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A CD8+ T Cell Heptaepitope Minigene Vaccine Induces Protective Immunity against *Chlamydia pneumoniae*¹

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An intact T cell compartment and IFN-γ signaling are required for protective immunity against *Chlamydia*. In the mouse model of *Chlamydia pneumoniae* (*Cpn*) infection, this immunity is critically dependent on CD8+ T cells. Recently we reported that *Cpn*-infected mice generate an MHC class I-restricted CD8+ Tc1 response against various *Cpn* Ags, and that CD8+ CTL to multiple epitopes inhibit *Cpn* growth in vitro. Here, we engineered a DNA minigene encoding seven H-2b-restricted *Cpn* CTL epitopes, the universal pan-DR epitope Th epitope, and an endoplasmic reticulum-translocating signal sequence. Immunization of C57BL/6 mice with this construct primed IFN-γ-producing CD8+ CTL against all seven CTL epitopes. CD8+ T cell lines generated to minigene-encoded CTL epitopes secreted IFN-γ and TNF-α and exhibited CTL activity upon recognition of *Cpn*-infected macrophages. Following intranasal challenge with *Cpn*, a 3.6 log reduction in mean lung bacterial numbers compared with control animals was obtained. Using a 20-fold increase in the *Cpn* challenging dose, minigene-vaccinated mice had a 60-fold reduction in lung bacterial loads, compared with controls. Immunization and challenge studies with β2-microglobulin /mice indicated that the reduction of lung *Cpn* burdens was mediated by the MHC class I-dependent CD8+ T cells to minigene-included *Cpn* CTL epitopes, rather than by pan-DR epitope-specific CD4+ T cells. This constitutes the first demonstration of significant protection achieved by immunization with a CD8+ T cell epitope-based DNA construct in a bacterial system and provides the basis for the optimal design of multicomponent anti-*Cpn* vaccines for humans. *The Journal of Immunology*, 2005, 174: 5729–5739.

The human pathogens *Chlamydia pneumoniae* (*Cpn*)³ and *Chlamydia trachomatis* (*Ct*) belong to a group of obligate intracellular Gram-negative bacteria that infect many vertebrate hosts and cause a wide spectrum of diseases of significant medical and veterinary importance worldwide. Although most infections are mild or subclinical, *Cpn* is a common cause of sinusitis, pharyngitis, bronchitis, and pneumonia, and *Ct* is responsible for several ocular and sexually transmitted diseases (1). Repeated and persistent *Cpn* infections have been incriminated in a number of inflammatory conditions such as atherosclerosis, chronic obstructive pulmonary disease, and asthma (2, 3). Similarly, chronic *Ct* infections can lead to complications that include blindness and infertility (1). When diagnosed early, chlamydial infections can be treated with antibiotics. However, the high costs required to identify and treat individuals with mild or no symptoms limits the feasibility of this control strategy. Moreover, cells can remain persistently infected despite chemotherapy, and some antibiotics may induce chlamydial persistence (4, 5). Thus, development of safe and effective vaccines represents a cost-effective approach that would have a greater impact on the prevalence of chlamydial infections and the prevention of severe long-term sequelae. To date, however, no vaccines against *Cpn* or *Ct* are available.

Like all chlamydialae, *Cpn* and *Ct* have a unique biphasic developmental cycle consisting of a metabolically inert and infectious elementary body, and a metabolically active and replicating reticulate body. Upon infection of susceptible cells, the elementary body remains within a nonacidified vacuole known as an inclusion, where it differentiates into a reticulate body, which multiplies by binary fission. The generated progeny differentiate back into elementary bodies that are released upon host cell lysis to infect other cells. During chlamydial infections, the immune system of the infected host encounters bacterial Ags from both developmental forms and from the inclusion membrane (6–10). Although this antigenic stimulation generates short-lived partial immunity against reinfection, it can also elicit a pathological response. Early attempts at vaccinating humans with whole inactivated chlamydial organisms also led to short-term protection and to the induction of a response that exacerbated disease upon reinfection (11). Thus, in the absence of genetic methods to generate stable live nonpathogenic chlamydial strains, current *Chlamydia* vaccine efforts are focused on elucidating the immune mechanisms and pathogen Ags or epitopes that lead to protection or tissue damage, and on developing subunit vaccines and delivery vehicles that can induce rapid, potent, and durable sterilizing immune responses.

Acquired host resistance against *Chlamydia* involves humoral and cell-mediated immune responses, with T cells and type 1 cytokines playing the predominant role (12–14). Although both CD4+ and CD8+ T cells contribute to protection, mainly through the secretion of IFN-γ (13–15), mouse studies indicate that during *Cpn* infection, CD8+ T cells play a major protective role compared

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³Abbreviations used in this paper: *Cpn*, *Chlamydia pneumoniae*; *Ct*, *Chlamydia trachomatis*; IFU, inclusion-forming unit; TCM, T cell medium; RCAS, rat Con A supernatant; PADRE, pan-DR epitope; SC, spleen cell; mAM, murine alveolar macrophage; SFC, spot-forming cell; β2m, β2-microglobulin; ER, endoplasmic reticulum; MOMP, major outer membrane protein.
with that of CD4+ T cells, while the opposite operates during Ct infection (13, 16, 17). Yet, for both Ct and Cpn, CD8+ T cell responses are elicited following infection, CD8+ Tc1 cells from infected mice exhibit MHC class I-restricted recognition of chlamydia-infected cells, and mice are protected by adoptive transfer of CD8+ Tc1 cells (10, 18–20). Because chlamydiae are intracellular, and CD8+ T cells typically recognize Ags processed from cytosolic proteins, we and others have begun to identify products from Cpn and Ct that access the cytosol of infected cells, become degraded by the MHC class I processing machinery, and subsequently induce CD8+ Tc1 cell responses (10, 20–24). However, thus far only modest protective immunity has been shown when a few Ct CD8+ CTL target Ags were tested through vaccination (21, 23).

