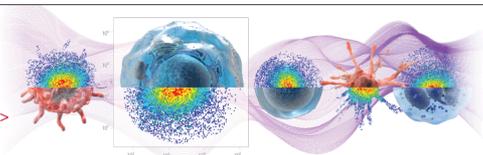


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An Excess of the Proinflammatory Cytokines IFN- γ and IL-12 Impairs the Development of the Memory CD8⁺ T Cell Response to *Chlamydia trachomatis*

Xuqing Zhang and Michael N. Starnbach

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. Transfer of cultured *Chlamydia*-specific CD8⁺ T cells or vaccination with recombinant virus expressing an MHC I-restricted *Chlamydia* Ag confers protection, yet surprisingly a protective CD8⁺ T cell response is not stimulated following natural infection. In this study, we demonstrate that the presence of excess IL-12 and IFN- γ contributes to poor memory CD8⁺ T cell development during *C. trachomatis* infection of mice. IL-12 is required for CD8⁺ T cell expansion but drives effector CD8⁺ T cells into a short-lived fate, whereas IFN- γ signaling impairs the development of effector memory cells. We show that transient blockade of IL-12 and IFN- γ during priming promotes the development of memory precursor effector CD8⁺ T cells and increases the number of memory T cells that participate in the recall protection against subsequent infection. Overall, this study identifies key factors shaping memory development of *Chlamydia*-specific CD8⁺ T cells that will inform future vaccine development against this and other pathogens. *The Journal of Immunology*, 2015, 195: 1665–1675.

There remains a pressing need for vaccines that induce robust CD8⁺ T cell-mediated immunity, specifically to combat pathogens that replicate within cells and evade the protective mechanisms mediated by Abs. A typical CD8⁺ T cell response is characterized by rapid expansion of rare Ag-specific T cells that contribute to the elimination of a specific pathogen. A vast majority of these cells then contract to maintain homeostasis of the immune system (1). The cells that survive contraction remain stable over time and mediate immunological memory (2). Stimulation of robust memory is critical for any successful vaccine. When effector CD8⁺ T cells expand and differentiate during a primary response, they do not equally acquire memory cell properties (3, 4). The signals that promote memory cell development are not thoroughly understood and usually occur early in the immune response (4, 5). In this article, we investigate the factors that drive memory CD8⁺ T cell fate following infection with the obligate intracellular bacterial pathogen *Chlamydia trachomatis*.

C. trachomatis infects >100 million people worldwide annually and is both the most prevalent bacterial genital tract infection

and the leading cause of preventable blindness (6). Chronic *C. trachomatis* genital tract infections lead to pelvic inflammatory disease (PID), which can cause fallopian tube scarring, infertility, and ectopic pregnancy (7, 8). Although human infection with *C. trachomatis* stimulates multiple elements of the immune system, these responses often fail to clear the infection or prevent subsequent reinfection (9). As with other pathogens that cause chronic infectious diseases, this lack of immune protection suggests a failure in adaptive immunity—specifically the memory responses that should provide long-lasting protection against reinfection. Therefore, an effective *Chlamydia* vaccine must induce a memory response better than that stimulated during natural infection.

Although Ab and CD4⁺ T cells clearly are required for full immunity to *C. trachomatis* (10, 11), CD8⁺ T cells should also be a major component of adaptive immunity against this pathogen. *C. trachomatis* infects epithelial cells in the genital tract, a cell type that expresses MHC I, but not usually MHC II. Because *C. trachomatis* translocates a subset of its proteins into the host cell cytosol, it allows for MHC I processing of these proteins and subjects the cell to recognition by CD8⁺ T cells (12, 13). CD8⁺ T cells have been shown to protect against infection when cultured *ex vivo* and transferred into naive animals, and immunization with recombinant vaccinia viruses expressing CD8⁺ T cell Ags from *C. trachomatis* also confers protection in mice (13). Yet, during natural infection of mice, the CD8⁺ T cell response does not play a significant protective role (14, 15). Previous studies from our laboratory have shown that CD8⁺ T cells respond well to primary *C. trachomatis* infection, but the memory cells that result from initial infection are impaired in their ability to respond to subsequent encounters with the pathogen (16, 17).

To better understand the failure of CD8⁺ T cell memory development following *C. trachomatis* infection, we compared the Ag-specific CD8⁺ T cells induced by *C. trachomatis* (poor recall) with those of the same Ag specificity induced by recombinant vaccinia virus expressing a *C. trachomatis* Ag, CrpA (robust recall) (17). We found that the proinflammatory cytokines IL-12 and

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Abbreviations used in this article: IFU, inclusion-forming unit; KLRG1, killer cell lectin-like receptor G1; p.i., postinoculation; SLO, secondary lymphoid organ; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{MPEC}, memory precursor effector T cell; T_{SLEC}, short-lived effector T cell; VacCrpA, recombinant vaccinia virus expressing the *Chlamydia* CrpA protein.

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IFN- γ drive effector CD8⁺ T cells stimulated by *C. trachomatis* into a short-lived fate [short-lived effector T cells (T_{SLEC})] and impair the development of memory cells. Transient blockade of these cytokines during priming increases the frequency of memory precursor effector T cells (T_{MPEC}) and memory CD8⁺ T cell numbers. Overall, this study identified factors that are critical for CD8⁺ T cell memory development following *C. trachomatis* infection, which should aid in vaccine development against this and other pathogens responsible for chronic infections.

Materials and Methods

Mice

C57BL/6J, B6.PL-Thy1^a/CyJ (CD90.1 congenic), B6.129S7-Irfn γ 1^{tm1Agt/J} (IFN- γ R^{-/-}), and B6.129S1-Il12rb1^{tm1Jm/J} (IL-12R β ^{-/-}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Tbet^{-/-} mice (C57BL/6 background) were kindly provided by L. Glimcher (Harvard School of Public Health) (18). Tbet^{+/-} mice were generated by crossing C57BL/6J and Tbet^{-/-} mice. PDL1^{-/-} mice (C57BL/6 background) have been described before and were generously provided by A. Sharpe (Harvard Medical School) (19). To generate *Chlamydia*-specific CD8⁺ TCR transgenic mice specific for CrpA₆₃₋₇₁, we cloned the rearranged genomic TCR α and TCR β sequences from *Chlamydia*-specific CD8⁺ T cell clone NR23.4 into expression vectors (20). The cloned TCR constructs were then linearized and injected into C57BL/6 fertilized oocytes. TCR transgenic founders were identified by PCR. Although NR23.4 transgenes were integrated into the genome of these founders, possible competition from endogenous TCR rearrangements inhibited efficient expression of the NR23.4 TCR. To restrict TCR expression, we crossed these mice onto a RAG1^{-/-} background (NR23.4 mice). The rearranged TCR from NR23.4 uses the V α 4JTA13 and V β 8.2J β 2.5 receptor chains. NR23.4 IL-12R β ^{-/-} and NR23.4 IFN- γ R^{-/-} were generated by crossing NR23.4 mice with IL-12R β ^{-/-} and IFN- γ R^{-/-} mice, respectively. Mice were maintained within the Harvard Medical School Center for Animal Resources and Comparative Medicine. All experiments in this report were approved by Harvard's Institutional Animal Care and Use Committee.

Growth, isolation, and detection of bacteria and virus

C. trachomatis serovar L2 (434/Bu; ATCC) was propagated within McCoy cell monolayers grown in Eagle's MEM (Invitrogen) supplemented with 10% FCS, 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. Infected monolayers were disassociated from flasks using 0.05% trypsin/EDTA and sonicated to disrupt the inclusion. Elementary bodies were purified by density gradient centrifugation, as previously described (21). Aliquots were stored at -80°C in sucrose-phosphate-glutamate buffer and thawed immediately before use. Construction of the recombinant vaccinia virus expressing the *Chlamydia* CrpA protein (VacCrpA) has been described previously (13). Virus preparations were treated with an equal volume of 0.25 mg/ml trypsin for 30 min at 37°C and diluted in PBS before infecting mice.

