An Inclusion Membrane Protein from *Chlamydia trachomatis* Enters the MHC Class I Pathway and Stimulates a CD8$^+$ T Cell Response[1]

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During its developmental cycle, the intracellular bacterial pathogen *Chlamydia trachomatis* remains confined within a protective vacuole known as an inclusion. Nevertheless, CD8$^+$ T cells that recognize *Chlamydia* Ags in the context of MHC class I molecules are primed during infection. MHC class I-restricted presentation of these Ags suggests that these proteins or domains from them have access to the host cell cytoplasm. *Chlamydia* products with access to the host cell cytoplasm define a subset of molecules uniquely positioned to interface with the intracellular environment during the pathogen's developmental cycle. In addition to their use as candidate Ags for stimulating CD8$^+$ T cells, these proteins represent novel candidates for therapeutic intervention of infection. In this study, we use *C. trachomatis*-specific murine T cells and an expression-cloning strategy to show that CT442 from *Chlamydia* is targeted by CD8$^+$ T cells. CT442, also known as CrpA, is a 15-kDa protein of undefined function that has previously been shown to be associated with the *Chlamydia* inclusion membrane. We show that: 1) CD8$^+$ T cells specific for an H-2D^b-restricted epitope from CrpA are elicited at a significant level (~4% of splenic CD8$^+$ T cells) in mice in response to infection; 2) the response to this epitope correlates with clearance of the organism from infected mice; and 3) immunization with recombinant vaccinia virus expressing CrpA elicits partial protective immunity to subsequent i.v. challenge with *C. trachomatis*. *The Journal of Immunology,* 2003, 171: 4742–4749.

Chlamydia trachomatis is responsible for several medically important diseases of humans (1), including ocular infections that lead to blindness, genital infections that lead to pelvic inflammatory disease and infertility, and infections of the lymphatic system that lead to lymphogranuloma venereum. *Chlamydia* infections may also contribute to increased risk of secondary infection with other pathogens including HIV (2, 3). It is estimated that the incidence rate of new cases of *Chlamydia* infections in the U.S. in the year 2000 was ~400 cases per 100,000 women and ~90 per 100,000 men (4).

Chlamydiae are obligate intracellular bacteria that differentiate from metabolically inactive, infectious elementary bodies to metabolically active, replicating reticulate bodies while remaining sequestered within a membrane-bound vacuole known as an inclusion. Following several rounds of replication, replicating reticulate bodies differentiate back again into infectious elementary bodies, which are released upon host cell lysis to begin the cycle again. At a molecular level, the inclusion membrane (Inc)[3] defines the critical interface between *Chlamydia* and the host cell. It is thought that pathogen-derived products are transported through this membrane, where they combine with host cell factors necessary for bacterial development (5). Despite the complete genomic sequencing of *C. trachomatis*, the identity and function of most *Chlamydia* gene products that interface with the host cell remain a mystery. Characterizing proteins that play an important role in development may provide avenues for intervention in the infectious cycle of this medically significant microorganism. Furthermore, pathogen-derived factors that require access to the host cytosol for their function are intriguing as vaccine candidates, as these proteins most likely serve as targets for MHC class I-restricted presentation to CD8$^+$ T cells.

Adaptive immune protection against *Chlamydia* has been clearly demonstrated and appears to involve both cellular and humoral responses (6, 7). Several recent reports have focused on the protective effects mediated by CD4$^+$ (8–10) and CD8$^+$ T cells (11, 12) during infection. Although the contribution of CD8$^+$ T cells to immunity following natural infection remains unclear, both CD4$^+$ and CD8$^+$ T cell lines specific for *Chlamydia* have been cultured from infected mice, and adoptive transfer of these lines into naive mice can afford protection against challenge with the pathogen (8–12). Therefore, it has been suggested that vaccine strategies designed to stimulate both T cell subsets may be required for optimal protection against infection.

Historically, studies to define the role of CD8$^+$ T cells in both natural immunity and following vaccination have been hampered by the paucity of known CD8$^+$ T cell Ags and difficulties in Ag delivery. Intuitively, CD8$^+$ T cells could play a significant role in protection against this intracellular bacterium, as they do against other intracellular pathogens. The response of CD8$^+$ T cells during natural infection suggests that there are stages in the developmental cycle in which a subset of *C. trachomatis* products accesses the

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Received for publication May 15, 2003. Accepted for publication August 21, 2003.

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[1] This work was supported by Grant AI10628 to W.P.L. and Grants AI31448 and AI39558 to M.N.S.

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[3] Abbreviations used in this paper: Inc, inclusion membrane; IFU, inclusion-forming unit; ORF, open reading frame.

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0022-1767/03/$02.00

cytosol of infected cells and encounters the MHC class I-processing machinery. We have previously used protective $CD8^+$ T cells to identify CT529, or Cap1, as one *C. trachomatis* Ag presented by MHC class I on infected cells (13). Vaccination of mice to specifically induce $CD8^+$ T cells against Cap1 appears to provide some measure of protective immunity. Although the function of this protein remains undetermined, we found that Cap1 associates with the Inc, even though it does not have targeting motifs predictive of other Inc proteins (14–16).

We report in this work the use of an expression-cloning system to identify a second $CD8^+$ T cell Ag, CT442 or CrpA, from *C. trachomatis*. Like Cap1, the function of CrpA is unknown, although it originally was described as a cysteine-rich protein thought to associate with the outer membrane. Recently, CrpA has been shown to associate with the Inc and contains a motif typical of other Inc proteins (14).

We also define an H-$2D^b$-restricted epitope from this Ag and show that during murine infection with *C. trachomatis*, ~4% of splenic $CD8^+$ T cells are specific for this D^b-restricted epitope. The response to this epitope is accompanied by a decrease in the number of organisms in the infected mice. In addition, immunization with recombinant vaccinia virus expressing CrpA elicits partial protective immunity against subsequent challenge with *C. trachomatis*.

Materials and Methods

Mice

Female C57BL/6 mice (H-2^b) and CByB6F_1 mice (H-$2^{b/d}$) were obtained from The Jackson Laboratory (Bar Harbor, ME) and used at 7–12 wk of age.