Considering the antigenic complexity of Chlamydia and other nonviral intracellular pathogens, it is almost certain that vaccination strategies that stimulate protective CD8+ T cells, among other arms of the immune system, will need to induce durable, vigorous, and broad responses directed against several Ags or epitopes. Of the available vaccine technologies, the multiepitope-based subunit approach is perhaps the best suited to mimic or augment whole-organism-induced immunity and prevent potential immunopathogenic or suppressive responses by incorporating into a construct a mix of protective T and B cell epitopes from multiple Ags. Studies in other pathogen and tumor model systems indicate that CD8+ T cell multiepitope constructs can simultaneously induce responses against different CTL determinants when delivered either as DNA plasmids, recombiant infectious vectors, or as poly-peptides (25–32). However, few reports using this multiepitope-based approach have evaluated CD8+ CTL-mediated protection following immunization, and none have assessed the induction of multispecific CTL immunity against a bacterial pathogen. Thus, with our recent demonstration that multiple Cpn Ags are targets of a potent anti-chlamydial H-2b-restricted CD8+ Tc1 response in infected mice (10), we sought to determine whether a DNA minigene encoding a CTL epitope from each of seven Cpn Ags, and optimized to maximize immunogenicity in vivo, could induce a CD8+ CTL response of the breadth and strength needed to protect mice against pulmonary Cpn infection. The data obtained in this study validate the multiepitope approach as a potential vaccination strategy against Cpn and lay the groundwork for future studies aimed at developing similar constructs against Ct and other intracellular bacterial pathogens.

### Materials and Methods

#### Mice

Six- to 10-week-old female C57BL/6J (B6) and MHC class I (β2-micro-globulin (β2m)-deficient C57BL/6-Jb2m1/1c2c (β2m−/−) mice (H-2b) (The Jackson Laboratory) were maintained in a pathogen-free facility and housed in microisolator cages. All mice were handled in accordance with institutional guidelines for care and use.

#### Chlamydia

Cpn isolate K6 (obtained from M. Puolakkainen, University of Helsinki, Helsinki, Finland) was grown within HL cells in minimal medium (25) supplemented with 10% HS, 2 mM HEPES, 2 mM L-glutamine, 0.1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 20 μg/ml gentamicin (Invitrogen Life Technologies). T cell medium (TCM) was prepared by supplementing complete RPMI 1640 with 50 μM of 2-ME (Invitrogen Life Technologies). TCM-rat Con A supernatant (RAS) was made by addition of 5% supernatant from Con A-stimulated rat spleenocytes (T-STIM without Con A; Collaborative Biomedical Products). Chlamydia medium consisted of complete DMEM/F12 (Invitrogen Life Technologies) with 0.5 mg/ml glucose and 0.26 mg/ml sodium bicarbonate. Cycloheximide (1 μg/ml; Sigma-Aldrich) was included when indicated.

#### Construction of multiepitope minigene DNA plasmid

The DNA minigene construct CpnCTL7 (Fig. 1A) was designed to include 7 H-2b-restricted epitopes from the Cpn CTL target Ags Omp5, DnaK, Omp85 homologue, inclusion membrane protein Cpn0585, OmpB, and the 43-kDa homologous proteins Cpn0562 and Cpn0928 (34). This construct, which also incorporated the pan-DR epitope (PADRE) universal Th cell epitope (35), an endoplasmic reticulum (ER)-translocating signal sequence (36), and spacer sequences, was optimized for mouse codon usage, the absence of internal restriction sites, and the efficient translation of the minigene product. The order of the CTL epitopes in the minigene and type of spacer sequences that favor proper proteasomal cleavage (37) were determined by a customized computer software program (Epimmune) that identifies the most favorable sequence for epitope processing and simultaneously minimizes the creation of new junctional H-2k* alleles (Table 1). To determine the affinity of 56 nM (Ref. 10 and J. Sidney, A. Sette, and B. Wizel, unpublished data). Other peptides used were Cpn20 K2 restricted epitopes Cpn0585 49 – 56 (peptide 39), and Cpn0928 185–192 (peptide 20), and D3-restricted epitopes Omp85 133–140 (peptide 37) and Cpn0562 162–170 (peptide 27) (10) (Fig. 1A). Omp5-derived peptide 32.2 (FSPMFEVL) represents the minimal Kb-restricted epitope within the previously reported CTL peptide 32 (SHYAFSPFMEL) and this octamer binds to soluble K* molecules with an affinity of 36 nM (Ref. 10 and J. Sidney, A. Sette, and B. Wizel, unpublished data).

