sequences of the Cpn genome for N-terminal peptides conforming to the H-2-M3 binding motif. Of 48 hexameric segments identified, 18 were selected for motif. Of 48 hexameric segments identified, 18 were selected for

We next sought to determine whether N-fM Cpn peptides were elicted in response to Cpn infection, we tested the CTL activity and IFN-γ-producing capacity of peptide-stimulated effector cells from Cpn-infected mice. Of the six peptides with high H-2-M3 binding affinity, five (P1, P4, P8, P10, and P12) generated CTL effectors with net peptide-specific lysis values ranging from 18 to 28% (E:T ratio 30:1) (Fig. 2A). However, only two N-fM Cpn CTL peptides (P1 and P4) stimulated SC and LMNC to secrete IFN-γ at levels that were respectively ~3- to 4-fold and ~5- to 8-fold higher than the levels released by control COI peptide-stimulated cells (Fig. 2B). No in vitro recall CTL or IFN-γ responses to the positive N-fM Cpn peptides were detected in mock-infected mice (data not shown).

We next sought to determine whether CD8+ T cells were the source of the IFN-γ released by P1- and P4-stimulated LMNC, and whether these cells were detectable without stimulation. Again, of the five N-fM Cpn CTL peptides, significant ex vivo IFN-γ responses were only detected for P1 and P4 (Fig. 2C). By ELISPOT, the numbers of IFN-γ spot-forming cells (SFC) counted for LMNC stimulated with peptides P1 and P4 were, respectively, ~2.5- to 3-fold and ~3.5- to 4-fold higher than those enumerated for cells incubated in the absence of peptide (DMSO) or with the control Fr38 peptide (96–133 vs 34–37 SFC/106 LMNC). Moreover, P1- and P4-specific cells were CD8+ because depleting LMNC of CD8+, but not of CD4+, T cells reduced the numbers of IFN-γ SFC to background levels (Fig. 2C). Together, these data established that the lungs and spleens of Cpn-infected B6 mice include CD8+ Tc1 cells that recognize H-2-M3-binding Cpn Ag-derived N-fM peptides and suggested that these effectors may participate in the immune response against this pathogen.

Kb−/−/Db−/− mice generate H-2-M3-restricted Cpn peptide-specific CD8+ Tc1 responses

To explore the role of MHC class Ia-restricted CD8+ T cells in anti-Cpn immunity and further characterize the CD8+ Tc1 response to N-fM Cpn peptides P1 and P4, we used Kb−/−/Db−/− mice. MHC class Ia-deficient mice represent a useful model to study the contribution of MHC class Ia-dependent Ag presentation in protective immunity against intracellular bacterial pathogens (29, 30, 32, 37, 44, 45). Upon Cpn infection, Kb−/−/Db−/− mice controlled pulmonary bacterial growth with delayed kinetics relative to B6 mice (Fig. 3A). However, a significant difference in lung Cpn loads was only detected at 21 days after infection (p < 0.01). At this time point, Kb−/−/Db−/− and B6 mice had, respectively, a 15- and 144-fold reduction in mean lung bacterial numbers when compared with the counts obtained at 10 days postinfection. At 34 days, low Cpn infectious titers were seen in the lungs of both mouse groups, with each representing a ~3 log reduction in mean pulmonary Cpn loads. Similar to the results obtained in B6 mice, pulmonary CD8+ T cells from Cpn-infected Kb−/−/Db−/− mice have immediate cytolytic activity against infected mAM. This lytic activity was inhibited by 43% in the presence of H-2-M3-blocking Ab (Fig. 3B) indicating that a significant component of the effector response is H-2-M3-restricted. These results indicate that an immune response with Cpn growth-inhibiting activity was generated in MHC class Ia-deficient mice. Although innate immune mechanisms likely played a role in the observed reduction of lung bacterial numbers, adaptive immunity was likely to dominate at the tested time points. Thus, these data suggest that Ag-specific, H-2-M3-restricted T cells contributed to the observed resistance against Cpn.

