CD8⁺ T Cells Define an Unexpected Role in Live-Attenuated Vaccine Protective Immunity against *Chlamydia trachomatis* Infection in Macaques

Norma Olivares-Zavaleta, William M. Whitmire, Laszlo Kari, Gail L. Sturdevant and Harlan D. Caldwell

*J Immunol* published online 7 April 2014
http://www.jimmunol.org/content/early/2014/04/05/jimmunol.1400120

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/04/05/jimmunol.1400120.DCSupplemental

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
**CD8⁺ T Cells Define an Unexpected Role in Live-Attenuated Vaccine Protective Immunity against *Chlamydia trachomatis* Infection in Macaques**

Norma Olivares-Zavaleta, William M. Whitmire, Laszlo Kari, Gail L. Sturdevant, and Harlan D. Caldwell

Trachoma, caused by the obligate intracellular organism *Chlamydia trachomatis*, is the world’s leading cause of preventable blindness for which a vaccine is needed. We have previously shown that a plasmid-deficient live-attenuated trachoma vaccine delivered ocularly to macaques elicited either solid or partial protective immunity against a virulent ocular challenge. Solidly protected macaques shared the same MHC class II alleles implicating CD4⁺ T cells in superior protective immunity. Understandably, we sought to define T cell immune correlates in these animals to potentially improve vaccine efficacy. In this study, following a 2-y resting period, these macaques were boosted i.m., with the live-attenuated trachoma vaccine and their peripheral T cell anamnestic responses studied. Both solidly and partially protected macaques exhibited a CD4⁺ and CD8⁺ T cell anamnestic response following booster immunization. CD8⁺ but not CD4⁺ T cells from solidly protected macaques proliferated against soluble chlamydial Ag. We observed a more rapid T cell inflammatory cytokine response in tears of solidly protected animals following ocular rechallenge. Most notably, depletion of CD8⁺ T cells in solidly protected macaques completely abrogated protective immunity. Collectively, our findings support the conclusion that CD8⁺ T cells play an important but unexpected role in live-attenuated trachoma vaccine-mediated protective immunity. *The Journal of Immunology, 2014, 192: 000–000.*

**Materials and Methods**

**Nonhuman primates, vaccination, and chlamydial challenge**

Six cynomolgus macaques (Macaca fascicularis) described in our original LATV vaccine study were used (7). The animals were housed separately for all experimental studies in the nonhuman primate section at the Rocky Mountain Laboratories veterinary branch. All experimental procedures were performed in accordance with the Guidelines of the Institutional Animal Care and Use Committee. After a 2-y resting period, macaques were administered a series of three simultaneous i.m. (2 × 10⁶ inclusion-forming units [IFU]) and ocular (2 × 10⁸ IFU/eye) immunizations using the *C. trachomatis* plasmid-deficient LATV strain (A2497P⁰). Eight weeks following the last immunization macaques were ocularly challenged with virulent *C. trachomatis* A2497P⁰ organisms (2 × 10⁸ IFU/eye).

**Infection and disease evaluation**

Clinical evaluation and specimen collection for culturing chlamydiae were performed weekly. Chlamydial infection of the macaque conjunctival...
surface results in inflammation of subconjunctival tissues clinically scored as hyperemia and follicle formation. Hyperemia and follicle formation on the upper and lower conjunctivae of both eyes were scored by a veterinary pathologist. Hyperemia was scored as follows: 0, no hyperemia; 1, mild hyperemia; and 2, severe hyperemia. Follicles were scored as follows: 0, no follicles; 1, 1–3 follicles; 2, 4–10 follicles; 3, >10 follicles; and 4, follicles too numerous to count. The clinical disease score for a given animal was the aggregate scores of both hyperemia and follicle formation. The maximum clinical disease score was 24. After clinical pathological scoring, the surfaces of the upper and lower conjunctivae of both eyes were swabbed using a Calgiswab (Puritan, Guilford, ME). Ocular swabs were used to monitor chlamydial shedding by culturing organisms in monolayers of cycloheximide-treated HeLa 229 cells as previously described (13).