Peptides

These studies used peptides representing Cpn K2-restricted epitopes Omp5 297–304 (peptide 32.2), DnaK 276–286 (peptide 13), Omp85 315–322 (peptide 36), Cpn0585 49–56 (peptide 39), and Cpn0928 185–192 (peptide 20), and D3-restricted epitopes Omp85 133–140 (peptide 37) and Cpn0562 162–170 (peptide 27) (10) (Fig. 1A). Omp5-derived peptide 32.2 (SHYAFSPFMEL) represents the minimal K2-restricted epitope within the previously reported CTL peptide 32 (SHYAFSPFMEL) and this octamer binds to soluble K* molecules with an affinity of 36 nM (Ref. 10 and J. Sidney, A. Sette, and B. Wizel, unpublished data). Other peptides used were Cpn K2- and D3-restricted CTL epitopes Cpn0585 49–56 (peptide 33; ISAFACQL) and outer membrane protein (MOMP) 193–201 (peptide 31; SLLGNNTAL), respectively (10), K2-restricted OVA epitope (SIINFEKL; OVA257), D3-restricted influenza A nucleoprotein epitope (ASNENMETM; FLUnp366), and the universal PADRE Th epitope (ASNENMETM; FLUnp366) (35). Peptides were synthesized by F-moc-based solid phase chemistry using an ABI 430A peptide synthesizer (Applied Biosystems) and purified by reversed-phase HPLC. To confirm their purity (>95%) and identity, peptides were analyzed by mass spectrometry. Peptides were dissolved in DMSO (Sigma-Aldrich) at 20 μg/ml and stored at −20°C. No cell toxicity was associated with any peptide.

### Assessment of minigene expression

Expression of the minigene was assessed using EL-4 cells transiently transfected with CpnCTL7 DNA. For transfections, 10 μg of DNA mixed with 30 μg of Lipofectin (Invitrogen Life Technologies) was added in a 200-μl volume to EL-4 cells seeded in six-well plates (Costar) at 2 × 10^6 cells per well in 2 ml of Opti-MEM I Reduced Serum Medium (Invitrogen Life Technologies). After 18 h of incubation at 37°C, 6% CO2, cells were diluted with an equal volume of complete RPMI 1640–20% FBS. Two days later, transfected EL-4 cells were used to isolate RNA for RT-PCR and as target cells for cytotoxicity assays. Total RNA was extracted from 3 × 10^6 transfected EL-4 cells using TRIzol LS (Invitrogen Life Technologies). Isolated RNA was treated with RNase-free DNase and RNasin RNase inhibitor (Promega), reextracted with phenol/chloroform, and then reverse transcribed using oligo(dT)15 (Promega) and Avian Myeloblastosis Virus reverse transcriptase (Roche Applied Science). cDNA was amplified by PCR using the minigene-specific primers 5′-TACGATATCGCCTCAGCATTAGC-3′ (forward) and 5′-ATTCCGATCCCTCAGACTCCAGG-3′ (reverse) to yield a 365-bp product. PCR products were analyzed on agarose gels.
Genetic immunizations

Mice were immunized three times at 3-wk intervals with 100 μg of CpnCTL7 or VR1012 plasmid DNA in 100 μl of PBS. Controls also included mice receiving PBS only. Each dose was delivered i.m. into each tibialis anterioris and intradermally at the tail base in three 33-μl injections using a 0.5-ml syringe with a 29-gauge needle. Each experiment included groups of 8–12 mice.

Challenge infections and quantitation of Cpn pulmonary loads

Twelve days after the third DNA immunizing dose, mice were infected by intranasal inoculation with 10^6 or 2 × 10^7 IFU of Cpn K6 in 40 μl of PBS under halothane anesthesia. The low dose was used to reinfect 35-day Cpn-infected mice. Ten days following challenge, the lungs of all mice were minced and mechanically homogenized in 3 ml of cold sucrose-phosphate-glutamate buffer using the BD Medimachine (BD Biosciences). For quantitation of Cpn loads, lung homogenates were serially diluted in Chlamydia medium and 0.5 ml of each dilution was centrifuged (500 g, 1 h, 35°C) onto duplicate cultures of HL cells grown in coverslips in 24-well plates (Costar). Plates were incubated for 1 h at 37°C, before replacing the inocula with 2 ml of cycloheximide-containing medium. After 72 h of incubation, cells were fixed with methanol and stained with a FITC-conjugated Chlamydia genus-specific mAb (Pathfinder Chlamydia Culture Confirmation System; Bio-Rad). Chlamydial inclusions were counted by fluorescence microscopy and the results expressed as IFU per lung. The limit of detection is 30 IFU per lung.

Histopathology

Lungs collected from DNA-immunized mice immediately before and 10 days after Cpn challenge infection were processed for histopathological analysis as previously described (10).

Generation of CTL effectors

Effector cells were generated from the spleens of genetically immunized and Cpn-infected mice 12–14 days after each DNA immunizing dose or following the second intranasal infection with 10^6 IFU of Cpn K6, respectively. Spleen cells (SC) were prepared in TCM and seeded in 24-well plates at 5 × 10^6 cells per well. Individual peptides (2 μM) were included in each 2-ml culture. After 2 days of incubation at 37°C, 6% CO2, cultures were made to 5% RCAS and incubated for 4 additional days. Peptide-specific CD8 CTL lines were generated as previously described (10). Briefly, 2–10 wk after the last dose, 4 × 10^7 SC from CpnCTL7-immunized mice were incubated with each of the minigene-encoded CTL peptides (2 μM) for 6 days in 10 ml of TCM using T25 flasks (Corning). RCAS was added on day 2 of culture. In each of three subsequent 6-day cycles of restimulation, 4 × 10^7 peptide-pulsed irradiated (3300 rad) syngeneic naive SC in 15 ml of TCM-RCAS.