Preparation of IL-2/anti-IL-2 complexes

IL2/anti-IL-2 complexes were prepared as previously described (22–24). A total of 1.5 μ g carrier-free mouse recombinant IL-2 (eBioscience) and 50 μ g anti-IL-2 mAb (S4B6, Bio X Cell) were mixed in 10 μ l HBSS at room temperature for 15 min before adding 190 μ l HBSS for each injection. Control groups were treated with IgG2a isotype control Abs (2A3, Bio X Cell).

Infection of mice and preparation of tissue

For systemic infection, mice were infected i.v. with 10⁷ inclusion-forming units (IFU) of *C. trachomatis* in 200 μ l sucrose-phosphate-glutamate buffer or 2 \times 10³ PFU VacCrpA in 200 μ l PBS, unless otherwise noted. To infect in the genital tract, mice were treated s.c. with 2.5 mg medroxyprogesterone acetate (Pfizer) and then infected 1 wk later trans-cervically with 5 \times 10⁶ IFU *C. trachomatis* or 5 \times 10⁵ PFU VacCrpA, as described previously (25). At specific times post infection, the iliac lymph nodes, spleen, and uterine horns were excised. Uteri were dissected free of the mesometrium and then finely minced with scalpels. Minced tissues were enzymatically dissociated in HBSS/Ca²⁺/Mg²⁺ containing 1 mg/ml type XI collagenase and 50 Kunitz/ml DNase for 30 min at 37°C, washed in Ca²⁺/Mg²⁺-free PBS containing 5 mM EDTA, and then ground between frosted microscope slides prior to filtration through a 70-cm mesh (26). Single-cell suspensions of secondary lymphoid organs (SLO) were pre-

pared by grinding the tissue between frosted microscope slides. RBCs in the splenocytes were lysed using ammonium chloride.

Flow cytometry

Cells were immediately stained for surface and activation markers or stimulated for 4–5 h with 10 μ M CrpA₆₃₋₇₁ peptide in the presence of brefeldin A (BioLegend) for intracellular cytokine staining. The D^p/ASFVNPIYL (CrpA₆₃₋₇₁) MHC I tetramer was generated at the National Institutes of Health Tetramer Facility. Abs were purchased from BioLegend except for CD16/CD32 (2.4G2; Bio X Cell), anti-CD62L-PE-Texas Red (Invitrogen), anti-CD95 (BD Biosciences), anti-CD127 (eBioscience), anti-CD4 Qdot⁶⁰⁵ (Invitrogen), anti-IL-18R α (R&D Systems), and anti-IFN- γ allophycocyanin-Cy7 (BD Biosciences). Cells were pre-incubated with CD16/CD32 (2.4G2) before staining with tetramer and fluorochrome (allophycocyanin, allophycocyanin-Cy7, FITC, PE, PerCP, PerCP-Cy5.5, PE-Cy7, Pacific Blue, PE-Texas Red) conjugated Abs against mouse B220 (RA3-6B2), CD4 (RM4-5 or GK1.5), CD8 (53-6.7), CD90.1 (OX-7), CD90.2 (53-2.1), CD27 (Lg.3A10), PDL1 (10F.9G2), CD3 (17A2), CD11b (M1/70), killer cell lectin-like receptor G1 (KLRG1) (2F1), CD25 (PC61), CD95 (Jo2), IL-18R α (112614), CD122 (TM- β 1), CD326 (G8.8), CD11c (N418), MHC I-A^b (AF6-120.1), or CD127 (A7R34). LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Invitrogen) was used along with other Abs to exclude dead cells from analyses. For intracellular staining, cells were permeabilized with the Cytofix/Cytoperm Plus Kit according to the manufacturer's instructions (BD Biosciences) and stained with anti-IFN- γ (XMG 1.2). The absolute cell number in each sample was determined using AccuCheck Counting Beads (Invitrogen). Data were collected on a LSR II (BD Biosciences) and analyzed using FlowJo (TreeStar, Ashland, OR). CrpA-specific CD8⁺ T cells were gated as LIVE/DEAD⁻ CD4⁻ B220⁻ CD11b⁻ MHC I-A^b-CD3⁺ CD8⁺ CrpA-tetramer⁺.

Detection of BrdU uptake

To determine the proliferation rate of T cells, mice were injected i.p. with 1 mg BrdU daily on days 30–35 post inoculation (p.i.). On day 35, splenocytes were isolated, surface stained, fixed, permeabilized, and stained with anti-BrdU mAb as recommended by the manufacturer of the BrdU flow kit (BD Biosciences).

Cytokine detection and depletion

Serum was extracted from peripheral blood, and cytokine levels in the serum were determined as recommended by the Luminex Kit (Millipore) or by ELISA, as previously described (13). To deplete cytokines, mice were injected i.p. with 200 μ g anti-IFN- γ (XMG1.2) together with 200 μ g anti-IL-12 (C17.8), or isotype control (200 μ g HRPN and 200 μ g 2A3) in 200 μ l PBS on day 4 p.i. Serum was extracted from these mice on day 7 p.i., and cytokine levels in the serum were determined by ELISA to check the efficiency of the depletion protocol. The IFN- γ level is below the limit of detection in depletion Ab-treated mice. The IL-12 level is significantly lower in mice treated with depletion Abs (12.99 \pm 0.02% of the levels in control mice) compared with control Ab-treated mice (p < 0.01). All isotype and neutralizing Abs were purchased from Bio X Cell.

Transfer of T cells

For transfer of transgenic cells, *C. trachomatis*-specific CD8⁺ T cells were isolated from the SLOs of donor NR23.4 mice. Recipient mice were injected with 10⁴–10⁵ cells i.v. into tail veins 1 d before infection. For transfer of immune T cells, SLOs were isolated on day 28 p.i. and homogenized into single-cell suspensions. CD8⁺ T cells were isolated using the Dynal Mouse CD8 Negative Isolation Kit according to the manufacturer's instructions (Invitrogen). Isolated cells were then labeled with 10 μ M CFSE (Invitrogen) as previously described (27). Unless otherwise stated, 5 \times 10⁶ CD8⁺ T cells were injected i.v. into naive mice 4 h prior to transcervical infection.

Quantitative PCR

The levels of *C. trachomatis* or VacCrpA in the spleens or the uteri of infected mice were quantified using a previously described quantitative PCR assay (28, 29). Briefly, total nucleic acid from infected spleen or uterus homogenates was prepared using the QIAamp DNA Mini Kit (QIAGEN). *Chlamydia* 16S DNA, vaccinia ribonucleotide reductase (Vv14L), and mouse GAPDH DNA content of individual samples were then quantified by quantitative PCR on an ABI 7000 sequence detection system (Applied Biosystems) using primer pairs and dual-labeled probes (IDT or Applied Biosystems). Standard curves were generated from known

amounts of *Chlamydia*, vaccinia, or mouse DNA, and these curves were used to calculate the amount (in picograms) of *Chlamydia* DNA or vaccinia DNA per unit weight (in micrograms) of mouse DNA in the samples.

Statistical analysis

A two-tailed Mann–Whitney *U* test was applied to determine statistical significance for bacterial burdens among groups. All other data were evaluated for statistical significance with an unpaired two-tailed *t* test. Differences were considered statistically significant if the *p* value was < 0.05; **p* < 0.05, ***p* < 0.01. Results were shown as mean ± SE.

