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A *Chlamydia*-specific TCR Transgenic Mouse Demonstrates Th1 Polyfunctionality with Enhanced Effector Function [1]

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Abstract

Chlamydia is responsible for millions of new infections annually, and current efforts focus on understanding cellular immunity for targeted vaccine development. The *Chlamydia*-specific CD4 T cell response is characterized by the production of IFNγ, and polyfunctional Th1 responses are associated with enhanced protection. A major limitation in studying these responses is the paucity of tools available for detection, quantification, and characterization of polyfunctional, antigen-specific T cells. We addressed this problem by developing a TCR transgenic mouse with CD4 T cells that respond to a common antigen in *Chlamydia muridarum* and *Chlamydia trachomatis*. Using an adoptive transfer approach, we show that naïve transgenic CD4 T cells become activated, proliferate, migrate to the infected tissue, and acquire a polyfunctional Th1 phenotype in infected mice. Polyfunctional Tg Th1 effectors demonstrated enhanced IFNγ production compared to polyclonal cells, protected immune deficient mice against lethality, mediated bacterial clearance, and orchestrated an anamnestic response. Adoptive transfer of *Chlamydia*-specific CD4 TCR Tg T cells with polyfunctional capacity offers a powerful approach for analysis of protective effector and memory responses against chlamydial infection, and demonstrates that an effective monoclonal CD4 T cell response may successfully guide subunit vaccination strategies.

Introduction

CD4 T cells contribute to cell-mediated immunity through effector functions mediated by the production of cytokines. Polyfunctional T-helper 1 (Th1) cells can sequentially produce IFNγ, IL-2, and TNF in response to T-cell receptor (TCR) stimulation (1). This phenotype has been reported in a variety of infectious disease models, including *Leishmania* (2) tuberculosis (3), HIV (4), *Plasmodium* (5), and *Chlamydia* (6). Polyfunctional Th1 cells demonstrate enhanced protective efficacy in comparison to IFNγ monofunctional cells (3),

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Disclosures

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potentially by producing higher levels of Th1 cytokines (7, 8). Th1 polyfunctionality represents a measure of immunogenicity in vaccine studies (9), and generation of durable polyfunctional Th1 memory will likely be critical for *Chlamydia* vaccine development (6).

Protective immunity against *Chlamydia* is primarily mediated through Th1 cells (10, 11), and the importance of *Chlamydia*-specific CD4 T cells has been demonstrated by adoptive transfer (12, 13) and depletion studies (14). Despite the importance of CD4 T cells in controlling chlamydial infection, little is known about the generation of polyfunctional Th1 cells and how they contribute to cell-mediated immunity. Previous studies showed that a *Chlamydia*-specific IFNγ monofunctional Th1 clone was not protective, whereas a clone producing IFNγ and TNF cleared *C. muridarum* infection in nude mice (15). Vaccine models have shown that antigens and adjuvants generating polyfunctional (IFNγ+ TNF+) Th1 cells were more protective than IFNγ monofunctional Th1 cells (16, 17), and this protection has been observed in immunogenicity studies investigating single (18, 19) or multiple chlamydial antigens (20–23).

We recently developed the first TCR transgenic (Tg) mouse specific for a conserved antigen in *C. muridarum* and *C. trachomatis* to investigate the polyfunctional Th1 response *in vivo*. Identification of a polyfunctional Th1 clone allowed us to isolate and clone the *Chlamydia*-specific TCR for Tg mouse generation. After adoptive transfer, naïve TCR Tg CD4 T cells proliferated in the iliac lymph nodes, migrated to the infected genital tract, and primarily differentiated into polyfunctional Th1 cells. Polyfunctional Tg Th1 cells exhibited enhanced effector function characterized by increased IFNγ production associated with improved bacterial clearance compared to polyclonal, predominately monofunctional, Th1 cells. These studies demonstrate the first transgenic TCR to protect against *C. muridarum* genital infection and exhibit *C. trachomatis* cross-reactivity, and further define antigen-specific, enhanced effector function afforded by Th1 polyfunctionality.

Materials and Methods

Strains, cell lines, and culture conditions

Chlamydia muridarum Nigg stock (AR Nigg) was obtained from Roger Rank at the University of Arkansas for Medical Sciences, and has been previously described (24). *C. trachomatis* D/UW-3/Cx (25) was obtained from the American Type Culture Collection (Manassas, VA) and plaque purified before use (24). Plaque-purified *C. trachomatis* D/UW-3/Cx, Nigg strain CM001 (26), and plasmid-deficient CM3.1 (27) were propagated in mycoplasma-free L929 cells (28), and titrated by plaque assay or as inclusion-forming units (29), using a fluorescently tagged anti-chlamydial lipopolysaccharide monoclonal antibody (Bio-Rad). UV-inactivated bacteria were prepared, as described (30).

Generation of Chlamydia-specific T Cell hybridoma and transgenic mice

Two eight-week-old female C57BL/6J mice were intravaginally infected with 3×10^5 inclusion forming units (IFU) of wild-type *Chlamydia muridarum* Nigg. Infected mice were allowed to resolve primary infection, and were re-challenged two months later. The spleen and lymph nodes were collected one-week post-secondary challenge, and single-cell

suspensions were stimulated *ex vivo* with reticulate body (RB)-enriched Nigg (1μg/mL) (31) for 5 days prior to fusion with murine BW5147 T cell lymphoma cells (32) in 50% PEG solution. Fused cells were cultured in HAT medium for an additional 7 to 9 days. Hybridomas were screened and sorted based on CD3, CD4, CD8, and TCRβ expression. Sorted CD4 T cell hybridomas underwent limiting dilution and were co-cultured with irradiated syngeneic splenocytes in the presence of Nigg elementary bodies (1μg/mL) or RB (1μg/mL) for 24–48 hours at 37°C. Harvested supernatants were tested for IL-2 and IFNγ levels by enzyme-linked immunosorbent assay (ELISA) from R&D Systems. The CD4 T cell clone with the highest co-production of IL-2 and IFNγ in the presence of Nigg elementary bodies (EB) was harvested and cultured for cloning of TCRα and TCRβ cDNA.

RNA from the CD4 T cell clone was made using the Qiagen RNAeasy method, and TCRα and TCRβ cDNA was obtained using the SuperTCRExpress™ Mouse TCR Vα/Vβ Repertoire Clone Screening Assay Kit (BioMed Immunotech), which contains 5′ RACE primers for all TCR Vα/Vβ. The cDNAs were cloned into the TOPO vector (Promega), sequenced, and identified as Vα6 and Vβ10. Genomic sequences corresponding to the mRNA sequences were used to map the variable, joining, and constant regions in the sequence. Primers with flanking XmaI site and NotI site, GATCCCGGGCAGAGCTGCAGCCTTCCCAAGGCTC and CATGCGGCCGCAGTGCTAGGAAGGGCGGCCTGGAC were generated for amplifying the variable region of Vα6 from genomic DNA. Primers with flanking XhoI site and SacII site, TCCGCTCGAGCCTTGACCCAACTATGGGCTGT and ATTCCCGCGGCTGGTCTACTCCAAACTACTCCAGG were generated to amplify the variable region of Vβ10. Vα6 amplicon was cloned into the pTαcass and Vβ10 amplicon into pTβcass vectors (33), which contain the respective promoters for Vα and Vβ expression and provided the joining and constant region, as a genomic clone (Fig S1 and S2). DNA constructs were sequenced for confirmation, linearized at SalI (Vα6) and KpnI (Vβ10) sites, respectively, purified and injected into the pronuclei of (C57BL/6J × SJL/J) F2 fertilized eggs.