Preparation of target cells and cytotoxicity assays

Forty-eight-hour transiently transfected EL-4 cells were labeled with 100 Ci Na_2^51CrO_4 (51Cr; Amersham) a night before their use as targets in CTL assays. Peptide-pulsed EL-4 and RMA-S target cells were prepared in 24-well plates (10^6/2 ml/well) by overnight incubation at 37°C in the presence of peptide (0.1 μM) and 51Cr. Cpn-infected and control uninfected mAM targets were prepared as described (10). Chlamydia-specific immunofluorescent staining of infected mAM incubated for 24 h more indicated that ~70% of the cells were infected. CTL activity of effector cells on target cells was assessed by standard 51Cr release cytotoxicity assays, as described (38). Spontaneous releases ranged between 12 and 23% of maximum releases. SEs were within 0.6 to 7% of the mean.
ELISPOT assay for IFN-γ-secreting CD8+ T cells

Enumeration of IFN-γ-producing CD8+ T cells specific for the CTL epitopes represented in the minigene was assessed by ELISPOT as described (10), using immunomagnetically purified (Miltenyi Biotec) splenic CD8+ T cells (>96% CD8+) as effectors, and peptide-pulsed (1 μM) irradiated naive SC (105/100 μl/well) as stimulators. CD8+ T cells stimulated with irradiated unspotted SC 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.

IFN-γ and TNF-α determinations

mAM monolayers prepared a day earlier in 48-well plates (5 × 10^4/well) were infected with Cpn (4 IFU per well) and cultured with CD8+ T cells (4 × 10^6/well) from short-term CTL lines. As controls, CD8+ T cells were cultured with mAM inoculated with HL cell-derived material. Control effectors were Treponema pallidum SSA-1-3C, specific CD8+ T cells (38). Supernatants were harvested 48 h later, and levels of IFN-γ and TNF-α were determined by ELISA as described (10). The lower detection limits for both cytokines were 40 pg/ml.

Lymphoproliferation assay

Splenic CD4+ T cells were purified (>95% CD4+) by positive selection using anti-L3T4 Ab-coupled magnetic beads (Miltenyi Biotec), adjusted to 2 × 10^6 cells/ml in TC medium, and seeded in triplicate at 100 μl/well in flat-bottom 96-well plates (Costar). Each well also received 100 μl of irradiated naive SC that had been RBC-lysed and adjusted to 5 × 10^5 cells/ml. Cultures were incubated for 4 days in the presence or absence of PADRE peptide (20 μg/ml). As a positive control, cells were stimulated with 1 μg/ml Con A (Sigma-Aldrich). During the final 18 h of culture, 1 μCi of [3H]thymidine (Amersham) was added to each well. Cells were harvested onto glass fiber filters using a 96-well plate cell harvester and [3H]Tdr incorporation was determined by using a scintillation counter. Results were expressed as mean cpm of triplicates.

Statistical analysis

Differences between experimental and control groups were assessed by using the Wilcoxon rank sum test and these were considered statistically significant when p values were <0.05.

Results

Epitope selection and CpnCTL7 minigene construct design

The DNA minigene construct CpnCTL7 was designed to include 7 of the 18 CTL epitopes recently defined in 12 target Ags of anti-Cpn murine CD8+ T cells (10). These determinants are five Kβ-restricted CTL epitopes from the envelope protein Omp5, and Omp85 homologue, chaperone DnaK, inclusion membrane protein Cpn0585, and the 43-kDa protein Cpn0928, and two Dβ-restricted CTL epitopes from the envelope protein OmpB and the 43-kDa protein Cpn0562 (34) (Fig. 1A). Each CTL epitope is derived from multiple epitopes from different proteins. We chose these seven epitopes because the most effective anti-chlamydial CD8+ CTL response is likely to be directed against multiple epitopes from different proteins. We chose these seven epitopes on the basis of their recognition by CD8+ T cells that are highly lytic to Cpn-infected cells, secrete significant levels of Th1 cytokines, and markedly inhibit chlamydial growth.

Because CD4+ T cell help is often needed to induce strong and durable CTL responses (39), and no CD4+ T cell epitope has been defined for Cpn, the CpnCTL7 minigene construct also incorporated the universal PADRE T cell epitope (Fig. 1A). This determinant binds with high affinity to molecules of a wide range of mouse and human MHC class II haplotypes (35).

To maximize epitope immunogenicity in vivo, the CpnCTL7 minigene also included the mouse Ig κ signal sequence at the 5’ end of the construct, and spacer sequences containing K residues flanking the C terminus of all but the last epitope (Fig. 1A). Whereas the former facilitates processing of the CTL epitopes in the ER (36), the latter favors proper posttranslational processing and prevents the formation of junctional H-2Kb epitopes (37).

CpnCTL7 was optimized for mouse codon usage, synthesized, and a clone with the correct sequence was subcloned into the VR1012 eukaryotic expression vector (Fig. 1B).

Expression of CpnCTL7 and processing of encoded CTL epitopes by transfected cells

Before assessing the immunogenicity of CpnCTL7, we determined whether the minigene was transcribed, and whether the different CTL epitopes in the translated product were processed. By RTPCR, a product with the predicted size of 365 bp was amplified using cDNA from EL-4 cells transiently transfected with CpnCTL7 but not from EL-4 cells transfected with the unmodified VR1012 vector (Fig. 2A, lane 4 vs lane 5). No amplification was obtained when the RNA template was not reverse transcribed (Fig. 2A, lanes 1 and 2) or when no RNA template was used (Fig. 2A, lane 3). These results established that the minigene is transcribed.