If P1- and P4-specific CD8+ T cells participated in the anti-Cpn response of Kb−/−/Db−/− mice, then these specificities would have
to be induced following Cpn infection. Indeed, analysis of the activation status of P1- and P4-stimulated SC from Cpn-infected K\(^{b-/-}\)D\(^{b-/-}\) and B6 mice revealed that the percentages of CD8\(^+\) T cells with a CD44\(^{high}\)CD69\(^{high}\) activated phenotype were 4- to 6-fold higher than those found in peptide-stimulated naive SC (Fig. 4). Parallel studies with markers of memory cells showed that the percentages of CD62L\(^{low}\)CD11a\(^{high}\) CD8\(^+\) T cells were 4- to 16-fold higher in P1- and P4-stimulated SC from infected K\(^{b-/-}\)D\(^{b-/-}\) and B6 mice compared with SC from naive animals (Fig. 4). As reported by others (45, 46), a high percentage of CD8\(^+\) T cells in naive K\(^{b-/-}\)D\(^{b-/-}\) mice had an activated/memory phenotype. However, a higher frequency of these T cells changed their phenotype even further after Cpn infection (Fig. 4). Despite these changes, it should be noted that the frequencies of splenic CD8\(^+\) T cells did not significantly vary in naive and Cpn-infected K\(^{b-/-}\)D\(^{b-/-}\) (3 vs 4%) and B6 (14 vs 17%) mice (data not shown).

We then asked whether the N-fM Cpn peptides P1 and P4 represent H2-M3-restricted Tc1 epitopes that are presented on the surface of Cpn-infected cells. Effector cells generated from P1- and P4-stimulated K\(^{b-/-}\)D\(^{b-/-}\) SC lysed Cpn-infected mAM and peptide-pulsed RMA-S cells, but not mock-infected or COI peptide-pulsed target cells (Fig. 5A). Target cell lysis was H2-M3-restricted, as the anti-H2-M3 mAb 130 blocked the killing of P1- and P4-specific CTL (Fig. 5A). Moreover, the supernatants from SC that were stimulated with each of the two peptides contained IFN-\(^\gamma\) and TNF-\(\alpha\), but not IL-4 (Fig. 5B). Thus, P1 and P4 represent peptides with sequences that are totally or partially represented among the naturally processed peptides displayed by Cpn-infected mAM.

**FIGURE 4.** Activation phenotypes of CD8\(^+\) T cells from Cpn-infected B6 and K\(^{b-/-}\)D\(^{b-/-}\) mice. SC from naive and 7-day Cpn-infected B6 and K\(^{b-/-}\)D\(^{b-/-}\) mice were stimulated for 24 h with Cpn CTL peptides (each at 2 \(\mu M\) each) and then tested for the expression of the indicated activation and memory markers. Cells were gated on CD8\(\alpha\) and analyzed for the expression of each marker by flow cytometry. The numbers in the quadrants of each dot plot indicate the mean percentage of cells \(\pm\) SEM from three independent experiments.

**FIGURE 5.** Type 1 cytokine-secreting N-fM Cpn peptide-specific splenic CD8\(^+\) T cells lyse Cpn-infected and peptide-pulsed target cells in an H2-M3-restricted manner. SC from K\(^{b-/-}\)D\(^{b-/-}\) mice (\(n = 5\)) were obtained 12 days after Cpn infection and pooled. A, After 6 days of stimulation with CTL peptides (2 \(\mu M\)), effector cells were tested for their ability to kill Cpn-infected and mock-infected mAM, and RMA-S cells pulsed with the homologous Cpn CTL peptide or negative control COI peptide (2 \(\mu M\)) in a CTL assay at an E:T ratio of 30:1. Infected and Cpn CTL peptide-pulsed target cells were treated with mAb130 (anti-H2-M3) or control mAb before and during coculture with effectors. *, Differences vs lytic activity inhibited by mAb 130 are significant (\(p \leq 0.001\), Student’s \(t\) test). B, Levels of IFN-\(\gamma\), TNF-\(\alpha\), and IL-4 were determined by ELISA in supernatants from pooled SC stimulated for 4 days with Cpn CTL peptide-pulsed (5 \(\mu M\)) irradiated RMA-S cells. Peptide-stimulated SC from mock-infected mice did not secrete detectable amounts of cytokines. Values in both panels represent mean \(\pm\) SEM of five independent experiments, each consisting of triplicate cultures with pooled SC.
Pulmonary Cpn H2-M3 peptide-specific CD8+ T cells exhibit CTL activity in vivo