Animals

Female C57BL/6J (Stock No: 000664), B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ (CD45.1+; Stock No: 002014), B6.129S7-Rag1^{tm1Mom}/J (*Rag1*$^{-/-}$; Stock No: 002216), and B6.129S2-Tcra^{tm1Mom}/J (*Tcra*$^{-/-}$; Stock No: 002116) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were given food and water ad libitum in an environmentally controlled pathogen-free room with a cycle of 12 h of light and 12 h of darkness. TCR transgenic mice generated as described above at the University of Pittsburgh were subsequently backcrossed onto C57BL/6J for over 10 generations. Transgenic mice were screened for expression of Vα6 and Vβ10 on CD4+ T cells from peripheral blood by PCR and FACS. Experimental mice were age-matched and used between 8 and 12 weeks of age. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and University of North Carolina.

Generation of bone marrow-derived DCs

Dendritic cells were generated from the tibias/femurs of C57BL/6J mice as previously described (34). Briefly, erythrocytes were lysed with ACK lysis buffer, and bone marrow precursors were cultured for 7 days in complete media (RPMI containing 10% fetal bovine serum, 2 mM glutamine, 10 mM HEPES, pH 7.4, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and 50 μg/ml gentamicin) supplemented with 1000 U/mL recombinant murine GM-CSF and 1000 U/mL recombinant murine IL-4 (both from Peprotech). CD11c+ DCs were isolated using specific beads (Miltenyi Biotech), according to the manufacturer's protocol.

Antigen-specific T-cell proliferation, activation, and cytokine analysis

The spleens of littermate or TCR transgenic mice were processed into a single cell suspension, as described previously (35). Splenocytes (1×10^5 cells/well) were seeded in a 96-well flat-bottomed tissue culture plate in complete media with 5 μg/ml *C. muridarum* AR Nigg, plasmid-deficient CM 3.1, *C. trachomatis* D/UW-3/Cx, or recombinant ovalbumin (Sigma) for 6 days. Splenocytes were treated with 20 U/mL murine IL-2 (Peprotech) over the final 48 hours. Cells were treated with 20 μl of Alamar Blue (Biosource) 6 hours before the end of the 6-day culture, and proliferation was measured at 530-nm excitation/590-nm emission with a Biotek fluorescence microplate reader.

Alternatively, transgenic or polyclonal CD4+ T cells were isolated from the spleens of naïve TCR transgenic or wild-type C57BL/6J mice by negative magnetic selection (EasySep™ Mouse CD4 T cell). Isolated CD4+ T cells were co-cultured at a 1:5 ratio with bone marrow-derived dendritic cells (BMDCs) for 3 days in the presence or absence of *C. muridarum* AR Nigg (5μg/mL). Expression of CD69 and Ki67 was determined by FACS surface and nuclear staining respectively, as described previously (36). Supernatants from dendritic-cell stimulated CD4+ T cells were collected and IL-2 concentrations determined by ELISA.

Murine Chlamydia infection and monitoring

For genital tract infection, female mice at least 8 weeks old were s.c. injected with 2.5 mg medroxyprogesterone (Depo-Provera®; Upjohn) 5–7 days prior to infection to induce a state of anestrous (37). Mice were intravaginally inoculated with 3×10^5 inclusion-forming units (IFU) CM001 diluted in 30 μl sucrose-sodium phosphate-glutamic acid buffer. Mice were monitored for cervicovaginal shedding via endocervical swabs (38), and IFUs were calculated, as described previously (39). Prior to reinfection, mice were treated intraperitoneally with 0.3 mg doxycycline in 100 μl phosphate buffered saline for 5 days (40). Animal welfare was monitored daily. Genital tract gross pathology, including presence of hydrosalpinx, was examined and recorded at sacrifice.

Lymphocyte isolation and flow cytometry

Spleen, iliac lymph nodes, oviducts, uterine horns, and cervical tissues were isolated from sacrificed mice. Cervical tissue and uterine horns were minced separately and incubated with 1 mL of collagenase I (Sigma) for 20 minutes at 37°C before neutralization with EDTA (10 μM). Single-cell suspensions were prepared by dispersing tissues through a 70-micron

tissue strainer (Falcon). Cell suspensions were treated with erythrocyte lysis buffer (VitaLyse®; BioE), incubated in Fc block (5 μg/ml) for 10 minutes, and stained with LIVE/DEAD Fixable Yellow (Life Technologies) plus various combinations of the following fluorochrome-labeled antibodies: anti-CD3 (clone 17A2) anti-CD3e (145-C211), anti-CD4 (GK1.5, RM4-5, H129.19), anti-CD8a (53-6.7), anti-TCRVβ10 (V21.5), anti-TCRβ (H57–597), anti-CD45 (30-F11) anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD44 (IM7), anti-CD62L (MEL-14), and anti-CD69 (H1.2F3), from BD Biosciences. The samples were analyzed on a CyAN ADP (Beckman Coulter) or LSR II flow cytometer (BD Bioscience), and data were analyzed with FlowJo software.

CFSE-Labeling and adoptive transfer

Transgenic or polyclonal CD4+ T cells were negatively separated, and a sample of isolated cells was analyzed by flow cytometry to confirm >93% purity. The indicated numbers of transgenic or wild-type CD4+ T cells were injected i.v. into Depo-Provera®-treated CD45.1+, *Rag1*$^{-/-}$, or *Tcra*$^{-/-}$ mice 5–6 days prior to intravaginal infection. In some experiments, Tg CD4+ T cells were labeled with 1 μM CFSE (Thermo Fisher) for 5 minutes at 37°C prior to intravenous transfer and analysis.

Intracellular cytokine detection

Lymphocytes isolated from infected mice as described above were incubated in a 96-well plate at a concentration of 1×10^6 cells per well in the presence of UV-irradiated *C. muridarum* AR Nigg (5 μg/ml) or media alone for 6 hours at 37°C; GolgiPlug (BD Biosciences) was added, and incubation was continued for an additional 12–16 hours. Control samples were stimulated for 4–6 hours in the presence of PMA/ionomycin (Cell Stimulation Cocktail; eBioscience) and GolgiPlug. Surface staining was performed as described above, and the cells were fixed in BD Bioscience Cytofix/Cytoperm for 20 minutes. For detection of intracellular cytokines, cells were incubated for 30 minutes in BD Bioscience Perm/Wash with various combinations of the following fluorochrome-labeled antibodies: anti-IL-2 (JES6-5H4), anti-TNFα (MP6-XT22), and anti-IFNγ (XMG1.2) from BD Bioscience.

