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Chlamydia trachomatis Infection Alters the Development of Memory CD8$^+$ T Cells[1]

Wendy P. Loomis and Michael N. Starnbach[2]

The obligate intracellular bacterium *Chlamydia trachomatis* is the most common cause of bacterial sexually transmitted disease in the United States and the leading cause of preventable blindness worldwide. Prior exposure to *C. trachomatis* has been shown to provide incomplete protection against subsequent infection. One possible explanation for the limited immunity afforded by prior *C. trachomatis* infection is poor activation of *Chlamydia*-specific memory CD8$^+$ T cells. In this study, we examined the development of CD8$^+$ memory T cell responses specific for the *Chlamydia* Ag CrpA. The percentage of CrpA$_{63–71}$-specific T cells expressing an effector memory T cell phenotype (IL-7R$^+$ CD62^{low}) was dramatically diminished in mice immunized with *C. trachomatis*, compared with mice immunized with vaccinia virus expressing the CrpA protein. These alterations in memory T cell development were correlated with a significant reduction in the capacity of convalescent mice to mount an enhanced recall response to *Chlamydia* Ags, compared with the primary response. CrpA-specific memory T cells primed during VacCrpA infection also failed to respond to a challenge with *Chlamydia*. We therefore investigated whether *C. trachomatis* infection might have a global inhibitory effect on CD8$^+$ T cell activation by coinfecting mice with *C. trachomatis* and *Listeria monocytogenes* and we found that the activation of *Listeria*-specific naive and memory CD8$^+$ T cells was reduced in the presence of *C. trachomatis*. Together, these results suggest that *Chlamydia* is able to alter the development of CD8$^+$ T cell responses during both primary and secondary infection, perhaps accounting for the incomplete protection provided by prior *Chlamydia* infection. *The Journal of Immunology,* 2006, 177: 4021–4027.

Model pathogens such as *Listeria monocytogenes* and lymphocytic choriomeningitis virus (LCMV)[3] have been extremely useful for characterizing the in vivo dynamics of both primary and secondary CD8$^+$ T cell responses to infection. Using these model systems, it has been shown that, following recognition of Ag, naive CD8$^+$ T cells become activated and undergo a massive expansion that peaks at around 7 days postinfection (p.i.). The subsequent contraction phase results in the elimination of ~90% of the T cells, leaving a stable number of memory T cells. These memory cells are then maintained for extended periods of time and are able to respond much more quickly and to a greater magnitude than naive cells when challenged with the same Ag (1–4).

The characterization of memory CD8$^+$ T cell development has recently become an active area of investigation. Several reports have shown that, following initial activation of a CD8$^+$ T cell response, a small percentage of the primary effector cell population up-regulates cell surface IL-7R (CD127) expression (5, 6). The IL-7R$^+$ cells can be detected within 7 days of immunization, and it is this T cell population that is believed to give rise to memory cells. The IL-7R$^+$ memory cell population can be further subdivided based on expression of other cell surface markers, such as the lymph node homing receptor CD62L (L-selectin) (5, 7). Central memory T cells (T_{CM}), which express CD62L, reside preferentially in lymphoid organs, lack immediate effector functions, and are characterized by vigorous homeostatic and Ag-driven proliferation. Conversely, low-level expression of CD62L by peripheral effector memory T cells (T_{EM}) promotes their preferential migration to nonlymphoid tissues. T_{EM} cells proliferate poorly but are able to mediate immediate effector functions, such as IFN-γ secretion and lysis of infected cells, upon re-encountering Ag in the peripheral tissues (8).

Both CD4$^+$ and CD8$^+$ T cells have been investigated for their protective role in immunity to the intracellular bacterial pathogen *Chlamydia trachomatis*. Mice lacking a CD4$^+$ T cell response, as a result of mutations in either CD4 or MHC class II, were found to be considerably more susceptible to *C. trachomatis* infection than mice carrying a mutation in β_2-microglobulin (which lack MHC class I and are therefore impaired in CD8$^+$ T cell responses), suggesting a more dominant role for CD4$^+$ T cells (9). Others have compared the role of CD4$^+$ and CD8$^+$ cells using spleen cells isolated from convalescent animals (10). Abs were used in vitro to deplete either the CD4$^+$ or CD8$^+$ T cells and then the resulting CD8$^+$- or CD4$^+$-enriched spleen cell populations were returned i.v. to infected mice. In these experiments, only the CD4$^+$ cells were able to reduce the time course of infection. These experiments and others suggest that CD4$^+$ T cells may be the main effectors of both primary and secondary immunity following natural infection (11).

The limited contribution of CD8$^+$ T cells to clearance was unexpected given what we know about the biology of *Chlamydia* infection. *C. trachomatis* is a vacuolar pathogen that primarily infects genital tract epithelial cells. Unlike professional APCs, such as macrophages and dendritic cells, epithelial cells are unable to present pathogen-derived Ags in the context of MHC class II to CD4$^+$ T cells. Therefore, CD8$^+$ T cell surveillance should be the

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[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; p.i., postinfection; T_{EM}, effector memory T cell; T_{CM}, central memory T cell; EB, elementary body; IFU, inclusion-forming unit; KO, knockout; Treg, regulatory T cell.

