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An Excess of the Pro-inflammatory cytokines IFNγ and IL12 Impairs the Development of the Memory CD8+ T cell Response to *Chlamydia trachomatis*

Xuqing Zhang and **Michael N. Starnbach**

Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA

Abstract

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 a MHCI-restricted *Chlamydia* antigen 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 IL12 and IFNγ contributes to poor memory $CD8^+$ T cell development during *C. trachomatis* infection of mice. IL12 is required for $CD8^+$ T cell expansion but drives effector $CD8^+$ T cells into a short-lived fate whereas IFNγ signaling impairs the development of effector memory cells. We show that transient blockade of IL12 and IFNγ 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.

Introduction

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 antibodies. A typical $CD8^+$ T cell response is characterized by a 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 cells development are not thoroughly understood and usually occur early in the immune response (4, 5). Here we investigate the factors that drive memory $CD8^+$ T

Correspondence: Michael N Starnbach, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA, 02115, Tel: 617 432 1873, starnbach@hms.harvard.edu.

cell fate following infection with the obligate intracellular bacterial pathogen, *Chlamydia trachomatis*.

C. trachomatis infects over 100 million people worldwide annually (WHO, 2008), and is both the most prevalent bacterial genital tract infection and the leading cause of preventable blindness. Chronic *C. trachomatis* genital tract infections lead to pelvic inflammatory disease (PID), which can cause fallopian tube scarring, infertility, and ectopic pregnancy (6, 7). 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 (8). 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 antibody and $CD4^+$ T cells clearly are required for full immunity to *C. trachomatis* (9, 10), $CD8^+$ 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 MHCI but not usually MHCII. Because *C. trachomatis* translocates a subset of its proteins into the host cell cytosol it allows for MHCI processing of these proteins and subjects the cell to recognition by $CD8^+$ T cells (11, 12). $CD8^+$ T cells have been shown to protect against infection when cultured *ex vivo* and transferred into naïve animals, and immunization with recombinant vaccinia viruses expressing $CD8^+$ T cell antigens from *C. trachomatis* also confers protection in mice (12). Yet during natural infection of mice, the $CD8^+$ T cell response does not play a significant protective role (13, 14). 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 (15, 16).

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 antigen specificity induced by recombinant vaccinia virus expressing a *C. trachomatis* antigen, CrpA (robust recall) (16). We found that the proinflammtory cytokines IL12 and IFNγ drive effector $CD8^+$ T cells stimulated by *C. trachomatis* into a short-lived fate (T_{SLEC}) and impair the development of effecter memory cells. Transient blockade of these cytokines during priming increases the frequency of memory precursor $CD8^+$ 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-*Ifngr1*^{tm1Agt}/J ($IFN\gamma R^{-/-}$), and B6.129S1-*Il12rb1*^{tm1Jm}/J ($IL12R\beta^{-/-}$) 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) (17). 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) (18). To generate *Chlamydia*-specific CD8^{+} TCR transgenic mice specific for CrpA$_{63\text{-}71}$, we cloned the rearranged genomic TCRα and TCRβ sequences from *Chlamydia*-specific CD8^{+} T cell clone NR23.4 into expression vectors (19). The cloned TCR constructs were then linearized and injected into C57BL/6 fertilized oocytes. TCR tg 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. In order to restrict TCR expression, we crossed these mice onto a RAG1$^{-/-}$ background (NR23.4 mice). The rearranged TCR from NR23.4 uses the Vα4JTA13 and Vβ8.2Jβ2.5 receptor chains. NR23.4 IL12Rβ$^{-/-}$ and NR23.4 IFNγR$^{-/-}$ were generated by crossing NR23.4 mice with IL12Rβ$^{-/-}$ and IFNγR$^{-/-}$ 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 (EBs) were purified by density gradient centrifugation as previously described (20). Aliquots were stored at −80 °C in sucrose-phosphate-glutamate buffer (SPG) and thawed immediately before use. Construction of the recombinant vaccinia virus expressing the *Chlamydia* CrpA protein (VacCrpA) has been described previously (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 before infecting mice.

Preparation of IL2-anti-IL2 complexes

IL2-anti-IL2 complexes were prepared as previously described (23–25). 1.5 μg carrier-free mouse recombinant IL2 (eBioscience) and 50 μg anti-IL2 monoclonal antibody (S4B6, BioXCell) were mixed in 10 μl HBSS at room temperature for 15 minutes before adding 190 μl HBSS for each injection. Control groups were treated with IgG2a isotype control antibodies (2A3, BioXCell).

Infection of mice and preparation of tissue

For systemic infection, mice were infected i.v. with 10^7 inclusion-forming units (IFU) of *C. trachomatis* in 200 μl SPG, 2×10^3 PFU of VacCrpA in 200 μl PBS, or 10^3 CFU of *Lm*CrpA in 200 μl of 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 one week later transcervically with 5×10^6 IFU of *C. trachomatis* or 5×10^5 PFU of VacCrpA as described previously (26). 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/Ca^{2+}/Mg^{2+} containing 1 mg/ml type XI collagenase and 50 Kunitz/ml DNase for 30 minutes at 37° C, washed in Ca^{2+}/Mg^{2+}-free PBS containing 5 mM EDTA, and then ground between frosted microscope slides prior to filtration through a 70-cm mesh (27). Single cell suspensions of secondary lymphoid organs (SLO) were prepared by grinding the tissue between frosted microscope slides. Red blood cells in the splenocytes were lysed using ammonium chloride.