Statistical analysis

Differences between the means of experimental groups after infection were calculated using two-way repeated measures (RM) ANOVA. Significant differences in flow cytometric data were determined by one-way and two-way ANOVA. Comparisons of animal survival were performed by an exact log rank test. Prism software (GraphPad Software) was utilized for statistical analyses, and values of $P \leq 0.05$ were considered significant.

Results

Generation of a Chlamydia-specific TCR transgenic mouse

Previous studies demonstrate that the frequency of IFNγ-producing CD4 Th1 cells correlate with enhanced chlamydial clearance from the genital tract (10, 11, 13, 41). To directly monitor CD4 T cell responses during murine infection, we generated a Tg mouse strain with a TCR reactive with *C. muridarum,* which demonstrated cross-reactivity with *C. trachomatis*

(Fig. 1A). The TCR genes were isolated from a hybridoma expressing Vα6 and Vβ10 chains specific for *C. muridarum* elementary and reticulate bodies. These genes (Fig. S1 and S2) were cloned into an expression vector used to generate germline Tg mice. Founder Tg mice almost uniformly express Vβ10 on the surface of autologous CD4 T cells (Fig. 1B), and were backcrossed to C57BL/6J mice for over ten generations. Tg mouse splenocytes demonstrated a 6–8-fold increase in *Chlamydia*-specific proliferation compared to littermates, with minimal proliferation induced by ovalbumin (Fig. 1A). *C. muridarum* Nigg plasmid-competent and -deficient (CM3.1) strains stimulated TCR Tg splenocytes equally. To further confirm the specificity of Tg CD4 T cells, we analyzed their ability to activate and proliferate in comparison to wild-type polyclonal CD4 T cells *in vitro*. Transgenic CD4 T cells demonstrated the ability to co-express high levels of CD69 and Ki67 when cultured with BMDC and *C. muridarum* elementary bodies, with over 40% being double-positive and ~50% expressing CD69 (Fig. 1C). In contrast, minimal activation was observed with polyclonal CD4 T cells. This enhanced proliferation was associated with significantly increased levels of IL-2 in the supernatants of stimulated TCR Tg CD4 T cells that were 29 times higher than polyclonal CD4 T cells (Fig. 1D). We next examined the ability of Tg CD4 T cells to become activated and proliferate *in vivo*.

C. muridarum genital infection initiates TCR Tg CD4 T cell proliferation and activation in vivo

To determine the ability of TCR Tg CD4 T cells to proliferate and become activated in response to intravaginal *C. muridarum* infection, we utilized an adoptive transfer approach. To first test if these cells proliferate *in vivo*, we labeled naïve CD45.2+ TCR Tg CD4 T cells with CFSE and intravenously transferred 1×10^6 cells into congenic CD45.1+ mice. An increased percentage of CD45.2+ TCR Tg CD4 T cells were detectable on day 5 post-infection in the iliac lymph nodes compared to mock-infected controls (Fig. 2A), and infection resulted in their loss of CFSE expression consistent with proliferation (Fig. 2B). We then compared the activation state of endogenous and Tg CD4 T cells after infection by examining expression of the activation markers CD44, CD69, and CD62L on CD45.2+ TCR Tg and endogenous CD45.1+ CD4 T cells in the spleen, iliac lymph nodes, and oviducts. The gating strategy is shown in Fig. 2C. Comparison of the surface marker frequency between all CD45.1+ CD4 T cells or CD45.1+ CD4 T cells expressing the Vβ10 chain did not significantly alter the frequency of surface marker-positive endogenous cells (data not shown). TCR Tg CD4 T cells upregulated CD44 and CD69 concomitantly with down-regulation of CD62L by day 5 in the iliac lymph nodes and demonstrated greater percentages of activated cells in peripheral and secondary lymphoid organs (SLO) compared to the endogenous pool by day 8 post-infection (Fig. 2D, 2E). TCR Tg cells expressed significantly higher levels of CD69 in the ILN (iliac lymph node) on each day analyzed after primary infection, compared to endogenous cells. This was further associated with significantly decreased percentages of $CD62L^{hi}$ TCR Tg cells on days 5, 8, 22, and 44 post-primary infection. Similar CD62L kinetics was observed in the spleen.

$CD44^{hi}$ expression is used as a marker of T cell activation and Th1 memory (42, 43), and this memory phenotype was significantly increased among TCR Tg CD4 T cells in the iliac nodes throughout the course of primary infection, and on day 13 of secondary infection,

compared to endogenous cells. Similar CD44 expression was observed for splenic TCR Tg cells. On day 13 post-secondary challenge, 85–98% of splenic Tg T cells were CD44^{hi}, and these Tg cells comprised ~7% of the total splenic CD44^{hi} CD4 T cell pool (Fig. 2F). The kinetics reflect the enhanced ability of Tg cells to adopt an activated effector and/or effector memory phenotype in the lymphoid tissues throughout infection compared to the endogenous T cell repertoire, and by day 8 in the infected peripheral tissues. These data collectively support other studies demonstrating *Chlamydia*-specific CD4 T cell priming and proliferation in the ILN (44, 45) and the presence of activated cells in the genital tract one-week post-infection (46).

Naïve TCR Tg CD4 T cells confer protection, acquire effector function, and demonstrate a recall response

After demonstrating that TCR Tg CD4 T cells become activated, proliferate, and migrate to the infected genital tract, we investigated whether they would provide protection and produce IFNγ upon challenge. Prior data from our lab had revealed the plaque-purified strain of *C. muridarum* Nigg, CM001, resulted in disseminated lethal infection in *Rag1*$^{-/-}$ mice after intravaginal inoculation (26). *Rag1*$^{-/-}$ mice received adoptive transfers of 10^3 Tg or 10^3, 10^4, and 10^6 polyclonal CD4 T cells 5 days prior to infection (Fig. 3A). A precursor frequency of 10^6 polyclonal CD4 T cells was used as a positive control, based on previous observations that this dose conferred protection (data not shown). Mice receiving 10^3 Tg CD4 T cells survived infection, whereas 10^3 and 10^4 polyclonal CD4 T cells were not protective. TCR Tg CD4 T cells demonstrated a recall response characterized by the production of inflammatory cytokines. Transgenic CD4 T cells isolated from the uterine horns and oviducts on day 7 post-secondary challenge produced IFNγ and TNF in response to *in vitro* re-stimulation with UV-irradiated *C. muridarum* (Fig. 3B). These data indicate that TCR Tg CD4 T cells prevent death from disseminating infection, migrate to infected tissues, and acquire Th1 effector functions post-infection.

