The Journal of Immunology

Multiple *Chlamydia pneumoniae* Antigens Prime CD8 $^{+}$ Tc1 Responses That Inhibit Intracellular Growth of This Vacuolar Pathogen

Benjamin Wizel, Barry C. Starcher, Buka Samten, Zissis Chroneos, Peter F. Barnes, John Dzuris, Yuichiro Higashimoto, Ettore Appella and Alessandro Sette

J Immunol 2002; 169:2524-2535; ;
doi: 10.4049/jimmunol.169.5.2524
http://www.jimmunol.org/content/169/5/2524

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Multiple *Chlamydia pneumoniae* Antigens Prime CD8$^+$ Tc1 Responses That Inhibit Intracellular Growth of This Vacuolar Pathogen[1]

Benjamin Wizel,[2]*† Barry C. Starcher,‡ Buka Samten,* Zissis Chroneos,‡ Peter F. Barnes,*†§ John Dzuris,‖ Yuichiro Higashimoto,¶ Ettore Appella,¶ and Alessandro Sette‖

CD8$^+$ T cells play an essential role in immunity to *Chlamydia pneumoniae* (*Cpn*). However, the target Ags recognized by *Cpn*-specific CD8$^+$ T cells have not been identified, and the mechanisms by which this T cell subset contributes to protection remain unknown. In this work we demonstrate that *Cpn* infection primes a pathogen-specific CD8$^+$ T cell response in mice. Eighteen H-2^b binding peptides representing sequences from 12 *Cpn* Ags sensitized target cells for MHC class I-restricted lysis by CD8$^+$ CTL generated from the spleens and lungs of infected mice. Peptide-specific IFN-γ-secreting CD8$^+$ T cells were present in local and systemic compartments after primary infection, and these cells expanded after pathogen re-exposure. CD8$^+$ T cell lines to the 18 *Cpn* epitope-bearing peptides were cytotoxic, displayed a memory phenotype, and secreted IFN-γ and TNF-α, but not IL-4. These CTL lines lysed *Cpn*-infected macrophages, and the lytic activity was inhibited by brefeldin A, indicating endogenous processing of CTL Ags. Finally, *Cpn* peptide-specific CD8$^+$ CTL suppressed chlamydial growth in vitro by direct lysis of infected cells and by secretion of IFN-γ and other soluble factors. These studies provide information on the mechanisms by which CD8$^+$ CTL protect against *Cpn*, furnish the tools to investigate their possible role in immunopathology, and lay the foundation for future work to develop vaccines against acute and chronic *Cpn* infections. ***The Journal of Immunology*, 2002, 169: 2524–2535.**

Chlamydia pneumoniae (*Cpn*)[3] is an obligate intracellular bacterial pathogen that is estimated to cause at least one infection during the lifetime of nearly every human being (1). Although most infections are mild or subclinical, *Cpn* is a common cause of community-acquired pneumonia, bronchitis, pharyngitis, and sinusitis (2). Like *Chlamydia trachomatis* and *Chlamydia psittaci*, the two other chlamydial human pathogens, *Cpn* can persist in the host and cause chronic infection (3), which is associated with many inflammatory conditions, including asthma, chronic obstructive pulmonary disease, and multiple sclerosis (4–6). Of greatest significance is the compelling association of *Cpn* infection with atherosclerosis and cardiovascular events (7, 8). Although antibiotics can treat acute *Cpn* infection, cells can remain persistently infected despite treatment (9). Therefore, a logical approach to reduce respiratory and systemic morbidity from *Cpn* is to develop an effective vaccine to prevent or ameliorate acute and chronic infection from this pathogen. However, developing vaccines against *Chlamydia* has been hindered by the limited knowledge of pathogen Ags and immune mechanisms that lead to protective or adverse immune responses.

CD8$^+$ T cells play a critical role in protection against most intracellular pathogens, including *Chlamydia.* Pathogen-derived Ags from organisms that replicate in the host cell cytosol, such as *Listeria monocytogenes* and *Trypanosoma cruzi*, readily induce a CD8$^+$ T cell response, as microbial proteins are directly accessible to the MHC class I Ag-processing machinery. In contrast, for *Chlamydia*, which resides in a membrane-bound vacuole termed an inclusion, and for other intravacuolar pathogens, such as *Mycobacterium tuberculosis*, Ags need to traffic into the cytosol for CD8$^+$ CTL induction. Nevertheless, CD8$^+$ CTL responses are induced to mycobacterial Ags (10), and CD8$^+$ T cells primed during *C. trachomatis* infection lyse chlamydia-infected cells (11, 12). Moreover, depletion and adoptive transfer of CD8$^+$ T cells have, respectively, abrogated and conferred protection to *C. psittaci*- and *C. trachomatis*-challenged mice (12–14). Despite the clear role of CD8$^+$ T cells in resistance to chlamydial pathogens, only two *C. trachomatis* CD8$^+$ CTL target Ags have been identified to date (15, 16).

Information on immunity to *Cpn* is sparse, but studies using a mouse model that faithfully mimics important aspects of human *Cpn* infection (17–19) indicate that CD8$^+$ T cells and IFN-γ are critical for protection (20–22). In the absence of CD8$^+$ T cells, *Cpn*-infected mice have increased bacterial burdens and disease severity (20, 21), and in animals lacking IFN-γ signaling, bacterial loads are higher, and clearance of organisms is greatly hampered (22). Nevertheless, it is uncertain whether CD8$^+$ T cells recognize *Cpn*-infected cells and whether this T cell subset contributes to protection through cytokine production or a lytic mechanism, as

*Center for Pulmonary and Infectious Disease Control, Departments of †Microbiology and Immunology, ‡Biochemistry, and §Medicine, University of Texas Health Center, Tyler, TX 75708; ¶National Cancer Institute, Bethesda, MD 20892; and ‖Epimmune Corp., San Diego, CA 92121

Received for publication April 3, 2002. Accepted for publication July 1, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the National Institutes of Health (RO1 HL70641-01), the American Heart Association Texas Affiliate, the Center for Pulmonary and Infectious Disease Control, and the Cain Foundation for Infectious Disease Research. B.W. is a recipient of the Lyndon Baines Johnson Research Award from the American Heart Association.

[2] Address correspondence and reprint requests to Dr. Benjamin Wizel, Department of Microbiology and Immunology, Center for Pulmonary and Infectious Disease Control, University of Texas Health Center, 11937 U.S. Highway 271, Tyler, TX 75708. E-mail address: bwizel@uthct.edu

[3] Abbreviations used in this paper: *Cpn*, *Chlamydia pneumoniae*; BFA, brefeldin A; HPF, high powered field; IFU, inclusion-forming units; i.n., intranasal; LMNC, lung mononuclear cell; mAM, murine alveolar macrophage; MFI, mean fluorescence intensity; MOMP, major outer membrane protein; RCAS, rat Con A supernatant; SC, spleen cell; SFC, spot-forming cell; TCM, T cell medium; TSA, trypomastigote surface Ag.

0022-1767/02/$02.00

cytokine-producing $CD8^+$ CTL have not been documented during *Cpn* infection. Furthermore, the *Cpn*-derived Ags contributing to MHC class I-restricted $CD8^+$ T cell responses remain unidentified.

We report in this work that *Cpn*-infected mice generate pathogen-specific $CD8^+$ CTL with a type 1 cytokine secretion pattern and that these effector cells recognize multiple MHC class I-restricted epitopes from *Cpn* Ags endogenously processed by productively infected macrophages. We also show that *Cpn* peptide-specific $CD8^+$ CTL and their soluble factors significantly inhibit chlamydial growth in vitro.

Materials and Methods

Bacteria

These studies used *Mycoplasma*-free stocks of the *Cpn* Kajaani 6 (K6) (obtained from Dr. M. Puolakkainen, University of Helsinki, Helsinki, Finland), AR39 (University of Washington, Seattle, WA), and CWL029 (obtained from Dr. C. M. Black, Centers for Disease Control, Atlanta, GA) strains. For propagation to high titers, each bacterial strain diluted in *Chlamydia* medium was centrifuged (500 × *g*, 1 h, 35°C) onto monolayers of HL cells (23) grown in 12-well plates (BD Biosciences, Franklin Lakes, NJ). Plates were incubated for 1 h at 37°C in 6% CO_2 before replacing the inocula with cycloheximide-containing medium that was then used to incubate infected cultures for 72 h. Infected monolayers were harvested with glass beads and sonicated on ice for 20 s. After removing cell debris by low speed centrifugation (200 × *g*, 10 min, 4°C), bacteria were pelleted (33,100 × *g*, 35 min, 4°C), resuspended in cold sucrose-phosphate-glutamate solution, and aliquoted for titration and storage at −70°C. Similarly processed uninfected HL cell monolayers were used to prepare control material.

Mice and infections

Six- to 10-wk-old female C57BL/6J (B6) mice (H-2^b) (The Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were kept in microisolator cages and housed in a pathogen-free environment. B6 mice were infected by intranasal (i.n.) inoculation with 10^6 inclusion-forming units (IFU) of *Cpn* K6 in 40 μl PBS under methoxyflurane anesthesia. In most experiments animals were reinfected i.n. with the same infectious dose 35–100 days after the initial infection. Control mice were inoculated with material prepared from uninfected cells. To induce *T. cruzi* trypomastigote surface Ag (TSA)-1-specific CTL, B6 mice were infected with this parasite as previously described (24). The institutional animal care and use committee approved all procedures involving animals.

Cell lines and culture media

RMA-S (H-2^b; TAP2$^-$, T cell lymphoma; provided by Dr. H.-G. Ljundggren, Karolinska Institute, Stockholm, Sweden) (25), mAM (H-2^b; murine alveolar macrophage cell line; Z. Chroneos, unpublished observations), HL (University of Washington) (23), and HEp-2 (ATCC CCL 23, American Type Culture Collection, Manassas, VA) were maintained in complete RPMI 1640 medium containing 10% heat-inactivated FBS (HyClone, Logan, UT), 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 20 μg/ml gentamicin (all from Invitrogen-Life Technologies, Gaithersburg, MD). T2K^b and T2D^b (T2 cells transfected with the K^b and D^b genes; supplied by Dr. P. Cresswell, Yale University, New Haven, CT) (26) were maintained in complete IMDM with 0.4 mg/ml Geneticin (Invitrogen). T cell medium (TCM) was prepared by supplementing complete RPMI 1640 with 50 μM 2-ME (Invitrogen). TCM-RCAS was made by addition of 5% supernatant from Con A-stimulated rat splenocytes (T-STIM without Con A; Collaborative Biomedical Products, Bedford, MA). *Chlamydia* medium consisted of complete DMEM/F-12 medium (Invitrogen) with an additional 0.5 mg/ml glucose and 0.26 mg/ml sodium bicarbonate. Cycloheximide (1 μg/ml; Sigma-Aldrich, St. Louis, MO) was included when indicated.

Histology and immunohistochemistry

Lungs collected at various time points between 2 and 165 days after primary and secondary *Cpn* or mock infections (three to five mice per time point) were perfused with 10 ml PBS via the right ventricle and inflated by intratracheal instillation with Excell fixative (American MasterTech Scientific, Lodi, CA). After postfixation, tissues were embedded in paraffin, and sections (6 μm) were stained with H&E for histological analysis. To detect *Cpn*, deparaffinized tissue sections were treated for 15 min with 3% H_2O_2, blocked for 20 min with 5% BSA, and then incubated overnight at 4°C with a 1/2000 dilution of the *Cpn* major outer membrane protein (MOMP)-specific mAb RR-402 (University of Washington) (27, 28). A 1/1000 dilution of biotinylated goat anti-mouse IgG (ICN, Costa Mesa, CA) was then applied to the sections for 30 min, followed by 15 min with a 1/10 dilution of streptavidin-HRP (Innovex Biosciences, Richmond, CA). Color development and counterstaining was achieved using Turbo AEC (Innovex) and Contrast Blue (KPL, Gaithersburg, MD), respectively. After each staining step, sections were rinsed with wash solution (KPL) and signal enhancing buffer (Innovex).