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primary mechanism for recognition of *Chlamydia*-infected epithelial cells. In fact, $CD8^+$ T cells have been shown to recognize *Chlamydia*-encoded Ags during *C. trachomatis* infection (12–14). We also have successfully protected mice against *C. trachomatis* infection by immunizing with recombinant vaccinia virus expressing these *Chlamydia*-specific $CD8^+$ T cell Ags and by adoptively transferring cultured *Chlamydia*-specific $CD8^+$ T cell lines into naive mice (12, 15). These results suggest that $CD8^+$ T cells are capable of controlling *Chlamydia* growth yet fail to act during natural infection.

Because we expected that $CD8^+$ T cells should be playing a more important role in controlling *C. trachomatis* infection, we decided to characterize the development of *Chlamydia*-specific $CD8^+$ T cell responses. In this study, we report that the T cell response to *Chlamydia* Ags does not follow the kinetics observed using model pathogens such as *L. monocytogenes* and LCMV. Instead, *C. trachomatis* infection appears to alter the development of memory $CD8^+$ T cells and, as a result, is able to suppress the recall response upon subsequent infection.

Materials and Methods

Infection of mice

Female C57BL/6 (H-2^b), CByB6F1 (H-$2^{b/d}$), and B6.129P2-*IL10*^{tm1Cgn} (IL10 knockout (KO)) mice were obtained from The Jackson Laboratory and used at 7–12 wk of age. Elementary bodies (EBs) of *C. trachomatis* serovar L2 434/Bu were propagated within and titered on McCoy cell monolayers, as previously described (12). EBs were diluted in sucrose-phosphate-glutamate buffer before immunization. Mice were infected i.v. with 10^7 inclusion-forming units (IFU) in 200 μl of buffer unless otherwise noted. We generated UV-inactivated *C. trachomatis* organisms by subjecting EBs to 10 min of UV irradiation. Construction of the recombinant vaccinia virus expressing the *Chlamydia* CrpA protein is described elsewhere (12). Virus preparations were treated with an equal volume of 0.25 mg/ml trypsin for 30 min at 37°C and diluted in PBS (10^6 PFU/200 μl) before immunization of mice. *Listeria monocytogenes* strain JJL-OVA (16) were grown to stationary phase in brain-heart infusion broth, aliquoted, titered, and stored at −80°C. Before immunization, bacteria were thawed on ice, grown to early exponential phase in brain-heart infusion broth, and diluted in PBS. Mice were infected i.v. with ~10^3 CFU of *Listeria* in 200 μl of PBS.

ELISPOT analysis

ELISPOT analysis of $CD8^+$ T cell responses following immunization with *C. trachomatis*, *L. monocytogenes*, or rVacCrpA was performed as described previously (12). The length of time postimmunization necessary to reach the peak of the $CD8^+$ T cell response and the peptides used to stimulate the T cells varied depending on the immunogen. For animals infected with *Chlamydia*, spleen cells were harvested 6 days postimmunization and stimulated on $CrpA_{63–71}$-coated EL4 thymoma (H-2^b) cells. For those infected with VacCrpA, spleen cells were harvested 7 days postimmunization and stimulated on $CrpA_{63–71}$-coated EL4 cells. Spleens from *Listeria* (JJL-OVA)-infected mice were harvested 7 days postimmunization and stimulated on EL4 cells coated with synthetic $OVA_{257–264}$ peptide. Peptides were purchased from Bio-Synthesis. The results are reported as the total number of spleen cells that secrete IFN-γ in response to the appropriate peptide.

Phenotypic characterization of memory cell populations using flow cytometry

The D^b/ASFVNPIYL ($CrpA_{63–71}$) MHC tetramer (allophycocyanin-conjugated) was generated at the National Institutes of Health Tetramer Facility (Bethesda, MD). Splenocytes were harvested from infected mice on the indicated days p.i. RBC were lysed, and the spleen cell suspension was enriched for $CD3^+$ T cells using mouse T cell enrichment columns (R&D Systems). The resulting T cell populations were stained with the MHC tetramer as well as Abs against surface markers IL-7R (CD127) and CD62L (BD Pharmingen) and then sorted on a FACSCalibur flow cytometer (BD Biosciences).

Doxycycline treatment of mice

C57BL/6 mice were infected i.v. with 10^7 IFU of *C. trachomatis* as described above. Half of the mice received daily i.p. injections of doxycycline on days 7–21 p.i. The course of treatment was based on experiments conducted by Caldwell and colleagues (17). Each mouse received 100 μl of 3 mg/ml doxycycline hyclate (30 g per mouse at 10 mg/kg; Sigma-Aldrich). Following antibiotic treatment, mice were allowed to rest for 7 days to allow for degradation of residual doxycycline before challenging both the treated and untreated mice with *C. trachomatis* on day 28. The number of $CrpA_{63–71}$-specific $CD8^+$ T cells was measured 5 days later using ELISPOT analysis.