Results

CD8⁺ T cells induced by C. trachomatis contract more than those induced by VacCpA

VacCpA is known to induce a more robust CrpA-specific recall response than is *C. trachomatis* (17). To determine why *C. trachomatis* induces an impaired *CD8⁺ T* cell population that fails to efficiently participate in recall, we compared CrpA-specific *CD8⁺ T* cells induced by *C. trachomatis* with those induced by VacCpA. To rule out the impact of pathogen level on memory *CD8⁺ T* cell development, the VacCpA challenge dose was carefully titrated so that similar numbers of CrpA-specific *CD8⁺ T* cells were induced at the peak of expansion following either *C. trachomatis* or VacCpA infection (Fig. 1A). Values of 2000 PFU of VacCpA and 10^7 IFU of *C. trachomatis* yielded no significant differences in the number of CrpA-specific *CD8⁺ T* cells at the peak of the primary response (day 7). Therefore, these doses were used to challenge animals throughout this study. Significantly more CrpA-specific *CD8⁺ T* cells were recovered from VacCpA-infected mice than *C. trachomatis*-infected mice at later times when stable memory had formed (Fig. 1B), indicating that CrpA-specific *CD8⁺ T* cells induced by *C. trachomatis* contracted more than the cells induced by VacCpA. A similar proportion of memory CrpA-specific *CD8⁺ T* cells from *C. trachomatis*- or

VacCpA-infected mice secreted IFN- γ following ex vivo restimulation (Fig. 1C), suggesting that the memory cells that did survive the contraction following *C. trachomatis* infection were similarly functional compared with cells stimulated by VacCpA infection. Although these memory cells proliferated as efficiently as VacCpA-induced memory cells, as measured by BrdU uptake (Fig. 1D), they expressed higher levels of CD95 (FasR) (Fig. 1E), suggesting that these cells are more prone to apoptosis. Moreover, compared with CrpA-specific memory *CD8⁺ T* cells from VacCpA-infected mice, CrpA-specific cells from *C. trachomatis*-infected mice expressed lower levels of CD27, CD122 (IL-2/IL-15R β), and IL-18R α , all of which are critical for the recall capacity of memory *CD8⁺ T* cells (Fig. 1F–H) (30–33). Overall, these data suggest that although CrpA-specific *CD8⁺ T* cells are expanded by *C. trachomatis* or VacCpA infection to a similar extent, *C. trachomatis*-stimulated cells contract more and the memory cells that do survive the contraction are of lower quality.

IL-2 α -IL-2 complexes are not sufficient to rescue the recall capacity of CD8⁺ T cells stimulated by C. trachomatis

The kinetics of CrpA-specific *CD8⁺ T* cells stimulated by *C. trachomatis* infection were indicative of helpless *CD8⁺ T* cells that are not durable and wane over time (34–38). One of the mechanisms by which helper T cells mediate help is to instruct DCs to produce cytokines that induce upregulation of IL-2R α (CD25) on Ag-specific *CD8⁺ T* cells, rendering them more responsive to IL-2 (39, 40). We therefore explored whether CD25 expression is differentially stimulated following infection with *C. trachomatis* versus VacCpA. Because CD25 expression peaks early, when the number of endogenous CrpA-specific *CD8⁺ T* cells is too low to be reliably detected, we transferred CrpA-specific transgenic cells before infection and examined the expression of CD25 on these cells. On day 3 p.i., significantly fewer

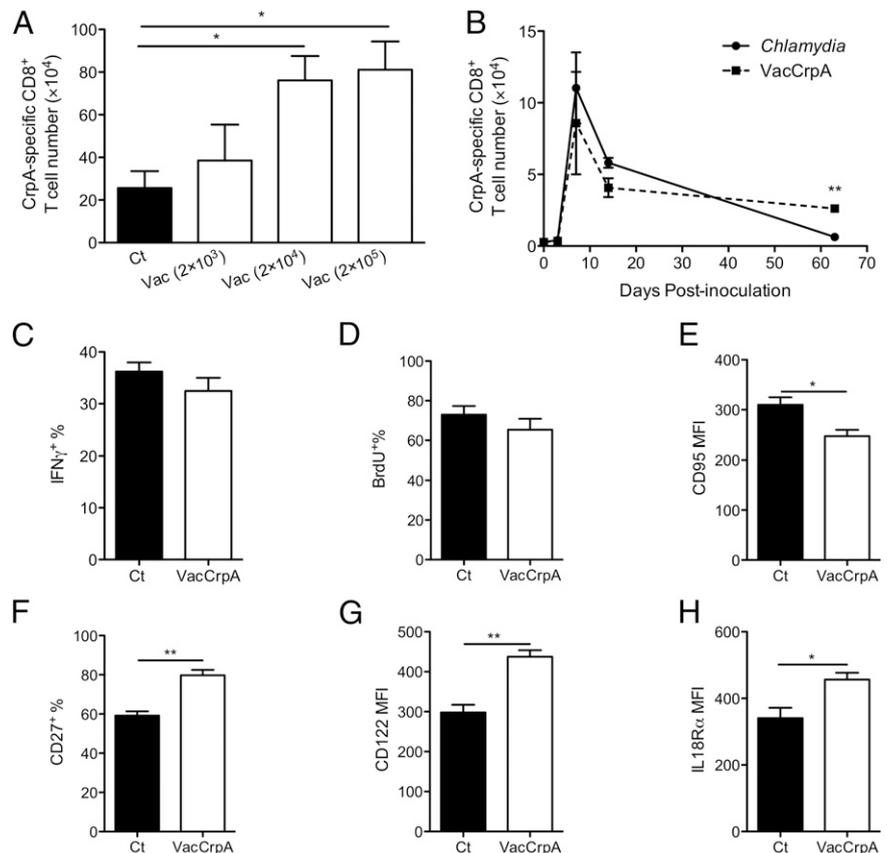


FIGURE 1. *C. trachomatis* induces impaired memory *CD8⁺ T* cells. **(A)** CrpA-specific *CD8⁺ T* cell numbers in the spleens of systemically infected mice on day 7 p.i. are shown. **(B)** CrpA-specific *CD8⁺ T* cell numbers in the spleens of mice systemically infected with *C. trachomatis* or 2×10^3 PFU of VacCpA on indicated days p.i. are shown. **(C)** IFN- γ %, **(D)** BrdU % , **(E)** CD95 mean fluorescence intensity (MFI), **(F)** CD27 % , **(G)** CD122 MFI, and **(H)** IL-18R α MFI of CrpA-specific *CD8⁺ T* cells in the spleen on days 26–30 p.i. are shown. Data are representative of at least two experiments, each with five to seven mice per group. **p* < 0.05, ***p* < 0.01.

CrpA-specific transgenic cells expressed CD25 as they divided in *C. trachomatis*-infected mice compared with VacCpA-infected mice. A similar trend was observed on day 4 p.i. By day 6 p.i., the *C. trachomatis*-stimulated cells had caught up, suggesting that the induction of CD25 on CrpA-specific CD8⁺ T cells was delayed in mice infected with *C. trachomatis* compared with those infected with VacCpA (Fig. 2A).

Previous reports have shown that IL-2 signaling not only augments the accumulation of CD8⁺ T cells but also programs the ability of memory cells to expand upon secondary challenge (39–42). To test whether IL-2 signaling differences were responsible for the poor recall capacity of *Chlamydia*-stimulated memory cells, we treated mice with IL-2/anti-IL-2 (IL-2/S4B6) complexes that have been shown to increase the recall capacity of CD8⁺ T cells (43). Mice were infected with *C. trachomatis*, treated with IL-2/anti-IL-2 complexes or isotype control Abs on days 3 and 5 (early) or on days 24 and 26 (late), and then rechallenged with VacCpA on day 28. The numbers of CrpA-specific CD8⁺ T cells in these mice were determined 5 d later. Numbers of CrpA-specific CD8⁺ T cells were similar among all rechallenged groups and were significantly lower than the primary control group (Fig. 2B), suggesting that stimulating IL-2 signaling early or late during memory development was not sufficient to rescue the recall capacity of CrpA-specific CD8⁺ T cells. Overall, these data suggest that the delayed upregulation of CD25 is not the primary reason why CrpA-specific CD8⁺ T cells induced by *C. trachomatis* fail to efficiently participate in the recall response.