**PBL immunophenotyping**

Fluorochrome-conjugated Abs were incubated with 100 μl EDTA anti-coagulated whole blood for 30 min at room temperature. Abs used were anti–CD3–Alexa 700 (SP34–2), anti–CD4–FITC (L200), anti–CD20–allophycocyanin (2H7) (all from BD Biosciences San Jose, CA), and anti–CD8–PE (DK25; Dako, Carpinteria, CA). Erythrocytes were lysed with multispecies RBC lysis buffer (eBioscience, San Diego, CA) following the manufacturer’s instructions. Lysed specimens were washed once with 3 ml flow cytometry buffer and centrifuged for 5 min at 1200 rpm. Samples were analyzed for four-color immunofluorescence and lymphocytes gated based on forward and side scatter parameters using an LSRII flow cytometer (BD Biosciences, San Jose, CA) and Flowjo software version 8.8.6 (Tree Star, Ashland, OR). Total blood counts were calculated using a 950 FS hematology analyzer (Drew Scientific, Dallas, TX).

**Chlamydial soluble Ag**

Buffalo green monkey kidney (BGMK) cells were infected with *C. trachomatis* A2497P+ using a multiplicity of infection of 1. Infected BGMK monolayers were fed with Dulbecco’s minimal essential medium (Cellgro, Manassas, VA) supplemented with 10% cytomolysin serum (Innovative Research, Novi, MI) and 10 μg/ml gentamicin. Infected cells were incubated at 42° at 37°C. The monolayers were washed with HBSS, removed by scraping, and disrupted by sonication. Host cell debris was removed by centrifugation at 1500 rpm for 15 min at 4°C. The supernatant was collected and centrifuged at 15,300 rpm for 30 min at 4°C to pellet chlamydial organisms. The clarified supernatant was then centrifuged at 100,000 × g for 1 h at 4°C. The supernatant was collected and concentrated 10-fold using an Amicon Ultracel-10K (Millipore, Billerica, MA). The protein concentration was adjusted to 10 mg/ml and aliquots were stored at −80°C.

**Analysis of chlamydial-specific T cell immune response**

Chlamydial-specific T cell expansion and cytokine production from PBMCs was done as described (14). Briefly, CFSE (Molecular Probes)–labeled PBMCs were incubated at 2 × 10⁶ cell/ml in deep-well tissue culture plates (96-well; Eppendorf) in 1 ml AIM V medium (Life Technologies) only or pulsed with *C. trachomatis* A2497P+-infected BGMK cells (Fig. 3). The clarified supernatant was then centrifuged at 15,300 rpm for 30 min at 4°C to pellet chlamydial organisms. The clarified supernatant was then centrifuged at 100,000 × g for 1 h at 4°C. The supernatant was collected and concentrated 10-fold using an Amicon Ultracel-10K (Millipore, Billerica, MA). The protein concentration was adjusted to 10 mg/ml and aliquots were stored at −80°C.

In vivo depletion of CD8+ T cells and chlamydial challenge

Macaques received by s.c. injection 50 mg/kg anti-CD8 rhesus recombi-nant Ab MT807R-IgG1 (CDR-g, Nonhuman Primate Reagent Resource, University of Massachusetts Medical School, Boston, MA). Ab was administered two times on days 0 and 21. Blood samples were withdrawn weekly to immunophenotype and quantify CD8+ and CD4+ T cells. Following the second Ab injection animals were ocularly challenged with 2 × 10⁶ IFU/eye of virulent *C. trachomatis* A2497P+. Chlamydial ocular shedding and clinical disease scores were monitored weekly as described above.

**Statistical analyses**

Differences were compared by an unpaired t test (two-tailed, unequal variance).