Flow cytometric analysis of cell surface markers

mAbs used for cell surface staining were FITC and PE anti-CD8α (53-6.7), FITC anti-CD4 (H129.19), PE anti-CD44 (IM7), PE anti-TCRαβ (H57-597), FITC anti-CD3ε (145-2C11), FITC- and PE isotype-matched control mAbs (all from BD PharMingen, San Diego, CA), purified anti-D^b (28-14-8S; ATCC HB176; ATCC), and purified anti-K^b (Y3; ATCC HB176; ATCC). Cells (5×10^5–1×10^6) were washed with cold FACS buffer (1% BSA/0.05% NaN_3 in PBS) and stained for 45 min at 4°C in 100 μl buffer with saturating concentrations of Abs. For purified mAbs, cells were then stained for 30 min on ice with a 1/50 dilution of FITC-F(ab′)$_2$ goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL). Cells were washed twice after each staining step and then analyzed on an EPICS C flow cytometer (Beckman Coulter, Hialeah, FL).

Intracellular IFN-γ staining of lung mononuclear cells

Single-cell suspensions of perfused lungs from *Cpn*- and mock-infected mice were prepared by homogenizing the organs in 100-μm pore size mesh cell strainers (BD Biosciences). After lysing RBC, cells were washed and resuspended at 10^7/ml in TCM. Isolated lung mononuclear cells (LMNC) were plated (10^6/100 μl/well) into TCM-washed, 40-h cultures of *Cpn* K6-infected and uninfected mAM monolayers prepared in 96-well, flat-bottom plates (Costar, Cambridge, MA). The mAM (2×10^4/well) were infected at 4 IFU/cell, then incubated in medium without cycloheximide. Parallel *Cpn*-infected mAM monolayers, fixed for 10 min in methanol and stained with an FITC-conjugated *Chlamydia* genus-specific mAb (Pathfinder *Chlamydia* Culture Confirmation System; Bio-Rad, Hercules, CA), indicated that ~60–70% of mAM had *Cpn* inclusions. After 1.5 h of coculture, 100 μl TCM with 2 μl/ml GolgiPlug (brefeldin A (BFA); BD PharMingen) was added to each well. LMNC were harvested 3.5 h later, washed once in FACS buffer, incubated for 15 min on ice with a 1/100 dilution of anti-CD16/CD32 (2.4G2) mAb (BD PharMingen), and then surface-stained with FITC anti-CD8α. Cells were washed, fixed, and permeabilized (Cytofix/Cytoperm kit, BD PharMingen), then incubated for 30 min on ice with a 1/100 dilution of PE-conjugated anti-IFN-γ (XMG1.2) before analysis by flow cytometry. An isotype-matched mAb (rat IgG1) was used to control for the specificity of intracellular cytokine staining.

Peptides

H-2^b motif-bearing *Cpn* and control peptides (Table I) were synthesized by F-moc-based solid phase chemistry using an ABI 430A peptide synthesizer (Applied Biosystems, Foster City, CA) and were purified by reverse phase HPLC. To confirm their purity (95%) and identity, peptides were analyzed by mass spectrometry. The control peptides used were K^b-restricted *T. cruzi* TSA-1_{515} epitope VDYNFTIV (*Tc*TSA) (24), K^b-restricted OVA_{257} epitope SIINFEKL (OVA) (29), and D^b-restricted influenza A NP_{366} epitope ASNENMETM (FLUnp) (30). Lyophilized peptides were dissolved in DMSO (Sigma) at 20 mg/ml and stored at −70°C. Before use, peptides were further diluted with RPMI 1640. No cell toxicity was associated with any peptide.

H-2^b peptide binding assay

Peptide binding to K^b or D^b was measured by the stabilization of class I molecules on the surface of RMA-S cells (31) and by a quantitative molecular binding assay that measures the inhibition of binding of a radiolabeled probe peptide to soluble K^b or D^b molecules (32). For the MHC class I stabilization assay, RMA-S cells (10^6/ml) were cultured at 26°C in 6% CO_2 for 24 h, followed by 1 h in the presence of peptide (0.1–50 μM). Cells were then transferred to 37°C for 2 h and washed with FACS buffer, and up-regulated cell surface expression of K^b and D^b was detected by FACS analysis after staining for immunofluorescence. Results were expressed as the mean fluorescence intensity (MFI) ratio: MFI of peptide-

Table I. Chlamydia pneumoniae *synthetic peptides: protein sources and H-2^b binding affinities*

ID	Peptide Sequence[a]	Protein(s)[b,c]	*Cpn* Genome Annotations[d]	H-2^b Motif[e]	Ratio MFI[f]	Binding (IC_{50} nM)[g]
1	NTVVFDAL	60 kDa Omp (Omp2, OmcB)[b,h]	CPn0557, CP0195	K^b	1.69	155,000
2	QESCYGRL			K^b	2.78	344
3	ISVSNPGDL			D^b	2.54	220
4	VLSFNLGDM			D^b	1.49	62,857
5	AEDTNVSLI			D^b	0.95	440,000
6	SKLQYKII	FKBP-type PPIASE (Mip)	CPn0661, CP0086	K^b	1.13	155,000
7	SSEGNNEPIL			D^b/D^b	1.01	293,333
8	QLPPNSLLI			D^b	1.07	293,333
9	DDEEYVIL	10 kDa chaperonin (GroES)[b,h]	CPn0135, CP0637	K^b	0.99	124,000
10	ANEGYDAL	60 kDa chaperonin (GroEL)[b,h]	CPn0134, CP0638	K^b	0.97	11,923
11	TAGANPMDL			D^b	1.47	22,000
12	ISANNDSEI			D^b	1.80	880
13	STEINQPFITM	DnaK (heat shock protein-70)[b,h]	CPn0503, CP0251	D^b/K^b	2.01/2.07	124,000/13,209
14	VLSTNGDTL			D^b	3.30	427
15	FLLFFEFLLV	76 kDa[b,h]	CPn0728, CP0018	K^b/K^b	1.85	20,667
16	LMSGFRQM			K^b	1.43	6,889
17	YASDNQAIL			D^b	2.89	480
18	GFKSNFNKI			K^b/D^b	1.2/0.95	155,000/440,000
19	LVYNYPGV	43 kDa homologs 1–4:4	CPn0929, CP0937	K^b	3.20	23
20	LIYNYPGV	3	CPn0928, CP0938	K^b	2.99	4.1
21	LLVFNYPGI	2	CPn0927, CP0939	D^b/K^b	1.69/1.48	5,167/62,857
22	LIFNYPGV	1	CPn0562, CP0188	K^b	2.81	40
23	HPYLYRLL	1	CPn0562, CP0188	K^b	2.10	194
24	HPTLFKVL	2	CPn0927, CP0939	K^b	2.87	620
25	SIILFLPL	2	CPn0927, CP0939	K^b	3.61	1.0
26	KICQNFILL	1	CPn0562, CP0188	D^b/K^b	2.80/2.95	2,480/2,200
27	ISNGNSDCL	1	CPn0562, CP0188	D^b	3.62	314
28	YSQGNSGLM	2	CPn0927, CP0939	D^b	2.35	889
29	TGKLNLENL	3	CPn0928, CP0938	D^b	1.79	17,600
30	QAPTNRWML	4	CPn0929, CP0937	D^b	3.07	5.6
31	SLLGNATAL	MOMP (OMP1, OmpA)[b,h]	CPn0695, CP0051	D^b	2.98	160
32	SHYAFSPMFEVL	Omp5[b,h] (Pmp10)	CPn0449, CP0303	K^b/K^b	2.66	689
33	ISFAFCQL			K^b	2.91	1.3
34	QPQNYLRL	Omp4[b,h] (Pmp11)	CPn0451, CP0302	K^b	1.54	51,667
35	HDQLFSLL			K^b	1.89	2,296
36	GTYHFTKL	Omp85 homolog (YaeT)	CPn0300, CP0458	K^b	2.79	18
37	FQLCNSYDL	OmpB (PorB)	CPn0854, CP1015	D^b	4.29	13
38	NHPVFSPL	IncA homolog	CPn0585, CP0163	K^b	2.61	56
39	LQQRYSRL			K^b	2.91	0.6
*Tc*TSA	VDYNFTIV	*T. cruzi* TSA1		K^b	3.37	36
OVA	SIINFEKL	OVA		K^b	3.38	11
FLUnp	ASNENMETM	Influenza A nucleoprotein		D^b	3.47	6.2

[a] Sequence in single-letter amino acid code.
[b] *Cpn* proteins from which H-2^b motif-bearing sequences were selected for peptide synthesis; synonyms shown in parentheses.
[c] Originally reported in *Cpn* genome sequencing Refs. 33–35.
[d] Gene name designations as annotated from the sequenced genomes of *Cpn* strains CWL029 (prefix CPn; Chlamydia Genome Project; reported in Ref. 33; http://chlamydia-www.berkeley.edu;4231) and AR39 (prefix CP; reported in Ref. 34; http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=bcp). *Cpn* strain J138 gene names are commonly those of CWL029 (http://w3.grt.kyusbu-u.ac.jp/J138/; http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=ntcp02).
[e] H-2^b binding motif present in synthetic peptides; H-2K^b binding motif is FY at position 5, and LMIV at position 8; H-2D^b binding motif is N at position 5, and MIL at position 9 (36).
[f] Binding affinity as assessed by ability of peptides (10 μM) to up-regulate and stabilize K^b or D^b molecules on RMA-S cells. Ratio MFI is MFI in the presence of test peptide over the fluorescence intensity in the absence of peptide (high, ≥2.5; intermediate, 2.0–2.4; low, 1.5–1.9; negative, ≤1.4).
[g] Binding affinity as measured by quantitative binding assay. IC_{50} is the nanomolar concentration of peptide capable of inhibiting by 50% the binding of ^{125}I-labeled index peptide to purified soluble H-2^b molecules (high, ≤50 nM; intermediate, 50–500 nM; low, 500–10,000 nM; negative, >10,000).
[h] Originally reported in Refs. 39–44.

-treated cells/MFI of untreated cells. For the quantitative binding assay, test peptides (1 nM to 100 μM) were coincubated with radiolabeled probe peptides (1–10 nM; SGPSNTYPEI for D^b; RGYVFQGL for K^b), purified soluble D^b or K^b H chain (5–500 nM), and β_2-microglobulin (1 μM; Scripps Laboratories, San Diego, CA) for 48 h at room temperature in the presence of protease inhibitors. The percentage of MHC-bound radioactivity was determined by gel filtration, and the concentration required to inhibit 50% (IC_{50}) of the binding of radiolabeled peptide was calculated.

Generation of effector cells

To generate *Cpn* peptide-specific CTL, mice were killed 2 wk to 6 mo after the first or second *Cpn* infection. Immune spleen cells (SC) were washed, resuspended in TCM, and seeded in 24-well plates (Costar) at 5×10^6 cells/well. Individual peptides (2 μM) were included in each 2-ml culture. After 2 days of incubation at 37°C, 6% CO_2, cultures were made to 5% RCAS and incubated for 4 additional days. Peptide-stimulated effectors were also generated from LMNC removed 1 wk to 45 days after primary or secondary infection.

Preparation of peptide-pulsed and Cpn*-infected target cells*

To prepare RMA-S targets, cells preincubated for 24 h at 26°C in 6% CO_2, were seeded into 24-well plates (10^6/2 ml/well) and incubated overnight in the presence of peptide (0.1 μM) and 100 μCi $Na_2{}^{51}CrO_4$ (^{51}Cr; Amersham, Arlington Heights, IL). Cells were shifted to 37°C for 2 h before processing for CTL assays. T2K^b and T2D^b targets were prepared by overnight incubation at 37°C with ^{51}Cr and peptide. To prepare *Cpn*-infected targets, 24-h mAM monolayers growing in 12-well plates (3×10^5/well) were centrifuged with 0.4 ml/well *Chlamydia* medium containing live or heat-killed *Cpn* (4 IFU/cell). Heat-killed *Cpn* was prepared by incubating bacteria at 60°C for 1 h. Control mAM targets were inoculated with HL

cell-derived material. After 1 h of incubation at 37°C, the inocula were removed, and 3 ml medium with 100 μCi ^{51}Cr was added to each well. The plates were incubated at 37°C for 20 h, at which time BFA (10 μg/ml) was added to a subset of wells containing *Cpn*-infected mAM. Two hours later, monolayers were washed with RPMI 1640 and treated with Cell Dissociation Buffer (Invitrogen) to prepare single-cell suspensions. *Chlamydia*-specific immunofluorescent staining of infected mAM incubated for 24 h more indicated that ~60–70% of the cells were infected. Similar rates of infection were achieved for the K6, AR39, and CWL029 *Cpn* strains. BFA treatment did not affect *Cpn* growth, as determined by subculture of 5-h BFA-treated infected mAM monolayers.