TCR Tg CD4 T cells can mediate bacterial clearance during primary and secondary infection

We next investigated whether adoptively transferring TCR Tg CD4 T cells to αβ TCR-deficient mice would lead to clearance of primary genital tract infection and enable resistance to challenge infection. Mice that did not receive T cells failed to clear infection, whereas adoptive transfer of 10^3 or 10^6 TCR Tg CD4 T cells to *Tcra*−/− mice resulted in equivalent rates of infection clearance, with a 3.5-log reduction in shedding being detected by day 10 post-infection (Fig. 3C). In addition, infection clearance after adoptive transfer of 10^3 or 10^6 TCR Tg CD4 T cells was accelerated when compared to groups that received either 10^3 or 10^6 polyclonal CD4 T cells, indicating that the Tg CD4 T cells are more efficient effectors (Fig. 3C).

We also investigated if TCR Tg CD4 T cells would contribute to a recall response upon secondary challenge. Immune mice that had received 10^3 or 10^6 TCR Tg CD4 T cells prior to primary infection exhibited a 4.5- and 3-log reduction in shedding, respectively, on day 3 post-challenge compared to primary infection (Figs. 3C, 3D). Mice that received 10^3 Tg CD4 T cells prior to primary infection were more resistant to challenge when compared to

mice that received 10^6 Tg CD4 T cells (Fig. 3D). This was consistent with findings in other TCR transgenic models, where lower numbers of adoptively transferred naïve Tg CD4 T cells induce better memory development. It is possible that decreased interclonal competition for antigen leads to enhanced differentiation of the fittest effectors into memory cells (47). In contrast, infectious burden during secondary infection was significantly lowered in mice receiving 10^6 but not 10^3 polyclonal CD4 T cells. In this instance, a broad array of antigen-specific cells avoids interclonal competition for peptide-MHC class II stimulation.

Although mice that were re-infected without prior receipt of adoptive T cells failed to exhibit any decline in infectious burden up to two weeks post inoculation, on day 3 post-challenge, their infectious burden was 2-log lower than that observed during primary infection. This transient protection may be a result of circulating T-cell independent antibody or memory γδ T cells that are not capable of clearing infection independent of conventional CD4 T cells.

TCR Tg CD4 T cells preferentially adopt a polyfunctional Th1 phenotype with increased IFNγ production

The TCR transgenic mouse was developed using a TCR that induced a Th1 response after chlamydial stimulation *in vitro*. We hypothesized that TCR Tg CD4 T cells would differentiate into polyfunctional Th1 cells *in vivo* since adoptive transfer of these cells led to enhanced chlamydial clearance during primary infection (Fig. 3C). We detected significantly increased percentages of IFNγ+TNF+ double-positive cells in the spleen and genital tissues of *Tcra*−/− mice receiving Tg CD4 T cells, on day 13 post-infection compared to mice receiving polyclonal CD4 T cells (Fig. 4A, 4B). Additionally, transgenic polyfunctional IFNγ+TNF+ CD4 T cells expressed significantly higher amounts of IFNγ compared to polyfunctional, polyclonal populations. TCR Tg polyfunctionality for TNF and IFNγ was associated with increased IFNγ production compared to cells singly positive for IFNγ (Fig. 4C). These data indicate that TCR Tg CD4 T cells preferentially adopt a polyfunctional phenotype characterized by high levels of IFNγ production. Furthermore, the percentage of triple-positive (IFNγ+TNF+IL-2+) CD4 T cells in the spleen was increased among the Tg CD4 T cell population when compared to polyclonal CD4 T cells on day 48 post-infection (Fig. 4D, 4E). Triple positive transgenic cells expressed significantly higher levels of IFNγ per cell compared with single- or double-positive cells (Fig. 4F), as previously described for pathogen-specific polyfunctional T cells in other models of infection (4, 8, 48). Triple positive cells also expressed significantly higher levels of TNF per cell compared to TNF monofunctional cells, but at a reduced magnitude (60% increase), and no differences were observed in IL-2 GMFI (data not shown). These data collectively show that Tg CD4 T cells have superior functional capacity with enhanced cytokine production, and this polyfunctional effector response is associated with enhanced bacterial clearance.

Discussion

Pathogen-specific TCR Tg mice have been utilized in a variety of infectious disease models (49–56), including NR1 mice that recognize *C. trachomatis* (46). Adoptive transfer of naïve

TCR Tg cells is a superior approach to transfer of *in vitro* maintained T cell lines, since naïve TCR Tg cells allow analysis of the initial antigen encounter and phenotypic differences between *in vivo* derived effector and memory populations. We developed a TCR Tg mouse that recognizes a conserved antigen between *C. trachomatis* and *C. muridarum*. The TCR Tg cells of this mouse react to *C. trachomatis* serovars D, F, H, and L2 (data not shown), and preliminary biochemical analyses reveal that they recognize a soluble, secreted protein enriched in reticulate bodies (data not shown). We have excluded commonly studied immunogenic antigens such as MOMP, OmcB, HSP60, and PmpG based on their inability to stimulate TCR Tg cellular proliferation *in vitro*. This report demonstrates the first TCR to protect against *C. muridarum* genital infection, and allowed us to analyze enhanced effector function afforded by Th1 polyfunctionality at a level that had not been previously attainable. Generation of this mouse has allowed for the unique ability to adoptively transfer TCR Tg cells for investigation of antigen-specific T cell responses to both mouse and human chlamydial strains in the murine model of genital tract infection.

Development of the *Chlamydia*-specific TCR Tg mouse was based on selection of a Th1 clone specific for both *C. muridarum* elementary bodies and reticulate bodies. We used a non-biased approach, by analyzing T cell clones demonstrating the strongest IFNγ and IL-2 production, which has been shown to be effective in TCR Tg mouse development. Selecting clones reactive against whole organism or crude antigen preparations has resulted in Tg CD4 T cells with the capacity to mount robust effector and memory responses following infection and vaccination (55), compared to model antigens (57). Our studies reveal that the Tg CD4 T cells possess a TCR, which confers protection against intravaginal *C. muridarum* infection.

These Tg CD4 T cells become activated, proliferate extensively, and produce high levels of IL-2 when stimulated with *C. muridarum*. Naïve and memory CD4 T cells require TCR stimulation in combination with IL-2 signaling to proliferate (58), and TCR engagement upregulates IL-2R subunits (59). The strength of IL-2 signaling also correlates with the magnitude of proliferation in Th1 cells (60), and IFNγ expression increases with successive cell divisions (61). Furthermore, IL-2 signaling during priming enhances differentiation of the effector pool into memory (62).