**Results**

A systemic T cell anamnestic response occurs in vaccinated rested macaques following a combined i.m. and ocular booster immunization

Our first goal was to boost those ocularly vaccinated macaques used in our original LATV report (7) to generate T cell anamnestic responses in their PBLs sufficiently to study LATV-mediated T cell immunity. LATV-vaccinated macaques that were either SP or PP were rested for a 2-2 period and then given a combined i.m. and ocular booster immunization with the LATV (Supplemental Fig. 1). Prior to and after booster immunization we analyzed by flow cytometry the frequency and total numbers of CD4+ and CD8+ T cells in the PBLs of these animals (Fig. 1). Total T cell numbers for individual animals in each group and the respective means of the groups showed a significant increase in both CD4+ (p = 0.049) and CD8+ T cells (p = 0.009) in the PBLs of SP and PP animals. Thus, the LATV booster immunization recalled a systemic T cell anamnestic response.

Protective immunity in rested LATV-boosted macaques

We next challenged the LATV-boosted macaques ocularly with virulent plasmid-bearing A2497P+ trachoma organisms to confirm their protective immune status (Fig. 2). PP and SP monkeys were inoculated ocularly with 2 × 10⁶ IFU onto the conjunctival surface of both eyes and their infection and ocular clinical pathology were monitored (Fig. 2A, 2B). This challenge dose was 10-fold higher than that used in our initial vaccine efficacy study. A historical naive control group of similarly A2497P+-infected macaques (13) was used (RML 124, 126, and 134) to compare the level of protective immunity conferred by the vaccine (Fig. 2C). The challenged animals retained their original immune status being either PP (RML 145, 147, and 643) or SP (RML 641, 642, and 647) despite the 10-fold increase in challenge inoculum. SP animals, although colonized, presented with transient infections of low burden that completely resolved by day 21 postinfection. We observed no clinical pathology in two of the SP macaques (RML 641 and 642) and animal 647 exhibited minimal hyperemia without detectable follicle formation. Compared to SP animals, PP macaques shed greater numbers of organisms for longer periods that were accompanied by moderate clinical pathology scores. Nevertheless, both infection and disease in PP animals was patently less severe compared with infected naive controls.

**CD8+ T cells from SP macaques proliferate in response to chlamydial soluble Ag**

To determine whether the T cell anamnestic response of PP- and SP-boosted macaques was Ag-specific, CFSE-labeled PBMCs were stimulated in vitro with *C. trachomatis* A2497P+ EBs or an SA prepared from A2497P+-infected BGMK cells (Fig. 3). The SA contains the two well-characterized cytosolically secreted chlamydial virulence factors: chlamydial protease activity factor.
(CPAF) (15) and plasmid-encoded Pgp3 protein (Pgp3) (16) but lacks chlamydial major outer membrane protein and heat shock protein 60 (Supplemental Fig. 2). CD8⁺ T cells from both PP and SP macaques proliferated in response to SA; however, the proliferative response of CD8⁺ T cells from SP macaques was noticeably greater (Fig. 3A). In contrast, CD4⁺ T cells from PP or SP macaques failed to proliferate in response to SA (Fig. 3A). The CD4⁺ and CD8⁺ T cell responses against chlamydial EBs were variable and inconsistent in magnitude both within and between the groups (Fig. 3B). Intuitively, the preferential stimulation of CD8⁺ T cells by chlamydial SA is puzzling. A possible explanation for these findings could be that cytosolic CPAF protease in the SA targets chlamydial proteins for proteolytic degradation and these processed proteins are exogenously loaded onto MHC class I molecules of APCs (17, 18).

We next asked whether SA-stimulated CD8⁺ T cells from PP and SP macaques exhibited different surface markers that correlated with protective immunity. Flow cytometry analysis of SA-stimulated...
CD8+ T cells revealed expression of IFN-γ, αβ7 integrin, and granzyme B but we observed no differences in the staining intensity of these molecules between CD8+ T cells of PP and SP animals (Supplemental Fig. 3). Nonetheless, this finding is consistent with the conclusion that SA-stimulated CD8+ T cells possess functional characteristics capable of secreting IFN-γ, homing to mucosal tissues, and potentially functioning as cytolytic T cells against chlamydial-infected conjunctival epithelial targets.

