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Human CD8⁺ T Cells Recognize the 60-kDa Cysteine-Rich Outer Membrane Protein from *Chlamydia trachomatis*¹

Ana L. Gervassi,*† Kenneth H. Grabstein,* Peter Probst,* Bruce Hess,* Mark R. Alderson,* and Steven P. Fling²*

The intracellular bacterial pathogen *Chlamydia* is sequestered from the host cell cytoplasm by remaining within an inclusion body during its replication cycle. Nevertheless, CD8⁺ T cells recognizing *Chlamydia* Ags in the context of MHCI molecules are primed during infection. We have recently described derivation of *Chlamydia*-specific human CD8⁺ T cells by using infected dendritic cells as a surrogate system to reflect *Chlamydia*-specific CD8⁺ T cell responses in vivo. These CD8⁺ T cell clones recognize chlamydial Ags processed via the conventional class I processing pathway, as assessed by treatment of infected APC with lactacystin and brefeldin A, suggesting that the Ags are translocated from the chlamydial inclusion into the host cell cytosol. In this study, outer membrane protein 2 (OmcB) was identified as the Ag recognized by one of these *Chlamydia*-specific human CD8⁺ T cells, and we defined the HLA*A0101-restricted epitope from this Ag. CD8⁺ T cell responses to this epitope were present at high frequencies in the peripheral blood of both of two HLA*A0101 donors tested. In vitro chlamydial growth was completely inhibited by the OmcB-specific CD8⁺ T cell clone independently of lytic mechanisms. OmcB is a 60-kDa protein that has been postulated to be associated with the *Chlamydia* outer membrane complex. The subcellular localization of OmcB to the cytosol of infected cells, as defined by conventional MHCI class I Ag processing and presentation, suggests the possibility of an additional, cytosolic-associated function for this protein. *The Journal of Immunology*, 2004, 173: 6905–6913.

*Chlamydiae* are obligate intracellular, Gram-negative bacteria responsible for a broad spectrum of clinically important diseases in humans. Colonization of the ocular mucosa with *Chlamydiae trachomatis* can lead to trachoma, the major cause of preventable blindness worldwide (1). Genital tract infection with *C. trachomatis* is the most commonly sexually transmitted bacterial infectious agent in the United States (1, 2). A notable proportion of chlamydial genital tract infections will go undiagnosed and untreated due to the lack of clinical symptoms. Untreated infections in women can lead to pelvic inflammatory disease (PID),² and sequelae often lead to infertility or ectopic pregnancy. Respiratory infections with *Chlamydia pneumoniae* are implicated in coronary artery diseases (3, 4). The pathological mechanisms by which *Chlamydia* induces conjunctival scarring, PID, or arteriosclerosis are not well understood. However, the pathology seems to be related to chronic inflammation due to persistent or repeated infections. An effective vaccine to prevent and treat chlamydial infections is highly desirable.

CD8⁺ T cells are important in the resolution of several intracellular bacterial infections through lysis of infected cells and the secretion of cytokines (5). However, the human CD8⁺ T cell response to *Chlamydia* is relatively poorly characterized. Although murine gene knockout and in vivo depletion experiments suggest little participation of CD8⁺ T cells in protection against a *C. trachomatis* infection, adoptive transfer studies indicate that CD8⁺ T cells are capable of mediating passive protection. This protection is dependent on IFN-γ production (6), not on lytic mechanisms (7–9). However, CD8⁺ T cells play a significant role in cellular immunity against *C. pneumoniae*, as shown by murine gene knockout experiments, and this protection is perforin independent (10–12). One potential limitation in interpreting murine models of chlamydial disease is that mice lack granulysin (13). Granulysin is found, along with perforin, in the cytotoxic granules of human cytolytic T cells and NK cells. Granulysin acts synergistically with perforin to kill intracellular microbes delivered in combination; perforin provides access to the intracellular compartment, allowing granulysin to directly kill intracellular pathogens, including Gram-positive and -negative bacteria, fungi, and parasites (14).

*Chlamydia*-reactive, peptide-specific CD8⁺ T cells are elicited during human infection and are detected in children resolving infection, in adults without scarring of the conjunctiva in a trachoma-endemic population (15), and in individuals who have acquired a genital tract infection (16, 17). The limitation of these studies is that these responses are specific to predicted class I binding peptides derived from the major outer membrane protein (MOMP) or the heat shock protein 60. The significance of these peptide-specific CD8⁺ T cell responses to the overall CD8⁺ T cell response to *Chlamydia* during natural infection is unknown. Similarly, the potential of human CD8⁺ T cell responses to contribute to the resolution of natural infections with *Chlamydia* has yet to be determined.

We have recently described a method for cloning *Chlamydia*-specific human CD8⁺ T cells using *C. trachomatis*-infected dendritic cells (DC) (18). Using this approach, the Ag and the restricting allele may reflect more closely the natural CD8⁺ T cell response in vivo. Two class Ia-restricted CD8⁺ T cell clones were derived from an exposed individual; one was shown to be HLA*A0101-restricted, and the second was B*1501-restricted.
Both T cell clones recognized cells infected with live Chlamydia, but not cells pulsed with inactivated elementary bodies. These CD8 T cell clones recognized chlamydial Ags processed via the conventional class Ia processing pathway, because responses were blocked by treatment of APC with lactacystin and brefeldin A, suggesting that the Ags recognized by these T cells are protein Ags that are translocated from the chlamydial inclusion into the host cell cytosol. Translocation of chlamydial proteins into the host cell cytosol could be accomplished through the recently identified type III secretion system (19). We report in this study the identification by expression cloning of the Ag recognized by the human, Chlamydia-specific, HLA*0101-restricted CD8 T cell clone. We show that this CD8 T cell clone is specific for the 60-kDa cysteine-rich outer membrane protein 2 (OmcB). We also define the HLA*0101-restricted epitope from this Ag, OmcB (or its homologue in C. pneumoniae) has previously been shown to be an Ag for the humoral (20, 21) and CD4 T cell (22, 23) immune responses to C. trachomatis and C. pneumoniae, as well as for murine CD8 T cell responses to C. pneumoniae (24, 25). These data are the first showing that OmcB is an Ag recognized by human CD8 T cells, and the results underscore the potential significance of OmcB as a target of Chlamydia-specific immunity.