Although the spleens of Cpn-infected Kb−/−Db−/− mice included IFN-γ-producing P1- and P4-specific CD8+ CTL, it remained to be determined whether the lungs of these animals also included the same T cell specificities and effector functions. LMNC obtained 7 days after Cpn infection were stimulated with peptide-pulsed RMA-S cells, and the frequencies of N-FM Cpn peptide-specific IFN-γ-producing CD8+ T cells were determined by ELISPOT. The frequencies of P1- and P4-specific IFN-γ SFC were ~3.5- to 5-fold higher compared with the IFN-γ-producing cells enumerated for the control P6, P13, and Fr38 peptides (115–162 vs 31–33 SFC/106 LMNC; Fig. 6A). Although the numbers of P1-specific IFN-γ-producing CD8+ T cells were similar in both Kb−/−Db−/− and B6 mice, the P4-specific IFN-γ response was ~20% higher in animals lacking MHC class Ia molecules (Figs. 2C and 6A). In mock-infected Kb−/−Db−/− mice, the IFN-γ responses to peptides P1 and P4 were within the background responses seen for control peptides (35–39 SFC/106 LMNC; data not shown).

To evaluate the lytic potential of pulmonary P1- and P4-specific CD8+ T cells, we measured epitope-specific cytolytic activity in Kb−/−Db−/− mice using an in vivo cytotoxicity assay. After transfer of peptide P1- or P4-pulsed CFSEhigh SC with an equal number of unpulsed CFSElow SC, in vivo CTL activity to N-FM Cpn peptide-pulsed targets was detected in Cpn-infected but not in naive mice (Fig. 6B). Interestingly, the lytic and IFN-γ responses to peptide P4 were, respectively, 1.6- and 1.4-fold higher than those observed to peptide P1. Taken together, these data confirm that CD8+ Tc1 responses to N-FM peptides P1 and P4 are primed upon Cpn infection, and show that the lungs of infected animals include H2-M3-restricted T cells that could control Cpn growth in vivo through secretion of IFN-γ and/or killing of infected cells.

Adoptively transferred Cpn H2-M3 peptide-specific CD8+ T cells reduce lung bacterial loads

Based on the foregoing results, we asked whether adoptive transfer of P1- and P4-specific CD8+ T cells into naive Kb−/−Db−/− mice could restrict pulmonary chlamydial growth following Cpn challenge. To generate sufficient numbers of effector cells, SC from Cpn-infected mice were expanded by two 6-day cycles of stimulation with each CTL peptide. The resulting P1- and P4-specific T cell lines were 85–87% CD8+ (Fig. 7A) and highly lytic to Cpn peptide-pulsed targets (data not shown). Before transfer, each T cell line was further enriched for CD8+ T cells to ensure that the purity of these effectors was comparable to that attained for the control population of naive CD8+ T cells (97% CD8+). Ten days postchallenge, the lungs of P1- and P4-specific CD8+ T cell recipients had, respectively, 5.2- and 16.5-fold fewer bacteria than the lungs of mice that received naive CD8+ T cells (p < 0.05; Fig. 7B). Thus, H2-M3-restricted CD8+ T cells can play a protective role in immunity against Cpn.