CTL assay

Cytotoxic activity of effector cells on target cells was assessed by ^{51}Cr release assays, as previously described (24). Briefly, peptide-sensitized, *Cpn*-infected, and control ^{51}Cr-labeled target cells (5×10^3/well) were incubated for 5-h with effector cells at various E:T cell ratios in 96-well, round-bottom plates (Corning, Corning, NY). BFA (10 μg/ml) was included in those wells containing *Cpn*-infected BFA-treated target cells. Effectors depleted of CD8$^+$ and CD4$^+$ T cells were only tested at the highest E:T cell ratio. Depletions were conducted with magnetic beads coated with anti-CD8 or anti-CD4 mAbs (Miltenyi Biotec, Auburn, CA) or by incubating cells on ice for 30 min with anti-CD8 (3.155; ATCC TIB 211, ATCC) or anti-CD4 (GK1.5; ATCC TIB 207; ATCC), followed by 30 min at 37°C with rabbit complement. Supernatants were harvested (Skatron SCS System; Molecular Devices, Sunnyvale, CA), and released ^{51}Cr was counted on a gamma counter. The percent specific lysis was calculated from the mean of triplicates as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release did not exceed 26% of the maximum release. SEs were <6% of the mean. A CTL response was considered positive when, at the highest E:T ratio, a difference of ≥10% lysis was obtained by subtracting the percentage lysis for control peptide from the percentage lysis for the test peptide.

Generation and maintenance of Cpn-specific CD8$^+$ T cell lines

Peptide-specific CD8$^+$ T cell lines were generated as previously described (24). In brief, 4×10^7 immune SC from *Cpn*-infected mice were incubated with CTL peptides (2 μM) for 6 days at 37°C in 6% CO_2 in 10 ml TCM using T25 flasks (Corning). RCAS (5%) was added on day 2 of culture. In each of two to four subsequent 6-day cycles of restimulation, 4×10^6 viable effector cells were cultured with 4×10^7 peptide-pulsed irradiated (3000 rad) syngeneic SC in 15 ml TCM-RCAS. A similar protocol was used to generate CD8$^+$ T cell lines from immune LMNC.

Cytokine determinations

Culture supernatants of peptide-stimulated SC were harvested 48 h into the first and second cycles of stimulation, and levels of IFN-γ, IL-4, and TNF-α were determined by sandwich ELISA. Capture and detection Ab pairs were R4-6A2/XMG1.2 (BD PharMingen) for IFN-γ, BVD4-1D11/BVD6-24G2 (Caltag, Burlingame, CA) for IL-4, and Ag affinity-purified goat polyclonal Ab/MP6-XT3 (R&D Systems and BD PharMingen) for TNF-α. The lower detection limits for IFN-γ, IL-4, and TNF-α were 40, 8, and 40 pg/ml, respectively.

ELISPOT assay for IFN-γ-secreting cells

Nitrocellulose-backed 96-well plates (MultiScreen MAHA S4510; Millipore, Bedford, MA) were coated overnight at 4°C with 75 μl PBS containing 10 μg/ml anti-IFN-γ mAb R4-6A2. After washing with PBS, the wells were blocked for 2 h at 37°C with 100 μl TCM. Two-fold serial dilutions of freshly isolated LMNC or SC starting at 10^7 cells/ml were added in TCM-RCAS in triplicate wells (100 μl/well) containing peptide-pulsed (1 μM) irradiated (16 krad) RMA-S cells (10^5/100 μl/well). Irradiated unpulsed RMA-S cells were used as a control for Ag-independent IFN-γ secretion. As positive controls, LMNC and SC containing unpulsed RMA-S cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml; Sigma). After incubation at 37°C in 5% CO_2 for 24 h, plates were washed three times with PBS, followed by three times with PBS/0.05% Tween 20 (PBS/T). Wells then received 75 μl of a solution of 3 μg/ml biotinylated anti-IFN-γ mAb XMG1.2 in PBS/T/0.5% FBS. After a 16-h incubation at 4°C, plates were washed six times with PBS/T, and 100 μl alkaline phosphatase-ExtrAvidin (Sigma; 1/800 dilution) was added to each well. Following 1 h at 26°C wells were washed four times with PBS/T and twice with PBS. Spots were developed with 75 μl/well 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium substrate (Sigma). After 20 min the plates were rinsed with water and dried, and spots in each well were counted under a stereomicroscope.

Inhibition of chlamydial inclusion development assay

To evaluate the inhibition of *Cpn* growth by CD8$^+$ T cell-derived soluble factors, 24-h HEp-2 cell monolayers prepared in 48-well plates (5×10^4/well) were treated with supernatants from CTL lines for 18 h before *Cpn* infection. Supernatants preincubated with anti-IFN-γ XMG1.2 or IgG1 control mAbs (20 μg/ml; BD PharMingen) were also used for HEp-2 cell pretreatment. After *Cpn* infection (3 IFU/cell), plates were incubated for 1 h at 37°C, the inocula were removed, and monolayers were incubated for 24 h more with new samples of the same supernatants. Following 48 h of incubation in *Chlamydia* medium, cultures were stained with the Pathfinder *Chlamydia* genus-specific mAb, and the number of inclusions in each well was counted in 10 ×400 high powered fields (HPF). Supernatants were tested in triplicate. The numbers of inclusions in *Cpn*-infected HEp-2 cells treated with supernatants from cultures lacking CD8$^+$ T cells, peptide, or both were also assessed. All results were compared with those obtained using supernatants from immunomagnetically purified naive CD8$^+$ T cells stimulated with *Cpn* CTL peptides.

To test the chlamydial growth-inhibiting activity of *Cpn* peptide-specific CTL, mAM monolayers prepared 1 day earlier in 48-well plates (4×10^4/well) were infected with *Cpn* (4 IFU/cell) and incubated for 20 h before adding graded numbers of CD8$^+$ T cells (2.5×10^4–4×10^5/ml/well). Cultures with or without effectors were washed 4 h later, and 1 ml *Chlamydia* medium with cycloheximide was added to each well. After 48 h at 37°C, mAM monolayers were scraped, plates were frozen and thawed once, and serial dilutions of cleared pooled material from triplicate wells were used to infect fresh HEp-2 cell monolayers. Total numbers of mAM recovered from parallel cultures were similar for all effector cell types and densities tested, ranging between $1–1.4 \times 10^5$/triplicate. Inoculated HEp-2 cells were incubated for 72 h in cycloheximide-containing medium and processed for immunofluorescence, and chlamydial inclusions were counted in 10 HPF. Control effector cells were *T. cruzi* TSA-1_{515}-specific CD8$^+$ CTL and purified naive CD8$^+$ T cells.

Results

Mononuclear cell infiltrates in the lungs of Cpn*-infected mice contain pathogen-specific IFN-γ-producing CD8$^+$ T cells*

Mouse models of *Cpn* infection indicate that CD8$^+$ T cells and IFN-γ play important roles in protective immunity (20–22). However, the induction of *Cpn*-specific CD8$^+$ T cells during infection has not been reported. Therefore, we first determined whether the pulmonary inflammatory response of B6 mice during primary and secondary *Cpn* infections included pathogen-specific CD8$^+$ T cells capable of IFN-γ production. Lung sections from mock-infected animals showed no evidence of pulmonary inflammation up to 100 days after each of two inoculations (Fig. 1*A*). In contrast, a mild interstitial mononuclear cell infiltration with a perivascular and peribronchiolar lymphoid cuffing was present in the lungs from *Cpn*-infected mice from days 5–60 after infection (Fig. 1, *C* and *D*). During reinfection, a moderate to marked pneumonia and lymphoid infiltrate was detected 2 days postinoculation, peaked on days 8–12 (Fig. 1*B*), and gradually declined, but remained present for up to 2 mo. Pulmonary bacterial loads commonly peaked 2–4 days before maximal inflammatory responses with mean IFUs per lung of $3 \times 10^5 \pm 9 \times 10^4$ after primary infection and $2.9 \times 10^4 \pm 8 \times 10^3$ after reinfection. Staining with a *Cpn* MOMP-specific mAb localized chlamydial Ag within epithelial and mononuclear cells throughout the interstitium, especially in the perivascular and peribronchiolar inflammatory foci (Fig. 1*D*). No staining was detected using an IgG3 control mAb (Fig. 1*C*). The MOMP-specific mAb did not stain lung tissue from mock-infected mice (data not shown).

Analysis of LMNC isolated 12 days postinfection revealed that the percentage of CD8$^+$ T cells in lungs from infected mice was almost twice that in lungs from mock-infected animals (Fig. 1, *G* and *H* vs *E* and *F*). When LMNC were cocultured for 5 h with mock-infected mAM, the percentage of CD8$^+$ T cells that were

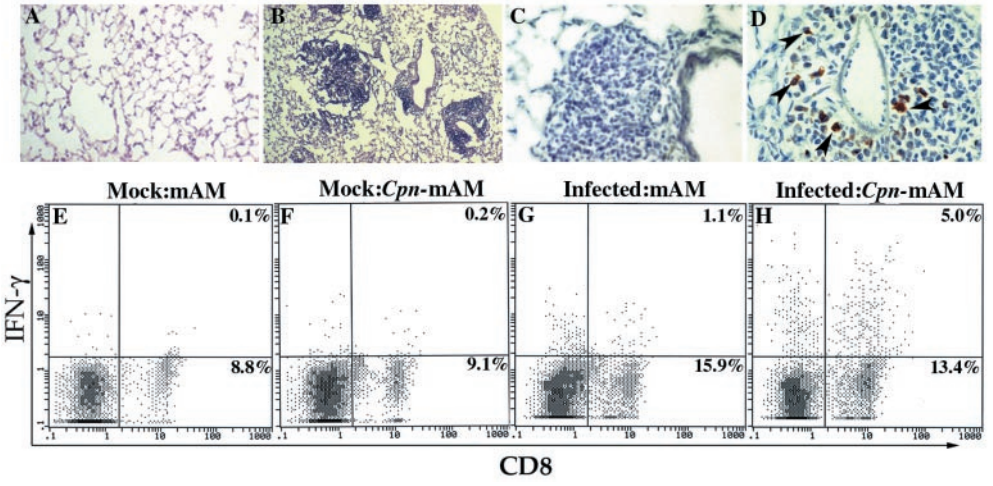

FIGURE 1. Detection of intracellular *Cpn* Ag and pathogen-specific IFN-γ-producing $CD8^+$ T cells in the lungs of infected mice. Lungs from *Cpn*- and mock-infected B6 mice were obtained 8–12 days after i.n. inoculation with 40 μl PBS containing 10^6 *Cpn* IFUs or material obtained from mock-infected HL cells, respectively. *A* and *B*, Histological analysis of representative 6-μm H&E-stained sections from the lungs of mock-infected (×200; *A*) and *Cpn*-infected (×100; *B*) mice 12 days postinoculation. *C* and *D*, Pulmonary sections from infected mice prepared 8 days postinoculation and immunostained with isotype-matched (IgG3) control mAb (×400; *C*) or *Cpn*-specific mAb RR-402 (×600; *D*; *arrowheads* point at some cells with positive intracellular staining for *Cpn* Ag). *E–H*, Pooled LMNC isolated 12 days postinoculation from mock-infected ($n = 8$; *E* and *F*) and *Cpn*-infected ($n = 4$; *G* and *H*) mice were stimulated in vitro for 5 h with mock-infected (*E* and *G*) and *Cpn*-infected (*F* and *H*) mAM. GolgiPlug was added for the last 3.5 h of culture. After staining for CD8, LMNC were fixed, permeabilized, stained for intracellular IFN-γ, and analyzed by flow cytometry. Results are representative of three experiments.

IFN-γ^+ was 6.5% in *Cpn*-infected animals ((1.1/17) × 100), but only 1.1% in mock-inoculated mice ((0.1/8.9) × 100; Fig. 1, *G* vs *E*). This level of IFN-γ production in pulmonary $CD8^+$ T cells from infected mice may reflect specific activation in vivo. After coculture with *Cpn*-infected mAM, the percentage of $CD8^+$ T cells expressing IFN-γ was 27.1% in cells from infected mice ((5/18.4) × 100), and only 2.2% in cells from mock-infected animals ((0.2/9.3) × 100; Fig. 1, *H* vs *F*). Coculture with *Cpn*-infected mAM elicited ~4-fold increase in the percentage of $CD8^+$ IFN-γ^+ T cells (Fig. 1, *H* vs *G*). These data establish that *Cpn*-specific $CD8^+$ T cells are present in the lungs from infected B6 mice and suggest that they may participate in the immune response against this pathogen by producing IFN-γ.