A more rapid recall of T cell cytokines occurs in the tears of solidly protected macaques following infectious challenge

Schenkel et al. (19) described a role for circulating and resident memory CD8+ T cells in the elimination of intracellular pathogens in front line mucosal tissues through two contact-independent effector mechanisms: 1) cytolysis and 2) secretion of antiviral cytokines. These authors suggested that vaccines should establish both populations to augment rapid recall of protective immunity. We were not able to directly address a role for cytolytic CD8+ T cells in the present study. However, we did investigate whether PP and SP had a measurable difference in the temporal appearance of T cell anti-inflammatory cytokines in their tears following chlamydial ocular challenge (Fig. 4). Tears were collected from PP and SP animals on days 3 and 7 postchallenge with virulent A2497P+ and subjected to immunoassay for the detection of IL-2, IFN-γ, IL-12, IL-17, IL-5, IL-10, and MCP-1. Similar low levels of cytokines were found in the tears of both groups of animals 3 d following infectious challenge. Notably however, there was an increase in tear cytokines in all three SP animals on day 7 postchallenge, a time when their infections were resolving. A similar increase in cytokines after challenge was not observed in the tears of PP macaques. Interestingly, and consistent with the differences in inflammatory pathology that existed between the groups, the inflammatory cytokine IL-8 was only found in the tears of PP animals at day 7 (Fig. 2A). These results support the conclusion that SP macaques generated a more rapid recall of T cells to submucosal conjunctival tissue capable of secreting protective antichlamydial cytokines, such as IFN-γ, that may function in infection resolution. Thus, a rapid recall of T cell–secreted cytokines is at least in part a plausible explanation for the superior level of protective immunity observed in SP macaques. We next sought to determine whether the source of the T cell cytokine response in SP macaques was the result of CD4+ or CD8+ T cells.

In vivo depletion of CD8+ T cells in SP macaques abrogates protective immunity

Collectively our findings implicated T cells, specifically CD8+ T cells, as an important protective phenotype in SP macaques. To directly test this hypothesis we challenged SP animals prior to and following in vivo depletion of their CD8+ T cells (Fig. 5). Animals were administered two doses of anti-CD8+ mAb and the total
numbers of CD8\(^{+}\) T and CD4\(^{+}\) T cells in peripheral blood were determined by flow cytometry prior to and following a secondary chlamydial challenge. Treatment with anti-CD8\(^{+}\) Ab significantly reduced and sustained reduced numbers of CD8\(^{+}\) T cells in all animals without affecting the numbers of circulating CD4\(^{+}\) T cells (Fig. 5A). Remarkably, protective immunity against ocular infection was abrogated in all three SP animals (RML 641, 642, and 647) depleted of their CD8\(^{+}\) T cells (Fig. 5B). Infections resulted in an

![Graphs showing cytokine levels in tears of LATV-challenged macaques](image)

**FIGURE 4.** A rapid appearance of inflammatory cytokines in the tears of LATV-challenged macaques correlates with protective immune status. Ocular secretions (tears) were collected at 3 and 7 d after chlamydial challenge and were analyzed with a Luminex bead-based multiplex assay to measure local cytokines and chemokines. The graphs show cytokines representing the Th1/Th17 (IL-2, IFN-\(\gamma\), IL-12, IL-17) and Th2 (IL-5, IL-10) profiles and the chemotactic and proinflammatory cytokines MCP-1 and IL-8, respectively. Each bar represents the average of cytokine levels detected in PP and SP monkeys \(\pm\) SD. The SP macaques produced a more intense and rapid (day 7) production of Th1/Th2 cytokines and the MCP-1 chemokine. Interestingly, the proinflammatory cytokine IL-8 was not detected in the tears of solidly protected macaques but was found in partially protected animals, findings that are consistent with the ocular pathology exhibited by these animals.