Tissue culture and cell lines

The SV40-transformed, H-D^b murine fibroblast cell line, K41 (17, 18) (provided by M. Michalek (University of Alberta, Edmonton, Alberta, Canada)), was maintained in DMEM supplemented with 10% FCS and antibiotics. All other cells were maintained in RP-10, consisting of RPMI 1640 supplemented with L-glutamine (Invitrogen, San Diego, CA), 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO), antibiotics (penicillin/streptomycin, Sigma-Aldrich; gentamicin sulfate, Irvine Scientific, Santa Ana, CA, except where noted), and 10% FCS (Invitrogen). The H-2^b cell line 1308.1 was derived from thymus epithelium (19). EL4 (H-2^b) and P815 (H-2^d) cell lines were used as stimulator cells in ELISPOT assays. H-$2D^b$ and H-$2K^b$ transfectants of P815 and of L929 cells were gifts of M. Bevan (University of Washington, Seattle, WA).

Growth, isolation, and detection of C. trachomatis

Elementary bodies of *C. trachomatis* serovar L2 434/Bu were propagated within human epithelial cell (HeLa 229 or McCoy) monolayers in RP-10 and purified by density-gradient centrifugation, as described (20). Aliquots of *C. trachomatis* were stored at −70°C in a buffer containing sucrose, phosphate, and glutamate (21).

To quantitate *C. trachomatis*, spleens from infected mice were homogenized; sonicated; diluted in sucrose, phosphate, and glutamate; and applied to McCoy cell monolayers. Inclusions were counted by immunofluorescent microscopy 36 h after infection and reported as inclusion-forming units (IFU) per spleen. Groups of five mice were used for all experimental and control groups for the challenge experiments.

Construction of a C. trachomatis *DNA expression library*

To allow eukaryotic expression of bacterial polypeptides, we cloned random fragments of *C. trachomatis* L2 chromosomal DNA into pcDNA4 HisMax (Invitrogen). The pcDNA4 HisMax A,B,C set of vectors allows expression of DNA in each reading frame by staggered insertion of DNA fragments downstream of a QBI SP163 translational enhancer. *C. trachomatis* L2 genomic DNA was prepared from elementary bodies, essentially as described previously (13). Briefly, DNA was purified by phenol/chloroform extraction of 5×10^9 IFU *Chlamydia* elementary bodies previously treated with proteinase K. For expression library construction, 2 μg of purified DNA was fragmented by incomplete restriction enzyme digestion using *Sau*3A1 to generate *Bam*HI-compatible ends. The resulting DNA fragments were ligated into the unique *Bam*HI site of pcDNA4 HisMax and transformed into ElectoMax DH10B *Escherichia coli* (Invitrogen) by electroporation. Characterization of a subset of clones revealed an insert frequency of >92%. The insert range was 0.1–3.0 kb, and the average insert size was 700 bp (data not shown). Approximately 81,000 independent clones (representing ~10-fold coverage of the genome) were arrayed in pools of ~70 clones/pool in twelve 96-well culture plates containing Luria-Bertani medium (with 50 μg/ml carbenicillin) and grown for 18 h at 37°C. Plasmid DNA for transfections was prepared from these cultures using Qiagen 96 Turbo-Prep plates (Qiagen, Valencia, CA).

Library screening

For transfections, plasmid DNA from each pool was prepared by mixing 100 ng of DNA with OptiMem (Life Technologies, Carlsbad, CA) and 1 μl of the transfection reagent Fugene-6 (Roche, Basel, Switzerland). These preparations were used to transfect individual wells of K41 cells, an SV40-transformed H-2^b fibroblast line that had been seeded at 5000 cells/well in a 96-well flat-bottom plate 24 h before transfection. We have previously observed that the K41 cell line allows efficient episomal amplification of vectors containing the SV40 origin of replication such as pcDNA4HisMax (22). Two hours after transfection, the wells were supplemented with medium. The efficiencies of transfections were estimated using flow cytometry to detect expression of a reporter gene in cells transfected with control plasmid. Two days later, 10^4 T cells/well were added to the transfected K41 cells. After incubating the cultures for 8 and 24 h at 37°C, supernatants from the cultures were collected and assayed for IFN-γ by ELISA.

ELISA

Standard IFN-γ ELISA protocols were used for detection of T cell activation in library screening. Briefly, plates were coated with 50 μl/well of rat anti-mouse IFN-γ (554431; BD Biosciences, San Jose, CA) diluted to 1 μg/ml in 0.1 M bicarbonate buffer, pH 9.6. Plates were blocked with PBS buffer containing 0.05% Tween and 1% BSA and then washed in PBS-Tween. Samples (undiluted) and standards (diluted in PBS with 0.05% Tween and 0.1% BSA) were added at 50 μl/well. Following incubation, plates were washed and then incubated again with 100 μl/well of biotinylated rat anti-mouse IFN-γ (BD Biosciences 554410) at 0.5 μg/ml. Following incubation, plates were again washed and then incubated with streptavidin-HRP conjugate (Zymed, San Francisco, CA) diluted 1/2500 in PBS with 0.05% Tween and 0.1% BSA. Following incubation, plates were washed and activity detected by adding 100 μl/well tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Reactions were stopped with 50 μl of 1 N sulfuric acid, and quantification was performed by spectrophotometric measurement of absorbance at 450 and 570 nm.

Stimulation and maintenance of C. trachomatis*-specific $CD8^+$ T cells*

C. trachomatis-specific $CD8^+$ T cell line 102 was cultured from a C57BL/6 mouse (H-2^b) infected i.p. with 10^7 IFU *C. trachomatis* L2. This T cell line was maintained and expanded by weekly stimulation on *C. trachomatis*-infected 1308.1 (H-2^b) cells, essentially as described for other similar lines (11).

Stimulation of CrpA-specific T cells from infected mice was conducted as follows. Briefly, C57BL/6 mice were infected i.v. with 10^7 IFU of *C. trachomatis* serovar L2. After 14 days, splenocytes from infected mice were washed once in RP-10 and then cultured with naive, irradiated (2000 rad), syngeneic splenocytes treated for 1 h with 10 μM sterile $CrpA_{63-71}$ peptide. Cultures containing 3×10^6 stimulator cells and 3×10^7 splenocytes (from immunized mice) were incubated upright for 5 days in a T-25 flask at 37°C in 7% CO_2 in a total volume of 20 ml of RP-10.

