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The Chlamydia Conundrum: Challenges to the Classical Th Subset Canon

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The Chlamydia Conundrum:
Challenges to the Classical Th Subset Canon

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Abstract

The obligate intracellular bacteria *Chlamydia trachomatis* is the cause of the most common bacterial sexually transmitted infection worldwide. Untreated *Chlamydia* infections can develop into severe pathological outcomes in the female reproductive tract (FRT). In order to control transmission rates and prevent pathology, the development of an effective vaccine is urgently required. The mouse model of FRT infection with the closely related *Chlamydia muridarum* species offers a way to determine what would be needed for a protective immune response. Researchers using this model have found that CD4 T helper cells are the critical component of the immune response to clear *Chlamydia* infection in the epithelium of the FRT. However, the particular polarization state needed for these CD4 T cells and the mechanism used to orchestrate clearance of infected epithelial cells has not been determined.

Here we show that, contrary to expectations, classical Th1 cells defined by high T-bet transcription factor expression are not required to mediate clearance of *Chlamydia*, as T-bet-deficient mice competently clear infection. Wild-type CD4 T cell responses to *Chlamydia* in this model do not express high levels of T-bet and do not resemble T-bet^{hi} Th1 cells that result from *Salmonella* infection. While the Th1 typical cytokine IFN-γ is produced during infection, it is not required for control of the majority of FRT infection. Instead, we found using bone marrow chimera mice that IFN-γ signaling plays an important role in controlling *Chlamydia* replication outside of the FRT in non-hematopoietic cells to protect mice from morbidity and mortality. Thus, the CD4 T cell response is not characterized by classical T-bet-driven IFN-γ production aimed at stimulating macrophages for killing intracellular bacteria. Instead, it is a Th1-like response in which IFN-γ is still a prominent marker despite low T-bet expression and IFN-γ is not a necessary component of an effective response in the mucosal barrier site of the FRT.

We also tested the involvement of Th2 cells in the FRT using a STAT6-deficient mouse model and determined that Th2 cells are not necessary to mediate clearance of *Chlamydia*. We then tested a requirement for Th17 cells using an RORγt mutant mouse and found that these mice experienced only a one week long delay in clearance, indicating that Th17 cells contribute to the early phase of clearance, but non-Th17 mechanisms swiftly recover the capacity for eliminating *Chlamydia*. Bulk RNA-sequencing and flow cytometry experiments confirmed a significant Th17 shift in T-bet-deficient mice and a high degree of similarity of RORγt mutant mice with wild-type, except for a reduction in some Th17 markers. This showed that either Th1-like or Th17 populations as the major responders were capable of controlling FRT infection.

In testing the potential for Th1 and Th17 pathways to compensate for each other, we eliminated both populations in multiple mouse models to assay for a delay in FRT clearance. In T-bet-deficient mice either given neutralizing anti-IL-6 and anti-TGF-β or T-bet-deficient mice bred to RORγt mutant mice, no significant delay was detected, indicating that clearance mechanisms operate outside of classical Th1 and Th17 specific fate programs. We additionally tested IL-12p40-deficient mice, as this cytokine subunit can promote both Th1 and Th17 associated responses, and found a severe delay in clearance multiple weeks beyond wild-type mice, though these mice do eventually clear infection very late. Data from flow cytometry and a scRNA-seq experiment illustrate a high degree of similarity between wild-type responses dominated by Th1-like CD4 T cells and the responding CD4 T cells in IL-12p40-deficient mice. Analysis of differential gene expression in the CD4 T cells from IL-12p40-deficient mice compared to wild-type and T-bet-deficient mice has revealed a set of genes specifically downregulated in IL-12p40-deficient mice. Further work is needed to determine which of these genes is mediating the clearance of *Chlamydia* from the FRT. These studies have revealed an effector module regulated by IL-12p40 and unrelated to Th1 or Th17 cell fates that is effective against obligate intracellular bacteria in a

mucosal epithelial layer. This provides specific information on what CD4 T cell characteristics will need to be elicited by an effective vaccine against *Chlamydia* reproductive tract infection.

Chapter 1

Introduction

Public Health Background

Chlamydia trachomatis is the most common bacterial sexually transmitted infection (STI), accounting for 1.6 million cases in 2020 in the United States alone [1]. Two other major STIs, *Gonorrhea* and *Syphilis,* together in comparison totaled only half that amount, around 800,000 cases. In addition to an already high case incidence, infection rates have been increasing for years, dipping briefly in 2020 coincident with the beginning of the SARS-CoV-2 pandemic, when screening rates declined. Preliminary data from 2021 indicates that since then, infection rates have begun to increase once again [2]. The bulk of *Chlamydia* infections occurs in adolescents and younger adults, particularly 15-24 year old individuals, who represent two-thirds of total cases [1, 3]. Contributing to the large infection rate is the fact that infections are asymptomatic in the majority of cases, making screening necessary to ensure that all infected individuals can receive treatment and prevent transmission [4]. Treatment is simple and usually consists of antibiotics, though reinfection is unfortunately common [5]. Untreated infections can result in severe pathologies particularly in individuals with a female reproductive tract (FRT). The bacteria initially infect the epithelium of the cervix, but ascend into the upper reproductive tract and cause pelvic inflammatory disease resulting in chronic pain, infertility due to blocked oviducts, and ectopic pregnancies [4].

Chlamydia trachomatis was first isolated in the early 1900s by researchers investigating incidences of trachoma, where scarring on the inside of the eyelid degrades the cornea and can cause blindness [6]. Later in the 1970s, it was also recognized as an STI and linked to reproductive tract pathologies [6]. Vaccine trials for *Chlamydia trachomatis* in the context of ocular infection were started in the 1960s, though found increased pathology in some subjects [7, 8] and other studies observed increased infection rates in the vaccinated group [9]. Together this stalled

vaccination efforts, especially to avoid vaccine enhanced disease. Investigators revisiting these vaccination efforts have since reanalyzed this early work and determined that the risk of increasing pathological outcomes due to vaccination is potentially not significant [10, 11]. Regardless, despite research efforts in the intervening decades, a successful vaccine for *Chlamydia* infection has not been developed, though a recent formulation has completed a Phase I trial [9, 12, 13]. Should this or future efforts prove effective going forward, use of such a vaccine would be vitally important for reducing transmission of infection and preventing the public health consequences discussed above.

Chlamydia **Microbiology**

Chlamydiales is an order of gram-negative bacteria of which the family *Chlamydiaceae* contains *Chlamydia trachomatis* (the pathogen that causes trachoma and reproductive disease in humans), *Chlamydia muridarum* that only infects mice, as well as a number of other species that can infect various animals from other mammals like cows and koalas to birds and reptiles [14]. Chlamydiales are obligate intracellular bacteria wherein two major developmental forms operate at distinct stages of a biphasic developmental cycle. The first, extracellular form are termed elementary bodies (EBs) and are infectious but not replicative. Initially considered a generally inactive state, work in the past two decades has found that EBs are not completely metabolically inert and actually play an active role in invasion of host cells [15]. The second form are reticulate bodies (RBs) that can replicate but are not infectious. EBs differentiate into RBs while inside a host cell and undergo multiple rounds of replication. RBs then differentiate back to EBs, which are released either by extrusion or lysis of the host cell [16].

The replication cycles occur within a specific membrane-bound compartment inside the host epithelial cell established upon infection [17, 18]. This compartment, termed an inclusion, provides a protective niche for *Chlamydia* from within which the bacteria can manipulate the host cell. The inclusion avoids fusion with the lysosome and instead associates with the endoplasmic

reticulum. Key to this manipulation of the host cell is the type 3 secretion system (T3SS), a protein complex that allows for injection of bacterial factors across both the bacterial membranes and through the host membrane [14, 17]. Many effectors translocated by the T3SS are inclusion membrane proteins (Incs), that can modulate host responses. These Incs contribute to recruiting nutrients to the inclusion, interacting with the cytoskeleton to determine positioning within the host cell, modulating fusion events with other membrane-bound compartments, inhibiting apoptotic pathways, and blocking host immune mechanisms [17, 19].

The Mouse Model and *Chlamydia muridarum*

Mouse models can be used to interrogate immune responses to *Chlamydia* infection in the female reproductive tract. The human pathogen *Chlamydia trachomatis* can be used to infect mice, though with some limitations. If infected cervicovaginally as would mimic a natural exposure, *Chlamydia trachomatis* can be cleared without adaptive immune responses, as illustrated in Rag1-/- mice [20]. In order to establish a more robust infection in the upper reproductive tract that requires adaptive immune responses to clear, *Chlamydia trachomatis* must be introduced transcervically directly into the upper reproductive tract [21]. This infection route also induces FRT pathology that results in reduced fertility, as seen in humans [22].

Alternatively, mice can be productively infected intravaginally using the mouse-specific species *Chlamydia muridarum*, referred to in earlier literature as *Chlamydia trachomatis* mouse pneumonitis (MoPn). Intravaginal infection with *Chlamydia muridarum* establishes a robust infection that ascends the upper reproductive tract, requires adaptive immune responses to clear, and induces pathology of the FRT related to pathology seen in human infections [20, 23-26].This species of *Chlamydia* was originally isolated from mice in the 1940s by researcher Clara Nigg [27]. Other than this Nigg strain, multiple strains of this species have been isolated that have varying degrees of virulence [28]. The Weiss strain, for example, has been shown to cause more morbidity and mortality in respiratory infections than the Nigg strain [28]. Our lab uses a derivative

of Weiss that was deposited at ATCC courtesy of Dr. Julius Schacter and is thus likely more virulent than Nigg, which has also been used by other labs in immunology related mouse experiments [28].

One caveat of these models is the requirement for pre-treatment of the mice with medroxyprogesterone acetate, a derivative of progesterone used for contraceptive purposes, one week prior to infecting mice. This step is necessary in order to establish a productive infection [29, 30]. The compound is introduced as a depot injection administered sub-cutaneously and halts the progression of the estrus cycle in diestrus for a prolonged time [31]. A similar effect of increasing susceptibility to *Chlamydia* infection after treatment with this compound has also been documented in humans [32].

Hormonal changes can have dramatic effects on the immune state of the reproductive system over the course of the reproductive cycle, from epithelial cells and fibroblasts to immune cells [31, 33, 34]. This can alter the role of epithelial cells in secreting antimicrobial factors, translocating IgG and IgA antibodies into the lumen of the FRT, and acting as sentinels to infection [35]. The proportions of various adaptive and innate immune cells present can fluctuate over the course of the cycle along with cytokine and chemokine levels [36, 37]. Thus, pre-treatment of experimental mice with medroxyprogesterone acetate changes the context of the FRT to be more permissive to *Chlamydia* infection likely through stabilizing the estrous cycle as well as exerting immunosuppressive effects.

Innate Immune Responses

Chlamydia must be able to evade innate immune mechanisms in order to survive and replicate within host epithelial cells and create a productive infection. The host has an array of pattern recognition receptors (PRRs) that may detect signals generated directly from *Chlamydia* or detect signs of perturbation of host processes. Some of these PRRs are located on the surface of cells or within endosomal compartments. TLR4 recognizes gram-negative bacteria via

lipopolysaccharide (LPS), though *Chlamydia* LPS has been to be significantly weaker at eliciting an inflammatory response than *Salmonella* and other gram-negative bacteria because of unique structural changes in lipid A [38, 39]. TLR4 has also been shown to recognize *Chlamydial* Hsp60, which is linked to pathological inflammatory responses [40, 41]. TLR2, however, seems to play a more important role than TLR4 as a PRR in *Chlamydia* infection. TLR2 has been shown to localize to the inclusion and have a critical role in the maximal release of inflammatory cytokines and resulting pathology [42-45]. Lipoproteins are a major ligand of TLR2, though *Chlamydia* major outer membrane protein (MOMP) has also been shown to be an agonist for this receptor, similar to other bacterial porins [46]. MOMP is abundant on the surface of EBs, making up approximately 60% of the protein component of the outer membrane [15]. TLR3, which senses DNA products located in endosomal compartments, has also been implicated in *Chlamydia* infection in signaling to produce inflammatory cytokines in some cells [47, 48].

Cytosolic PRRs can also detect signals of *Chlamydia* infection. NOD1 may directly sense *Chlamydia*-derived peptidoglycans that escape the inclusion [49]. NOD1/2 can additionally be activated by ER stress caused by *Chlamydia* infection, which promotes pro-inflammatory signaling through NF-κB [50]. Cytosolic DNA released into the cytosol can be detected by cGAS, which activates STING, shown to associate with the inclusion, to induce type I interferon (IFN) [51]. STING may also be activated directly by cyclic di-AMP made by *Chlamydia* [52]. Interestingly, mice deficient in type I IFN receptor (IFNAR-/-) experience a reduced bacterial burden, indicating that *Chlamydia* induction of type I IFN may assist to skew immune responses to a more favorable state for *Chlamydia* replication [53].

Chlamydia must maintain the integrity of the inclusion long enough to produce new EBs, which means preventing fusion with lysosomal or other intracellular compartments and preventing autophagy-mediated destruction of the inclusion. Vesicle trafficking and fusion events are controlled by host RAB GTPases, SNARE proteins, and sorting nexins. *Chlamydia* inclusion proteins interact with these components to preserve the independence of the inclusion [17, 54-

58]. Autophagy can be instigated through tagging of the inclusion via immunity related GTPases (IRGs) and guanylate binding proteins (GBPs), which are inducible by IFN-γ signaling, though *Chlamydia* appears to have mechanisms to avoid the action of these proteins [59-61].

In order to avoid host mechanisms of apoptosis that might terminate the capacity of the host cell to support the developmental cycle, *Chlamydia* can block apoptotic pathways [62-64]. The inclusion protein IncG, for example, has been shown to sequester pro-apoptotic factor BAD to the inclusion to prevent its action [65]. *Chlamydia* can also promote anti-apoptotic factors and block caspase-8 function [17]. Taken together, it is clear that *Chlamydia* has developed strategies for evading innate immune responses that would allow destruction of the bacteria within host cells or the elimination of infected host cells entirely.

Once *Chlamydia* has infected epithelial cells in the FRT, innate immune cells have the chance to affect the course of the infection. Cells capable of antigen presentation are critical for eliciting an adaptive immune response. Tissue macrophages, monocytes, and dendritic cells can all be important for this process. Multiple macrophage and monocyte cell lines have been shown to allow *Chlamydia* infection and replication, though to a limited degree, and macrophages polarized towards a killing M1 phenotype are better able to restrict growth [66-68]. In macrophages that were able to eliminate *Chlamydia*, cathepsin B, reactive oxygen species (ROS), and inducible nitric oxide synthase (iNOS) were all critical factors [69]. Despite this, mice deficient in iNOS have not shown an inability to clear FRT infection and thus this mechanism is not a major determinant of the course of FRT infection [70, 71]. *Chlamydia* can also infect dendritic cells, though may enter a latent persistence state instead of productively replicating, and these infected dendritic cells can still activate CD4 T cells [66, 72]. Dendritic cells that encounter EBs become activated, upregulating MHC class II and costimulatory molecules CD80, CD86, CD40, and ICAM-1, as well as the proinflammatory cytokine IL-12 [73].

The role of neutrophils in *Chlamydia* clearance has been difficult to determine. Neutrophil depletion using α-Ly6G has been reported in one study to increase early *Chlamydia* burden,

though mice did eventually clear the bacteria [74]. In another study, depletion had no effect on clearance and increased pathology, though immature neutrophils were still present [75]. Further research is required to determine the extent of neutrophil involvement in the early phase of infection. NK cells and ILC1s also respond early in infection to make IFN-γ [76, 77]. These cells are important for restricting *Chlamydia* dissemination from the FRT to other organs during this early phase, as Rag1-/- mice given depleting α-NK1.1 or α-IFN-γ and Rag2-/-γc-/- mice lacking ILCs all experienced accelerated death rates, though clearance in the FRT itself does not depend on this response [76].

Adaptive Immune Responses

As mentioned earlier, adaptive immune responses are critical for controlling *Chlamydia* infection. Rag1-/- mice that lack the ability to form B or T cell responses cannot clear *C. muridarum* infection [20]. Within the organized structures of secondary lymphoid organs, these cells encounter *Chlamydia*-derived antigens and an inflammatory context created by activated innate immune cells and are activated in turn [78-80]. They can then in an antigen-specific manner contribute to bacterial clearance through various mechanisms.

Because the primary site of *Chlamydia* replication is within FRT mucosal epithelial cells, one might expect cytotoxic programs to play a critical role in eliminating infected epithelial cells, similar to what is seen in enteric infections of the intestinal epithelium like rotavirus [81, 82]. Intraepithelial lymphocytes (IELs) are well studied in the gut, another mucosal surface with a simple columnar epithelium that encounters many microbes. At this site, IELs patrol the epithelial layer and recognize various classical and non-classical MHC presenting antigen. IELs comprise a few different populations, including γδ T cells, αβ T cells negative for CD4 and CD8, as well as CD4+ and CD8+ single positive αβ T cells [81, 83]. These IELs in proximity with epithelial cells can patrol for infected cells and enact cytotoxic programs as well as release cytokine signals [84].

IELs have also been found within the murine female reproductive tract [85]. However, previous work has shown that β_2-microglobulin-deficient mice that cannot form MHC class I nor some non-classical MHC and thus cannot mediate cytotoxic CD8+ T cell responses and other IEL responses are not delayed in primary clearance of *Chlamydia* from the FRT [86]. Additionally, $\gamma\delta$ T cells were ruled out due to competent clearance in mice lacking the TCR δ-chain [87]. Despite this, cytotoxicity remains a conceptually attractive method for T cells in proximity to infected epithelial cells to control infection. The major cytotoxic pathways of Fas/FasL and perforin have also been shown to not be critical for clearance in gene deficient mice [88]. Other cytotoxic pathways are still possible, but require further investigation, though as mentioned above *Chlamydia* may produce factors that aid escape of pathways to induce apoptosis.

The development of antibody responses by B cells could be helpful in neutralizing *Chlamydia* EBs to prevent infection of other host cells as well as aid in complement-mediated or phagocyte-mediated destruction of the bacteria. However, B cells have been shown to be dispensable for primary clearance, as B cell-deficient μMT mice clear FRT infection normally [89-91]. Interestingly, Li et. al. observed a role of B cells in preventing systemic dissemination of *Chlamydia* outside of the FRT during primary infection [90]. In the context of secondary infection, μMT mice do show a deficiency in clearance [91], indicating a much stronger role for B cells during the memory phase. This has been attributed to IgG opsonization allowing for neutrophil-mediated elimination of bacteria [92].

In contrast to cytotoxic CD8 T cells and B cells, CD4 T helper cells are critical for clearance of primary infection from the FRT, as shown by the inability of MHC class II-deficient mice that cannot mediate CD4 T cell responses to clear *Chlamydia* [86]. CD4 T cells provide help to other immune cells to activate various effector modules specialized towards different functionalities and classes of pathogens [93-95]. Activated dendritic cells that have received innate signals from these pathogens can activate naive CD4 T cells and skew their differentiation towards these

specific fate programs. Major CD4 Th subsets are Th1, Th2, Th17, follicular helper T cells (Tfh), and regulatory T cells (Treg). Th1 cells classically express the master transcription factor T-bet and make IFN-γ to activate macrophages to increase their capacity to kill intracellular bacteria [96, 97]. Th2 cells likewise express GATA3 and make IL-4, IL-5, and IL-13 to activate eosinophils and control helminth infections [98-100]. Th17 cells are defined by RORγt expression and make IL-17 and IL-22 to activate neutrophils to phagocytose and kill extracellular bacteria and fungi, as well as support mucosal barrier function [101, 102]. Tfh express Bcl-6 and make IL-21 to support germinal centers and the development of antibody responses by B cells [103-106]. Treg are defined by Foxp3 expression and make IL-10 to dampen immune responses [107-109].

In *Chlamydia muridarum* FRT infection, evidence from experiments in the 1990s pointed towards Th1 cells being the critical Th subset involved in primary clearance, consistent with the general rule that Th1 cells target intracellular bacteria. Depleting IL-12, which promotes Th1 differentiation, *in vivo* led to a significant delay in FRT shedding, though mice eventually cleared bacteria around day 50 [87]. The α-IL-12 clone used in this experiment was specific for IL-12p40, which is shared between IL-12 and IL-23. While IL-12 promotes Th1 cells, IL-23 promotes the expansion of Th17 cells. This experiment was done prior to the recognition of Th17 cells and thus whether this effect was due to Th1 or Th17 cells was not investigated at this time. In addition to this piece of evidence, the Th1 cytokine IFN-γ was shown to be important for full clearance of FRT infection, as IFN-γ-/- mice maintained a low level of chronic infection after a couple weeks [87, 110]. At the same time, these mice showed evidence of dissemination to organs outside of the FRT and exhibited signs of sickness, some requiring euthanasia due to severe illness [87, 110]. This demonstrates the critical importance of IFN-γ to preserve the host. However, both of these early papers also clearly show that IFN-γ-/- mice clear the majority of FRT infection in the first weeks of infection like wild-type mice. It is only at later time points that these mice experience dissemination coincident with the development of morbidity. Nevertheless, IFN-γ is a major

cytokine produced during *Chlamydia* FRT infection both in mice and in humans [90, 111-113]. Since these early experiments, further evidence has emerged showing that IFN-γ-deficient CD4 T cells are sufficient to control FRT infection while innate sources of IFN-γ are important for controlling systemic dissemination [76].

In the mouse model of infection with the human-specific species *Chlamydia trachomatis,* IFN-γ seems to play a more important role for FRT clearance than the above experiments with *Chlamydia muridarum*. Bacterial shedding is significantly more delayed when IFN-γ-/- mice are infected with *Chlamydia trachomatis* than *Chlamydia muridarum* [114]. Additionally, Helble et. al. found in this model that IFN-γ production by CD4 T cells did contribute to FRT clearance [115]. Notably, *Chlamydia trachomatis* and *Chlamydia muridarum* differ in their sensitivity to specific IFN-γ-mediated mechanisms of clearance depending on the species of the host cell. *Chlamydia muridarum* is sensitive to IFN-γ-mediated upregulation of indoleamine dioxygenase (IDO) that restricts the availability of tryptophan, though this pathway is not upregulated in mice [116, 117]. *Chlamydia trachomatis* has a tryptophan operon to synthesize tryptophan from indole when this nutrient is limited upon IDO induction in human hosts [117, 118]. Instead of limiting tryptophan, the mouse host can upregulate IRGs and GBPs as described above. These proteins utilize ubiquitin tagging to direct vacuoles containing pathogens within a cell for destruction via autophagolysosomes [119]. When infecting mouse host cells, *Chlamydia muridarum* can evade the action of these proteins while *Chlamydia trachomatis* cannot [59, 61, 120]. Therefore, each of these *Chlamydia* species *trachomatis* and *muridarum* are host adapted to humans and mice respectively and specialized to evade IFN-γ induced immune responses in their natural host, explaining the differential susceptibility to IFN-γ observed in mouse experiments.

Evidence for the involvement of Th2 cells and their cytokines in clearance is conflicting. Some researchers have associated Il-4, eosinophils, and IL-13 with clearance, and Th2 responses have been found in human patients [121-123]. On the other hand, other research has

found that IL-13 is associated with susceptibility to infection and GATA3 expression is low in the CD4 T cells in infected mice [90, 124]. Further experiments are required to determine how critical Th2 pathways are in *Chlamydia* clearance.

The involvement of some Th17 associated cytokines has been tested in mouse models. Mice deficient in Il-23p19, a subunit of Il-23 that promotes Th17 development, showed no delay in shedding kinetics [125]. Likewise, deficiency in the Th17 effector cytokine Il-22 and the receptor for IL-17 also did not result in a delay in clearance [125, 126]. In flow cytometry experiments, a small population of Th17 cells can be found in the FRT, represented by RORγt+ cells [90]. However, it is unclear if these cells play a significant role, particularly outside of the Th17 cytokines tested so far.

This project aims to fully characterize which CD4 Th subsets are present during *Chlamydia* primary infection in the FRT and determine which are necessary to mediate clearance. Here, we more robustly test the participation of these CD4 Th subsets, with a particular focus on the transcription factors that define them. Conceptually, it is unclear how *Chlamydia* infection fits into the Th1/2/17 paradigm. Th1 cells are known to target intracellular bacteria through activating macrophages, and while *Chlamydia* is an obligate intracellular bacterium, it primarily replicates in epithelial cells instead of macrophages. Th17 cells are important in controlling extracellular bacteria and maintaining the mucosal barrier, especially seen in the context of the gastrointestinal tract. *Chlamydial* EBs are released extracellularly and therefore may be targeted by Th17 effectors, but it is unclear how Th17 cells may target *Chlamydia* within epithelial cells. Is it important to delineate how CD4 T cell responses to *Chlamydia* do or do not fit into this paradigm and which mechanisms contribute to clearance.

How CD4 T cells mechanistically support secondary infection clearance is still unclear, though a role in T-dependent antibody production likely contributes, as mentioned above. The requirement for memory CD4 T cells that are circulating versus resident in the FRT has also been explored. Memory lymphocyte clusters including CD4 T cells have been observed to form in the

FRT after primary infection [127]. Tissue resident cells (Trm) have the potential to provide an early antigen-specific response to further challenge from pathogens at barrier sites and may be important components of secondary immunity at mucosal sites [128]. However, work from Labuda et. al has established that in this mouse model of *Chlamydia* infection, circulating memory CD4 T cells resulting from intranasal infection, which does not generate Trm in the FRT, are sufficient to provide protection in secondary challenge [129]. The work from this project in primary infection will provide a starting point to investigate how circulating CD4 T cells can mechanistically aid clearance of *Chlamydia* secondary FRT infection. This in turn will inform vaccine development by providing target markers to elicit from CD4 T cells for effective clearance.

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Chapter 2

Th1 cells are dispensable for primary clearance of *Chlamydia* from the female reproductive tract of mice

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Introduction

Chlamydia trachomatis is a gram-negative bacterium with an atypical obligate intracellular developmental cycle wherein replication occurs primarily in epithelial cells of the urogenital tract [1,2]. This chronic bacterial infection can initiate marked pathology at this mucosal surface and eventually lead to severe reproductive problems in otherwise healthy individuals [3]. In 2018, over 1.7M cases of *Chlamydia* infection were reported to the Centers for Disease Control and Prevention, with almost two-thirds of these cases found in young adults (ages 15-24 years) [4]. *Chlamydia* infections are often asymptomatic and are therefore difficult to detect and treat as part of a public health approach to reducing disease incidence [4]. Untreated *Chlamydia* infection can often lead to pelvic inflammatory disease, ectopic pregnancy, and sterility [5,6]. Given the high incidence and impact of this disease on the US population, development of a *Chlamydia* vaccine is now a public health priority [7].