Based on the ability of these cells to recognize chlamydia *in vitro*, we used an adoptive transfer approach to analyze the proliferation and activation kinetics *in vivo*. Similar to the *C. trachomatis* model, our approach revealed that *C. muridarum* infection induced significant TCR Tg CD4 T cell activation and expansion in the iliac lymph nodes by day 5 and Tg cells expressed an activated phenotype ($CD44^{hi}CD69+CD62L^{lo}$). The $CD44^{hi}$ $CD62L^{lo}$ phenotype was also observed in the infected oviducts. Expression of CD69 on Tg cells in the spleen and oviducts on day 8, in our model, is likely due to the ability of CM001 to quickly disseminate to the distal organs and rapidly ascend the genital tract. Increased CD69 expression on CD4 T cells early in CM001 infection may be a result of local priming events, prior to tissue infiltration of activated T cells primed in the ILN. At later time points, CD44 expression in SLOs steadily increased, particularly during re-infection, consistent with the formation of memory T cells. These kinetics are similar to other infectious disease models of CD4 T cell activation and memory (46, 51, 56, 63). After priming in the ILN, Tg

cells made up ~7% of all CD44^{hi} CD4 T cells in the spleen, which consistently decreased through infection, until mice received a secondary challenge. This is consistent with other systems demonstrating that peak CD4 T cell expansion typically occurs after one week (64), and is followed by CD4 T cell contraction over 1–2 weeks, where 90–95% of the expanded population undergoes cell death (65, 66). Late in the course of *C. muridarum* infection and during reinfection, a majority of Tg cells expressed high levels of the memory marker CD44. Additional phenotyping experiments are required to determine the proportions of Tg T cells in the terminal effector, effector memory, and central memory pools.

TCR Tg cells prevented death in immunocompromised mice infected with CM001, and these cells were recalled to the infected tissues and produced IFNγ and TNF upon secondary challenge. These results parallel other infectious disease models demonstrating that adoptive transfer of antigen-specific naïve CD4 T cells can protect against lethality (56). Adoptive transfer to T- and B-cell deficient *Rag1*$^{-/-}$ hosts illustrates the CD4 T helper-independent protective function of TCR Tg cells, likely mediated through their production of IFNγ (67). Furthermore, transfer of these cells into αβ TCR-deficient mice led to enhanced protection against primary infection and equivalent protection against a secondary challenge compared to the polyclonal response. Comparable levels of oviduct gross pathology were observed between the TCR Tg and polyclonal groups (100% and 95% hydrosalpinx, respectively), which was not surprising given the ability of CM001 to induce severe pathology in wild-type mice (68). Future studies utilizing vaccination or adoptive transfer of *in vitro* primed TCR Tg cells should help reveal their capacity to protect against oviduct pathology.

Reduced bacterial burden mediated by TCR Tg cells was associated with increased frequencies of double- and triple-positive Th1 populations producing higher levels of IFNγ compared to polyclonal CD4 T cells. IFNγ is a critical effector molecule for controlling chlamydial replication (13, 69–72), and enhanced frequencies of polyfunctional Tg cells producing IL-2 could allow for enhanced Th1 effector proliferation. Our TCR Tg cells clearly recognize an antigen that drives a favorable response that leads to enhanced bacterial clearance and resistance to challenge infection. Persistent antigen and antigen depots reduce the memory pool leading to non-protective responses from terminally differentiated, exhausted T cells. Removal of antigen drives T cell transition to memory (73), and these cells remain plastic and heterogeneous (74, 75). Thus, triple-positive Th1 Tg cells could be a consequence of improved effector function leading to lower bacterial load (76, 77). In addition, Tg cells may also demonstrate greater functional avidity, which has been linked with improved disease outcomes (78) and expression of decreased levels of inhibitory receptors (79). High avidity T cells are less susceptible to activation-induced cell death (80) and demonstrate increased polyfunctionality (81, 82).

Our analyses were limited to the study of CD4 T cells and focused on profiling three major Th1 cytokines. A comprehensive analysis of TCR Tg cell production of cytokines, chemokines, and cytotoxic effectors, as well as their helper function for antibody production by B cells is needed to fully delineate their protective, or pathological, mechanisms. Alternative effectors (83) and antibody (84, 85) have been shown to play a significant role in mediating chlamydial clearance. Additional analysis of the recall response is needed to determine the mechanisms whereby equivalent protection from reinfection occurred in

Tcra$^{-/-}$ mice that had received 10^3 polyclonal T cells or 10^3 monoclonal TCR Tg T cells. Potentially, polyclonal, polyfunctional T cells were maintained and IFNγ monofunctional cells culled, or monofunctional cells responding to a variety of antigens elicit similar protection to polyfunctional TCR Tg cells recognizing a single antigen.

The TCR Tg C57BL/6 background presents a caveat. Some TCR Tg mice are backcrossed to *Rag* backgrounds to eliminate endogenous TCR subunit expression, however this can result in deletion of the TCR Tg cells due to the requirement of a second endogenous TCR alpha chain for thymic emigration (56). Endogenously activated CD4 T cells expressing a second TCR can be present in TCR Tg mice. However, the spleen population exists at a small frequency (86) and at similar numbers in wild-type mice (87). This phenomenon led us to utilize equal and greater numbers of wild-type CD4 T cells as controls for adoptive transfer experiments.

The finding that a single immunogenic antigen that elicits polyfunctional T cells can successfully induce a protective response is encouraging from a subunit vaccinology perspective. Viral models demonstrate that primary and secondary effectors share organ-specific expression patterns, but secondary effectors are more polyfunctional (triple positive); polyfunctional cells also express higher levels of genes associated with survival and migration (88). Whether CD4 T cell polyfunctionality can predict memory generation and subsequent, enhanced secondary effector functions is an important area to be addressed. Once we have identified the antigen recognized by the TCR Tg T cells, we can determine if vaccination with this antigen drives induction of a protective polyfunctional response, and whether adoptive transfer of *in vitro* antigen-primed Tg T cells can protect from infection and disease.

In conclusion, we have demonstrated that adoptive transfer of Tg CD4 T cells specific for a single *Chlamydia* antigen induces polyfunctional CD4 T cells that provide enhanced immunity against *Chlamydia.* Transgenic CD4 T cells specific for *Chlamydia* can be further used to directly monitor differentiation of antigen-specific effector and memory responses during infection and to better delineate protective responses upon challenge. The development of a successful vaccine will be facilitated by better understanding of how CD4 T cell polyfunctionality is generated, sustained, and provides immunity at the mucosal surface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

IFNγ	interferon gamma

TCR	T cell receptor
Th1	T-helper 1
Tg	transgenic
IL-2	interleukin 2
HIV	human immunodeficiency virus
TNF	tumor necrosis factor
EB	elementary body
RB	reticulate body
IFU	inclusion forming units
HAT	hypoxanthine-aminopterin-thymidine
PEG	polyethylene glycol
ELISA	enzyme-linked immunosorbent assay
CM	*Chlamydia muridarum*
BMDC	bone marrow-derived dendritic cell
ACK	ammonim-chloride-potassium
UV	ultraviolet
PMA	Phorbol 12-myristate 13-acetate
SLO	secondary lymphoid organ
ILN	iliac lymph node
MHC	major histocompatibility complex
GMFI	geometric mean fluorescent intensity
MOMP	major outer membrane porin
OmcB	outer membrane complex protein B
HSP60	heat shock protein 60
PmpG	polymorphic membrane protein G

