B Cell Presentation of *Chlamydia* Antigen Selects Out Protective CD4 γ 13 T Cells: Implications for Genital Tract Tissue-Resident Memory Lymphocyte Clusters

Raymond M. Johnson,^a Hong Yu,^c Norma Olivares Strank,^a Karuna Karunakaran,^c Ying Zhu,^b Robert C. Brunham^c

^aSection of Infectious Diseases, Department of Medicine, Yale University School of Medicine, New Haven, Connecticut, USA

^bDepartment of Biostatistics, Yale University School of Medicine, New Haven, Connecticut, USA

^cVaccine Research Laboratory, University of British Columbia Centre for Disease Control, Vancouver, Canada

ABSTRACT Surveillance and defense of the enormous mucosal interface with the nonsterile world are critical to protecting the host from a wide range of pathogens. *Chlamydia trachomatis* is an intracellular bacterial pathogen that replicates almost exclusively in the epithelium of the reproductive tract. The fallopian tubes and vagina are poorly suited to surveillance and defense, with limited immune infrastructure positioned near the epithelium. However, a dynamic process during clearing primary infections leaves behind new lymphoid clusters immediately beneath the epithelium. These memory lymphocyte clusters (MLCs) harboring tissue-resident memory (Trm) T cells are presumed to play an important role in protection from subsequent infections. Histologically, human *Chlamydia* MLCs have prominent B cell populations. We investigated the status of genital tract B cells during *C. muridarum* infections and the nature of T cells recovered from immune mice using immune B cells as antigen-presenting cells (APCs). These studies revealed a genital tract plasma B cell population and a novel genital tract CD4 T cell subset producing both gamma interferon (IFN- γ) and interleukin-13 (IL-13). A panel of CD4 T cell clones and microarray analysis showed that the molecular fingerprint of CD4 γ 13 T cells includes a Trm-like transcriptome. Adoptive transfer of a *Chlamydia*-specific CD4 γ 13 T cell clone completely prevented oviduct immunopathology without accelerating bacterial clearance. Existence of a CD4 γ 13 T cell subset provides a plausible explanation for the observation that human peripheral blood mononuclear cell (PBMC) *Chlamydia*-specific IFN- γ and IL-13 responses predict resistance to reinfection.

KEYWORDS *Chlamydia*, CD4, IL-13, B cells, Trm

Chlamydia trachomatis infections of the reproductive tract have evaded public health interventions for the past several decades. In the United States and Canada, the incidence of *C. trachomatis* infections continues to climb despite effective antibiotics and public health measures that increased screening, partner notification, and treatment. In fact, the attempt to control *C. trachomatis* infection likely aborts the development of herd immunity and results in the need to treat even great numbers of individuals (1, 2); arrested immunity due to doxycycline treatment is demonstrable in the *Chlamydia muridarum* mouse model (3). It is widely accepted by researchers and public health officials that the only intervention likely to reduce the incidence of disease and the human toll and expense inflicted by *C. trachomatis*-induced infertility and ectopic pregnancy is a *Chlamydia* vaccine. While much progress has been made, the immunologic goals of a *Chlamydia* vaccine remain elusive, and no human vaccine against the urogenital serovars has been attempted. The finding that untreated hu-

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Address correspondence to Raymond M. Johnson, raymond.johnson@yale.edu.

mans can self-clear genital tract infections (4–6) and that those who do are less likely to be reinfected (7) provides proof in principle for a *Chlamydia* genital tract vaccine.

The immunologic goal of vaccination for protective immunity against urogenital serovars is likely a multifunctional Th1 response (8). The role of antibodies in a future *C. trachomatis* vaccine is unclear, with animal model data supporting (9–12) and refuting (13–15) a role for *Chlamydia*-specific antibodies in protective immunity absent a preexisting T cell response. In human studies, we and others have shown that IgG and IgA antibody responses measured in serum do not correlate with protective immunity (16–18), and a prospective human clinical investigation showed a linear positive correlation between antichlamydial antibody titers and future infertility (19). In mice, CD8 T cell responses are associated with immunopathology rather than protection (20–23), although there are caveats to this statement, including evidence for CD8 protection with a trachoma vaccine in macaques (24) and the identification of CD8 epitopes that correlate with self-resolution in humans (25). While many questions remain about the pathophysiology of protection versus immunopathology, it is generally accepted that the reliably protective arm of the adaptive immune response is the CD4 T cell response (26, 27).

A critical component for rational vaccine development is a surrogate biomarker for protective immunity. For early successful vaccines like the hepatitis B virus vaccine, the surrogate biomarker was a relatively easily determined antibody titer to the hepatitis B surface antigen. A practicable surrogate biomarker for protective immunity is defined as a testable parameter that can be reasonably and reliably measured after administration of a vaccine that correlates with resistance to infection. Currently there are only two such surrogate biomarkers for *C. trachomatis* immunity defined by Cohen et al. in a longitudinal study of Kenyan sex workers (18): a peripheral blood mononuclear cell (PBMC) gamma interferon (IFN- γ) response to *Chlamydia* heat shock protein 60 (HSP60), which is not useful in the context of vaccines as HSP60 is an unlikely candidate component of a subunit vaccine, and a PBMC interleukin-13 (IL-13) response to the elementary body (EB [i.e., the infectious form of *C. trachomatis*]). The latter has been an enigma as IL-13 is a Th2 cytokine, and Th2 responses are associated with negative outcomes in animal models of *Chlamydia* infection (28, 29).

In the context of an emerging new understanding of mucosal host defense based on local adaptive immunity mediated by tissue-resident memory (Trm) T cells, we recently revisited the *Chlamydia* genital tract pathogenesis paradigm with a Trm rather than cytokine polarization Th1/2/17 framework and reported our unpublished observation that the *Chlamydia*-specific CD4 T cell response includes a population of CD4 T cells that produce IFN- γ and IL-13 (30). We postulated that CD4 γ 13 T cells reflected a Trm response and, based on the data of others (31–33), that the *Chlamydia* memory lymphocyte clusters include immune plasma B cells as antigen-presenting cells (APCs). We present the discovery and characterization of CD4 γ 13 T cells here.

RESULTS

Plasma cells in the genital tract. We recently revisited the *Chlamydia* pathogenesis literature through the lens of tissue-resident immunity rather than cytokine polarization (Th1/2/17), highlighting human studies by others showing B lymphocytes and plasma B cells are prominent in *Chlamydia* infection-associated memory lymphocyte clusters (c-MLCs) (30). B lymphocyte data in the *C. muridarum* mouse model are inconclusive due to utilization of staining with B220, a marker downregulated when B lymphocytes transition to immune plasma B cells. To address the discrepancy between human and mouse data, we determined B cell dynamics in the genital tract over the course of a *C. muridarum* infection, gating on CD79a and measuring the relative levels of B lymphocytes (high B220 expression) and plasma B cells (low B220 expression) (Fig. 1A; [see the gating strategy in Fig. S1 in the supplemental material]). Gating on CD79a allows detection of plasma B cells that do not express B220 (34). In naive mice, very few plasma cells reside in the genital tract. During the course of a *C. muridarum* genital tract infection, the percentage of plasma cells increases from a baseline of 3% to 13%, with

FIG 1 B cell dynamics in the genital tract during *C. muridarum* infection and differential expansion of memory T cell subsets. (A) Single-cell suspensions of genital tracts from the following conditions were gated on CD79a (B cells) and analyzed for the level of B220: high expression of B220 indicates B lymphocytes and low expression indicates plasma B cells. Uninfected mice (Naïve), day 7 primary infection (D7_pri_inf), day 35 primary infection (D35_pri_Cm inf), and day 5 secondary infection (D5_sec_inf) were investigated. Mice were pretreated with medroxyprogesterone and infected 1 week later with 1,500 IFU of *C. muridarum*; representative data are shown from a minimum of 2 experiments for each experimental group. (B) Analysis of B cell APC-derived and immune splenocyte-derived *Chlamydia*-specific polyclonal T cell lines for production of IL-13. T cell lines were activated for 5 h with PMA-ionomycin-brefeldin A-monensin and stained for CD8 versus IL-13. CD4 T cells are identified as CD8^{neg} in this assay (see Materials and Methods).

a further expansion to 22% during rechallenge infections. The results in Fig. 1A show that plasma B cells are nearly absent in a naïve genital tract and expand as demonstrable immunity develops over the course of a primary infection.