Mice infected with Cpn *generate a CTL response that recognizes peptides from multiple chlamydial Ags*

Based on the foregoing results, we asked: Which *Cpn* Ags reach the cytosol of infected cells and are recognized as MHC class I-peptide complexes by $CD8^+$ T cells? From the available *Cpn* genome sequence databases (33–35) we selected 35 proteins that included outer and inclusion membrane proteins, chaperones, and selected proteins unique to *Chlamydia* or *Cpn*. In the sequences of these proteins we identified 461 segments of 8–9 aa conforming to the murine H-2K^b and D^b class I binding motifs (36). Of these, 39 sequences from 16 proteins were selected for peptide synthesis (Table I). When the ability to bind to K^b and D^b was examined by the RMA-S stabilization assay (31), 22 peptides were classified as high or intermediate binders (MFI ≥ 2.0). Using a quantitative molecular binding assay (32), which provides precise measurements of binding affinity that better correlate with $CD8^+$ T cell immunogenicity (37), 17 peptides bound with high or intermediate affinity ($IC_{50} \leq 500$) to purified K^b or D^b molecules. Four additional peptides with affinities of 500 nM but ≤1000 nM were considered good binders, as $CD8^+$ T cell immunogenicity has also been reported in this affinity range (38). Overall, 20 peptides were identified as high to intermediate binders by both binding assays (Table I).

All synthetic peptides were next assayed for their ability to target H-2^b-bearing cells for lysis by SC from *Cpn*-infected mice obtained after culture with each individual peptide. Of the 39 peptides, 18 generated effector cells that specifically lysed RMA-S cells (H-2^b; MHC class II^-) pulsed with the respective peptide, but not RMA-S cells pulsed with control OVA (K^b) and FLUnp (D^b) CTL peptides (Fig. 2). The 18 peptides represent sequences in 12 *Cpn* Ags: five outer membrane proteins (omp), a 76-kDa protein, a family of four hypothetical 43-kDa proteins, heat shock protein-70, and an inclusion membrane protein (33–35, 39–44) (Table I and Fig. 2). At the highest E:T cell ratio, net peptide-specific lysis for effectors stimulated with the 18 CTL peptides ranged from 10–27.9% after primary infection and from 10.4–34.2% after reinfection. As illustrated for a subset of peptides, CTL activity was detectable after primary infection (Fig. 2*A*). For most of the 18 positive peptides, the in vitro recall CTL responses were slightly enhanced in reinfected mice, and absent in mock-infected animals (Fig. 2, *B* and *C*). Thus, most peptides capable of binding to H-2^b molecules with high to intermediate affinity elicit functional CTL, and the polyclonal multi-Ag-specific CTL response is induced as a result of infection.

Splenic CTL recognize Cpn *peptides in a specific, K^b- or D^b-restricted, and $CD8^+$ T cell-dependent fashion*

To further characterize the lytic response to the positive *Cpn* CTL peptides, cell depletion and MHC class I restriction analyses were conducted. As illustrated for a subset of positive peptides, effector cells generated from SC of *Cpn*-reinfected mice lysed RMA-S cells pulsed with *Cpn* peptide but not with irrelevant peptides, and target cell lysis was eliminated by depleting effectors of $CD8^+$, but not of $CD4^+$ T cells (Fig. 3). T2 target cells pulsed with K^b-binding CTL peptides (e.g., peptides 19, 20, and 39) were lysed by

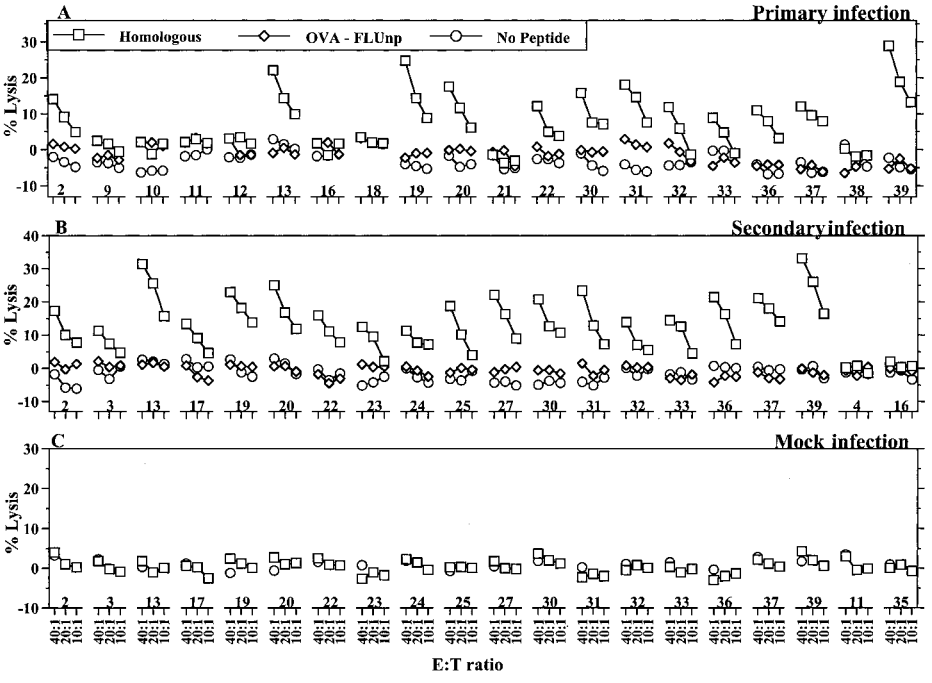

FIGURE 2. *Cpn* infection primes a CTL response to multiple chlamydial Ags. SC from *Cpn*-infected (10^6 IFU) and mock-infected B6 mice were stimulated in vitro with H-2^b motif-bearing peptides (2 μM) from pathogen-derived Ags. After 6 days effector cells were tested for recognition of RMA-S cells pulsed with homologous peptide, negative control K^b OVA or D^b FLUnp peptides (0.1 μM), or no peptide in a CTL assay at the indicated E:T ratios. *A*, Lytic activity of immune SC from 40-day *Cpn*-infected mice (20 of 39 peptides are shown). *B*, Lytic activity of immune SC from 65-day *Cpn*-infected mice tested 1 mo after reinfection (10^6 IFU; 18 positive peptides and negative peptides 4 and 16 are shown). *C*, Lytic activity of SC from 80-day mock-infected mice tested 1 mo after the second inoculation (18 positive peptides and negative peptides 11 and 35 are shown).

the respective peptide-specific effectors only if the targets expressed K^b but not D^b molecules. In contrast, effectors generated with D^b-binding CTL peptides (e.g., peptides 27, 30, 31, and 37) lysed $T2D^b$ but not $T2K^b$ cells sensitized with the homologous peptide (Fig. 3). These results demonstrate that SC from *Cpn*-infected B6 mice recognize pathogen-derived peptides in an Ag-specific, K^b- or D^b-restricted, and $CD8^+$ T cell-dependent manner.

Cpn *peptide-specific* $CD8^+$ *CTL display a memory Tc1 phenotype*

To further characterize *Cpn* peptide-specific T cells, SC from *Cpn*-infected mice were expanded by three weekly cycles of stimulation with each peptide and cell lines were evaluated for cytokine secretion profiles and surface phenotypes (Table II). During the first cycle, supernatants contained IFN-γ but not IL-4. After the second cycle, IFN-γ levels were 4- to 9-fold higher, but IL-4 levels remained undetectable. All the CTL peptides elicited significant TNF-α production. IFN-γ and TNF-α levels varied for each CTL peptide, but were significantly higher than the levels produced by stimulation with four *Cpn* peptides without CTL activity (Table II). All T cell lines were >90% $CD3^+CD8^+TCR\alpha\beta^+CD44^{high}$ (Table II) and highly lytic to *Cpn* peptide-pulsed targets (data not shown). These results indicate that the 18 *Cpn* peptides stimulate memory $CD8^+$ T cells in the spleens of infected mice displaying cytolytic function and a Tc1 cytokine secretion pattern.

Infected murine alveolar macrophages are lysed by Cpn *peptide-specific* $CD8^+$ *CTL*

To ascertain whether the 18 *Cpn* peptides represent CTL epitopes that are presented on the surface of infected cells, we used *Cpn* K6-infected mAM as targets for peptide-stimulated effectors. As shown for seven *Cpn* CTL peptides, effector cells generated after two cycles of stimulation with individual peptides lysed *Cpn*-infected but not mock-infected or BFA-treated infected mAM (Fig. 4). Short term CTL lines

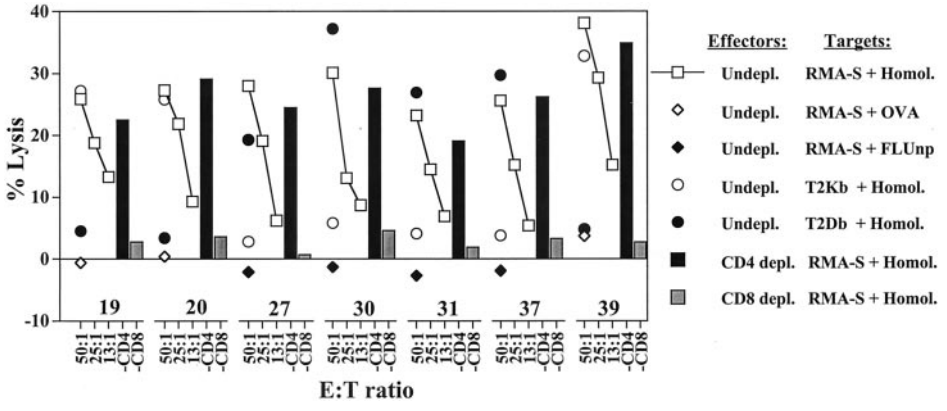

FIGURE 3. Recognition of *Cpn* peptides by splenic CTL is specific, K^b- or D^b-restricted, and $CD8^+$ T cell-dependent. Immune SC from 3-mo infected B6 mice were obtained 2 mo after reinfection (10^6 IFU) and cultured for 6 days with *Cpn* CTL peptides (2 μM). The lytic activity of undepleted effector cells was tested against RMA-S cells pulsed with the homologous CTL peptide or with negative control K^b OVA or D^b FLUnp peptides (0.1 μM). $T2K^b$ and $T2D^b$ target cells were used to define the H-2^b class I restricting molecule. After depletion of $CD4^+$ and $CD8^+$ T cells, the cytotoxicity of depleted effectors was measured at the highest E:T ratio (50:1) against RMA-S sensitized with homologous peptide. Results for seven of 18 *Cpn* CTL peptides are shown.

Table II. Chlamydia pneumoniae *peptide-stimulated effector cells: cytokine and cell surface phenotype profiles*

		IFN-γ (ng/ml)[a]		IL-4 (pg/ml)[a]		TNF-α (pg/ml)[a]	TCRαβ$^+$CD8$^+$CD44^{+b}
		Stimulation cycle		Stimulation cycle		Stimulation cycle	Stimulation cycle
ID	Peptide	1	2	1	2	2	3 (%)
Cpn CTL peptides							
2	QESCYGRL	2.5	18.5	<8	<8	243.8	93.7
3	ISVSNPGDL	1.9	10.8	<8	<8	198.4	93.1
13	STEINQPFITM	12.5	61.2	<8	<8	597.1	98.9
17	YASDNQAIL	3.0	15.4	<8	<8	206.3	96.8
19	LVYNYPGV	5.1	24.6	<8	<8	281.0	97.0
20	LIYNYPGV	4.3	24.8	<8	<8	275.2	97.7
22	LIFNYPGV	2.8	11.5	<8	<8	211.9	90.0
23	HPYLYRLL	3.0	13.9	<8	<8	187.7	90.2
24	HPTLFKVL	2.8	18.1	<8	<8	266.5	93.3
25	SIILFLPL	6.1	39.9	<8	<8	494.1	95.9
27	ISNGNSDCL	3.2	26.1	<8	<8	289.9	94.1
30	QAPTNRWML	5.2	30.3	<8	<8	328.6	95.8
31	SLLGNATAL	1.9	13.3	<8	<8	185.3	90.7
32	SHYAFSPMFEVL	2.9	19.9	<8	<8	292.0	94.0
33	ISFAFCQL	3.2	30.1	<8	<8	338.7	92.8
36	GTYHFTKL	4.9	22.6	<8	<8	303.8	97.6
37	FQLCNSYDL	5.6	49.5	<8	<8	386.7	98.9
39	LQQRYSRL	12.8	57.9	<8	<8	504.6	99.2
Negative *Cpn* peptides							
11	TAGANPMDL	0.4	1.1	<8	<8	67.2	15.1
12	ISANNDSEI	0.05	0.6	<8	<8	55.4	10.6
16	LMSGFRQM	<0.04	0.6	<8	<8	<40.0	11.7
21	LLVFNYPGI	0.9	1.6	<8	<8	62.5	14.9
*Tc*TSA	VDYNFTIV	5.3	38.4	<8	<8	354.3	96.2

[a] Levels of IFN-γ, IL-4, and TNF-α were determined by ELISA as described in *Materials and Methods.* Culture supernatants were harvested 48 h poststimulation with peptide (2 μM) and kept at −20°C until assayed. Negative and positive control supernatants were obtained from *Cpn*- and *T. cruzi*-infected mice using SC stimulated with *Cpn* peptides with no CTL activity and *Tc*TSA, respectively. Supernatants from wells containing *Cpn* CTL peptide-stimulated naive SC contained minimal to undetectable levels of cytokines.