Initial attempts at developing human *Chlamydia* vaccines were derailed by the detection of increased pathology in some vaccinated individuals, impeding additional human trials of vaccine candidates [8]. Despite the fact that these problems occurred almost 60 years ago, an

effective *Chlamydia* vaccine remains elusive today [7,8]. Although much has been learned about *Chlamydia* pathogenesis and host immunity during this time [5,9-11], deeper understanding of the mechanisms of immune clearance from the reproductive tract are needed to assist vaccine development. Central to these efforts is the use of mouse models of *Chlamydia* infection which recapitulate many of the central features of human disease [10]. In one common model, inbred mice are infected with *C. muridarum*, a mouse pathogen that is delivered vaginally and naturally ascends the reproductive tract to cause significant pathology [10]. This experimental approach in mice recapitulates key features of human disease and has been widely used to study the immune response to *Chlamydia* infection [3,10].

Foundational work from several laboratories has determined that CD4 T cells are the critical lymphocyte population that orchestrate clearance of *Chlamydia* infection from the female reproductive tract [12-14]. Severe Combined Immune Deficient (SCID)-, RAG-deficient-, TCR-α-, and MHC class-II-deficient mice all fail to resolve a primary vaginal infection with *C. muridarum* [13,15,16]. Thus, functioning MHC class-II restricted CD4 T cell responses are the primary requirement for bacterial clearance. In marked contrast, gene-deficient mice lacking MHC class-I or B cells resolve primary *Chlamydia* infection [13,17], although systemic infection has been detected in B cell-deficient mice before clearance occurs [18,19].

The central role of CD4 T cells in combating any microbial infection relies on the capacity to develop “helper” activity, where they assist macrophages, neutrophils, B cells, CD8 T cells, or mast cells/eosinophils to kill particular classes of pathogen [20]. After infection, naïve CD4 T cells mature into Th1, Th2, Th17, Tfh, or Treg lineages that can help or impede other immune cells to kill microbes. In the case of *Chlamydia* infection, it is widely believed that CD4 Th1 cells are the major effector lineage that orchestrates bacterial clearance from the reproductive tract [10,11,21-23]. Th1 cells are often required to coordinate the immune response against intracellular pathogens via their capacity to activate macrophages through secretion of IFN-γ [24]. Th1 cells

are defined by their expression of the T-box transcription factor TBX21 (T-bet), which controls expression of IFN-γ and related genes [25]. In the *C. muridarum* mouse model, IFN-γ has been demonstrated to play a vital role in host control of infection [26,27]. Additionally, several human studies have correlated the capacity to generate an IFN-γ responses during *Chlamydia trachomatis* infection with a lower risk of reinfection [28,29]. Together, these animal and human data provide a simple model for understanding *Chlamydia* clearance in human and murine infection, wherein Th1 cell production of IFN-γ allows the control of bacterial growth within the FRT. Despite the attraction of this Th1 cell-centric view, there are some obvious caveats to the model as it pertains to *Chlamydia* immunity.

First, published studies have reported uncontrolled growth of certain intracellular pathogens in T-bet-deficient mice in the absence of Th1 cells [30-32], but this has not yet been studied in the *Chlamydia* model. Second, looking closely at published data, mice lacking IFN-γ do not always exhibit the same level of severe deficiency observed in MHC-class-II-deficient mice [26,27,33]. Indeed, *Chlamydia* shedding from the FRT is often reduced by multiple logs from peak infection in these mice, suggesting that IFN-γ-independent mechanisms play a major role in bacterial clearance. Third, a number of recent reports have uncovered a potential role for non-Th1 cells in *Chlamydia* immunity, or in tissue repair after infection, including Type-II immune cells [34,35], Th17 cells [36,37], and CD4 T cells secreting IL-13 [38]. Furthermore, IFN-γ can also be secreted by some of these non-Th1 (T-bet-negative) CD4 T cell populations [39]. It is therefore vitally important to clearly define the contribution of CD4 T-bet$^+$ Th1 cells to *Chlamydia* clearance in the mouse model.

In this study, we examined the role of IFN-γ versus T-bet in reproductive tract immunity against *Chlamydia muridarum.* Contrary to expectations, T-bet expression was low and transient in all CD4 T cells within the reproductive tract and draining iliac lymph nodes of *Chlamydia*-infected mice, despite the fact that high levels of IFN-γ production were detected. Furthermore,

loss of T-bet expression in CD4 T cells, or in other host cells, did not completely eliminate IFN-γ production or impede the ability of mice to clear *Chlamydia* infection from the FRT. Interestingly, CD4 T cells in T-bet-deficient mice displayed a profound shift towards Th17 responses, suggesting compensation between these two CD4 effector subsets in the control of *Chlamydia* infection. Despite the lack of an essential requirement for Th1 cells, mouse models deficient in IFN-γ or IFN-γR displayed fatal systemic infection. Additionally, T-bet-deficient mice administered depleting anti-IFN-γ also experienced bacterial dissemination and mortality, indicating that IFN-γ is essential for resolving systemic infection, regardless of which effector mechanisms are operational in the FRT. Together, these data highlight the importance of non-Th1 cell secretion of IFN-γ outside the FRT to control systemic disease and suggest the potential for compensatory CD4 effector responses for bacterial clearance within the FRT.

Results

Chlamydia infection of the FRT induces IFN-γ-producing CD4 T cells

Previous work has established that IFN-γ is a common component of the CD4 T cell response to *Chlamydia* infection of the FRT [10]. We initially sought to confirm this finding by examining the production of IFN-γ by CD4 T cells in C57BL/6 mice infected vaginally with *C. muridarum*. CD4 T cells recovered from the FRT at day 14 post vaginal infection were assessed by flow cytometry following brief PMA/ionomycin stimulation in the presence of Brefeldin A. As expected, CD4 T cells in the FRT of *Chlamydia*-infected mice produced significant amounts of IFN-γ, but negligible IL-4 (Fig. 2.1A and B). Indeed, CD4 T cell production of IFN-γ to both *Chlamydia* and *Salmonella* infection was similar (Fig. 2.1A and B), reinforcing the concept that both of these bacterial infections drive strong Th1 responses. This was reflected in increased percentages and total numbers of IFN-γ+ IL-4- activated CD4 T cells, as well as increased staining intensity for IFN-γ, while staining for IL-4 was minimal. Additionally, at 15 days post infection, CD4 T cells isolated from the FRT of *Chlamydia*-infected mice produced significant levels of IFN-γ after ex vivo stimulation with heat-killed *C. muridarum* elementary bodies (HKEBs) (Fig. 2.1C). Thus, vaginal *Chlamydia* infection drives robust CD4 IFN-γ production within the FRT.

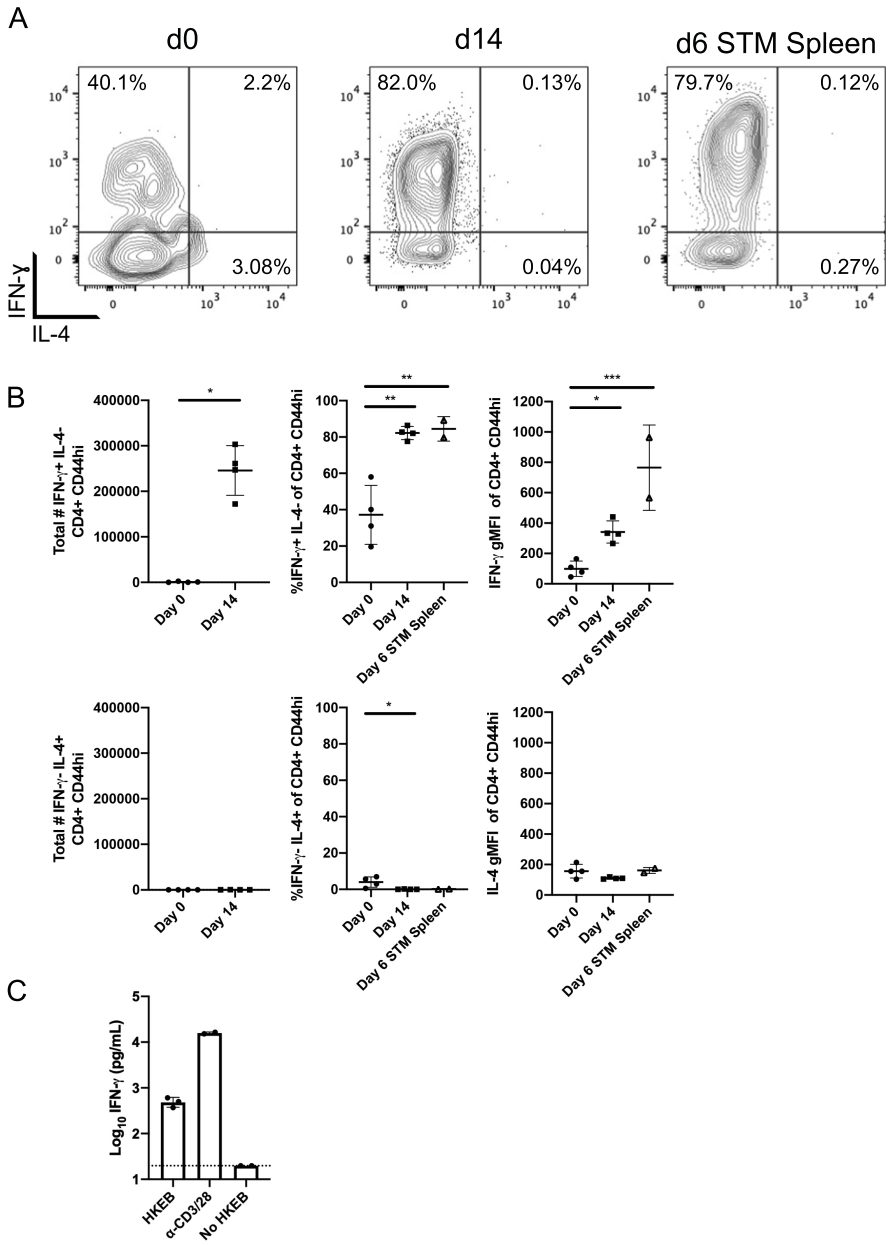

Figure 2.1: CD4 T cells respond during primary FRT infection in wild-type mice and have a strong IFN- ɣ signature

Mice either received 2.5mg Depo-Provera 7 days before infection with $1x10^5$ IFU *Chlamydia muridarum* i.vag. or $5x10^5$ CFU *Salmonella* i.v. Lymphocytes were isolated from the FRT on day 14 post infection for *Chlamydia*-infected mice or from the spleen day 6 post *Salmonella* infection and stimulated with PMA/ionomycin in the presence of Brefeldin A before staining for flow

cytometry. n=4 for naïve and *Chlamydia* groups, n=2 for *Salmonella* group. (A) Example flow cytometry plots of IFN-ɣ and IL-4 expression gated on CD4+ $CD44^{hi}$ cells. (B) Summary of flow analysis. (C) IFN-ɣ ELISA results for CD4 T cells isolated from the FRT of *Chalmydia* infected mice 15 days post infection and stimulated with HKEBs (Heat-Killed Elementary Bodies), controls stimulated with α-CD3 and α-CD28, or controls that did not receive HKEBs. n=3 for HKEB stimulation and n=2 for control groups due to low cell recovery from the third FRT. Results (B and C) are shown as mean +/- SD.

Most IFN-ɣ-producing CD4 T cells responding to Chlamydia lack T-bet expression

Since Th1 cells are defined by the expression of the master transcription factor T-bet (*Tbx21*) [25], we hypothesized that IFN-γ-producing CD4 T cells responding to *Chlamydia* infection would express T-bet. Activated $CD44^{+}$ CD4 T cells initially expanded in the ILN and could be detected infiltrating the FRT during the first week of infection (Fig. 2.2A). As the bacteria were cleared from the FRT during the second and third week of infection, the number of these responding CD4 T cell declined (Fig. 2.2A). In the ILN, CD4 T cell production of IFN-γ peaked at day 7 post-infection, while the peak of IFN-γ production in the FRT occurred later, at day 14 (Fig. 2.2B and C). Again, the magnitude of this CD4 IFN-γ response approximated the response of CD4 Th1 cells in *Salmonella*-infected mice (Fig. 2.2B). However, IFN-γ-producing CD4 T cells in *Chlamydia*-infected mice expressed low levels of T-bet at all time points in the ILN, with only a transient signal detected at day 7 during the peak of the IFN-γ response (Fig. 2.2B and D). More strikingly, in the FRT, IFN-γ-producing CD4+ T cells exhibited little to no T-bet expression over the full course of primary clearance of bacteria from the tissue (Fig. 2.2B and D). In marked contrast, robust T-bet expression was detected in IFN-γ-producing CD4 T cells from *Salmonella*-infected mice (Fig. 2.2B). Together, these data suggest that CD4 T cell IFN-γ production during

Chlamydia infection is largely T-bet-independent and contrasts sharply with the response to *Salmonella* infection.

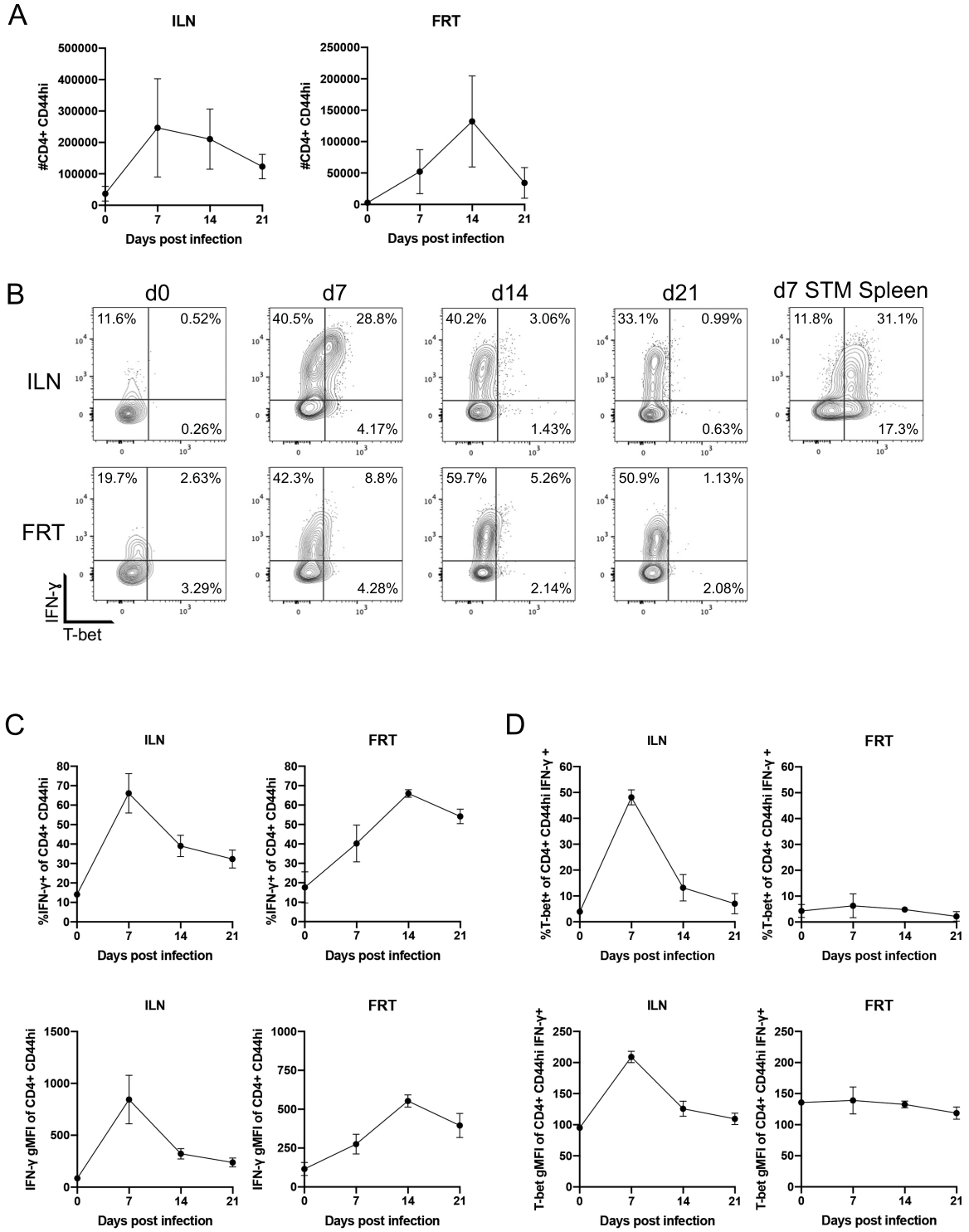

Figure 2.2: IFN-ɣ Producing Th1 cells in the FRT and draining lymph node during primary infection do not express high levels of T-bet

Mice received 2.5mg Depo-Provera 7 days before infection with $1x10^5$ IFU *Chlamydia muridarum* i.vag. or $5x10^5$ CFU *Salmonella* i.v. Lymphocytes were isolated from the FRT or ILN for *Chlamydia*-infected mice the spleen for *Salmonella* infection and stimulated with PMA/ionomycin in the presence of Brefeldin A before staining for flow cytometry. n=3 for each group. Infections were staggered so all time points were processed on the same day. (A) Total number of CD4+CD44^{hi} cells in the ILN and FRT. (B) IFN-ɣ and T-bet expression in CD4+CD44^{hi} cells. (C) IFN-ɣ production of activated CD4s and geometric MFI of IFN-ɣ expression. (D) T-bet expression in IFN-ɣ+CD4+CD44hi cells and geometric MFI. Data is representative of two experiments. Results are shown as mean ± SD.

Lack of T-bet expression does not impair resolution of primary Chlamydia infection

Given the virtual absence of T-bet expression among *Chlamydia*-specific CD4 T cells within the FRT of infected mice, we explored whether T-bet expression was required to resolve *Chlamydia* infection. We initially utilized a Cre-loxP system to selectively delete T-bet in CD4 T cells by crossing CD4-Cre mice to a *Tbx21* floxed mouse line. Consistent with the absence of CD4 T-bet expression in the FRT (Fig. 2.2), CD4-Cre *Tbx21*f/f mice resolved *Chlamydia* infection with similar kinetics to Cre-negative littermate controls (Fig. 2.3A). The proportion of mice shedding bacteria at any given point during *Chlamydia* infection was also comparable between CD4-Cre *Tbx21*f/f mice and littermate controls (Fig. 2.3B). These data conclusively demonstrate that Th1 cells are not required to resolve primary infection with *C. muridarum*. Since T-bet is also expressed by other immune cell lineages, such as ILC1 and NK cells [39], we explored whether T-bet expression in any cell population is necessary for resolving *Chlamydia* infection. Again, mice deficient for T-bet expression in all cell types resolved FRT *Chlamydia* infection, while mice lacking MHC class-II-restricted T cells were severely deficient in clearing bacteria (Fig. 2.3C). This

efficient FRT clearance was evident whether monitoring bacterial shedding or the proportion of mice shedding bacteria at any given point (Fig. 2.3D). Consistent with previous reports [40,41], these same T-bet deficient mouse lines displayed a marked deficiency in resolving *Salmonella* infection and had an obvious reduction in IFN-γ production (Suppl. Fig. 2.1). These data uncover an important difference between *Chlamydia* and *Salmonella* infection models. T-bet and Th1 cells are required to resolve primary *Salmonella* infection while T-bet expression is not necessary for primary clearance of *C. muridarum* infection from the FRT.

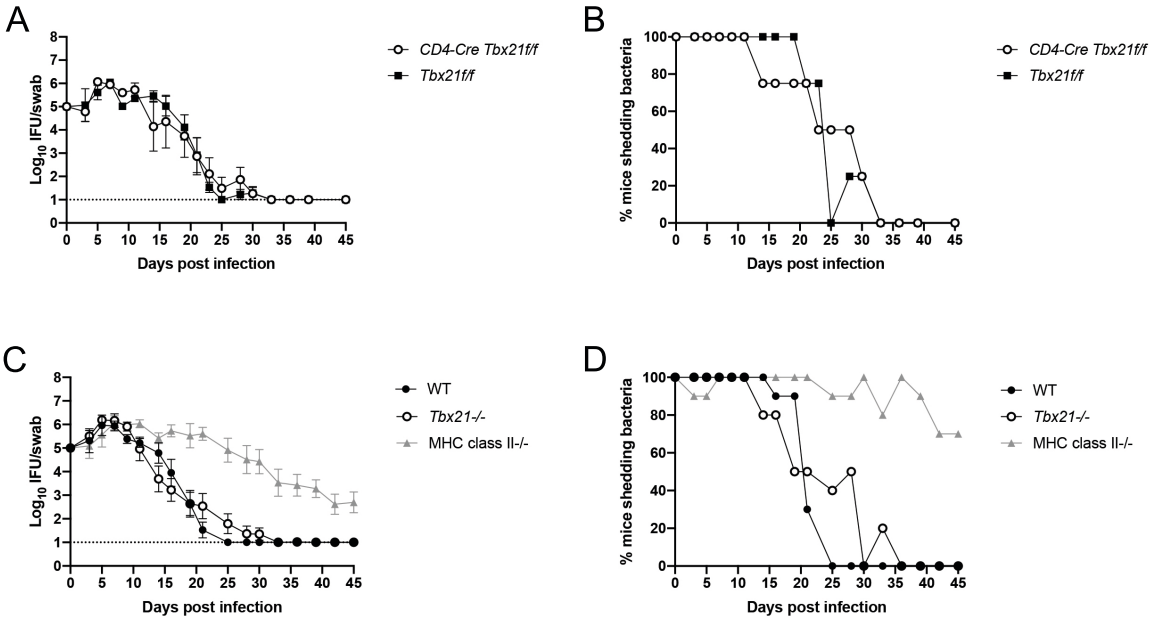

Figure 2.3: Expression of the Th1 master transcription factor T-bet is not required for clearance from the reproductive tract

Mice received 2.5mg Depo-Provera 7 days before infection with $1x10^5$ IFU *Chlamydia muridarum* i.vag. (A) IFU per vaginal swab over time. n=4 for both groups. (B) Percent of mice from A with culture-positive swabs. (C) IFU per vaginal swab over time. Results are from two pooled experiments with a total of 10 mice per group. (D) Percent of mice from C with culture-positive swabs. (A) and (C) are shown as mean ± SEM

Chlamydia-infected T-bet deficient mice retain IFN-ɣ-producing CD4 T cells but display enhanced Th17 responses

Given the capacity of T-bet-deficient mice to efficiently resolve C*hlamydia* infection, we examined the effector response of CD4 T cells in these mice in more detail. Although CD4 T cells from T-bet-deficient mice displayed reduced capacity for IFN-γ production compared to wild-type mice, IFN-γ production was retained in 20-40% of CD4 T cells (Fig. 2.4A and B). A similar reduction in IFN-γ producing capacity was observed in CD8 T cells (Suppl. Fig. 2.2). While wild-type mice had barely detectable Th17 responses (Fig. 2.4A and B), CD4 T cells in *Chlamydia*-infected T-bet-deficient mice displayed high levels of RORγt and IL-17A, comprising 60-70% of all CD4 T cells (Fig. 2.4A and B). This marked shift towards Th17 development in T-bet deficient mice was confirmed by bulk RNA-sequencing comparing sorted CD4 T cells from the FRT of wild-type and T-bet-deficient mice. T-bet-deficient samples exhibited significant downregulation of RNA for Th1 markers compared to wild-type mice (Table 2.1 and Suppl. Table 2.1). Additionally, notable upregulation of Th17 markers *Il17a* and *Il23r* was detected, although RORγt (gene name *Rorc*) was not significantly altered (Table 2.1 and Suppl. Table 2.1). This compensatory Th17 response is reminiscent of IFN-γ-deficient mice where a previous report documented increased Th17 activity and corresponding tissue damage [37]. To determine if the Th17 shift in T-bet deficient mice was a pathological response, we analyzed the FRTs of mice at day 61 post-infection for signs of acute or chronic inflammation, fibrosis, erosion or ulceration, and luminal dilation. Surprisingly, the composite lesion scores and histology of T-bet deficient mice did not significantly differ from wild-type mice (Fig. 2.4C and D). In summary, CD4 T cells in *Chlamydia*-infected T-bet deficient mice show a perturbed immune response with marked inhibition of IFN-γ responses and a prominent shift toward a dominant Th17 response, however this did not increase FRT pathology during primary infection.

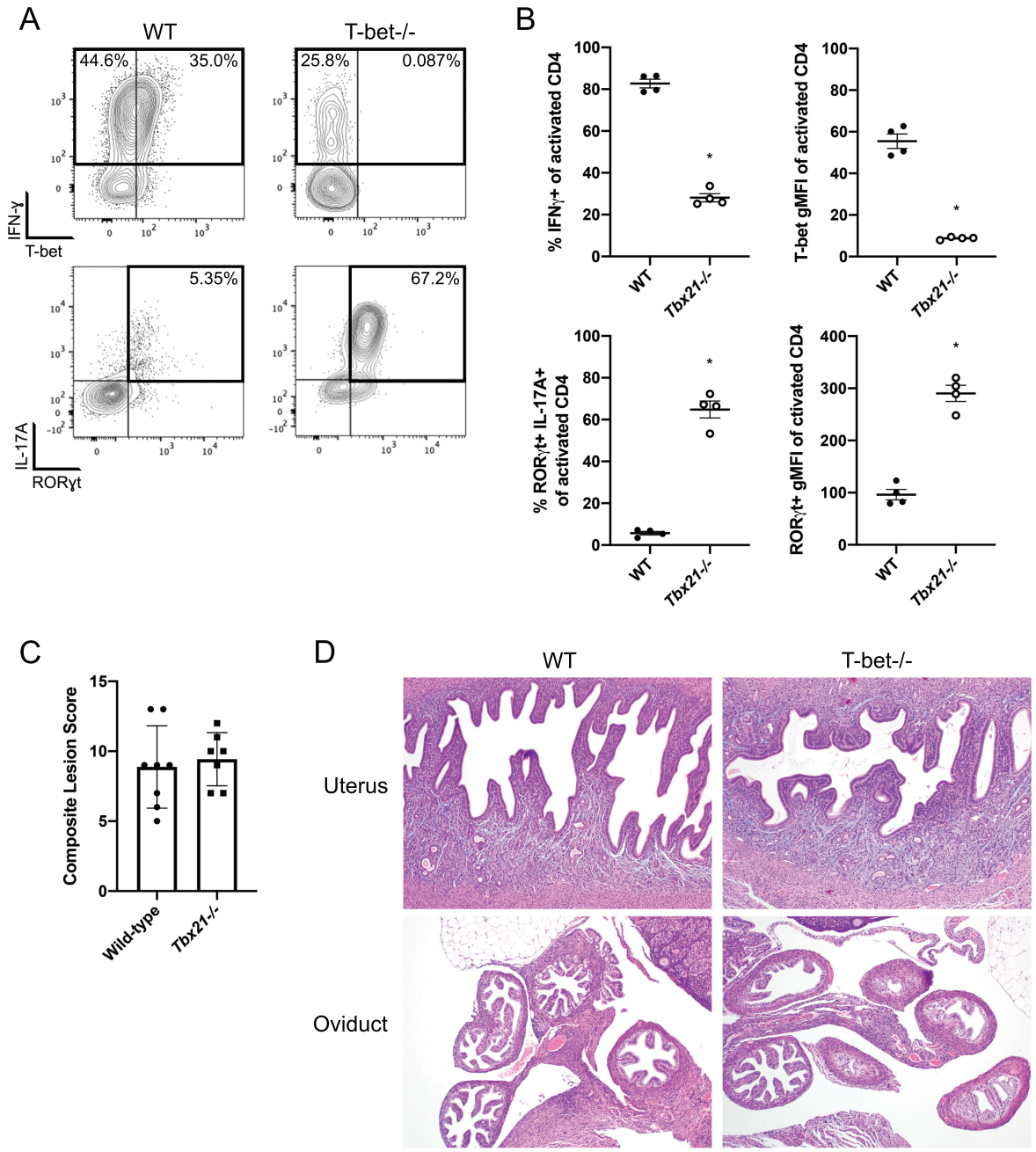

Figure 2.4: T-bet deficient mice make T-bet independent IFN-ɣ and shift towards Th17 responses

Mice received 2.5mg Depo-Provera 7 days before infection with 1x10^5 IFU *Chlamydia muridarum* i.vag. (A) 17 days post infection lymphocytes were isolated and stimulated with PMA/ionomycin and Brefeldin A and stained for flow cytometry. n=4 for both groups. Plots are gated on CD44^{hi}

CD62L^{lo} CD4 T cells. Results are representative of two experiments. (B) Summary plots from (A). (C) Summary of composite lesion scores for wild-type and *Tbx21-/-* FRT tissue sections on day 61 post infection. Graph comprises two experiments, total n=8 for both groups though one mouse from *Tbx21-/-* group was excluded due to suspected imperforate vagina and non-productive infection. p=0.549, Mann-Whitney test. (D) Example histology slides from part (C).