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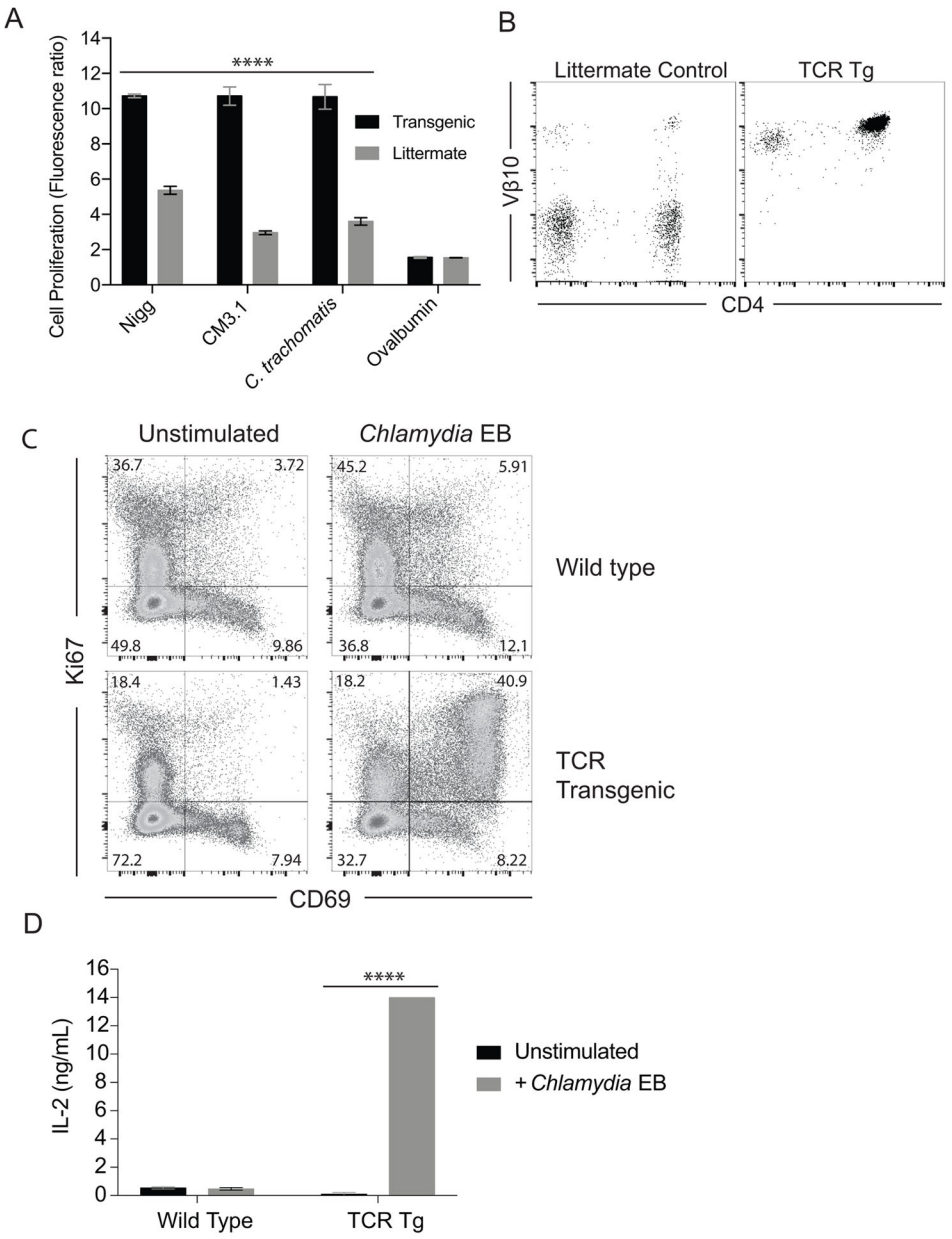

Figure 1. Generation of a *Chlamydia*-specific TCR transgenic mouse

(A) TCR Tg or littermate splenocytes were stimulated with 5 μg/ml *C. trachomatis*, *C. muridarum* AR Nigg, plasmid-deficient Nigg (CM 3.1), or recombinant ovalbumin, as indicated. Splenocytes were stimulated for 4 days, followed by 2 additional days in the presence of 20 U/mL IL-2. Change in proliferation was determined by the ratio of Alamar Blue fluorescent intensity compared to unstimulated controls (**** $P < 0.0001$ determined by two-way ANOVA). (B) Peripheral blood from C57BL/6J backcross progeny of Tg founder mice or littermate controls was stained with antibodies against CD3, CD4, and Vβ10. The right dot plot is representative of the Vβ10 expression on CD3+CD4+ T cells from Tg mice. (C) CD4+ T cells from Tg mice or wild type mice were incubated for 3 days with BMDC pulsed with and without 5 μg/ml *C. muridarum*. The right dot plots show CD69

and Ki67 expression from Tg and polyclonal CD4 T cells after stimulation. (D) Supernatants from dendritic cell-stimulated CD4 T cells were analyzed for IL-2 by ELISA (**** $P < 0.0001$ determined by two-way ANOVA).