[b] Effector cells purified at the end of the third cycle of stimulation with *Cpn* CTL peptides were analyzed by flow cytometry for surface expression of CD3, TCRαβ, CD8, CD4, and CD44 after staining with FITC- and PE-conjugated mAbs. Negative and positive control effector cell lines were SC from *Cpn*- and *T. cruzi*-infected mice stimulated for three cycles with *Cpn* peptides negative for CTL activity and *Tc*TSA, respectively. Similar cytokine levels and cell surface phenotypes were obtained in two to four additional experiments.

did not lyse mAM inoculated with heat-killed *Cpn* (Fig. 4). Similar results were obtained when mAM targets were treated with the *Cpn* strains AR39 and CWL029 (data not shown). Thus, all 12 *Cpn* CTL Ags are processed by the endogenous pathway, and the 18 epitope-bearing peptides are presented to CTL only in productively infected cells.

Cpn *CTL peptides are also recognized by CD8$^+$ T cells from the lungs of infected mice*

To determine whether *Cpn* peptide-specific CD8$^+$ CTL were present in the lungs of infected mice, we purified LMNC 12 days after reinfection and stimulated them with *Cpn* CTL peptides. Six days later, the lytic activity of undepleted and CD8$^+$ T cell-depleted effectors was tested against peptide-pulsed target cells. For five representative peptides, each from a different *Cpn* CTL target Ag, resulting effectors displayed CD8$^+$ T cell-dependent CTL activity against RMA-S cells sensitized with homologous peptide, but not against the same cells pulsed with control CTL peptides or with no peptide (Fig. 5*A*). As shown for a D^b- and a K^b-restricted *Cpn* CTL peptide, T cell effectors generated after four cycles of stimulation were highly enriched for peptide-specific, H-2^b-restricted lytic activity and a CD8$^+$ CD44^{high} memory phenotype. Moreover, these LMNC-derived CTL lines lysed

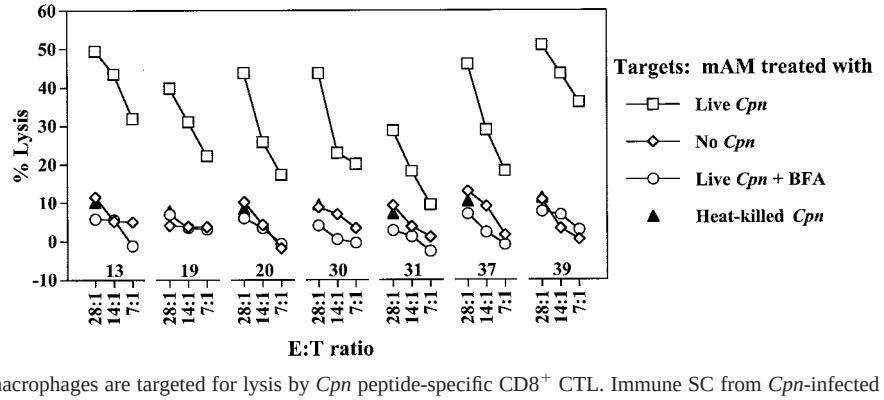

FIGURE 4. Infected macrophages are targeted for lysis by *Cpn* peptide-specific CD8$^+$ CTL. Immune SC from *Cpn*-infected B6 mice inoculated 6 and 4 mo (10^6 IFU each dose) before sacrifice were stimulated for two 6-day cycles with CTL peptides (2 μM). At the end of the second cycle, effectors (79–86% CD8$^+$) were tested for their ability to kill mAM inoculated 22 h earlier with live *Cpn* (□), HL cell-derived material (◇), or heat-killed bacteria (▲). Lytic activity of effector cells was also tested against BFA-treated *Cpn*-infected mAM (○). BFA (10 μg/ml) was added for the last 2 h of infection and during the CTL assay. Results for seven of 18 *Cpn* peptide-specific, short term CTL lines are shown.

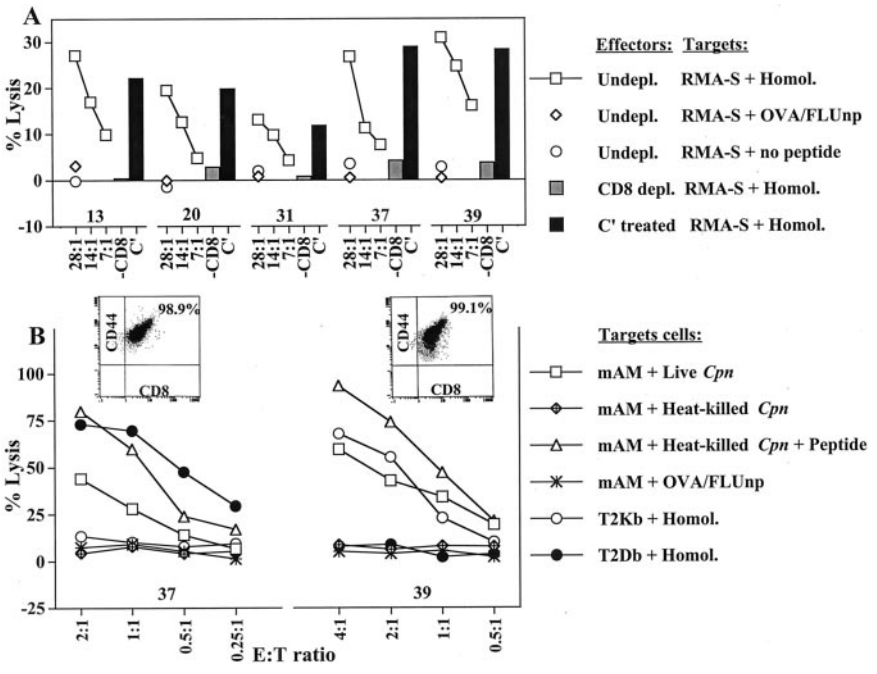

FIGURE 5. Presence of *Cpn* peptide-specific CD8$^+$ CTL in the lungs of infected mice. Immune LMNC from 2-mo infected B6 mice were obtained 12 days after reinfection (10^6 IFU) and cultured for four 6-day cycles with *Cpn* CTL peptides (2 μM). *A*. At the end of the first cycle, the lytic activity of undepleted effector cells was tested against RMA-S (H-2^b) cells pulsed with the homologous CTL peptide or with negative control K^b OVA or D^b FLUnp peptides (0.1 μM) in a CTL assay at the indicated E:T ratios. The cytotoxicity of CD8$^+$ T cell-depleted effectors and complement-treated controls was measured at the highest E:T ratio (28:1) against RMA-S sensitized with homologous peptide. *B*, After four cycles of stimulation, effector cells were analyzed for surface expression of CD8 and CD44 by flow cytometry and for their ability to lyse the indicated target cells inoculated 22 h earlier with live or heat-killed *Cpn* AR39 bacteria or were sensitized for 16 h with CTL or control peptide (0.1 μM). Results for five (*A*) and two (*B*) of 18 *Cpn* peptide-specific CTL lines are shown.

mAM inoculated with live AR39 *Cpn* bacteria, but not with heat-killed organisms unless they were also sensitized with peptide (Fig. 5*B*). These results demonstrate that *Cpn*-specific CD8$^+$ CTL are present in the lungs of infected animals and further support the natural presentation of identified epitopes by *Cpn*-infected cells.

Cpn *infection increases the precursor frequencies of epitope-specific IFN-γ-secreting CD8$^+$ T cells*

We initially observed that LMNC from *Cpn*-infected B6 mice contained CD8$^+$ T cells capable of rapidly producing IFN-γ in response to *Cpn*-infected cells (Fig. 1, *E–H*). Once *Cpn* CTL determinants became defined, we asked whether epitope-specific CD8$^+$ T cells were detectable in the lungs and spleens of infected mice without stimulation, and if so, we determined precursor frequencies of these cells during primary and secondary *Cpn* infections. Immune LMNC and SC from B6 mice were obtained 11 days after infection or reinfection and were stimulated for 24 h with peptide-pulsed and unpulsed RMA-S cells, followed by enumeration of IFN-γ secreting CD8$^+$ T cells by ELISPOT. We measured the CD8$^+$ T cell precursor frequencies for peptides 13, 37, and 39, which were associated with the highest IFN-γ production (Table II). The mean number of LMNC isolated at 11 days postinfection ($5.3 \times 10^6 \pm 8 \times 10^5$) or reinfection ($6.7 \times 10^6 \pm 1.4 \times 10^6$) was 6- to 7-fold higher than the yields obtained for mock-infected animals ($9 \times 10^5 \pm 3 \times 10^5$). Based on these data and the frequencies of IFN-γ spot-forming cells (SFC) obtained by ELISPOT, the lungs of infected animals contained an estimated 165, 145, and 250 peptide 13-, 37-, and 39-specific IFN-γ-producing CD8$^+$ T cells, respectively. For each peptide the number of IFN-γ SFCs was 2- to 2.5-fold higher after reinfection (Fig. 6*A*). Parallel studies with SC showed that the precursor frequencies of peptide 13-, 37-, and 39-specific CD8$^+$ T cells were 2- to 5-fold higher after reinfection compared with primary infection (69–172 vs 20–39 IFN-γ SFCs/10^6 cells; Fig. 6*B*). IFN-γ secretion was specific, as spots were rarely observed when immune cells were incubated with unpulsed RMA-S cells. Furthermore, peptide-specific IFN-γ SFCs were not detected using cells from mock-infected animals. Thus, *Cpn* CTL peptide-specific, IFN-γ-secreting CD8$^+$ T cells are present in local and systemic compartments after primary chlamydial infection, and these cells expand after reinfection.

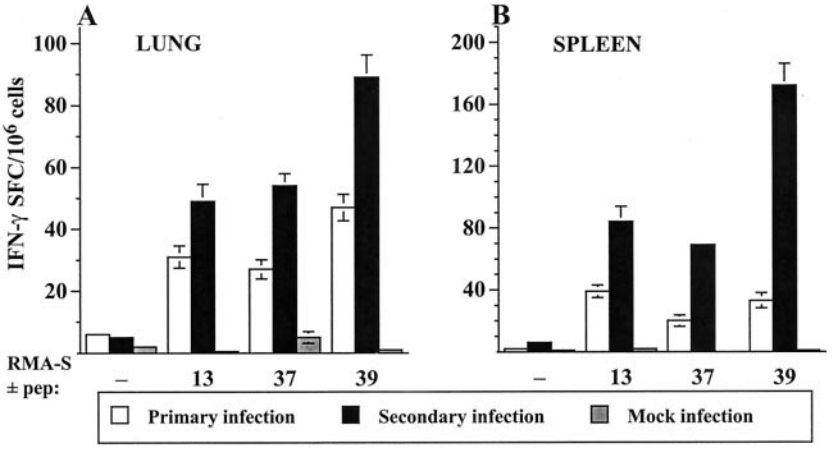

FIGURE 6. Enumeration of pulmonary and splenic *Cpn* CTL peptide-specific IFN-γ-producing CD8$^+$ T cells by ex vivo ELISPOT. *Cpn*-infected ($n = 5$) and mock-infected ($n = 25$) B6 mice were sacrificed 11 days after each of two i.n. inoculations with 10^6 *Cpn* IFUs or with material obtained from mock-infected HL cells, respectively. *A* and *B*, Irradiated RMA-S cells pulsed (1 μM), or not, with the indicated CTL peptides were cocultured for 24 h with pooled isolated LMNC (*A*) and SC (*B*) under the conditions described in *Materials and Methods*, and the number of specific IFN-γ SFC (CD8$^+$ T cells) was quantified. Values represent the mean ± SEM of triplicate determinations from a representative of three experiments.