C. trachomatis-stimulated effector CD8⁺ T cells are enriched for the short-lived phenotype

Early in priming, the differential expression of KLRG1 and CD127 (IL-7R α) has been shown to mark two effector T cell populations

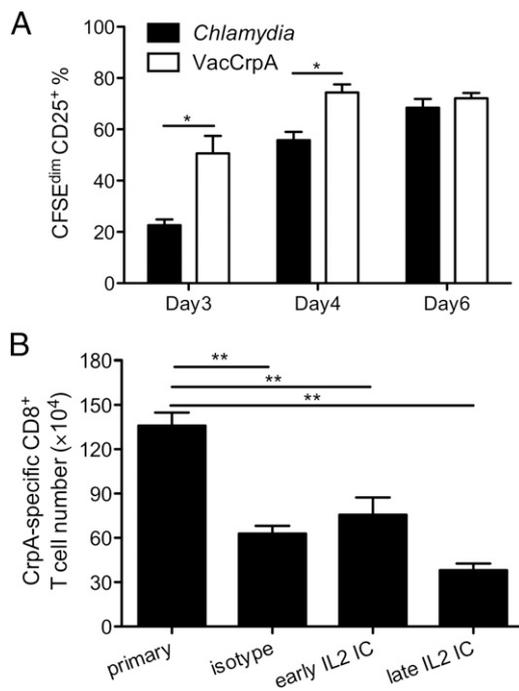


FIGURE 2. IL-2/anti-IL-2 immune complex treatment is not sufficient to rescue the blunted recall response. **(A)** CFSE^{dim}CD25⁺ % of NR23.4 cells in the spleen are shown. **(B)** Mice were infected i.v. with *C. trachomatis*, treated with IL-2/anti-IL-2 immune complex (IL-2 IC) or isotype control Abs on days 3 and 5 p.i. (early) or on days 24 and 26 p.i. (late), and rechallenged with VacCpA on day 28 p.i. CrpA-specific CD8⁺ T cell numbers 5 d after secondary challenge are shown. Representative data from two experiments are shown, each with five to seven mice per group. * $p < 0.05$, ** $p < 0.01$.

with distinct memory potential. The CD127^{low}KLRG1^{high} T_{SLEC} do not gain memory T cell potential. Rather, primarily the descendants of CD127^{high}KLRG1^{low} T_{MPEC} participate in secondary responses upon reinfection (44). We hypothesized that *C. trachomatis* infection may favor the development of T_{SLEC} because the kinetics of the *Chlamydia*-specific CD8⁺ T cell response resembles the kinetics of T_{SLEC}. To test whether *C. trachomatis* and VacCpA differentially stimulate T_{SLEC} versus T_{MPEC} among the CrpA-specific CD8⁺ T cells, we compared CD127 and KLRG1 expression on CrpA tetramer⁺ CD8⁺ T cells following infection with *C. trachomatis* versus VacCpA. At the peak of expansion, more CrpA-specific CD8⁺ T cells induced by *C. trachomatis* were T_{SLEC} than those induced by VacCpA (Fig. 3A, 3B). In contrast, VacCpA infection favored the formation of T_{MPEC} (Fig. 3A, 3B). We quantified the total number of CrpA-specific T_{SLEC} and T_{MPEC} cells and found that this trend also held true over the time course of infection (Fig. 3C, 3D). Together, these data suggest that *C. trachomatis* infection favors the formation of T_{SLEC} CD8⁺ T cells in contrast to VacCpA infection.

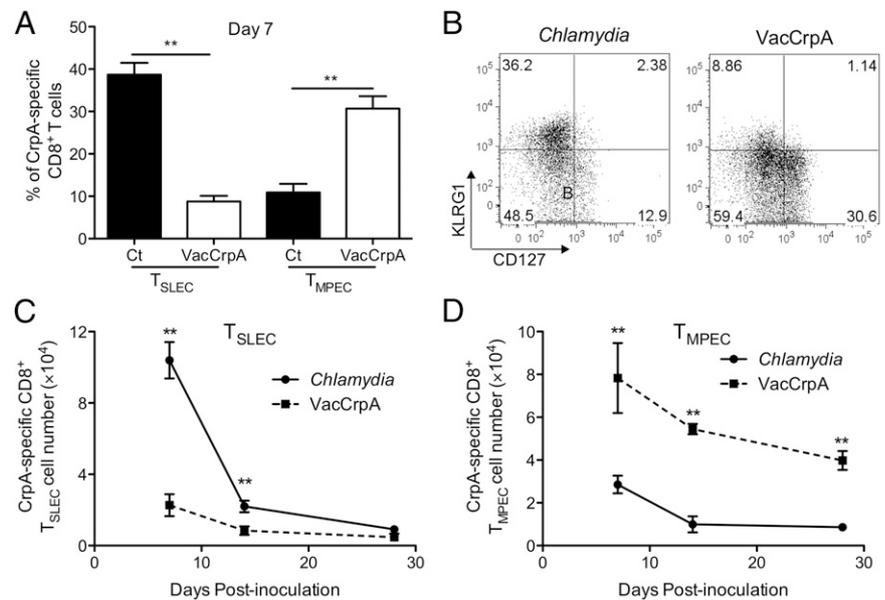
Transient reduction of IFN- γ and IL-12 levels increases the proportion of memory precursor cells

One of the mechanisms that drive effector cells into a short-lived fate during viral infection is overwhelming inflammation (44). To assess whether *C. trachomatis*- and VacCpA-infected mice experience differential levels of inflammation, we measured levels of several cytokines in the serum of these mice, including IFN- γ , IL-12, IL-6, IL-10, IL-7, and IL-2. Among the cytokines tested, the levels of two proinflammatory cytokines, IFN- γ and IL-12, were higher in serum from *Chlamydia*-infected mice than in serum from VacCpA-infected mice between day 2 and day 5 p.i. (Fig. 4A, 4B). To test whether the increased levels of these cytokines in *C. trachomatis*-infected mice are responsible for the dominance of the T_{SLEC} phenotype in pathogen-specific CD8⁺ T cells, we treated infected mice with a single dose of neutralizing Abs against IFN- γ and IL-12 or isotype control Abs on day 4 p.i. This transient treatment did not alter *C. trachomatis* burden (data not shown) or the absolute number of CrpA-specific CD8⁺ T cells at the peak of expansion (Fig. 4C). However, this treatment did reduce the percentage and number of T_{SLEC} and increased the percentage and number of T_{MPEC} among CrpA-specific CD8⁺ T cells at the peak of expansion (Fig. 4D, 4E). A previous report from our laboratory has shown that the development of *Chlamydia*-specific effector memory T cells (T_{EM}) (CD127⁺CD62^{low}) is inhibited during *C. trachomatis* infection (17). Cytokine-neutralizing Ab treatment increased the percentage and number of T_{EM} among CrpA-specific CD8⁺ T cells without sacrificing the development of central memory T cells (T_{CM}) (Fig. 4F, 4G), suggesting that these two cytokines also inhibit the development of T_{EM}. More importantly, when CrpA-specific CD8⁺ T cell number was quantified a month after inoculation, more CrpA-specific CD8⁺ T cells were recovered from neutralizing Ab-treated mice (Fig. 4H). Similar percentages of CrpA-specific CD8⁺ T cells secreted the effector cytokine IFN- γ in mice treated with depletion and control Abs (Fig. 4I). Overall, these data suggest that transient ablation of proinflammatory cytokines early during priming increases the number of T cells that survive contraction without affecting the functionality of memory T cells, consistent with an overall increase of memory potential at the peak of expansion.

Genetically reducing *Tbet* expression increases the memory potential of *Chlamydia*-induced CD8⁺ T cells

IFN- γ and IL-12 are known to regulate the expression of Tbet, a transcription factor critical for regulating CD8⁺ T cell memory

FIGURE 3. CrpA-specific CD8⁺ T cells induced by *C. trachomatis* infection show T_{SLEC} characteristics. Mice were infected i.v. with *C. trachomatis* or VacCrpA. **(A)** The percentage of T_{SLEC} (CD127⁻KLRG1⁺) and T_{MPEC} (CD127⁺KLRG1⁻) among CrpA-specific CD8⁺ T cells on day 7, **(B)** representative flow cytometry analysis of KLRG1 and CD127 expression on CrpA-specific CD8⁺ T cells on day 7, **(C)** the absolute number of CrpA-specific T_{SLEC}, and **(D)** T_{MPEC} overtime are shown. Data are representative of at least three experiments, each with five to seven mice per group. ***p* < 0.01.



development (44, 45). To determine whether IFN- γ and IL-12 regulate memory development through Tbet following *Chlamydia* infection, we assessed the phenotype of CrpA-specific CD8⁺ T cells in Tbet^{+/-} mice. We chose Tbet^{+/-} mice instead of Tbet^{-/-} mice to avoid the impact of the complete loss of Tbet on Th1 CD4⁺ T cell development with the resulting increase in *C. trachomatis* burden (data not shown). Moreover, the dose dependency of Tbet on CD8⁺ T cell memory development has been previously described (44). Consistent with the cytokine-neutralizing data, a genetic reduction in the expression of Tbet increased the number of CrpA-specific CD8⁺ T cells on day 21 p.i., when a stable memory pool had formed (Fig. 5A), without significantly altering *C. trachomatis* burden (data not shown) or the number of CrpA-specific CD8⁺ T cells at the peak of expansion (Fig. 5A). Consistent with the cytokine neutralization results, reducing Tbet expression also altered the T_{MPEC} versus T_{SLEC} ratio in favor of T_{MPEC} at the peak of CD8⁺ T cell expansion (Fig. 5B) and when a stable memory pool had formed (Fig. 5C). Moreover, reducing Tbet expression resulted in an overall increase of CD127⁺ memory T cells within which the formation of a CD62L^{low} T_{EM} population was favored (Fig. 5D). Taken together with the IFN- γ and IL-12 ablation experiments (Fig. 4), these results suggest that proinflammatory cytokines, IFN- γ and IL-12, modulate Tbet to alter pathogen-specific CD8⁺ T cell development following *C. trachomatis* infection.