Materials and Methods

Human subjects

D48, a male volunteer recruited from employees of Corixa, was believed to have been previously exposed to C. trachomatis, as measured by positive CD4 T cell proliferation and IFN-γ production in response to C. trachomatis inactivated elementary bodies. The HLA class I haplotype of D48 is A01,02:B08,15. Donors CT18 and CT13 were recruited from the Public Health-Seattle and King County STD Clinic at Harborview Medical Center. CT18 is a 34-year-old female subject, infected with C. trachomatis serovar E (as determined by ligase chain reaction detection of C. trachomatis in urine), presenting cervicitis and discharge and seropositive for C. pneumoniae at the time of leukapheresis. The HLA class I haplotype of CT13 is A02,03;B07,60. Institutional review board-approved protocols were used for venipuncture, apheresis, and skin testing (as determined by microimmunofluorescence) at the time of leukapheresis. The HLA class I haplotype of CT13 is A02,03:B07,18. CT13 is a 18-year-old female, coinfected with C. pneumoniae and Chlamydia trachomatis, presenting cervicitis, vaginal discharge, and dysuria (as determined by ligase chain reaction detection of Chlamydia in urine), presenting cervicitis and discharge and seropositive for C. pneumoniae (as determined by microimmunofluorescence) at the time of leukapheresis.

The HLA class I haplotype of CT13 is A02,03:B07,60. Institutional review board-approved protocols were used for venipuncture, apheresis, and skin biopsies. HLA typing was performed by the Puget Sound Blood Center (Seattle, WA).

Cells and cell lines

The human HeLa cervical epithelial cell line (HeLa 229, CCL-75.1) and the SV40-transformed human fibroblast line VA13 (CCL-75.1) were purchased from the American Type Culture Collection (Manassas, VA). Human PBMC were isolated by Ficoll-Hypaque 1.077 (Nycomed, Oslo, Norway) centrifugation of a leukapheresis product, followed by aliquoting and cryopreservation. Previously frozen PBMC were subsequently used for CD8 T cell separations and generation of DC. Primary human fibroblasts (Raritan, NJ) in 200,000/well RPMI 1640 and 10% human sera. Cultures were incubated at 37°C in 5% CO2, and 20 U/ml IL-2 was added on days 1, 4, 7, and 10 after stimulation. After 10–14 days of culture, wells were retested for T cell separations and generation of DC. Primary human fibroblasts were grown from skin biopsies in Weymouth’s Opti-MEM medium (Invitrogen Life Technologies) and supplemented with L-glutamine (Invitrogen Life Technologies).

Chlamydia

C. trachomatis and C. pneumoniae. The human HeLa cervical epithelial cell line (HeLa 229, CCL-75.1) and the SV40-transformed human fibroblast line VA13 (CCL-75.1) were purchased from the American Type Culture Collection (Manassas, VA). Human PBMC were isolated by Ficoll-Hypaque 1.077 (Nycomed, Oslo, Norway) centrifugation of a leukapheresis product, followed by aliquoting and cryopreservation. Previously frozen PBMC were subsequently used for CD8 T cell separations and generation of DC. Primary human fibroblasts were grown from skin biopsies in Weymouth’s Opti-MEM medium (Invitrogen Life Technologies, Grand Island, NY) containing 15% FCS (HyClone, Logan, UT). All media were supplemented with gentamicin and L-glutamine (Invitrogen Life Technologies).

Chlamydia

C. trachomatis (strain L2) was cultured in HeLa cell monolayers as previously described (26). Briefly, HeLa cell monolayers were infected with C. trachomatis L2 by centrifugation. Elementary bodies (EB) were purified by Hypaque-70 two-step gradient ultracentrifugation (Nycomed, Princeton, NJ) and frozen in sucrose-phosphate-glutamate buffer. Determination of inclusion-forming units (IFU; per milliliter) of C. trachomatis preparations was performed by titration on HeLa cells. Chlamydia preparations were then tested for T cell separations and generation of DC. Primary human fibroblasts were grown from skin biopsies in Weymouth’s Opti-MEM medium (Invitrogen Life Technologies) and supplemented with L-glutamine (Invitrogen Life Technologies). A DNA expression library was constructed for eukaryotic expression of bacterial polypeptides for expression cloning, random fragments of C. trachomatis L2 chromosomal DNA were cloned into the pCDN4A HisMax set of vectors (Invitrogen Life Technologies). The pCDNA4 HisMax A, B, and C vectors allow expression of DNA in each reading frame by staggered insertion of DNA preparations was ligated into the unique BamHI site of pCDNA4 HisMax and transformed into ElectroMax DH10B Escherichia coli (Invitrogen Life Technologies) 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 12 96-well culture plates containing Luria Bertoni medium (with 50 µg/ml carbenicillin) and grown for 18 h at 37°C. Plasmid DNA for transfections was prepared from these cultures using 96 Turbo-Prep plates (Qiagen, Valencia, CA).