Results

Chlamydia-*specific CD8$^+$ T cell recall responses appear to be inhibited*

C. trachomatis infection has been shown to stimulate a robust CrpA-specific T cell response in naive mice with ~4% of the splenic $CD8^+$ T cells specific for $CrpA_{63–71}$ at the peak of the response (Ref. 12 and Fig. 1*A*). The number of CrpA-specific T cells then declines ~10-fold, leaving a population of memory cells. To test the magnitude and kinetics of a T cell response to secondary *Chlamydia* infection, immune mice were challenged with *C. trachomatis* on day 28 postimmunization. The magnitude

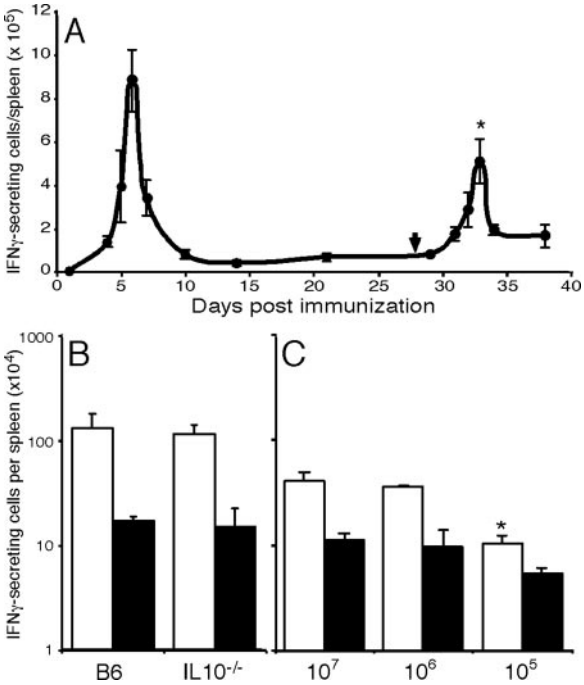

FIGURE 1. $CrpA_{63–71}$-specific T cell responses are suppressed during secondary infection with *C. trachomatis*. *A*, CByB6F1 mice were infected i.v. with 10^7 IFU of *C. trachomatis*. On various days p.i., spleens were harvested from three mice, and the number of CrpA-specific T cells was measured using IFN-γ ELISPOT analysis. To monitor the kinetics of the secondary response, immune mice were challenged with *Chlamydia* on day 28 p.i. (arrow), and the number of CrpA-specific T cells was measured on various days postchallenge. *, $p < 0.05$ (paired *t* test). *B*, C57BL/6 (B6) and IL10 KO mice were infected with *Chlamydia*, and the number of CrpA-specific T cells was measured on day 6 p.i. (□). The remaining immune mice were then challenged with *Chlamydia*, and the CrpA-specific T cell response was measured on day 5 postchallenge (■). *C*, C57BL/6 mice were infected with decreasing doses of *C. trachomatis*, and the number of CrpA-specific T cells was measured on day 6 p.i. (□). **, $p < 0.02$ (paired *t* test comparing 10^5 with 10^7 values). Immune mice were challenged with *Chlamydia*, and the CrpA-specific T cell response was measured on day 5 postchallenge (■).

of the $CrpA_{63–71}$-specific secondary response was found to be significantly smaller than that of the primary response (Fig. 1*A*). In addition, the recall response to *Chlamydia* did not peak much earlier than it did during the primary response (day 5 vs day 6 postimmunization). These observations were in contrast to what has been shown for other pathogen-specific responses where recall is significantly enhanced, compared with the primary response. The diminished recall response also was seen with a second *Chlamydia* Ag, Cap1, suggesting that this is a general phenomenon of *Chlamydia*-specific T cell responses (data not shown).

One possible explanation for reduced expansion of memory cells is that *C. trachomatis* infection induces the activation of *Chlamydia*-specific regulatory T cells (T_{reg}). Upon challenge, these T_{reg} would reactivate and secrete immunosuppressive cytokines, such as IL-10 and TGFβ, that are capable of suppressing the proliferation of CrpA-specific memory T cells. To test whether the secretion of IL-10 by T_{reg} is responsible for the diminished secondary response to *Chlamydia* infection, we compared the CrpA-specific T cell response in wild-type C57BL/6 mice to the recall response in IL-10 KO mice (Fig. 1*B*). If IL-10-mediated suppression is involved, we would expect to see increased expansion of the CrpA-specific memory cells following challenge of the KO animals. However, the absence of IL-10 had no effect on the magnitude of the secondary response. Although we have not yet tested the role of TGFβ in this process, the results in the IL-10 KO mice suggest that regulatory T cells are not responsible for the suppression of the secondary T cell responses.