IL-12 is critical for T_{MPEC} versus T_{SLEC} development, whereas IFN- γ affects T_{EM} versus T_{CM} formation among C. trachomatis-specific CD8⁺ T cells

We next explored whether cell-intrinsic IFN- γ or IL-12 signaling in pathogen-specific CD8⁺ cells is responsible for altering T_{SLEC} versus T_{MPEC} or T_{EM} versus T_{CM} development following *C. trachomatis* infection. We crossed *Chlamydia*-specific CD8⁺ TCR transgenic mice (NR23.4) onto the IL-12R β ^{-/-} or IFN- γ R^{-/-} backgrounds to create CrpA-specific CD8⁺ T cells that do not respond to IL-12 or IFN- γ . The transgenic cells lacking IL-12R β did not expand as efficiently as did wild-type cells (Fig. 6A), suggesting that IL-12 signaling is required for efficient expansion of pathogen-specific CD8⁺ T cells following *C. trachomatis* infection. Nevertheless, the transgenic cells that do not respond to IL-12 did shift toward a T_{MPEC} phenotype (Fig. 6B), consistent with the cytokine depletion experiments described above. IL-12

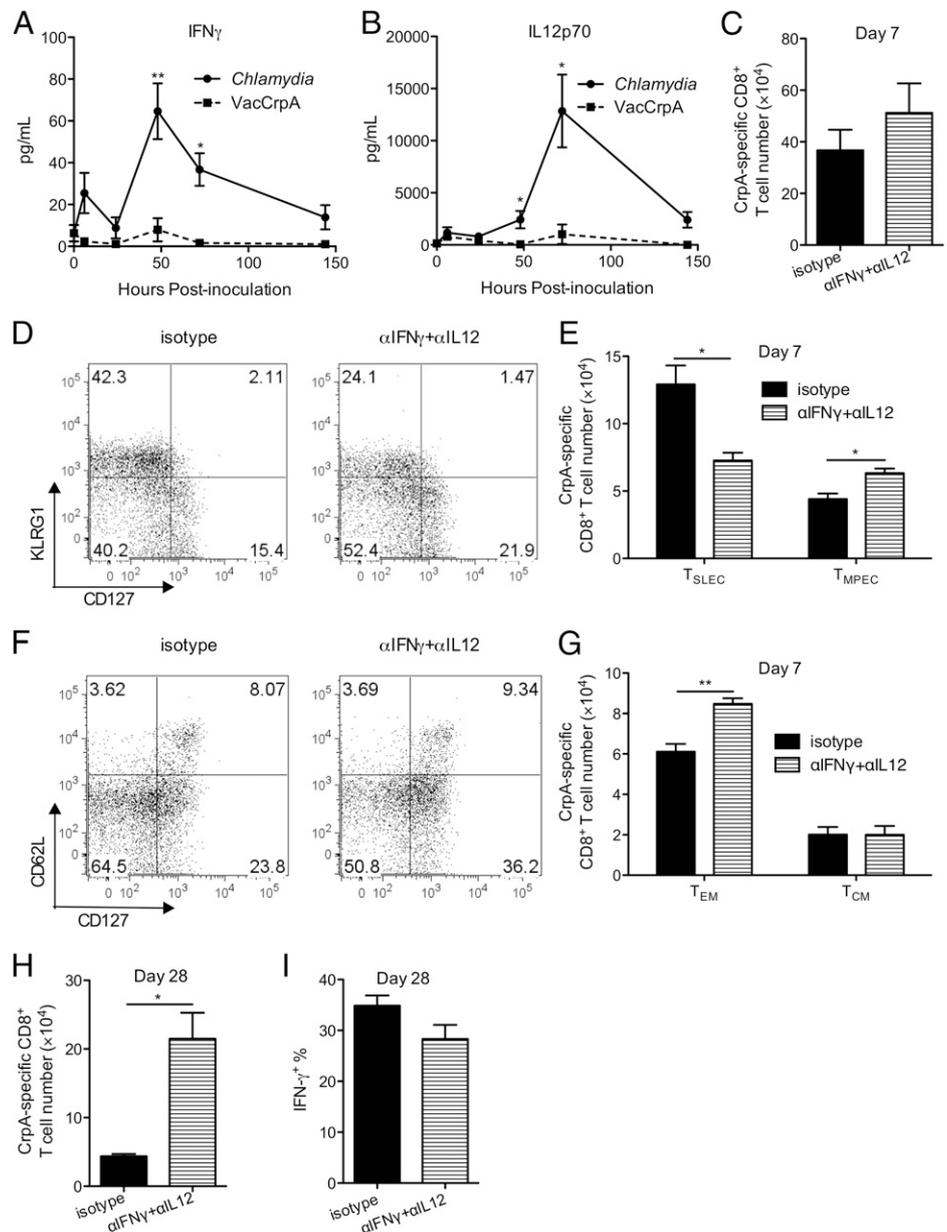
signaling did not seem to affect T_{EM} versus T_{CM} development because the percentages of T_{EM} and T_{CM} were similar between wild-type and IL-12R β ^{-/-} cells (Fig. 6C). In contrast, similar numbers of wild-type and IFN- γ R^{-/-} transgenic cells were recovered at the peak of expansion (Fig. 6D), suggesting that IFN- γ signaling is not required for pathogen-specific CD8⁺ T cell expansion following *C. trachomatis* infection. The transgenic cells that do not respond to IFN- γ did not show an obvious shift toward T_{MPEC} (Fig. 6E) but did show an increase in T_{EM} numbers (Fig. 6F). Overall, these data suggest that IL-12 signaling is important for T_{MPEC} versus T_{SLEC} differentiation, whereas IFN- γ is involved in T_{EM} versus T_{CM} development.

Transient ablation of proinflammatory cytokines during mucosal infection also favors the formation of memory precursor CD8⁺ T cells

To test whether reducing proinflammatory cytokine signaling can improve *Chlamydia*-specific CD8⁺ T cell memory development following mucosal infection, we conducted cytokine depletion experiments in mice infected with *C. trachomatis* in the genital tract. Transient reduction of IFN- γ and IL-12 did not significantly alter *Chlamydia* burden in the uterus of infected mice (Fig. 7A). This treatment did shift CD8⁺ T cells in the spleens toward a T_{MPEC} phenotype (Fig. 7B, 7C). A similar trend was observed in the uterine tissues and draining lymph nodes, although the differences did not reach statistical significance (Fig. 7B, 7C). Transient proinflammatory cytokine ablation also increased the number of T_{EM} in the spleens and uterine tissues of mucosally infected mice without affecting the numbers of T_{CM} (Fig. 7D, 7E). Overall, in mice infected in the genital tract, transient reduction of IFN- γ and IL-12 levels shifted the CD8⁺ T cells toward a T_{MPEC} and a T_{EM} phenotype, consistent with what was observed in systemically infected mice.