Cpn H2-M3-restricted CD8+ T cells are primed and maintained in the absence of CD4+ T cells

The induction of strong and durable MHC class Ia-restricted T cell responses often requires CD4+ T cell help, which acts by promoting the development and preservation of a functional memory
CD8\(^+\) T cell pool (47–49). It is unclear, however, whether CD4\(^+\) T cells are needed to induce non-MHC class Ia-restricted CD8\(^+\) T cell responses and to support the persistence and functional activity of nonclassically restricted memory CD8\(^+\) T cells.

To begin to investigate the role that CD4\(^+\) T cells play in the priming, maintenance, and recall of Cpm-specific H2-M3-restricted CD8\(^+\) T cell responses, the frequencies of CD8\(^+\) IFN-\(\gamma\)-producing T cells to peptides P1 and P4 were assessed by ELISPOT using SC from Cpm-infected K\(^b\)-/-D\(^b\)-/- mice that had been depleted of CD4\(^+\) T cells (Fig. 8A). Seven days after the primary Cpm infection, the numbers of P1- and P4-specific IFN-\(\gamma\)-producing CD8\(^+\) T cells in CD4\(^+\) T cell-depleted mice paralleled those enumerated in control undepleted animals (29–35 vs 27–32 SFC/10\(^6\) SC). A week after reinfection, the response to each peptide increased (48–35 vs 55–37 SFC/10\(^6\) SC), but only the numbers of P1-reactive CD8\(^+\) T cells differed significantly in both CD4\(^+\) T cell-deficient (\(p < 0.05\)) and control (\(p < 0.01\)) mice. Interestingly, a month after the primary infection, recall peptide-specific IFN-\(\gamma\)-producing T cells did not show any sign of erosion regardless of whether the generated memory CD8\(^+\) T cells had or had not been exposed for the three preceding weeks to a CD4\(^+\) T cell-depleted environment (37–31 vs 36–38 SFC/10\(^6\) SC). The similar numbers of peptide-specific CD8\(^+\) IFN-\(\gamma\)-producing T cells found in CD4\(^+\) T cell-deficient and -sufficient mice did not result from compensatory CD8\(^+\) T cell increases in the former, as the percentages of splenic CD8\(^+\) T cells in both groups were similar at each tested time point (4 vs 4.4%; 3.1 vs 3.3%; 6.8 and 7.2%). Together, these data imply that H2-M3-restricted CD8\(^+\) T cell responses to Cpm are independent of CD4\(^+\) T cells at both the priming and recall stages, and that functional Cpm-primed memory CD8\(^+\) T cells can persist in the absence of CD4\(^+\) T cell help.

Additional studies were conducted in CD4\(^+\) T cell-depleted K\(^b\)-/-D\(^b\)-/- mice to determine whether their ability to control lung Cpm growth was altered by a concurrent lack of CD4\(^+\) and MHC class Ia-restricted CD8\(^+\) T cells (Fig. 8B). In mice depleted of CD4\(^+\) T cells before Cpm infection, a ~0.5 log reduction in pulmonary Cpm loads was observed 1 wk postinfection when compared with undepleted mice (\(p < 0.05\)). By contrast, when CD4\(^+\) T cell depletion was conducted before a secondary infection, the lungs of CD4\(^+\) T cell-depleted mice had on average ~2-fold more chlamydia than those of control animals. The lungs of all Cpm-reinfected mice, however, had significantly lower infectious loads than those of primarily infected animals. Of note, compared with CD4\(^+\) T cell-deficient mice, the percentages of pulmonary CD8\(^+\) T cells in CD4\(^+\) T cell-deficient mice were 5-fold higher (6 vs 31%) and unchanged (6.3 vs 6%) following primary and secondary infections, respectively. Thus, these results suggest that in the absence of CD4\(^+\) and classically restricted CD8\(^+\) T cells, non-MHC class Ia-dependent responses, including those mediated by MHC class Ib-restricted CD8\(^+\) T cells, can control Cpm infections.