Immune B cells as antigen-presenting cells. B cells from immunized mice bearing endogenous immunoglobulins (single specificity) and a sampling of serum IgG (multiple specificities) bound to their cell surfaces via Fc receptors (FcRs) can activate T cells at cognate antigen concentrations 1,000-fold lower than do naïve B cells (35): i.e., are 1,000× more potent as APCs. For the remainder of the article, we refer to B cells purified from mice that previously self-cleared *C. muridarum* genital tract infections (immune mice) as “immune B cells.” We investigated the nature of *Chlamydia*-specific T cells recovered from immune mice using immune B cells as APCs; the use of splenocytes rather than genital tract lymphocytes was based on limited cell numbers in genital tract tissue and the need to develop untested methodologies.

We purified splenic B cells from an immune mouse, pulsed them with UV-inactivated *Chlamydia muridarum* (uvMoPn), and then washed them extensively (400,000-fold) to remove all antigen not bound to or internalized by the immune B cells, thereby limiting antigen presentation to B cells. We cocultured antigen-pulsed/washed B cells (immune B cell APC) with splenocytes from the same immune mouse in two primary wells to expand T cells. In parallel, for comparison, from the same mouse, we expanded T cells in 2 primary wells using uvMoPn and unfractionated immune splenocytes (immune splenocyte APC) as we have previously published (36). At passage 3, we did flow cytometry to determine relative CD4/CD8 numbers: >95% of the resulting T cell populations from the expansions based on the immune B cell APC line and the expansions based on the immune splenocyte APC line were CD4 T cells (see Fig. S2 in the supplemental material). Although none of our published or unpublished *Chlamydia*-specific CD4 T cell murine studies had evidence for a CD4 IL-13 T cell response, we were interested in IL-13 because of its association with immune protection and pathology. Upon activation, the two immune B cell APC-derived polyclonal T cell lines produced IL-13, while the two immune splenocyte APC-derived T cell populations did not (data not shown). We propagated the two immune B cell APC-derived T cell lines and generated immune splenocyte APC-derived polyclonal T cell lines from four additional mice that previously cleared *C. muridarum* genital tract infections. We activated two immune B cell APC-derived and four immune splenocyte APC-derived polyclonal T cell lines with phorbol myristate acetate (PMA)-ionomycin and stained CD8 versus IL-13 (negative CD8 staining was intentionally used to identify CD4 T cells in this assay [see Materials and Methods]) (Fig. 1B). The immune B cell APC-derived polyclonal lines included a subset of CD4 T cells producing IL-13; none of the four immune splenocyte APC-derived polyclonal T cell lines had a CD4 IL-13 T cell subset. We activated one immune B cell APC-derived T cell line and one immune splenocyte APC-derived T cell line from the same mouse and did intracellular staining for IFN- γ and IL-13 (Fig. 2A). The immune splenocyte APC-derived CD4 T cell line did not stain for IL-13; all the T cells in the immune B cell APC-derived T cell line that produced IL-13 also produced IFN- γ . We activated an immune B cell APC-derived T cell line (B2) and five immune splenocyte APC-derived T cell lines (spl1 to -5) with purified uvMoPn-pulsed immune B cells and measured IFN- γ , IL-13, and IL-4 in culture supernatant (Fig. 2B). All of the polyclonal T cell lines were *Chlamydia* specific: when activated by antigen-pulsed B cells, they all produced IFN- γ . Only the immune B cell APC-derived T cell line B2 produced IL-13. None produced IL-4. We empirically noted that IL-13 production in immune B cell APC-derived T cell lines faded with increasing passage number (not shown) and determined that durable IL-13 production by immune B cell APC-derived polyclonal T cell lines required addition of transforming growth factor β 1 (TGF- β 1) to the medium (see Fig. S3 in the supplemental material) and that the TGF- β 1 effect was specific to T cell lines derived from the immune B cell APC combination: i.e., addition of TGF- β 1 to immune splenocyte APC-derived T cell lines did not result in IL-13 production (data not shown).

We next investigated whether there was a CD4 γ 13 T cell response to *Chlamydia* infection systemically (spleen) and locally (genital tract). CD4 T cell responses in the genital tract and spleen, quantified for IFN- γ , tumor necrosis factor alpha (TNF- α), and IL-13, were determined for naive, PmpG-immunized, and immune mice (i.e., mice that had cleared prior infection) on day 6 post-*C. muridarum* infection (Fig. 2C; see the gating strategy in Fig. S4 and data for individual mice in Table S1 in the supplemental material). CD4 γ 13 T cells were present but rare in the spleen. In the genital tract during primary infection, 1 to 2% of CD4 T cells were IL-13 $^{+}$, which increased to 5 to 10% with PmpG-dimethyldioctadecylammonium bromide (DDA)-trehalose dibehenate (TDB) immunization and were maximal during secondary infection at 4 to 15%. In naive mice, roughly half of the IL-13 $^{+}$ CD4 T cells were IFN- γ positive: 70 to 80% in PmpG-immunized mice during a primary response and >90% dual cytokine positive in mice during a secondary response (Fig. 2D). CD4 γ 13 T cells are a significant component of

FIG 2 Cytokine profile of B cell-derived polyclonal B2 versus immune splenocyte-derived polyclonal CD4 T cell lines, shown by the presence of CD4 γ 13 T cells in the genital tract and spleen. (A) Polyclonal B cell-derived line 2 (B2) and splenocyte-derived line 2 (spl2) were activated for 5 h with PMA-ionomycin-brefeldin A-monensin and then stained for CD8 (negative stain for CD4 T cells) versus IL-13 and IFN- γ versus IL-13. (B) Specificity and cytokine profiles for B2 and five splenocyte-derived T cell lines (spl1 to -5). 2.5×10^4 T cells were coincubated with 5×10^4 purified immune B cells unpulsed (APCs) or pulsed with uvMoPn (antigen [Ag]) in triplicate. Cell culture supernatants harvested at 48 h, and levels of IFN- γ , IL-13, and IL-4 were determined by ELISA. Shown are comparisons between control (Con) and antigen-pulsed (Ag) B cell activation for each cytokine and T cell line. *, $P < 0.05$; ****, $P < 0.0005$. (C) Frequency of IL-13 $^{+}$ CD4 T cells in the spleen and genital tract. C57BL/6 female mice in experimental groups of 4 mice each were PmpG immunized (PmpG Imm), unimmunized (Prim. Infect), or previously infected with *C. muridarum* (1,500 IFU vaginally [Sec. Infect]). Four weeks after the last immunization or *C. muridarum* primary infection, mice were challenged with intravaginally with *C. muridarum*. Six days later, single-cell suspensions prepared from individual mice (except naive uninfected control) and CD4 T cells were scored for IFN- γ , TNF- α , IL-13, and IFN- γ /IL-13. (D) For genital tract CD4 γ 13 T cells, the left panel shows the percentage that also produce IFN- γ and the right panel shows the intensity of IFN- γ production. Data for individual mice are shown in Table S1.

the local mucosal, but not the systemic, CD4 T cell response to primary genital tract infections, and their numbers were enhanced in the setting of preexisting immunity due to prior infection or protective PmpG vaccination.