Table 2.1: Selected gene expression analysis of *Tbx21*-deficient CD4+ T cells compared to wild-type. CD4+ T cells were FACS sorted from FRTs 17 days post infection and processed for bulk RNA sequencing. Table shows differential gene expression analysis of selected markers for Th1, Th2 and Th17 responses in *Tbx21-/-* samples compared to wild-type. p<0.05 are shown in bold. Average expression is across all samples in log2 counts per million reads. Adjusted p-value is adjusted with the Benjamini-Hochberg false discovery rate.

Gene name	Log2 Fold Change	Average Expression	Adjusted p Value	Gene description
Th1-associated				
Tbx21	-3.79	5.67	**2.79E-07**	T-box 21 [Source:MGI Symbol;Acc:MGI:1888984]
Stat1	1.04	8.31	**0.0006**	signal transducer and activator of transcription 1 [Source:MGI Symbol;Acc:MGI:103063]
Stat4	-0.42	7.06	0.2952	signal transducer and activator of transcription 4 [Source:MGI Symbol;Acc:MGI:103062]
Ifng	-1.91	8.32	**4.28E-07**	interferon gamma [Source:MGI Symbol;Acc:MGI:107656]
Ifngr2	2.96	2.81	**0.0036**	interferon gamma receptor 2 [Source:MGI Symbol;Acc:MGI:107654]
Il12rb2	-4.89	6.72	**1.12E-10**	interleukin 12 receptor, beta 2 [Source:MGI Symbol;Acc:MGI:1270861]
Ccr5	-3.63	6.14	**1.07E-06**	chemokine (C-C motif) receptor 5 [Source:MGI Symbol;Acc:MGI:107182]
Cxcr3	-6.72	5.09	**5.07E-08**	chemokine (C-X-C motif) receptor 3 [Source:MGI Symbol;Acc:MGI:1277207]
Th17-associated				
Rorc	3.1	0.59	0.1547	RAR-related orphan receptor gamma [Source:MGI Symbol;Acc:MGI:104856]
Stat3	0.05	9.53	0.9387	signal transducer and activator of transcription 3 [Source:MGI Symbol;Acc:MGI:103038]
Il17a	5.48	5.43	**0.0102**	interleukin 17A [Source:MGI Symbol;Acc:MGI:107364]
Il22	0.59	2.58	0.8698	interleukin 22 [Source:MGI Symbol;Acc:MGI:1355307]
Il23r	2.54	3.13	**0.0488**	interleukin 23 receptor [Source:MGI Symbol;Acc:MGI:2181693]
Th2-associated				
Gata3	0.41	6.99	0.4171	GATA binding protein 3 [Source:MGI Symbol;Acc:MGI:95663]
Il5	3.97	-0.7	0.1088	interleukin 5 [Source:MGI Symbol;Acc:MGI:96557]

Il13	2.91	0.03	0.2837	interleukin 13 [Source:MGI Symbol;Acc:MGI:96541]

IFN-γ signaling is required to prevent fatal dissemination of Chlamydia

Our data show that non-Th1 CD4 T cells secrete IFN-γ in the FRT during *Chlamydia* infection (Fig. 2.2B and D). In order to understand the contribution of these CD4 T cells and other non-CD4 sources of IFN-γ during *Chlamydia* infection, we infected IFN-γ- and IFN-γR1-deficient mice vaginally with *C. muridarum*. In accordance with a key role for IFN-γ signaling in *Chlamydia* defense, the mortality rate for both gene-deficient mouse lines was 100%, with median survival of 30 days for IFN-γ-, and 28 days for IFN-γR1-deficient mice (Fig. 2.5A). Interestingly, FRT shedding was similar between wild-type, IFN-γ-, and IFN-γR1-deficient mice during the first 2 weeks of infection (Fig. 2.5B). Indeed, initial FRT infection was reduced by at least 2 orders of magnitude in the complete absence of IFN-γ signaling (Fig. 2.5B). However, after this initial period, FRT shedding plateaued in the absence of IFN-γ signaling, coincident with bacterial dissemination to systemic tissues (Figure 2.5C). Thus, despite the ability of mice to control *Chlamydia* infection for 2 weeks in in the FRT without Th1 cells or IFN-γ, IFN-γ signaling remains an essential component of *Chlamydia* immunity in this mouse model. However, the pattern of infection observed in these studies would suggest that the predominant role of IFN-γ is to prevent bacterial dissemination and death, rather than resolve local infection within the FRT.

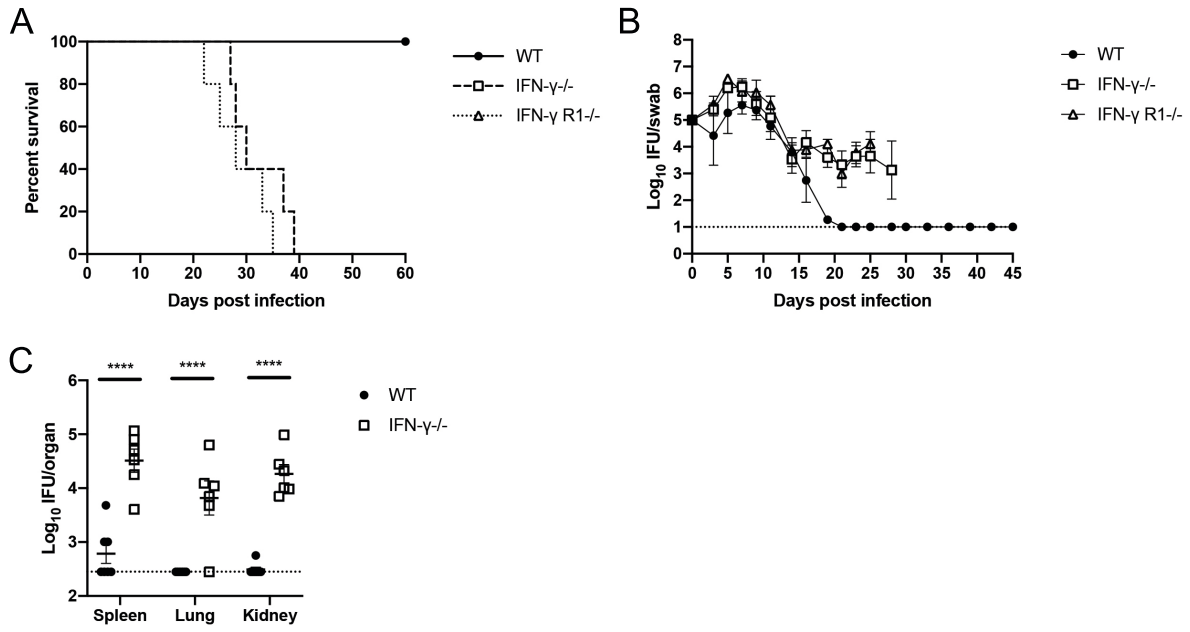

Figure 2.5: IFN-γ-deficient mice die from systemic disease, but clear the majority of FRT infection

Mice received 2.5mg Depo-Provera 7 days before infection with $1x10^5$ IFU *Chlamydia muridarum* i.vag. (A) Survival curve. (B) IFU per vaginal swab over time of mice from (A). IFN-ɣ-/- and IFN-ɣ Receptor-/- data was truncated at days 28 and 25 respectively when the number of mice surviving dropped below 50%. n=5 for all groups in (A) and (B). Results are shown as mean with SEM. (C) Bacterial load per organ on day 14 (2-way ANOVA). Data is compiled from two separate experiments with n=3 and 4 per group, though one IFN-ɣ-/- mouse died prior to day 14 and could not be included. **** = $p<0.0001$

IFN-γ production contributes to resolution of systemic Chlamydia infection in the absence of Th1 cells

Since T-bet-deficient mice lacking Th1 cells fully resolved *Chlamydia* infection of the FRT, it was of interest to determine whether this clearance required IFN-γ, since T-bet-independent pathways of CD4 IFN-γ production have been detected in other infection models [39]. Wild-type

and T-bet-deficient mice were infected with *C. muridarum* and administered neutralizing antibody against IFN-γ or isotype control antibody. T-bet-deficient mice given anti-IFN-γ displayed signs of systemic disease around the same time as IFN-γ-deficient mice, approximately two weeks into infection. These symptoms included marked weight loss and decreased body conditioning and progressed until all mice in both of these groups had to be euthanized in compliance with IACUC regulations (Fig. 2.6A). In contrast, mice in isotype control groups and wild-type administered anti-IFN-γ displayed no overt signs of systemic disease and survived until the end of the study. Interestingly, all groups exhibited similar shedding from the FRT (Fig. 2.6B), consistent with earlier observations (Fig. 2.5), suggesting again that IFN-γ signaling is not crucial to reducing bacterial burdens during initial FRT infection. To examine the extent of systemic dissemination of *Chlamydia* from the FRT, spleen, lung, and kidneys were examined for bacterial burdens either at the time of euthanasia or at 21 days for groups not showing symptoms of disease. As in IFN-γ-deficient mice, IFN-γ depletion by antibody treatment in T-bet-deficient mice led to increased dissemination (Fig. 2.6C). In contrast, wild-type mice administered depleting antibody did not show the same degree of dissemination or systemic signs of disease (Fig. 2.6C), suggesting their greater IFN-γ response was incompletely depleted by antibody. T-bet-deficient mice given isotype control antibody displayed detectable dissemination to the lung and spleen, but T-bet-deficient mice depleted for IFN-γ had significantly higher burdens than IFN-γ-deficient mice, suggesting T-bet further supports the immune response against disseminated bacteria outside of IFN-γ. Thus, in the absence of Th1 cells in T-bet-deficient mice, other sources of IFN-γ play a crucial role in protection from dissemination and death during *Chlamydia* infection.

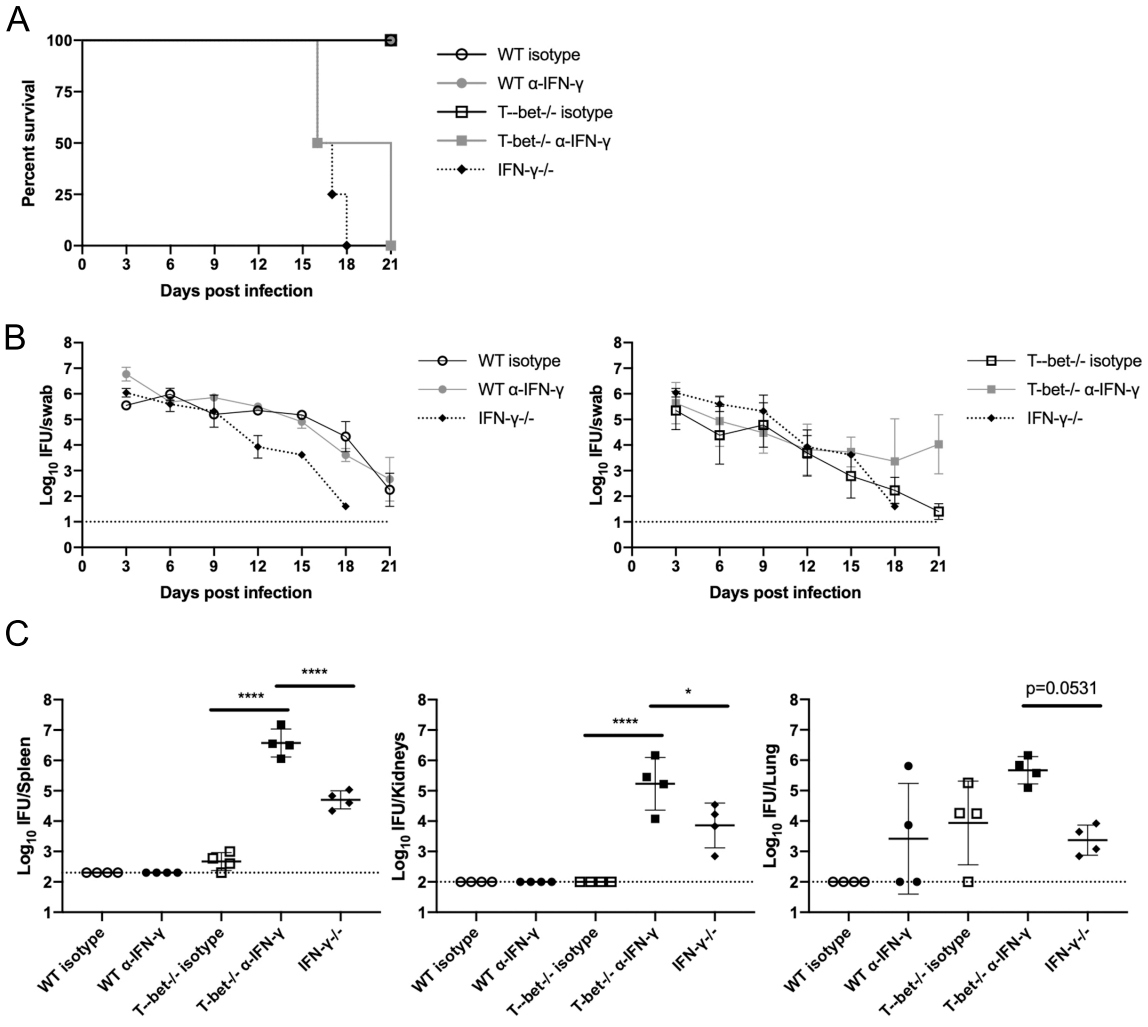

Figure 2.6: T-bet deficient mice given depleting α-IFN-ɣ are not able to control disseminated bacteria

Mice received 2.5mg Depo-Provera 7 days before infection with 1x10^5 IFU *Chlamydia muridarum* i.vag. (A) Survival curve. (B) IFU per vaginal swab over time of mice from (A) Data has been split into two graphs for ease of visualization. Results are shown as mean with SEM. (C) Bacterial load per organ at time of euthanasia (1-way ANOVA). All mice surviving in WT α-IFN-ɣ, WT isotype, and T-bet-/- groups were euthanized as controls despite showing no signs of disease at day 21 when the last mice in the T-bet-/- α-IFN-ɣ were euthanized. n=4 for all groups (A-C). Data is from one experiment.

Discussion

Pathogen-specific CD4 Th1 cells develop from naïve precursors that are initially activated in the draining lymph node by dendritic cells bringing foreign antigen from the local site of infection [42]. These activating signals include ligation of the T cell receptor and CD28 by short peptides presented in the context host MHC class-II and CD80/CD86 on the surface of an appropriate dendritic cell [43]. Local cytokines (including IFN-γ and IL-12) allow these newly activated T cells to initiate programing events that commit to future secretion of IFN-γ upon secondary TCR ligation or non-cognate signals such IL-18 and IL-12 or TL1A [44,45] . These Th1 cells are able to combat a variety of intramacrophage infections via the production of local IFN-γ which causes macrophage activation and killing of internalized pathogens [32,41,46]. Key to the Th1 lineage commitment is the intracellular expression of the master Th1 transcription factor T-bet [25]. Thus, mice lacking T-bet are unable to resolve intramacrophage infections such as *Leishmania* or *Salmonella* [30,41,47].

Although *Chlamydia* is often referred to as a "Th1 pathogen" [10,11,21-23] due to prominent CD4 T cell IFN-γ production and the high susceptibility of IFN-γ-deficient mice, our data show that mice lacking T-bet can control FRT infection with essentially normal kinetics. Clearly, *Chlamydia muridarum* infection is not being controlled by classical T-bet Th1 cells in this mouse model. The involvement of *Chlamydia*-specific Th1 cells is further questioned by the absence of T-bet expression in CD4 T cells within the FRT during the period of infection control. Surprisingly, around 25% of CD4 T cells in the FRT of *Chlamydia*-infected T-bet-deficient mice also retained the capacity to produce IFN-γ. These data are reminiscent of the *M. tuberculosis* models where T-bet was not essential for protection, but CD4 T cells displaying a phenotype approximating a Th1 signature could mediate bacterial clearance [48]. Similarly, CD4 T cells in T-bet-deficient mice infected with *Toxoplasma gondii* retain the capacity to generate a prominent population of

CD4 T cells producing IFN-γ, although these mice were still highly susceptible to infection [32]. Greater investigation of non-Th1 CD4 T cells in the *Chlamydia* model will be required in order to determine whether this population contributes significantly to pathogen clearance from the FRT.

Despite the high susceptibility of IFN-γ- and IFN-γR-deficient mice, it could be argued that the Th1 signature cytokine IFN-γ does not appear to play a prominent role in bacterial clearance within the FRT itself, since IFN-γ-deficient mice exert multiple logs of control on FRT shedding. The hypothesis that IFN-γ is largely irrelevant to FRT clearance in this model would also be supported by evidence that *Chlamydia* can escape IFN-γ-mediated mechanisms of defense. Previous work has shown that inclusions of the human pathogen *Chlamydia trachomatis* grown in mouse cells are susceptible to tagging by IFN-γ-induced immunity related GTPases that lead to destruction of inclusions, while *Chlamydia muridarum* escape these functions [49,50]. In addition, *Chlamydia trachomatis* contains a tryptophan synthase that allows escape of IFN-γ-induced reduction of available tryptophan via IDO expression in human host cells. *Chlamydia muridarum* is a tryptophan auxotroph that cannot escape the same IDO induction, though this is not induced in the mouse [51]. Together, these lines of evidence indicate that the respective *Chlamydial* species have adaptations specific to their natural host to escape IFN-γ-mediated defenses. *Chlamydia muridarum*, originally isolated out of a mouse host [52], retains host-specific adaptations to evade IFN-γ-mediated mechanisms in the murine host, while *Chlamydia trachomatis* as a human adapted pathogen may be able to mediate the similar evasion in the human host, but not in mice. In summary, there is insufficient evidence to show that IFN-γ producing CD4 T cells significantly contribute to the control of infection within the FRT during *C. muridarum* infection. However, it is important to note that there could still be a tissue protective role for IFN-γ that was unable to be assessed in our study due to high fatality rates [37]. Even if this was the case, any tissue protective role does not seem to require Th1 cells since T-bet-deficient mice do not display enhanced pathology.

In contrast, at sites outside of the FRT, IFN-γ consistently plays an essential role in limiting bacterial dissemination and preventing mortality. Mice lacking either IFN-γ or IFN-γR experience terminal disease with overt symptoms appearing within 2 weeks of vaginal infection. Similarly, neutralization of IFN-γ in T-bet-deficient mice caused a similar systemic infection, but did not affect wild-type mice presumably because their higher levels of IFN-γ were not fully depleted by antibody. The 100% mortality we observed in IFN-γ-deficient mice was greater than previous reports where evidence of persistent infection in surviving mice was detected. However, this disparity is likely explained by the use of different *Chlamydia* strains between these studies. Our laboratory uses the ATCC Weiss derivative which has been reported to have increased virulence over the Nigg strain used in prior studies [53]. Indeed, a recent paper by *Li et al.* described similar levels of fatality to our study and confirmed the primary importance of IFN-γ for systemic control of infection [54]. Interestingly, this same report also found that ILCs and NK cells were a major source of IFN-γ and prevented early systemic bacterial dissemination, a finding consistent with our observations of systemic spread in T-bet-deficient mice depleted of IFN-γ. Although the systemic anatomical niche of *Chlamydia* replication in the absence of IFN-γ is yet to be fully defined, it seems possible that tissue macrophages allow limited bacterial replication in the absence of innate IFN-γ. Macrophage M1 polarization, typically induced via IFN-γ signaling, has been shown to restrict intracellular *Chlamydia* replication while unpolarized or M2 macrophages allow growth. A role for innate IFN-γ working in concert with systemic antibody and possibly other phagocytes to prevent bacterial growth and dissemination has been suggested [33,55]. However, our data would argue that these mechanisms could be completely distinct from the mechanisms responsible for primary clearance within the FRT.

The question of what mechanism of protection exerts control of primary *Chlamydia* infection within the FRT remains unresolved. While IFN-γ-producing non-Th1 cells are present in the infected FRT, the evidence for local IFN-mediated protection is not entirely convincing.

Similarly, we did not detect development of a Type-II response with IL-4 secreting CD4 T cells. Since *Chlamydia* replication is dependent on residence within a host cell inclusion, adaptive immune mechanisms involving cytotoxicity would be an attractive theoretical solution for control of intracellular replication. If *Chlamydia*-specific cytotoxic T cells could identify infected host epithelial cells and eliminate them before the completion of a 48-hour *Chlamydial* developmental cycle, progression of infection could be effectively curtailed. However, despite the conceptual appeal of this moidel, protection against *C. muridarum* within the FRT is CD4-dependent and mice lacking cytotoxic CD8 T cells control infection [13]. It is therefore tempting to speculate that *Chlamydia*-specific CD4 T cells gain the capacity for cytotoxic killing of the infected epithelium, a possibility previously raised by other laboratories examining Nitric oxide-dependent and Nitric oxide-independent mechanisms of epithelial cell killing [56-58]. An alternative or complementary mechanism might involve the recruitment of neutrophils via the development of Th17 cells. Indeed, we detected a sizable shift in T-bet-deficient mice towards CD4 T cells expressing RORγt and IL-17 expression. Enhanced Th17 responses have previously been associated with increased FRT pathology [37], but this was not evident in our study. These data might suggest that Th17 cells have been somewhat overlooked as a protective population in restraining local *Chlamydia* infection. Th17 type responses are typified by an influx of neutrophils that might phagocytose extracellular *Chlamydia* as well as support the barrier integrity of the mucosal epithelium. However, it should be noted that very low Th17 responses were detected in the FRT of wild-type mice perhaps suggests that this subset is unlikely to be the major component of reproductive tract defense in the presence of T-bet. Studies are underway to examine the possibility of compensatory control of FRT infection by Th17 cells in the absence of T-bet. It is clear that further studies are required to pinpoint the precise mechanisms of CD4-mediated control of *Chlamydia* replication within the FRT.

In summary, our data show that, contrary to previous assumptions, classical T-bet-expressing Th1 cells are not required for clearance of primary infection with *C. muridarum* in the

mouse FRT. In contrast, IFN-γ is essential to prevent fatal systemic proliferation of *Chlamydia*. In the absence of Th1 development within the FRT, we detected the emergence of T-bet-independent IFN-γ secreting CD4 T cells and a large population of Th17 cells, coincident with bacterial clearance without worsening pathology. Defining the contribution of CD4 T cell populations and others to immune-mediated clearance of *Chlamydia* will help further understanding of T cell-mediated bacterial clearance and FRT pathology.

Methods

Mice

C57BL/6 (JAX stock no. 000664), MHC class II-deficient (JAX stock no. 003584), IFN-γ-deficient (JAX stock no. 002287), IFN-γ R1-deficient (JAX stock no. 003288), *Tbx21*-deficient (JAX stock no. 004648), CD4-Cre (JAX stock no. 017336), *Tbx21*f/f (JAX stock no. 022741), CD45.1 (JAX stock no. 002014), mice were purchased at 6-8 weeks old from The Jackson Laboratory (Bar Harbor, ME) and used for experiments at 7-12 weeks old. Breeding colonies for many of these strains were established at UC Davis and used in several experiments. All experiments were performed in agreement with regulations set by the University of California, Davis Institutional Animal Care and Use Committee.

Chlamydia Infection and Burden

Chlamydia muridarum was purchased from ATCC and propagated as described previously [18]. The estrous cycle of the mice was synchronized via administration of 2.5mg of medroxyprogesterone acetate (Depo-Provera) subcutaneously one week prior to infection. For vaginal infections, $1x10^5$ IFU of *Chlamydia muridarum* was pipetted in SPG buffer into the vaginal vault of the mice. To assess in vivo bacterial shedding, vaginal swabs were taken and deposited into 500uL SPG buffer with two glass beads in 2mL microcentrifuge tubes. The tubes were shaken at 1400rpm for 5min at 4°C, after which the swab was removed. A dilution series of the bacterial suspension was used to infect a monolayer of HeLa cells in 96-well plates, allowed to grow into inclusions overnight, then fixed and stained for counting to determine IFU per swab. To determine IFU burden per organ, the organs of *Chlamydia muridarum* infected mice were homogenized in 1-2mL SPG. 1mL of the homogenate was shaken with glass beads at 1400rpm and 4°C for 5min, then centrifuged at 500g and 4°C and for 10min. The supernatant was isolated for use in the counting assay described above.

Cytokine ELISAs

For ELISA quantification of IFN-γ production, CD4 T cells were isolated from the FRT of infected mice using a negative MACS CD4 T cell isolation kit on LS columns (Miltenyi Biotech). $2x10^4$ T cells were incubated overnight with $0.8x10^6$ irradiated splenocytes and $1x10^5$ heat-killed IFU *Chlamydia muridarum* elementary bodies per well, or α-CD3 (0.5μg/mL) and α-CD28 (1μg/mL) for controls. Culture supernatant was collected and ELISAs performed using the Invitrogen ELISA kit for IFN-ɣ, according to manufacturer's instructions.

Salmonella Infection

Salmonella enterica Typhimurium strain BMM50 (SL1344 Δ*aroA*) was given to mice intravenously at a dose of $5x10^5$ CFU. A stock culture was streaked onto MacConkey agar and one colony was used to inoculate a culture grown overnight at 37°C in Luria-Bertani broth before dilution in PBS prior to administration.