Flow cytometry

Cells were immediately stained for surface and activation markers or stimulated for 4–5 hours with 10μM $CrpA_{63\text{-}71}$ peptide in the presence of brefeldin A (Biolegend) for intracellular cytokine staining. The D^b/ASFVNPIYL ($CrpA_{63\text{-}71}$) MHC tetramer was generated at the National Institutes of Health Tetramer Facility. Antibodies 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 (ebiosciences), anti-CD4 $Qdot^{605}$ (Invitrogen), anti-IL18Rα (R&D systems), and anti-IFNγ APC-Cy7 (BD Biosciences). Cells were pre-incubated with CD16/CD32 (2.4G2) before staining with tetramer and fluorochrome (APC, APC-Cy7, FITC, PE, PerCP, PerCP-Cy5.5, PE-Cy7, Pacific Blue, PE-Texas Red) conjugated antibodies 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), IL18Rα (112614), CD122 (TM-β1), 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 antibodies 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-IFNγ (XMG 1.2). The absolute cell number in each sample was determined using AccuCheck Counting Beads (Invitrogen). Data were collected on a LSRII (BD Bioscience) and analyzed using FlowJo (Tree Star Industries, 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 proliferation rate of T cells, mice were injected i.p. with 1 mg of 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 (12). To deplete cytokines, mice were injected i.p. with 200 μg anti-IFNγ (XMG1.2) together with 200 μg anti-IL12 (C17.8), or isotype control (200 μg HRPN and 200 μg 2A3) in 200 μl 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 depletion protocol. IFNγ level is below the limit of detection in depletion antibody treated

mice. IL12 level is significantly lower in mice treated with depletion antibodies (12.99±0.02% of the levels in control mice) compared to control antibody treated mice ($p < 0.01$). All isotype and neutralizing antibodies 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 10^4–10^5 cells i.v. into tail veins one day 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 Dynal Mouse CD8 Negative Isolation Kit according to manufacturer's instructions (Invitrogen). Isolated cells were then labeled with 10 μM CFSE (Invitrogen) as previously described (28). Unless otherwise stated, 5×10^6 $CD8^+$ T cells were injected i.v. into naïve mice 4 hours 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 (qPCR) (29, 30). 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 (Vvl4L), and mouse GAPDH DNA content of individual samples were then quantified by qPCR 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 pg) of *Chlamydia* DNA or vaccinia DNA per unit weight (in μg) 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 ± standard error.

Results

$CD8^+$ T cells induced by *C. trachomatis* contract more than those induced by VacCrpA

VacCrpA is known to induce a more robust CrpA-specific recall response than *C. trachomatis* (16). 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* to those induced by VacCrpA. To rule out the impact of pathogen level on memory $CD8^+$ T cell development, the VacCrpA 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 VacCrpA infection (Fig. 1A). Two thousand PFU of VacCrpA 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 VacCrpA 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 VacCrpA. A similar proportion of memory CrpA-specific CD8$^+$ T cells from *C. trachomatis*- or VacCrpA-infected mice secreted IFNγ following *ex vivo* restimulation (Fig. 1C), suggesting that the memory cells that did survive the contraction following *C. trachomatis* infection were similarly functional compared to cells stimulated by VacCrpA infection. Although these memory cells proliferated as efficiently as VacCrpA-induced memory cells as measured by BrdU uptake (Fig. 1D), they expressed higher level of CD95 (FasR) (Fig. 1E), suggesting that these cells are more prone to apoptosis. Moreover, compared to CrpA-specific memory CD8$^+$ T cells from VacCrpA infected mice, CrpA-specific cells from *C. trachomatis* infected mice expressed lower levels of CD27, CD122 (IL2/IL15 receptor β), and IL18Rα, all of which are critical for the recall capacity of memory CD8$^+$ T cells (Fig. 1F–H) (31–34). Overall, these data suggest that although CrpA-specific CD8$^+$ T cells are expanded by *C. trachomatis* or VacCrpA infection to a similar extent, *C. trachomatis*-stimulated cells contract more and the memory cells that do survive the contraction are of lower quality.

IL2-α-IL2 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 (35–39). One of the mechanisms by which helper T cells mediate help is to instruct DCs to produce cytokines that induce up-regulation of IL2Rα (CD25) on Ag-specific CD8$^+$ T cells, rendering them more responsive to IL2 (40, 41). We therefore explored whether CD25 expression is differentially stimulated following infection with *C. trachomatis* vs. VacCrpA. Since 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 CrpA-specific transgenic cells expressed CD25 as they divided in *C. trachomatis*-infected mice compared to VacCrpA-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 to those infected with VacCrpA (Fig. 2A).