Cytotoxicity assays

Standard chromium release assays were used to determine the percentage of specific lysis of peptide-pulsed target cells, as previously described (11).

DNA sequencing

Sequencing of cloned plasmid DNA derived from immunoreactive clones was done on an automated sequencer (ABI 377) using sequence-specific forward primers 5′-TAATACGACTCACTATAGGG-3′ and 5′-ATGACTGGTGGACAGCAAATG-3′, and reverse primers 5′-TAGAAGGCACAGTCGAGG-3′ and 5′-CAGGAAACAGCTATGAC-3′.

Peptides

Peptides used in this study were synthesized using a Rainin Symphony system, according to the guidelines of the manufacturer. Each peptide was purified by HPLC to >95% before use.

Generation of recombinant vaccinia virus

A DNA fragment containing the entire crp*A* gene without the stop codon was amplified from *C. trachomatis* L2 genomic DNA using 5′ and 3′ primers that included terminal *Stu*I restriction sites. The product was digested with *Stu*I and ligated into plasmid pAS1008 that also had been digested with *Stu*I. Plasmid pAS1008 is a modified version of pSC11ss (23) that allows fragments to be cloned into an engineered *Stu*I site. This vector contains stop codons in three frames downstream of the cloning site. The resulting plasmid, pDR1505, was used to generate recombinant vaccinia virus Vac1505 using the protocol described previously (13). Virus preparations were treated with an equal volume of 0.25 mg/ml trypsin for 30 min at 37°C and diluted in PBS before immunization of mice.

D^b tetramer staining

For tetramer staining and quantitation, cells were stained with CD4 CyChrome (BD Biosciences), B220 CyChrome (BD Biosciences), CD8 APC (BD Biosciences), and CD44 FITC (BD Biosciences) as an activation marker, or PE-labeled D^b/$CrpA_{63-71}$ tetramer (for Ag specificity). As a negative control, cells were stained with D^b/$Mtb32_{309-318}$ tetramer. $Mtb32_{309-318}$ is a D^b-restricted peptide (GAPINSATAM) from MT0133, a *Mycobacterium tuberculosis* Ag that elicits a strong $CD8^+$ T cell response in B6 mice (unpublished observations, M. Alderson). Cells were analyzed using a BD Biosciences FACScan flow cytometer. For quantitation, $CD4^-B220^-CD8^+$ cells were gated and those cells then plotted for specific tetramer binding vs CD44 FITC signal. D^b tetramers were prepared, essentially as described by others (24).

ELISPOT analysis

$CByB6F_1$ mice were infected i.v. with 10^7 IFU of *C. trachomatis* L2. At various days postimmunization, the number of $CrpA_{63-71}$-specific and $Cap1_{139-147}$-specific spleen cells was determined using an IFN-γ ELISPOT assay modified from that described previously (25). Briefly, nitrocellulose-backed 96-well plates were coated overnight with 10 μg/ml rat anti-mouse IFN-γ Ab (clone R4-6A2; BD Biosciences) and then washed with PBS + 0.25% Tween 20 (PBS-T) and blocked with PBS + 5% FBS.

Spleens from *C. trachomatis*-immune mice (three per group) were harvested at various times after infection and homogenized in medium. A fraction of the spleen homogenate was frozen and later used to determine the number of *C. trachomatis* IFU per spleen. The remainder was transferred into RP-10 medium, pelleted, resuspended in RBC lysis buffer, and then washed with RP-10. Five-fold dilutions of spleen cells (from 5×10^5 to 2×10^4 cells/50 μl) were added to ELISPOT wells (in triplicate) containing 1×10^5 cells of one of the following stimulator cell types: P815 cells, P815 cells coated with $Cap1_{139-147}$ peptide, EL4 cells, or EL4 cells coated with $CrpA_{63-71}$ peptide and coincubated for 24 h at 37°C. The stimulator cells had been coated with 1 μM peptide for 1 h, then washed with RP-10 and irradiated (1.4×10^4 rad).

After incubation, plates were washed with PBS-T, then incubated with a biotinylated rat anti-mouse IFN-γ Ab (clone XMG1.2; BD Biosciences) diluted to 5 μg/ml, followed by incubation with streptavidin-HRP (BD Biosciences) diluted 1/400. The ELISPOT plate was developed using hydrogen peroxide and 1 mg/ml diaminobenzidine (Bio-Rad, Hercules, CA). Spots resulting from IFN-γ-secreting cells were counted in each well, and, using the dilution factor in each well, the number of IFN-γ-secreting cells per spleen was calculated.

ELISPOT analysis of CrpA-specific T cells from animals immunized with recombinant vaccinia virus expressing CrpA was conducted as described for the *C. trachomatis*-infected animals.

Results

$CD8^+$ T cell line 102 is C. trachomatis *specific and H-$2D^b$ restricted*

The *C. trachomatis*-specific $CD8^+$ T cell line 102 was cultured from a C57BL/6 mouse (H-2^b) infected with *C. trachomatis* L2 and is >95% $CD8^+$ (data not shown). As shown in Fig. 1, line 102 is specific for *C. trachomatis* and is MHC class I restricted. To determine the actual MHC restriction element for line 102, we tested this line for recognition of *C. trachomatis*-infected L929 cells that were stably transfected with cDNA encoding either D^b or K^b. L929 cells transfected with the D^b gene and infected with *C. trachomatis* stimulated T cell line 102 to secrete IFN-γ, while infected L929 cells transfected with the K^b gene did not stimulate the line (Fig. 1). Uninfected L929 cells that were transfected with either D^b or K^b genes did not stimulate the line (Fig. 1). We have also shown that modest levels (~3-fold reduction in IFU in spleens) of protection against *C. trachomatis* challenge are conferred to mice receiving line 102 by adoptive transfer (data not shown).