A recent report from our laboratory has shown that the PDL1-PD1 pathway also contributes to the suppression of CD8⁺ T cell memory development during *Chlamydia* infection of the genital tract (16). To test whether reducing proinflammatory cytokines modulates memory development by regulating PDL1 expression, we transcerivally infected mice with *C. trachomatis*, treated the mice with IFN- γ and IL-12 neutralizing Abs, and then determined PDL1 expression on various cell populations. We found that neutralization of the proinflammatory cytokines reduced PDL1

FIGURE 4. Transient reduction of proinflammatory cytokines early during priming improves the development of memory CrpA-specific CD8⁺ T cells. (A and B) Serum IFN- γ (A) and IL-12p70 (B) levels in mice systemically infected with *C. trachomatis* or VacCrpA are shown. (C–I) Mice were infected i.v. with *C. trachomatis* and treated i.p. with isotype control or IFN- γ and IL-12 neutralizing Abs (α IFN- γ + α IL-12) on day 4 p.i. (C) CrpA-specific CD8⁺ T cell numbers, (D) representative flow cytometry analysis of KLRG1 and CD127 expression on CrpA-specific CD8⁺ T cells, (E) total numbers of CrpA-specific T_{SLEC} and T_{MPEC}, (F) representative flow cytometry analysis of CD62L and CD127 expression on CrpA-specific CD8⁺ T cells, and (G) total numbers of CrpA-specific T_{EM} and T_{CM} in the spleen on day 7 p.i. are shown. (H) CrpA-specific CD8⁺ T cell numbers in the spleen and (I) IFN- γ ⁺ % among CrpA-specific CD8⁺ T cells on day 28 p.i. are shown. Data are representative of at least two experiments, each with five to seven mice per group. * $p < 0.05$, ** $p < 0.01$.



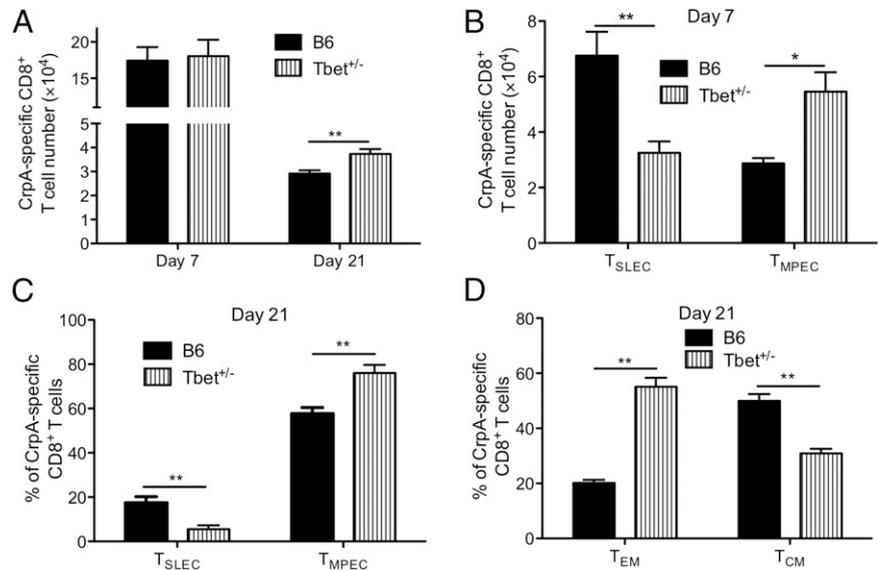
expression on uterine epithelial cells (Fig. 7F). The numbers of uterine dendritic cells were too few to reliably examine the differences in PDL1 expression among groups; however, we did observe a reduction in PDL1 level on splenic dendritic cells in mice treated with the neutralizing Ab compared with the control mice. A similar trend was observed in dendritic cells from the draining lymph node, although the difference did not reach statistical significance (Fig. 7F). Together, these results suggest that the improvement in memory CD8⁺ T cell development may be driven through a reduction of PDL1 expression.

Reducing inflammation during priming increases the protective capacity of *Chlamydia*-specific CD8⁺ T cells

To test whether reducing IFN- γ and IL-12 levels during priming increases the recall and protective capacity of *Chlamydia*-specific CD8⁺ T cells, we treated systemically infected mice with IFN- γ and IL-12 neutralizing Abs or isotype control Abs, waited a month for memory T cells to develop in these mice, then isolated and transferred similar numbers of purified CD8⁺ T cells from these two groups of mice into naive mice. The recipient mice were then

challenged transcervically with *C. trachomatis*. Five days later, more CrpA-specific CD8⁺ donor T cells were recovered from uteri of mice that had been given T cells from donor mice that experienced lower levels of proinflammatory cytokines during priming (Fig. 8A). The donor cells that experienced lower levels of proinflammatory cytokines proliferated more in SLOs and uterine tissues (Fig. 8B). These cells also showed downregulation of CD62L in SLOs (Fig. 8C), enabling them to migrate to infected tissues. Moreover, CD8⁺ T cells from neutralizing Ab-treated donors conferred significantly more protection against transcervical *Chlamydia* infection than did CD8⁺ T cells from isotype-control Ab-treated donor mice (Fig. 8D). Because the number but not functionality of memory CrpA-specific CD8⁺ T cells increases in depleted mice (Fig. 4H, 4I), we believe the increased protective capacity conferred by the transferred CD8⁺ T cells from depleted mice is due to the increased percentage and therefore number of *Chlamydia*-specific CD8⁺ T cells among transferred cells, but not alteration in per cell functionality. Finally, to determine whether transient reduction of proinflammatory cytokine signaling also increases the protection conferred by *Chlamydia*-specific CD8⁺

FIGURE 5. *Tbet*^{+/-} mice have more T_{MPEC} and fewer T_{SLEC}. C57BL/6 (B6) or *Tbet*^{+/-} mice were infected i.v. with *C. trachomatis*. (A) CrpA-specific CD8⁺ T cell numbers on days 7 and 21 p.i., (B) numbers of CrpA-specific T_{SLEC} and T_{MPEC} on day 7, (C) T_{SLEC} % versus T_{MPEC} %, and (D) T_{EM} % versus T_{CM} % among CrpA-specific CD8⁺ T cells on day 21 p.i. are shown. Data are representative of two experiments, each with five to six mice per group. **p* < 0.05, ***p* < 0.01.



T cells primed in the genital tract, we transcutaneously inoculated mice with *C. trachomatis*, treated the mice with IFN- γ and IL-12 neutralizing Abs or isotype control Abs on day 4 p.i., allowed the mice to rest for a month, and then rechallenged these mice or naive mice transcutaneously with VacCrpA. Five days later, the vaccinia burden was determined in the uterine tissue of these mice. In this heterologous challenge experiment, the protective effect of primary *C. trachomatis* infection against secondary VacCrpA infection should be mainly mediated by the cross-reactive CrpA-specific CD8⁺ T cells. Consistent with the memory CD8⁺ T cell transfer experiment (Fig. 8D), transient reduction of IFN- γ and IL-12 levels during primary mucosal infection renders the CrpA-specific CD8⁺ T cells more protective against secondary infection (Fig. 8E). Overall, these results suggest that transient dampening of IFN- γ and IL-12 levels during priming not only shifts the phenotype of *Chlamydia*-specific CD8⁺ T cells to favor memory formation but also increases protection conferred by these cells against a secondary challenge.

Discussion

C. trachomatis-specific CD8⁺ T cells can confer protection in mice following immunization with recombinant vaccinia viruses expressing CD8⁺ T cell Ags or when transferred into naive mice from ex vivo culture (13). In a primate trachoma model, the protective immunity elicited by a live-attenuated trachoma vaccine also has been shown to be mediated by CD8⁺ T cells (46). Yet, memory CD8⁺ T cells capable of participating in secondary protection are not stimulated during natural *C. trachomatis* infection in mice. With the goal of understanding why *C. trachomatis* infection does not stimulate protective CD8⁺ T cells, we compared CD8⁺ T cells generated by *C. trachomatis* infection with those generated by VacCrpA. We demonstrated that the proinflammatory cytokines IL-12 and IFN- γ drive *C. trachomatis*-specific CD8⁺ T cells into a short-lived fate and hinder T_{EM} development. A transient blockade of these cytokines during priming not only shifts CD8⁺ effector T cells toward a memory precursor phenotype but also increases memory T cell numbers after stable memory has formed.