Generation and maintenance of Chlamydia-reactive CD8 T cell clones

Chlamydia-reactive CD8 T cell clone was cloned by the limiting dilution approach described by Lewinsohn et al. (28) and Gervassi et al. (18). Briefly, CD8 T cells were purified from previously frozen PBMC by TCRγδ and CD4 depletion, followed by CD8 enrichment through magnetic bead-conjugated Abs according to manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). Flow cytometric analysis confirmed that cells were >98% positive for CD8 and were negative for TCRγδ. Purified CD8 T cells were plated in a 96-well cloning plate (Nunclon; Nunc, Roskilde, Denmark) at various cell numbers (100, 400, 1000, and 3500) per well in RPMI 1640 and 10% human sera. DC from the same donor were grown from adherent monocytes in GM-CSF- and IL-4 (10 ng/ml)-containing medium. DC were harvested on day 7, plated in a 24-well plate at 2 × 105 cells/well, and placed overnight at 37°C. DC were infected with Chlamydia L2 at a multiplicity of infection (MOI) of 10:1, and plates were centrifuged for 1 h at 1400 × g. A higher MOI was used for DC (10:1) than for other cell types (1:1) to accomplish similar rates of infection. Infection rates typically ranged from 30–70% of plated DC. Chlamydia-infected DC were harvested 24 h postinfection by centrifugation, and plated at 10 000 cells/well along with autologous irradiated PBMC (10 000 cells/well) as feeders. Cultures were incubated at 37°C in 5% CO2. Recombinant IL-2 (10 U/ml) was added on days 1, 4, 7, and 10 after stimulation. After 10–14 days of culture, wells were screened for Chlamydia specificity by ELISPOT split-well analysis. The CD8-30 clone was picked because it showed reactivity against autologous DC infected with C. trachomatis L2 and had lost reactivity against both noninfected autologous DC or HLA-mismatched, C. trachomatis L2-infected DC. CD8-30 was expanded by anti-CD3 stimulation in 96-well cloning plates by seeding T cells (1 × 105/well) with 1 × 107 gamma-irradiated B lymphoblastoid cell line (B-LCL)/well, 7.5 × 105 gamma-irradiated PBMC/well, and 30 ng/ml anti-CD3 Ab (OKT3; Ortho Biotech, Raritan, NJ) in 200 µl/well RPMI 1640 and 10% human sera. Cultures were incubated at 37°C in 5% CO2, and 20 U/ml IL-2 was added on days 2, 6, and 10. After 14 days of culture, wells were retested for Chlamydia specificity.

Library screening

For transfections, 100 ng (per pool) of library DNA and the HLA*0101 allele CNNA (100 ng) were mixed with Opti-MEM (Invitrogen Life Technologies) and a 1 µl dilution of the transfection reagent FuGene-6 (Roche, Basel, Switzerland). HLA*0101 was cloned from the original donor (D48) into a modified pCDNA3 vector using the Gateway system (Invitrogen Life Technologies). This DNA/EfGene preparation was used to transfect individual wells of VA13 cells, an SV40-transformed human fibroblast line (29), that were seeded at 2 × 104 cells/well in 96-well, flat-bottom plates (Costar, Cambridge, MA) 24 h before transfection. Wells were supplemented with medium 0.5 h after transfection and were incubated for an additional 48 h at 37°C. Transfection efficiencies were assessed by flow cytometric analysis of cells infected with a control gene encoding a reporter gene. Positive controls consisted of Chlamydia-infected VA13 cells transfected with HLA*0101. Cells were directly infected in these plates with C. trachomatis L2 (MOI: 1:1) 24 h after transfection. Infection...
rates typically ranged from 80–100% of VA-13 cells. Twenty-four hours after infection (48 h after transfection), T cells were added (4 × 10^6 cells/well) to APC with 2 U/ml IL-2. T cell activity was measured on 72-h supernatants by assaying for IFN-γ secretion by ELISA.

**DNA sequencing**

Sequencing of cloned plasmid DNA derived from immunostimulatory clones was performed on an automated sequencer (ABI 377; Applied Biosystems, Foster City, CA) using sequence-specific forward primers (5′-TAATACGACTCACTATAGGG-3′ and 5′-ATGACTGTGACACAGCAATACT-3′) and reverse primers (5′-TAGAGGCCACGTCTGAGG-3′ and 5′-CAGGAAAAACATGATGAC-3′).

**Peptide synthesis**

Peptides were synthesized on a Rainin/PTI (Woburn, MA) Symphony peptide synthesizer using 9-fluorenylethoxycarbonyl batch chemistry with 2-(1H-benzotriazole-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate activation. Peptides were analyzed by reverse phase HPLC, and molecular masses were verified using a MALDI-TOF mass spectrometer. The VSD-TENTHY peptide was synthesized by SynPep (Dublin, CA).

**Peptide epitope mapping**

Gamma-irradiated autologous fibroblasts were seeded in a 96-well, flat-bottom plate at 15,000 cells/well in Weymouth’s medium containing 15% FCS. After an overnight incubation at 37°C, medium was replaced with RPMI 1640 and 10% human serum containing 1 U/ml IL-2, and peptides were added to reach a final concentration of 1 µg/ml (unless otherwise noted). Clone CD8-30 T cells were added to reach 50,000 cells/well. Seventy-two-hour supernatants were assayed for IFN-γ not noted. Clone CD8-30 T cells were added to reach 50,000 cells/well. Seventy-two-hour supernatants were assayed for IFN-γ by ELISA. For assays using B-LCL as APC, gamma-irradiated B-LCL were peptide-pulsed for 1 h at 37°C, washed, and plated in a round-bottom, 96-well plate at 50,000 cells/well before adding 25,000 CD8-30 T cells and 2 U/ml rIL-2. Seventy-two-hour supernatants were analyzed for IFN-γ production by ELISA.