When the SC from Cpn-infected mice were stimulated with peptides representing each of the seven minigene-encoded CTL epitopes, the generated effector cells displayed lytic activity against CpnCTL7- but not against VR1012-transfected EL-4 target cells (Fig. 2B). In contrast, effector cells generated to peptides 31 and 33, which represent MOMP- and Omp5-derived Cpn CTL epitopes not included in the minigene, failed to lyse both types of target cells. However, all peptide-stimulated effectors lysed EL-4 cells pulsed with the homologous Cpn peptide but not the same cells sensitized with the Kβ-restricted OVA257 or Dβ-restricted FLUnp[566] control CTL peptides. These data indicate that CpnCTL7 is translated, that the product is processed, and that the recognition of minigene-transfected cells by Cpn-primed CD8+ CTL is specific.

CpnCTL7 is immunogenic for cytolytic and IFN-γ-producing CD8+ T cells

Based on the foregoing results, we then examined the capacity of the CpnCTL7 construct to induce CTL responses in vivo. Following the second and third DNA doses, each of the seven CTL epitopes generated effectors from CpnCTL7-immunized mice that lysed RMA-S cells sensitized with homologous peptide but not with the irrelevant control CTL peptides (Fig. 3A). At the highest E:T ratio, the range and mean net peptide-specific lysis for effectors generated in three independent experiments increased from 14–61% and 42% after the second dose to 20–79% and 65% after the third dose. This increase in overall lytic activity after the third immunizing dose was largely attributed to amplified recall CTL responses against epitopes represented by peptides 32.2, 36, and 37, as responses to the other four peptides were unchanged or only marginally augmented. Whereas the strongest CTL responses were generally elicited against peptides 13 and 39, the weakest was consistently induced against peptide 20. Nevertheless, the magnitude of recall CTL responses to each of the seven epitopes in minigene-immunized mice was higher than the lytic activity detected for effectors of the same specificity obtained from Cpn-infected or reinfected mice (10). Importantly, minigene-induced CTL responses were still broad and strong 5 mo after the last dose (data not shown). The CTL responses observed in CpnCTL7-immunized animals did not result from in vitro CTL induction and were only primed by minigene-encoded CTL epitopes, as peptide-stimulated SC from VR1012-immunized mice exhibited minimal or no lytic activity against target cells pulsed with the stimulating peptides, and nonminigene encoded Cpn CTL peptides 31 and 33 failed to stimulate lytic activity in SC from minigene immunized-mice (Fig. 3A). These results demonstrate that CpnCTL7 is immunogenic and that it can simultaneously elicit long-lasting CD8+
T cells with cytolytic activity to all seven minigene-encoded CTL epitopes.

Mouse models of Cpn infection indicate that CD8+ T cells and IFN-γ play important roles in protective immunity (13, 14, 16), and that Cpn epitope-specific IFN-γ-secreting CD8+ T cells are present in Cpn-infected mice (10). To ascertain whether the CpnCTL7-primed CD8+ T cells can produce IFN-γ, the precursor frequencies of splenic CD8+ IFN-γ+ T cells specific to minigene-encoded CTL epitopes were assessed by ex vivo ELISPOT (Fig. 3B). Twelve days after the third dose, the IFN-γ responses induced by the minigenes were in the range of 120–271 spot-forming cells (SFC)/10⁶ cells for the seven CTL epitopes. In contrast, 65–185 SFC/10⁶ cells were obtained for the same epitopes using CD8+ T cells from Cpn-infected mice 12 days after secondary infection.

Compared with Cpn infection, CpnCTL7 immunization induced precursor frequencies that were higher (2–4.1-fold) for peptides 32.2 and 36 (178–185 vs 120–149 SFC/10⁶ cells), and relatively unchanged for peptides 27 and 20 (119–135 vs 128–158 SFC/10⁶ cells). Again, IFN-γ responses to the irrelevant Cpn CTL peptides 31 and 33 were detected only for infection, but not for CpnCTL7-primed CD8+ T cells. IFN-γ secretion was specific because the mean background response induced by the VR1012 vector was within the range of 6–18 spots enumerated for CD8+ T cells incubated in the absence of peptides.

CpnCTL7-immunized mice are recognized by CpnCTL7-primed CD8+ T cells

Cpn infection-primed CD8+ T cells are lytic and secrete Tc1 cytokines upon interaction with Cpn-infected cells (10). To confirm that the findings using peptide-loaded cells were relevant to minigene-primed CD8+ T cells exposed to live Cpn, we generated short-term CD8+ T cell lines specific for each of the CpnCTL7-encoded CTL epitopes and evaluated their lytic potential and capacity to secrete Tc1 cytokines upon exposure to Cpn-infected macrophages. When cocultured with Cpn-infected mAM, the levels of IFN-γ and TNF-α released by effector cells from the seven T cell lines (77–92% CD8+ TCRαβ+CD4−) were respectively 4- to 26-fold and ~6- to 8-fold higher than the levels released when cocultured with mock-infected mAM (Fig. 4A). All the CD8+ T cell lines were also lytic to Cpn-infected but not mock-infected mAM (Fig. 4B). CD8+ T cell lines were also lytic for EL-4 cells that were either transfected with CpnCTL7 or pulsed with the respective Cpn CTL peptide but not for EL-4 cells transfected with the empty VR1012 vector or sensitized with the control peptides (Fig. 4B). These results demonstrate that minigene-primed, epitope-specific CD8+ T cells recognize endogenously generated epitopes on Cpn-infected cells and express the cytokines and CTL activity of classical Tc1 cells.