Flow cytometry

Lymphocytes were isolated from the female reproductive tract of mice 16 days post infection. The FRT was harvested into complete RPMI, diced, then incubated while stirring with collagenase IV (386mg/L MP Biomedicals) for 1 hour at 37°C. Cells were filtered (70µm cell strainer, Corning), then lymphocytes were isolated out of the supernatant using a Percoll gradient (GE Healthcare). Cells were stimulated with PMA (0.2 mM, Millipore Sigma) and Ionomycin (1ug/mL, Millipore Sigma) with Brefeldin A (71.4uM Millipore) for 3.5 hours at 37°C 5% CO_2. Cells were stained for viability with Zombie Yellow (BioLegend) and surface markers B220-APC-eF780 (RA3-6B2, eBioscience), CD11b-APC-eF780 (M1/70, eBioscience), CD11c-APC-eF780 (N418, eBioscience), F4/80-APC-eF780 (BMB, eBioscience), CD4-PE (RM4-4, eBioscience), CD4-

eF450 (RM4-5, eBioscience), CD8-PerCPCy5.5 (2.43, Tonbo), CD44-APC (IM7, eBioscience) CD62L-PETexasRed (MEL-14, Invitrogen), followed by intracellular stains IFN-γ-BV785 (XMG1.2, BioLegend), IL-4-PE (11B11, eBioscience), T-bet-PECy7 (4B10, eBioscience), RORγt-BV421 (Q31-378, BD Biosciences), and IL-17A-FITC (17B7, eBioscience) using the Foxp3 Transcription Factor Staining Kit (eBioscience). Data was acquired on an LSRFortessa (BD) and analyzed using FlowJo (Tree Star, San Carlos, CA). Contour plots are shown with 5% outliers.

RNA-seq analysis

For each group, five samples were prepared by pooling three individual mice each. Lymphocytes were isolated from the FRT, as described above. CD4 T cells were enriched using a negative MACS CD4 T cell isolation kit on LS columns (Miltenyi Biotech). These cells were stained for subsequent FACS sorting using the viability stain Zombie Yellow (BioLegend) and antibodies for surface markers APC-B220 (RA3-6B2, eBioscience), APC-F4/80 (BM8.1, Tonbo Biosciences), APC-CD11b (M1/70, Tonbo Biosciences), APC-CD11c (N418, Tonbo Biosciences, eF450-CD4 (RM4-5, eBioscience), and PerCP-Cy5.5-CD8a (53-6.7, eBioscience). Events passing through the sequential gates for single cell, live, dump negative, and CD4+CD8- were collected and processed to isolate RNA (Qiagen RNeasy Mini Kit). Gene expression profiling was carried out using a 3'Tag-RNA-Seq protocol. Barcoded sequencing libraries were prepared using the QuantSeq FWD kit (Lexogen, Vienna, Austria) for multiplexed sequencing according to the recommendations of the manufacturer using also the UMI Second-Strand Synthesis Module (Lexogen). The fragment size distribution of the libraries was verified via micro-capillary gel electrophoresis on a Bioanalyzer 2100 (Agilent, Santa Clara, CA). The libraries were quantified by fluorometry on a Qubit fluorometer (LifeTechnologies, Carlsbad, CA), and pooled in equimolar ratios. Up to forty-eight libraries were sequenced per lane on a HiSeq 4000 sequencer (Illumina, San Diego, CA) with single-end 100 bp reads to 4-7 million reads per sample. Analysis of the

sequencing data and differential gene expression was performed by UC Davis Bioinformatics Core. Raw reads were processed with HTStream v.1.1.0 (https://s4hts.github.io/HTStream/) to perform raw sequence data QA/QC, remove adapter contamination and low-quality bases/sequences. The trimmed reads were aligned to the *Mus musculus* GRCm38 primary assembly genome with GENCODE v.M23 annotation, using the aligner STAR v. 2.7.0f (Dobin, et al. 2013, Reference at https://www.ncbi.nlm.nih.gov/pubmed/23104886) to generate raw counts per gene. Differential expression analyses were conducted using limma-voom [59] (edgeR version 3.20.9, limma version 3.34.9, R version 3.4.4). The model used in limma included effects for treatment, RNA extraction batch, number of cells, and age at death. *Mus musculus* Ensembl gene identifiers and annotations were used in this study [60]. Raw data is available in NCBI's Gene Expression Omnibus [61], and are accessible through GEO Series accession number GSE193909 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193909). Supplemental Table 2.1 contains the full list of differentially expressed genes.

Histopathology

Mice were euthanized at day 60 or 61 post infection and the FRT (vagina, uterus, oviducts, and ovaries) collected and immersion fixed in 10% neutral buffered formalin. The tissues were then embedded in paraffin and sectioned at 5μm before staining with hematoxylin and eosin. A board certified veterinary anatomic pathologist then analyzed and scored sections of each of the vagina, uterus, oviducts, and ovaries for acute inflammation (neutrophilic infiltration and edema), chronic inflammation (lymphohistiocytic infiltration), erosion (loss of mucosal epithelial cells regardless of breach of basement membrane), dilation (luminal distention), and fibrosis (increased fibroblasts or increased collagenous connective tissue). Ordinal scores on a 0 to 4 point scale reflect the severity and distribution of lesions. The cumulative lesion score for each individual is the sum of the scores for the oviduct and uterus.

IFN-γ depletion in vivo

Mice were administered 0.2mg anti-IFN-γ antibody (R4-6A2, BioXCell) or isotype control antibody (Rat IgG1 anti-horseradish peroxidase, BioXCell) i.p. on days -1, 1, and every 3 days after infection with Chlamydia and monitored for bacterial shedding and the presence of bacteria in tissues at later time points.

Statistics

Statistics were performed using GraphPad Prism version 8 (GraphPad Software, LLC). Either Mann-Whitney U test or one-way ANOVA were used. * $p<0.05$, ** $p<0.01$, *** $p<0.01$, and **** $p<0.0001$.

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Author Contributions

Rixon (Planned experiments, gathered data, interpreted data, wrote paper). Depew (Planned experiments, gathered data). McSorley (Planned experiments, interpreted results, wrote paper).

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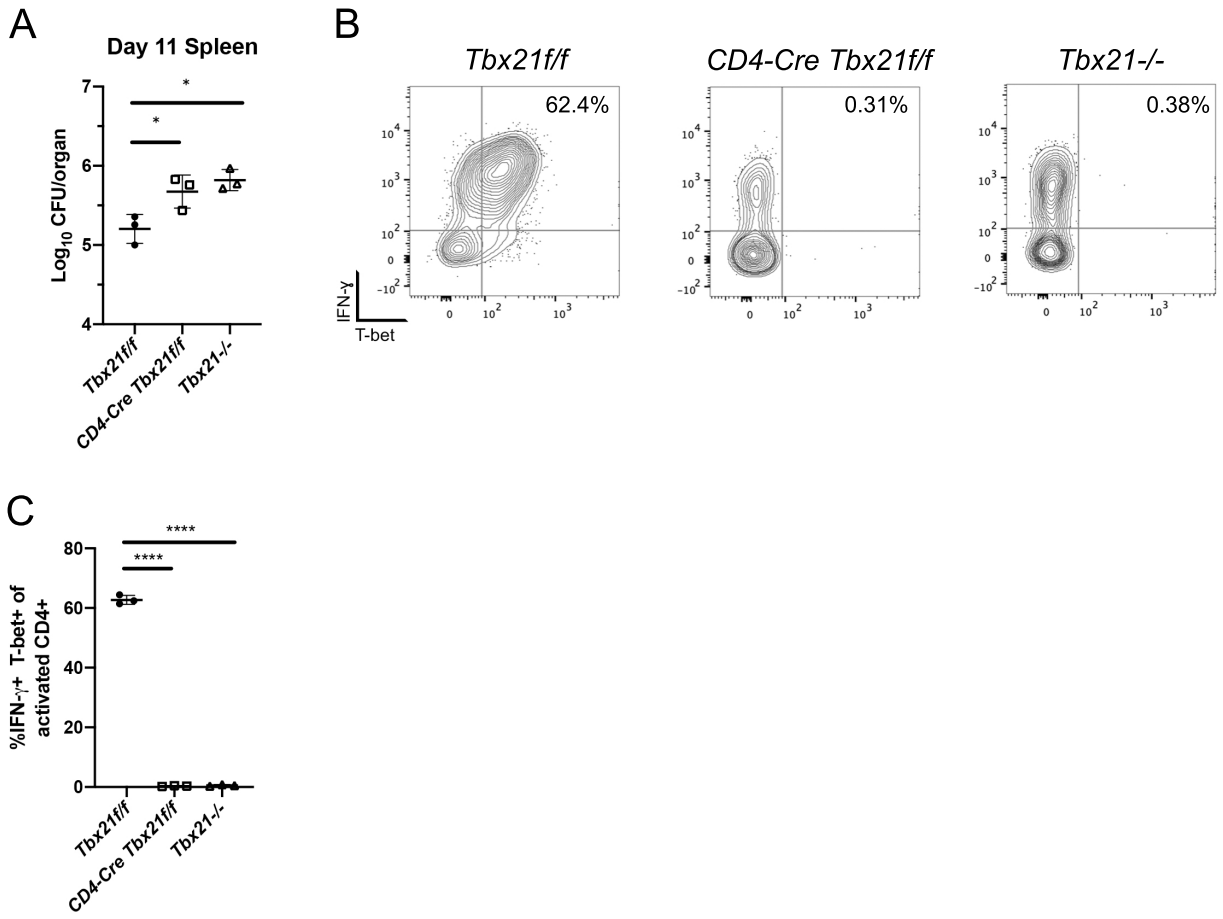
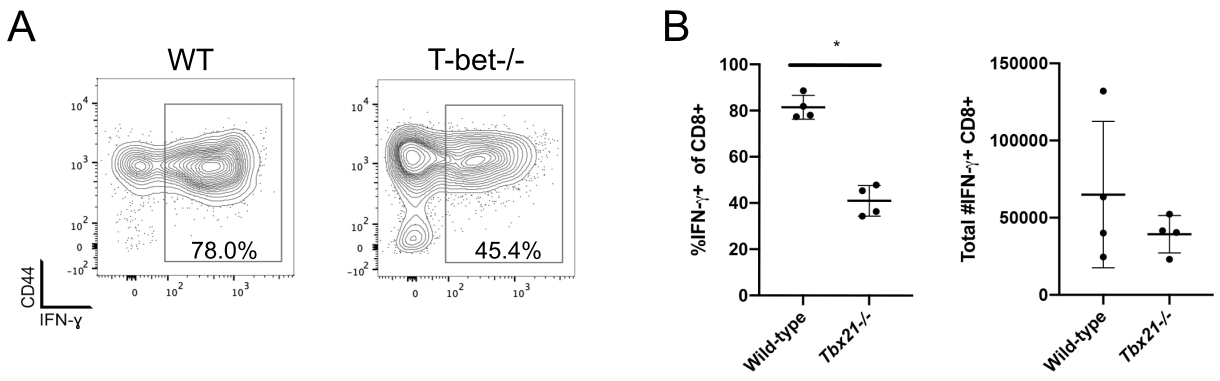


Supplemental Figure 2.1: T-bet deficient mouse models exhibit deficiencies during *Salmonella* infection and decreased Th1 staining. Mice received 5x10^5 CFU *Salmonella* i.v. (A) Bacterial burdens isolated from the spleen 11 days post infection. n=3 for each group. Graph displays mean ± SD. (B) Example flow plots of T-bet and IFN-γ expression 11 days post infection. Cells are gated on lymphocytes, singlets, live cells, dump negative, and CD44hi CD62Llo. (C) Summary of plots from (B). n=3 for all groups. Graphs depicts mea ± SD, 1-way ANOVA.



Supplemental Figure 2.2: CD8+ T cells from the FRT also exhibit a reduced capacity for IFN-γ production in T-bet deficient mice. Mice either received 2.5mg Depo-Provera 7 days before infection with $1x10^5$ IFU *Chlamydia muridarum* i.vag Lymphocytes were isolated from the FRT on day 17 post infection for *Chlamydia*-infected mice and stimulated with PMA/ionomycin in the presence of Brefeldin A before staining for flow cytometry. n=4 for both groups. Cells are gated on the lymphocyte population, singlets, live cells, dump negative, and CD8+ CD4-. (A) Example flow plots of IFN-γ and CD44 expression. (B) Summary of (A). Graphs display mean ± SD, 1-way ANOVA.

Supplemental Table 2.1: Complete list of differential gene expression between Tbx21-/- and Wild-Type samples.

(Supplemental Table 2.1 is available from doi: 10.1371/journal.ppat.1010333)

Raw data is available in NCBI's Gene Expression Omnibus (Edgar *et al*., 2002) and are accessible through GEO Series accession number GSE193909 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193909). LogFC is the log2 fold change with the Tbx21-/- samples as the numerator of the fold change. AveExpr is the average expression across all samples in log2 counts per million reads. P.Value is the raw p-value from the test that the log fold change differs from 0. Adj.P.Val is the Benjamini-Hochberg false discovery rate adjusted p-value.

Chapter 3

Clearance of *Chlamydia* from the female reproductive tract fails to correlate with local CD4 T helper subset development

Jordan A. Rixon, Alana Nguyen, Claire E. Depew, Stephen J. McSorley

Introduction

Chlamydia trachomatis is a common cause of genital tract infection and represents an increasing public health burden in the US [1]. *Chlamydia* is the most common notifiable bacterial infection to the Centers for Disease Control and Prevention (CDC), amounting to 1.5 million cases in 2020. The bulk of these genital tract infections are initially asymptomatic in young females (15-24 years old), but can cause significant reproductive harm if not appropriately treated [2]. Chronic *Chlamydia* infections are a cause of pelvic inflammatory disease, ectopic pregnancy, and serious reproductive complications. Prevention methods for *Chlamydia trachomatis* infection do not include an effective vaccine [3]. Early attempts to develop a whole cell or live vaccine ended in failure when some clinical trials detected increased pathological outcomes for a subset of vaccinated individuals [4]. Although the validity of these adverse vaccine events has since been questioned [5], the perception that *Chlamydia* vaccines can harm healthy young adults has persisted.

Mouse models provide an excellent opportunity to interrogate immunological responses to *Chlamydia* infection in the context of the female reproductive tract (FRT) [6-8]. Indeed, vaccine-mediated protection and immune suppression have each been detected within the murine reproductive tract [9], providing confidence that this model reproduces clinical observations. Cervicovaginal infection of mice with *Chlamydia muridarum* initiates an ascending reproductive

tract infection, causing pathology in a similar manner to human *Chlamydia trachomatis* infections [2, 10].

CD4 T cells are an essential component of the host immune response and mediate the clearance of *Chlamydia muridarum* from the FRT. As might be expected, SCID and RAG-deficient mice are unable to clear bacteria, and TCRα-deficient and MHC class II-deficient mice that lack CD4 T cells fail to successfully resolve primary *Chlamydia* infection of the FRT[11-15]. In marked contrast, mice lacking B cells or CD8 T cells can resolve primary infection with *Chlamydia*, although the pattern of systemic infection differs from wild-type mice in B cell-deficient mice[12, 16].

CD4 T helper cell responses are generally understood to differentiate into a few different modalities (Th1/Th2/Th17,Tfh,Treg) that exhibit unique effector strategies adapted to combat particular types of pathogens and regulate the immune response [17, 18]. These differentiated helper lymphocytes can provide help to cells of the innate immune system to encourage pathogen clearance. Each subset has a particular profile that includes the expression of a master transcription factor and secretion of certain cytokines. The Th1 subset of CD4 T cells is defined by the master transcription factor T-bet and secretion of IFN-γ, a cytokine that activates macrophages for increased killing intracellular bacteria. Similarly, Th2 cells express GATA3 and secrete IL-4, IL-5, and IL-13 to activate eosinophils against helminths. Th17 cells are defined by RORγt expression and secrete IL-17 which function to recruit neutrophils for killing extracellular bacteria and fungi. Although this model provides a simple framework for understanding host responses to intramacrophage infections, helminths, and extracellular bacteria, the microbiology of *Chlamydia* does not fit neatly into either of these categories.

Chlamydia are obligate intracellular bacteria that primarily replicate in epithelial cells. The bacteria exist in two basic forms, an elementary body (EBs) that is metabolically inactive but highly infectious, and a reticulate body that is metabolically active and replicates but is non-infectious

[19, 20]. EBs within infected epithelial cells generate an inclusion within the cell that allows bacterial replication and EB to RB conversion. Thus, most bacteria replicate inside an epithelial vacuole as RBs or are released from the cell as EBs when the cell is lysed. It remains unclear which CD4 T helper subset is important for coordinating immune defense against a pathogen with this particular lifecycle. CD4 T cells responding to *Chlamydia* infection in humans have been observed secreting significant levels of IFN-γ [21-24]. Similarly, mice lacking IFN-γ suffer from systemic bacterial dissemination that leads to early mortality [25-27]. These observations have led to the idea that CD4 Th1 cells regulate the immune defense to *Chlamydia* within the FRT. However, previous work from our laboratory using the mouse model have demonstrated that *Chlamydia*-specific CD4 T cells do not express high levels of T-bet, and mice lacking T-bet expression entirely can clear infection similar to wild-type mice [27]. At present, it is unclear whether another CD4 T cell subset is required to resolve *Chlamydia* infection or whether another model is needed to understand bacterial clearance in the FRT. Here, we look more closely at the role of IFN-γ in *Chlamydia* defense and investigate the role of alternative CD4 Th fates within the FRT. Our data rule out a requirement for Th2 cells and uncover an essential role for Th17 cells in rapid bacterial clearance. Surprisingly, compensation between Th1 and Th17 responses was insufficient to explain the role of CD4 T cells in *Chlamydia* clearance. Instead, our data suggest that CD4 T cell responses to *Chlamydia* involve common effector mechanisms accessible to multiple T helper subsets. Thus, the specific axis of differentiation that T helper subset generation takes does not appear to define the protective response to *Chlamydia* within the FRT.

Results

Bacterial clearance requires non-hematopoietic cells to sense IFN-γ

Studies done by our laboratory and others show that the loss of IFN-γ production or IFN-γ responsiveness adversely affects host control of *C. muridarum*, especially disseminated replication outside the FRT [25, 27, 28]. Indeed, mice deficient in IFN-γ experience up to 100% mortality in some studies of FRT infection [27]. It is not clear whether this requirement is due to systemic phagocytes requiring IFN-γ signaling to kill disseminated *Chlamydia* or for epithelial cells to control infection within the FRT via IFN-γ sensing. In order to determine which of these mechanisms was essential we generated bone marrow chimeras where donor hematopoietic cells lacked IFN-γR while non-hematopoietic cells retained IFN-γR expression, or vice versa. After *Chlamydia* cervicovaginal infection, wild-type mice with wild-type bone marrow retained normal body weight and displayed no overt signs of sickness (Fig. 3.1A and B). As expected, these mice also had very low levels of systemic bacteria (Fig. 3.1C). In contrast, IFN-γR-deficient mice with IFN-γR-deficient bone marrow lost body weight in the second week of infection (Fig. 3.1A) and succumbed to infection (Fig. 3.1B), similar to unmanipulated IFN-γ deficient control mice (Fig. 3.1A and B). Both of these groups also exhibited increased bacterial burdens in the spleen, lungs, and kidney (Fig. 3.1C). Wild-type mice reconstituted with IFN-γ receptor-deficient bone marrow did not lose body weight or exhibit signs of sickness and were able to control bacterial growth within the FRT and elsewhere (Fig. 3.1A, B, C, and D). Thus, resolution of *Chlamydia* infection does not require IFN-γ responsiveness from hematopoietic cells. In contrast, IFN-γ receptor-deficient mice reconstituted with wild-type bone marrow still suffered weight loss, mortality, and systemic dissemination (Fig. 3.1A, B, and C) indicating that IFN-γ signaling is primarily required in non-hematopoietic cells. All groups were able to reduce shedding from the FRT (Fig. 3.1D), supporting the idea that IFN-γ signaling is not necessary for clearance within the FRT itself.

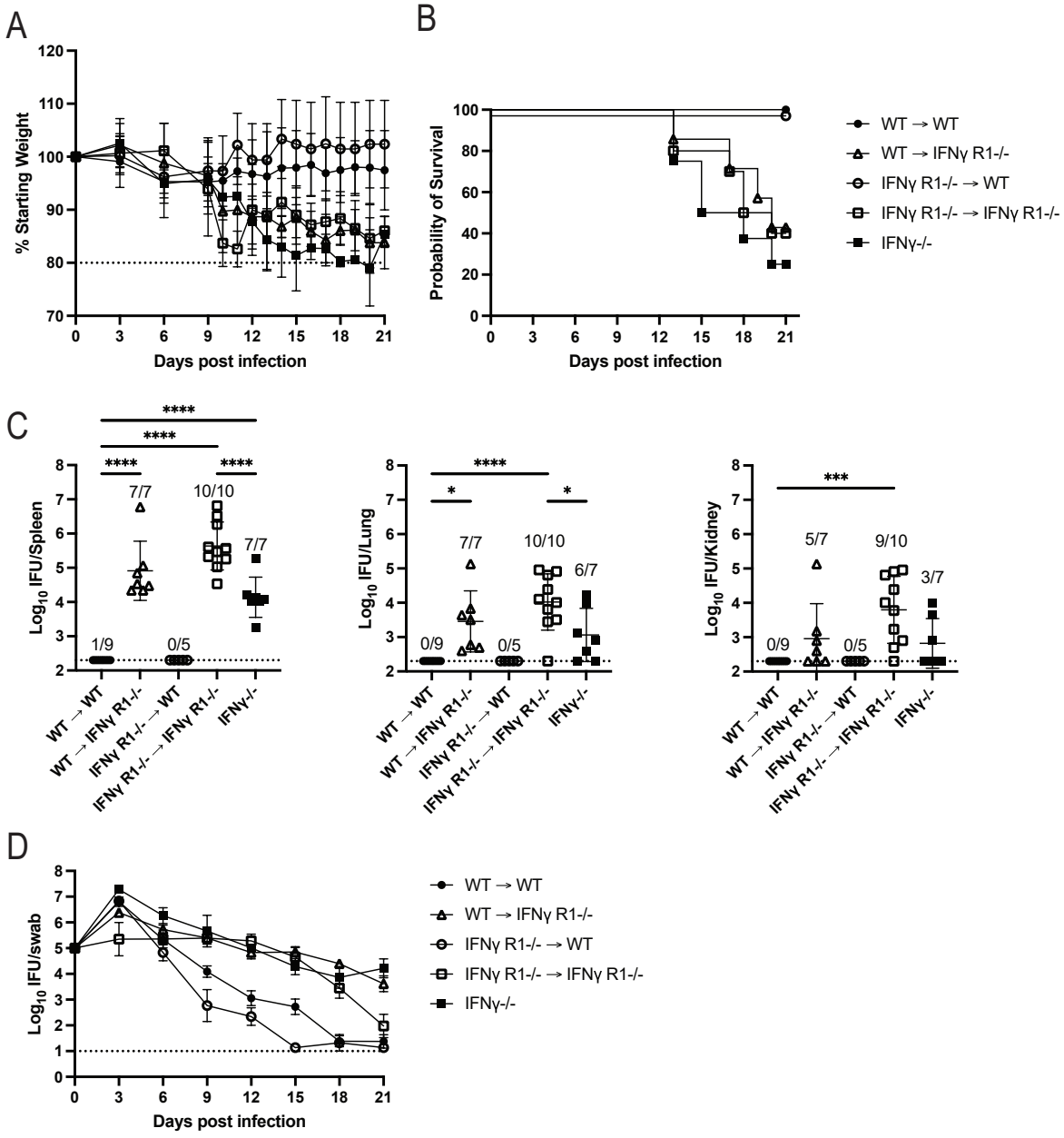


Figure 3.1: Bone marrow chimera mice require expression of IFN-γ receptor by the recipient host tissues, but not donor bone marrow cells to control systemic *Chlamydia* infection.

Four groups of bone marrow chimera mice were generated as follows: CD45.1 wild-type bone marrow was transferred into CD45.2 wild-type recipients (WT→WT), CD45.1 wild-type bone marrow was transferred into CD45.2 IFNγR1-/- recipients (WT→IFNγ R1-/-), CD45.2 IFNγR1-/-

bone marrow was transferred into CD45.1 wild-type recipients (IFNγ R1-/-→WT), and CD45.2 IFNγR1-/- bone marrow was transferred into CD45.2 IFNγR1-/- recipients (IFNγ R1-/-→IFNγ R1-/-). These groups were synchronized and infected alongside an additional, unmanipulated group of IFNγ-/- mice. The weight of each mouse was monitored over the course of the experiment and mice were euthanized before day 21 post infection if their weight was below 80% of the starting value or their condition otherwise became too severe. All remaining mice were euthanized at day 21. Upon euthanasia, spleen, lung, and kidneys were harvested for counting *Chlamydia* burdens. A) The percentage of starting weight of each mouse over time ± SD. B) Survival curve. C) Bacterial load measured in each organ at the time of euthanasia ± SD (1-way ANOVA). D) IFUs isolated from vaginal swabs over the course of infection ± SEM. Data is combined from two experiments, total n are 9 for WT→WT, 7 for WT→IFNγ R1-/-, 5 for IFNγ R1-/-→WT, 10 for IFNγ R1-/-→IFNγ R1-/-, and 8 for IFNγ-/-.

Mice lacking T-bet display a profound shift of CD4 T cells towards a Th17 signature

Although CD4 T cells in *Chlamydia*-infected mice secrete IFN-γ, T-bet levels remained low in the FRT and draining iliac lymph nodes (ILN), compared to the classical Th1 response detected in *Salmonella* infected mice (Fig. 3.2A and B). Despite low T-bet, the archetypal Th1 cytokine IFN-γ was produced by 60-80% of CD4 T cells in the FRT, while Th17 markers RORγt and IL-17A only represented a small fraction of the population, (Fig. 3.2C and D). We previously reported that T-bet deficiency has no impact on bacterial shedding from the FRT [27], but the CD4 T cells isolated from the FRT of T-bet-deficient mice displayed clear differences in CD4 T cell differentiation compared to wild-type mice. Specifically, the proportion of IFN-γ producing CD4 T cells fell to less than half of that observed in wild-type mice (Fig. 3.2A and B), while RORγt and Il-17A expression increased to around 60% of the population in the FRT and 40% in the ILN (Fig. 3.2C and D). Thus, a drastic increase Th17 type responses occurs in mice lacking T-bet.