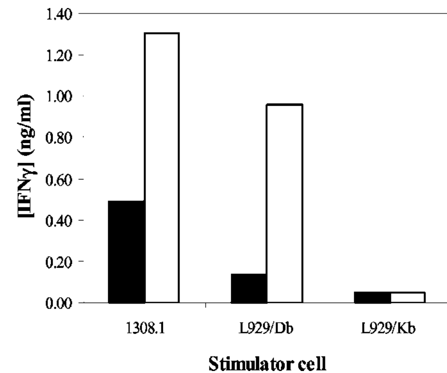

FIGURE 1. The *C. trachomatis* $CD8^+$ T cell line 102 is H-$2D^b$ restricted. The 1308.1 cells (H-2^b) or L929 cells (H-2^k) transfected with either the D^b or K^b gene were infected with *C. trachomatis* L2 (open bars) or left uninfected (filled bars), and were incubated with *Chlamydia*-specific $CD8^+$ T cell line 102. Following 48 h of incubation, the culture supernatants were assayed by ELISA for IFN-γ.

Identification of a Chlamydia*-specific $CD8^+$ T cell Ag*

To identify the Ag(s) recognized by the B6-derived T cell line, we undertook a transfection-based expression-cloning strategy, using for library transfections the syngeneic cell line, K41 (17, 18). These SV40-transformed cells were used based upon our determination that line 102 T cells recognize *Chlamydia*-infected K41 cells (see Fig. 3*A*) and our previous experience with these transformed cells in expression-cloning strategies (22). To determine the specificity of T cell line 102, we transfected the H-2^b cell line K41 with 4.8×10^4 independent clones of the *Chlamydia* expression library and screened these transfectants to identify genomic DNA fragments encoding polypeptides able to stimulate line 102. This screen represented ~5.6-fold coverage of the *C. trachomatis* genome. The initial screen identified six pools of clones that stimulated IFN-γ secretion by line 102. This frequency of positive pools is consistent with our estimated frequency of independent DNA fragments within the library. To identify clones expressing the individual Ags contained within the pools, cells were independently transfected with plasmid DNA prepared from 96 individual clones taken from each of the original positive pools. These transfectants were then screened using the same method as used for the pools. Inserts contained within plasmids from individual clones that stimulated line 102 were sequenced, and the DNA sequences were searched by BLAST analysis of available databases. Two clones (2A7.H7 and clone 5B4.B5) contained an identical restriction fragment of genomic DNA corresponding to a 411-bp portion of the predicted open reading frame (ORF) designated CT442 in the published genome sequence of *C. trachomatis* (26). This same 411-bp *Sau*IIIA1 fragment was also found within each of four other independent clones that stimulated line 102, but that contained multiple *C. trachomatis* genomic DNA fragments. CT442 corresponds to a protein previously designated CrpA (27, 28). Based on these observations, we hypothesized that the portion of

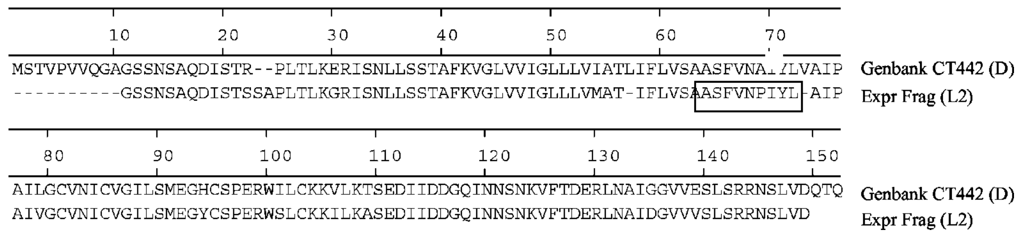

FIGURE 2. CrpA (CT442) polypeptide sequence identified by expression cloning. Alignment of the polypeptide sequence from *C. trachomatis* L2 predicted from the common 411-bp fragment of the CT442 ORF found in each of the clones recognized by line 102 (Expr Frag (L2)) with the published sequence of full-length CT442 from serovar D (GenBank CT442 (D)) (26). Boxed residues show the position within this sequence of the peptide from the CT442 ORF with highest predicted binding to H-2D^b (see Table I).

CrpA expressed by these clones most likely contained the CD8$^+$ T cell epitope recognized by line 102. The portion of the CrpA polypeptide predicted to be encoded by the 411-bp *Sau*IIIA1 fragment is shown in Fig. 2 and is aligned with the full-length version of CrpA from serovar D.

A nonamer peptide from CrpA (ASFVNPIYL) is recognized by CD8$^+$ T cell line 102

To determine the epitope within CrpA recognized by CD8$^+$ T cell line 102, synthetic peptides from this sequence were constructed.

FIGURE 3. CrpA contains the D^b-restricted epitope, ASFVNPIYL. *A*, Stimulation of line 102 with overlapping 15-mer peptide corresponding to the sequence of the 411-bp fragment of CT442 identified in the library screen. K41 cells were pulsed with the indicated peptides at a final concentration of 1 μg/ml (in 50 μl of RP-10) for 1.5 h at 37°C and then incubated with line 102. Positive controls included K41 cells infected with *C. trachomatis* L2, and K41 cells transfected with either clone 2A7.H7 or clone 5B4.B5 that were identified in the library screen. After 48 h, culture supernatants were assayed for IFN-γ by ELISA. *B*, Stimulation of CD8$^+$ T cell line 102 with peptides from CrpA shown in Table I. K41 cells were pulsed with the indicated peptides (♦, ASFVNPIYL; ●, AASFVNPIYL; □, GQINNSNKV; △, NSLVDPSSL; ○, NAIDGVVVSL) at the indicated concentrations and then incubated with line 102. After 48 h, culture supernatants were assayed for IFN-γ by ELISA. *C*, H-2D^b-restricted presentation of CrpA$_{62-71}$. P815 cells or P815 cells transfected with either the D^b or K^b gene were tested in a ^{51}Cr release assay using line 102. An E:T ratio of 2:1 was used. Open bars represent cells treated with no peptide; filled bars represent cells treated with 0.25 μg/ml of CrpA$_{62-71}$.