**ELISAs**

Standard IFN-γ ELISA protocols were used for detection of T cell activation in library screening. Briefly, plates were coated with anti-human IFN-γ (BD Biosciences) in 0.1 M bicarbonate buffer, pH 9.6. Plates were blocked with PBS buffer containing 5% nonfat dry milk, then washed in PBS-Tween. Samples and standards were added. After incubation, plates were washed, then incubated again with biotinylated rabbit anti-human IFN-γ (generated at Corixa) in PBS-10% goat serum. Plates were washed and incubated with donkey anti-rabbit-HRP conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in PBS with 5% nonfat dry milk. Plates were again washed, and activity was detected by 3,3′,5′-tetramethylbenzidine peroxidase substrate (Kierkegaard & Perry Laboratories, Keene, NH). Reactions were stopped by addition of 1 N sulfuric acid, and quantification was performed by spectrophotometric measurement of absorbance at 450 and 570 nm.

**CTL assay**

For *Chlamydia* infection of target cells, autologous fibroblasts were plated at 1 × 10^5 cells/well in a 24-well plate (Costar) in 15% FCS-Weymouth’s medium. Cells were incubated overnight before infection. Medium was replaced with 0.3 ml containing the infection inoculum (C. trachomatis L2; MOI, 1:1). Infection rates typically ranged from 80–100% of fibroblasts. Plates were centrifuged for 1 h at 1400 × g. Medium was aspirated after centrifugation and replaced with 1 ml of fresh medium containing 150 µCi of 51Cr (NEN, Boston, MA)/well, and plates were placed at 37°C in 5% CO2 overnight. Fibroblasts were washed with PBS before detaching with trypsin. After additional washes, fibroblasts were plated in a 96-well, round-bottom plate at 5 × 10^5 cells/well. T cells were added to obtain the E:T cell ratios described. Peptides were added to respective wells at this point. Plates were incubated at 37°C in 7% CO2 for 18 h before harvesting supernatants with the Skatron harvesting system (Molecular Devices, Lier, Norway). 51Cr was quantified in the supernatants with the Skatron harvesting system (Molecular Devices, Lier, Norway). Breakdown of infected fibroblasts was quantified by measurement of absorbance at 450 and 570 nm.

**Inhibition of chlamydial growth by CD8-30**

To evaluate the inhibition of chlamydial growth by CD8-30, VA-13 monolayers were prepared by plating gamma-irradiated (3500 rad) VA-13 cells in a 12-well plate at 2 × 10^5 cells/well in RPMI 1640 and 10% FCS. The inhibitors were added at 1 × 10^5 cells/well before adding 25,000 CD8-30 T cells and 2 U/ml rIL-2. Seventy-two-hour supernatants were analyzed for IFN-γ not noted. Clone CD8-30 T cells were added to reach 50,000 cells/well. Seventy-two-hour supernatants were assayed for IFN-γ by ELISA. For assays using B-LCL as APC, gamma-irradiated B-LCL were peptide-pulsed for 1 h at 37°C, washed, and plated in a round-bottom, 96-well plate at 50,000 cells/well before adding 25,000 CD8-30 T cells and 2 U/ml IL-2. Seventy-two-hour supernatants were analyzed for IFN-γ production by ELISA.

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and blocked 96-well nitrocellulose-backed plate (Multiscreen HA; Millipore, Bedford, MA). Plates were incubated overnight at 37°C in 5% CO₂ before developing and quantifying IFN-γ spots.

Results

Identification of a Chlamydia-specific CD8⁺ T cell Ag

The Chlamydia-specific, human CD8⁺ T cell clone CD8-30 was derived by limiting dilution using C. trachomatis-infected DC as stimulator cells (18). CD8-30 T cells recognize C. trachomatis-infected APC in the context of HLA*A0101. For Ag identification, an expression library constructed from C. trachomatis genomic DNA (30) was cotransfected along with an HLA*A0101 cDNA into an SV40-transformed human fibroblast cell line, VA13 (29). This cell line allows episomal replication of SV40 origin of replication-containing expression vectors. To identify the genomic DNA fragment(s) encoding the Ag recognized, ~48 x 10⁶ independent clones (672 pools) of the expression library were screened by transfection of VA13 cells, followed by incubation with CD8-30 T cells. This screen represents ~5- to 6-fold coverage of the C. trachomatis genome. From this primary screen, two positive pools were defined, 2A1 (Fig. 1A) and 6D4 (data not shown). This is somewhat less than the estimated frequency of individually expressed positive clones expected within the library and less than that observed using other T cells to screen this library (30). To identify individual clones representing the Ag expressed in the pools, plasmid DNA was prepared from 96 individual clones from each of the original pools, and wells of VA13 cells were independently cotransfected with each clone along with HLA*A0101 cDNA. These transfectants were then screened using the same method as that used for the primary screen. One Ag positive clone (2A1.C10) from pool 2A1 was identified (Fig. 1B). No Ag-positive clones were identified from the 96 individual clones of pool 6D4. Plasmid DNA from Ag positive clone 2A1.C10 was sequenced.

By BLAST analysis, clone 2A1.C10 was determined to contain three genomic Sau3A1 DNA fragments representing portions of the predicted C. trachomatis open reading frames (ORFs; schematically shown in Fig. 2A), designated CT314, CT811, CT443, and CT442 within the published C. trachomatis genome sequence (19).

The three genomic DNA fragments were in random orientation. They were joined by Sau3A1-compatible cohesive ends, as would be expected from the protocols used in library construction. However, within clone 2A1.C10, the fragments combined to form a contiguous ORF of 308 aa that is in-frame with the translational start site of pcDNA4HisMax (Fig. 2). Specifically, this combined ORF encoded a fusion protein comprised of a short N-terminal sequence encoded by the vector, a short antisense portion of CT314, a short portion of CT811 (34 aa), and a long portion of CT443 (Fig. 2B). The CT443 fragment corresponds to the C-terminal 205 aa residues of OmcB and is aligned with this portion of OmcB in Fig. 2B for reference. When the predicted fusion polypeptide for this entire combined ORF was analyzed in an MHC peptide-binding algorithm (31), only the OmcB-encoded sequence was predicted to encode potential HLA*A0101-binding peptides. Based on these observations, we hypothesized that the OmcB fragment probably encoded the CD8⁺ T cell epitope recognized by CD8-30 T cells. OmcB (CT443) is a 553-aa residue protein also referred to as the 60-kDa cysteine-rich outer membrane protein. This protein is highly conserved among the C. trachomatis serovars (19, 32–35), sharing between 98 and 99% aa sequence identity. OmcB from C. trachomatis shares ~71% aa sequence identity with its homologue in C. pneumoniae (36).