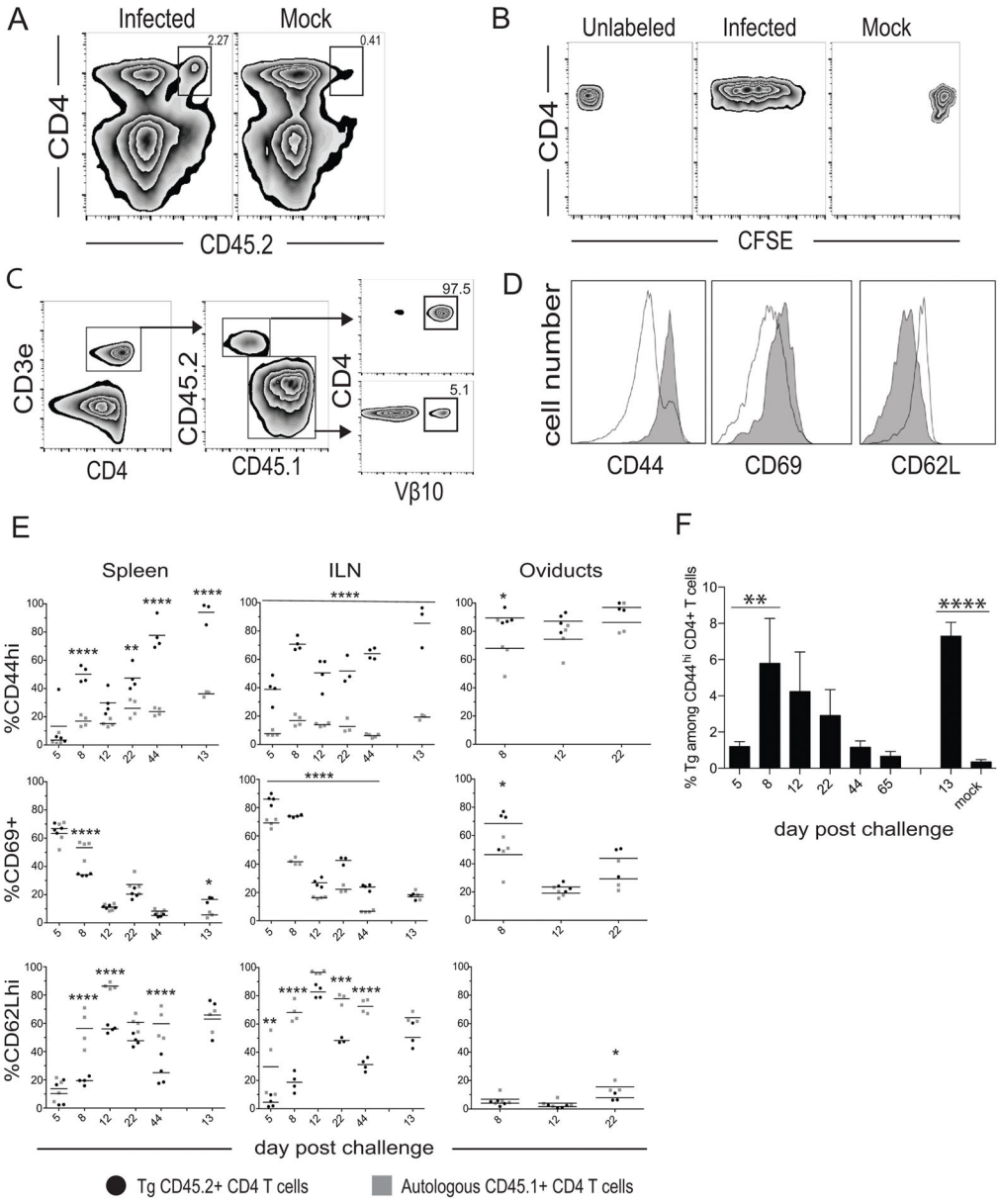

Figure 2. Proliferation and activation kinetics of TCR Tg CD4 T cells during *C. muridarum* genital tract infection

One million CFSE-labeled Tg T cells were transferred into CD45.1+ female recipients, which were mock infected or infected with CM001. (A) Iliac lymph nodes from infected (top left) or mock-infected (top right) mice were examined for the presence of Tg T cells, and (B) Tg cells were examined for CFSE fluorescence. (C) Diagram of the flow cytometric gating strategy used to analyze CD62L, CD69, and CD44 expression by CD45.2+ Tg and CD45.1+ endogenous, polyclonal CD4 T cells. (D) Representative histograms comparing surface marker expression between Tg (grey) and endogenous (white) CD4 T cells during early infection. (E) Expression of CD62L, CD69, and CD44 on donor Tg and endogenous host CD4 T cells in the spleen, iliac lymph node, and oviducts on the indicated days post primary and secondary infection. Data points are representative of individual mice.

Horizontal bars indicate the mean of 3–4 mice per group. Statistical significance was noted relative to autologous CD45.1+ CD4 T cells and indicated by asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ by two-way ANOVA. (F) Percentage of transgenic cells comprising the $CD44^{hi}$ CD4 T cell population from the spleen on the indicated days post-infection (** $P < 0.01$, **** $P < 0.0001$ by RM one-way ANOVA).

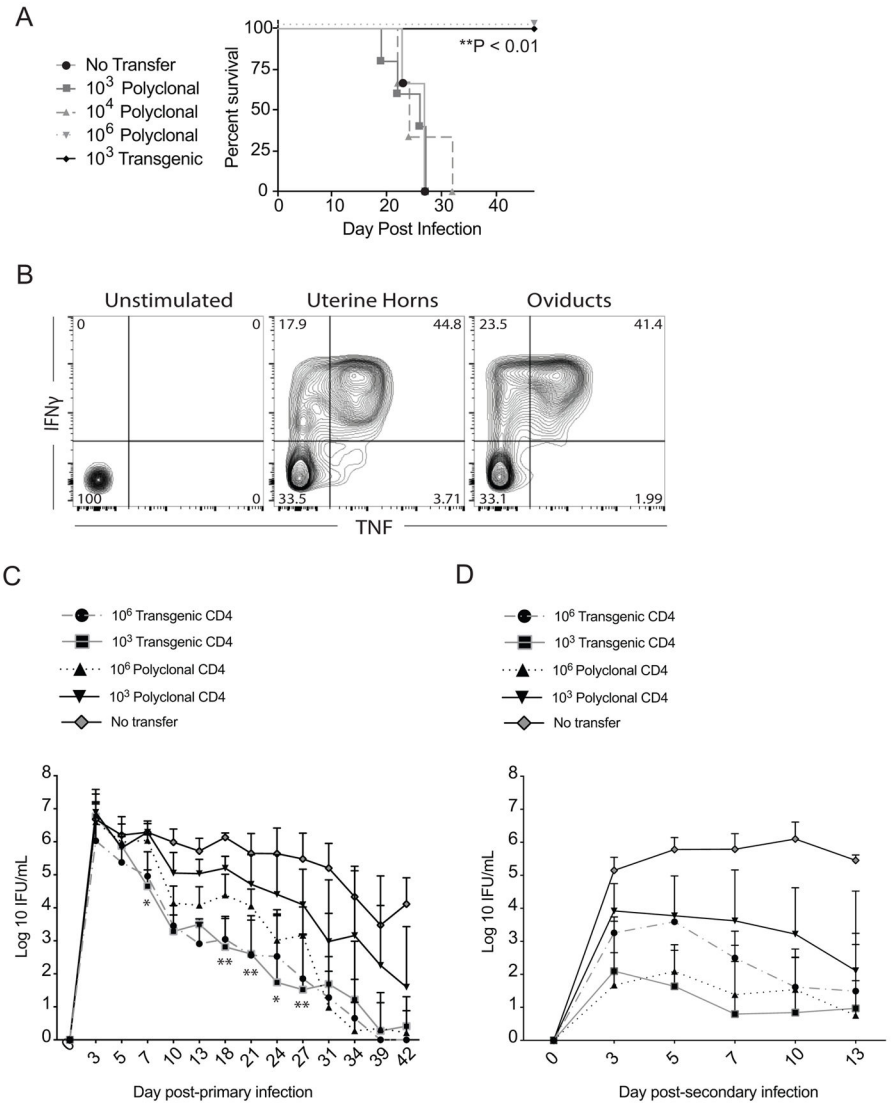

Figure 3. TCR Tg CD4 T cells mediate protection and demonstrate a recall response following challenge