**Discussion**

CD8\(^+\) T cells are a central component of anti-Cpm immunity (6, 10). Although CD8\(^+\) Tc1 cells have been shown to respond to Cpm Ags in an MHC class Ia-restricted fashion (11–13, 50), the participation and significance of MHC class Ib molecules as Ag-presenting structures for Cpm infection-primed CD8\(^+\) T cells remained unknown. In this study, we demonstrate that Cpm-infected mice generate CD8\(^+\) Tc1 cells to Cpm and show that the two Cpm Ag-derived N-IM epitopes P1 and P4 are presented by H2-M3 molecules on the surface of infected macrophages. We also provide evidence that Tc1 cells to both determinants contribute to protection against Cpm infection, and that CD4\(^+\) T cells are not required for the development and maintenance of 

Cpm-specific lytic activity of LMNC is comprised of class Ia- and non-class Ia-restricted components. Although NK- and class Ib-restricted CD8\(^+\) T cells are known to lyse targets in a class Ia-nonrestricted manner, we chose to investigate the latter because CD8\(^+\) T cells are critical for controlling Cpm growth (6, 10). Of the murine class Ib molecules, we studied H2-M3 as this Ag-presenting structure only binds peptides from bacterial and mitochondrial proteins (24–26). Analysis of all predicted Cpm protein coding sequences yielded 48 N-terminal peptides fitting the H2-M3-binding motif. Of the 18 peptides studied, 6 up-regulated H2-M3 cell surface expression, 5 generated effector cells with lytic activity, and 2 (P1 and P4) stimulated IFN-\(\gamma\) production from SC and LMNC of Cpm-infected mice. Despite similar levels of lytic activity, 3 of the 5 peptides that generated CTL effectors did not stimulate IFN-\(\gamma\) production. Because secretion of this cytokine was measured ex vivo or after brief stimulation, this dichotomy in effector function may be due to low precursor frequencies of CD8\(^+\) T cells to these three epitopes. However, because CD8\(^+\) T cell
cytotoxicity and IFN-γ production are regulated independently (51), it is possible that these H2-M3-restricted Cpn-specific effectors do not produce IFN-γ.

Cpn N-FM peptides P1 and P4 represent, respectively, the N-terminal sequences of CP0021, a conserved hypothetical protein, and CP0421, the conserved SsrA (small stable RNA A)-binding protein, also known as small protein B (SmpB) (39, 52). Although orthologs of CP0021 exist in other chlamydial species, the sequence of P1 (IMFFAPL) is an N-terminal peptide found only in Cpn and in a hypothetical protein from the facultative intracellular bacterial pathogen Campylobacter jejuni (53). In regard to CP0421, sequence searches have revealed SmpB orthologs in all complete bacterial genomes (54). However, the sequence of P4 (IMFLYWFL) is unique to Cpn. Although CP0021 does not have a predicted signal peptide, the fact that it is secreted by the Shigella flexneri type III secretion apparatus (55) suggests that this Ag may also be translocated to the host cell cytosol by the homologous system in Cpn. CP0421 could also represent an effector molecule secreted by this pathway as there is no accurate method to predict which proteins are targeted for a type III-dependent secretion. However, SmpB is known to remain in the bacterial cell cytosol during its ascribed function of recognizing and delivering SsrA to ribosomes (54). Because bacterial cytosolic proteins are prone to deformylation, and the N-terminal formyl group is required for high-affinity binding to H2-M3 (56), it will be important to determine the mechanism by which the N-FM peptides of CP0021 and CP0421 are presented by H2-M3 on infected cells. It should be noted, that H2-M3-restricted CD8⁺ T cells responses are elicited against proteins not predicted to be secreted, such as L. monocytogenes AtTm and M. tuberculosis Rv0227c and Rv0749, and against membrane proteins with a putative Nlp topology, such as the mycobacterial Ag Rv1021 (20, 22). Although these proteins lack a direct route to the host cell cytosol, it is clear that nonsecreted bacterial Ags can also be a source of H2-M3 epitopes. In fact, the lysis of infected macrophages by P1- and P4-specific CD8⁺ T cells indicates that professional APCs can process CP0021 and CP0421 and present P1 and P4. These results also strongly suggest that priming of CD8⁺ T cells occurs by infected professional APCs. Whether nonphagocytic Cpn-infected cells are also capable of processing and presenting P1 and P4 to CD8⁺ T cells remains unclear. However, because it is unlikely that lysis of infected DC and macrophages alone accounts for the decrease in pulmonary Cpn loads observed in mice adaptively transferred with P1- and P4-specific CD8⁺ T cells, nonprofessional APCs may also be capable of processing Cpn Ags for presentation in the context of H2-M3.