OmcB epitope mapping

The portion of the OmcB polypeptide predicted to be encoded within clone 2A1.C10 is shown in Fig. 2. To determine whether the epitope recognized by the CD8⁺ T cell clone CD8-30 is found

FIGURE 2. Expression fragment clone 2A1.C10. A, Schematic summary of DNA sequencing results of Ag-positive clone 2A1.C10. Clone 2A1.C10 contains three random Sau3A1 genomic DNA fragments from C. trachomatis. The relative positions of the ORFs within clone 2A1.C10 are diagrammatically oriented 5’ to 3’ with respect to the MCS of expression vector pcDNA4HisMax, as indicated. Heavy horizontal line indicate DNA fragments oriented 5’ to 3’ (not to scale). Arrows indicate the relative orientation of the normal translational frames of the individual partial ORFs. Bold italics show ORFs contained within the fusion polypeptide. B, Alignment of the 2A1.C10 fusion polypeptide with the C-terminal portion of CT443. For CT443, only aa residues 201–553 from the published sequence of serovar D are shown. Homology begins at aa residue 349 of OmcB (position 104 of 2A1.C10; shown at the triple arrow). Positions 70–103 of 2A10 represent the CT811 fragment; positions 34–69 represent the CT314 antisense fragment; positions 1–33 represent pcDNA4HisMax encoded. The box indicates the HLA*A 0101 epitope defined by CD8⁺ T cell clone CD8-30.
within OmcB, synthetic 15-mer peptides, overlapping by 11 aa and covering the entire portion of the OmcB sequence found in clone 2A1.C10, were synthesized. Autologous fibroblasts were pulsed with each of these peptides and assessed for recognition by CD8-30 T cells by IFN-γ production. As shown in Fig. 3, CD8-30 produced IFN-γ only in response to fibroblasts pulsed with peptide TLTVPSVDTENTHY representing aa 539–553 of OmcB. No other 15-mer peptide was recognized. In addition, nonamer and decamer peptides from the OmcB portion in 2A1.C10 predicted by MHC peptide binding algorithms (31) to bind HLA*A0101 with high affinity were synthesized (Table I) and were tested for their ability to stimulate CD8-30. As shown in Fig. 4A, CD8-30 T cells also recognized peptide VSDTENTHY, which represents aa 544–553 of OmcB. Consistent with data using the longer peptides (Fig. 3), this shorter sequence is fully contained within the 15-mer peptide recognized by CD8-30. To further define the minimal epitope, C- and N-terminal-truncated synthetic peptides of VSDTENTHY were tested for their ability to sensitize autologous fibroblasts. As shown in Fig. 4B, truncations of one amino acid residue from either side of VSDTENTHY significantly diminished recognition by CD8-30 T cells, suggesting that the minimal HLA*A0101 epitope from OmcB is the 10-mer encoded by aa 544–553. This portion of OmcB is also found in the C. pneumoniae sequence (36), differing only at aa position 552 (C. trachomatis numbering) with a conserved change from isoleucine to valine. To determine whether CD8-30 T cells cross-react with the C. pneumoniae homologue, synthetic peptides representing both sequences were titrated on autologous B-LCL and compared for their ability to stimulate CD8-30 T cells. As shown in Fig. 4C, both the C. trachomatis (VSDTENTHY) and the C. pneumoniae (VSDTENTHY) peptides induced the production of IFN-γ by CD8-30. In addition, position 552 is critical because even a conserved change to leucine at this position abrogated T cell recognition (Fig. 4C).

OmcB-specific clone CD8-30 can lyse peptide-pulsed targets

We have previously shown that CD8-30 does not lyse Chlamydia-infected autologous fibroblasts (18), even though those targets are recognized, as assessed by cytokine secretion. Whether infection with live Chlamydia plays a role in this observation has remained unresolved due to the absence of a defined Ag. Because the epitope recognized by these T cells has now been identified, we addressed this issue and asked whether CD8-30 is capable of lysing peptide-pulsed fibroblasts. As shown in Fig. 5, CD8-30 T cells efficiently lysed VSDTENTHY peptide-pulsed fibroblasts. Killing of peptide-pulsed targets also occurred when targets were simultaneously infected with Chlamydia (Fig. 5). However, CD8-30 T cells did not lyse targets infected with Chlamydia in the absence of peptide. Furthermore, there was no difference in CD8-30 lytic activity of Chlamydia-infected or noninfected autologous fibroblasts when VSDTENTHY peptide was titrated (Fig. 5). These data show that live infection with Chlamydia does not actively inhibit lytic activity of these T cells, as has been shown to occur in vitro after stimulation of infected cells with a variety of proapoptotic agents (37). One possible explanation for these disparate observations is to invoke epitope density differences. Specifically, higher surface epitope densities are achieved by peptide pulsing. It is known that cytokine release and T cell cytolytic activity can be differentially responsive to epitope densities (38). To investigate epitope density in relation to our data, peptide VSDTENTHY was titrated, and both killing and IFN-γ assays were run in parallel. As shown in Fig. 6, CD8-30 T cells are equally sensitive to peptide concentrations in both the IFN-γ and 51Cr release assays, with half-maximal responses achieved with ~10 ng/ml peptide in both assays. To determine whether the lytic activity of Chlamydia-infected targets is specific for fibroblasts, additional target cell types were also tested (e.g., HeLa and U937 cells transduced with HLA*A0101). Chlamydia-infected and noninfected HeLa and U937 cells were efficiently lysed by CD8-30 when pulsed with peptide, but not when infected with Chlamydia only. However, both targets stimulated secretion of IFN-γ when they were infected with Chlamydia (data not shown).