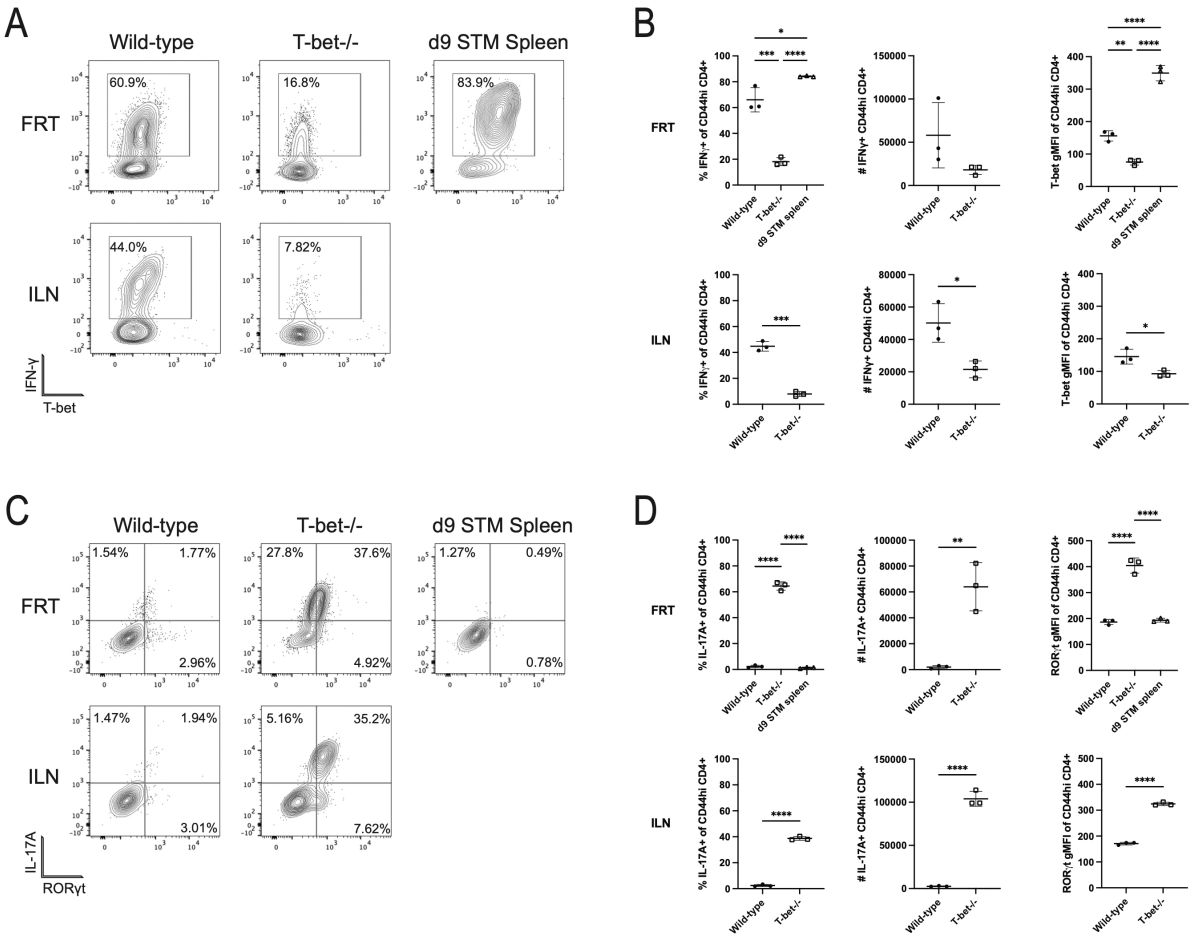


Figure 3.2: T-bet deficient mice display a significant shift towards Th17 responses.

Lymphocytes isolated from the FRT were stimulated with PMA and ionomycin with Brefeldin A before staining for flow cytometry. Results are gated on CD4+ CD44hi cells. A) Expression of Th1 markers T-bet and IFN-γ. B) Summary graphs from A. C) Flow cytometry of expression of Th17 markers RORγt and IL-17A. D) Summary graphs from C. All graphs are displayed as mean ± SD. Data is representative of two experiments.

Mice lacking Th17 responses display a reduced ability to clear *Chlamydia*

Since Th17 cells represented the major component of an effective CD4 T cell response in T-bet-deficient mice, we examined whether this subset was necessary for bacteria clearance. RORγt null mice have impaired lymph node formation and peripheral T cell development that

prevent their use in examining Th17 deficiency [29, 30]. In contrast, RORγt mutant mice were generated that have a two amino acid mutation that allows lymph node and peripheral T cell development, while impeding Th17 responses [31]. When these mice were infected with *Chlamydia*, they exhibited a significant delay in bacterial clearance compared to wild-type mice (Fig. 3.3A and B). However, all RORγt mutant mice eventually cleared bacteria from the FRT, demonstrating that other effector mechanisms can also participate in the resolution of *Chlamydia* infection. Th17 responses appear to play an obligate role in initiating clearance from the FRT before these other CD4-dependent mechanisms are operational.

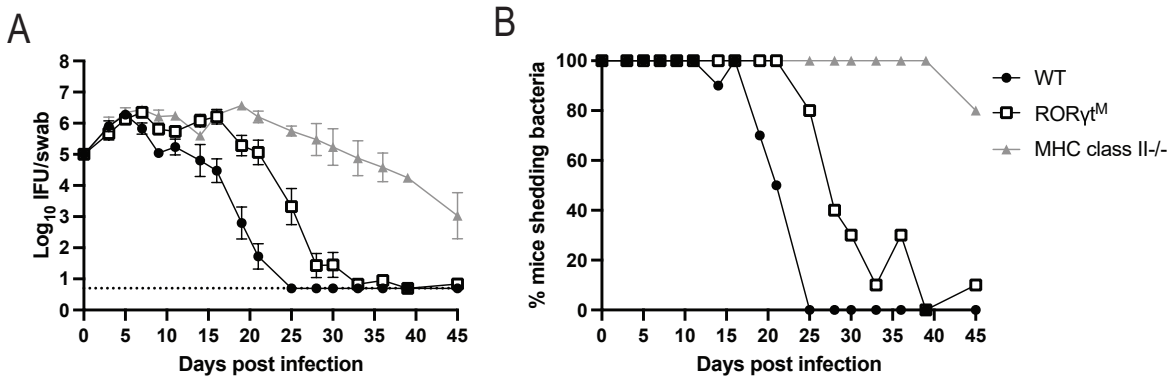


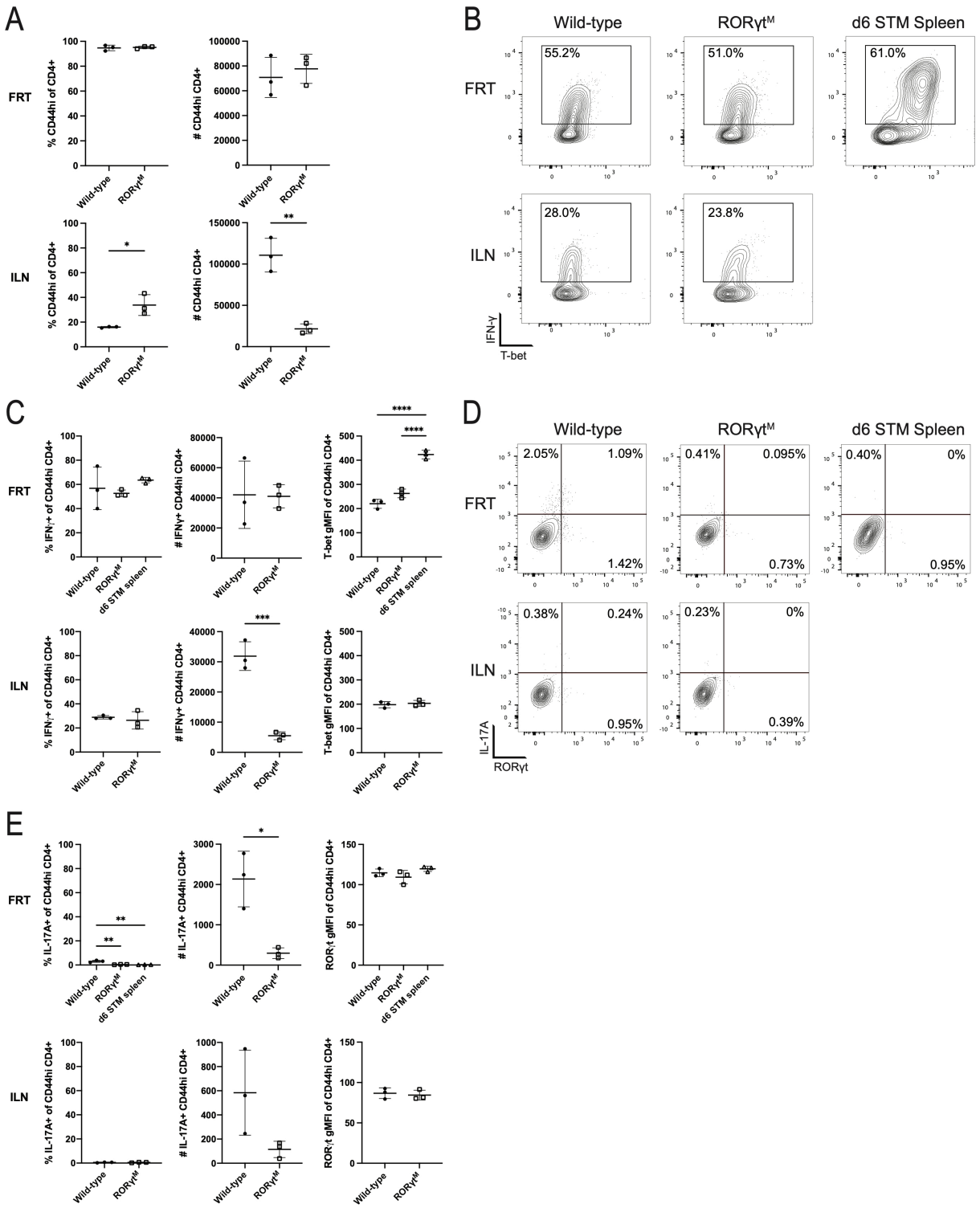
Figure 3.3: RORγt mutant mice show a minor delay in clearance kinetics.

A) IFUs isolated from vaginal swabs at various time points after infection. For wild-type mice n=10, RORγt mutant mice n=10, and MHC class II-/- mice n=5. Data is combined from two experiments, MHC class II-/- group was included in one experiment. Graph is displayed as mean ± SEM. B) Data from A expressed as the percent of mice with culture positive vaginal swabs.

We next investigated the effector potential of CD4 T cells responding to Chlamydia in RORγt mutant mice compared to wild-type mice. An overall reduction in the total number of activated CD4 T cells was observed in the draining ILN of RORγt mutant mice, reflecting lower

LN number as previously seen in the original work describing these mice [31], but this did not affect total CD4 T cell numbers in the FRT (Fig. 3.4A). The proportion of CD4 T cells expressing IFN-γ was comparable in the FRT and ILN of both mouse groups (Fig. 3.4B and C). In contrast, the low level of IL-17A-producing CD4 T cells detected in the FRT of wild-type mice was further reduced in RORγt mutant mice, falling from 2% in wild-type to less than 1% in RORγt mutant mice (Fig. 3.4D and E). Overall, these data show that RORγt mutant mice have relatively subtle differences to wild-type mice, most notably a shift away from Th17 responses.

Given the small difference in the CD4 T cell response between wild-type and RORγt mutant mice by flow cytometry, we sought confirmation of this difference by gene expression analysis. We used bulk RNA-seq to interrogate overall gene signatures in CD4 T cells isolated from the FRTs of wild-type and RORγt mutant mice 17 days post infection. At this time point, wild-type mice successfully cleared the bulk of *Chlamydia* infection while RORγt mutant mice retained a high burden (Fig. 3.3). The top ten differentially expressed genes between wild-type and RORγt mutant CD4 T cells are listed in Table 3.1, ordered by adjusted p-value. Only three genes, *Il1r1*, *Il17re*, and *Ramp1*, have adjusted p-values below 0.05, indicating that these two samples are highly similar. With *Il22* being the fourth ranked gene (p-value of 0.087), along with *Il1r1* and *Il17re*, this data supports flow cytometry findings showing that RORγt mutant CD4 T cells have constrained Th17 responses. Thus, expression analysis suggests that Th17 polarization is reduced in RORγt mutant CD4 T cells, supporting flow cytometry analysis above.


A
FRT
ILN
% CD44hi of CD4+
# CD44hi CD4+
Wild-type
RORγt^M
B
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61.0%
28.0%
23.8%
IFN-γ
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IL-17A
RORγt
E
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# IL-17A+ CD44hi CD4+
RORγt gMFI of CD44hi CD4+

Figure 3.4: CD4 T cells from RORγt mutant mice have a similar expression pattern of Th1 and Th17 markers as wild-type mice.

Lymphocytes isolated from the FRT were stimulated with PMA and ionomycin with Brefeldin A before staining for flow cytometry. A) Percentage and number of total activated CD4 T cells isolated from FRT and ILN. B) Expression of Th1 markers T-bet and IFN-γ in activated CD4 T cells. C) Summary graphs from B. D) Flow cytometry of expression of Th17 markers RORγt and IL-17A in activated CD4 T cells. E) Summary graphs from D. All graphs are displayed as mean ± SD. Data is representative of two experiments.

Table 3.1: Bulk RNA-seq on CD4 T cells from the FRTs of RORɣt mutant mice show few differences from wild-type mice. At 17 days post infection, CD4+ T cells were FACS sorted from infected FRTs of RORɣt mutant and wild-type mice and RNA sequencing. This table lists differential gene expression analysis of RORɣt mutants compared to wild-type, including the top 10 genes as ordered by adjusted p-value. Bold values are $p<0.05$. Average expression is across all samples in log2 counts per million reads and the adjusted p-value is uses the Benjamini-Hochberg false discovery rate.

Gene Name	Log2 Fold Change	Average Expression	Adjusted p value	Gene Description
Il1r1	-3.08	5.26	**0.0071**	interleukin 1 receptor, type I [Source:MGI Symbol;Acc:MGI:96545]
Il17re	-2.53	5.43	**0.0148**	interleukin 17 receptor E [Source:MGI Symbol;Acc:MGI:1889371]
Ramp1	-3.13	4.56	**0.0148**	receptor (calcitonin) activity modifying protein 1 [Source:MGI Symbol;Acc:MGI:1858418]
Il22	-5.48	2.58	0.0876	interleukin 22 [Source:MGI Symbol;Acc:MGI:1355307]
Ighg2c	2.73	2.29	0.1707	immunoglobulin heavy constant gamma 2C [Source:MGI Symbol;Acc:MGI:2686979]
Myl12b	-0.97	6.71	0.2665	myosin, light chain 12B, regulatory [Source:MGI Symbol;Acc:MGI:107494]
Nrgn	-3.04	4.03	0.3077	neurogranin [Source:MGI Symbol;Acc:MGI:1927184]

Tmem176a	-2.13	5.33	0.3077	transmembrane protein 176A [Source:MGI Symbol;Acc:MGI:1913308]
Ccl5	0.85	7.45	0.3077	chemokine (C-C motif) ligand 5 [Source:MGI Symbol;Acc:MGI:98262]
Ccdc66	1.87	4.15	0.308	coiled-coil domain containing 66 [Source:MGI Symbol;Acc:MGI:2443639]

Mice deficient in Th2 cells can clear *Chlamydia* infection similarly to wild-type mice

Neither Th1 nor Th17 fates appear to be necessary for clearance of primary *Chlamydia* infection from the FRT, although lack of Th17 responses significantly delays clearance (Fig. 3.3). Previous reports have detected Th2 responses during *Chlamydia* infection in human samples [32]. In mice, Th2 responses have been associated with protection against pathology and the type 2 cytokine IL-13 has been associated both with clearance and susceptibility to infection [33-35]. Thus, it was of interest to examine bacterial clearance in mice lacking Th2 cells. The master transcription factor of Th2 fate is GATA3, but since *Gata3* null mutations are embryonic lethal, we used STAT6-deficient mice which are deficient for Th2 development [36, 37]. This transcription factor is downstream of IL-4 and IL-13 signaling and promotes GATA3 expression [38, 39]. After infection with *Chlamydia*, STAT6-deficient mice shed bacteria from the FRT with the same kinetics as wild-type mice (Fig. 3.5A). Bacterial burdens followed wild-type clearance kinetics whether expressed as the number of IFU isolated from vaginal swabs or as the percentage of mice that were culture positive at any given time (Fig. 3.5A and B). Thus, Th2 development is not critical for clearance of *Chlamydia* from the FRT of infected mice.

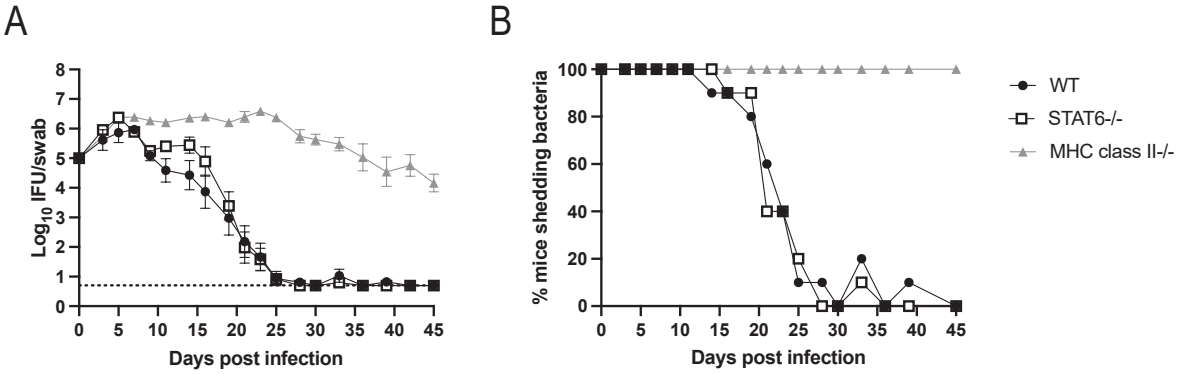

Figure 3.5: STAT6-deficient mice do not show a delay in clearance.

A) IFUs isolated from vaginal swabs at various time points after infection. For wild-type mice n=10, STAT6-/- mice n=10, and MHC class II-/- mice n=5. Data is combined from two experiments, MHC class II-/- group was included in one experiment. Graph is displayed as mean ± SEM. B) Data from A expressed as the percent of mice with culture positive vaginal swabs.

Blocking Th1 and Th17 pathways simultaneously does not prevent bacterial clearance

Our data demonstrates that individually blocking any of three common helper T cell pathways (Th1, Th2 and Th17) is insufficient to inhibit primary clearance of *Chlamydia* from the FRT. Given the dominance on Th1 responses in wild-type mice and Th17 responses in T-bet-deficient mice, it seemed possible that these two CD4 subsets provided effective overlapping protection. In order to examine this possibility, we developed two approaches to limiting Th1 and Th17 programs simultaneously. First, we used depleting antibodies for IL-6 and TGF-β to prevent Th17 development in T-bet-deficient mice. As controls, wild-type mice or T-bet-deficient mice were administered isotype antibodies. Neither wild-type nor T-bet-deficient mice administered antibodies depleting IL-6/TGF-β displayed evidence of delayed bacterial clearance from the FRT (Fig. 3.6A and B). Thus, simultaneously blocking both type 1 and type 17 responses was insufficient to eliminate the development of a protective response in the FRT. As a complementary approach, we crossed T-bet-deficient mice to RORγt mutant mice. Similarly, clearance kinetics in these mice did not deviate significantly from wild-type, where bacterial burden began to reduce in the second week of infection (Fig. 3.6C). However, there was a delay in clearing bacteria from some mice at the late stage of infection (Fig. 3.6D). Together, this data suggests that type 1 and type 17 responses do not compensate for each other in clearance of *Chlamydia*.

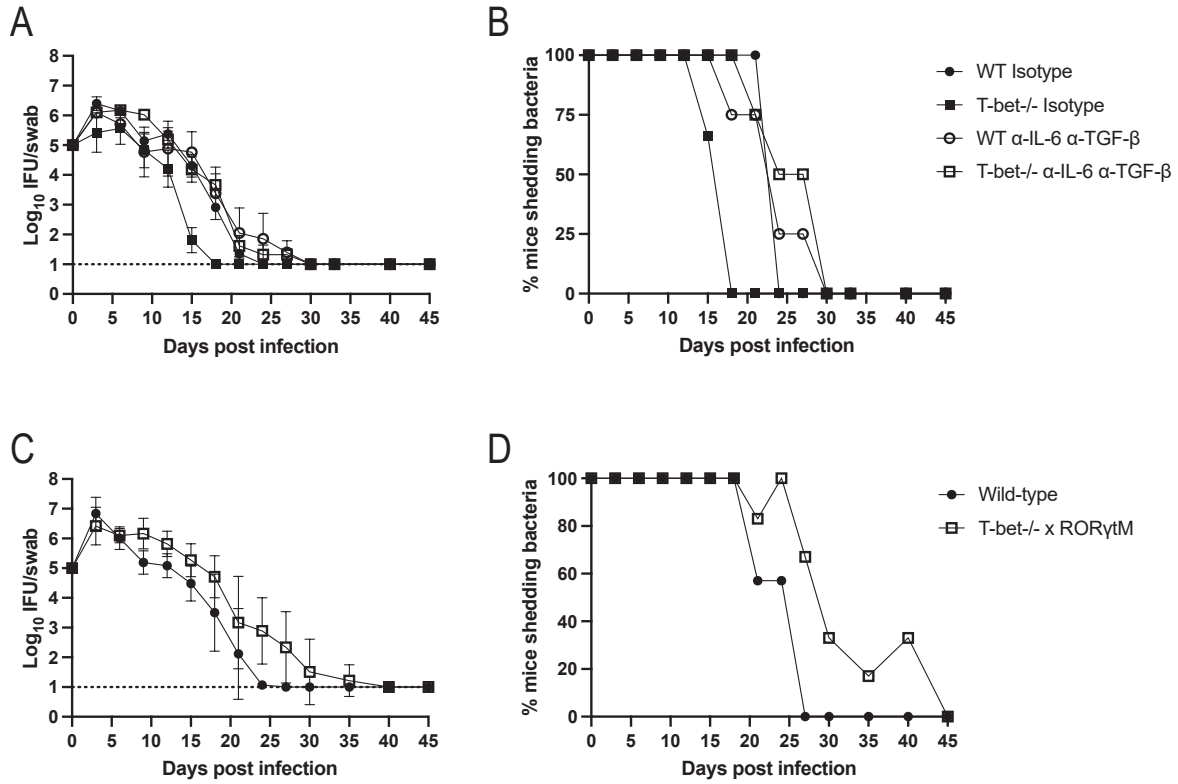

Figure 3.6: Two models of depleting Th1 and Th17 cells concurrently are able to mediate FRT clearance.

A and C) IFUs isolated from vaginal swabs at various time points after infection. B and D) Data from A expressed as the percent of mice with culture positive vaginal swabs. A and B) Wild-type or T-bet-/- mice were given isotype or depleting antibodies for IL-6 and TGF-β. C and D) T-bet-/- mice were bred with RORγt mutant mice and infected with *Chlamydia*.

Discussion

Chlamydia infection has long been understood as a Th1 disease, where Th1 cells making IFN-γ restrict *Chlamydia* growth in the female reproductive tract epithelium. This work along with already published data challenge this model and further support evidence that IFN-γ does not mediate bacterial clearance within the FRT, but rather has important functions preventing proliferation of *Chlamydia* in other, systemic sites.

Our lab has already established that expression of the Th1 defining transcription factor T-bet is low in CD4 T cells isolated from *Chlamydia* infected murine FRTs and showed that knocking out T-bet entirely in the mouse had no effect on clearance kinetics [27]. While CD4 T cells from the FRT do have the capacity to produce a significant amount of IFN-γ (Fig. 3.2), mice lacking this cytokine still exhibit a clear ability to control bacterial levels within the FRT and clear most of the infection before succumbing to problems related to systemic spread of *Chlamydia* (Fig. 3.1) [25, 27]. Experiments done by Mercado et al. show that transfer of IFN-γ-deficient CD4 T cells into TCRβ-deficient mice is sufficient to protect mice from death and control FRT infection [28]. Taken together, the role of IFN-γ against *Chlamydia* in this mouse model appears to be restricted to controlling replication after escape from the primary site of infection in the FRT mucosa.

Our data show that mice with wild-type bone marrow derived cells and IFN-γ receptor deficient non-hematopoietic cells are still susceptible to death from systemic proliferation. On the other hand, mice with IFN-γ receptor deficient hematopoietic cells and wild-type non-hematopoietic cells are functionally wild-type in their ability to control infection both at the local FRT site and systemically. Thus, in the first case, *Chlamydia* is replicating in non-hematopoietic cells that are usually able to control infection via IFN-γ receptor signaling in wild-type mice, rather than replicating within phagocytes. The source of IFN-γ is likely to be NK cells and ILC1s, as indicated by Mercado et al. [28].This does not rule out that phagocytes may initially transport bacteria to secondary locations in the first week of the infection. In vitro studies have shown an ability of *Chlamydia* to infect and survive or proliferate within macrophages unless they are polarized to the activated M1 killing state by signals such as IFN-γ [40, 41]. Infected phagocytes during this early time may travel to a secondary location before *Chlamydia* replication results in the lysis of the cell and release of infectious elementary bodies.

Further work is required to determine which secondary sites of infection result in the morbidity and mortality of mice deficient in IFN-γ signaling. Wild-type mice have been shown to

support *Chlamydia* infections in other organs. In the gastrointestinal tract, IFN-γ from CD4 T cells is able to clear *Chlamydia* in the small intestine, but not the large intestine where infection becomes chronic [42]. Transient lung infection has also been observed after intravaginal infection [43]. Clearly, different host cell types in different tissue contexts have variable capacities to allow *Chlamydia* proliferation. It will be interesting to dissect which IFN-γ-mediated pathways are active and effective or blocked and evaded in these different host cells.

In summary, we and others have shown that the IFN-γ-producing Th1 paradigm is dispensable for clearance in the FRT [25, 27, 28]. Additionally, neither Th2 nor Th17 responses are critical to mediate clearance in this model (Fig. 3.5 and 3.3), though early Th17 responses may contribute to initial control. The lack of Th2 involvement agrees with previous observations of low to no Th2 related markers in CD4 T cells isolated from infected FRTs [16, 27]. Surprisingly, RORγt mutant mice that exhibited a limited deficiency in early clearance displayed very similar patterns of Th1/17 markers compared to wild-type mice (Fig. 3.4 and Table 3.1). In comparing mRNA expression between CD4 T cells from infected wild-type and RORγt mutant mice, two of the significantly differentially expressed genes are *Il1r1* and *Il17re*. *Il1r1* has been shown to be regulated directly by RORγt [44]. *Il17re* is part of the receptor for IL-17C, which has been shown to be produced by epithelial cells in other organs in response to microbes, and can be stimulated by IL-1 [45]. As has been shown in a mouse model of *C. rodentium* infection [46], IL-17C may act as an early signal for *Chlamydia* infected epithelial cells in the FRT to activate T cells, a pathway inhibited by the RORγt mutation here, resulting in the delay in shedding in these mice.