![Graphs showing CD3+ T cell depletion](image)

**FIGURE 5.** Depletion of CD8\(^{+}\) T cells in solidly protected macaques abrogates LATV-mediated protective immunity. (A) The number of peripheral CD4\(^{+}\) and CD8\(^{+}\) T cells in SP macaques (RML 641, 642, and 647) after s.c. administration of two doses of anti-CD8 Ab. A marked decrease (\(\geq 88\%\)) of CD3\(^{+}\) CD8\(^{+}\) (triangles), but not CD3\(^{+}\)CD4\(^{+}\) (squares) T cells was detected following administration of the first dose of Ab. Following the second dose, specific depletion of CD8\(^{+}\) was sustained over the entire experimental period (90 d). Macaques were challenged with \(2 \times 10^{4}\) IFU virulent *C. trachomatis* A2497P\(^{+}\) EBs prior to and after depletion of CD8\(^{+}\) T cells. (B) Infection pre- and postdeletion of CD8\(^{+}\) T cells. (C) Ocular pathology pre- and postdeletion of CD8\(^{+}\) T cells. Depletion of CD8\(^{+}\) T cells completely abrogated the ability of SP macaques to resist challenge infection. Infections in CD8\(^{+}\)-depleted animals resulted in greater chlamydial conjunctival burdens that persisted for up to 56 d after challenge. In contrast, the same LATV-immunized macaques infected prior to depletion of CD8\(^{+}\) T cells shed less organisms from their conjunctive and spontaneously resolved infection between 21 and 28 d after challenge.
increased burden of 10-fold in two animals (RML 641 and 647) and there was dramatic extension in shedding duration in all animals that persisted for 42–56 d postinfection. Surprisingly, these exacerbated infections produce a more variable outcome in ocular pathology (Fig. 5C). A single animal (RML 647) presented with severe clinical disease score over the entire infection period; in contrast, only moderate to minimal ocular pathology was observed in animals RML 641 and 642 despite their high infectious burdens. This discordance between infection and ocular disease is not understood.

Discussion
In summary, we present evidence that solid protective immunity elicited by the LATV is mediated by CD8+ T cells. It is unclear how this immunity is generated by the attenuated vaccine. Circulating CD8+ T cells are separated into two functional subsets termed central memory T and effector memory T cells (20). Central memory T cells are long-lived with a greater proliferative capacity upon re-exposure to pathogens that have the capacity to permanently reside in peripheral tissues including the mucosa (18, 21), termed tissue-resident memory T cells. Thus, for reasons not understood at this time, SP-vaccinated macaques may have selectively generated central memory T cell and tissue-resident memory T cells capable of trafficking rapidly to and being retained at the ocular mucosa that were then highly efficient in controlling chlamydial infection. The rapid appearance of T cell inflammatory cytokines in the tears of SP macaques following infection challenge, consisting of IFN-γ, a potent in vitro antichlamydial inhibitory cytokine (22), supports this hypothesis. A role for CD4+ T cells in SP LATV protective immunity, either independently or collectively with CD8+ T cells, cannot be ignored, however, as we previously found a correlation between MHC class II alleles and CD8+ T cells become unconventional but superior targets for Ag presentation occurs without internalization or processing of exogenous antigenic peptides. J. Immunol. 168: 191–200.

References
8. Su, H., and H. D. Caldwell. 1995. CD4+ T cells play a regulatory role in the differentiation or retention of protective tissue-resident memory T cells in the conjunctival mucosa.

The principal questions that emerge from our past (7) and present study are: 1) what is the molecular basis for the dramatic infection attenuation of the trachoma plasmid-deficient vaccine strain, and 2) how does this strong attenuation phenotype play a role in the enhanced ability of the host to generate a protective CD8+ T cell immune response? Indisputably, in models of murine urogenital tract infection with virulent plasmid-bearing chlamydial organisms CD8+ T cells are not an important protective phenotype despite the obligate intracellular life-style of the pathogen (11, 23). In contrast, infection of mice generates a protective immune response that is dominated by CD4+ T cells (8–12). Although CD4+ are capable of conferring protective immunity they may be inferior to CD8+ T cell–mediated immunity elicited by the LATV. A logical conclusion from these contrasting immune outcomes is that virulent plasmid-bearing chlamydiae avoid CD8+ T cell immunity by an unknown plasmid function. Perhaps in the absence of plasmid-encoded virulence factors infected epithelial cells become unconventional but superior targets for Ag presentation by dendritic cells that are very proficient in the generation of protective memory CD8+ T cells (18). Interestingly, a recent report (24) found that the immunomodulatory ligand PD-L1 contributes to this defective CD8+ T cell response in mice, as inhibition of PD-L1 restores protective antichlamydial CD8+ T cell immunity. It is not clear how these observations might be related to our findings but it is tempting to speculate that they are associated with plasmid genes or plasmid-regulated genes (25–27) that upregulate PD-L1 in chlamydial-infected target cells.