Previous reports have shown that IL2 signaling not only augments the accumulation of CD8$^+$ T cells, but also programs the ability of memory cells to expand upon secondary challenge (40–43). To test whether IL2 signaling differences were responsible for the poor recall capacity of *Chlamydia*-stimulated memory cells, we treated mice with IL2-anti-IL2 (IL2/S4B6) complexes that have been shown to increase the recall capacity of CD8$^+$ T cells (44). Mice were infected with *C. trachomatis*, treated with IL2-anti-IL2 complexes or isotype control antibodies on days 3 and 5 (early) or on days 24 and 26 (late), and then rechallenged with VacCrpA on day 28. The numbers of CrpA-specific CD8$^+$ T cells in these mice were determined 5 days 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 IL2 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 up-regulation 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 (IL7Rα) has been shown to mark two effector T cell populations with distinct memory potential. The $CD127^{low}KLRG1^{high}$ short-lived effector cells (T_{SLEC}) do not gain memory T cell potential. Rather, it is primarily the descendents of $CD127^{high}KLRG1^{low}$ memory precursor effector cell (T_{MPEC}) that participate in secondary responses upon reinfection (45). We hypothesized that *C. trachomatis* infection may favor the development of T_{SLEC} since the kinetics of the *Chlamydia*-specific $CD8^+$ T cell response resembles the kinetics of short-lived effector cells. To test whether *C. trachomatis* and VacCrpA differentially stimulate T_{SLEC} vs. 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* vs. VacCrpA. At the peak of expansion, more CrpA-specific $CD8^+$ T cells induced by *C. trachomatis* were T_{SLEC} than those induced by VacCrpA (Fig. 3A, 3B). In contrast, VacCrpA 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 VacCrpA infection.

Transient reduction of IFNγ and IL12 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 (45). To assess whether *C. trachomatis* and VacCrpA infected mice experience differential levels of inflammation, we measured levels of several cytokines in serum of these mice, including IFNγ, IL12, IL6, IL10, IL7, and IL2. Among the cytokines tested, the levels of two pro-inflammatory cytokines, IFNγ and IL12, were higher in serum from *Chlamydia*-infected mice compared to serum from VacCrpA-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 is responsible for the dominance of the T_{SLEC} phenotype in the pathogen-specific $CD8^+$ T cells, we treated infected mice with a single dose of neutralizing antibodies against IFNγ and IL12 or isotype control antibodies 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 T_{EM} ($CD127^+CD62^{low}$) is inhibited during *C. trachomatis* infection (16). Cytokine-neutralizing Ab treatment increased the percentage and number of T_{EM} among CrpA-specific $CD8^+$ T cells without sacrificing the development of 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 percentage of CrpA-specific $CD8^+$ T cells secreted the effecter cytokine, IFNγ, in mice treated with depletion and control antibodies (Fig. 4I). Overall, these data suggest that transient ablation of pro-inflammatory 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

IFNγ and IL12 are known to regulate the expression of Tbet, a transcription factor critical for regulating $CD8^+$ T cell memory development (45, 46). To determine whether IFNγ and IL12 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 (45). 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} vs. 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 $CD62L^{low}$ T_{EM} population was favored (Fig. 5D). Taken together with the IFNγ and IL12 ablation experiments (Fig. 4), these results suggest that pro-inflammatory cytokines, IFNγ and IL12, modulate Tbet to alter pathogen-specific $CD8^+$ T cell development following *C. trachomatis* infection.

IL12 is critical for T_{MPEC} vs. T_{SLEC} development while IFNγ affects T_{EM} vs. T_{CM} formation among *C. trachomatis*-specific $CD8^+$ T cells

We next explored whether cell intrinsic IFNγ or IL12 signaling in pathogen-specific $CD8^+$ cells is responsible for altering T_{SLEC} vs. T_{MPEC} or T_{EM} vs. T_{CM} development following *C. trachomatis* infection. We crossed *Chlamydia*-specific $CD8^+$ TCR transgenic mice (NR23.4) onto the $IL12R\beta^{-/-}$ or $IFN\gamma R^{-/-}$ backgrounds to create CrpA-specific $CD8^+$ T cells that do not respond to IL12 or IFNγ. The transgenic cells lacking IL12Rβ did not expand as efficiently as wild-type cells (Fig. 6A), suggesting that IL12 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 IL12 did shift toward a T_{MPEC} phenotype (Fig. 6B), consistent with the cytokine depletion experiments described above. IL12 signaling did not seem to affect T_{EM} vs. T_{CM} development since the percentages of T_{EM} and T_{CM} were similar between wild-type and $IL12R\beta^{-/-}$ cells (Fig. 6C). In contrast, similar numbers of wild-type and $IFN\gamma R^{-/-}$ transgenic cells were recovered at the peak of

expansion (Fig. 6D), suggesting that IFNγ signaling is not required for pathogen-specific $CD8^+$ T cell expansion following *C. trachomatis* infection. The transgenic cells that do not respond to IFNγ did not show an obvious shift toward T_{MPEC} (Fig. 6E) but did show an increase of T_{EM} numbers (Fig. 6F). Overall, these data suggest that IL12 signaling is important for T_{MPEC} vs. T_{SLEC} differentiation while IFNγ is involved in T_{EM} vs. T_{CM} development.