OmcB-specific clone CD8-30 inhibits Chlamydia replication

Our data show that OmcB enters the class I processing pathway of infected cells and stimulates CD8+ T cells. Whether these CD8+ target cells are infected is critical because even a conservative change to leucine at this position abrogated T cell recognition (Fig. 4C).

Table I. Peptides from the polypeptide sequence of OmcB contained within clone 2A1.C10 that are predicted to bind HLA*A0101

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>MHC Binding Score*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CARATYYWK</td>
<td>36</td>
</tr>
<tr>
<td>ELNPGESLOQY</td>
<td>172.5</td>
</tr>
<tr>
<td>GNESEIFTFFF</td>
<td>31.1</td>
</tr>
<tr>
<td>SSDTLTVPVS</td>
<td>10.35</td>
</tr>
<tr>
<td>TCCDPVCAGDEN</td>
<td>34.5</td>
</tr>
<tr>
<td>TVFESVTQLKA</td>
<td>31.1</td>
</tr>
<tr>
<td>TVFESVTNLK</td>
<td>18</td>
</tr>
<tr>
<td>VSDTENTHY</td>
<td>258.8</td>
</tr>
</tbody>
</table>

* Estimate of the half-time of dissociation (minutes) from HLA-A1 of a molecule of this sequence. Relative binding affinity is indicated using an algorithm based on Ref. 31.
T cells will mediate a protective effect in humans remains to be determined, especially in light of the lack of in vitro lytic activity against infected targets. However, to evaluate the functionality of CD8-30 T cells and the potential for a CD8-specific T cell response against OmcB to be immunoprotective, we tested the inhibition of chlamydial growth by CD8-30 T cells when stimulated by infected and peptide-pulsed cells. As shown in Fig. 7, chlamydial growth in HLA-A0101-positive infected cells (but not HLA-A0101-negative infected cells) was significantly inhibited when the infected cells expressing endogenous Ag were cultured with CD8-30 T cells. Specifically, the total IFU per milliliter in supernatant recovered from HLA-A0101-positive, Chlamydia-infected VA13 cells was reduced by >20-fold relative to that from control infected cells. Furthermore, inclusion of VSDTENTHY peptide, but not control peptide, led to at least a 100-fold additional inhibition of chlamydial growth (Fig. 7). These data suggest that a T cell response against OmcB could significantly decrease chlamydial replication in vivo.

OmcB peptide VSDTENTHY is recognized in primary cultures of CD8+ T cells from two independent HLA-A0101 donors

To determine whether an OmcB-specific CD8+ T cell response could also be detected in other Chlamydia-exposed individuals, we assayed primary CD8+ T cells from a second HLA-A0101 donor (CT18) who was assessed to have current exposure to C. trachomatis at the time of leukapheresis. As shown in Table II, the VSDTENTHY peptide stimulated an easily detectable response in primary CD8+ T cells from both donors D48 and CT18. The strength of the response to VSDTENTHY in primary cultures from CT18 (~180 spots/500K CD8+ T cells) suggests a high frequency (1 in 3200) of OmcB-specific CD8+ T cells in this donor. These results also show that the HLA-A0101-restricted CD8+ T cell response in D48 used to identify OmcB was not unique to this donor. Finally, detection of the response to peptide VSDTENTHY in primary cultures suggests that the CD8-30 T cell clone used in these experiments was not derived by a spurious in vitro priming event, but, rather, was present in the exposed donor, presumably arising from in vivo priming after chlamydial infection.

Discussion

In this study we describe the identification of an HLA-A0101-restricted, CD8+ T cell Ag (OmcB) from Chlamydia by screening an expression library containing fragments of C. trachomatis genomic DNA. The human CD8+ T cell clone was derived by limiting dilution of CD8+ T cells from an exposed individual stimulated with C. trachomatis-infected DC. We also describe the 10-aa HLA-A0101-restricted epitope, VSDTENTHY, from OmcB. There are to date a very limited number of identified Ags from Chlamydia that enter the MHC class I pathway. Indeed, this paucity of known chlamydial Ags known to stimulate human CD8+ T cells has hindered characterization of this aspect of the human immune response to this intracellular pathogen. Our report

FIGURE 4. Epitope mapping of the HLA-A0101b peptide from OmcB. A, IFN-γ production in response to autologous fibroblasts pulsed with (1 μg/ml) of each of the HLA-A0101-binding peptides. The positive control was T cells stimulated with C. trachomatis L2-infected fibroblasts. Peptide VSDTENTHY corresponds to aa 544–553 of the full-length OmcB sequence. B, IFN-γ production in response to autologous fibroblasts pulsed with (1 μg/ml) of the indicated, truncated versions of the VSDTENTHY peptide. C, Autologous B-LCL (HLA-A0101 positive; ● and ▽) and allogeneic, MHC-mismatched B-LCL (HLA-A0101 negative; □ and ○) were pulsed with the indicated concentrations of either the C. trachomatis peptide VSDTENTHY (● and □) or the C. pneumoniae homologue peptide VSDTENTHY (▽ and ○), a negative control peptide ELNPGESLQY (▲), or the position 552-substituted peptide VSDTENTHY (▼). Pulsed BLCL were then used to stimulate CD8-30 T cells for IFN-γ production.