In one set of experiments, we synthesized 15-mer peptides, each overlapping by 11 aa and covering the entire sequence found in this fragment. We then assayed these for their ability to stimulate line 102 by pulsing syngeneic APC with each peptide. Only the 15-mer peptide SAASFVNPIYLAIPA (CrpA$_{61–75}$) stimulated a T cell response (Fig. 3*A*). In a second set of experiments, 9- and 10-mer peptides corresponding to peptides within CrpA that are predicted by MHC peptide-binding algorithms (29) to bind H-2D^b with high affinity (Table I) were synthesized and tested. Syngeneic APC were pulsed with each of these peptides and assayed for recognition by line 102 T cells. As shown in Fig. 3, *A* and *B*, line 102 produced IFN-γ in response to peptides ASFVNPIYL (CrpA$_{63–71}$) and AASFVNPIYL (CrpA$_{62–71}$), which represent nonamer and decamer versions of the same epitope, and are the peptides with highest predicted affinity for H-2D^b. No other peptides predicted to bind D^b stimulated line 102. These data suggest that CrpA$_{63–71}$ represents the minimal H-2D^b epitope recognized by line 102.

CrpA is recognized in the context of H-2D^b

The specificity of line 102 for *C. trachomatis* L2-infected cells is restricted by H-2D^b class I molecules (Fig. 1). As described above, the CrpA$_{63–71}$ and CrpA$_{62–71}$ peptides are also predicted to bind H-2D^b with high affinity. To verify that line 102 recognition of this epitope is H-2D^b restricted, we tested the ability of line 102 to recognize CrpA$_{62–71}$ peptide-pulsed P815 (H-2^d) cells that were stably transfected with the genes encoding either the D^b or K^b molecule. As shown in Fig. 3*C*, only peptide-pulsed P815 cells transfected with the D^b gene were recognized and lysed by line 102. These data show that the H-2D^b-restricted recognition of *Chlamydia* by line 102 is at least in part directed against CrpA$_{62–71}$.

CrpA$_{63–71}$-specific T cells are primed during murine infection with *C. trachomatis*

To confirm that murine infection with *C. trachomatis* primes a CrpA$_{63–71}$-specific T cell response, mice were infected with *C. trachomatis* and allowed to recover for 2 wk. The mice were then sacrificed, and splenocytes were stimulated on irradiated syngeneic spleen cells pulsed with CrpA$_{63–71}$. After 5 days of stimulation, the cultures were used in a ^{51}Cr assay to determine whether there were CrpA$_{63–71}$-specific T cells present in these cultures. As shown in Fig. 4, these cultures contained significant levels of CrpA$_{63–71}$-specific cytolytic activity, demonstrating that CrpA$_{63–71}$-specific T cells are primed during murine infection with *C. trachomatis*.

Infection of mice with C. trachomatis *stimulates a significant expansion of CrpA$_{63–71}$-specific CD8$^+$ T cells that correlates with clearance of infectious organisms*

Because of the strong CrpA$_{63–71}$-specific response in primary cultures from infected mice, we analyzed in greater detail the frequency and kinetics of the T cell response to CrpA$_{63–71}$ in infected mice using a D^b/CrpA$_{63–71}$-specific tetramer and also by ELISPOT analysis. In one set of experiments summarized in Fig. 5*A*, groups of C57BL/6 mice (three mice/group) were infected i.v. with *C. trachomatis* L2, and, at the indicated times postinfection, spleen cells were stained with T cell markers and a CrpA$_{63–71}$-specific D^b tetramer. In Fig. 5*A*, the number of CrpA$_{63–71}$-specific T cells peaked between 6 and 7 days after primary infection. The responding T cells comprised 4% of the splenic CD8$^+$ T cell population at the peak of the response and remained a significant component of the CD8$^+$ T cell population 10 days after infection. Although these CrpA$_{63–71}$-specific cells quickly decrease in number, we have found that they persist at modest, but significant levels (~0.6% of the CD8$^+$ T cell population at day 21 postinfection; data not shown).

We also investigated whether the expansion of CrpA$_{63–71}$-specific CD8$^+$ T cells corresponded to clearance of *C. trachomatis* from infected mice. At various times postinfection, spleens from infected mice described in Fig. 5*A* were simultaneously analyzed for the magnitude of cells bound by the D^b/CrpA$_{63–71}$ tetramer and for the number of *C. trachomatis* IFU in the spleens of infected mice. As shown in Fig. 5*B*, there is an inverse correlation between these two measures, suggesting that the D^b-restricted CD8$^+$ T cell response to CrpA may play a role in pathogen clearance.

Given the marked expansion of CD8$^+$ T cells specific for CrpA$_{63–71}$ in *Chlamydia*-infected mice, we next analyzed the T cell response to CrpA$_{63–71}$ during the course of infection in CByB6F$_1$ (C57BL/6 × BALB/c) H-2$^{b/d}$ mice. This allowed us to compare the response to CrpA$_{63–71}$ with the response to another CD8$^+$ T cell epitope, Cap1$_{139–147}$. Cap1 was previously described as an H-2^d-restricted CD8$^+$ T cell Ag recognized by *Chlamydia*-specific T cells during infection of BALB/c mice (13). CByB6F$_1$ were infected with *C. trachomatis* and sacrificed at various times postinfection. CrpA$_{63–71}$-specific and Cap1$_{139–147}$-specific T cells present in the spleens of these animals were enumerated using an IFN-γ ELISPOT assay. As shown in Fig. 6, the maximal response to both epitopes is seen 6 days after infection. The magnitude of the CrpA$_{63–71}$-specific response is ~9 × 10^5 IFN-γ-secreting cells