CD4 γ 13 T cell clones. Having established that CD4 γ 13 T cells were a physiologic component of the host response to *Chlamydia* genital tract infections, we generated CD4 T cell clones using immune B cell antigen presentation to assemble a panel of immune B cell APC-derived multifunctional Th1 and CD4 γ 13 T cell clones for comparison to each other and to our existing CD4 T cell clones. Using conditions mimicking the polyclonal derivation, modified to incorporate TGF- β 1 and using both uvMoPn and soluble *Chlamydia* antigen, we were able to derive a panel of T cell clones from immune mice. A working panel of six CD4 T cell clones was carried forward including two immune B cell APC-derived CD4 γ 13 clones (sBT13-7 and sBT16-8), two immune B cell APC-derived multifunctional Th1 clones that did not produce IL-13 (BT12-7 and sBT13-11), an immune B cell APC-derived CD4 clone that lost IL-13 production over time (BT12-17), and a multifunctional Th1 clone derived with unfractionated splenocyte

FIG 3 Cytokine profiles of *Chlamydia*-specific CD4 clones, IL-13 production, and replication control. 2.5×10^4 T cells were activated with immobilized anti-CD3 antibody in 96-well plates; 5×10^4 T cells/well were used for IL-4 to increase sensitivity. Twenty-hour culture supernatants were analyzed for IL-2 (yellow), IFN- γ (black), IL-13 (green), IL-10 (gray hatched), TNF- α (light blue hatched), IL-17 (red), IL-22 (dark blue), IL-5 (orange), and IL-4 (pink). All visible bars are significant ($P < 0.05$) compared to parallel wells lacking anti-CD3 antibodies. The data presented are aggregated from two independent experiments. (B) The CD4 γ 13 IL-13 pathway is partially calcineurin independent. 2.5×10^4 T cells were activated by immobilized anti-CD3 in the absence and presence of 500 nM CsA without or with small molecule inhibitors of CrTh2 (CrTh2-1 and CrTh2-2) at $5 \mu\text{M}$ ($\sim 50\times$ the 50% inhibitory concentration [IC_{50}]). The top panel shows IL-13 production, the middle panel IFN- γ production, and the bottom panel relative IL-13 versus IFN- γ in the presence of 500 nM CsA. Shown are aggregated data from two independent experiments. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$. (C) Termination of *C. muridarum* replication. C57epi.1 epithelial cells, untreated (top) or pretreated with 10 ng/ml IFN- γ for 6 h (middle), were washed and then infected with 2 IFU *C. muridarum* per cell. Four hours later, 1.5×10^5 T cells (1:1 ratio) were added to each well and to uninfected wells as controls. Twenty-eight hours later, 100 μl supernatant was removed for IFN- γ analysis and wells were harvested into SPG buffer. IFN- γ levels were determined by ELISA and IFU number by culture on McCoy cells. All IFN- γ values (picograms per milliliter) in parentheses are significant ($P < 0.05$); IFN- γ levels for all CD4 clones cultured on uninfected epithelial cells were < 100 pg/ml. Shown are aggregated data from two independent experiments. The bottom panel shows the cytolytic capability of the CD4 clones versus a *Chlamydia*-specific CD8 T cell clone in redirected lysis. Ten thousand T cells were incubated with 10,000 Fc receptor (FcR)-bearing P815 cells in the absence (spontaneous release) and presence of 0.5 $\mu\text{g/ml}$ anti-CD3 antibody (activation/lysis) in quadruplicate in a 4-h assay based on LDH release. The percentage of specific lysis is shown for each of the T cell clones (single experiment done as quadruplicates).

APCs that we previously described (4uvmo-3) (37). The CD4 T cell clones were activated with immobilized anti-CD3, and then levels of IL-2, IFN- γ , IL-13, IL-10, TNF- α , IL-17, IL-22, IL-4, and IL-5 in culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA) (Fig. 3A). The two CD4 γ 13 clones shared IL-2, IFN- γ , IL-13, IL-10, and TNF- α and split on IL-17 plus IL-22 versus IL-4 plus IL-5. Only one of the CD4 γ 13 T cell clones produced IL-4: sBT16-8. Even at high cell density, no IL-4 was detectable upon activation of the CD4 γ 13 clone sBT13-7. No overarching statement can be made about CD4 T cell cytokine polarization in the panel, except that each clone had a unique profile. Th2 cells have a T cell receptor (TCR)-independent pathway for IL-13 production based on prostaglandin D binding the CrTh2 receptor (38). As CD4 γ 13 T cells have not been previously described, we tested whether IL-13 production was calcineurin dependent and whether the known calcineurin-independent prostaglandin D-CrTh2 pathway could account for IL-13 production (Fig. 3B). Compared to IFN- γ , IL-13 production was significantly less inhibited by cyclosporine (CsA). Based on small molecule inhibitors, the residual IL-13 production in the presence of CsA was not due to the CrTh2 pathway.

We investigated the CD4 T cell clones' ability to recognize and terminate *C. muridarum* replication in epithelial cells (Fig. 3C, top and middle panels) and their

relative cytolytic ability compared to a *Chlamydia*-specific CD8 cytotoxic T lymphocyte (CTL) clone (8uvmo-2) using redirected lysis (Fig. 3C, bottom panel). All the CD4 T cell clones recognized infected epithelial cells and to various degrees terminated *C. muridarum* replication in them; only the CD4 γ 13 clone sBT16-8 fell below 50% inhibition. Two CD4 clones terminated *C. muridarum* without IFN- γ pretreatment; 4uvmo-3 was nearly IFN- γ independent (did not require epithelial cells be pretreated with IFN- γ), consistent with our prior publication (37). All CD4 clones produced *Chlamydia*-specific IFN- γ under the conditions of the replication termination assay; IL-13 was not detected under these conditions. No conclusions can be drawn about IFN- γ production versus termination efficiency as termination likely reduces T cell activation, as suggested by lower levels of IFN- γ for all CD4 T cell clones when epithelial cells were pretreated with IFN- γ (improved termination efficiency); the highest level of IFN- γ was for sBT13-11 with untreated infected epithelial cells (8,196 pg/ml), an experimental condition in which sBT13-11 did not significantly terminate replication. The CD4 clones were less cytolytic than a conventional CD8 CTL clone, but all had some ability to kill in a short-term assay.

Gene expression microarray analysis of CD4 γ 13 T cells. Having a panel of CD4 γ 13 and multifunctional Th1 T cell clones offered the possibility of defining CD4 γ 13 T cells at the molecular level using gene expression microarray analysis. We chose to do the initial investigation using T cells in their “rested” state as that condition was more likely to reflect T cell differentiation biology (i.e., biomarkers that may be useful in peripheral blood and uninfected tissue). T cells at the end of the usual 7-day culture cycle were purified by Ficoll-Hypaque and plated without antigenic stimulation for an additional 48 h in medium containing recombinant IL-7. Two days later, the wells were harvested and total RNA isolated; the experiment was repeated 4 times to minimize false discovery. The comparators were sBT13-7 and sBT16-8 (CD4 γ 13), BT12-7 and sBT13-11 (multifunctional Th1 derived with B cell APCs that do not produce IL-13), BT12-17 (multifunctional Th1 that initially made and then lost IL-13 production), and 4uvmo-3 (multifunctional Th1 derived with unfractionated splenocyte APC). The value of BT12-17 was unclear: it represents either plasticity in the CD4 γ 13 phenotype or a breakthrough dominant second clone from incomplete limiting dilution. At worst, BT12-17 was a third multifunctional CD4 T cell that did not produce IL-13. sBT13-7 and sBT16-8 were derived with soluble *Chlamydia* antigen and the other clones with uvMoPn. The microarray comparisons were as follows: (i) sBT13-7+sBT16-8 versus BT12-7+sBT13-11 for the comparison CD4 γ 13 versus multifunctional Th1 (all B cell derived), (ii) sBT13-7+sBT16-8 versus BT12-17 for possible unique insight into IL-13 biology (loss of function), and (iii) sBT13-7+sBT16-8 versus 4uvmo-3 for the comparison CD4 γ 13 versus conventional multifunctional Th1 (splenocyte APC derived).