FIGURE 5. Lysis of peptide-pulsed, but not Chlamydia-infected, fibroblasts by CD8-30 CD8+ T cell clone. Lytic activity was measured by 18-h ⁵¹Cr release assay of C. trachomatis L2-infected (▲ and ▽) and/or peptide-pulsed (● and ▪) autologous fibroblasts. Data are the mean ± SD of two separate experiments performed in triplicate. ○, fibroblasts only; •, fibroblasts and VSDTENTHY; △, fibroblasts and L2; ▲, fibroblasts, L2, and VSDTENTHY.

FIGURE 6. Sensitivity of IFN-γ production and lysis assays. A, IFN-γ production; B, lytic activity by CD8-30 T cell clone against peptide-pulsed autologous fibroblasts. The assays were run in parallel with equal numbers of effector and target cells. ●, peptide VSDTENTHY; ▲, control peptide (ELNPGESLQY).
is the first using cloned human CD8\(^+\) T cells to find such an Ag, as opposed to screening peptides from hypothesized Ags, an approach that has a number of biases and limitations. Identification of OmcB as another Ag from *Chlamydia trachomatis* that enters the MHC class I pathway and stimulates human CD8\(^+\) T cells represents one step in elucidating the human immune response to this pathogen.

*Chlamydiae* are obligate intracellular bacterial pathogens that remain sequestered, by means of an inclusion body, from the host cell cytoplasm throughout their life cycle. During this cycle, *Chlamydiae* differentiate from metabolically inactive, infectious EB to metabolically active, replicating reticulate bodies (RB) before differentiating back into infectious EB. Because all the early stages of chlamydial infection (attachment, endocytosis, and avoidance of lyosomal fusion) are dependent on products present on the EBs (39), we speculate that translocation of some of these preformed products into the host cell cytosol will be necessary for development. These protein products will either be translocated completely into the host cell cytosol or incorporated into the inclusion membrane. In earlier studies we found that subcellular location to the inclusion membrane is apparently sufficient to efficiently target *C. trachomatis* Ags for CD8\(^+\) T cell recognition (27, 30). Previous results with the T cells described in this study show that recognition of OmcB by the CD8\(^+\) T cell clone CD8-30 requires processing by the conventional class I pathway (18). Reports from others have identified OmcB from *C. pneumoniae* as a murine CD8\(^+\) T cell Ag (24, 25). OmcB has also been reported to be a *C. trachomatis* and *C. pneumoniae* CD4\(^+\) T cell Ag in both mice (40) and humans (22, 23). Processing of OmcB for presentation to the murine CD8\(^+\) T cells is also sensitive to brefeldin A (25), suggesting that OmcB is indeed processed via the conventional class I processing pathway in both humans and mice.

The portion of OmcB (C terminus) recognized by CD8-30 T cells is also found in the *C. pneumoniae* sequence (36), with a conserved change from isoleucine to valine at aa position 552 (*C. pneumoniae* numbering). Because the prevalence of *C. pneumoniae* is high in the population (3), a positive read-out in our assays could be due to a past *C. pneumoniae* infection. To determine this possibility, both *C. trachomatis* and *C. pneumoniae* homologues were analyzed for stimulation of specific IFN-\(\gamma\) production. CD8-30 recognized OmcB peptides derived from both chlamydial species. Thus, even though endogenous OmcB from *C. trachomatis*-infected cells clearly enters the MHC class I pathway, and CD8-30 T cells were cloned by stimulating in vitro with *C. trachomatis*, we cannot rule out the possibility that the response was primed by a previously undetected *C. pneumoniae* infection. Indeed, it is possible that the high frequency CD8\(^+\) T cell response directed toward OmcB in donor CT18 could be a result of either this patient’s exposure to *C. pneumoniae* or the noted *C. trachomatis* infection at the time of leukapheresis. In any case, our results highlight the potential of cross-protective CD8\(^+\) T cell responses between *C. trachomatis* and *C. pneumoniae*.

The location and function of OmcB are not completely understood. The fact that OmcB is a CD8\(^+\) T cell Ag in infected cells and is processed via the conventional class I pathway implies that OmcB gains access to the host cell cytosol. How it breaches this compartment is unclear, given its hypothesized function. OmcB and OmcA (9-kDa cysteine-rich protein) are postulated to reside in the periplasmic space of EBs and to be functionally associated with the outer membrane complex (41). Both OmcB and OmcA contain numerous cysteine residues and, along with MOMP, may confer rigidity and osmotic integrity to chlamydial EBs by creating a lattice of disulfide bond cross-linked structure, forming the functional equivalent of peptidoglycan (42, 43). OmcB as well as OmcA have been identified in EBs and only in the late cycle of RBs (42). Part of the differentiation process of EBs into RBs would involve the reduction of these cross-links and the loss of OmcA (9-kDa cysteine-rich protein).

Table II. OmcB-specific, CD8\(^+\) T cell responses in primary cultures derived from Chlamydia-exposed donors\(a\)

<table>
<thead>
<tr>
<th>Donor</th>
<th>ELISPOT/s/well</th>
<th>Precursor Frequency to VSDTENTHIY</th>
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<tbody>
<tr>
<td>D48(^+)</td>
<td>18(^d)</td>
<td>1</td>
</tr>
<tr>
<td>CT13(^+)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CT18(^-)</td>
<td>165</td>
<td>0</td>
</tr>
</tbody>
</table>

\(a\) Purified CD8\(^+\) T cells (5 \times 10\(^3\)/well) from donor D48 or donor CT18 were stimulated with gamma-irradiated DC (5 \times 10\(^3\)/well). CD8\(^+\) T cell responses were quantified by IFN-\(\gamma\) ELISPOT.

\(b\) DC were pulsed with 0.5 \(\mu\)g/ml of the indicated peptides from OmcB.

\(c\) HLA* A0101-positive donor.

\(d\) Counts are the average of duplicate wells.