Interestingly, one of the significantly differentially expressed genes in this comparison was *Ramp1*, which encodes the RAMP1 protein. Receptor activity-modifying proteins (RAMPs) are known to modulate the signaling and trafficking of a variety of G-protein coupled receptors, including chemokine receptors [47, 48]. RAMP1 has been shown to be expressed in T cells and RAMP1 deficiency can alter T cell trafficking patterns [49]. Additionally, RAMP1-deficiency has

been linked to suppression of Th17 function and IL-17 production [50]. In our model, the RORγt mutation preventing enforcement of a Th17 program may lead to an inability to promote RAMP1 expression, which in turn may either affect the ability of responding T cells to localize to inflammatory sites or prevent the execution of type 17 effector functions.

The week-long delay in shedding in these RORγt mutant mice is not also observed when wild-type mice are given depleting anti-IL-6 and anti-TGF-β to prevent Th17 development (Fig. 3.4). The reason for this discrepancy is unclear and may be due to idiosyncrasies of the RORγt mutation or incomplete blockade of the Th17 program by depleting antibodies.

After ruling out any of Th1, Th2 or Th17 programs as being individually critical for the response, we turned to testing compensation between the two subsets Th1 and Th17, as wild-type mice display Th1-like low T-bet and high IFN-γ production while T-bet-deficient mice display Th17 identity (Fig. 3.2). Th1-like programs could operate in wild-type mice orchestrating activated killing of *Chlamydia* taken up by macrophages and activation or cytotoxicity directed towards infected cells. Th17 programs in T-bet deficient mice could be organizing phagocytosis of extracellular bacteria and support of mucosal barrier integrity and antibacterial products protecting the epithelium from infection. We hypothesized that removal of both axes could prevent any effective responses from developing. Both methods we tested for this, using antibody depletion and genetically blocking transcription factor expression and function, did not result in deficiencies in clearing infection (Fig. 3.6). We are thus left with an insufficient explanation for clearance using the classical Th subset model after having ruled out the likely candidates.

Instead, we propose that these subset designations may not be a useful way of classifying Th cells that can mediate clearance in *Chlamydia* infection in the female reproductive tract. Even in other infection models, Th1 cells are not all equivalent. In comparing Th1 development in influenza versus *Salmonella* infection, Krueger et al. found that *Salmonella* infection drove further programming of Th1 cells into a secondary module of higher T-bet and IFN-γ expression over

what was observed in influenza infection[51]. *Chlamydia* infection here seems to behave similarly to influenza with regards to inducing only low expression of T-bet, though IFN-γ remains a salient marker. Other evidence in the influenza model observes similar phenomena where T-bet-/- cells are shown to shift towards Th17 responses, but can still mediate protective effects against infection [52]. In summary, either the CD4 Th cells responding to *Chlamydia* infection are not polarizing strongly to any one subset, or all possible fates allowed in each model tested here have access to a common effector mechanism not constrained to one subset. Future work is required to determine what this mechanism may be and the degree to which it can be accessed by CD4 T cells of different differentiation patterns.

Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The University of California Davis is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal experiments were approved by University of California Davis Institutional Animal Care and Use Committee (IACUC) (Protocol number 21869)

Mice

C57BL/6 (JAX stock no. 000664), IFN-γ R1-deficient (JAX stock no. 003288), CD45.1 (JAX stock no. 002014), IFN-γ-deficient (JAX stock no. 002287), MHC class II-deficient (JAX stock no. 003584), *Tbx21*-deficient (JAX stock no. 004648), RORγt mutant (JAX stock no. 031393)[31], and STAT6-deficient (JAX stock no. 005977) mice were purchased at 6–8 weeks old from The Jackson Laboratory (Bar Harbor, ME) and used for experiments at 7–12 weeks old. For many of these strains, breeding colonies were established to supply experiments. Mice were handled and used according to regulations of the Institutional Animal Care and Use Committee at University of California, Davis.

Generation of bone marrow chimeras

Recipient mice were irradiated at 800rad in an X-ray irradiator and placed on antibiotics by diluting 6mL of Pediatric Suspension Cherry Flavor (NDC 65862-496-47) into 250mL water bottles. Approximately 16 hours later, bone marrow was harvested from donor mice by flushing the marrow from femurs, tibias, and humeruses with 2.5% FBS in PBS. The cell suspension was run through 70μm cell strainers and red blood cells lysed by treating with ACK lysis buffer for 2min at room temperature before lysis was stopped upon addition of further 2.5% FBS in PBS. Cell

suspensions were washed twice with PBS before being suspended in PBS for injection via tail vail. Recipient mice received $6x10^6$ donor bone marrow cells in 0.2mL total volume. Mice were kept on antibiotics for 6 weeks. To assess chimerism via congenic markers, blood was collected via retro-orbital bleeds from recipient mice at week 8, RBCs lysed with ACK lysis buffer as described above, and samples stained for flow cytometry. The markers used were CD8-BV785 (53-6.7, BioLegend), CD4-BV786 (RM4-5, BD Biosciences), CD11b-FITC (M1/70, Tonbo), CD11c-PECy7 (N418, Biolegend), B220-FITC (RA3-6B2, eBioscience), CD45.1-PerCPCy5.5 (A20, BioLegend), and CD45.2-PE (104, eBioscience).

Chlamydia infections

One week prior to infection, mice were given 2.5mg Depo-Provera (medroxyprogesterone acetate, Pfizer) s.c. in a 0.1mL volume. Mice received $1x10^5$ IFU of *Chlamydia muridarum* intravaginally in 5uL SPG buffer. The strain used for all experiments was derived from ATCC Nigg II stock.

Salmonella infections

Mice infected with *Salmonella enteria* Typhimurium strain BMM50 (SL1344 Δ*aroA*) received $5x10^5$ CFU i.v. Stock bacterial suspensions were streaked onto MacConkey agar, from which one colony was used to inoculate an overnight culture of Luria-Bertani broth. This culture was used to prepare the infection suspension by diluting into 1X PBS for a total injection volume of 0.2mL.

Counting *Chlamydia* burden

To monitor vaginal shedding of *Chlamydia muridarum*, vaginal swabs were taken and placed into 2mL microcentrifuge tubes containing 500uL SPG buffer and two glass beads. The tubes were shaken at 1400rpm for 5min at 4°C, and the swab subsequently discarded. Samples were frozen at -80°C until used for the counting protocol. To determine IFU burden in the organs, spleen, lung,

and kidney harvested from infected mice, organs were homogenized in 2mL SPG. 1mL was transferred to a 2mL microcentrifuge tube with two glass beads and samples were shaken at 1400rpm and 4°C for 5min. To remove debris from suspension, samples were centrifuged at 500g and 4°C for 10min and the supernatant removed and frozen at -80°C. To count the bacteria, samples were diluted in a series used infect a monolayer of HeLa cells in 96-well plates. These were cultured into inclusions overnight, then fixed and stained before counting to calculate IFU per swab.

Flow cytometry

To isolate lymphocytes from lymph nodes, frosted slides were used to break up lymph nodes and washed wish PBS containing 2% fetal bovine serum. To isolate lymphocytes form the female reproductive tract, the FRT was harvested into complete RPMI, then minced into small pieces and incubated with collagenase IV (386mg/L MP Biomedicals) for 1 hour at 37°C. The resulting suspension was filtered (70µm cell strainer, Corning) and lymphocytes isolated on a Percoll gradient (GE Healthcare). For intracellular staining, cells were cultured in stimulating conditions with PMA (0.2 mM, Millipore Sigma) and Ionomycin (1ug/mL, Millipore Sigma) along with Brefeldin A (71.4uM Millipore) for 3.5 hours at 37°C 5% CO_2. A viability stain was performed first using Zombie Yellow (BioLegend), then surface markers were stained, including B220-APC-eF780 (RA3-6B2, eBioscience), CD11b-APC-eF780 (M1/70, eBioscience), CD11c-APC-eF780 (N418, eBioscience), F4/80-APC-eF780 (BMB, eBioscience), CD4-PE (RM4-4, eBioscience), CD4-eF450 (RM4-5, eBioscience), CD8-PerCPCy5.5 (2.43, Tonbo), CD44-APC (IM7, eBioscience), and CD62L-PETexasRed (MEL-14, Invitrogen). Then intracellular stains IFN-γ-BV785 (XMG1.2, BioLegend), T-bet-PECy7 (4B10, eBioscience), RORγt-BV421 (Q31-378, BD Biosciences), and IL-17A-FITC (17B7, eBioscience) were used with the Foxp3 Transcription Factor Staining Kit (eBioscience). Data was acquired on an LSRFortessa (BD) and analyzed using FlowJo (Tree Star, San Carlos, CA). Contour plots are shown with 5% outliers.

Bulk RNA-seq

The bulk RNA-seq experiment was performed as previously described[27]. Briefly, five samples per group were prepared by combining three mice per sample. Lymphocytes were isolated out of the FRT 17 days post infection as described above before running negative MACS CD4 T cell isolation with LS columns (Miltenyi Biotech). CD4s were further enriched by flow sorting on a BD FACSAria and sorted cells used for RNA isolation using a Qiagen RNeasy Mini Kit. Library construction, sequencing, and analysis are as described in detail in prior study. Raw data can be accessed via the NCBI's Gene Expression Omnibus[53] through GEO Series accession number GSE193909.

Cytokine depletion *in vivo*

Mice were given 250ug each of anti-IL-6 (MP5-20F3, cat. BE0046, BioXCell) and anti-TGF-β (1D11.16.8, cat. BE0057, BioXCell), or isotype controls IgG1 anti-horseradish peroxidase (HRPN, cat. BE0088, BioXCell) and IgG1 unknown specificity (MOPC-21, cat. BE0083, BioXCell) i.p. starting on day 0 and every other day for 6 weeks.

Statistics

Statistics were performed using GraphPad Prism version 9 (GraphPad Software, LLC). Either t-test or one-way ANOVA were used. * $p<0.05$, ** $p<0.01$, *** $p<0.01$, and **** $p<0.0001$.

Acknowledgements

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Chapter 4

Chlamydia clearance in the FRT is dependent on IL-12p40-driven mechanisms

Jordan A. Rixon, Claire Morris, and Stephen J. McSorley

Introduction

Thus far, it is clear that CD4 T cells are required for clearing primary *Chlamydia muridarum* infection in the female reproductive tract (FRT) [1], but it is unknown which Th subset or subsets are able to enact an effector module effective for clearance. We have determined that Th1, Th2, and Th17 cells alone are not necessary for clearance from the FRT (Chapter 2 [2], Chapter 3). Additionally, blocking compensation between Th1 and Th17 modules using T-bet-deficient mice and either treating with neutralizing anti-IL-6 and anti-TGF-β or breeding them with RORγt mutant mice [3] was unable to recapitulate the severe delay in clearance seen in mice unable to mediate CD4 T cell responses entirely. Clearly, other factors are at play regulating an effective CD4 T cell response in *Chlamydia* infection.

The fact that Th1 cells are not required for clearance and indeed show little Th1 identity, with very low expression of the master Th1 transcription factor T-bet (Chapter 2 [2]), conflicts with a prior experiment using neutralizing anti-IL-12p40 ostensibly to block Th1 development that showed a significant delay in clearance [4]. Here, we revisited the involvement of IL-12 signaling to determine if IL-12p40 is involved in instructing the CD4 T cell response even though T-bet-defined Th1 cells are not. This cytokine subunit is a part of the IL-12 family of heterodimeric cytokines [5]. IL-12p40 can pair with IL-12p35, to form IL-12, or with IL-23p19 to form IL-23. The receptor for IL-12 is comprised of IL-12Rβ1 and IL-12Rβ2 subunits and signals through STAT4 to promote Th1 responses, while the receptor for IL-23 is comprised of IL12Rβ1 and IL-23R and signals through STAT3 and STAT4 to promote Th17 responses [6, 7]. Therefore, blocking IL-12p40 signaling also represents an alternative method to blocking both Th1 and Th17 pathways

simultaneously, though IL-12p40 could also regulate gene expression independently of Th1 and Th17 master transcription factors [8].

Surprisingly, unlike the previous two models we tested to block Th1 and Th17 responses (Chapter 3), IL-12p40-deficient mice exhibited a severe deficiency in the ability to clear FRT infection, though were eventually able to mediate clearance at a late time point. This is despite the capacity for CD4 T cells from these mice to secrete IFN-γ, further indicating that IFN-γ is not the critical cytokine mediating clearance in the FRT. This delay in clearance was dependent on the *Chlamydia muridarum* strain used, as the less virulent Nigg strain showed only a slight delay, as observed previously by another laboratory [9]. Analyzing these mice with scRNA-seq shows that overall, the IL-12p40-deficient mice have a surprisingly similar CD4 T cell transcriptional response to wild-type mice. The deficiency in clearance may be explained by an expanded Treg response or the decreased expression of certain effector molecules. In summary, our studies have showed that IL-12p40 drives critical components of the CD4 T cell response to *Chlamydia* in the FRT, which in wild-type mice is not acting through classical T-bet^{hi} IFN-γ+ Th1 pathways. This could represent a particular Th response pathway geared towards intracellular bacteria that reside in mucosal epithelial cells rather than macrophages that are a part of classical Th1 responses.

Results

IL-12p40-deficient mice cannot efficiently clear *Chlamydia* FRT infection despite IFN-γ expression

Our laboratory has previously used two mouse models to block access simultaneously to Th1 and Th17 programming without finding a severe delay in shedding (Fig. 3.7). These methods involved treating T-bet-deficient mice with neutralizing anti-IL-6 and anti-TGB-β, as well as breeding T-bet-deficient mice to RORγt mutant mice. The depleting antibody method as well as the particularities of the RORγt mutation have a potential for incomplete blockade of RORγt. Here, we used a third mouse model aiming for the same effect of Th1/17 blockade in an effort to avoid these potential issues. These mice are deficient in the cytokine subunit IL-12p40, which is shared between IL-12 and IL-23 that respectively promote Th1 and Th17 cells. Infecting these mice with *Chlamydia muridarum* resulted in a severe delay in clearance (Fig. 4.1), unlike the other two models previously tested (Fig. 3.7). The level of bacteria shed from the FRT remained at peak levels for the first three weeks of infection, equivalent to MHC class II-deficient controls. After this, IL-12p40-deficient mice did begin to slowly clear and were largely culture negative by 80 days post infection (Fig. 4.1A and B), while MHC class II-deficient mice were still robustly shedding bacteria. Thus, IL-12p40-deficient mice show the most severe delay in shedding observed so far in mice that can still generate CD4 T cell responses to *Chlamydia*.

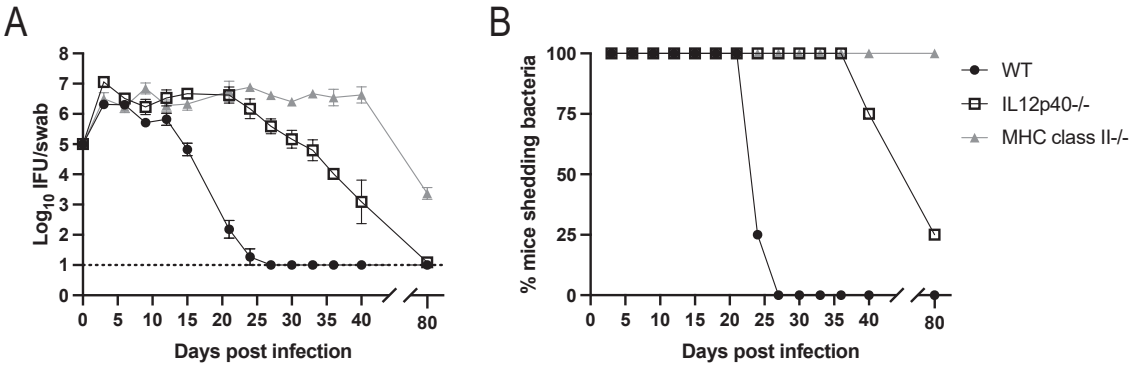

Figure 4.1: IL-12p40-deficient mice exhibit a severe delay in *Chlamydia* clearance in the FRT.

A) IFUs isolated from vaginal swabs at various time points after infection. n=4 for all groups. Graph is displayed as mean ± SEM. B) Data from A expressed as the percent of mice with culture positive vaginal swabs. Data is representative of two experiments.

We next investigated whether the responding CD4 T cells in these mice expressed Th1 and Th17 markers using flow cytometry. As observed previously, T-bet-deficient controls showed impaired IFN-γ production and upregulated RORγt and IL-17A, supporting a dramatic shift towards Th17 responses, while RORγt mutant controls retained similar expression of all markers as wild-type mice (Fig. 4.2A-D). T-bet-deficient X RORγt mutant mice and IL-12p40-deficient mice showed a similar reduction in the percent of IFN-γ+ cells and T-bet expression level as T-bet-deficient mice, indicating that both are inhibited for Th1 type programs (Fig. 4.2A and B). Compared to the high level of Th17 markers seen in T-bet-deficient mice, however, the T-bet-deficient X RORγt mutant mice did show a much reduced percentage of cells expressing IL-17A, indicating that the RORγt mutation is indeed blocking Th17 pathways, though the level remained above that in wild-type mice in the FRT (Fig. 4.2C and D). This may mean that the RORγt mutation cannot block all of the signaling promoting Th17 pathways in the absence of T-bet. In contrast, IL-12p40-deficient cells were not significantly different from wild-type with respect to the percentage of IL-17A producing cells or the expression level of RORγt (Fig. 4.2C and D). None of these groups showed significantly different total numbers of CD44hi CD4+ T cells in the FRT (Fig. 4.2E), indicating that the mild deficiency in clearance in the T-bet-deficient X RORγt mutants (Fig. 3.7) and severe deficiency in IL-12p40-deficient mice (Fig. 4.1) are not due to lower CD4+ T cell infiltration. Interestingly, the T-bet-deficient X RORγt mutant mice did have a higher percentage

and number of activated CD4 T cells in the draining iliac lymph node, potentially pointing to a tendency to retain T cells in the draining lymph node.

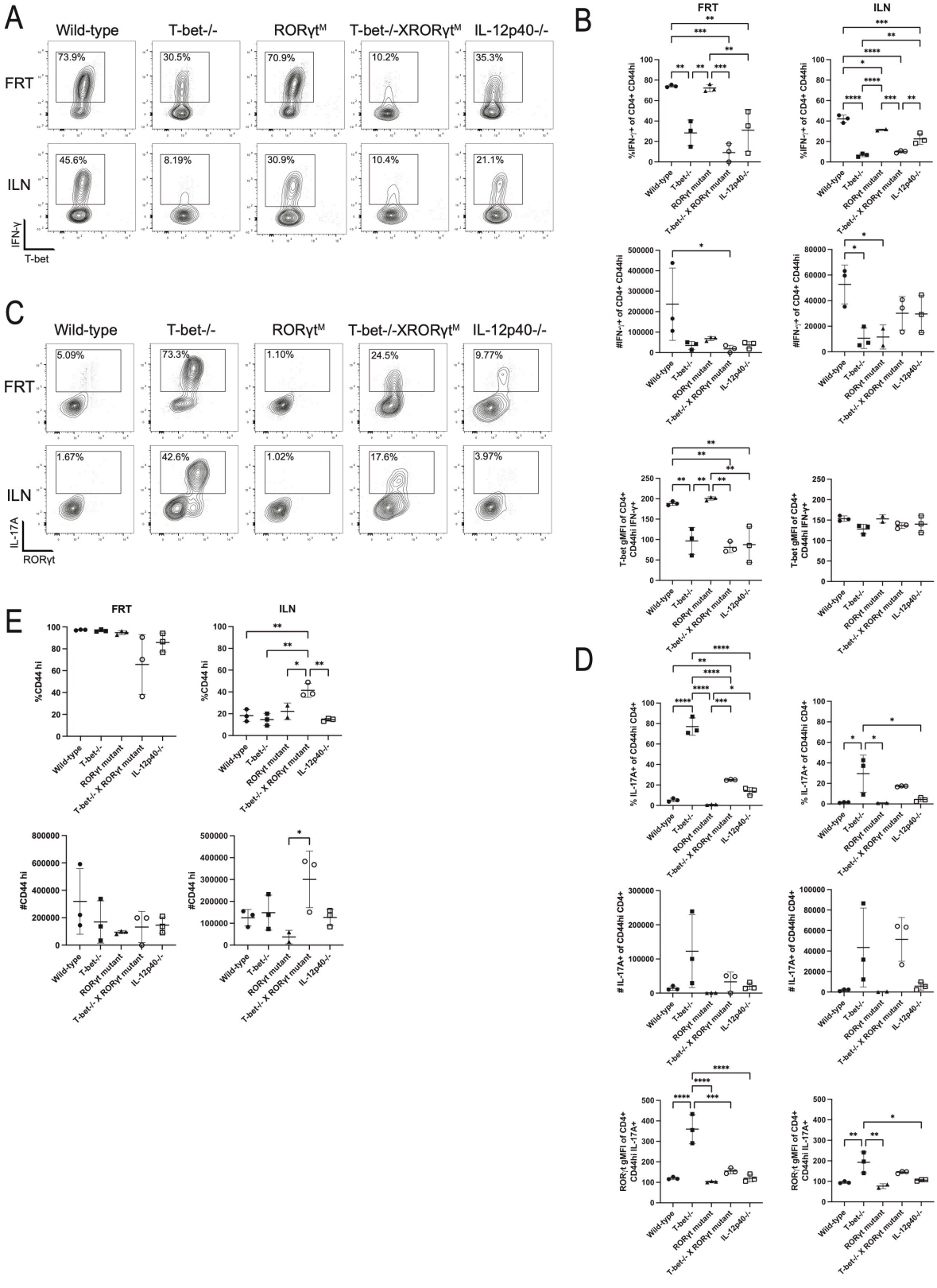
A
Wild-type
T-bet-/-
RORγt^M
T-bet-/-XRORγt^M
IL-12p40-/-
FRT
ILN
73.9%
30.5%
70.9%
10.2%
35.3%
45.6%
8.19%
30.9%
10.4%
21.1%
IFN-γ
T-bet
B
FRT
ILN
%IFN-γ+ of CD4+ CD44hi
#IFN-γ+ of CD4+ CD44hi
T-bet gMFI of CD4+ CD44hi IFN-γ+
T-bet gMFI of CD4+ CD44hi IFN-γ+
C
Wild-type
T-bet-/-
RORγt^M
T-bet-/-XRORγt^M
IL-12p40-/-
FRT
ILN
5.09%
73.3%
1.10%
24.5%
9.77%
1.67%
42.6%
1.02%
17.6%
3.97%
IL-17A
RORγt
D
% IL-17A+ of CD44hi CD4+
# IL-17A+ of CD44hi CD4+
RORγt gMFI of CD4+ CD44hi IL-17A+
RORγt gMFI of CD4+ CD44hi IL-17A+
E
FRT
ILN
%CD44 hi
#CD44 hi
Wild-type
T-bet-/-
RORγt mutant
T-bet-/- X RORγt mutant
IL-12p40-/-

Figure 4.2: T-bet-deficient X RORγt mutant mice and IL-12p40-deficient mice show altered patterns of Th1 and Th17 markers.

Lymphocytes isolated from the FRT and ILN were stimulated with PMA and ionomycin with Brefeldin A before staining for flow cytometry. Results are gated on CD4+ CD44hi cells. n=3 for all groups except RORγt mutant ILN, where n=2 where ILN were unable to be recovered in one mouse. A) Expression of Th1 markers T-bet and IFN-γ. B) Summary graphs from A. C) Expression of Th17 markers RORγt and IL-17A. D) Summary graphs from C. E) Percentages and numbers of total CD44hi CD4 T cells. All graphs are displayed as mean ± SD. Data is representative of two experiments.

The delayed clearance phenotype of IL-12p40-deficient mice is dependent on the *Chlamydia* strain

The observed severe delay in shedding in IL-12p40-deficient mice was a surprise because these mice had previously been tested by Chen et. al. in *Chlamydia* FRT infection, where shedding was only mildly delayed [9]. In comparing the methods, our lab used the strain of *Chlamydia muridarum* derived from ATCC stock while Chen et al. used the Nigg strain. The ATCC stock is a derivative of a Weiss strain of *Chlamydia muridarum* that is more virulent than Nigg [10]. We acquired the Nigg strain and tested it in parallel with the ATCC strain to see if this explained the differences observed between these experiments. Indeed, using the less virulent Nigg strain was able to recapitulate a mild delay in shedding while the more virulent ATCC strain resulted in a more severe delay in IL-12p40-deficient mice (Fig. 4.3A). The mild delay phenotype with the Nigg strain indicated that it was possible that *Chlamydia* clearance from the FRT for this less virulent strain was independent of CD4 T cell responses. We thus additionally tested these strains in MHC class II-deficient mice to make sure there was no difference and indeed observed similar bacterial kinetics (Fig. 4.3B). In conclusion, the increased virulence of the ATCC derived bacterial

strain likely increases dependence on the IL-12p40-driven mechanism mediating clearance than the Nigg strain, while both require CD4 T cells for resolution.

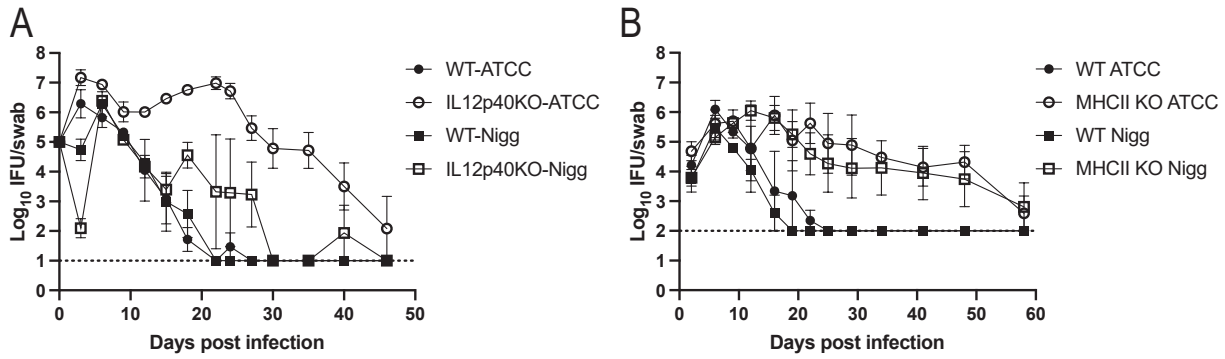

Figure 4.3: The severe delay in clearance in IL-12p40 deficient mice is dependent on the *Chlamydia* strain used, though MHC class II deficient mice do now show a difference in response.