The criteria applied to identify genes of interest were (i) a log₂ fluorescence signal of >5.0 (mRNA signal above background), (ii) a statistically significant ($P < 0.01$) 3-fold difference (up or down) between the two CD4 γ 13 clones in aggregate versus non-CD4 γ 13 clones in all three comparisons, and (iii) the log₂ fluorescence signal for both CD4 γ 13 clones had to be greater than the individual log₂ signals for all the other T cell clones in the array (which eliminates genes skewed by very high or low expression by one of the CD4 γ 13 T cell clones). Analysis of the microarray data showed that the CD4 γ 13 T cell clones had more genes in common with each other than the other clones (Fig. 4). Genes with significantly enhanced mRNA signal in CD4 γ 13 T cells are shown in Table 1, and those with significantly reduced mRNA signal are shown in Table 2. All gene mRNA differences in Table 1 and Table 2 are statistically significant, with the highest false-discovery rates (FDRs) being 7×10^{-4} (*Trib2*) for the upregulated genes and 1×10^{-4} (*S1pr1*) for the downregulated genes. The cytokine data for CD4 γ 13 T cell clones (Fig. 3A) showed unusual combinations of Th1/2/17/22 cytokines, so we parsed out CD4 γ 13 T cell clone differentiation markers/transcription factors from the microarray (Table 3) and did Western blotting for *Tbet* (Th1), *Gata3* (Th2), *Eomes*, and *Fhl2* on the two CD4 γ 13 T cell clones (sBT13-7 and sBT16-8) and three IL-13-negative controls

FIG 4 Clustering of the top 1,000 genes by the ANOVA *P* value (i.e., genes that were differentially expressed in at least one clone) shows the two CD4 γ 13 T cell clones sBT13-7 and sBT16-8 (top two vertical bars on left) are the most alike among the six CD4 T cell clones in the panel (from left to right, blue-red-blue-red-blue pattern).

(4uvmo-3, BT12-7, and BT12-17) on day 5 of their usual 7-day culture cycle (peak T cell numbers in well). Differentiation markers/transcription factors in the microarray with mRNA signals that were negligible, *ROR γ T* (Th17), or low and mismatched between the two CD4 γ 13 clones, *Ahr* (Th22), were not included in Western blot analysis; blotting with commercial antibodies for *Epas1* generated low-quality blots, and therefore the results are not included in Fig. 5. Transcriptionally the CD4 γ 13 T cell clones look like Trm (*Klrg1*^{neg}, *Klf2*^{neg}, *Hnfla*^{neg}, *S1pr1*^{neg}, *Ccr7*^{neg}; *Hobit*^{neg}, *Blimp-1*^{pos}, *Rgs1*^{pos}, *Rgs2*^{pos}, *Cd69*^{pos}, and *Cd44*^{pos}). Based on limited data, *Chlamydia*-specific multifunctional Th1 derived from mice that self-cleared a genital tract infection universally express *Gata3* and *Eomes*. Interestingly Tbet expression, as detectable at the level of Western blotting, does not appear to be required for IFN- γ production, although its relative absence may be required for IL-4 production (e.g., sBT16-8), and *Gata3* in *Chlamydia*-specific multifunctional Th1 does not denote a conventionally defined Th2 phenotype or IL-4 production. *Fhl2* appears to be the transcription factor that qualitatively and perhaps quantitatively, denotes a CD4 γ 13 T cell's ability to uniquely produce IL-13.

Adoptive transfer of multifunction Th1 versus CD4 γ 13. To determine whether CD4 γ 13 T cells were capable of protecting or causing genital tract pathology during *C. muridarum* infections, we adoptively transferred them into naive C57BL/6 mice and challenged the next day with genital tract infections. For comparison, we adoptively transferred 4uvmo-3, the conventional multifunctional Th1 comparator that we'd previously predicted to be protective based on *Plac8* positivity, early and relatively IFN- γ -independent recognition of infected epithelial cells, and efficient termination of *Chlamydia* replication in them (39). Our initial experiment and a staggered/stacked-replicate second experiment were focused on 4uvmo-3 (splenic APC-derived multifunctional Th1) versus sBT16-8 (CD4 γ 13 with the highest IL-13 production), piloting smaller numbers of mice with sBT13-7 (other CD4 γ 13 clone) and BT12-17 (B cell-derived multifunctional Th1 without IL-13). When the first experiment reached 8 weeks and was scored for pathology, it was clear that sBT13-7 was likely the most protective T cell clone (zero pathology in three mice; control incidence of $\geq 60\%$). A third cohort of control and sBT13-7 mice was initiated to complete the data set. Mice were monitored for bacterial shedding through day 30 (Fig. 6A and B) and killed on day 56 to score immunopathology (Fig. 6C [dissected genital tracts in panel D]). The multifunctional

TABLE 1 Genes with significantly enhanced mRNA signal in CD4 γ 13 T cells

Gene	Result for comparison						Gene product and function
	sBT13-7 + sBT16-8 vs BT12-7 + sBT13-11		sBT13-7 + sBT16-8 vs BT12-17		sBT13-7 + sBT16-8 vs 4uvmo-3		
Gene	P value	Fold change	P value	Fold change	P value	Fold change	Gene product and function
<i>Bace2</i>	2.29E−12	16.89	6.73E−11	14.76	1.59E−10	12.78	Beta-site APP-cleaving enzyme 2: transmembrane protease
<i>Eomes</i>	1.62E−12	14.73	7.94E−14	45.01	6.07E−14	47.92	Eomesodermin homolog (<i>Xenopus laevis</i>): prevents CD4 differentiation into Treg and makes CD8 less cytolytic
<i>Trat1</i>	5.03E−08	10.11	4.80E−07	10.13	1.31E−07	12.74	T cell receptor associated transmembrane adaptor 1: adjusts TCR signaling
<i>Acvr2a</i>	6.70E−11	7.72	3.46E−10	8.71	1.31E−08	5.49	Activin receptor IIA: T cell differentiation (Th17) IL-6 + TGF-β
<i>Nrn1</i>	7.39E−11	6.35	3.69E−09	5.41	3.33E−09	5.47	Neuritin 1: transcript found in CD8 TIL (tumor infiltrating lymphocytes) and Tregs
<i>Ctse</i>	1.41E−10	6.29	4.52E−08	4.37	3.59E−07	3.57	Cathepsin E: releases TRAIL in Tregs
<i>Cpa3</i>	4.97E−09	6.26	7.83E−10	11.46	6.68E−08	6.09	Carboxypeptidase A3, mast cell: released granule protease
<i>Lrrc32</i>	4.70E−07	6.19	1.77E−06	6.96	4.28E−07	8.67	Leucine-rich repeat containing 32: binds latent TGFb complex
<i>Wls</i>	2.40E−11	5.96	4.71E−10	5.69	8.16E−14	19.78	wntless homolog (<i>Drosophila</i>): wnt ligand secretion vehicle; IL-13, IL-4
<i>2900026A02Rik</i>	9.62E−11	5.40	1.78E−10	6.73	2.42E−09	5.00	RIKEN cDNA 2900026A02 gene: intracellular protein unknown function
<i>Gpx8</i>	2.26E−11	5.23	3.66E−13	12.36	3.52E−13	12.43	Glutathione peroxidase 8 (putative): ER ^α protein, disulfide bonds, regulated by EPas1
<i>Ccr8</i>	6.27E−10	5.17	9.44E−11	8.88	7.88E−15	49.24	Chemokine (C-C motif) receptor 8
<i>Lrrc16a</i>	1.61E−10	4.73	3.68E−09	4.44	1.10E−08	4.00	Leucine rich repeat containing 16A: uncaps actin and slows migration
<i>Rgs10</i>	6.69E−12	4.68	1.11E−14	14.67	3.61E−15	17.72	Regulator of G-protein signaling 10: knockout with reduced CD4 with aging
<i>Oit3</i>	3.98E−10	4.52	3.55E−10	5.98	4.36E−07	3.00	Oncoprotein-induced transcript 3: secreted protein
<i>Fhl2</i>	8.86E−09	4.05	1.96E−09	6.16	3.82E−10	7.56	Four and a half LIM domains 2: knockout mouse has decreased IL-13 response
<i>Epas1</i>	1.40E−07	3.95	8.68E−11	14.14	4.44E−08	5.77	Endothelial PAS domain protein 1: transcription factor associated with cytokines
<i>Trib2</i>	2.23E−06	3.56	4.73E−05	3.18	3.08E−05	3.33	Tribbles homolog 2 (<i>Drosophila</i>): cell survival
<i>Qpct</i>	1.37E−06	3.45	4.81E−09	8.81	3.55E−09	9.21	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase): N-terminal protein modification
<i>Bcl2l11</i>	2.28E−10	3.32	1.60E−09	3.47	1.72E−15	18.58	BCL2-like 11 (apoptosis facilitator): cytokine withdrawal upregulates apoptosis
<i>Afap1l1</i>	2.33E−06	3.31	2.40E−05	3.20	2.21E−05	3.23	Actin filament associated protein 1-like 1: rounded cell shape
<i>Gnb4</i>	1.30E−09	3.08	2.41E−12	7.21	1.54E−13	10.36	Guanine nucleotide binding protein (G protein), beta 4: LFA-1 activation

^aER, endoplasmic reticulum.