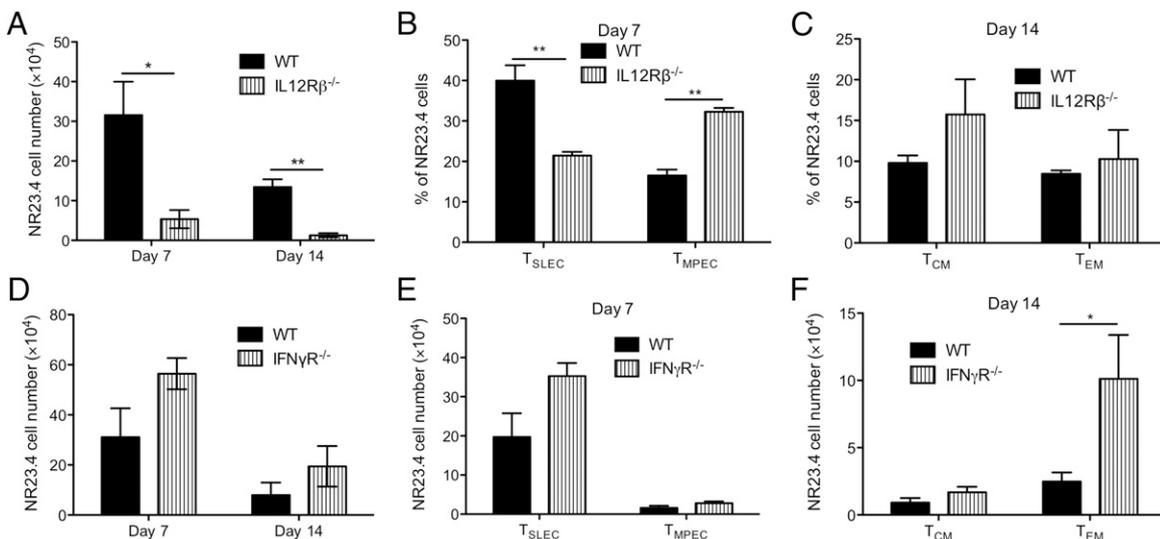


FIGURE 6. Cell-autonomous IL-12 signaling is required for expansion and T_{SLEC} versus T_{MPEC} formation, whereas IFN- γ signaling affects T_{EM} versus T_{CM} formation in *Chlamydia*-specific CD8⁺ T cells. CD90.1 C57BL/6 mice received CD90.2 wild-type (WT), IL-12R β ^{-/-} (A-C), or IFN- γ R^{-/-} (D-F) NR23.4 1 d before i.v. infection with *C. trachomatis*. (A and D) NR23.4 cell numbers in the spleen on days 7 and 14 p.i., (B) T_{SLEC} % and T_{MPEC} % among NR23.4 cells on day 7 p.i., (C) T_{EM} % and T_{CM} % among NR23.4 cells on day 14 p.i., (E) T_{SLEC} and T_{MPEC} NR23.4 cell numbers on day 7 p.i., and (F) T_{EM} and T_{CM} NR23.4 cell numbers on day 14 p.i. are shown. Data are representative of two experiments, each with five to six mice per group. **p* < 0.05, ***p* < 0.01.

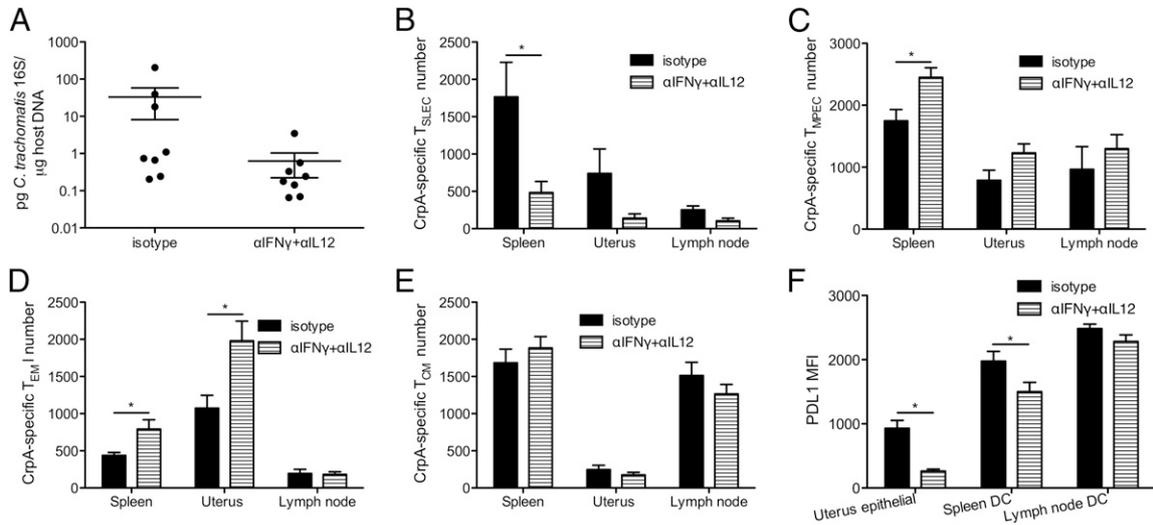
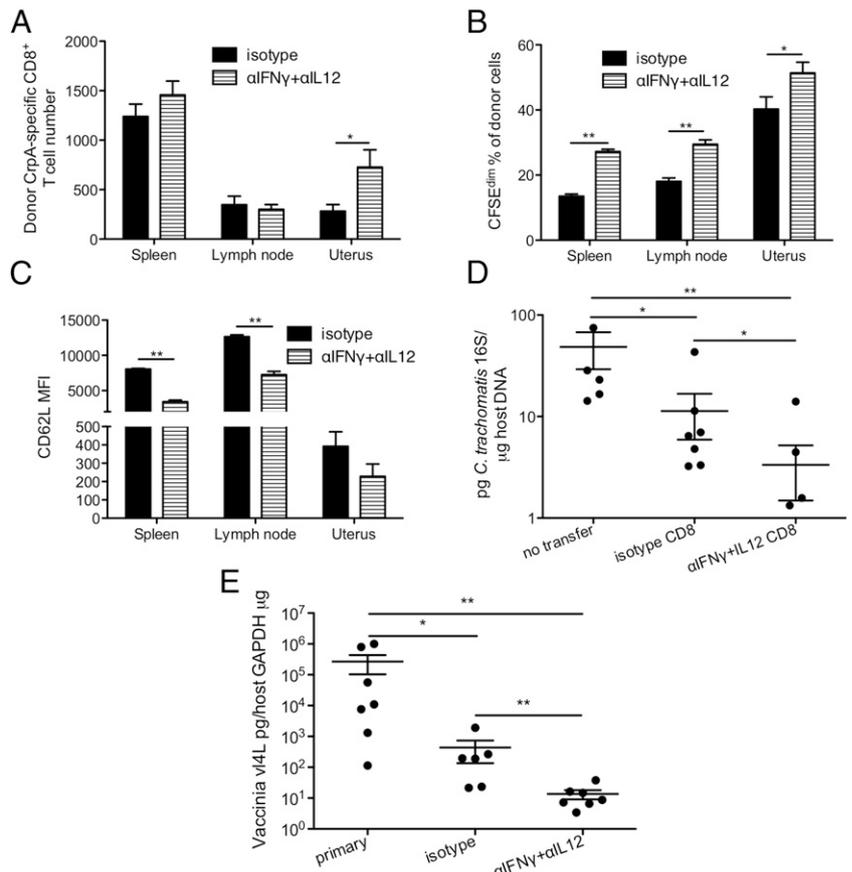


FIGURE 7. Transient reduction of proinflammatory cytokine signaling during transcervical infection improves memory development of CD8⁺ T cells. Mice were transcervically infected with *C. trachomatis* and treated with isotype control or IFN- γ and IL-12 neutralizing Abs on day 4 p.i. (A) *Chlamydia* burden in the uterus on day 7 p.i., (B) CrpA-specific T_{SLEC}, (C) T_{MPEC} numbers on day 7 p.i., and (D) CrpA-specific T_{EM}, (E) T_{CM} numbers on day 14 p.i. are shown. (F) The PDL1 mean fluorescence intensity (MFI) of uterine epithelial cells (CD326⁺) and dendritic cells (CD3⁻CD11c⁺) in the spleen and lymph node on day 7 p.i. is shown. Data are representative of at least two experiments, each with six to eight mice per group. **p* < 0.05.