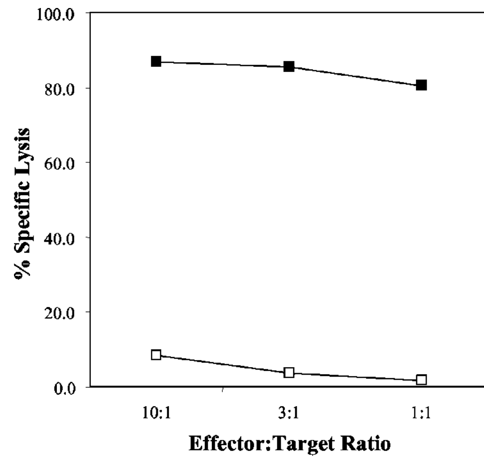

FIGURE 4. CrpA$_{63–71}$-specific CTLs are primed during murine infection with *C. trachomatis*. C57BL/6 mice were immunized i.v. with 1 × 10^7 IFU of *C. trachomatis* serovar L2. Two weeks after immunization, spleen cells were harvested from the infected mice and cultured in the presence of syngeneic spleen cells pulsed with 100 nM CrpA$_{63–71}$ peptide. After 5 days, the cultured cells were tested for their ability to specifically lyse CrpA$_{63–71}$-coated EL4 cells (■) and untreated EL4 cells (□) in a standard ^{51}Cr release assay. The data shown are one representative of results obtained from four T cell cultures.

Table I. *Predicted H-2D^b-binding peptides from clones containing CT442*

Peptide	Score[a]
ASFVNPIYL	343.2
NSLVDPSSL	47.0
GOINNSNKV	30.0
AASFVNPIYL	286.0
NAIDGVVVSL	30.2

[a] Estimate of the half-life of dissociation (min) from D^b of a molecule of this sequence.

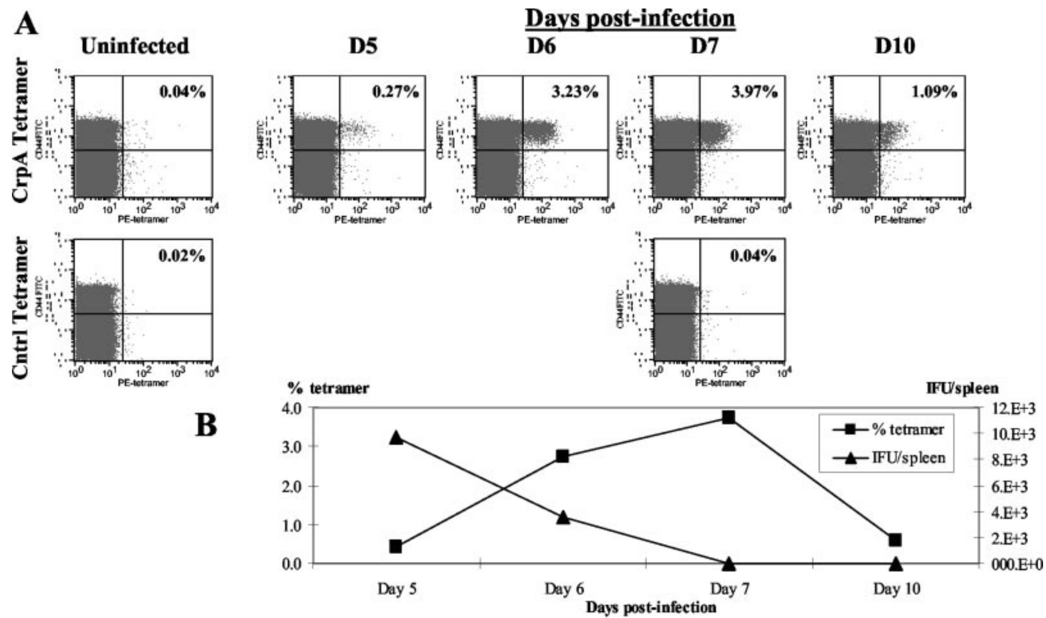

FIGURE 5. Tetramer staining of spleen cells from *C. trachomatis*-infected mice. *A*, D^b/CrpA$_{63-71}$ tetramer staining of spleen cells from *C. trachomatis* serovar L2-infected mice. C57BL/6 were infected with 5×10^6 IFU *C. trachomatis* serovar L2 i.v. Mice were sacrificed 6 and 7 days after infection. Spleens were excised and divided to provide cells for both D^b/CrpA$_{63-71}$ tetramer analysis and *C. trachomatis* culture to confirm infection. For FACS, single-cell suspensions of spleens were prepared and treated with ammonium chloride to lyse RBCs, and 4×10^6 spleen cells were then stained with the following reagents: 1) CD4 CyChrome and B220 CyChrome for negative gating; 2) CD8 APC for positive gating; 3) CD44 FITC as an activation marker; and 4) PE-labeled D^b/CrpA$_{63-71}$ tetramer for Ag specificity. For analysis, $CD4^-B220^-CD8^+$ were gated and plotted for D^b/CrpA$_{63-71}$ tetramer binding on the *x*-axis and CD44 on the *y*-axis. Three C57BL/6 mice per time point were infected, and spleens from each mouse were stained and analyzed individually. Data shown are from one representative mouse. Maximal response to CT442 epitope is between 6 and 7 days postinfection. Control tetramer D^b/MT0133$_{309-318}$ is a tetramer that contains the D^b-restricted peptide (GAPINSATAM) from the *M. tuberculosis* Ag MT0133. *B*, Kinetics of CrpA$_{63-71}$-specific T cell expansion and concomitant clearance of *C. trachomatis* L2. D^b/CrpA$_{63-71}$ tetramer staining of spleen cells from mice described in *A* vs kinetics of IFU. Points indicate mean IFU for three animals at each time point and mean percentage of D^b/CrpA$_{63-71}$ tetramer-positive $CD8^+$ T cells.

per spleen, 11-fold higher than the Cap1$_{139-147}$-specific response. The difference in the CrpA$_{63-71}$-specific response and the Cap1$_{139-147}$-specific response persists 21 days after infection when the response to CrpA$_{63-71}$ is 13-fold higher than the response to Cap1$_{139-147}$.