CpnCTL7 immunization reduces pulmonary bacterial loads and disease severity in Cpn-challenged mice

Having established that CpnCTL7-immunized B6 mice mounted a strong and broad Tc1 response against Cpn-infected cells, we next examined whether DNA immunization with this construct could prevent infection or reduce chlamydial loads in the lungs of mice. Ten days after intranasal challenge with 10⁶ IFU of the K6 strain of Cpn, B6 mice genetically immunized with three doses of CpnCTL7 had, on average, a dramatic 3.7 log reduction in pulmonary bacterial loads when compared with bacterial numbers present in the lungs of mice immunized with the vector alone or with PBS (p < 0.001; Fig. 5A). Remarkably, 75% of the CpnCTL7-immunized mice had no recoverable organisms in their lungs. The results of this and three additional experiments had an overall 3.6 log reduction in the mean lung bacterial counts and no isolatable Cpn in 59% (17 of 29) of minigene-immunized mice. By
Protective immunity conferred by CpnCTL7 immunization is MHC class I-dependent

To begin to investigate the mechanism by which CpnCTL7 confers significant protection against Cpn infection, we conducted immunization-challenge studies using β2m−/− mice. These animals lack both MHC class Ia and Ib molecules and consequently have a drastically reduced number of functional CD8+ T cells (40). After the third plasmid DNA dose, CD4+ T cells from CpnCTL7- but not VR1012-immunized β2m−/− and B6 mice exhibited strong lymphoproliferative responses to the PADRE peptide (Fig. 7A), indicating efficient priming of Th epitope-specific cells. However,
minigene-immunized $\beta_2m^{-/-}$ animals failed to generate CTL responses to all CpnCTL7-encoded CD8$^+$ T cell epitopes (data not shown), and upon Cpn challenge, their lungs had on average 3.6 log more chlamydia than those of similarly immunized B6 animals (Fig. 7B). As the minigene only encodes seven Cpn CTL epitopes and a Th determinant, and PADRE-specific CD4$^+$ T cells did not directly reduce pulmonary Cpn loads, the CpnCTL7-induced protection was thus mediated by MHC class I-dependent CD8$^+$ T cell responses.

Discussion

Subunit vaccines against Chlamydia have the potential to improve the suboptimal immunity conferred by previous exposure to chlamydial agents and to reduce the safety concerns of whole organism-based immunization. Ideally, such vaccines should include chlamydial components that when properly delivered rapidly induce strong, broad, and sustained immune responses that eliminate infected cells or render developing bacteria noninfectious, thereby preventing microbial growth, spread, and persistence in the infected host. Because immunity to Chlamydia mainly depends on T cells and IFN-$\gamma$ (12–14), we believe that infection-induced resistance is nonsterilizing because of poor priming, expansion, and maintenance of type 1 T cells to multiple Ags. Thus, immunization strategies that enhance the magnitude and quality of responses against T cell epitopes from several Ags could be superior to protection induced by whole organisms. Herein, we provide the first evidence that immunization of mice with a multiepitope-based DNA minigene encoding H-2$^b$-restricted CTL epitopes from seven Cpn Ags induces a potent, durable CD8$^+$ Tc1 response that provides an unprecedented level of protection against infectious Cpn challenge.

With the availability of the complete genome sequences of Cpn and Ct (34, 41), and of animal models that mimic the immune mechanisms thought to control these pathogens in humans (12), the number of potential vaccine candidate Ags has increased (10, 20–24). We recently defined 18 H-2$^b$-restricted CTL epitopes in 12 target Ags of the CD8$^+$ T cell response in Cpn-infected mice, and showed that epitope-specific Tc1 cells inhibit Cpn growth in vitro (10). Based on these data, we asked whether protective anti-Cpn Tc1 responses could be induced in vivo through vaccination and chose the multiepitope approach to construct a Cpn CTL epitope-based subunit vaccine.

Multiepitope vaccines offer several advantages over the use of whole Ags. First, extensive antigenic complexity can be packed into a single immunogen. Second, these constructs can induce a broader and more potent response and exclude potentially immunosuppressive epitopes. Third, the immune response can be focused on selected conserved epitopes, a key advantage for pathogens with high sequence variability but that may also prove valuable for Chlamydia given the reported intraspecies and interspecies variation in Cpn (42, 43) and the evidence supporting selection of Ct escape mutants by anti-MOMP T cell responses (44).

Fourth, potentially immunopathogenic epitopes can be excluded such as those in chlamydial Omp2 and in the Cpn0483 gene product, that respectively cause autoimmune myocarditis in mice and encephalomyelitis in rats (45, 46). Finally, the use of epitopes can incorporate determinants from Ags that might be differentially expressed at specific stages of pathogen development or during persistence, a factor of crucial importance in the case of Chlamydia.