We next sought to exclude the possibility that the reduced magnitude of the secondary response was simply a result of efficient clearance of infected cells before significant numbers of the memory T cells had an opportunity to respond. It has been shown that the magnitude of the primary response dictates the level of resting memory cells in immune mice, with a higher primary response leading to a greater number of memory cells (16). These memory cells respond to a secondary infection by proliferating and lysing infected cells. If the bacterial load is too low (or the number of memory cells is too high), the bacteria will be cleared before all the memory cells are reactivated, thus diminishing the apparent magnitude of the secondary response. To test whether the recall response could be enhanced by decreasing the number of CrpA-specific memory cells, we immunized naive mice with decreasing doses of *C. trachomatis*. The number of $CrpA_{63–71}$-specific T cells was measured at the peak of the primary response on day 6 p.i. using ELISPOT analysis (Fig. 1*C*, □). As expected, the number of CrpA-specific T cells decreased as the immunizing dose was reduced. We then challenged the groups of mice that had been immunized with the decreasing doses of organisms with 10^7 IFU of *C. trachomatis* and measured the number of CrpA-specific T cells at the peak of the secondary response (Fig. 1*C*, ■). Mice that had been immunized with 10^5 IFU of *C. trachomatis* showed a lower recall response to $CrpA_{63–71}$ than mice that had received higher doses. Because reducing the number of memory cells did not enhance the subsequent recall response, we concluded that the suppression of the recall response results from diminished reactivity of the memory T cells, and not from efficient clearing of Ag.

Chlamydia-*primed CD8$^+$ T cells are limited in their capacity to develop into memory cells*

The apparent defect in memory cell function led us to test whether *C. trachomatis* infection alters the development of memory cells specific for *Chlamydia* Ags. We measured the activation of memory T cells specific for $CrpA_{63–71}$ following immunization with *C. trachomatis* and compared it to the level of activation of the same population of naive CrpA-specific T cells observed following infection with a recombinant vaccinia virus expressing CrpA (VacCrpA) (12). Splenocytes were harvested from mice infected with either *C. trachomatis* or VacCrpA and stained with $D^b/CrpA_{63–71}$ MHC tetramer and Abs against IL-7R and CD62L. On day 14 p.i., *Chlamydia*-immunized mice possessed a significantly lower percentage of CrpA-specific IL-$7R^+$ memory cells than VacCrpA-immunized mice (Fig. 2*A*). This reduction in memory cell activation was most pronounced in the $CD62L^{low}$ subpopulation, suggesting that it is the development of *Chlamydia*-specific effector memory T (T_{EM}) cells that is inhibited during *Chlamydia* infection.

It is conceivable that normal memory cell populations are simply slower to develop in *Chlamydia*-infected animals than in VacCrpA-infected animals. If this were true, we would expect to see a higher number of CrpA-specific memory cells if we waited longer. To determine whether the percentage of T_{EM} cells in *Chlamydia*-infected mice increased over time, memory T cell populations were examined on days 21 and 35 p.i. when T cell memory should be firmly established and fully ready to respond to challenge. As expected, we observed a steady increase in the IL-$7R^+CD62L^{low}$ T_{EM} population between days 14 and 35 p.i. in the *Chlamydia*-infected animals, though the percentage of T_{EM} cells was still lower than in the VacCrpA-infected mice (Fig. 2*A*). Because the memory cell populations were increasing with time, it seemed possible that the magnitude of the recall response might improve if the challenge dose was given later following the primary infection. To test whether the day of the challenge had an effect on the secondary response, groups of mice were immunized with 10^7 IFU of *C. trachomatis* and then challenged after 14, 21, 28, or 35 days. The number of $CrpA_{63–71}$-specific T cells was then measured. As shown in Fig. 2*B*, waiting longer to challenge the mice had no effect on the magnitude of the CrpA-specific recall response. These data suggest that it is not simply the number of memory cells present that dictates the magnitude of the secondary response. Instead, there appears to be a functional defect in the *Chlamydia*-specific memory T cells that prevents them from responding to a subsequent challenge.

It has recently been shown that infections with persistent pathogens, such as HIV and certain strains of LCMV, lead to a decrease in the number of IL-$7R^+$ memory cells and a subsequent loss of memory cell function (18–20). Interestingly, there is evidence that *Chlamydia* may also persist in vivo (21, 22) suggesting the possibility that the reduced activation of *Chlamydia*-specific T_{EM} cells and subsequent failure of immune mice to mount a robust recall response may result from continuous low level T cell stimulation by persistent *Chlamydia* Ag. To test whether the elimination of organisms that may be persisting below detectable levels would improve the ability of *Chlamydia*-specific memory cells to respond to antigenic challenge we treated mice with the antibiotic doxycycline before challenge. Treatment of mice with doxycycline within the first 3 days of infection has been shown to alter development of primary $CD4^+$ T cell responses by reducing the levels of available Ag (17). To ensure adequate priming of naive *Chlamydia*-specific $CD8^+$ T cells, we waited 7 days after we infected mice with *C. trachomatis* before initiating antibiotic treatment. We then treated the mice daily with doxycycline from days 7–21 p.i.. Animals were removed from antibiotic treatment for 7 days and then both doxycycline-treated and untreated mice were re-infected with *C. trachomatis*. When we measured the number of $CrpA_{63–71}$-specific T cells we found that doxycycline treatment had no effect on the magnitude of the CrpA-specific secondary response (Fig. 2*C*), suggesting that reducing the number of persistent organisms using antibiotic treatment failed to improve the ability of *Chlamydia*-specific memory cells to respond to challenge.