Cpn epitope-specific CD8$^+$ CTL and their soluble factors inhibit chlamydial growth

IFN-γ contributes to protection against *Cpn* infection (21, 22) and inhibits chlamydial growth in vitro (45). Our results suggested that *Cpn*-specific CD8$^+$ T cells could participate in protective immunity through secretion of IFN-γ and/or killing of infected cells. To evaluate the ability of CD8$^+$ T cell-derived soluble factors to inhibit bacterial growth, we counted the number of *Cpn* inclusions in infected HEp-2 cells that had been treated with supernatants collected from CD8$^+$ T cell cultures (Fig. 7*A*). As shown for peptides 13, 20, 37, and 39, undiluted culture supernatants from peptide-specific CD8$^+$ T cells inhibited inclusion development by 95–100% compared with undiluted culture supernatant from *Cpn* peptide-stimulated naive CD8$^+$ T cells. Serial dilutions of the supernatants resulted in 70–92% (1:5) and 8–51% (1:25) inhibition, indicating a dose-dependent effect on chlamydial growth. Addition of anti-IFN-γ mAb to the undiluted supernatants from peptide-specific CD8$^+$ T cells reduced inhibition by 18–65%. No reversal of inhibition was observed using control mAb (data not shown). Supernatants from cultures containing peptide alone or peptide-stimulated naive CD8$^+$ T cells failed to inhibit chlamydial multiplication. Minimal inhibition was detected using supernatant from unstimulated peptide-specific CD8$^+$ T cells, perhaps due to accumulation of inhibitory factors during the 48 h before collection of the supernatants for testing. Thus, *Cpn*-specific CD8$^+$ CTL suppress chlamydial growth by production of IFN-γ and other unidentified soluble factors.

To examine whether *Cpn* peptide-specific CD8$^+$ T cells directly inhibit chlamydial growth, we evaluated inclusion development in HEp-2 cell monolayers inoculated with lysates of chlamydia-infected mAM that had been briefly cocultured with CD8$^+$ T cells (Fig. 7*B*). At high CD8$^+$ T cell densities, *Cpn* peptide 13-, 20-, 37-, and 39-specific CTL inhibited 94–100% the number of *Cpn* inclusions that developed in the presence of naive CD8$^+$ T cells, and the extent of inhibition decreased with decreasing numbers of *Cpn* peptide-specific CD8$^+$ T cells. Significant inhibition was observed for all *Cpn* peptide-specific CD8$^+$ CTL lines compared with studies in the absence of CD8$^+$ T cells or in the presence of irrelevant *T. cruzi* TSA-specific CD8$^+$ T cells. These data indicate that *Cpn*-specific CD8$^+$ CTL decrease the output of viable organisms from infected cells.

Discussion

Like all chlamydiae, *Cpn* has an intracellular biphasic developmental cycle, alternating between the infectious, nonreplicating, and relatively metabolically inert elementary body and the noninfectious, replicating, and metabolically active reticulate body. Hundreds of infectious progeny are released from each infected cell. Like *C. trachomatis* and *C. psittaci*, *Cpn* can persist for long periods in infected cells without dividing before resuming a productive cycle (46, 47). Thus, the induction of an early potent immune response that eliminates *Chlamydia*-infected cells or renders developing bacteria noninfectious would reduce the pathogen's ability to grow and spread in the infected host. Herein, we provide

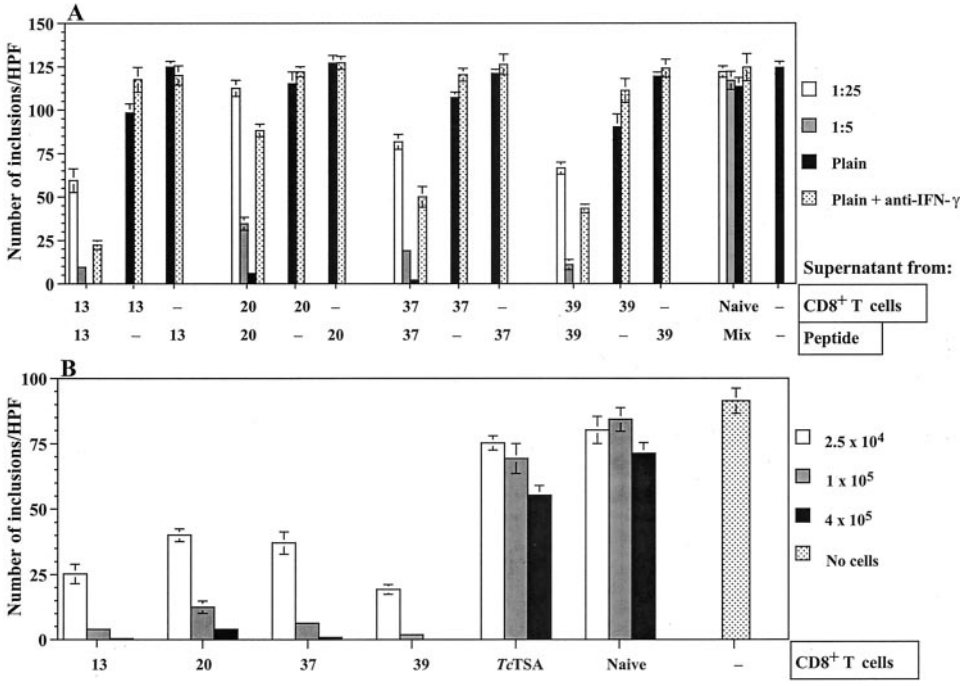

FIGURE 7. Inhibition of chlamydial multiplication by soluble factors and cells from *Cpn* peptide-specific CD8$^+$ CTL lines. *A*, Culture supernatants from *Cpn*-immune LMNC-derived CD8$^+$ T cell lines were obtained 48 h into the third cycle of stimulation with the indicated CTL peptides (2 μM) and irradiated syngeneic SC. Serial dilutions and undiluted supernatants preincubated, or not, with anti-IFN-γ neutralizing or isotype-matched mAbs (20 μg/ml) were used to pretreat triplicate HEp-2 cell cultures for 18 h before *Cpn* infection. Infected monolayers were treated for 24 h with new samples of the same supernatants and cultured for an additional 48 h in *Chlamydia* medium. Cultures were fixed and stained with a *Chlamydia* genus-specific mAb, and the number of developing chlamydial inclusions in 10 HPF/well was counted. Supernatants collected from cultures lacking CD8$^+$ T cells, peptide, or both were also tested. All results were compared with those obtained with the respective dilution of culture supernatant from immunomagnetically purified naive CD8$^+$ T cells stimulated with a mixture of the indicated CTL peptides. *B*, The indicated numbers of CD8$^+$ T cells obtained at the end of the third cycle of stimulation with *Cpn* CTL peptide were cocultured for 4 h with *Cpn*-infected mAM monolayers. Washed cultures were incubated for an additional 48 h and passed into fresh HEp-2 cell monolayers as described in *Materials and Methods*. After 72 h, the number of inclusions in 10 HPF/well was counted. *T. cruzi* TSA-1_{515} (*Tc*TSA)-specific CD8$^+$ CTL and purified naive CD8$^+$ T cells were used as control effector cells. Values represent the mean ± SEM. Data are representative of three experiments.

the first evidence that *Cpn*-infected mice mount pathogen-specific $CD8^+$ T cell responses that exhibit potent anti-chlamydial activity through lysis of infected cells and secretion of soluble factors, including IFN-γ. We also report the identification of 18 H-2^b-restricted Tc1 epitope-bearing sequences in the first 12 bona fide *Cpn* target Ags of the $CD8^+$ T cell response induced in infected mice.

Development of vaccines that induce $CD8^+$ T cell responses against *Cpn* requires knowledge of the mechanisms by which these cells mediate resistance to infection. Equally important for the design of anti-*Cpn* vaccines is to determine the response kinetics and functional attributes of $CD8^+$ T cells found in the lungs and systemic compartments of *Cpn*-infected hosts and whether these cells associate exclusively or collectively with protection, persistence, or immunopathology. However, understanding *Cpn*-specific $CD8^+$ T cell responses and designing vaccines to maximize protection and minimize tissue damage are contingent upon the ability to assess such responses and on the identification of the *Cpn* Ags or epitopes that are recognized by $CD8^+$ T cells. As a first step to characterize the anti-*Cpn* $CD8^+$ T cell response, we structured a strategy based on three components: 1) the mouse model of *Cpn* infection, which is an excellent system to study the immune mechanisms thought to control this pathogen in humans; 2) the *Cpn* genome sequence databases, from which putative target Ags of $CD8^+$ T cells were selected; and 3) the *Cpn*-competent mAM cell line, which served to stimulate and detect the activity of *Cpn*-specific $CD8^+$ T cells. We selected the macrophage because *Cpn* disseminates from the respiratory tract by infecting this cell (48), and a similar mechanism may permit this pathogen to spread systemically in humans.

We first asked whether chlamydial infection primes *Cpn*-specific $CD8^+$ T cells in the lungs of infected B6 mice. To select a time point when we could detect T cells, we conducted a kinetic analysis of histopathologic changes and bacterial loads in the lungs of *Cpn*-infected mice. From this analysis we determined that mononuclear cells are the main inflammatory cell type during infection, peak inflammatory responses are stronger and emerge faster after reinfection than after primary infection, and fewer pulmonary bacteria are recovered from *Cpn*-reinfected mice than from infected animals. These results indicated that *Cpn* infection induces immunoprotective responses. Because *Cpn* $MOMP^+$ foamy mononuclear cells were seen adjacent to lymphoid cells composing the inflammatory foci, we postulate that these consist of macrophages priming pathogen-specific $CD8^+$ T cells. Indeed, LMNC from 12-day infected mice contained *Cpn*-specific $CD8^+$ T cells, and nearly one-fourth of the infiltrating $CD8^+$ T cells produced IFN-γ upon stimulation with *Cpn*-infected mAM. Furthermore, $CD8^+$ T cells play a key role in early and late resistance to *Cpn* infection (20, 21). Early in the infection, $CD8^+$ T cells are thought to contribute to protection by modifying the $CD4^+$ T cell cytokine pattern from a Th2 to a protective Th1 phenotype (20). We speculate that *Cpn*-specific IFN-γ-producing $CD8^+$ T cells contribute to the type 1 cytokine milieu required to control early *Cpn* growth in the lungs of infected B6 mice.

We next conducted a genome-wide search for sequences encoding potential target Ags of murine anti-*Cpn* $CD8^+$ T cells, selecting *Cpn* proteins that we believed were most likely to reach the cytosol of infected cells for processing and MHC class I presentation. These included membrane proteins specific to *Chlamydia* or with relatives in other organisms, chaperones, and Ags unique to *Cpn*, or with orthologs in other chlamydial species. Within the primary sequences of 16 *Cpn* Ags, 39 of 146 K^b- or D^b motif-fitting segments were synthesized as peptides and tested for the capacity to sensitize target cells for lysis by peptide-stimulated SC and LMNC from *Cpn*-infected mice. Remarkably, a specific, H-2^b-restricted, and $CD8^+$ T cell-dependent CTL response was detected for 18 peptides in 12 *Cpn* Ags. Of the 18 $CD8^+$ CTL epitope-bearing peptides, seven represent sequences within five outer membrane protein complex Ags, namely, Omp2, MOMP, OmpB, Omp85 homolog, and Omp5 (Pmp10) (33–35, 39, 43, 44). In the group of four chaperones, only a peptide from DnaK (41) elicited recall $CD8^+$ CTL responses. Interestingly, DnaK is associated with the outer membranes of *C. trachomatis* (49). Finally, in the group of *Cpn* Ags with or without chlamydial orthologs, eight CTL peptides represent sequences within a family of four 43-kDa protein homologs, one within a *C. trachomatis*-like 76-kDa protein and another within an inclusion membrane protein similar to *C. psittaci* IncA (33–35, 42). The 43- and 76-kDa proteins may also be surface exposed, as *Cpn* Ags of these molecular masses are recognized by human and rabbit immune sera, the 43-kDa primary sequences revealed potential transmembrane domains, and a specific anti-76-kDa antisera neutralizes *Cpn* infectivity in vitro (42, 50, 51).

