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 Oliva-res-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 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.

*C. trachomatis* is an obligate intracellular bacterial pathogen and the etiologic agent of blinding trachoma, the world’s leading cause of infectious blindness. Trachoma affects hundreds of millions of people in sub-Saharan Africa and Asia and is recognized as one of the major neglected tropical infectious diseases of the developing world (1). Current trachoma control strategies of the World Health Organization are focused on mass antibiotic treatment of infected children in hyperendemic areas in attempts to reduce infection rates and burdens in children, interrupting the cycle of transmission and reinfection in adults who are at the greatest risk of developing blinding disease. Despite its benefits, there is debate on its overall effectiveness and sustainability (2). An alternative strategy for trachoma control is vaccination (3). In the face of decades of efforts toward this end, there has been little meaningful progress in the development of a trachoma vaccine using subunit immunogens (4–6).

Recently, we described a plasmid-deficient live-attenuated trachoma vaccine (LATV) that was safe, immunogenic, and protective in macaques (7). We reported that macaques immunized with the LATV were either solidly protected (SP) or partially protected (PP) following challenge with virulent trachoma organisms. SP macaques exhibited transient ocular infections that cleared spontaneously without detectable ocular pathology. SP macaques shared the same MHC class II alleles, implicating CD4+ T cells in superior vaccine-mediated immunity, a finding consistent with the paradoxical but unambiguous role of CD4+, not CD8+, T cells in chlamydial murine models of infection (8–12). Regardless, because of the exceptional level of protective immunity generated by the LATV in a relevant nonhuman primate animal model, we sought to better define the role of T cells in vaccine-mediated immunity. We deemed this to be an important goal since it could lead to knowledge for improving LATV efficacy in humans and the future development of a more conventional subunit trachoma vaccine.

In this study, previously LATV-vaccinated macaques were rested for a period of 2 years and then administered simultaneous i.m. and ocular vaccine booster immunizations to facilitate the study of chlamydial-specific T cell anamnestic responses in their PBls. Unexpectedly, we report CD8+ T cells play a critical role in LATV-mediated solid protective immunity.

**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 × 10³ inclusion-forming units [IFU]) and ocular (2 × 10⁸ IFU/eye) immunizations using the *C. trachomatis* plasmid-deficient LATV strain (A2497P). Eight weeks following the last immunization macaques were ocularly challenged with virulent *C. trachomatis* A2497P 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 conjunctiva 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 conjunctiva of both eyes were swabbed using a Calgiswab (Puritan, Guilford, ME). Ocular swabs were used to monitor chlamydial shedding by culturingorganisms 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-PE-TC (L200), anti-CD20-PE-Cy5 (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 for 42 h 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 13,500 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 cells/ml in deep-well tissue culture plates (96-well; Eppendorf) in 1 ml AIM V medium (Life Technologies) for 3 days. Infected cells were incubated for 42 h 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 13,500 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 then 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.

**Collection of tears and inflammatory cytokine analyses**

A polyvinyl acetal ophthalmologic mini sponge (Merocel, Beaver Visitec, Waltham, MA) was placed on the outer third of the lower eyelid margin of both eyes. After 5 min of tear collection, the sponge was recovered and placed in a 2-ml screw cap tube containing 500 μl PBS. The sponges were incubated for 30 min at 4°C and the liquid sample was eluted by centrifugation and stored at −80°C until use. Cytokines and chemokines were detected using a multiplexed microsphere-based suspension array (Luminex xMAP). Tear samples were analyzed in duplicate using the nonhuman primate cytokine magnetic bead panel PCYTMG-40K-PX23 (EMD Millipore, Billerica, MA).

**In vivo depletion of CD8⁺ T cells and chlamydial challenge**

Macques received by s.c. injection 50 mg/kg anti-CD8 rhesus recombinant 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 immuneophenotype and quantify CD8⁺ and CD4⁺ T cells. Following the second Ab injection animals were ocularly challenged with 2 × 10⁷ 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-y 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 × 10⁷ 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. 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, α4β7 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 strain 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.

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](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-γ, IL-12, IL-17) and Th2 (IL-5, IL-10) profiles and the chemotactant and proinflammatory cytokines MCP-1 and IL-8, respectively. Each bar represents the average of cytokine levels detected in PP and SP monkeys ± 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 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 (∼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 postdepletion of CD8+ T cells. (C) Ocular pathology pre- and postdepletion 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 conjunctivae 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 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 cytokine 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 SP animals, whereas no correlation was found with any MHC class I or class II alleles (7). Perhaps 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 cells–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 Reimmann for assistance in T cell depletion experiments, the Rocky Mountain Veterinary Branch of Rocky Mountain Laboratories for assistance with nonhuman primates, Aaron Carmdowy for flow cytometry assistance, Drs. Guangming Zhong and Lihua Song for CPAF and Pgp3 mAbs, respectively, Heather Murphy and Anita 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.

References

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$^+$, GraB$^+$ and IFN-γ, α4b7$^+$ GraB$^+$ 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.