Transient ablation of pro-inflammatory cytokines during mucosal infection also favors the formation of memory precursor $CD8^+$ T cells

To test whether reducing pro-inflammatory 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 IFNγ and IL12 did not significantly alter *Chlamydia* burden in the uterus of infected mice (Fig. 7A). This treatment did shift $CD8^+$ T cells in the spleens towards a T_{MPEC} phenotype (Fig. 7B, C). A similar trend was observed in the uterine tissues and draining lymph nodes although the differences did not reach statistical significance (Fig. 7B, C). Transient pro-inflammatory cytokine ablation also increased the number of T_{EM} cells in the spleens and uterine tissues of mucosally infected mice without affecting the numbers of T_{CM} cells (Fig. 7D, E). Overall, in mice infected in the genital tract, transient reduction of IFNγ and IL12 levels shifted the $CD8^+$ T cells towards a T_{MPEC} and a T_{EM} phenotype, consistent with what was observed in systemically infected mice.

A recent report from our lab 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 (15). To test whether reducing pro-inflammatory cytokines modulates memory development by regulating PDL1 expression, we transcervically infected mice with *C. trachomatis*, treated the mice with IFNγ and IL12 neutralizing antibodies, and then determined PDL1 expression on various cell populations. We found that neutralization of the pro-inflammatory cytokines reduced PDL1 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 of PDL1 level on splenic dendritic cells in mice treated with the neutralizing antibody compared to 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 cells 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 IFNγ and IL12 levels during priming increases the recall and protective capacity of *Chlamydia*-specific $CD8^+$ T cells, we treated systemically infected mice with IFNγ and IL12 neutralizing antibodies or isotype control antibodies, 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 naïve 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 pro-inflammatory cytokines during priming (Fig. 8A). The donor cells that experienced lower levels of pro-inflammatory 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 antibody treated donors conferred significantly more protection against transcervical *Chlamydia* infection than $CD8^+$ T cells from isotype-control antibody treated donor mice (Fig. 8D). Since 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 pro-inflammatory cytokine signaling also increases the protection conferred by *Chlamydia*-specific $CD8^+$ T cells primed in the genital tract, we transcervically inoculated mice with *C. trachomatis*, treated the mice with IFNγ and IL12 neutralizing antibodies or isotype control antibodies on day 4 p.i., allowed the mice rest for a month, and then re-challenged these mice or naïve mice transcervically 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 IFNγ and IL12 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 IFNγ and IL12 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 antigens or when transferred into naïve mice from *ex vivo* culture (12). 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 (47). 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 to those generated by VacCrpA. We demonstrated that the pro-inflammatory cytokines IL12 and IFNγ 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 towards a memory precursor phenotype but also increases memory T cell numbers after stable memory has formed.

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 (38, 39). We observed all of these characteristics in *Chlamydia*-specific memory $CD8^+$ T cells, suggesting that a lack of $CD4^+$ T cell 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 following *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 co-receptor expression on $CD8^+$ T cells or their ligand expression on APCs were noted following *C. trachomatis* vs. VacCrpA infection (data not shown). $CD4^+$ T cells can also directly interact with $CD8^+$ T cells through interactions of membrane-bound molecules, such as CD40-CD40L (48), or through soluble factors, such as IL2 (25) and/or IL15 (49). Although we did not observe differences in CD40 expression on $CD8^+$ T cells (data not shown), we did observe delayed IL2Rα expression on *C. trachomatis*-stimulated $CD8^+$ T cells. Nevertheless, boosting IL2 mediated signaling by IL2-anti-IL2 immune complex treatment did not rescue the $CD8^+$ T cell response.

Naïve 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. 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* vs. VacCrpA. Although both pathogens express CrpA, it is not straightforward to control for the level of antigen 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* vs. VacCrpA (data not shown), suggesting that signal 2 potency was similar.

Accumulating evidence suggests that although signal 3 provided by pro-inflammatory cytokines, mainly IL12, and Type 1 and 2 IFNs, promote antigen-specific $CD8^+$ T cell expansion (50–53), they can also induce terminal differentiation and thus shorten the lifespan of these cells (45, 54). This effect appears to be pathogen specific. For instance, IL12 promotes terminal maturation at the expense of memory precursor subpopulation differentiation following *Listeria* infection and during *Toxoplasma* vaccination (54–57). In contrast, no significant differences in T_{SLEC}/T_{MPEC} formation between wild-type and $IL12R\beta^{-/-}$ T cells were observed in the context of lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus or vaccinia virus infection (55). We observed a significant switch from T_{SLEC} to T_{MPEC} phenotype in $IL12^{-/-}$ mice (data not shown) and in transgenic cells lacking IL12Rβ following *C. trachomatis* infection. IFNγ production during the first 24 hours of infection has been shown to regulate the program of $CD8^+$ T cell contraction during *Listeria* infection through down-regulation of IL7R (58–60). IFNγR is also required in a $CD8^+$ T cell autonomous manner for memory $CD8^+$ T cell formation during LCMV infection (61). We did not observe significant differences in T_{SLEC} vs.