Rag1$^{-/-}$ mice (4–5 per group) were mock treated or intravenously injected with indicated numbers of CD4 T cells isolated from the spleens of naïve C57BL/6J wild type or Tg mice, and intravaginally inoculated with CM001 5 days later. Survival was monitored daily, and an exact log rank test was used to analyze survival differences between polyclonal and Tg groups (** $P < 0.01$). (B) Recall response of CD4+Vβ10+ Tg CD4 T cells to secondary infection. Following primary infection, Tg mice were treated with doxycycline, rested for 9 weeks, and re-challenged with CM001. On day 7 post-challenge, genital tract CD4 T cells were harvested, stimulated with 5 μg/ml *C. muridarum*, and analyzed for IFNγ and TNF production by intracellular cytokine staining. (C) Indicated numbers of naïve Tg or polyclonal CD4 T cells were adoptively transferred to *Tcra*−/− mice 5 days prior to intravaginal infection with CM001, and the course of primary infection was monitored by culture of lower genital tract swabs. Significance was determined by two-way RM ANOVA. Data represent the mean ± SEM of 10 mice per group. Comparison of individual days for

10^3 Tg versus 10^6 polyclonal: * $P < 0.05$, ** $P < 0.01$. Comparison of primary infection course between groups: P=NS for 10^6 Tg versus 10^3 Tg. *** $P = 0.0001$ for 10^3 Tg versus 10^6 Polyclonal. **** $P < 0.0001$ for all remaining group comparisons. (D) Immune mice were treated with doxycycline on days 52–56 post-infection, rested for 5 weeks, re-challenged with CM001, and infection monitored by culture of vaginal swabs. Significance was determined by two-way RM ANOVA. Data represent the mean + SD of 4–5 mice per group. Comparison of groups over primary infection course: P=NS for 10^3 Tg versus 10^6 polyclonal, 10^6 Tg versus 10^3 or 10^6 polyclonal. * $P < 0.05$ for 10^3 Tg versus 10^6 Tg. **** $P < 0.0001$ for all remaining group comparisons.

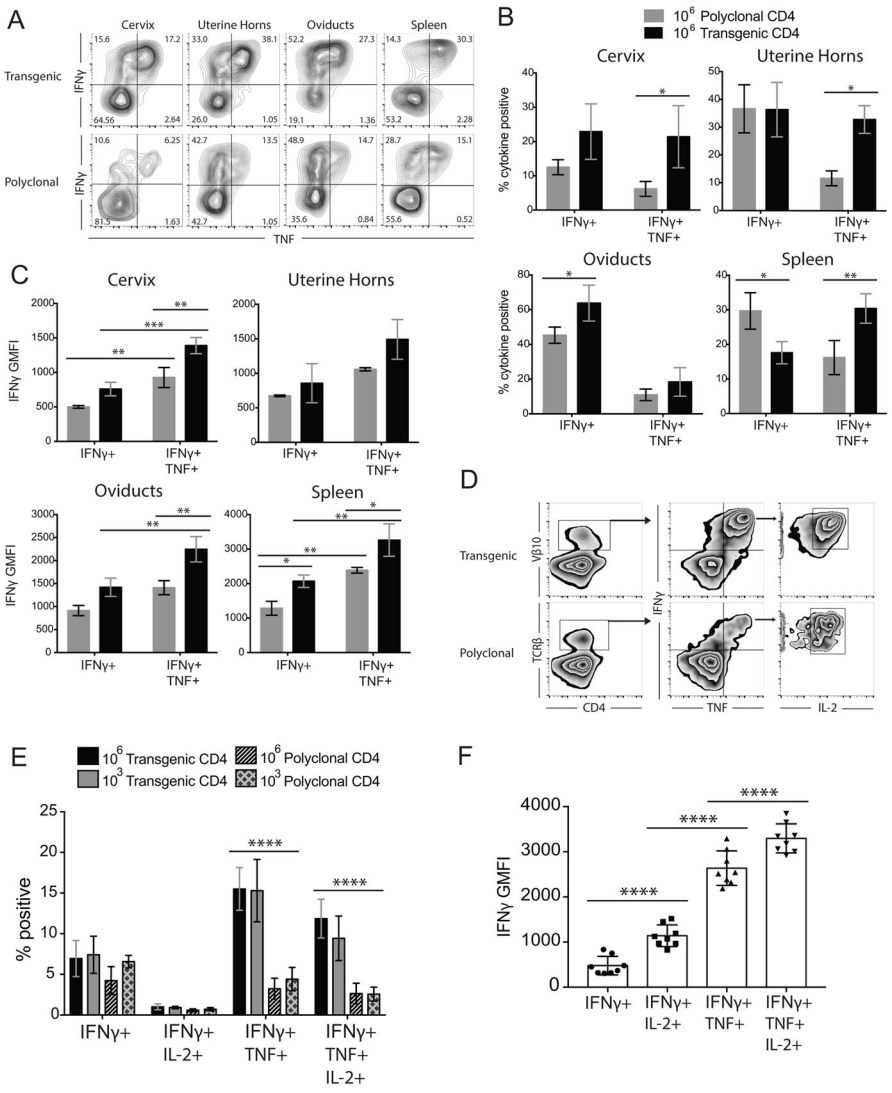

Figure 4. Naïve TCR Tg CD4 T cells differentiate into polyfunctional Th1 cells with increased IFNγ production

Tcra$^{-/-}$ mice receiving polyclonal or Tg CD4 T cells were analyzed for polyfunctional Th1 responses on Day 13 (A–B) and Day 48 (D–E) post-infection. (A) CD4 T cells isolated from indicated tissues were stimulated with 5 μg/ml *C. muridarum* and evaluated for intracellular cytokine production. Contour plots show representative IFNγ and TNF co-production by Tg CD3e+CD4+Vβ10+ cells (top) and polyclonal CD3e+CD4+TCRβ+ cells (bottom). (B) Comparison of the percentage of cytokine positive cells between single positive (IFNγ+) and double-positive (IFNγ+ TNF+) polyclonal and Tg CD4 T cells (C) Associated IFNγ geometric mean fluorescent intensities (GMFI). Data represent the mean ± SD of 3 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ determined by two-way ANOVA. (D) Diagram of the flow cytometric gating strategy used to analyze CD4 T cell polyfunctionality on day 48 post-infection. (E) The frequency of single positive (IFNγ+), double positive (IFNγ+ TNF+), and triple positive (IFNγ+ TNF+IL-2+) Tg or polyclonal CD4 T cells on day 48 post-infection. Data represent the mean ± SD of 4–5 mice per group (**** $P <$

0.0001). (F) Comparison of the IFNγ GMFI between spleen-isolated Tg CD4 T cell single, double, and triple-positive populations. Data represent the mean ± SD of 8 mice receiving 10^3 and 10^6 Tg cells. **** $P < 0.0001$ determined by two-way RM ANOVA.