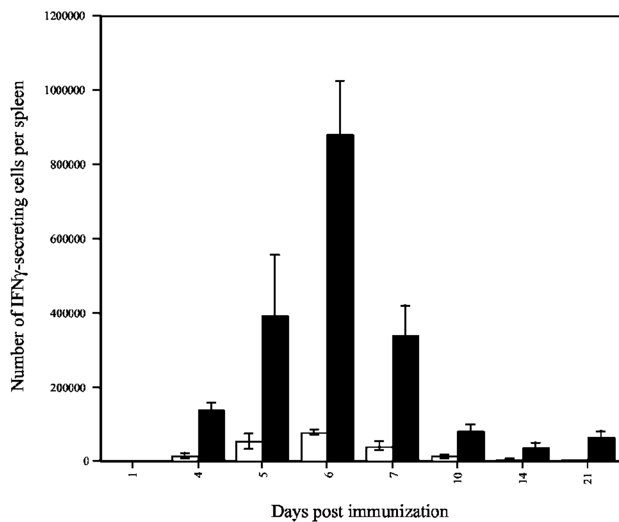

FIGURE 6. Kinetics of the $CD8^+$ T cell responses directed against CrpA$_{63-71}$ and Cap1$_{139-147}$ peptide Ags. CByB6F$_1$ mice were immunized i.v. with 1×10^7 IFU of *C. trachomatis* serovar L2. On the indicated days postimmunization, spleens were harvested from groups of three mice. The number of peptide-specific, IFN-γ-secreting cells per spleen was determined by ELISPOT analysis. CrpA$_{63-71}$ peptide-coated EL4 cells (filled bars) and Cap1$_{139-147}$ peptide-coated P815 cells (open bars) were used as stimulator cells.

Immunization with recombinant vaccinia virus expressing CrpA affords protection against C. trachomatis *challenge*

To evaluate whether an immunization strategy designed to stimulate CrpA$_{63-71}$-specific T cells would protect mice against *C. trachomatis* challenge, we constructed a recombinant vaccinia virus, designated Vac1505, which expresses the CrpA protein. Groups of eight C57BL/6 mice were immunized with either 10^6 PFU of wild-type vaccinia (i.p.), 10^6 PFU of Vac1505 (i.p.), or 10^7 IFU of *C. trachomatis* (i.v.). Twenty days after immunization, spleens were harvested from three mice in each group, and the number of CrpA$_{63-71}$-specific $CD8^+$ memory T cells in each spleen was determined by ELISPOT analysis. The remaining five mice in each group were challenged i.v. with 10^7 IFU of *C. trachomatis*. Three days after challenge, the mice were sacrificed and the number of *C. trachomatis* IFU per spleen was determined. As shown in Fig. 7*A*, significantly higher numbers of CrpA$_{63-71}$-specific memory cells were detected in mice immunized with Vac1505 compared with mice that recovered from infection with *C. trachomatis*. As shown in Fig. 7*B*, immunization of C57BL/6 mice with Vac1505 afforded significant protection against *C. trachomatis* challenge. The mean number of organisms present in the spleens of Vac1505-immunized mice was 18-fold lower than in control animals injected with 10^6 PFU of wild-type vaccinia. This reduction in bacterial colonization was comparable to that resulting from prior infection with *C. trachomatis*. This protective effect was most likely attributable to the CrpA-specific T cells primed by Vac1505, suggesting that

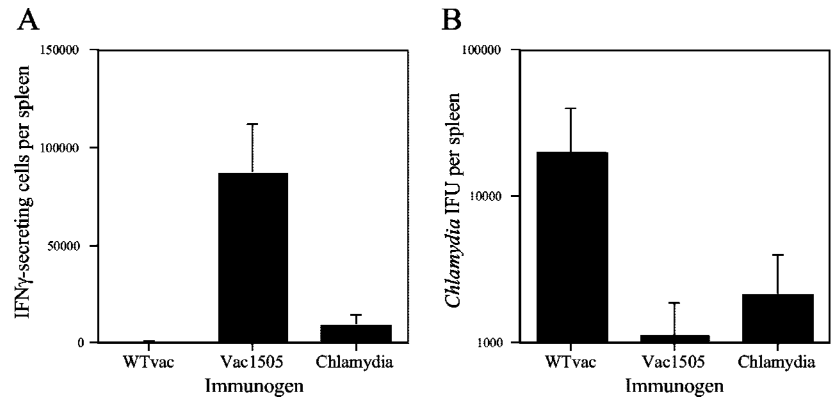

FIGURE 7. Immunization with recombinant vaccinia virus expressing $CrpA_{63–71}$ protects mice against challenge with *C. trachomatis*. Groups of eight C57BL/6 mice were immunized with 10^6 PFU of wild-type vaccinia (WTvac), 10^6 PFU of Vac1505, or 10^7 IFU of *C. trachomatis*. *A*, The number of CrpA-specific memory T cells per spleen 20 days postimmunization was determined by IFN-γ ELISPOT in three mice from each group. *B*, The remaining five mice from each immunization group were challenged with 10^7 IFU of *C. trachomatis* i.v. 20 days after immunization. Bars represent the number of *C. trachomatis* IFU per spleen 72 h after challenge.

CrpA-specific T cells can protect mice against challenge with *C. trachomatis*.

When protection of F_1 mice afforded by vaccinia expressing CrpA was compared with the protection seen following immunization with Cap1, far less protection was observed in mice immunized with Cap1 (∼3-fold; data not shown). This level of protection with Cap1 is consistent with previous data describing the ability of vaccinia expressing Cap1 to protect in BALB/c mice (13). Interestingly, this difference parallels the difference in magnitude of the $CrpA_{63–71}$-specific response when it is compared with the $Cap1_{139–147}$-specific response shown in Fig. 6.

Discussion

In this study, we describe the use of an expression library containing fragments of *C. trachomatis* genomic DNA to identify an H-2D^{b}-restricted CD8^{+} T cell Ag, CT442 or CrpA. This Ag was identified by screening the library with a polyclonal CD8^{+} T cell line cultured from the spleen of a C57BL/6 mouse previously infected with *C. trachomatis*. Moreover, we identify a 9-aa H-2D^{b}-restricted epitope from CrpA recognized during *Chlamydia* infection of C57BL/6 mice and show that a protective effect can be observed when mice vaccinated with this Ag are challenged with *C. trachomatis*.