T_{MPEC} formation between wild-type and IFNγR-deficient *Chlamydia*-specific $CD8^+$ T cells. Overall, we found that IL12 but not IFNγ is critical for T_{SLEC} vs. 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 antigen dose, duration of antigenic stimulation, and inflammatory stimuli. In the case of *C. trachomatis*, clearance largely depends on IFNγ secreted by T cells (10, 26). Because IL12, IFNγ, 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 antigen load. Therefore, in this study we 1) transiently treated animals with antibodies 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 antigen load while still manipulating the level of inflammation. Although we cannot rule out the impact of subtle changes in antigen load/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 towards a memory precursor phenotype. Overall our study indicates that excess induction of IL12, not excess antigen 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} (62). T_{EM} cells are thought to provide immediate effector function at the portal of pathogen entry but exhibit reduced proliferative capacity (63). T_{CM} cells migrate through SLOs and are efficient in homeostatic renewal and secondary proliferative responses (64). 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 (64–67). We found that reducing IFNγ signaling promotes T_{EM} formation without sacrificing T_{CM} formation. This increase of T_{EM} cell 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} vs. 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 pro-inflammation 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 antigens/adjuvants and their doses such that there is a balance in the cytokine milieu that favors effector cell expansion without driving $CD8^+$ T cells into terminal differentiation.

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Abbreviations used in this article

KLRG1	killer cell lectin-like receptor G1
T_{SLEC}	short-lived effector T cells
T_{MPEC}	memory precursor effector cells
IFU	inclusion-forming units
SLO	secondary lymphoid organ
p.i	post-infection
T_{EM}	effecter memory T cells
T_{CM}	central memory T cells
LCMV	lymphocytic choriomeningitis virus

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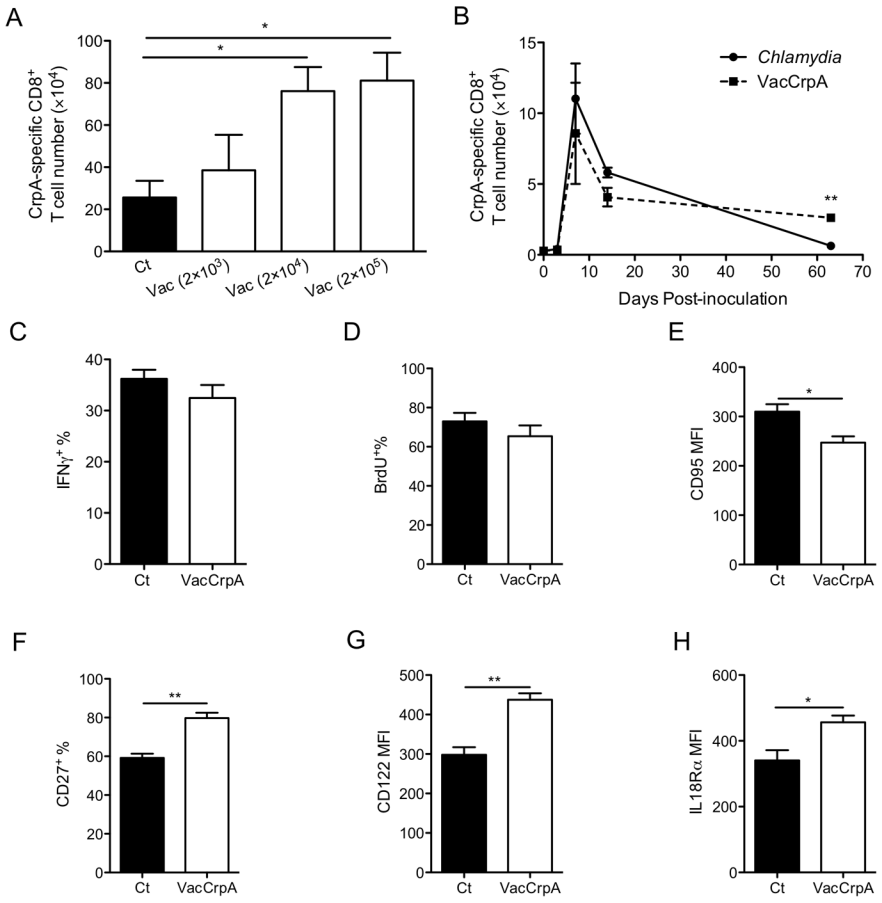

Figure 1.

C. trachomatis induces impaired memory CD8$^+$ T cells. (**A**) CrpA-specific CD8$^+$ T cell numbers in the spleen of systemically infected mice on day 7 p.i are shown. (**B**) CrpA-specific CD8$^+$ T cell numbers in the spleen of mice systemically infected with *C. trachomatis* or 2×10^3 PFU of VacCrpA on indicated days p.i. are shown. (**C**) IFNγ^+ %, (**D**) BrdU$^+$ %, (**E**) CD95 MFI, (**F**) CD27$^+$ %, (**G**) CD122 MFI, and (**H**) IL18Rα 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 5–7 mice per group.