Th1 clone 4uvmo-3 was partially protective, reducing the frequency of hydrosalpinx from ~60% of oviducts to ~20%. sBT13-7, a CD4 γ 13 T cell clone, dramatically protected mice from *C. muridarum* immunopathology, preventing damage to uteri in 8 of 9 mice and preventing hydrosalpinx in 9 of 9 mice. sBT16-8, the other CD4 γ 13 T cell clone, was neither protective or pathological. Interestingly the protection afforded to the murine genital tract by sBT13-7 and 4uvmo-3 was largely limited to oviducts and did not correlate with rapidity of bacterial clearance.

DISCUSSION

We investigated B cells, CD4 T cells, and IL-13 during *Chlamydia* infections of the genital tract in the context of tissue-resident mucosal immunity. The impetus for this research was the paradoxical data regarding Th2 cells and IL-13 in *Chlamydia* host defense. On one hand, Th2 responses to *Chlamydia* infections have been associated

TABLE 2 Genes with significantly reduced mRNA signal in CD4 γ 13 T cells

Gene	Result for comparison						Gene product and function
	sBT13-7 + sBT16-8 vs BT12-7 + sBT13-11		sBT13-7 + sBT16-8 vs BT12-17		sBT13-7 + sBT16-8 vs 4uvmo-3		
	P value	Fold change	P value	Fold change	P value	Fold change	
<i>Clec5a</i>	4.11E-07	-5.47	2.43E-09	-17.37	2.19E-04	-3.25	C-type lectin domain family 5, member a: activated by viruses
<i>Fbxo27</i>	1.47E-10	-4.95	4.55E-10	-5.75	1.72E-12	-12.04	F-box protein 27: targeting for ubiquitin ligase (function unknown)
<i>Ggt5</i>	5.93E-10	-3.80	1.36E-11	-7.37	1.17E-08	-3.63	γ -Glutamyltransferase 5: leukotriene D4 production
<i>Lima1</i>	2.34E-12	-3.79	2.39E-14	-7.97	5.58E-14	-7.19	LIM domain and actin binding 1: actin depolymerization
<i>Ptgdr2</i>	6.04E-09	-3.36	2.60E-08	-3.64	5.91E-13	-13.03	Prostaglandin D2 receptor 2: CrTh2 TCR-independent IL-13 production
<i>S1pr1</i>	2.57E-06	-3.31	2.33E-12	-36.54	9.88E-12	-26.83	Sphingosine-1-phosphate receptor 1: recruits T cells into circulation
<i>Tln2</i>	2.63E-10	-3.15	2.89E-11	-4.72	1.97E-12	-6.26	Talin 2: assembly of actin filaments

with ineffectual or worsened pathological outcomes in mouse models (28, 29), including a study showing that IL-13 knockout mice cleared infections more rapidly with less pathology than wild-type mice (40). On the other hand, our human clinical investigation showed that a PBMC IL-13 response to EBs prospectively identified individuals resistant to reinfection with *C. trachomatis* (18), supporting a protective role for presumably T cells polarized to produce IL-13. Data presented here show these disparate results are biologically compatible and call into question the utility of the Th1/2/17 cytokine polarization framework for understanding genital tract immunity during *Chlamydia* infections.

Mice deficient in B cells clear primary *C. muridarum* infections in the usual time frame, with the caveat that they develop a transient peritonitis and early dissemination (41); mice deficient in B cells remain susceptible to reinfection with a clearance time only slightly faster than that of naive mice during primary infections (14). Published immunohistochemical analysis of lymphoid aggregates in human and mouse genital tract and conjunctival tissues in the setting of current or prior *Chlamydia* infections documents abundant B cells in *Chlamydia*-specific memory lymphocyte clusters (c-MLCs) (reviewed in reference 30). The combination of B cell knockout mouse susceptibility to reinfection and the abundance of B cells in c-MLCs suggest that B cells play an important role in protective secondary immune responses (42). In the original mouse work by Morrison and Morrison based on B220 immunohistochemical staining, B cells were present during the first 2 weeks of infection but disappeared in subsequent weeks. B220 is downregulated as activated B lymphocytes transition to immune plasma B cells (34). We analyzed the status of B lymphocytes and plasma B cells over the time course of infection by doing flow cytometry on single-cell suspensions generated from uteri and oviducts. We used the pan-B cell marker CD79a to identify B cells *in toto* and B220 to characterize them as B lymphocytes (high B220 expression) or plasma B cells (low B220 expression). We found that plasma B cells are nearly absent in naive genital tract tissue, become detectable during primary infection, and markedly expand during infections in mice with preexisting immunity generated either by PmpG-DDA-TDB vaccination or prior infection. The kinetics of plasma B cell expansion mirror the time course of demonstrable T cell immunity to *C. muridarum* (3) and are compatible with plasma B cells playing a role in *Chlamydia*-specific MLC (c-MLC) and protective immunity. These results suggest that our novel T cell recovery/expansion protocol based on immune B cell APC is physiologically relevant.

Immune B cell presentation of *Chlamydia* antigens to T cells from mice that previously cleared genital tract infections preferentially expanded CD4 T cells over CD8 T cells, including a subset polarized to produce IL-13 and IFN- γ . Analysis of CD4 T cells in the mouse genital tract and spleen in the naive state, vaccinated state, and

TABLE 3 Expression of CD4 γ 13 differentiation markers and transcription factors

Lineage and differentiation markers	Mean CD4 γ 13 T cell clone mRNA signal (AU)		Score ^a	Gene product
	sBT13-7	sBT16-8		
Lineage				
Cd4 T cells	7,651	9,484	++++	CD4 antigen
Cd8 T cells	51	56	0	CD8 antigen, beta chain 1
NK/NKT cells	82	27	0	Killer cell lectin-like receptor subfamily B mem 1C
B cells	40	41	0	CD79A antigen (immunoglobulin-associated alpha)
Cytokine polarization				
Th1	523	402	+	T-box 21 (T-bet)
Th2	1,677	4,184	++/+++	GATA binding protein 3 (Gata3)
Th17	62	45	0	RAR-related orphan receptor gamma (ROR γ T)
Th22	283	96	+/0	Aryl-hydrocarbon receptor (Ahr)
?	298	855	+	Endothelial PAS domain protein 1 (Epas1)
?	1,456	3,741	++/+++	Eomesodermin (Eomes)
Trm				
<i>Klrg1</i>	64	57	0	Killer cell lectin-like receptor subfamily G, mem 1
<i>Klf2</i>	113	88	0	Kruppel-like factor 2 (lung)
<i>Hnf1a</i>	90	102	0	HNF1 homeobox A
<i>S1pr1</i>	90	61	0	Sphingosine-1-phosphate receptor 1
<i>Ccr7</i>	252	268	+	Chemokine (C-C motif) receptor 7
<i>Zfp683</i>	52	71	0	Zinc finger protein 683 (Hobit)
<i>Prdm1</i>	1,121	772	++/+	PR domain containing 1, (Blimp-1)
<i>Rgs1</i>	2,318	2,972	++/+++	Regulator of G-protein signaling 1
<i>Rgs2</i>	1,032	2,238	++	Regulator of G-protein signaling 2
<i>Cd69</i>	3,526	3,169	+++	CD69 antigen
<i>Cd44</i>	5,222	4,794	+++	CD44 antigen
<i>Itgal</i>	5,688	6,527	+++	Integrin alpha L
<i>Itga4</i>	1,605	214	++/+	Integrin alpha 4
<i>Itgb7</i>	3,184	2,423	+++	Integrin beta 7
<i>Itga1</i>	1,021	701	++/+	Integrin alpha 1
<i>Itgae</i>	265	784	+	Integrin alpha E, epithelial associated
Treg				
<i>Cd27</i>	25	23	0	CD27 antigen
<i>Il2ra</i>	1,070	1,285	++	IL-2 receptor, alpha chain
<i>Foxp3</i>	108	136	0	Forkhead box P3
<i>Ctla4</i>	2,127	1,519	++	Cytotoxic T-lymphocyte-associated protein 4
Th22				
<i>Ahr</i>	283	96	+/0	Aryl-hydrocarbon receptor
<i>Foxo4</i>	179	156	+/+	Forkhead box O4
<i>Bnc2</i>	43	39	0	Basonuclin 2

^aScores are defined as follows: <150 arbitrary units (AU [$\sim 3 \times$ the CD8b signal]) = 0, 150 to 999 AU = +, 1,000 to 2,499 AU = ++, 2,500 to 7,499 AU = +++, and 7,500 to 10,000 AU = ++++.

postclearance of a primary *C. muridarum* infection showed that CD4 γ 13 T cells were localized to the genital tract in physiologically relevant levels: up to 15% of *Chlamydia*-specific T cells, a 500-fold enrichment of CD4 γ 13 T cells in the genital tract tissue versus the spleen. Importantly, immunization with the protective PmpG in DDA-TDB vaccine at the base of the tail enhanced the presence of CD4 γ 13 T cells in the genital tract.