Aside from macrophages, *Cpn* can infect and multiply in nonprofessional APC, including airway epithelial, endothelial, and smooth muscle cells (52, 53). However, macrophages and dendritic cells are the APC most likely responsible for priming a *Cpn*-specific $CD8^+$ CTL response. Because in addition to the endogenous MHC class I pathway, professional APC can process and present exogenous Ag to $CD8^+$ T cells (54), detected *Cpn*-specific CTL could have been primed by processed Ags from phagocytosed bacteria and not from chlamydial Ags accessing the cytosol of infected APC. Arguing against this possibility was the finding that all 18 peptide-specific $CD8^+$ CTL effectors killed mAM only when inoculated with live *Cpn* bacteria. Moreover, the fact that BFA inhibited this lytic activity strongly suggested that the 12 *Cpn* Ags access the endogenous MHC class I processing machinery. The cytotoxic mechanism that prevailed in the killing of *Cpn*-infected mAM was probably not Fas/Fas ligand dependent, as BFA inhibits this pathway in CTL effectors (55). BFA did not affect *Cpn* growth, a finding consistent with that described in a study in which the endogenous Ag processing pathway was shown to target *C. trachomatis*-infected fibroblasts for lysis by pathogen-specific $CD8^+$ CTL (11). In support of our findings, *C. trachomatis* MOMP and the inclusion membrane protein Cap1 are the targets of infection-primed $CD8^+$ CTL that kill *C. trachomatis*-infected nonprofessional APC (15, 16). Thus, although our results do not rule out the contribution of alternative pathways to the processing of *Cpn* Ags by professional APC, they imply that $CD8^+$ CTL primed by endogenously processed Ags are the most likely to recognize all *Cpn*-infected cells.

Despite their intravacuolar location, chlamydiae interact with multiple host cell processes to ensure that the inclusion is a safe niche for their survival and replication. These interactions are needed to acquire nutrients, avoid fusion with lysosomes, obtain membrane components from Golgi-derived exocytic vesicles, and modify host cell functions (56, 57). The chlamydial products that control these processes are thought to be proteins translocated through or inserted into the inclusion membrane via a type III secretion apparatus (58). However, of the 12 identified *Cpn* CTL Ags, none is known to be secreted into the host cell cytosol, and only one, the Ag similar to *C. psittaci* IncA, is likely to belong to the Inc family of inclusion membrane proteins. Identified Inc proteins have a unique 50- to 60-aa hydrophobic region and domains that localize to the cytoplasmic face of the inclusion (59–61). These exposed domains may be cleaved by proteases in the cytosol or on the membranes of interacting vesicles, and after proteasomal processing, Inc-derived CTL epitopes, such as peptide 39, may be generated. How do the *Cpn* CTL target Ags located or associated with the envelopes of developing chlamydiae become accessible to the endogenous MHC class I presentation pathway? As no report

has localized *Cpn* envelope Ags in the cytosol of infected cells, we speculate that these and other envelope Ags may reach the cytosol in a denatured or preprocessed form. Unfolded envelope Ags or fragments thereof may arise during the extensive membrane remodeling that occurs during chlamydial replication and differentiation, from the "ghost-like" membranous material present alongside organisms within a typical inclusion (47), or from a small fraction of developing chlamydiae undergoing autolysis.

There is little variation in the sequences of various genes from multiple *Cpn* isolates, and nearly identical sequences were reported for the genomes of *Cpn* strains CWL029 and AR39 from the United States and J138 from Japan (33–35). Our CTL data are in line with these findings, as all 18 peptide-specific $CD8^+$ T cell effectors were generated from mice infected with the Finnish K6 *Cpn* strain, and these CTL lysed mAM infected with *Cpn* strains K6, AR39, or CWL029. These results are encouraging, as the CTL Ags or epitopes included in vaccines against *Cpn* will require this level of sequence conservation among strains from different geographic locations. Moreover, because 80% of the predicted coding sequences for *Cpn* have an ortholog in *C. trachomatis*, and, on average, orthologs from the two species share 62% aa identity (33), some CTL epitopes may be conserved between orthologous chlamydial Ags. Indeed, the sequence of *Cpn* Omp2-derived peptide 3 is identical with its ortholog in *C. trachomatis*. In contrast, the six *Cpn* CTL peptides derived from the 76-kDa protein, Omp85 homolog, DnaK, OmpB, and MOMP differed by one to eight residues from the respective sequences in the orthologous *C. trachomatis* Ags. Nevertheless, these Ags could still be the targets of CTL responses during human or experimental *C. trachomatis* infections, as recently shown for *C. trachomatis* MOMP (16).

Lung-derived *Cpn* peptide-specific $CD8^+$ T cells lysed *Cpn*-infected cells, expressed a $CD44^{high}$ memory phenotype and produced Tc1 cytokines. IFN-γ is crucial for the control of chlamydial infections (21, 22, 62), and IFN-γ-producing $CD44^{high}$ $CD8^+$ CTL mediate protection in murine models of *M. tuberculosis* and *T. cruzi* infection (24, 63). Similarly, we found that peptide-specific $CD8^+$ T cells can inhibit *Cpn* inclusion development by producing soluble factors that limit *Cpn* growth and by direct effects of $CD8^+$ T cells themselves on the infectious titers produced by *Cpn*-infected macrophages. The capacity of supernatants to suppress *Cpn* growth correlated with the levels of IFN-γ. However, neutralization of IFN-γ only partially restored inclusion development, suggesting that other factors inhibit *Cpn* multiplication. *Cpn* peptide-specific $CD8^+$ T cells produce TNF-α, which may contribute to the suppressive effect, as it synergizes with IFN-γ in inhibiting *Cpn* growth (45). *Cpn* multiplication may also be influenced by other $CD8^+$ T cell-derived factors, including RANTES, macrophage inflammatory proteins-1α and -1β, and IL-16, which suppress HIV replication (64, 65). The anti-chlamydial activity of purified *Cpn* peptide-specific $CD8^+$ T cells was probably due to direct lysis of *Cpn*-infected mAM. Because most organisms released by lysed mAM were most likely in the form of noninfectious reticulate bodies, *Cpn* viability may have been affected by the release of molecules with antimicrobial activity, similar to the human $CD8^+$ T cell-derived granulysin (66). This possibility is being evaluated using *Cpn*-infected cells, where organisms have differentiated into infectious elementary bodies. Altogether these results suggest that *Cpn*-specific $CD8^+$ T cells may also inhibit *Cpn* growth in vivo through the combined actions of both effector functions. We are conducting adoptive transfer experiments to determine whether $CD8^+$ T cells to each of the identified *Cpn* CTL epitopes can reduce pulmonary chlamydial loads in *Cpn*-challenged mice and to define the mechanisms by which CTL protect against infection.

Although $CD8^+$ CTL might protect against *Cpn*, they may also be partially responsible for inducing persistent infection and immunopathology. IFN-γ may have a dual role in controlling the outcome of chlamydial infections in vivo, as exposure of infected cells to high IFN-γ concentrations irreversibly inhibits chlamydial replication, while lower concentrations induce the formation of persistent forms (45). Thus, production of IFN-γ by *Cpn*-specific $CD8^+$ T cells may be critical to achieve the local threshold concentration required to inhibit *Cpn* growth and prevent persistence. Persistent *Cpn* infection in humans and rodents (3, 19) may be due to a suboptimal $CD8^+$ T cell response during the course of an active *Cpn* infection. Nevertheless, we believe that an efficacious anti-*Cpn* $CD8^+$ CTL response can be induced through vaccination. Vaccines against *Cpn* will most likely include multiple determinants from various chlamydial Ags that induce $CD8^+$ CTL capable of eliminating productively and possibly persistently infected cells without causing serious tissue inflammation. In the current studies we provide direction for the development of such vaccines, as we have demonstrated the generation of *Cpn*-specific MHC class I-restricted $CD8^+$ CTL with anti-chlamydial growth activity in infected mice and have identified 12 CTL target molecules. Work is in progress to further delineate the dynamics of the murine $CD8^+$ T cell response to the *Cpn* CTL epitope-bearing sequences described in this study and to determine whether *Cpn*-infected humans also generate CTL responses against the Ags identified using the murine model. Such information is important to validate the use of the mouse model to provide insight into the aspects of the CTL response that contribute to protection and those that mediate immunopathology, and to identify and test CTL target Ags that may ultimately be used to develop vaccines against *Cpn*.

References

1. Grayston, J. T. 2000. Background and current knowledge of *Chlamydia pneumoniae* and atherosclerosis. *J. Infect. Dis. 181(Suppl. 3):S402.*
2. Kuo, C. C., L. A. Jackson, L. A. Campbell, and J. T. Grayston. 1995. *Chlamydia pneumoniae* (TWAR). *Clin. Microbiol. Rev. 8:451.*
3. Hammerschlag, M. R., K. Chirgwin, P. M. Roblin, M. Gelling, W. Dumornay, L. Mandel, P. Smith, and J. Schachter. 1992. Persistent infection with *Chlamydia pneumoniae* following acute respiratory illness. *Clin. Infect. Dis. 14:178.*
4. Hahn, D. L., R. W. Dodge, and R. Golubjatnikov. 1991. Association of *Chlamydia pneumoniae* (strain TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma. *JAMA 266:225.*
5. Von Hertzen, L., H. Alakarppa, R. Koskinen, K. Liippo, H. M. Surcel, M. Leinonen, and P. Saikku. 1997. *Chlamydia pneumoniae* infection in patients with chronic obstructive pulmonary disease. *Epidemiol. Infect. 118:155.*
6. Sriram, S., C. W. Stratton, S. Yao, A. Tharp, L. Ding, J. D. Bannan, and W. M. Mitchell. 1999. *Chlamydia pneumoniae* infection of the central nervous system in multiple sclerosis. *Ann. Neurol. 46:6.*
7. Saikku, P., M. Leinonen, K. Mattila, M. R. Ekman, M. S. Nieminen, P. H. Makela, J. K. Huttunen, and V. Valtonen. 1988. Serological evidence of an association of a novel *Chlamydia*, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet 2:983.*
8. Elkind, M. S., I. F. Lin, J. T. Grayston, and R. L. Sacco. 2000. *Chlamydia pneumoniae* and the risk of first ischemic stroke: The Northern Manhattan Stroke Study. *Stroke 31:1521.*
9. Gieffers, J., H. Fullgraf, J. Jahn, M. Klinger, K. Dalhoff, H. A. Katus, W. Solbach, and M. Maass. 2001. *Chlamydia pneumoniae* infection in circulating human monocytes is refractory to antibiotic treatment. *Circulation 103:351.*
10. Lalvani, A., R. Brookes, R. J. Wilkinson, A. S. Malin, A. A. Pathan, P. Andersen, H. Dockrell, G. Pasvol, and A. V. Hill. 1998. Human cytolytic and interferon γ-secreting $CD8^+$ T lymphocytes specific for *Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. USA 95:270.*
11. Beatty, P. R., and R. S. Stephens. 1994. $CD8^+$ T lymphocyte-mediated lysis of *Chlamydia*-infected L cells using an endogenous antigen pathway. *J. Immunol. 153:4588.*
12. Starnbach, M. N., M. J. Bevan, and M. F. Lampe. 1994. Protective cytotoxic T lymphocytes are induced during murine infection with *Chlamydia trachomatis. J. Immunol. 153:5183.*
13. Buzoni-Gatel, D., L. Guilloteau, F. Bernard, S. Bernard, T. Chardes, and A. Rocca. 1992. Protection against *Chlamydia psittaci* in mice conferred by Lyt-2^+ T cells. *Immunology 77:284.*