A number of studies have characterized the expansion and contraction of CD8^{+} T cells in response to model Ags and viral pathogens. The lack of defined Ags for many bacterial pathogens has hampered investigation into the role of CD8^{+} T cells during bacterial infection. Among bacterial pathogens, only the CD8^{+} T cell response to the cytoplasmic bacterium *Listeria monocytogenes* has been well characterized (30). These studies in *Listeria* have been greatly aided by the identification of a number of protective CD8^{+} T cell epitopes. The identification of $CrpA_{63–71}$ has allowed us to focus on the response of CD8^{+} T cells to the important vacuolar pathogen *C. trachomatis*. The peak of the CD8^{+} T cell response to $CrpA_{63–71}$ was shown to occur on day 6–7 after primary infection by tetramer and ELISPOT analyses. Significantly, the magnitude of the CrpA-specific CD8^{+} T cell response in mice is high, reaching 4% of the splenic CD8^{+} T cells at the peak of the response. As expected, this response diminished over time, but was still readily detected 11 wk postinfection (data not shown). Furthermore, a comparison in F_1 mice of CD8^{+} T cell responses to $CrpA_{63–71}$ and a second *Chlamydia*-derived epitope ($Cap1_{139–147}$) showed that the $CrpA_{63–71}$-specific response was considerably dominant relative to the $Cap1_{139–147}$-specific response. The kinetics of the $CrpA_{63–71}$ response in infected mice correlated with clearance of the pathogen, suggesting that CD8^{+} T cells specific for this epitope may play a role in control of the infection. In support of this, vaccination to elicit a CD8^{+} T cell response to CrpA conferred protection when animals were later challenged with the pathogen. Although the recombinant vaccinia virus used to immunize the mice in this study stimulates a robust CD8^{+} T cell response to $CrpA_{63–71}$, other cell types may be stimulated by the immunization with full-length CrpA that contribute to protection.

It is unlikely that there are specificities for additional *Chlamydia* Ags within line 102. In screening the library with line 102, we estimated that the T cells were used to screen a DNA library representing ∼6-fold complete coverage of the genome. Consistent with this estimate, six independent DNA fragments were identified, and each contained the same Sau3A1 fragment from CT442. We cannot rule out that certain epitopes are not represented in the library, or were lost in its construction. We also cannot rule out that additional specificities in the line were lost during in vitro culture. However, screening the same pools used above with a human CD8^{+} T cell line that is *Chlamydia* specific has identified a completely different Ag, suggesting that the library most likely represents at least a majority of peptides that can be expressed in the context of MHC class I (unpublished observations). Finally, $CrpA_{63–71}$ appears to be the only epitope within CrpA recognized by line 102, as no other epitopes were found in screening overlapping peptides representing CrpA.

The function of CrpA is not yet known. Due to some historical confusion regarding the actual number and sizes of the CRPs, and because subsequent genomic sequencing placed CT442 adjacent to the 9- and 60-kDa cysteine-rich proteins, CT442 was originally designated as a cysteine-rich protein (15-kDa Cys Rich, CrpA) (27, 28). However, the 9- and 60-kDa proteins contain numerous Cys residues and are hypothesized to cross-link with other proteins, via disulfide linkages at the outer membrane, to form a pseudopeptidoglycan structure (31). By contrast, CT442 (CrpA) contains only four Cys residues, suggesting a different role unrelated to such cross-linking. Indeed, more recently it has been shown that CT442 fits a motif conserved among Inc proteins and has been shown by immunochemistry to localize to the Inc (14).

We have previously shown that Cap1 (CT529), another protein from *C. trachomatis* that is recognized by CD8^{+} T cells, is also an Inc-associated protein (13). These data suggest that other Inc-associated proteins may have access to the class I MHC-processing pathways of infected cells, and therefore may serve as targets for CD8^{+} T cells. This is consistent with the concept that CD8^{+} T cells can help define *Chlamydia* products interfacing with the host. There are numerous Inc proteins, and perhaps unique aspects of Cap1 and CrpA enhance their presentation to CD8^{+} T cells. In contrast to CrpA, Cap1 does not fit the hydrophobicity profile typical of Inc proteins. Cap1 has also recently been shown to associate with actin fibers in the cytosol (32), an observation consistent with its access to the MHC class I Ag-processing machinery. It will be

informative to investigate further the exact subcellular localization of CrpA and whether it also can be shown to directly access the cytosol. Such proteins most likely have unique or critical functions that define host-pathogen interactions during the *Chlamydia* developmental cycle.

The data presented in this work suggest that $CD8^+$ T cell responses can be stimulated during infection and that the Ags recognized by this T cell subset can afford significant protection against subsequent challenge. Although the $CD8^+$ T cell response to the $CrpA_{63-71}$ epitope is quite robust, sterilizing immunity was not achieved in vaccinated mice using this model. Several factors may contribute to the observation. First, the vaccination protocol may not induce an optimal effector phenotype in the $CrpA_{63-71}$-specific T cells. Second, optimal protection may likely require activation of additional effector cells, e.g., $CD4^+$ T cells. Similarly, vaccinating simultaneously with several $CD8^+$ T cell epitopes might enhance protection. Finally, different levels of protection may be observed when the challenge uses a different route of infection (e.g., mucosal). Whether or not the number of $CD8^+$ T cells responding to this epitope will confer protection against disease in different models of infection remains to be determined. In that regard, we are establishing a genital infection model of *Chlamydia trachomatis* infection in B6 mice to test the role of this epitope in protection.

Although it remains unclear the extent to which $CD8^+$ T cells contribute to adaptive immunity following natural infection with *C. trachomatis*, natural immunity alone does not afford robust protection against rechallenge. Therefore, it seems likely that a strategy that stimulates multiple arms of the immune system will be necessary to provide sterilizing or prolonged protective immunity. This is likely to involve methods that stimulate Ab production, $CD4^+$ T cells, and $CD8^+$ T cells. Vaccines that stimulate the immune system, yet fail to clear the pathogen, may exacerbate the immune pathology that is the major consequence of infection with this genus. Using the genital infection model, we will also assess the potential pathology of this $CD8^+$ T cell response.

Acknowledgments

We thank Bill Rees (Corixa) for generously proving D^b tetramer, and Majek Michalek for the kind gift of the murine line K41.

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