Using the immune B cell APC protocol, we generated a panel of CD4 T cell clones from the spleens of immune mice (with prior genital tract infection) that included two CD4 γ 13 T cells, thereby providing an opportunity to define the CD4 γ 13 T cell subset at the molecular level using gene expression microarray analysis. The microarray data suggest a CD4 γ 13 memory T cell subset with Trm-like differentiation (*Klrg1*^{neg}, *Klf2*^{neg}, *Hnf1a*^{neg}, *S1pr1*^{neg}, *Ccr7*^{neg}; *Hobit*^{neg}, *Blimp-1*^{pos}, *Rgs1*^{pos}, *Rgs2*^{pos}, *Cd69*^{pos}, and *Cd44*^{pos}) (43). We postulate that the CD4 γ 13 T cell clones are progeny of the contraction of the CD4 T cell response during the primary genital tract infection—i.e., Trm T cells trafficked to the spleen to serve as a Trm repository reflecting events that occurred in the genital

FIG 5 Protein levels of selected differentiation/transcription factors. On day 5 of the usual culture cycle, the indicated CD4 T cell clones were purified by Ficoll-Hypaque, and whole-cell lysates were prepared and utilized for immunoblotting for *Tbet*, *Gata3*, *Eomes*, and *Fhl2*. Blots were stripped and reprobed with anti-β-actin as the loading control. Molecular mass markers (M) in kilodaltons are indicated in the right margin.

tract. Within the microarray, CD4γ13 T cell clones were more like each other than the other T cells in the panel, but were not homogenous. At the cytokine level, sBT13-7 and sBT16-8 shared at least 5 cytokines (IL-2, IFN-γ, IL-13, IL-10, and TNF-α), with sBT13-7 adding IL-17 and IL-22 and sBT16-8 adding IL-4 and IL-5. Unlike IL-2, IL-4, or IFN-γ, T cell production of IL-13 is partially resistant to cyclosporine (CsA). In human peripheral blood CD4 T cells, CsA-resistant IL-13 production occurs only at low concentrations, <30 nM, through a MEK-dependent pathway (44). The CD4γ13 T cells we report here continued to preferentially produce IL-13 versus IFN-γ in experiments using 500 nM CsA, >10 times the concentration that completely blocked IL-13 in human peripheral blood CD4 T cells. We tested whether the Th2 calcineurin-independent (CsA-resistant) prostaglandin D-CrTh2 pathway (38) was responsible for CsA-resistant IL-13 production in the CD4γ13 T cell clones. It was not, and the microarray showed diminished CrTh2 mRNA transcripts in CD4γ13 compared to multifunctional Th1 clones (Table 2). These data suggest that CD4γ13 T cells have a TCR signaling pathway that regulates IL-13 production independent of calcineurin/nuclear factor of activated T cells (NFAT) activation.

The differentiation/cytokine polarization of *Chlamydia*-specific IFN-γ-producing CD4 T cells generated during clearance of *Chlamydia* genital tract infections appears to be based on the transcription factors *Gata3* and *Eomes* and, usually but not always, *Tbet*. A previous study showed few *Gata3*⁺ CD4 T cells in spleen and lymph nodes during *C. muridarum* infection without investigation of genital tract tissue (41). A human study showed CD4 *Gata3*⁺ T cells in presumed memory lymphocyte clusters in endometrial biopsies from *C. trachomatis*-infected women but not uninfected controls (45). In isolation, two CD4γ13 T cell clones and four multifunctional CD4 T cell clone controls are not sufficient to draw definitive conclusions about T cell biology. However, in combination with published cytokine regulation data, it is reasonable to extrapolate to preliminary conclusions. Our results are consistent with the existing paradigm that *Tbet* (Th1) must be downregulated for a CD4γ13 T cell clone to produce IL-4 (Th2): e.g., sBT16-8. More importantly we saw that *Fhl2* was CD4γ13 associated at the transcript level (microarray) and protein level (Western blot). Because *Fhl2* knockout mice have a

FIG 6 Adoptive transfer as shown by bacterial shedding and pathology scoring. (A) IFU shedding in the lower genital tract. (B) Percentage of mice shedding at each time point. (C) On day 56, mice were killed and uterine and oviduct pathology was scored *in situ* (see Materials and Methods). (D) Digital images of the oviducts; black arrows indicate scored pathology. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. Shown are aggregated data from 3 experiments: control (14 mice), 4uvmo-3 (9 mice), sBT13-7 (9 mice), and sBT16-8 (8 mice). Txf, adoptive transfer.

deficit in IL-13 production (46), it is reasonable to postulate that *Fhl2* is the transcription factor that denotes a CD4 γ 13 T cell's ability to produce IL-13, and *Fhl2* identifies an ideal candidate pathway for calcineurin-independent IL-13 production.

Although not conventionally quantifiable, we empirically discovered that immune B cell antigen presentation and exogenous TGF- β 1 were necessary to recover CD4 γ 13 T cells and maintain their IL-13 production *ex vivo* (Fig. S3). Absent TGF- β 1, IL-13 production seen in early passages of polyclonal T cells faded with serial passage suggesting that *in vivo* CD4 γ 13 memory T cells reside in a microenvironment with active TGF- β 1. Epithelial cells make latent TGF- β constitutively, and TGF- β in the latent form is abundant in mucosal tissues. The CD4 γ 13 T cell molecular fingerprint (microarray) includes genes relevant to TGF- β biology, including *Lrrc16a*, coding for the binding receptor for the latent TGF- β complex, *Bace2* and *Cpa3*, coding for proteases that potentially process latent TGF- β into the active form for use in an autocrine/paracrine fashion, and *Avr2a*, coding for the receptor for activins that share some TGF- β signaling pathways. These genes may provide new insights into the unique biology of immune B cells and memory lymphocyte clusters within the genital tract.