Subunit constructs comprising multiple epitopes can be immunogenic (28, 29, 31, 32) and protective (25–27, 30) when delivered using plasmid DNA, infectious live vectors, or as recombinant or synthetic polypeptides. We opted for the DNA plasmid format because they are inexpensive, noninfectious, and can be readily optimized to enhance immunogenicity and protective efficacy. Furthermore, DNA vaccines can induce robust and durable CD8$^+$ T cell responses, and are well suited to deliver large numbers of CTL epitopes (32). As we believe that protective CD8$^+$ T cell-based immunity against Cpn results from additive or synergistic responses against a cadre of epitopes, we engineered CpnCTL7, a minigene construct with a core of 7 Cpn CTL determinants. CpnCTL7 was designed to include a panel of K$^b$- and D$\alpha$-restricted epitopes that are recognized by CD8$^+$ T cells with strong Cpn growth inhibitory capacities (10). Moreover, each CTL epitope was selected from a different Ag, which together represent diverse groups of proteins, namely, chaperones (DnaK), outer membrane proteins (Omp5, OmpB, and Omp85 homologue), inclusion membrane proteins (Cpn0585), and Ags of unknown function (Cpn0928, Cpn0562). With these selection criteria, we hoped to prime a focused Tc1 response with a broad enough coverage of specificities so that CD8$^+$ T cells to these epitopes could potentially impair Cpn viability at any stage of its intracellular existence.

Many variables can influence the in vivo potency of multiepitope constructs arranged as a string-of-beads. Incorrect epitope position, aliphatic or aromatic residues flanking the C terminus of an epitope, and the creation of junctional epitopes, can all adversely affect the magnitude and frequency of CTL responses (29, 31, 37). Thus, the sequence of CpnCTL7 was optimized by defining the most favorable order of epitopes and the type of spacers that could maximize proper processing and minimize the creation of neoepitopes. Also, because efficient epitope processing can occur in the ER (29, 47), CpnCTL7 included the murine Ig $\kappa$ signal peptide.
sequence, an ER-targeting module shown to improve the immunogenicity of a minigene with CTL epitopes (29). Finally, because codon modification increases the expression and immunogenicity of DNA vaccine-encoded Ags (48), CpnCTL7 was optimized for mouse codon usage. Altogether, these strategies may have favored the proper processing of minigene product in CpnCTL7-transfected EL-4 target cells and efficient presentation to immune CTL effectors from Cpn-infected mice.

Evidence indicates that epitopes presented at detectable levels in antigenicity assays using minigene-transfected APCs are also immunogenic in minigene-immunized mice (29, 37). Our results are in line with these findings, as Tc1 effectors were induced against all seven CTL epitopes in CpnCTL7-immunized animals. CTL responses increased in strength after each minigene dose and were on average 2- to 4-fold higher than in Cpn-reinfected mice (10). As shown for other minigenes (27–29, 32), induced CTL responses were superior but the levels varied among the encoded epitopes. This variation was not due to differences in MHC class I binding affinities (10). Rather, the CTL epitopes might have been processed with different efficiencies by the APCs in vivo. Despite the different profiles in CTL activity, comparable numbers of IFN-γ-secreting CD8+ T cells were obtained for six of the seven epitopes. It should be noted, though, that cytotoxicity and IFN-γ secretion are regulated independently (49), and under similar experimental conditions, only a fraction of perforin+ CD8+ T cells produce IFN-γ (50). Interestingly, the lowest numbers of CD8+ IFN-γ+ T cells in Cpn-reinfected mice were for peptides 13, 37, and 39, and these were the only epitopes for which an expansion of precursors was seen after minigene immunization. It will be of interest to see how the numbers of CD8+ IFN-γ+ T cells for each CTL epitope change over time in CpnCTL7-immunized and Cpn-reinfected mice, as the size and kinetics of each epitope-specific CD8+ T cell population may be dictated by the level of epitope expression on APCs (51). Moreover, because ER-targeted minigene products can increase the avidity of induced CD8+ T cells even when the numbers of precursors remain comparable to those primed by constructs without the ER signal sequence (52), it will also be interesting to determine whether CpnCTL7-primecd Tc1 cells are of higher avidity than those elicited by Cpn infection.

For anti-Cpn vaccines to be effective, induced CD8+ T cells should recognize Cpn-infected cells, and the memory phase of this response should remain stable and functional long after immunization. Thus, Tc1 immunogenicity of CpnCTL7 was also evaluated using Cpn-infected alveolar macrophages, the cells that disseminate the infection from the respiratory tract (53). We found that minigene-primed CD8+ T cells to each CTL epitope displayed cytolytic activity and secreted IFN-γ and TNF-α upon recognition of endogenously processed peptides on Cpn-infected mAMs. Because IFN-γ and cytotoxicity mediate the in vitro Cpn growth inhibitory capacities of infection-primed, epitope-specific CD8+ T cells, these results suggested an in vivo protective potential for CpnCTL7-induced Tc1 cells. Importantly, Tc1 activities could still be detected in minigene-vaccinated mice up to 20 wk after the last immunization. One explanation for their longevity is that CD4+ T cells to the universal PADRE Th epitope furnished help to induce the Tc1 response and to promote the development and maintenance of functional CD8+ T cell memory (29, 54). Although CTL responses can occur without CD4+ T cell help, these are often decreased in frequency and magnitude (29, 55), and they are generally impaired in memory function (54). Alternatively, plasmid DNA vaccines, which can persist long-term in vivo (56), contain CpG motifs that can lead APCs to provide CD8+ T cells with activating costimulatory signals (57). Also, when high numbers of CTL precursors are induced by cross-priming, which is how DNA vaccines generate CTL immunity (58), CD8+ T cells can make sufficient cytokines to facilitate their own expansion (59) and presumably to maintain functional memory.