A) Wild-type or IL-12p40 deficient mice were infected with either *Chlamydia* derived from ATCC stock or Nigg strain. IFUs were counted from vaginal swabs over the course of infection. n=3 for both wild-type groups, n=3 for IL-12p40KO-ATCC, and n=2 for IL-12p40KO-Nigg. B) Wild-type or MHC class II-deficient mice were infected with either *Chlamydia* derived from ATCC stock or Nigg strain. IFUs were counted from vaginal swabs over the course of infection. n=2 for wild-type ATCC, n=4 for MHCIIKO ATCC, n=3 for wild-type Nigg, and n=4 for MHCIIKO Nigg. Graphs are displayed as mean ± SEM.

scRNA-seq shows changes in inflammatory cell infiltrates present in different mouse models

To determine what was different in IL-12p40-deficient mice that prevented clearance, we turned to single cell RNA sequencing. Lymphocytes were isolated from the FRTs of wild-type, T-bet-deficient, T-bet-deficient X RORγt mutant, and IL-12p40-deficient mice at day 14 (early), as well as IL-12p40-deficient mice at day 30 (late) in two replicates. These time points were chosen

because IL-12p40-deficient mice at day 14 are not actively clearing infection from the FRT while all other groups are, enabling us to perform multiple comparisons between various groups to determine what might be driving or inhibiting clearance. All samples were visualized on a UMAP plot with cell assignments in Fig. 4.4A. The proportions of cell types amongst each experimental group are represented in Fig. 4.4B. In the wild-type group, most cells are T cells, neutrophils, and macrophages, with some B cells, NK cells, other immune cells, and some non-immune cells from the FRT that likely contaminated the lymphocyte prep. The T-bet-deficient group had largely the same cell proportions as wild-type, while T-bet-deficient X RORγt mutant samples had less CD4 T cells (not seen in Fig. 4.2) and more neutrophils and other assorted T cells that are possible eosinophils or basophils. This contraction of T cells and expansion of neutrophils was also seen in the IL-12p40-deficient early group, and thus this shift does not provide an explanation why T-bet-deficient X RORγt mice can clear while IL-12p40-deficient cannot at this time point. The IL-12p40-deficient late group showed a similar proportion of T cells as the early group, but fewer neutrophils and macrophages, and expanded B cells and NK cells. This may either be a product of the progression of time during *Chlamydia* infection or may be related to why IL-12p40-deficient mice can clear at a much later time point. An increase in B cells making antibody responses and NK cell-mediated killing of infected cells may contribute to clearance at this late time point, though further work will be required to determine if this is the case.

On the UMAP plot of all samples, the T cell population may be visualized by expression of *Cd3g* (Fig. 4.4C). Within this region, CD4 and CD8 T cells can be generally described by expression of *Cd4* and *Cd8a* and describe mutually exclusive regions. This enables identification of clusters on the UMAP corresponding to the transcriptional programs of CD4 and CD8 T cells during *Chlamydia* infection.

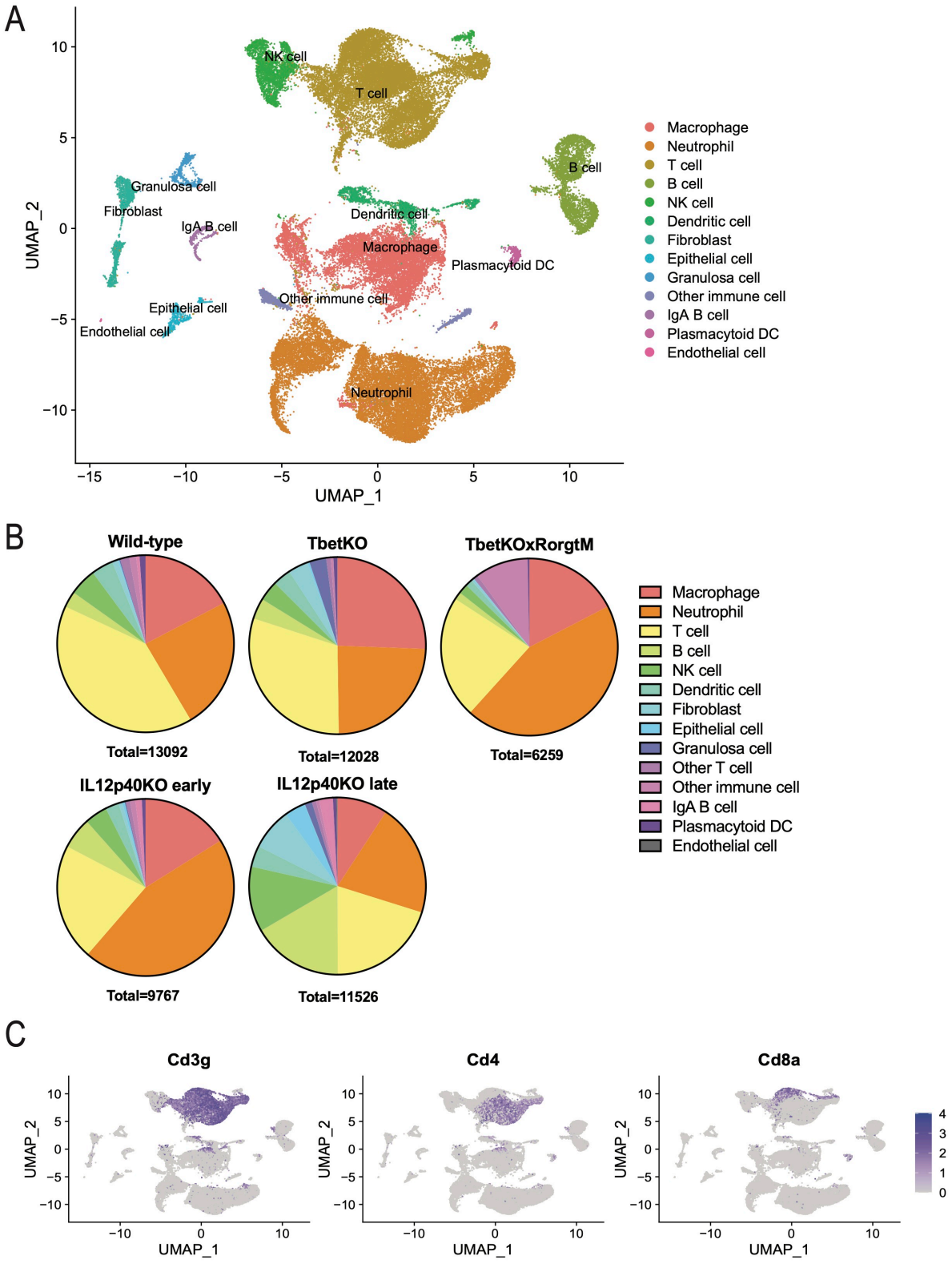
A
NK cell
T cell
B cell
Granulosa cell
Fibroblast
IgA B cell
Dendritic cell
Macrophage
Plasmacytoid DC
Other immune cell
Epithelial cell
Endothelial cell
Neutrophil
UMAP_1
UMAP_2
Macrophage
Neutrophil
T cell
B cell
NK cell
Dendritic cell
Fibroblast
Epithelial cell
Granulosa cell
Other immune cell
IgA B cell
Plasmacytoid DC
Endothelial cell
B
Wild-type
TbetKO
TbetKOxRorgtM
Total=13092
Total=12028
Total=6259
IL12p40KO early
IL12p40KO late
Total=9767
Total=11526
Macrophage
Neutrophil
T cell
B cell
NK cell
Dendritic cell
Fibroblast
Epithelial cell
Granulosa cell
Other T cell
Other immune cell
IgA B cell
Plasmacytoid DC
Endothelial cell
C
Cd3g
Cd4
Cd8a
UMAP_1
UMAP_2

Figure 4: scRNA-seq shows altered proportions of immune cell infiltrates in different mouse models.

Lymphocytes were isolated from the FRT of Wild-type, T-bet deficient, IL-12p40 deficient, and T-bet deficient mice crossed with RORγt mutants on day 14 post infection as well as IL-12p40 deficient mice 30 days post infection. Infections were staggered such that all samples were processed on the same day. Samples were processed for scRNA-seq using 10X Genomics technology. A) UMAP of all cells from all samples with assigned cell identities. B) The proportions of cell types across each experimental group. C) Feature plots of *Cd3g*, *Cd4*, and *Cd8a*.

The CD4 T cell population contains four different clusters

We next isolated clusters corresponding to the transcriptional signature of CD4 T cells in order to compare differences within these cells, as they are the critical mediators of clearance. After reclustering the data, four distinct clusters became apparent (Fig. 4.5A). The top 10 genes defining each cluster are listed in Fig. 4.5B. The Th1-like population, most represented in wild-type and the two IL-12p40-deficient groups, has the strongest signature for T-bet and IFN-γ (gene names *Tbx21* and *Ifng*) (Fig. 4.6A, 4.5C). We have termed them Th1-like rather than true Th1 due to the low expression of T-bet previously observed in wild-type mice, particularly compared to T-bet+ Th1 cells resulting from *Salmonella enterica* infection (Chapter 2 and 3). The Th17 population, most represented in the T-bet-deficient and T-bet-deficient X RORγt mutant groups, has the strongest signature of RORγt and IL-17A (gene names *Rorc* and *Il17a*) (Fig. 4.6A, 4.5C). These observations are in line with what was previously seen using flow cytometry in Figure 4.2. Interestingly, the Th17 cells from the T-bet-deficient group and T-bet-deficient X RORγt mutant group form neighboring clusters on the UMAP and do not overlap completely (Fig. 4.6A), further supporting that the RORγt mutant allele is able to block some Th17 function compared to cells in the T-bet-deficient group. The additional two clusters are Tregs and an “Other” population of

indeterminate type. The top ten markers of the Treg cluster notably contains *Foxp3*, the master transcription factor for Tregs (Fig. 4.5B).

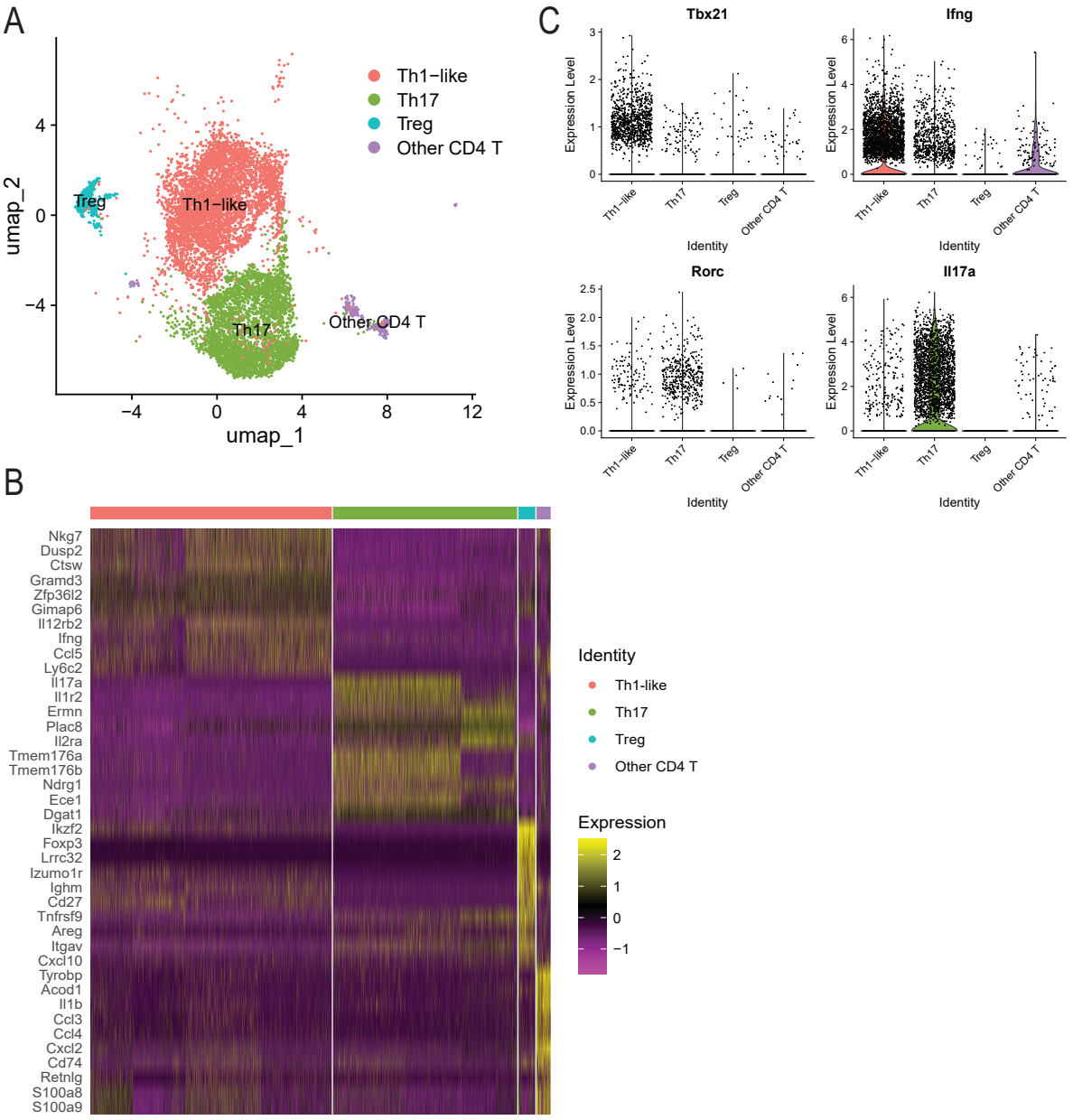

Figure 4.5: The CD4 T cell population shows four distinct clusters.

CD4 T cells from all samples were isolated in the scRNA-seq data set. A) UMAP of reclustered CD4 T cell subset of the scRNA-seq data. B) Heatmap of top 10 marker genes for each cluster. C) Violin plots of expression of *Tbx21*, *Ifng*, *Rorc,* and *Il17a* across each of the four clusters.

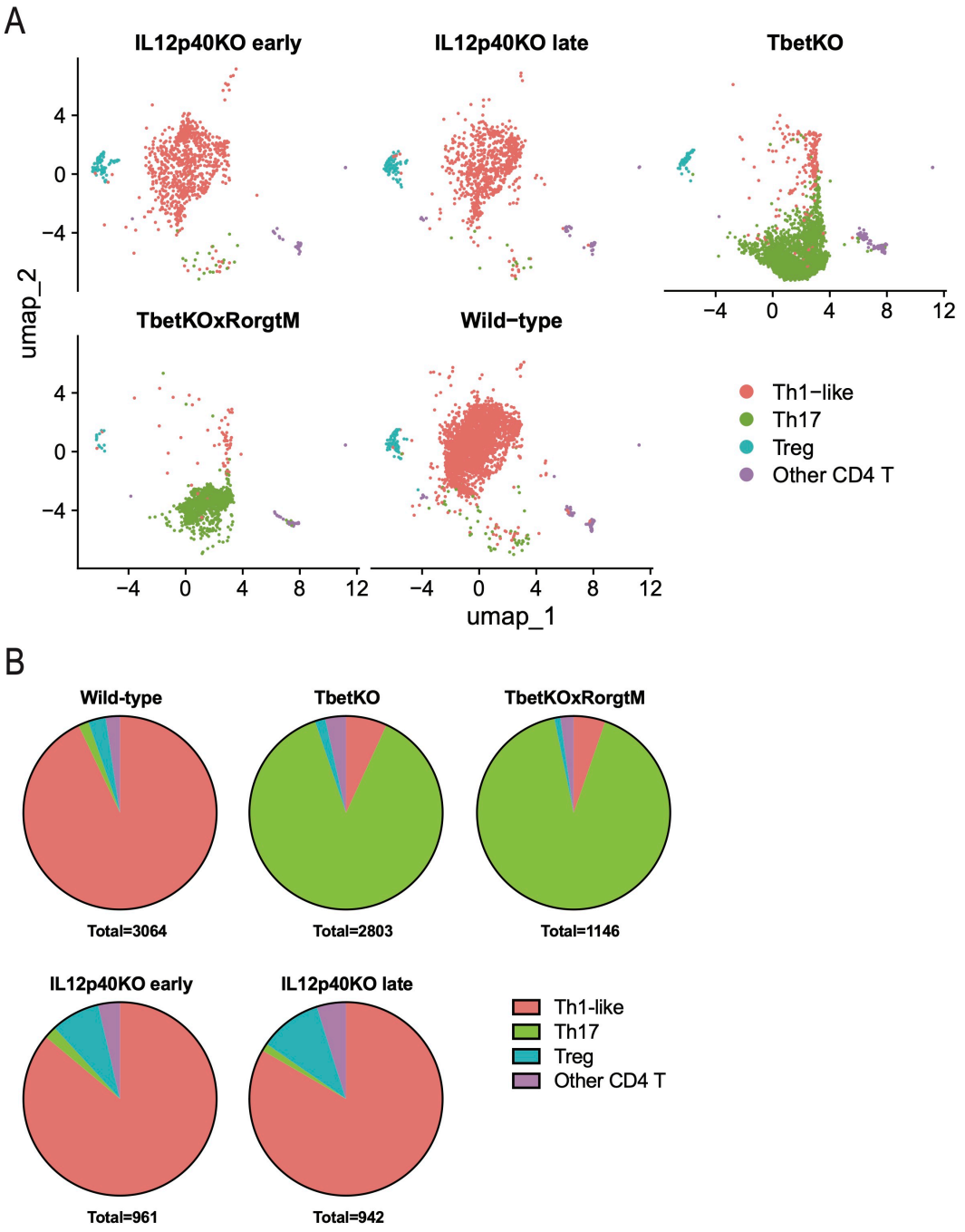

Figure 4.6: Different mouse models show different proportions of CD4 Th subsets.

A) UMAP from Fig. 4.5A split amongst experimental group. B) The proportions of assigned CD4 Th identity across each experimental group.

IL-12p40-deficient mice have an expanded Treg population

The Treg cluster was minor and represented in all samples, though appeared to be somewhat expanded in the IL-12p40-deficient early and late groups in this scRNA-seq data. Tregs represent 3% of CD4 T cells in wild-type mice and 8 and 11% in IL-12p40-deficient early and late groups respectively (Fig. 4.6B). We confirmed this expansion using flow cytometry and saw that IL-12p40-deficient mice had about 5% percent Foxp3+ CD4 T cells in the FRT 14 days post infection compared to 3% in wild-type mice (Fig. 4.7A). The total number of Foxp3+ cells was reduced in IL-12p40-deficient mice in this experiment (Fig. 4.7B), which reflected a lower total number of CD4+ cells isolated from this experiment compared to wild-type. This is in contrast to Figure 4.2E, potentially representing experimental variability. In summary, an increased proportion of anti-inflammatory Tregs to pro-inflammatory cells in the FRT may contribute to the delay in clearance in IL-12p40-deficient mice.

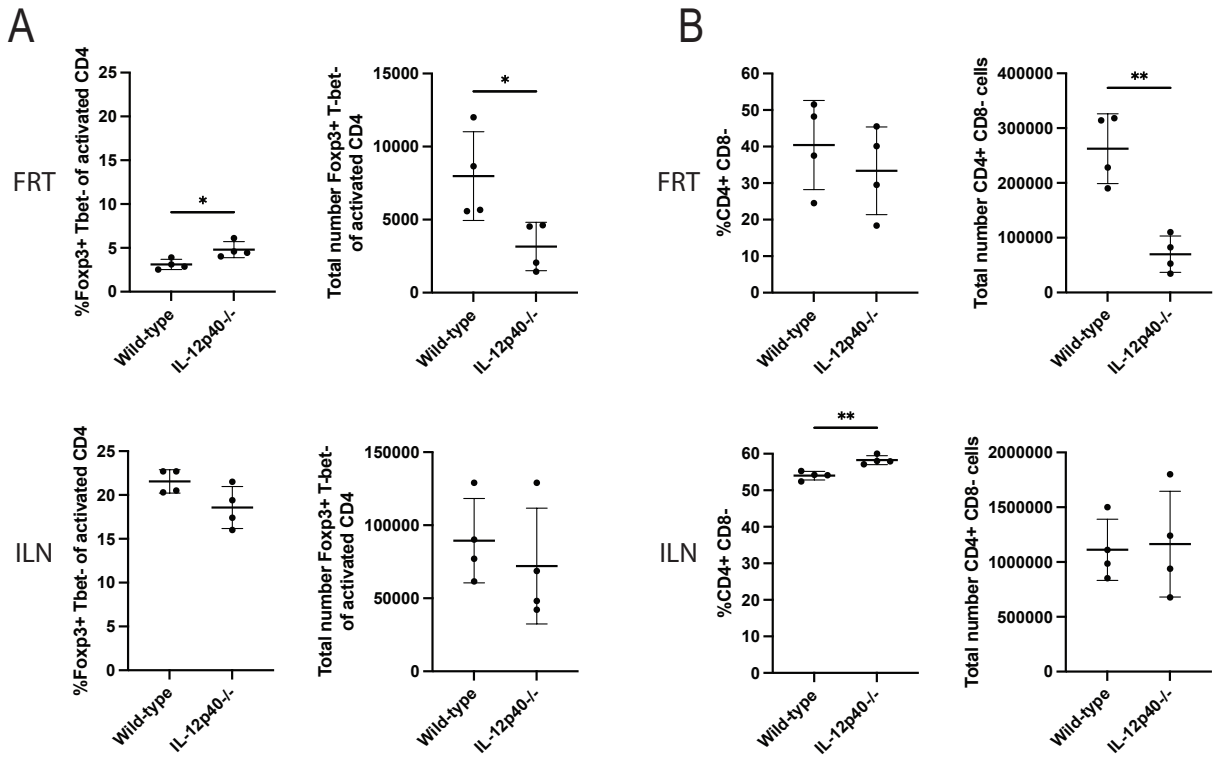

Figure 4.7: IL-12p40-deficient mice have an increased population of Tregs.

Lymphocytes were isolated from the FRT and ILN of Wild-type and IL-12p40-/- mice 14 days post infection and stimulated with PMA and ionomycin with Brefeldin A before staining for flow cytometry. Results are gated on CD4+ CD44hi cells. n=4 for each group. A) Expression of Foxp3+. B) Proportion of total CD4+ cells.

Differential gene expression analysis shows that T-bet-deficient CD4 T cells have a Th17-shifted signature compared to wild-type

We next analyzed differential gene expression between the CD4 T cell populations of different experimental groups and analyzed expression patterns using Gene Ontology (GO) terms. Table 4.1 lists selected GO terms of biological processes associated with genes found to have a positive fold change in CD4 T cells from T-bet-deficient mice versus wild-type. Table 4.2 lists specific differentially expressed genes from this comparison related to these terms. Significant GO terms include Th1 and Th17 regulation and differentiation, as well as factors associated with each subset like IFN-γ for Th1 and TGF-β, IL-6, CXCL1, IL-23, and IL-17 for Th17, though *Il6* and *Cxcl1* themselves were not differentially expressed. *Il17a* had the largest fold change in this comparison, in agreement with the large shift to IL-17A production as seen in previous flow cytometry experiments (Fig. 4.2C-D). IFN-γ production was also seen to be decreased in Fig. 4.2A-B, reflected in the decreased fold change here. Decreased expression of T-bet (*Tbx21*) was indeed detectable in the T-bet-deficient group. The upregulation of *Il15ra* and the corresponding GO term identifying a response to IL-15 may be reflective of a negative feedback pathway for Th17 cell development, as IL-15 signaling has been shown to downregulate IL-17A levels [11]. IL-1-mediated signaling was also identified by the GO analysis and both *Il1r1* and *Il1r2* were upregulated in T-bet-deficient mice. IL1R1 can support Th17 development, while IL1R2 is a decoy receptor that can block IL-1R1 signaling [12, 13]. IL-2 signaling can restrict Th17 development

[14], but IL-2 has also been shown to promote cytotoxicity in CD4 T cells [15]. Here, *Il2ra* (CD25) and *Il2rb* are also upregulated and may represent a pathway for restricting Th17 cells and pathological responses or may be driving such a cytotoxic module for eliminating infected epithelial cells. Additional Th17 functionalities are also listed in the GO terms for the regulation of antimicrobial responses, potentially pointing to T-bet-deficient mice contributing to *Chlamydia* clearance through upregulation of antimicrobial peptides from the FRT epithelial layer. Further experiments will be required to validate these differences and delineate which pathways are dominant in T-bet-deficient mice.

Table 4.1: Selected GO terms associated with differentially expressed genes with a positive fold change between the CD4 T cells of the T-bet-deficient experimental group versus wild-type.

p value	GO Term ID	Term Name
1.14E-07	GO:0007179	transforming growth factor beta receptor signaling pathway
2.57E-05	GO:0030217	T cell differentiation
8.72E-05	GO:0034341	response to interferon-gamma
8.32E-03	GO:0071352	cellular response to interleukin-2
1.01E-02	GO:0032675	regulation of interleukin-6 production
1.25E-02	GO:0072566	chemokine (C-X-C motif) ligand 1 production
1.25E-02	GO:0002826	negative regulation of T-helper 1 type immune response
1.28E-02	GO:0034612	response to tumor necrosis factor
1.56E-02	GO:0032479	regulation of type I interferon production
1.67E-02	GO:0071350	cellular response to interleukin-15
2.13E-02	GO:0002760	positive regulation of antimicrobial humoral response
2.13E-02	GO:0002784	regulation of antimicrobial peptide production
2.13E-02	GO:2000659	regulation of interleukin-1-mediated signaling pathway
3.66E-02	GO:0072539	T-helper 17 cell differentiation
3.69E-02	GO:0032667	regulation of interleukin-23 production
4.84E-02	GO:0097400	interleukin-17-mediated signaling pathway

Table 4.2: Selected genes differentially expressed in the CD4 T cells of T-bet-deficient versus wild-type mice.

Gene	Protein	Average Log2 Fold Change	Percent of T-bet-/- cells expressing	Percent of WT cells expressing	Percent difference	Adjusted p value
Il17a	IL-17A	3.98	0.67	0.02	0.65	0.0E+00
Il17f	IL-17F	0.96	0.14	0.01	0.13	9.8E-83
Il22	IL-22	0.71	0.05	0.01	0.04	4.0E-16
Il23r	IL-23R	0.63	0.25	0.03	0.22	1.3E-134
Tbx21	T-bet	-0.57	0.02	0.23	-0.21	2.1E-122
Ifng	IFN-ɣ	-1.48	0.19	0.60	-0.41	7.1E-219
Il15ra	IL-15Rα	0.27	0.157	0.086	0.071	1.7E-13
Il1r2	IL-1RB	2.27	0.735	0.162	0.573	0.0E+00
Il1r1	IL-1RA	0.90	0.41	0.085	0.325	3.1E-183
Il2ra	CD25/IL-2Rα	1.14	0.578	0.295	0.283	1.3E-130
Il2rb	IL-2Rβ	0.52	0.934	0.867	0.067	2.4E-96
Tgfb1	TGF-β	0.43	0.757	0.642	0.115	6.9E-40

IL-12p40-deficient CD4 T cells show deficiencies in expression of various genes compared to wild-type and T-bet-deficient groups

We likewise analyzed differential gene expression in CD4 T cells between IL-12p40-deficient mice at day 14 (early) and wild-type to identify deficiencies in the IL-12p40-deficient mice that resulted in these mice being unable to clear bacteria in the first phase of infection. In accordance with the positioning of the clusters for these samples in the same location on the UMAP plot (Fig. 4.6A), differences between these groups were small and only 160 total genes were determined to be differentially expressed (Supplemental Table 4.1). The fold changes and differences between the percentage of the population of each group expressing a given gene are also relatively small across much of this list, further underlining the similarity between them.