One of the most important findings in our present investigation is that CD4 γ 13 T cell clone sBT13-7 completely protected oviducts from pathology in adoptive transfer-*C. muridarum* challenge experiments, as did to a lesser extent the multifunctional Th1 clone 4uvmo-3. Those results link multifunctional Th1 and CD4 γ 13 T cells to protective immunity, with the interesting difference being that CD4 γ 13 sBT13-7 had a Trm mRNA fingerprint (*Klf2*^{neg} and *S1pr1*^{neg}) compared to Th1 4uvmo-3 (*Klf2*^{pos} and *S1PR1*^{pos}). The CD4 γ 13 phenotype in and of itself was not sufficient for genital tract protection as the CD4 γ 13 sBT16-8 clone was not protective; sBT16-8 was also relatively ineffective at terminating *Chlamydia* replication in epithelial cells *in vitro*. The mechanism of oviduct protection for 4uvmo-3 and sBT13-7 was not accelerated bacterial clearance as measured in the lower genital tract: 4uvmo-3 mice cleared at same rate as naive mice, while sBT13-7 mice had greater bacterial shedding at late time points, a result that may eventually provide insights into why IL-13 knockout mice clear infections faster than wild-type mice (40). At the level of individual T cell clone-mediated immunity, in wild-type mice with normal immune systems, this study shows that protection from pathology and bacterial shedding can be dissociated. This dissociation has been previously demonstrated in mouse models with plasmid-deficient *C. muridarum* (47) and knockouts of TLR2 (48) and IL-1b (49). sBT13-7 protection from immunopathology implies a regulatory mechanism that influenced the naive wild-type adaptive immune response, perhaps moderating the primary CD8 T cell response associated with immunopathology (20, 22, 23). Both CD4 γ 13 T cell clones have enhanced mRNA transcript levels for several regulatory T cell (Treg)-associated genes, including *Nrn1*, *Ctse*, and *Lrrc32*; however, they are not Tregs as they produced IL-2 upon activation and neither had an mRNA signal for *Foxp3*. In our opinion, it is unlikely likely that Treg or induced Treg (iTreg) mechanisms fully account for protection from pathology mediated by sBT13-7. We suspect sBT13-7 somehow reduced neutrophil recruitment and/or promoted a more beneficial healing response within the TNF- α -IL-13-TGF- β axis (50, 51). Recent work by Li et al. demonstrated that CCR7 homing contributes to the paucity of T lymphocytes in the naive murine female genital tract, presumably by lymph node sequestration. Naive CCR7 knockout mice have an aberrant immune architecture with many more T cells localized in genital tract tissue and cleared *C. muridarum* more rapidly and with less acute inflammation than did wild-type mice (52). In our study, adoptive transfer of the CD4 γ 13 T cell clone sBT13-7 into wild-type mice with their usual paucity of immune architecture and T cells prevented oviduct immunopathology. The CD4 γ 13 T cell clones had enhanced CCR8 (5- to 12-fold higher [Table 1]) with reduced CCR7 mRNA transcripts (Table 3 and GEO data) compared to the four non-CD4 γ 13 clones. Therefore, it is possible that CCR8, associated with skin homing (53), plays a role in c-MLC. While the mechanism of sBT13-7 protection remains to be determined, during primary genital tract infections in wild-type mice, it is reasonable to postulate that “how” is at least as or more important than “how fast” *Chlamydia* is cleared, with implications for assessment of future vaccines.

It is unlikely that IL-13 directly participates in the physical termination of *Chlamydia* cells replicating in reproductive tract epithelium, as we have shown that IL-13 modestly enhances *C. muridarum* replication in an upper reproductive tract epithelial cell line (36), and others have shown that IL-13 knockout mice show accelerated bacterial clearance from the genital tract (40). Instead we postulate that IL-13 is a biomarker for a CD4 Trm subset capable of preventing immunopathology during clearance of genital tract infections and that small numbers of CD4 γ 13 T cells in circulation are the source of EB-stimulated PBMC IL-13 production that predicted resistance to *C. trachomatis* infection in the study by Cohen et al. of Kenyan female sex workers. We anticipate that studies of CD4 γ 13 T cells in the mouse model will provide the tools necessary to test those hypotheses in humans.

Recently it has been proposed that protective/healing Th2 immunity explains how the majority of humans clear *Chlamydia* genital tract infections without fertility-limiting immunopathology based on *Gata3*-centered data (45, 54–56). That Th2 conclusion is consistent with the existing Th1/2 paradigm and reasonable as long as *Gata3* is tightly

associated with the Th2 phenotype. However, our data, generated in a productive *Chlamydia* genital tract infection model that reproduces human pathology, including infertility and hydrosalpinx, shows that *Chlamydia*-specific CD4 T cell clones universally expressed *Gata3* and produced IFN- γ upon activation—even a CD4 clone with little or no *Tbet* expression—violating the basic mutual exclusivity tenets of the Th1/2 paradigm.

MATERIALS AND METHODS

Mice. Four- to 5-week-old female C57BL/6 mice were purchased from Harlan Labs (Indianapolis, IN) and Jackson Laboratory (Bar Harbor, MA). Mice were housed in Indiana University Purdue University—Indianapolis (IUPUI) and Yale University specific-pathogen-free (SPF) facilities. The Institutional Animal Care and Utilization Committees at Indiana University, Yale University, and University of British Columbia approved all experimental protocols.

Cells and bacteria. McCoy fibroblasts were cultured as previously described (37). Mycoplasma-free *Chlamydia muridarum* Nigg, previously known as *C. trachomatis* strain mouse pneumonitis (MoPn), was grown in McCoy cells as previously described (57). Soluble *Chlamydia* antigen (infected cell lysate depleted of EBs by centrifugation) was prepared as previously described (36), aliquoted, and stored at -80°C .

Chlamydia-specific CD4 T cells. Conventional multifunction *Chlamydia*-specific Th1 clone 4uvmo-3 was previously described (37). For the new B cell APC-derived T cells, C57BL/6 mice were treated with 2.5 mg of medroxyprogesterone (Pfizer) delivered subcutaneously and then infected 7 days later with 5×10^4 inclusion-forming units (IFU) of *C. muridarum*. Mice that cleared infection (≥ 6 weeks postinfection) were used as the source of immune B and T cells. Initial *Chlamydia*-specific immune B cell-derived polyclonal T cell populations and clones were derived as follows. Splenocytes were harvested from immune mice. Immune B cells were purified from a portion of those splenocytes by “untouched” magnetic bead separation (Miltenyi Biotec). Immune B cells were pulsed with UV-MoPn (3.5×10^6 IFU equivalents per 7.5×10^5 B cells suspended at $7.5 \times 10^6/\text{ml}$ [~ 5 IFU/cell]) or soluble antigen ($7.5 \mu\text{l}$ per 7.5×10^5 B cells suspended at $7.5 \times 10^6/\text{ml}$) for 1 h at 37°C . Antigen-pulsed immune B cells were transferred to 7.5 ml of RPMI complete medium and pelleted, medium containing antigen was removed, and then the cells were washed two more times with 7.5 ml of medium ($\sim 400,000$ -fold) to eliminate all non-cell-bound or internalized *Chlamydia* antigen; the purpose of extended washing was to ensure that antigen presentation was limited to immune B cells. Primary stimulation wells were set up with 2.5×10^6 immune splenocytes plus 7.5×10^5 antigen-pulsed immune B cells in 0.75 ml RPMI complete medium supplemented with recombinant cytokines and conditioned medium as previously described (36); later T cell derivations during the course of the project included addition of 5 ng/ml recombinant murine TGF- $\beta 1$ to the medium. Limiting dilution cloning was done in medium supplemented with recombinant cytokines/conditioned medium with 10 to 20 ng/ml TGF- $\beta 1$. Restimulation/maintenance of T cell clones was done weekly in 48-well plates by adding 100,000 to 200,000 T cell clone cells to 1.5×10^6 irradiated naive splenocytes and 7.5×10^5 irradiated relevant antigen-pulsed, washed immune B cells as feeders in medium supplemented with recombinant cytokines/conditioned medium, including 2.5 to 10 ng/ml TGF- $\beta 1$. Recombinant mouse cytokines were purchased from the same vendor (R&D Systems; Minneapolis, MN), except for TGF- $\beta 1$ (Ebioscience, San Diego, CA).

Flow cytometry and intracellular cytokine staining. For B cell staining, single-cell suspensions of genital tracts pooled from four mice per experimental group were surface stained using anti-mouse B220 (RA3-6B2 coupled to fluorescein isothiocyanate [FITC]; BD Pharmingen) as well as with the viability dye aqua fluorescent reactive dye (L34957; Molecular Probes), followed by intracellular staining using anti-mouse CD79a (24C2.5 coupled to eFluor660; eBioscience). The experiment was repeated 1 or 2 times for individual experimental groups.