Collectively, defining a role for the chlamydial plasmid in modulating CD8+ T cell immunity is a challenging goal toward understanding chlamydial pathogenesis as it relates to avoidance of adaptive T cell immunity. Studies aimed at molecularly defining the role of plasmids in host cell interactions that impact immunity might come from performing comparative transcriptional and proteomic studies on plasmid-bearing and plasmid-deficient infected epithelial cells, as well as employing similarly infected epithelial cells as targets to assay CD8+ T cell cytotoxic function.

Acknowledgments
We thank Dr. Keith Reimann for assistance in T cell depletion experiments, the Rocky Mountain Veterinary Branch of Rocky Mountain Laboratories for assistance with nonhuman primates, Aaron Carmody for flow cytometry assistance, Drs. Guangming Zhong and Lihua Song for CPAF and Pgp3 mAbs, respectively, Heather Murphy and Aniita Mora for graphical art assistance, and Kelly Matteson for administrative assistance in preparation of this manuscript.

Disclosures
The authors have no financial conflicts of interest.
LATV vaccination of macaques (7)

Simultaneous boost (intramuscular/ocular) with LATV

Challenge with *C. trachomatis* A2497P+ high dose (2x10^5 IFU/eye)

Anti-CD8 antibody injections

Challenge with *C. trachomatis* A2497P+ (2x10^4 IFU/eye)

---

**Figure S1. A schematic illustration of the experimental design used in this study.**
The cynomolgus macaques described in the initial study were used (7). After two years of rest (time= 0), animals were repeatedly boosted with a simultaneous intramuscular/ocular dose of LATV (filled triangles). Eight weeks after the second booster immunization, macaques were challenged ocularly with a ten fold higher dose of the virulent plasmid-bearing *C. trachomatis* strain A2497P+ (filled arrow). To evaluate the CD8^+ T cell function in the LATV conferred protection, SP macaques received a subcutaneous injection of an anti-CD8 rhesus recombinant antibody (open squares). Three weeks following the initial injection, animals received a second dose of anti-CD8 antibody (open square) and simultaneously were ocularly challenged with 2 x10^4 IFU/eye (open arrow).
Figure S2. Western blots of *C. trachomatis* A2497 SA and EBs probed with monoclonal antibodies specific for chlamydial secreted and structural antigens. Western blotting of SA and EBs probed with Mabs specific to chlamydial protease activity factor (anti-CPAF), plasmid-encoded Pgp3 protein (anti-Pgp3), serovar A major outer membrane protein (anti-MOMP), and chlamydial heat shock protein 60 (anti-HSP60). Note that the secreted chlamydial protease CPAF is found primarily in the SA antigen preparation.
Figure S3. Chlamydial CD8⁺ T cells responding to SA express IFN-γ, granzyme B and α4β7 integrin. CFSE-PBMC antigen stimulated for five days and re-stimulated for additional six hours were subjected to a multi-color flow cytometry assay. Frequencies for CD8⁺ antigen-specific IFN-γ⁺, α4β7⁺, GrB⁺ and IFN-γ⁺, α4b7⁺ GrB⁺ responses were determined. The percentages of these T cell phenotypes found in PP (Fig. S3A) and SP (Fig. S3B) animals are shown in the pie charts. No differences in the frequency of individual surface markers or a combination of markers in SA stimulated CD8⁺ T cells were detected between the groups.