Cpn infection of B6 mice primes a strong MHC class Ia-restricted CD8⁺ T cell response which includes specificities for protective Cpn epitopes (11, 42). To study anti-Cpn MHC class Iβ-restricted CD8⁺ T cell responses in the absence of the dominant response to epitopes presented by class Ia molecules, we used Kᵇ⁻/-⁻Dᵢᵢ⁻/-⁻ mice. MHC class Ia-deficient mice have been used to investigate class Iβ-restricted CD8⁺ T cell responses to other intracellular pathogens (29, 30, 32, 37, 44, 45). Although Cpn numbers were greater in Kᵇ⁻/-⁻Dᵢᵢ⁻/-⁻ than in B6 mice 21 days postinfection, pulmonary bacteria were cleared with similar kinetics in both mouse strains. Because CD8⁺ T cells are critical for control of Cpn infection (6, 10), these results indicate that in the absence of class Ia-restricted CD8⁺ T cells, class Iβ-restricted CD8⁺ T cells can control Cpn infection. Phenotypic analysis of CD8⁺ T cell activation and memory markers show that naive Kᵇ⁻/-⁻Dᵢᵢ⁻/-⁻ mice have a larger percentage of CD8⁺ T cells with an activated phenotype than B6 mice, a finding that is consistent with previous reports (45, 46). However, there is a 4- to 6-fold increase in activated CD8⁺ T cells after peptide stimulation of SC from Cpn-infected B6 and Kᵇ⁻/-⁻Dᵢᵢ⁻/-⁻ mice, indicating that P1- and P4-specific CD8⁺ T cells are primed upon infection. Further analysis of peptide-stimulated effector cells from Kᵇ⁻/-⁻Dᵢᵢ⁻/-⁻ mice demonstrated that, similar to class Ia-restricted Cpn-specific CD8⁺ T cells (11), these effectors produce TNF and IFN-γ and are lytic for peptide-coated and Cpn-infected targets. Therefore, we found no appreciable differences in the class Ia- and class Iβ-restricted CD8⁺ T cell response to Cpn infection with regards to the kinetics of bacterial clearance, the activation of Cpn-specific CD8⁺ T cells, or the Tc1 effector functions used by these T cells.

Due to the suppressive effect of alveolar macrophages (57), pulmonary CD8⁺ T cells may have impaired CTL activity and cytokine production compared with peptide-stimulated effectors tested ex vivo. The fact that we detected in vivo lysis of P1- and P4-coated cells, and that this lytic activity correlated with the number of ex vivo enumerated epitope-specific IFN-γ-producing CD8⁺ T cells suggested that the effector functions of these epitope-specific Tc1 cells also operate in vivo. Nonetheless, the lung microenvironment may have down-regulated to some extent the lytic activity of CD8⁺ T cells, as the in vivo pulmonary elimination of P1- and P4-pulsed cells is ~3-fold lower than the splenic in vivo lytic activity against cells coated with listerial H2-M3-restricted epitopes (58, 59). Although this discrepancy could be explained by pathogen and T cell precursor frequency differences, lung-, but not spleen-, derived CD8⁺ T cells from respiratory syncytial virus-infected mice exhibit impaired CTL activity (60). Thus, it is possible that pathogen-specific CTL effector mechanisms may also vary for distinct anatomic locations.