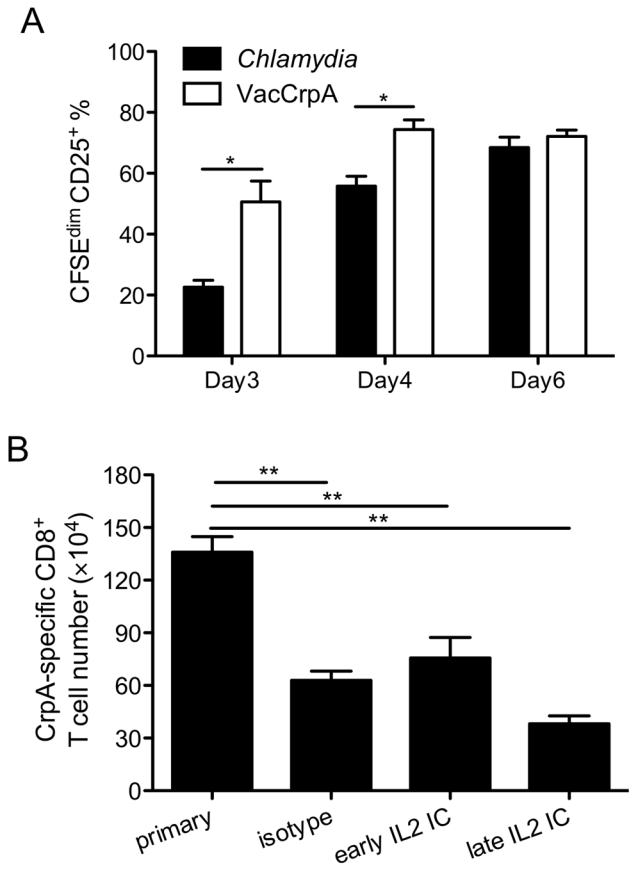

Figure 2.

IL2-anti-IL2 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 IL2-anti-IL2 immune complex (IL2 IC) or isotype control antibodies on days 3 and 5 p.i. (early) or on days 24 and 26 p.i. (late), and re-challenged with VacCrpA on day 28 p.i. CrpA-specific $CD8^{+}$ T cell numbers 5 days after secondary challenge are shown. Representative data from two experiments are shown, each with 5–7 mice per group.

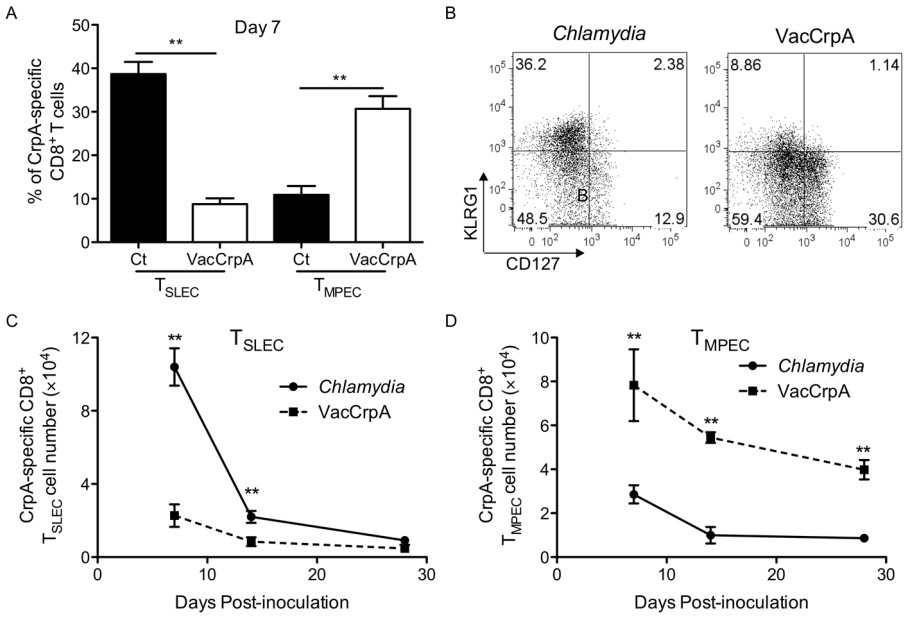

Figure 3.

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 5–7 mice per group.