T cell surface phenotypes were determined using antibodies to CD4 (GK1.5 coupled to phycoerythrin [PE]) and CD8a (53-6.7 coupled to FITC). T cells were stained for 20 min at 4°C with $1 \mu\text{g}$ per 1 million T cells in RPMI complete medium with 10% fetal bovine serum (FBS), fixed with 1% paraformaldehyde, and analyzed by flow cytometry (BD FacsCalibur or LSRII). For intracellular staining for IL-13 (eBio13A coupled to PE) and IFN- γ (XMG1.2 coupled to allophycocyanin), T cells were activated for 5 h in a cocktail of phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A, and monensin (cell stimulation cocktail; Ebioscience), stained for CD8a, and then fixed and permeabilized (Fix/Perm buffer; Ebioscience), stained for IL-13-PE/IFN- γ -allophycocyanin or control antibody (eBRG1-PE/IFN- γ -allophycocyanin) in the presence of 2 mg/ml donkey IgG (Jackson ImmunoResearch) for 30 min at room temperature, washed, suspended in 1% paraformaldehyde, and analyzed. All of the T cell populations are $>90\%$ CD4 T cells; negative staining based on CD8a was chosen because PMA-ionomycin activation resulted in shedding of cell surface CD4 and diminished CD4 staining; CD8a staining was not affected (data not shown).

Cytokine ELISAs and signaling reagents. 2.5×10^4 Ficol-Hypaque-purified T cell clones (5×10^4 purified T cells for IL-4 determination) cultured overnight in RPMI medium with 3 ng/ml IL-7 were activated in 96-well tissue culture plates by immobilized anti-CD3 monoclonal antibody 145-2c11, at 0.5 $\mu\text{g}/\text{ml}$ in phosphate-buffered saline (PBS) overnight at 4°C (washed once), in RPMI medium containing 1 ng/ml recombinant murine IL-7 (R&D Systems, Minneapolis, MN) for 20 h. Relative levels of IL-2, IFN- γ , IL-13, IL-10, TNF- α , IL-17, IL-22, IL-4, and IL-5 in culture supernatants were determined by ELISA using capture and biotinylated monoclonal antibody pairs with recombinant murine standards according to the manufacturer's protocols: for IL-2, JES6-1A12/Jes6-5H4; for IFN- γ ELISA, XMG1.2; (Pierce-Thermo Fisher, Rockford IL); for IL-13 ELISA, eBio13A/eBio1316H (Ebioscience); for IL-10, Jess-16E3/Jess-2A5; for

TNF- α , TN3-19.12/rabbit anti-mouse/rat polyclonal (BD Biosciences); for IL-17, 17CK15A5/17B7 (Ebioscience); for IL-22, polyclonal 5164 (Biolegend); for IL-5, TRFK5/TRFK4; and for IL-4, 11b11/BVD6-24g2 (Ebioscience). Detection was accomplished with streptavidin-horseradish peroxidase (HRP) (BD Biosciences) and tetramethylbenzidine (TMB) substrate (Sigma Chemical Co).

Cyclosporine was purchased from Sigma and dissolved in ethanol. CrTh2 inhibitors I [(4-chloro-2-((2-methyl-5-(propylsulfonyl)phenyl)ethynyl)phenoxy)acetic acid] and II [(R)-(5-chloro-1'-(5-chloro-2-fluorobenzyl)-2,2',5'-trioxospiro(indole-3,3-pyrrolidin)-1(2H)-yl)acetic acid] were purchased from EMD Millipore (Temecula, CA) and dissolved in dimethyl sulfoxide (DMSO).

Redirected lysis. Redirected lysis was performed as described by Leo et al. (58). 10,000 P815 cells (ATCC TIB-64; American Type Culture Collection, Manassas, VA), from a mastocytoma cell line expressing Fc receptors (FcRs), were incubated with 10,000 CD4 T cells in the presence of 0.5 μ g/ml anti-CD3e (clone145-2c11, NA/LE; BD Biosciences, San Jose, CA) in 96-well V-bottom plates, spun for 1 min at 300 $\times$ g, and then incubated for 4 h. Killing was quantified using a nonradioactive cytotoxicity assay measuring release of lactate dehydrogenase activity in culture supernatant (cyto 96; Promega, Madison, WI) following the manufacturer's protocol. The lysis assays were done using RPMI complete medium with 1% heat-inactivated serum (68°C for 30 min to inactivate lactate dehydrogenase activity present in FBS). The percentage of specific lysis was calculated as [(experimental release of T cells + P815 + anti-CD3) – (spontaneous release of T cells + P815 without antibody)]/(maximal release by Triton X-100 treatment of P815)] $\times$ 100.

Adoptive transfer and genital tract infections. T cells were purified with Ficoll-Hypaque (Histopaque 1083; Sigma Chemical Co, St. Louis, MO) on day 5 of the culture cycle and maintained in RPMI complete medium with 3 ng/ml murine recombinant IL-7 for 2 days prior to adoptive transfer. One week prior to infection, mice were treated with 2.5 mg of medroxyprogesterone (Pfizer) delivered subcutaneously. Six days later, 1×10^6 T cell clone cells were adoptively transferred via retro-orbital injection into fully anesthetized mice; controls were injected with an equivalent volume of phosphate-buffered saline (PBS). The day following adoptive transfer, lightly anesthetized mice were infected vaginally with 5×10^4 IFU of *C. muridarum* in 10 μ l of SPG buffer (10 mM sodium phosphate [8 mM Na₂HPO₄-2 mM NaH₂PO₄], 220 mM sucrose, 0.50 mM L-glutamic acid). Mice were serially swabbed through day 30 postinfection, and IFU were determined on McCoy cells to quantify bacterial shedding. On day 56 postinfection, the mice were killed and genital tracts scored for pathology as previously described (39). Briefly, each mouse genital tract has 2 uteri and 2 oviducts; one point is assigned for macroscopic (visible) thinning to dilatation of each site for a maximum score of 4 per mouse. Scoring is done *in situ*; the genital tracts are then excised and photographed for a digital record (qualitative data). The adoptive transfer experiments were aggregated for Chi-square analysis.

Gene expression microarray analysis. For the “resting” phenotype microarrays, *Chlamydia*-specific CD4 T cell clones 4uvmo-3, BT12-7, BT12-17, sBT13-11, sBT13-7, and sBT16-8 were purified by Ficoll-Hypaque at the end of their usual 7-day culture cycle and then maintained in RPMI complete medium with 3 ng/ml IL-7 for 48 h without antigen stimulation. Total RNA was isolated from each T cell clone using a protocol that included a genomic DNA removal step (G-eliminator; RNeasy; Qiagen, Valencia, CA). RNA isolation under these culture conditions was repeated 4 times (independent experiments) for each clone to minimize false discovery. With assistance from The Indiana University Center for Medical Genomics, gene expression patterns were analyzed using Affymetrix Clariom S mouse arrays, which analyze >20,000 well-annotated genes. Samples were labeled using the standard Affymetrix protocol for the Affymetrix WT Plus kit using 100 ng of total RNA. Individual labeled samples were hybridized to the Mouse Clariom S GeneChips for 17 h and then washed, stained, and scanned with the standard protocol using Affymetrix GeneChip Command Console software (AGCC) to generate data (CEL files). Arrays were visually scanned for abnormalities or defects. CEL files were imported into Partek Genomics Suite (Partek, Inc., St. Louis, MO).

Western blots. Polyclonal rabbit antiserum specific for *Eomes* (Thermo Fisher Scientific catalog no. 720202), *Tbet/Gata3/Fhl2* (Proteintech catalog no. 13700-1-AP/10417-1-AP/21619-1-AP), and HRP-coupled monoclonal antibody to β -actin (Sigma-Aldrich catalog no. A3854) were obtained from commercial vendors. Ten micrograms of whole-cell lysate protein was run on 4 to 12% gradient gels and transferred to nitrocellulose using a dry blotting system (iBlot; Thermo Fisher). Membranes were rinsed in Tris-buffered saline with Tween 20 (TBST) and blocked with 5% nonfat milk in TBST. Rabbit antisera were detected with a rabbit-specific chemiluminescent kit (Thermo Fisher catalog no. WB7106). Detection was performed with a commercial chemiluminescent substrate (Thermo Fisher catalog no. 34080).

Statistical methods. As indicated in each figure legend, aggregated data were analyzed by two-sample Student's *t* test using Origin 8.0 software. Exceptions were Fig. 3B and 4 (analysis of variance [ANOVA]) and Fig. 6 (Dunnett's test), which were analyzed with R software, and Fig. 6C, which underwent chi-square analysis.

Accession number(s). The microarray data presented here are available in the Gene Expression Omnibus database under accession no. [GSE104743](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104743).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00614-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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We have no conflicts of interest related to the content of this article.

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