Naïve recipients of P1- or P4-specific Tc1 cells were partially protected against Cpn infection. The moderate reduction in pulmonary bacterial numbers may be due to the fact that recipient mice were given 2-fold fewer cells from a short-term line than animals infused with an fMIGWII-specific CD8⁺ T cell clone (31), and ~8- to 10-fold fewer cells than mice that received immune SC from Listeria-infected, class Ia-deficient mice (29, 32). Furthermore, recipients of P1- and P4-specific effectors received cells of a single specificity while the highly protected recipients of Listeria-specific, class Ia-deficient immune SC, received CD8⁺ T cells of multiple specificities. Therefore, as shown for classically restricted CD8⁺ T cells (61), inducing Cpn multispecific MHC class Iβ-restricted CD8⁺ T cell responses is likely to translate into increased protective immunity.

Recent reports indicate that CD4⁺ T cells can be expendable for the priming of MHC class Iα-restricted CD8⁺ T cells but not for the development and maintenance of functional CD8⁺ T cell memory (47-49). In Cpn-infected Kᵇ⁻/-⁻Dᵢᵢ⁻/-⁻ mice depleted of CD4⁺ T cells, the number of P1- and P4-specific T cells remains constant through the memory phase and increases after a secondary infection. The apparent failure of both CD8⁺ T cell populations to undergo contraction in CD4⁺ T cell-deficient and -sufficient environments may be due to several factors, including the chronic nature of Cpn infection and systemic dissemination of the pathogen (62, 63). However, a detailed kinetic analysis will be required to determine how P1- and P4-specific responses vary during Cpn infection. The secondary expansion of P1- and P4-specific T cells may result from the absence of class Ia molecules, which are known to inhibit MHC class Iβ-restricted CD8⁺ T cell proliferation (64). Interestingly, a 3-fold reduction in pulmonary Cpn was noted after primary infection in CD4⁺ T cell-depleted Kᵇ⁻/-⁻Dᵢᵢ⁻/-⁻ mice. Because these animals show a 5-fold increase in pulmonary CD8⁺ T cells (data not shown), the reduction in Cpn infectious load may be due to an increase in the number of Tc1 effectors. However, it has been shown that CD4⁺ T cells have a
Th2-related detrimental effect early after Cpn infection (6). In CD8+ T cell-deficient, Cpn-infected mice, the transcript levels of IL-4 and IL-10 increase with a concurrent decrease in IFN-γ mRNA levels (6), suggesting that CD8+ T cells may play a role in regulating the polarization of Th1 CD4+ T cells. Recent reports have demonstrated that class Ib-restricted CD8+ T cells are an early source of IFN-γ (65). Thus, the early production of IFN-γ by H2-M3-restricted CD8+ T cells may polarize CD4+ T cells to a Th1 phenotype during Cpn infection. In fact, H2-M3- and Qa-1b-restricted CD8+ T cells that are known to contribute to antilisterial protection (66). Therefore, a better understanding of the functions that nonclassical MHC class Ib molecules can present CD4+ and class Ia-restricted CD8+ T cell responses (66).

It is becoming increasingly evident that MHC class Ib-restricted CD8+ T cells play an important role in immunity to intracellular pathogens through the expression of Tc1 effector functions as well as by augmenting the number of Ag-specific CD4+ and MHC class Ia-restricted CD8+ T cells (22, 29, 30, 32, 45, 58–65). Therefore, a better understanding of the functions that nonclassical MHC class Ib molecules have during immune responses against infectious diseases is needed to generate effective vaccines. The inclusion of MHC class Ib-restricted epitopes in subunit vaccines may result in increased numbers of Ag-specific T cells, improved type 1 cytokine responses, and enhanced population following vaccination. Although no human homolog for H2-M3 has yet been discovered, the results reported here led us to initiate human studies which have thus far shown that in Cpn-exposed humans, over 50% of peripheral blood-derived, pathogen-specific IFN-γ-producing CD8+ T cells are non-class Ia restricted. Thus, it will be important to determine whether murine class Ib molecules can present Cpn Ags to CD8+ T cells, and whether these effectors participate in infection protection against such infections. This information will be critical for the selection of Ags or epitopes that will ultimately be used for the development of anti-Cpn vaccines.

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