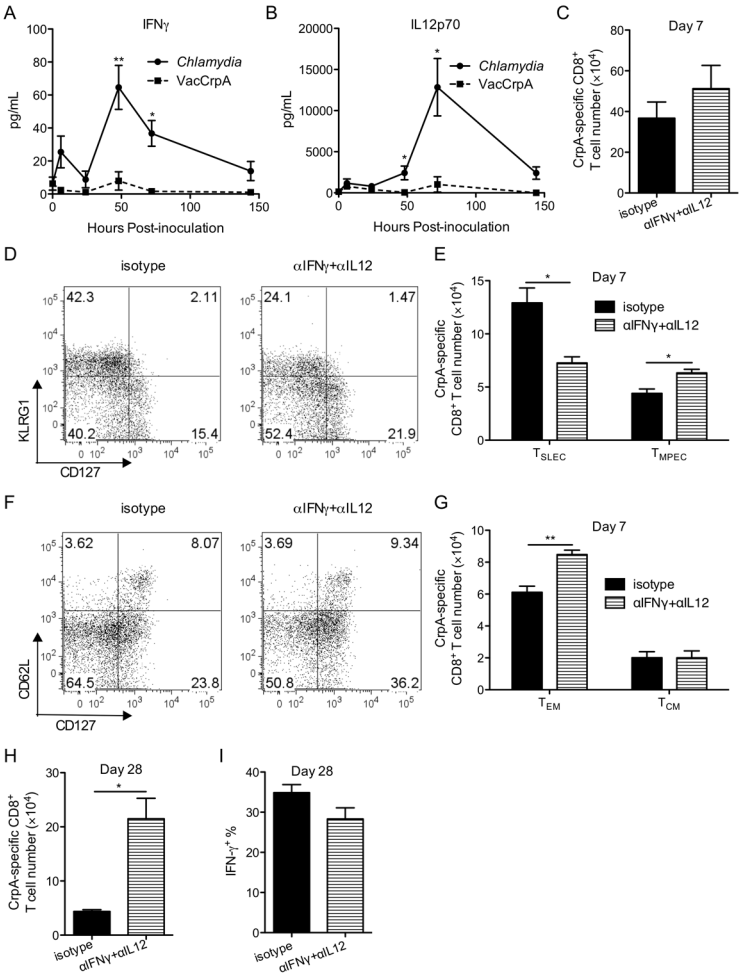

Figure 4.

Transient reduction of pro-inflammatory cytokines early during priming improves the development of memory CrpA-specific CD8$^+$ T cells. (**A, B**) Serum IFNγ (A) and IL12p70 (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 IFNγ and IL12 neutralizing antibodies (αIFNγ+αIL12) 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, (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) IFNγ $^+$ % among CrpA-specific CD8$^+$ T cells on day 28 p.i. are shown. Data are representative of at least two experiments, each with 5–7 mice per group.

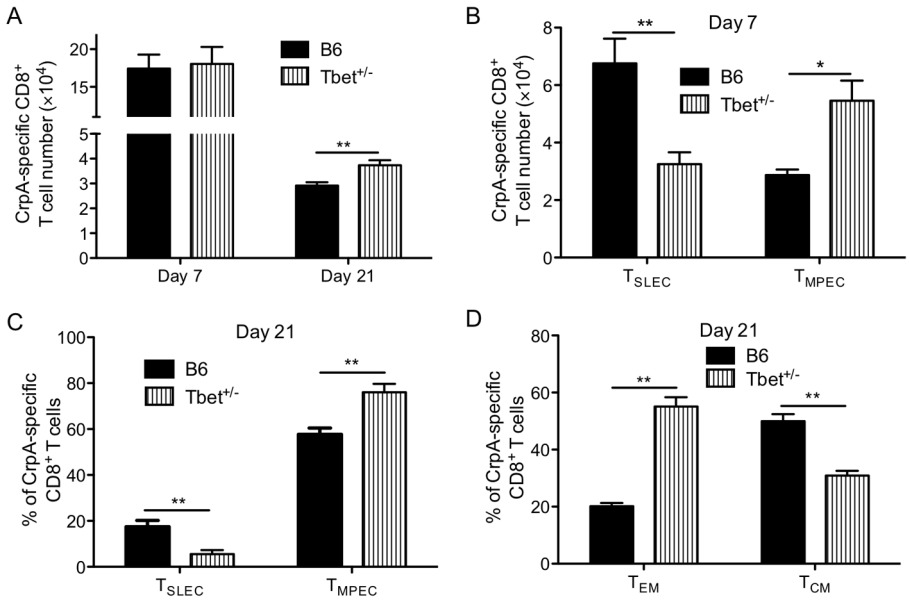

Figure 5.

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} % vs. T_{MPEC} %, and (**D**) T_{EM} % vs. T_{CM} % among CrpA-specific CD8^{+} T cells on day 21 p.i. are shown. Data are representative of two experiments, each with 5–6 mice per group.

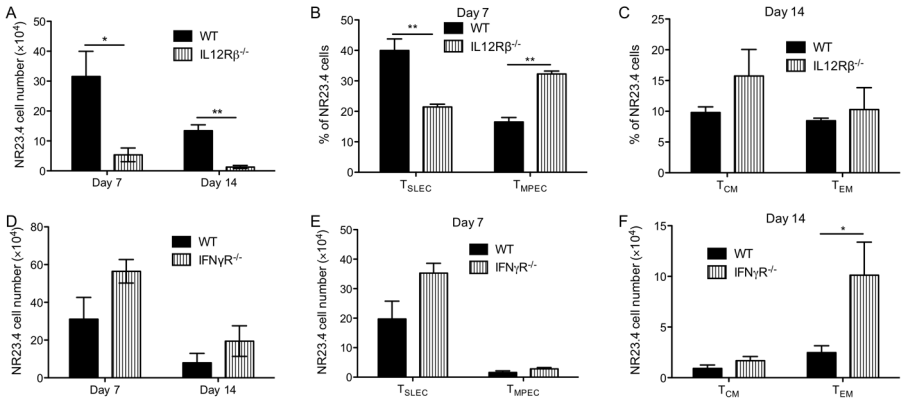

Figure 6.