Helpless CD8⁺ T cells typically fail to be efficiently maintained, and those that are maintained tend to have elevated KLRG1 expression and reduced CD127 and CD27 expression (37, 38). We observed all of these characteristics in *Chlamydia*-specific memory CD8⁺ T cells, suggesting that a lack of CD4⁺ T cells help might contribute to the faulty memory development of *C. trachomatis*-stimulated CD8⁺ T cells. However, our data suggest that the faulty CD8⁺ T cell memory development fol-

lowing *C. trachomatis* infection might not result from a lack of direct CD4⁺ T cell help. A number of mechanisms have been described to mediate help for CD8⁺ T cells. For example, CD4⁺ T cells can license APCs to become more potent in activating CD8⁺ T cells. However, no significant differences in stimulatory or inhibitory coreceptor expression on CD8⁺ T cells or their ligand expression on APCs were noted following *C. trachomatis* versus VacCrpA infection (data not shown). CD4⁺ T cells can

FIGURE 8. Reducing proinflammatory cytokine signaling during priming increased the protective capacity of CD8⁺ T cells following transcervical *Chlamydia* infection. (A–D) CD90.1 mice were i.v. infected with *C. trachomatis* and treated with isotype control or Abs to deplete cytokines on day 4 p.i. On day 28 p.i., CD8⁺ T cells were purified from pooled SLOs, CFSE-labeled, and transferred into naive CD90.2 mice. The recipient mice and control mice that did not receive any T cells (no transfer) were transcervically infected with *C. trachomatis*. (A) The number of donor cells, (B) CFSE^{dim} % among donor cells, (C) CD62L mean fluorescence intensity (MFI) of donor cells, and (D) *Chlamydia* burden in the uterus on day 6 p.i. are shown. (E) Mice were transcervically infected with *C. trachomatis* and treated with isotype control or Abs to deplete cytokines on day 4 p.i. On day 28 p.i., these mice and naive mice (primary) were challenged transcervically with VacCrpA. Viral burden in the uterus on day 6 p.i. is shown. Data are representative of at least two experiments, each with six to seven mice per group. **p* < 0.05, ***p* < 0.01.



also directly interact with CD8⁺ T cells through interactions of membrane-bound molecules, such as CD40-CD40L (47), or through soluble factors, such as IL-2 (25) and/or IL-15 (48). Although we did not observe differences in CD40 expression on CD8⁺ T cells (data not shown), we did observe delayed IL-2R α expression on *C. trachomatis*-stimulated CD8⁺ T cells. Nevertheless, boosting IL-2-mediated signaling by IL-2/anti-IL-2 immune complex treatment did not rescue the CD8⁺ T cell response.

Naive T cell activation, effector differentiation, and subsequent memory T cell development are regulated by TCR signals, costimulation, and inflammation, which are usually referred to as signals 1, 2, and 3, respectively. To understand the mechanisms underpinning faulty memory CD8⁺ T cell development during *C. trachomatis* infection, we compared these three signals experienced by CrpA-specific CD8⁺ T cells when stimulated by *C. trachomatis* versus VacCrpA. Although both pathogens express CrpA, it is not straightforward to control for the level of Ag presentation, given their different replication niches. We chose to challenge with doses of *C. trachomatis* and VacCrpA that expand CrpA-specific CD8⁺ T cells to a similar extent, and compared the strength of signal 2 and 3 under these conditions. We found no significant differences in costimulatory or inhibitory molecule (CD28, 4-1BB, OX40, and PD1) expression on CrpA-specific CD8⁺ T cells or their ligand expression on APCs following infection with *C. trachomatis* versus VacCrpA (data not shown), suggesting that signal 2 potency was similar.

Accumulating evidence suggests that signal 3 provided by proinflammatory cytokines, mainly IL-12 and type 1 and 2 IFNs, promotes Ag-specific CD8⁺ T cell expansion (49–52). However, this signal can also induce terminal differentiation and thus shorten the lifespan of these cells (44, 53). This effect appears to be pathogen specific. For instance, IL-12 promotes terminal maturation at the expense of memory precursor subpopulation differentiation following *Listeria* infection and during *Toxoplasma* vaccination (53–56). In contrast, no significant differences in T_{SLEC}/T_{MPEC} formation between wild-type and IL-12R β ^{-/-} T cells were observed in the context of lymphocytic choriomeningitis virus, vesicular stomatitis virus, or vaccinia virus infection (54). We observed a significant switch from T_{SLEC} to T_{MPEC} phenotype in IL-12^{-/-} mice (data not shown) and in transgenic cells lacking IL-12R β following *C. trachomatis* infection. IFN- γ production during the first 24 h of infection has been shown to regulate the program of CD8⁺ T cell contraction during *Listeria* infection through downregulation of IL-7R (57–59). IFN- γ R is also required in a CD8⁺ T cell autonomous manner for memory CD8⁺ T cell formation during lymphocytic choriomeningitis virus infection (60). We did not observe significant differences in T_{SLEC} versus T_{MPEC} formation between wild-type and IFN- γ R-deficient *Chlamydia*-specific CD8⁺ T cells. Overall, we found that IL-12, but not IFN- γ , is critical for T_{SLEC} versus T_{MPEC} fate determination of *C. trachomatis*-stimulated CD8⁺ T cells.

The terminal differentiation of effector CD8⁺ T cells during infection is inextricably linked to Ag dose, duration of antigenic stimulation, and inflammatory stimuli. In the case of *C. trachomatis*, clearance largely depends on IFN- γ secreted by T cells (11, 25). Because IL-12, IFN- γ , and Tbet play a protective role during *C. trachomatis* infection, a comparison of T cell responses in mice that lack these molecules is complicated by differences in *Chlamydia* burden and therefore Ag load. Therefore, in this study, we 1) transiently treated animals with Abs to neutralize cytokines, 2) determined the developmental phenotypes of transgenic cells lacking receptors for these cytokines, and 3) challenged Tbet heterozygous animals to avoid significantly changing Ag load

while still manipulating the level of inflammation. Although we cannot rule out the impact of subtle changes in Ag load or duration on effector CD8⁺ T cell differentiation, we did not observe a significant change in bacterial burden in all three experimental manipulations described above. Yet, we observed a shift of the *Chlamydia*-specific CD8⁺ T cells toward a memory precursor phenotype. Overall, our study indicates that excess induction of IL-12, not excess Ag load, during priming might drive terminal differentiation of effector *Chlamydia*-specific CD8⁺ T cells.

Memory T cell populations are heterogeneous, and two of the best characterized subsets are T_{EM} and T_{CM} (61). T_{EM} are thought to provide immediate effector function at the portal of pathogen entry but exhibit reduced proliferative capacity (62). T_{CM} migrate through SLOs and are efficient in homeostatic renewal and secondary proliferative responses (63). The results of experiments comparing the protective capacities of T_{CM} and T_{EM} have been mixed and might depend on the route of infection, pathogen dose, or tropism (63–66). We found that reducing IFN- γ signaling promotes T_{EM} formation without sacrificing T_{CM} formation. This increase of T_{EM} numbers is associated with increased protection conferred by memory CD8⁺ T cells against either *C. trachomatis* or a heterologous vaccinia virus genital tract challenge. Future experiments comparing the per cell protective capacity of T_{EM} versus T_{CM} CD8⁺ T cells stimulated by *C. trachomatis* will further clarify the role of each memory population in protection against this pathogen.

Developing effective vaccines is critical for preventing infection and/or immunopathology induced by *C. trachomatis*. It is important to note that preferentially inducing the T_{MPEC} CD8⁺ T cells might be as critical as inducing a large number of CD8⁺ T cells. Our data show that proinflammation cytokine signaling has a negative impact on memory CD8⁺ T cell development following *C. trachomatis* infection. Thus, future vaccine design for *C. trachomatis* will benefit from a careful choice of Ags/adjuvants and their doses to achieve a balance in the cytokine milieu that favors effector cell expansion without driving CD8⁺ T cells into terminal differentiation.

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Disclosures

The authors have no financial conflicts of interest.

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