The most notable finding of these studies was that immunization with CpnCTL7 afforded mice a remarkable level of protection against respiratory Cpn infection. With a challenging dose of 10^6 IFU, the mean Cpn lung titer at a peak time of pathogen growth was nearly 4,000-fold lower in minigene-vaccinated animals than in mice immunized with the vector alone or with PBS. The titers seen with this challenging dose and with a 20-fold higher inoculum were respectively, 2.1–2.9 log and 2–12 times superior to the levels achieved by immunization with single Ag-based anti-Cpn subunit vaccines (60–64). A critical finding was that lung bacteria were recovered from all reinfected animals but only from 41% of
minigene-immunized mice. Thus, among the subunit vaccines tested in murine models of Cpn and Ct lung infections (60–66), CpnCTL7 is the only construct that protects against infectious challenge better than whole live organism-induced immunity. Our results also represent the first evidence of a multiepitope-based construct conferring protective immunity against a bacterial pathogen.

The lack of recoverable Cpn from most CpnCTL7-vaccinated mice strongly suggested that T cells primed systemically could home to the infected lungs and induce sterile immunity. Although infection with noncultivable persistent bacteria is possible, this is unlikely for various reasons. First, the high levels of IFN-γ produced by CpnCTL7-primed CD8+ T cells probably created a strong type 1 cytokine bias in the lungs of minigene-immunized mice before or immediately after Cpn challenge. It is known that exposure of infected cells to high IFN-γ concentrations irreversibly inhibits chlamydial replication, while lower concentrations induce the formation of persistent forms (67). Second, the recall response to challenge of an already high number of CD8+ T cells might have strengthened the reported ability of this T cell subset to modify the CD4+ T cell cytokine pattern from a Cpn growth promoting Th2 to a protective Th1 phenotype (13). Third, this abundance of Cpn-specific CD8+ T cells might have also increased the chances that these effectors would find and destroy productively or persistently Cpn-infected cells, as Chlamydia-specific CTL can recognize and kill cells harboring either normal or persistent forms (68). Interestingly, the biggest difference in numbers of IFN-γ-producing CD8+ T cells between CpnCTL7-vaccinated and Cpn-reinfected mice were for peptides 37 and 39, and these epitopes are respectively from the OmpB and Omp0585 Ags, whose genes are up-regulated during Cpn persistence (69). Finally, the lungs of minigene-immunized Cpn-challenged mice showed minimal inflammatory pathology, unlike the chronic inflammation associated with persistent chlamydial infections (5). In this regard, TNF-α secreted by minigene-prime CD8+ T cells may have reduced lung inflammation by synergizing with IFN-γ to inhibit chlamydial growth (67).

Given the CTL epitope-based design and Tc1 immunogenicity of CpnCTL7, it was reasonable to infer that MHC class I-restricted T cells curbed Cpn growth in minigene-vaccinated B6 mice. This was supported by the finding that CpnCTL7-immunized β2m−/− animals showed unabated pulmonary Cpn replication after challenge, and lacked CD8+ T cell responses to all 7 CTL epitopes but responded to the PADRE Th epitope. Whether CpnCTL7-induced protection involved CD8+ T cells to all or just a few of the seven CTL epitopes, and whether it correlated with the expression of a defined Tc1 effector function, remains to be determined. The use of minigene constructs encoding fewer epitopes will be critical to precisely define the Cpn CTL epitopes responsible for the observed protection. Nevertheless, the fact that all seven CD8+ T cell specificities inhibit Cpn growth in vitro (10), and that additive or synergistic protection from challenge is achieved in mice adaptively transferred with defined pairs of Cpn epitope-specific Tc1 lines (J. Tvinneem and B. Wizel, unpublished data) suggest that CD8+ T cells to all minigene-encoded CTL epitopes acted in concert to attain the critical threshold of effector activities needed to control pulmonary Cpn replication. Based on available evidence (14, 15, 19), IFN-γ secretion was presumably the main mechanism by which CpnCTL7-primed Tc1 cells exerted their protective effect. Although CD8+ T cell-mediated lysis of infected cells is thought to play little or no role in anti-chlamydial immunity (13, 19), we speculate that an increase in the number of CD8+ T cell precursors to several chlamydial epitopes before or soon after infection increases the odds that infected cells will be found and lysed before they can host the development of infectious progeny.

A question of practical importance is the applicability of our current findings using a single inbred mouse strain to the development of vaccines for a genetically heterogeneous human population. Although the high degree of HLA polymorphism is often cited as a major hindrance to the use of epitope-based vaccines, this limitation can be addressed through the inclusion of supertype-restricted epitopes, which are recognized in the context of several related HLA alleles, and by designing vaccines with higher epitope densities (32, 70). A multiepitope-based anti-Cpn minigene vaccine could then for instance include three times the number of CD8+ CTL epitopes present in CpnCTL7, and such epitopes could be chosen to represent at least the HLA-A2, -A3, and -B7 supertypes, which would provide recognition in nearly 90% of the global population, regardless of ethnicity (70). Hence, work is now in progress to identify HLA class I degenerate Tc1 epitopes in Cpn Ags targeted by CTL from Cpn-infected humans and HLA transgenic mice. These epitopes, along with similarly identified HLA-DR supertype-restricted Cpn CD4+ Th1 epitopes, would provide the database needed to develop a multiepitope-based anti-Cpn minigene vaccine that is broadly efficacious in the majority of all racial and ethnic populations.

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