Cell autonomous IL12 signaling is required for expansion and T_{SLEC} vs. T_{MPEC} formation while IFNγ signaling affects T_{EM} vs. T_{CM} formation in *Chlamydia*-specific $CD8^+$ T cells. CD90.1 C57BL/6 mice received CD90.2 wild-type (WT), IL12Rβ$^{-/-}$ (**A–C**), or IFNγR$^{-/-}$ (**D–F**) NR23.4 one day before i.v. infection with *C. trachomatis*. (**A, D**) NR23.4 cell numbers in the spleen on day 7and 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 21 p.i. are shown. Data are representative of two experiments, each with 5–6 mice per group.

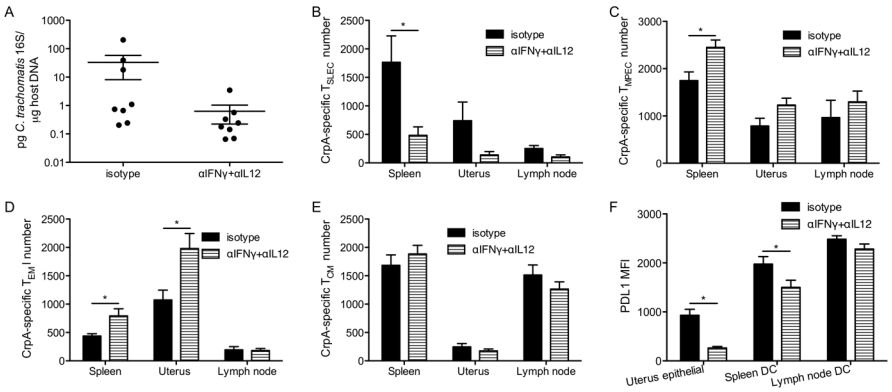

Figure 7.

Transient reduction of pro-inflammatory cytokine signaling during transcervical infection improves memory development of $CD8^+$ T cells. Mice were transcervically infected with *C. trachomatis*, treated with isotype control or IFNγ and IL12 neutralizing antibodies 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**) PDL1 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 6–8 mice per group.

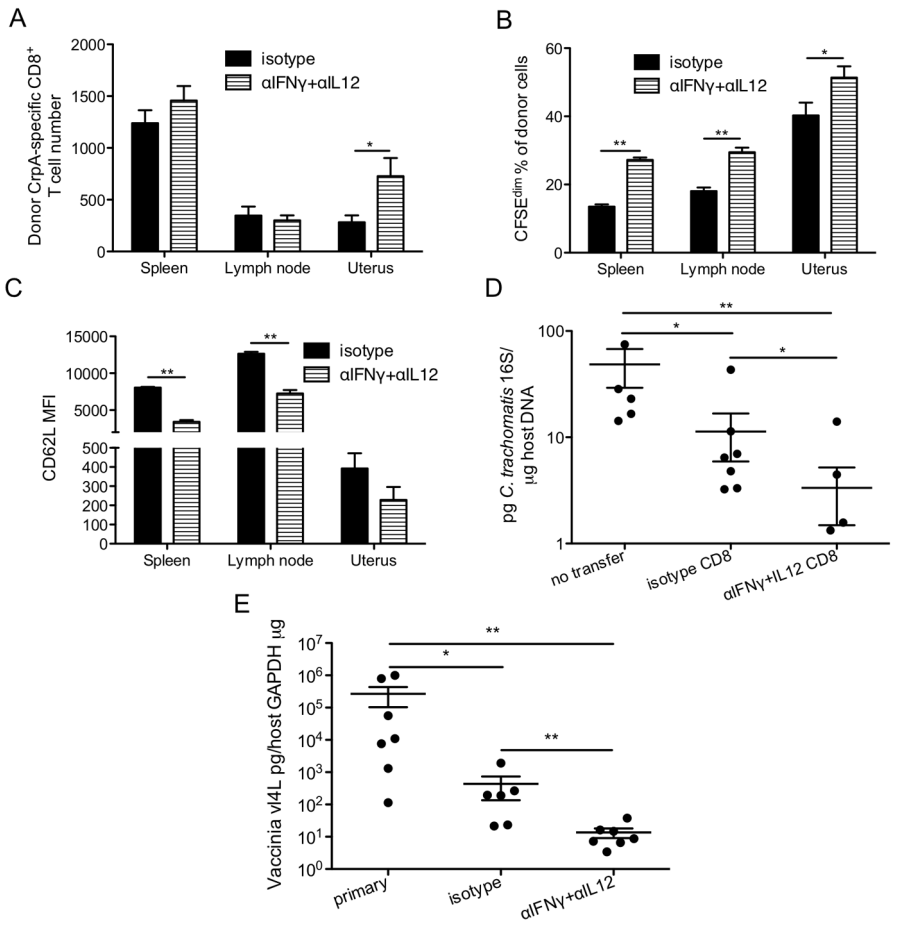

Figure 8.

Reducing pro-inflammatory 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 antibodies to deplete cytokines on day 4 p.i. On day 28 p.i., $CD8^+$ T cells were purified from pooled secondary lymphoid organs, CFSE-labeled, and transferred into naïve 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 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 antibodies to deplete cytokines on day 4 p.i. On day 28 p.i., these mice and naïve 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 6–7 mice per group.