14. Igietseme, J. U., D. M. Magee, D. M. Williams, and R. G. Rank. 1994. Role for $CD8^+$ T cells in antichlamydial immunity defined by *Chlamydia*-specific T-lymphocyte clones. *Infect. Immun. 62:5195.*
15. Fling, S. P., R. A. Sutherland, L. N. Steele, B. Hess, S. E. D'Orazio, J. Maisonneuve, M. F. Lampe, P. Probst, and M. N. Starnbach. 2001. $CD8^+$ T cells recognize an inclusion membrane-associated protein from the vacuolar pathogen *Chlamydia trachomatis. Proc. Natl. Acad. Sci. USA 98:1160.*
16. Kim, S. K., M. Angevine, K. Demick, L. Ortiz, R. Rudersdorf, D. Watkins, and R. DeMars. 1999. Induction of HLA class I-restricted $CD8^+$ CTLs specific for the major outer membrane protein of *Chlamydia trachomatis* in human genital tract infections. *J. Immunol. 162:6855.*
17. Yang, Z. P., C. C. Kuo, and J. T. Grayston. 1993. A mouse model of *Chlamydia pneumoniae* strain TWAR pneumonitis. *Infect. Immun. 61:2037.*
18. Penttila, J. M., M. Anttila, M. Puolakkainen, A. Laurila, K. Varkila, M. Sarvas, P. H. Makela, and N. Rautonen. 1998. Local immune responses to *Chlamydia pneumoniae* in the lungs of BALB/c mice during primary infection and reinfection. *Infect. Immun. 66:5113.*
19. Malinverni, R., C. C. Kuo, L. A. Campbell, and J. T. Grayston. 1995. Reactivation of *Chlamydia pneumoniae* lung infection in mice by cortisone. *J. Infect. Dis. 172:593.*
20. Penttila, J. M., M. Anttila, K. Varkila, M. Puolakkainen, M. Sarvas, P. H. Makela, and N. Rautonen. 1999. Depletion of $CD8^+$ cells abolishes memory in acquired immunity against *Chlamydia pneumoniae* in BALB/c mice. *Immunology 97:490.*
21. Rottenberg, M. E., A. C. Gigliotti Rothfuchs, D. Gigliotti, C. Svanholm, L. Bandholtz, and H. Wigzell. 1999. Role of innate and adaptive immunity in the outcome of primary infection with *Chlamydia pneumoniae*, as analyzed in genetically modified mice. *J. Immunol. 162:2829.*
22. Rottenberg, M. E., A. Gigliotti Rothfuchs, D. Gigliotti, M. Ceausu, C. Une, V. Levitsky, and H. Wigzell. 2000. Regulation and role of IFN-γ in the innate resistance to infection with *Chlamydia pneumoniae*. *J. Immunol. 164:4812.*
23. Kuo, C. C., and J. T. Grayston. 1990. A sensitive cell line, HL cells, for isolation and propagation of *Chlamydia pneumoniae* strain TWAR. *J. Infect. Dis. 162:755.*
24. Wizel, B., M. Nunes, and R. Tarleton. 1997. Identification of *Trypanosoma cruzi* trans-sialidase family members as targets of protective $CD8^+$ TC1 responses. *J. Immunol. 159:6120.*
25. Ljunggren, H. G., and K. Karre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants: analysis of the mechanism. *J. Exp. Med. 162:1745.*
26. Alexander, J., J. A. Payne, R. Murray, J. A. Frelinger, and P. Cresswell. 1989. Differential transport requirements of HLA and H-2 class I glycoproteins. *Immunogenetics 29:380.*
27. Puolakkainen, M., J. Parker, C. C. Kuo, J. T. Grayston, and L. A. Campbell. 1995. Further characterization of *Chlamydia pneumoniae* specific monoclonal antibodies. *Microbiol. Immunol. 39:551.*
28. Wolf, K., E. Fischer, D. Mead, G. Zhong, R. Peeling, B. Whitmire, and H. D. Caldwell. 2001. *Chlamydia pneumoniae* major outer membrane protein is a surface-exposed antigen that elicits antibodies primarily directed against conformation-dependent determinants. *Infect. Immun. 69:3082.*
29. Rotzschke, O., K. Falk, S. Stevanovic, G. Jung, P. Walden, and H. G. Rammensee. 1991. Exact prediction of a natural T cell epitope. *Eur. J. Immunol. 21:2891.*
30. Townsend, A. R., J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell 44:959.*
31. Ljunggren, H. G., N. J. Stam, C. Ohlen, J. J. Neefjes, P. Hoglund, M. T. Heemels, J. Bastin, T. N. Schumacher, A. Townsend, and K. Karre. 1990. Empty MHC class I molecules come out in the cold. *Nature 346:476.*
32. Vitiello, A., L. Yuan, R. W. Chesnut, J. Sidney, S. Southwood, P. Farness, M. R. Jackson, P. A. Peterson, and A. Sette. 1996. Immunodominance analysis of CTL responses to influenza PR8 virus reveals two new dominant and subdominant K^b-restricted epitopes. *J. Immunol. 157:5555.*
33. Kalman, S., W. Mitchell, R. Marathe, C. Lammel, J. Fan, R. W. Hyman, L. Olinger, J. Grimwood, R. W. Davis, and R. S. Stephens. 1999. Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis. Nat. Genet. 21:385.*
34. Read, T. D., R. C. Brunham, C. Shen, S. R. Gill, J. F. Heidelberg, O. White, E. K. Hickey, J. Peterson, T. Utterback, K. Berry, et al. 2000. Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Res. 28:1397.*
35. Shirai, M., H. Hirakawa, M. Kimoto, M. Tabuchi, F. Kishi, K. Ouchi, T. Shiba, K. Ishii, M. Hattori, S. Kuhara, et al. 2000. Comparison of whole genome sequences of *Chlamydia pneumoniae* J138 from Japan and CWL029 from USA. *Nucleic Acids Res. 28:2311.*
36. Falk, K., O. Rotzschke, S. Stevanovic, G. Jung, and H. G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature 351:290.*
37. Sette, A., A. Vitiello, B. Reherman, P. Fowler, R. Nayersina, W. M. Kast, C. J. Melief, C. Oseroff, L. Yuan, and J. Ruppert. 1994. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J. Immunol. 153:5586.*
38. Wentworth, P. A., A. Vitiello, J. Sidney, E. Keogh, R. W. Chesnut, H. Grey, and A. Sette. 1996. Differences and similarities in the A2.1-restricted cytotoxic T cell repertoire in humans and human leukocyte antigen-transgenic mice. *Eur. J. Immunol. 26:97.*
39. Watson, M. W., S. al-Mahdawi, P. R. Lamden, and I. N. Clarke. 1990. The nucleotide sequence of the 60 kDa cysteine rich outer membrane protein of *Chlamydia pneumoniae* strain IOL-207. *Nucleic Acids Res. 18:5299.*
40. Kikuta, L. C., M. Puolakkainen, C. C. Kuo, and L. A. Campbell. 1991. Isolation and sequence analysis of the *Chlamydia pneumoniae* GroE operon. *Infect. Immun. 59:4665.*
41. Kornak, J. M., C. C. Kuo, and L. A. Campbell. 1991. Sequence analysis of the gene encoding the *Chlamydia pneumoniae* DnaK protein homolog. *Infect. Immun. 59:721.*
42. Perez Melgosa, M., C. C. Kuo, and L. A. Campbell. 1994. Isolation and characterization of a gene encoding a *Chlamydia* pneumoniae 76-kDa protein containing a species-specific epitope. *Infect. Immun. 62:880.*
43. Perez Melgosa, M., C. C. Kuo, and L. A. Campbell. 1991. Sequence analysis of the major outer membrane protein gene of *Chlamydia pneumoniae*. *Infect. Immun. 59:2195.*
44. Knudsen, K., A. S. Madsen, P. Mygind, G. Christiansen, and S. Birkelund. 1999. Identification of two novel genes encoding 97- to 99-kDa outer membrane proteins of *Chlamydia pneumoniae*. *Infect. Immun. 67:375.*
45. Summersgill, J. T., N. N. Sahney, C. A. Gaydos, T. C. Quinn, and J. A. Ramirez. 1995. Inhibition of *Chlamydia pneumoniae* growth in HEp-2 cells pretreated with γ interferon and tumor necrosis factor α. *Infect. Immun. 63:2801.*
46. Beatty, W. L., R. P. Morrison, and G. I. Byrne. 1994. Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. *Microbiol. Rev. 58:686.*
47. Kutlin, A., C. Flegg, D. Stenzel, T. Reznik, P. M. Roblin, S. Mathews, P. Timms, and M. R. Hammerschlag. 2001. Ultrastructural study of *Chlamydia pneumoniae* in a continuous-infection model. *J. Clin. Microbiol. 39:3721.*
48. Moazed, T. C., C. C. Kuo, J. T. Grayston, and L. A. Campbell. 1998. Evidence of systemic dissemination of *Chlamydia pneumoniae* via macrophages in the mouse. *J. Infect. Dis. 177:1322.*
49. Raulston, J. E., C. H. Davis, D. H. Schmiel, M. W. Morgan, and P. B. Wyrick. 1993. Molecular characterization and outer membrane association of a *Chlamydia trachomatis* protein related to the hsp70 family of proteins. *J. Biol. Chem. 268:23139.*
50. Iijima, Y., N. Miyashita, T. Kishimoto, Y. Kanamoto, R. Soejima, and A. Matsumoto. 1994. Characterization of *Chlamydia pneumoniae* species-specific proteins immunodominant in humans. *J. Clin. Microbiol. 32:583.*
51. Puolakkainen, M., C. C. Kuo, A. Shor, S. P. Wang, J. T. Grayston, and L. A. Campbell. 1993. Serological response to *Chlamydia pneumoniae* in adults with coronary arterial fatty streaks and fibrolipid plaques. *J. Clin. Microbiol. 31:2212.*
52. Gaydos, C. A., J. T. Summersgill, N. N. Sahney, J. A. Ramirez, and T. C. Quinn. 1996. Replication of *Chlamydia pneumoniae* in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect. Immun. 64:1614.*
53. Jahn, H. U., M. Krull, F. N. Wuppermann, A. C. Klucken, S. Rosseau, J. Seybold, J. H. Hegemann, C. A. Jantos, and N. Suttorp. 2000. Infection and activation of airway epithelial cells by *Chlamydia pneumoniae*. *J. Infect. Dis. 182:1678.*
54. Jondal, M., R. Schirmbeck, and J. Reimann. 1996. MHC class I-restricted CTL responses to exogenous antigens. *Immunity 5:295.*
55. Kataoka, T., N. Shinohara, H. Takayama, K. Takaku, S. Kondo, S. Yonehara, and K. Nagai. 1996. Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. *J. Immunol. 156:3678.*
56. Al-Younes, H. M., T. Rudel, and T. F. Meyer. 1999. Characterization and intracellular trafficking pattern of vacuoles containing *Chlamydia pneumoniae* in human epithelial cells. *Cell. Microbiol. 1:237.*
57. Wolf, K., and T. Hackstadt. 2001. Sphingomyelin trafficking in *Chlamydia pneumoniae*-infected cells. *Cell. Microbiol. 3:145.*
58. Hsia, R. C., Y. Pannekoek, E. Ingerowski, and P. M. Bavoil. 1997. Type III secretion genes identify a putative virulence locus of *Chlamydia*. *Mol. Microbiol. 25:351.*
59. Rockey, D. D., D. Grosenbach, D. E. Hruby, M. G. Peacock, R. A. Heinzen, and T. Hackstadt. 1997. *Chlamydia psittaci* IncA is phosphorylated by the host cell and is exposed on the cytoplasmic face of the developing inclusion. *Mol. Microbiol. 24:217.*
60. Scidmore-Carlson, M. A., E. I. Shaw, C. A. Dooley, E. R. Fischer, and T. Hackstadt. 1999. Identification and characterization of a *Chlamydia trachomatis* early operon encoding four novel inclusion membrane proteins. *Mol. Microbiol. 33:753.*
61. Bannantine, J. P., R. S. Griffiths, W. Viratyosin, W. J. Brown, and D. D. Rockey. 2000. A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane. *Cell. Microbiol. 2:35.*
62. Lampe, M. F., C. B. Wilson, M. J. Bevan, and M. N. Starnbach. 1998. γ interferon production by cytotoxic T lymphocytes is required for resolution of *Chlamydia trachomatis* infection. *Infect. Immun. 66:5457.*
63. Bonato, V. L., V. M. Lima, R. E. Tascon, D. B. Lowrie, and C. L. Silva. 1998. Identification and characterization of protective T cells in hsp65 DNA-vaccinated and *Mycobacterium tuberculosis*-infected mice. *Infect. Immun. 66:169.*
64. Cocchi, F., A. L. DeVico, A. Garzino-Demo, S. K. Arya, R. C. Gallo, and P. Lusso. 1995. Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by $CD8^+$ T cells. *Science 270:1811.*
65. Baier, M., A. Werner, N. Bannert, K. Metzner, and R. Kurth. 1995. HIV suppression by interleukin-16. *Nature 378:563.*
66. Stenger, S., D. A. Hanson, R. Teitelbaum, P. Dewan, K. R. Niazi, C. J. Froelich, T. Ganz, S. Thoma-Uszynski, A. Melian, C. Bogdan, et al. 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science 282:121.*