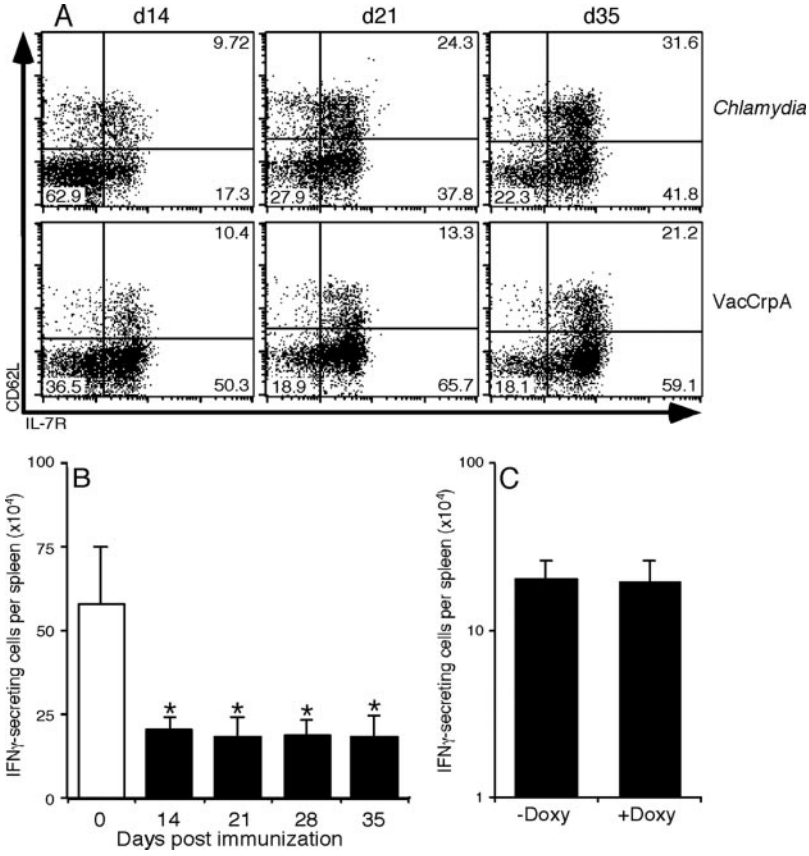

FIGURE 2. *Chlamydia*-specific memory T cell populations change over time, but increased IL-7R expression does not correlate with improved secondary T cell responses. *A*, C57BL/6 mice were infected with *C. trachomatis* (*top panels*) or VacCrpA (*bottom panels*) for 14, 21, or 35 days. T cell-enriched spleen cell populations were then stained with anti-IL-7R and anti-CD62L Abs and gated on $D^b/CrpA_{63–71}$ tetramer$^+$ cells. Numbers represent the percentage of tetramer$^+$ cells in each quadrant. *B*, Mice were immunized with 10^7 IFU of *C. trachomatis* for 0, 14, 21, 28, or 35 days and then challenged with *Chlamydia*. The number of $CrpA_{63–71}$-specific T cells was measured using ELISPOT analysis either on day 6 p.i. for the naive week 0 mice (□) or day 5 postchallenge for the immunized mice (■). ∗, $p < 0.01$ (paired *t* test). *C*, C57BL/6 mice were infected with *C. trachomatis* for 7 days, and then half of the mice were treated with doxycycline for 14 days. On day 28 p.i., both antibiotic-treated and untreated mice were challenged with *Chlamydia*, and the number of CrpA-specific T cells was measured 5 days later at the peak of the secondary response.

C. trachomatis *infection poorly reactivates memory cells primed by VacCrpA*

The results described above suggested that there is a functional defect in *Chlamydia*-induced memory cells that prevents them from responding to an antigenic challenge. However, it is also possible that the memory cells are functionally normal but that *C. trachomatis* infection fails to activate them. To test whether CrpA-specific memory cells are capable of responding to antigenic stimulation, *Chlamydia*-immune mice were challenged with either *C. trachomatis* or VacCrpA (Fig. 3). Significantly more CrpA-specific T cells were observed when the *Chlamydia*-immune mice were challenged with VacCrpA than when they were challenged with *C. trachomatis* ($p < 0.001$). We also found that *Chlamydia* infection poorly reactivated CrpA-specific memory cells in VacCrpA-immunized mice (Fig. 3). We were unable to compare the secondary response following *Chlamydia* infection to the same response following challenge with VacCrpA because expansion of immunodominant vaccinia-specific T cells overwhelms the CrpA-specific response (Ref. 23 and our unpublished observations). Together, these data suggest that the *Chlamydia*-induced memory cells are capable of responding reasonably well to Ag but that *C. trachomatis* infection may not provide an optimal environment for stimulating *Chlamydia*-specific T cells.