\(e\) HLA* A0101-negative donor.
early in the infection to invoke CD8+ T cell inhibition of chlamydial growth. Alternatively, OmcB may gain access late in infection, during synthesis in developing RBs. Even in this scenario, CD8+ T cells recognizing a late cycle Ag could alter the course of infection by mediating a protective effect via IFN-γ influences on surrounding cells not yet infected. In such a scenario, IFN-γ will arm additional aspects of an immune response, i.e., innate effectors, that could contribute to protection.

One can rule out an alternative processing pathway to explain how the OmcB-class I complex is formed because: 1) killed EBs are not recognized by the CD8-30 T cells; 2) recognition is sensitive to the proteasomal inhibitor lactacystin; and 3) recognition is also dependent on assembled class I complex transport through the Golgi (blocked by brefeldin A) (18). The hypothesized function of OmcB within the periplasmic space of EBs does not predict direct translocation to the host cytosol. However, this protein may have an unrecognized function in chlamydial biology, requiring its specific and directed translocation into the host cytosol. A dual function of chlamydial proteins is not unprecedented. For example, the structure and function of OmpA (MOMP) of Chlamydia vary dramatically between a highly disulfide-linked form in EBs (that functions possibly in adhesion) and a completely reduced form in RBs that functions (only in the non-cross-linked state) as a pore for ATP (44). Coincidently, MOMP has also been shown to be presented by MHC class I molecules (15–17), and consistent with MHC class I processing of this Ag, extracellular protein, cytosolic distribution of MOMP in infected cells has been recently demonstrated, where it is found associated with inclusion membrane protein A-laden filaments (45). Whether OmcB protein also directly localizes to the cytosol or has a cytosolic function in addition to its hypothesized membrane cross-linking function is unknown.

In previous studies we used murine CD8+ T cells to define two proteins (Cap1 and CrpA) from Chlamydia that also associate intracellularly with MHC class I (27, 30). Based on this observation, we correctly predicted their cytosolic localization; both these proteins directly interface with the host cytosol insofar as they have been found to be associated with the inclusion membrane. In addition, Cap1, like MOMP, has also been shown to be associated with cytosolic inclusion membrane protein A-laden filaments (45). Coincidently, the other CD8+ T cell Ag, CrpA (the CT442 product), was initially described in the same way as OmcB, as a cysteine-rich (15 kDa) protein (34), and CrpA maps adjacent to OmcB (CT443) in the Chlamydia genome (19). Whether these proteins are coregulated at some level or have related functions that explain their similar accessibility to the MHC class I processing pathway remains to be determined.

Identification of the OmcB HLA-A*0101 epitope allowed us to investigate further our initial observations that the CD8+ T cell clone CD8-30 does not lyse Chlamydia-infected cells, although it does secrete IFN-γ. This phenomenon does not seem to be due to a difference in epitope density on infected cells, because titration of the synthetic VSDTENTHYY peptide showed low levels of cytolytic activity at peptide concentrations that did not stimulate IFN-γ release. The ability of CD8-30 to lyse infected cells pulsed with synthetic peptide suggests that Chlamydiae are not actively interfering with lysis of infected cells, as has been shown to occur after stimulation of infected cells with several proapoptotic agents, including perforin/granzyme and Fas Abs (37). However, our experiments cannot rule out the possibility that live Chlamydia may actively inhibit killing of cells with the low levels of peptide associated with naturally processed Ag. Similarly, we cannot rule out that the naturally processed ligand is slightly altered from the synthetically defined epitope we have described, in a way that differentially activates the CTL.

Lytic activity is only one mechanism by which a CD8+ T cell can have a protective effect against an intracellular pathogen. As an in vitro surrogate measure of potential in vivo functionality of CD8-30 T cells, we tested whether this clone could inhibit chlamydial growth in infected cells. The CD8-30 T cells significantly inhibited chlamydial growth in HLA-A*0101-positive cells that were either infected or peptide-pulsed, but not in HLA-A*0101-negative cells. It is possible that inhibition of chlamydial replication by CD8-30 is mediated by secretion of IFN-γ. These findings correlate with the murine models of chlamydial infection, where CD8+ T cells play a role in protection by adaptive transfer, and this protection is mediated by secretion of IFN-γ (6). Furthermore, murine gene knockout experiments have shown that neither Fas nor perforin-mediated mechanisms are involved in the clearance of C. trachomatis (46) or C. pneumoniae (10) infections. The data presented in our report suggest that this may also be true in humans. OmcB-specific T cells could significantly decrease chlamydial replication in vivo through the production of IFN-γ, rather than by lytic mechanisms.

The data presented in this study show that CD8+ T cell responses specific to Chlamydia are present in exposed individuals. The possibility that the CD8-30 T cell clone was primed in vitro is unlikely, because a high peptide-specific precursor frequency was obtained from primary CD8+ T cells in both of the two HLA-A*0101 individuals tested. This finding also rules out the possibility that the OmcB-specific CD8+ T cell response was a spurious result from a single donor, because an even higher frequency was detected in an individual with a current C. trachomatis infection at the time of leukapheresis. Whether a natural, specific response to OmcB can contribute to immunity, or OmcB may, by proper formulation or delivery, invoke an immunoprotective response in humans remains to be determined. OmcB-specific CD8+ T cell precursor frequency analysis comparing either 1) individuals with PID vs asymptomatic Chlamydia-exposed subjects, or 2) individuals with scarring trachoma vs those with resolved trachoma, could give some insights into this question. To broaden the study population, other HLA epitopes within OmcB could be identified. The extent to which CD8+ T cells contribute to adaptive immunity in humans after natural infection with C. trachomatis has not been determined and may be limited. However, strategies to vaccinate and boost for specific CD8+ T cell responses may afford beneficial levels of protection, especially when combined with vaccination to invoke other arms of an adaptive response.

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References