Chlamydia *infection alters the development of* Listeria*-specific CD8$^+$ T cell responses*

To further test whether *C. trachomatis* infection can inhibit the development of CD8$^+$ T cell responses, we tested whether the presence of *Chlamydia* could affect the activation of CD8$^+$ T cells specific for *Listeria* monocytogenes. *Listeria* infection has been used extensively as a model system for studying T cell activation because the bacteria induce robust CD8$^+$ T cell responses that are crucial for clearing the infection (1, 24). Groups of mice were immunized with LmOVA, a recombinant strain of *Listeria* expressing OVA, either alone or in combination with various doses of *C. trachomatis*. After 7 days, the number of T cells specific for the $OVA_{257–264}$ (SIINFEKL) peptide was measured using ELISPOT analysis (Fig. 4*A*). Coinfection with *Chlamydia* led to a dose-dependent decrease in the $OVA_{257–264}$-specific T cell response stimulated by LmOVA, suggesting that *Chlamydia* can inhibit the priming of heterologous CD8$^+$ T cell responses. The

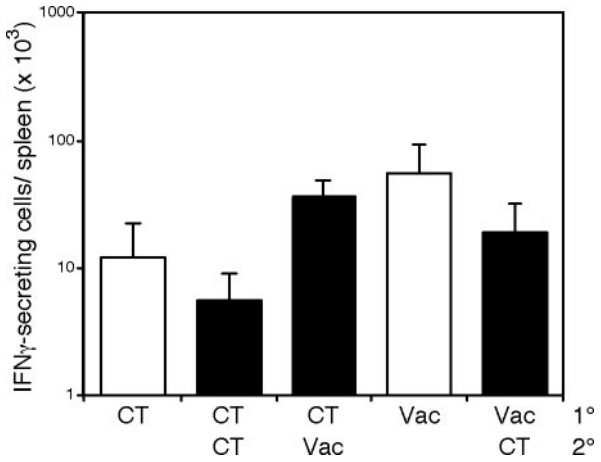

FIGURE 3. The expansion of CrpA-specific memory T cells is inhibited in the presence of *C. trachomatis*. C57BL/6 were infected with either *Chlamydia* or VacCrpA. The number of primary $CrpA_{63–71}$-specific T cells induced by *Chlamydia* or VacCrpA was measured on days 6 or 7 p.i., respectively (□). On day 21 p.i., the immune mice were challenged with either *C. trachomatis* or VacCrpA, and the CrpA-specific recall response was measured 5 days later (■).

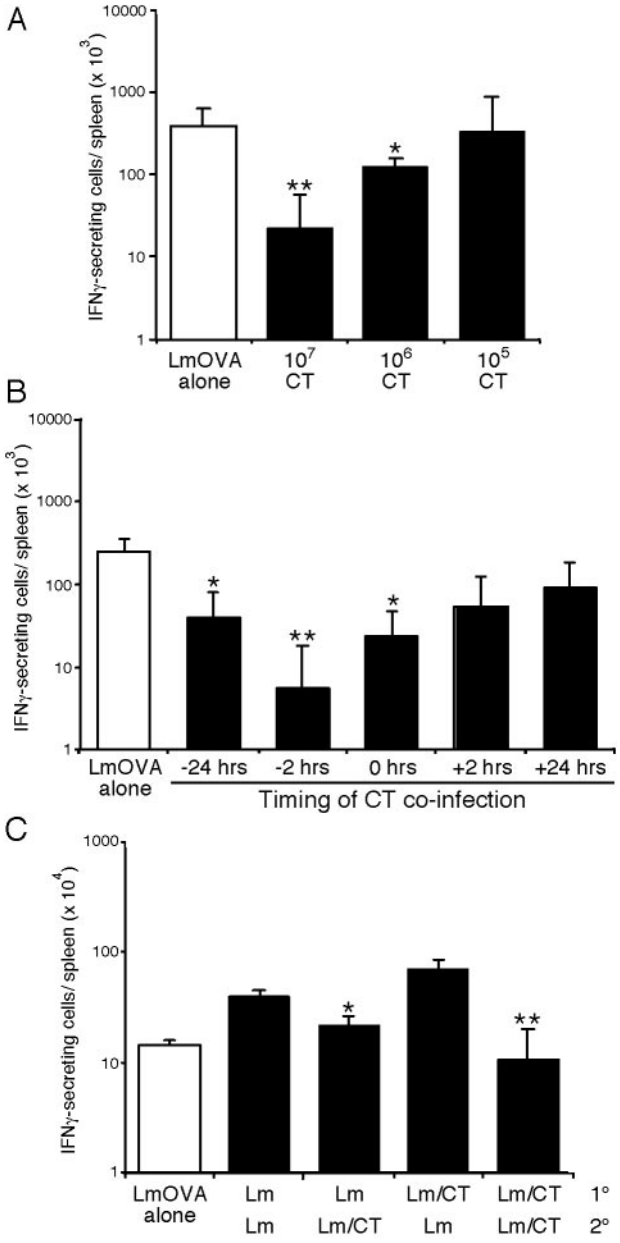

FIGURE 4. Coinfection with *Chlamydia* leads to reduced activation of *Listeria*-specific $CD8^+$ T cells. *A* and *B*, C57BL/6 mice were infected with a recombinant *Listeria* strain expressing OVA (LmOVA) either alone (□) or in combination with *C. trachomatis* (CT, ■), and the number of $OVA_{257–264}$-specific T cells was measured on day 7 p.i. using ELISPOT analysis. *A*, Mice were infected i.v. with 10^3 CFU of LmOVA either alone or in combination with various doses of *Chlamydia*. *B*, Mice were infected with LmOVA either alone or in combination with 10^7 IFU of *C. trachomatis*. The *Chlamydia* inoculum was delivered either before, after, or concurrent with the LmOVA injection. *C*, Mice were infected with LmOVA alone or coinfected with *Chlamydia*. On day 21 p.i., half of the immune mice from each group were challenged with LmOVA alone, while the rest of the mice were coinfected with LmOVA and *Chlamydia*. The number of OVA-specific T cells was measured on day 7 p.i. for the primary response (□) or day 5 for the secondary response (■). *, $p < 0.05$; **, $p < 0.01$ (paired *t* test).

inhibitory phenotype was dependent on coinfection with live *Chlamydia* because coinfection with UV-inactivated organisms failed to have a significant effect on T cell priming (data not shown).

It is unlikely that *Chlamydia* and *Listeria* are infecting the same APCs. Therefore, we predicted that *Chlamydia* infection exerts its inhibitory effect by altering the environment at the site of T cell priming. To determine how long this inhibitory phenotype might last, we delivered the *C. trachomatis* inoculum at various times relative to the injection of LmOVA (Fig. 4*B*). The maximum inhibition of OVA-specific T cell activation was seen when the *Chlamydia* were injected 2 h before LmOVA infection suggesting that *C. trachomatis* requires a short head start to establish the optimal inhibitory environment. Surprisingly, a significant reduction in LmOVA-specific responses was seen even 24 h after infection with *Chlamydia*. Introducing *C. trachomatis* after infecting the mice with LmOVA had little effect on the priming of OVA-specific T cells suggesting that once these T cells have been activated *Chlamydia* is unable to block their proliferation.

We then asked whether *Chlamydia* infection could reduce the reactivation of OVA-specific memory T cells. The stimulation of memory cells differs from naive cells in that activation of memory cells does not require costimulation. They simply need to recognize peptide-MHC complexes to proliferate. C57BL/6 mice were infected with LmOVA alone or in combination with *C. trachomatis*. After 21 days, each group of mice was divided with half being challenged with LmOVA alone (Lm) while the other half was coinfected with LmOVA and *Chlamydia* (Lm/CT)(Fig. 4*C*). LmOVA alone stimulated a robust secondary response to the $OVA_{257–264}$ peptide when compared with the LmOVA alone primary response (Fig. 4*C*, □). Challenging the Lm mice with Lm/CT resulted in a significant reduction in the OVA-specific recall response suggesting that the presence of *Chlamydia* inhibits the expansion of the *Listeria*-specific memory cells. The Lm/CT-immune mice responded more robustly to a challenge with Lm than did the Lm-immune mice perhaps as a result of there being fewer memory cells present to compete for available Ag (16). Consistent with there being fewer memory cells in the Lm/CT-immune mice, *Chlamydia* infection was better able to suppress memory cell expansion in mice that had been immunized with Lm/CT than in the Lm-immune mice. Together, these results suggest that *Chlamydia* infection is able to alter the environment at the site of T cell activation to inhibit the expansion of both naive and memory *Listeria*-specific $CD8^+$ T cells and it seems likely that a similar inhibitory mechanism could be acting on *Chlamydia*-specific T cells.

Discussion

We have faced a paradox regarding the activity of $CD8^+$ T cells during *Chlamydia* infection. On one hand, when mice are infected with *C. trachomatis* we see that $CD8^+$ T cells are limited in their contribution to protection against subsequent infection. On the other hand, if we culture $CD8^+$ T cells in vitro and transfer them into naive mice, or if we stimulate $CD8^+$ T cells through subunit vaccines rather than through natural infection, we can show that $CD8^+$ T cells have a protective effect. This discrepancy led us to explore the development of $CD8^+$ T cell responses in animals infected with *C. trachomatis* and to discover that memory responses are altered when mice are infected with *Chlamydia*.

Studies on the kinetics of $CD8^+$ T cell responses to *Chlamydia* Ags show a relatively robust primary response to $CrpA_{63–71}$ (12). However, upon challenge with *C. trachomatis* the magnitude of the CrpA-specific recall response was found to be quite small compared with the primary response (Fig. 1). This reduced secondary response was quite surprising considering the large body of evidence showing that reactivation of memory T cells results in rapid proliferation with a 5- to 10-fold increase in magnitude relative to the primary response. Interestingly, most of these studies were done using pathogens, such as *L. monocytogenes*, that cause acute infections (1–3). However, it is now becoming clear that this

model may not accurately reflect the response to persistent pathogens.

Several recent reports have examined persistent viral infections, such as HIV and LCMV, and found that the presence of persistent Ag leads to a decrease in the number of IL-7R$^+$ cells (18–20). The evidence that *Chlamydia* persists in vivo (22, 25) led us to look at the expression of IL-7R on CrpA$_{63–71}$-specific T cells. We saw a significant decrease in IL-7R$^+$ memory cells in *Chlamydia*-infected mice compared with mice that had been immunized with a recombinant vaccinia virus expressing the *Chlamydia* CrpA protein (Fig. 2). This suggested that *Chlamydia* infection was inhibiting the development of CD8$^+$ memory T cells, which could as a result decrease the ability of *Chlamydia*-specific memory cells to respond to a secondary challenge. However, this reduced recall response most likely did not result from persistent *Chlamydia* organisms because treating the infected mice with the antibiotic doxycycline had no effect on the magnitude of the CrpA-specific secondary response (Fig. 2*C*).

We show that it is specifically the T_{EM} population that is inhibited during *Chlamydia* infection, especially on day 14 p.i. (Fig. 2*A*). By day 35 p.i., it appeared that the memory population in *Chlamydia*-infected animals was skewed toward a T_{CM}, rather than a T_{EM}, phenotype. This result is interesting in light of recent work by Bachmann et al. (26) examining the relative roles of central vs effector memory T cells in mediating protection against viral infection. They found that the effectiveness of T_{CM} and T_{EM} cells in mediating antiviral protection depended on the site of viral replication and the relative contribution of cytokines vs perforin-mediated cell lysis. For example, vaccinia virus replicates predominantly in peripheral solid organs, such as ovaries, and the primary mechanism of protection is cytokine secretion, in particular the secretion of IFN-γ and TNF-α. To effectively control vaccinia infection, Ag-specific CD8$^+$ T cells had to be found in peripheral organs at the time of challenge and they had to secrete high levels of IFN-γ (26). The ability to migrate into peripheral tissues and to rapidly secrete cytokines are hallmarks of T_{EM} cells. Like vaccinia, *Chlamydia* infect peripheral tissues, namely the genital tract, and there is abundant evidence that IFN-γ secretion, rather than perforin-mediated lysis, is essential for controlling a *Chlamydia* infection (27–30). We would therefore predict that T_{EM} cells would be playing an important role in conferring protection. Yet this is the population that is most significantly altered during *Chlamydia* infection suggesting that the bacteria may be inhibiting the development of the T cell population most likely to control a secondary infection.

How is *Chlamydia* able to alter the development of memory CD8$^+$ T cells? We showed that the kinetics of memory cell development does not dictate whether *Chlamydia*-specific memory cells respond to challenge. We failed to improve recall by reducing the number of memory cells competing for Ag during a secondary challenge (Fig. 1*C*) or by waiting to challenge the mice until day 35 p.i. when a higher percentage of CrpA-specific cells express a memory phenotype (Fig. 2*B*). These data suggest that the memory cells that develop during *Chlamydia* infection are functionally defective and therefore unable to control bacterial growth.

Our results demonstrating that fewer *Listeria*-specific T cells are stimulated in the presence of *Chlamydia* infection suggest that inhibition is occurring at the time of initial T cell activation (Fig. 4). During the priming phase, many costimulatory signals are transmitted from APCs to naive CD8$^+$ T cells. Some lead to the generation of effector T cells that are capable of clearing a primary infection, while other signals direct naive T cells to undergo memory cell differentiation (31). It is tempting to speculate that reduced costimulatory signals could explain our results. However, reduced costimulation could only inhibit the priming of *Listeria*-specific T cells if *Listeria* and *Chlamydia* are infecting the same APCs. It is difficult to imagine that *Chlamydia* significantly inhibits *Listeria*-specific priming in this manner because it is unlikely that a sufficient number of APCs are coinfected, particularly when the *Chlamydia* was introduced 2–24 h before the *Listeria*. In addition, *C. trachomatis* infection was shown to affect the reactivation of Ag-experienced memory cells, which do not require costimulatory signaling to induce proliferation (Fig. 4*C*). Therefore the reduced priming of *Listeria*-specific T cells suggests that *Chlamydia* infection alters the local environment in such a way as to at least partially inhibit the priming of naive CD8$^+$ T cells.

Induction of regulatory T cells during infection has been shown in a number of systems to result in reduced memory responses (32–34). One possible explanation for the suppression of memory T cell responses in our system is the presence of *Chlamydia*-specific regulatory T cells. The induction of *Chlamydia*-primed T_{regs} might explain why *Chlamydia* coinfection has a greater impact on the reactivation of *Listeria*-specific T cells primed in the presence of *C. trachomatis*, but *Chlamydia* infection is also able to reduce the initial priming of *Listeria*-specific T cells in naive mice or mice that had been previously infected with LmOVA alone (Fig. 4). There is evidence that some pathogens, such as HSV, can activate natural T_{regs} and as a result regulate primary T cell responses (34). It is therefore possible that *Chlamydia* infection leads to diminished T cell responses through the activation of T_{reg}. We have begun to address the role of T_{reg} by examining whether IL-10 secretion contributes to suppression of the *Chlamydia*-specific T cell response. Infecting IL-10 KO mice failed to improve either the primary or secondary response against CrpA (Fig. 1*B*), suggesting that regulatory T cells are not necessary for this phenotype. However, IL-10 is not the only immunosuppressive cytokine implicated in T_{reg} function. Studies are currently underway to determine whether TGFβ is playing a role in this process.

It has long been appreciated that immunization with live *Chlamydia* provides minimal protection against subsequent challenge. Our data suggest that this limited protection during secondary infection may be due to the failure of CD8$^+$ T cells to respond adequately. Our goal is to understand the mechanism behind this apparent suppression. Ultimately, this knowledge could be used to develop more effective immunization protocols capable of overcoming this suppression.

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Disclosures

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