We ran GO analysis for the genes with a negative fold change in this comparison (Table 4.3). Here again can be seen terms related to Th1 responses, corresponding with flow cytometry-

based observations of lessened levels of Th1 markers IFN-γ and T-bet (Fig. 4.2). IFN-γ was the second most downregulated gene, though *Tbx21* (T-bet) was not found to be differentially expressed (Supplemental Table 4.1). Th17 responses were also listed in GO analysis, including IL-17 and IL-23 specifically. *Il17a* was upregulated in IL-12p40-deficient mice, while this is not a significant different difference seen via flow cytometry, nor was the Th17 cluster expanded in these mice (Fig. 4.2, Fig. 4.6B), Additionally, regulation of CD25+ Treg cells was identified by GO analysis, in accordance with the expansion of the Treg population in these mice (Fig. 4.6B, Fig 4.7A) and Treg transcription factor *Foxp3* was indeed upregulated (Supplemental Table 4.1).

Table 4.3: Selected GO terms associated with differentially expressed genes with a negative fold change between the CD4 T cells isolated on day 14 of IL-12p40-deficient versus wild-type mice.

p value	GO Term ID	Term Name
1.02E-08	GO:0042110	T cell activation
1.34E-09	GO:0001816	cytokine production
1.02E-05	GO:0032651	regulation of interleukin-1 beta production
4.73E-05	GO:0032729	positive regulation of interferon-gamma production
5.11E-05	GO:0071351	cellular response to interleukin-18
5.52E-05	GO:0032743	positive regulation of interleukin-2 production
5.55E-05	GO:0006809	nitric oxide biosynthetic process
6.25E-05	GO:1903039	positive regulation of leukocyte cell-cell adhesion
1.56E-04	GO:0032755	positive regulation of interleukin-6 production
3.81E-04	GO:0002294	CD4-positive, alpha-beta T cell differentiation involved in immune response
1.53E-03	GO:0035744	T-helper 1 cell cytokine production
1.56E-03	GO:0072538	T-helper 17 type immune response
9.09E-03	GO:0032753	positive regulation of interleukin-4 production
1.18E-02	GO:0150127	regulation of interleukin-33 production
1.18E-02	GO:0140460	response to Gram-negative bacterium
1.18E-02	GO:1901394	positive regulation of transforming growth factor beta1 activation
1.18E-02	GO:0032832	regulation of CD4-positive, CD25-positive, alpha-beta regulatory T cell differentiation involved in immune response
1.81E-02	GO:0032660	regulation of interleukin-17 production
1.81E-02	GO:0032735	positive regulation of interleukin-12 production
2.01E-02	GO:0032733	positive regulation of interleukin-10 production
2.62E-02	GO:0098758	response to interleukin-8
3.09E-02	GO:0001912	positive regulation of leukocyte mediated cytotoxicity
3.21E-02	GO:0072535	tumor necrosis factor (ligand) superfamily member 11 production

4.73E-02	GO:0032747	positive regulation of interleukin-23 production

Gene Set Enrichment Analysis (GSEA) of genes differentially regulated in IL-12p40-deficient CD4 T cells versus wild-type identified a set of genes previously found in differences between human CD4 T cells activated with IL-12 and TGF-β or activated without these cytokines (Fig. 4.8A) [16]. The gene set includes *Il12rb2*, *Il18r1*, *Man2a2*, *Fes, Il18rap*, *Ifng*, and *Furin*, all of which have a negative fold change in IL-12p40-deficient CD4 T cells relative to wild-type. IL-12Rβ2, part of the receptor for IL-12 but not IL-23, is upregulated after T cell activation and regulated by IFN-γ, which is also expressed at lower levels in IL-12p40-deficient CD4 T cells [17, 18]. The IL-12 receptor complex signals through STAT4 to mediate promotion of Th1 programs [17]. While *Stat4* here was not found to be differentially expressed in these groups, less phosphorylation by the lack of IL-12 signaling could be leading to the lack of upregulation of other factors in this data set. For example, Il-12 signaling can upregulate the receptor for IL-18, after which synergistic signaling can further enforce Th1 programming [19, 20]. The IL-18 receptor gene *Il18r1* and IL-18 receptor accessory protein *Il18rap* that participates in IL-18 signaling were also present on this list, indicating that the IL-12p40-deficient CD4 T cells are unable to take advantage of IL-18 signaling for further activation of effector programming.

Man2a2 encodes Mannosidase Alpha Class 2A Member 2, a mannosidase that has a role in N-glycan processing. Glycosylation patterns affect the function of many immune related proteins. This includes effects on T cell development and thymic function as well as T cell activation and regulation of fate determination [21]. Mannosidases are upregulated after TCR signaling, which can affect co-receptor function and surface levels [22]. Additionally, altering glycosylation patterns can change the balance between pro-inflammatory Th1 and Th17 cells and anti-inflammatory Treg cells through decreasing CD25 surface expression [21, 23, 24]. In *Chlamydia* infection *Man2a2* may be involved in either regulating effectors of bacterial clearance or may be involved in restricting the Treg expansion seen in IL-12p40-deficient mice.

Furin was the most downregulated gene in IL-12p40-deficient cells. The protein product Furin is a proprotein convertase whose basic function is to cleave a consensus sequence [25]. Furin is a type-I transmembrane protein that localizes to the trans-golgi network and can be found also in endosomal compartments and the cell surface as well as immature secretory granules. Cleavage of proproteins by Furin has important roles in embryogenesis and homeostasis pathways. In infection, Furin is key for the cleavage and activation of some pathogen products. Furin activates bacterial toxins like anthrax toxin and viral envelope proteins like influenza hemagglutinin among others. Specifically in immunity, Furin is necessary for cleavage of TNF family members like BAFF and APRIL as well as TGF-β. Mice with Furin-deficient T cells were shown to experience increased autoimmunity due to an inability to process TGF-β [26].

Furin expression has previously been shown to be induced in T cells by IL-12 via STAT4 signaling, upon which it regulates IFN-γ and other Th1 associated genes, in agreement with our observation here that IL-12p40-deficient mice cannot upregulate *Furin* and also have reduced IFN-γ expression [27, 28]. In the context of *Toxoplasma gondii* infection which requires Th1 responses for effective control of the pathogen, Furin-deficient T cells upregulated IL-4Rα and shifted towards Th2 responses [29]. *Furin* expression downstream of STAT4 signaling has also been shown to be independent of T-bet expression, which is low in *Chlamydia* infection [8]. Thus, Furin expression in CD4 T cells responding to *Chlamydia* infection could be involved in promoting anti-*Chlamydia* effector pathways without the involvement of T-bet-driven gene expression.

It is possible that Furin acts directly on extracellular *Chlamydia* elementary bodies. Furin has not been shown yet to directly have a protective effect when cleaving bacterial products, but it will be interesting to explore whether cleavage sites exist within proteins displayed on the *Chlamydia* elementary bodies such that Furin may neutralize the ability of *Chlamydia* to invade new host cells.

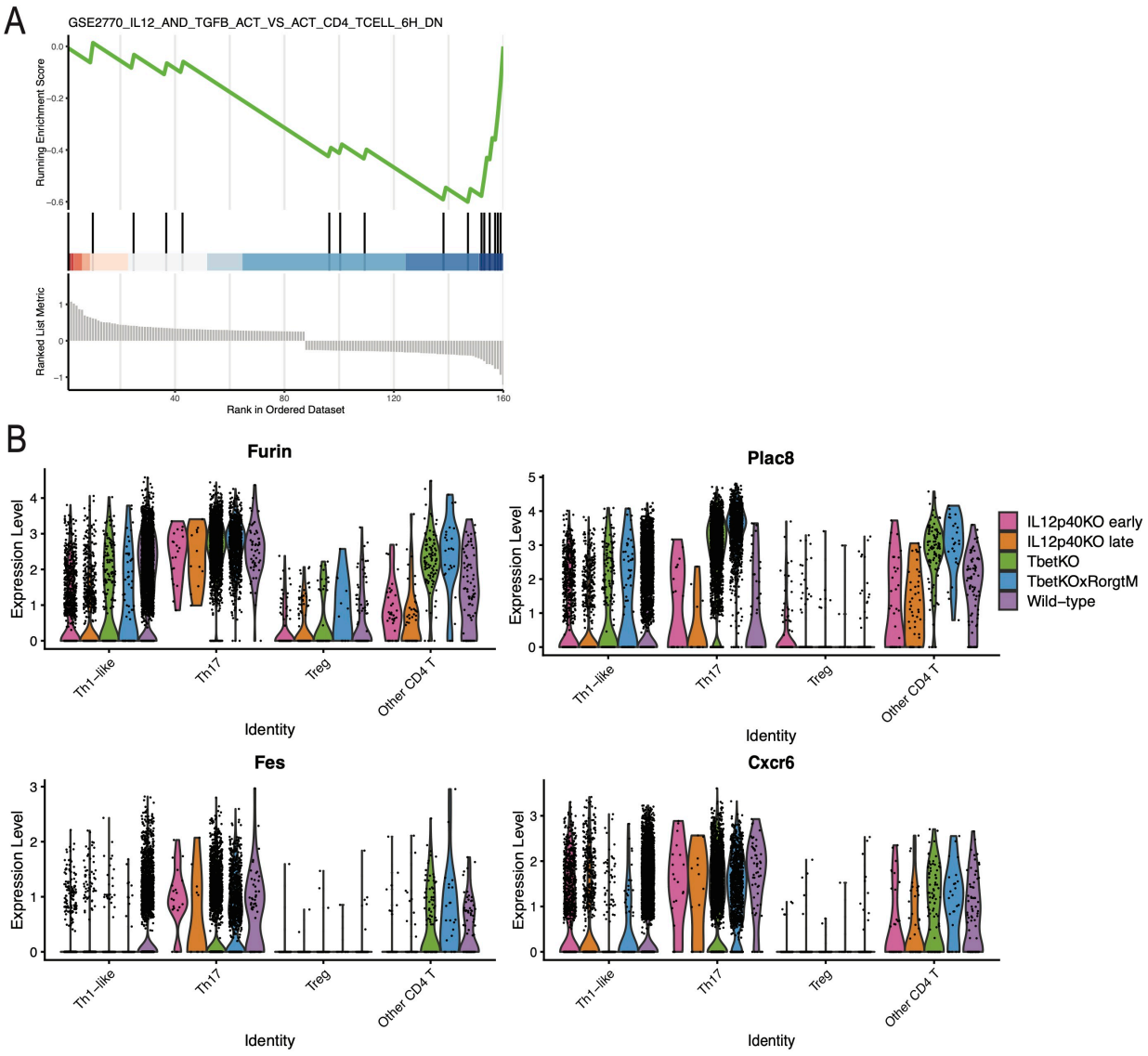

Figure 4.8: IL-12p40-deficient mice have distinctive deficiencies in gene expression.

A) GSEA results from ranked list of differentially expressed genes in IL-12p40-/- vs Wild-type CD4 T cells. Normalized enrichment score = -2.28. B) Violin plots showing expression of genes across CD4 T cell clusters and split by experimental group.

Also identified in the GSEA and in the top five most downregulated genes in the IL-12p40-deficient group is *Fes*, the gene for Feline Sarcoma Oncogene (Fes). Fes is a tyrosine kinase which is directly downstream of *Furin* on chromosome 7 and is regulated together with *Furin* [28].

Fes is expressed in hematopoietic cells and can regulate cytokine signaling. Fes was shown to activate STAT3 upon GM-CSF signaling and is also involved in IL-3 and IL-4 signaling [30-32]. Overall, Furin and Fes, regulated together downstream of IL-12p40 signaling, could be critical for promoting particular cytokine signaling pathways and STAT activation independently of T-bet.

The expression patterns across experimental groups were similar for *Furin* and *Fes* (Fig. 4.8B). These genes were upregulated in respective dominant cluster for the groups that are able to mediate clearance. This is the Th1-like population for wild-type mice and the Th17 population in T-bet-deficient and T-bet-deficient X RORγt mutant mice. However, in the IL-12p40-deficient group inhibited for clearance and mainly represented by Th1-like cells, expression was lower. This indicates that their expression is IL-12p40-dependent yet can be promoted in whichever Th subset is dominant. It will be important to explore this signaling pathway and the genes it regulates to determine which components are acting to mediate clearance of *Chlamydia*.

Other genes that were also downregulated in IL-12p40-deficient CD4 T cells in comparison to both wild-type and T-bet-deficient groups are listed in Table 4.4. The genes on this list not shown in Fig. 4.8B shared similar expression patterns to those that are depicted. *Plac8* (Placenta-specific 8) was originally identified in association with the placenta and has later been linked to other roles in embryonic development, organ development, cancer, autophagy, and apoptosis [33, 34]. In T cells, Slade et al. determined that *in vitro* IL-12 signaling induced *Plac8* more so in Th1 CD4 T cells than Th2, Th17 and Treg subsets and also upregulated basal expression in CD8 T cells [35]. This corresponds with our findings that it was downregulated in the absence of IL-12p40, though we saw this effect in both Th1 and Th17 subsets in the context of *Chlamydia* infection and more strongly in Th17 cells. *Plac8* expression has previously been observed in CD4 T cell clones isolated from *Chlamydia* infected mice [36]. Plac8-deficient mice experienced a late delay in clearance of *Chlamydia* beginning in the third week of infection, after the bulk of infection was cleared. This mild phenotype may be due to use of the Nigg strain in this

experiment and may be more severe in infection with a more virulent strain. The mechanism of Plac8 action in these T cells is unclear and requires further exploration.

Table 4.4: Top 10 differentially expressed genes with a negative fold change in both IL-12p40-deficient versus wild-type and IL-12p40-deficient versus T-bet-deficient comparisons, ordered by fold change in IL-12p40-deficient versus wild-type.

Gene	Average log2 Fold Change IL-12p40-/- versus WT	Average log2 Fold Change IL-12p40-/- versus T-bet-/-
Furin	-1.21	-1.86
Il18rap	-0.78	-0.35
Plac8	-0.77	-1.94
Fes	-0.67	-0.73
Serpinb1a	-0.64	-0.63
Man2a2	-0.64	-0.90
Il18r1	-0.56	-0.64
Dgat1	-0.45	-1.46
Pde4b	-0.40	-0.56
Cxcr6	-0.38	-0.29

Serpinb1a (protein SerpinB1, a serine protease inhibitor), has been associated with Th17 differentiation and Th17 cells driving pathogenesis in an experimental autoimmune encephalomyelitis model (EAE) [37, 38]. In our model, it may also be contributing to inflammatory pathways that promote bacterial clearance.

Cxcr6, encoding the chemokine receptor CXCR6, may affect the ability of responding CD4 T cells to optimally localize in the tissue to combat *Chlamydia*. IL-12 has been shown to upregulate CXCR6 in NK cells [39]. In CD8 T cells in a tumor model, CXCR6 was necessary to bring the cells in proximity to dendritic cells providing survival signals [40]. In an EAE model, CXCR6 was found to mark cytotoxic Th17 cells [41]. In this scRNA-seq data set, mRNA for CXCL16, the ligand for CXCR6, was most expressed in the macrophage and dendritic cell populations. CXCL16 has also

been shown to be produced by epithelial cells in the intestine, lung, and oviduct [42-44]. CXCR6 thus may be involved in either fine localization of CD4 T cells within the FRT such that they can receive signals for activation and survival from phagocytes, or localization to infected epithelial cells.

Dgat1 codes for Diacylglycerol O-Acyltransferase 1, an enzyme involved in metabolism. Dgat1-deficient mice were shown to have less pathology in EAE [45]. Dgat1 in T cells was determined to reduce available retinol and prevent Treg differentiation. In our model, lower *Dgat1* in IL-12p40-deficient mice could thus be contributing towards the expansion of the Treg population.

Pde4b is the gene for Phosphodiesterase 4B, which is involved in regulating cyclic nucleotide signaling. In T cells, cyclic nucleotides are important second messengers in control of T cell proliferation and cytokine production [46]. Inhibitors of phosphodiesterase 4 have been documented to have immunosuppressive effects. In one case, such an inhibitor suppressed IL-17 production by CD4 T cells [47]. The downregulation of phosphodiesterases and increased cAMP levels in T cells has also been linked to Treg formation [46, 48]. *Pde4b* may therefore also be a reflection of the increased Treg population in IL-12p40-deficient mice.

One transcription factor of note downregulated in IL-12p40-deficient CD4 T cells compared to wild-type is *Bhlhe40* (Supplemental Table 4.1). This transcription factor has been shown to be involved in the fate decision for pro- versus anti-inflammatory trajectories for Th1 cells [49]. Mice in which Bhlhe40 was conditionally knocked out in CD4+ cells expressed less IFN-γ independent of T-bet, similar to IL-12p40-deficient mice here (Fig. 4.2). Bhlhe40 has also been shown to be required for the pathogenic potential of CD4 T cells in the context of EAE, likely through regulation of GM-CSF and IL-10 production [50]. In our experiment, impaired *Bhlhe40* expression due to IL-12p40 deficiency could result in difficulties promoting effector responses required for clearing bacteria and reflect the shift towards Tregs.

Discussion

In this study we sought to investigate the involvement of IL-12p40 in *Chlamydia muridarum* primary infection of the FRT. We found that mice genetically deficient for this cytokine subset have a severe delay in FRT clearance (Fig. 4.1). This is in accordance with an observation in 1997 from Harlan Caldwell's lab in which neutralizing anti-IL-12 treatment was found to delay primary clearance [4]. This observation was a key component of the interpretation that Th1 cells are important to clear *Chlamydia* infection, as IL-12 is vital for promoting Th1 cells. The antibody used in this experiment for IL-12 depletion (clone C17.8) is specific for IL-12p40, and thus also depletes IL-23. However, this experiment was performed prior to the recognition of IL-23 and Th17 cells and thus the contribution of IL-12 versus IL-23 was not explored at this time. Later, Chen et al. tested primary infection in IL-12p40-deficient and IL-12p35-deficient mice and observed only a slight delay in clearance a few weeks into infection [9]. A major difference between these two experiments was the strain of *Chlamydia muridarum* each group used. The Caldwell lab received their strain from Julius Schachter's Weiss isolate, since deposited at ATCC and the source of the strain our lab uses [10]. The experiments from Chen et al. instead used a Nigg strain, noted to be less virulent [10]. In testing these strains against each other, we were indeed able to recapitulate the differences between these experiments. More work is required to determine the genetic determinants in these exact strains that determines these differences.

Therefore, IL-12p40 is a critical factor driving effector responses needed to control the more virulent Weiss-derived strain, while it is less important in the context of the less virulent Nigg strain. IL-12p40 may be driving CD4 T cells towards a more differentiated state with stronger effector responses targeted at *Chlamydia*. The putatively less differentiated CD4 T cells in IL-12p40-deficient mice may then be sufficient to control the less virulent Nigg strain. MHC class II-deficient mice do show the same degree of defect in clearing primary infection with both strains, indicating that even in infection with the Nigg strain, CD4 T cell responses are critical for clearance. Further investigation of how these strains act in different gene knockout mouse models may be

required, as this may result in discrepancies that make interpretation of the literature difficult. For example, neutrophil depletion was found to cause a slight delay in clearance in one case with the ATCC derived strain [51], similar to what our lab has seen in RORγt mutant mice, while another experiment depleting neutrophils with Nigg saw no difference [52]. Repeating previous experiments with alternate strains may discover similar patterns.

In our study, the IL-12p40-deficient mice do exhibit an ability to slowly clear FRT infection ahead of MHC class II-deficient mice, beginning in the third week of infection (Fig 4.1). This could be due to a late development of T-dependent antibody responses that don't develop in MHC class II-deficient mice and the increased infiltration of B cells in the FRT seen at the day 30 time point (Fig. 4.4B). Also observed at this late time point is an increase in NK cells that could also be contributing to clearance. Further work will be needed to test to what degree the same changes occur in wild-type mice at this later time during infection and what contributions they make.

Interestingly, the IL-12p40-deficient mice do not phenocopy prior experiments with T-bet-deficient X RORγt mutant mice or T-bet-deficient mice given neutralizing anti-IL-6 and anti-TGF-β, which did not show major delays in clearance (Chapter 3). All three of these models are aimed at simultaneously blocking Th1 and Th17 related pathways. It is likely, then, that IL-12p40 is driving mechanisms not dependent on T-bet or RORγt expression. This further underlines previous experiments determining that T-bet is only expressed a very low levels in wild-type CD4 T cells and is not required for clearance (Chapter 2) [2]. It will be important to test the involvement of the cytokine subunits that can pair with IL-12p40, IL-12p35 and IL-23p19 that form IL-12 and IL-23, in our model to determine if IL-12 or IL-23 are critical alone or together for this phenotype. Further investigation into downstream signaling through the STAT proteins that interact with the respective receptors for IL-12 and IL-23 may also shed light on this mechanism, particularly STAT4, which is activated by both IL-12 and IL-23 receptors.

The scRNA-seq experiment in this study has also underlined the complexity of CD4 T cell responses and the difficulty in categorizing responding CD4 Th cells as strictly Th1, Th2, Th17, or Treg. Breaking down heterogeneity within CD4 T cells can be difficult in single cell experiments and can require higher sequencing depth or greater cell numbers to discriminate differences [53]. Mapping classical subset descriptions based on surface markers to clusters defined by scRNA-seq is not direct and subsets with a particular set of surface markers may be represented across multiple clusters [54]. Additionally, Kiner et. al. observed that classical Th markers function as a continuum along scRNA-seq clusters and found that clusters were defined more by which microbe induced the T cell response [55].

In our data set, a clear axis can be drawn in this experiment from the Th1-like population to Th17 cluster following *Ifng* and *Il17a* expression respectively. Treg cells form a distinct cluster, but beyond this the discrete classifications begin to lose meaning (Fig. 4.5A). We have already established that the Th1-like population are not true T-bet-defined Th1 cells as can be observed in other infection models and *Chlamydia* clearance is not dependent on the primary Th1 markers T-bet and IFN-γ (Chapter 2). Within the Th17 cluster, the cells from T-bet-deficient mice versus T-bet-deficient X RORγt mutant mice occupy separate areas, indicating that RORγt mutation has altered the Th17 character of these cells in some way. The IL-12p40-deficient mice have the most dramatic phenotypic difference in shedding compared to wild-type, and yet both experimental groups are largely represented in the same Th1-like cluster. This could either mean the shifts in immune response are occurring in other cell types, or the subtle differences in CD4 T cells are enough to drive the phenotype.

A new framework for understanding CD4 T cell subset fate has been proposed where CD4 T cell responses are less defined by the particular marker cytokine products they make, but rather the class of functions and types of cells they provide help to [56]. *Chlamydia* as a class of pathogen does not fit neatly into the classical Th1-targeted functions toward killing intracellular

bacteria through IFN-γ-mediated activation of macrophages. *Chlamydia* instead replicates in a mucosal epithelial layer and can evade IFN-γ-mediated mechanisms in these cells. This new framework allows us to build the type of CD4 T cells responding to *Chlamydia* infection into the subset framework as CD4 T cells that target intracellular bacteria in epithelia. As such, we have found that IL-12p40 is necessary to drive a mechanism accessible to either Th1-like cells that are not true Th1 cells (T-bet low but IFN-γ+) in wild-type mice or Th17 cells (RORγt+ IL-17A+) in T-bet-deficient mice (Fig. 4.9). Treg programming is also suppressed by IL-12p40 in these mice, preserving a pro-inflammatory environment. IL-12p40-driven STAT4 activation then likely promotes expression of genes like *Furin* that promote bacterial clearance through a yet unidentified mechanism not dependent on IFN-γ production. This action of IL-12p40 is necessary to fully activate the CD4 T cells in order to control *Chlamydia* infection when challenged with a more virulent strain of the bacteria. This has important implications for what will be required to elicit effective CD4 T cell responses in the context of vaccines for human *Chlamydia* infections.

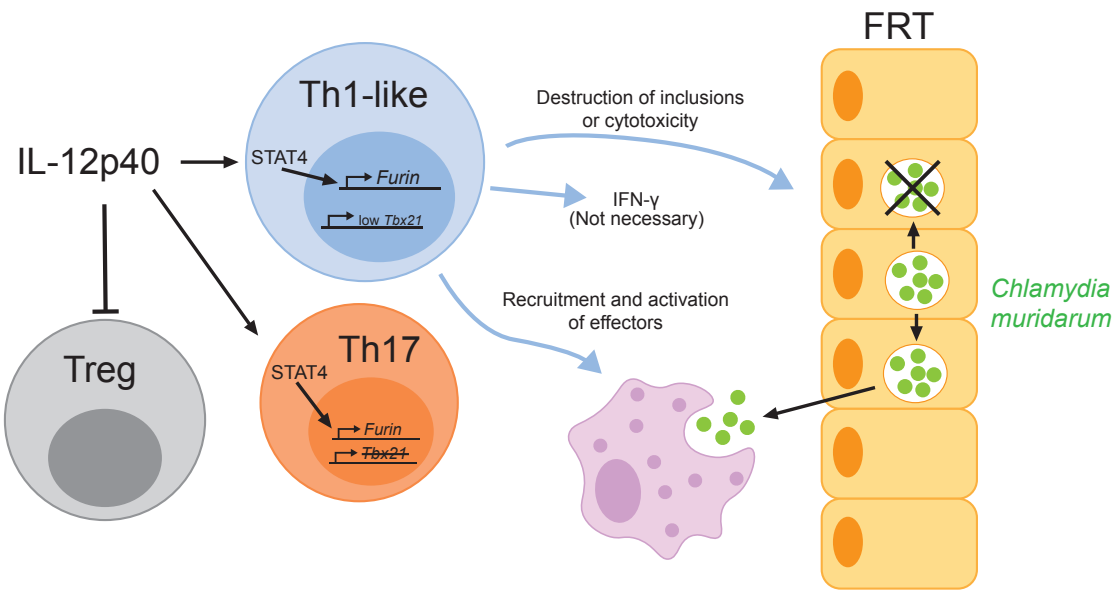

Figure 4.9: A model of CD4 T cell responses in *Chlamydia* infection in the FRT.

IL-12p40 signaling blocks Treg development and promotes an effector program in Th1-like cells in wild-type mice and Th17 cells in T-bet-deficient mice putatively through activation of STAT4. This drives the expression of T-bet-independent genes like *Furin*. This program mediates *Chlamydia* clearance through a yet unidentified mechanism that may be related to direct cytotoxicity of infected epithelial cells, activation of epithelial cells to destroy inclusions and resist infection, or recruitment of other effector cells for the same purposes as well as phagocytosis of extracellular *Chlamydia*.

Methods

Mice

C57BL/6 (JAX stock no. 000664), MHC class II-deficient (JAX stock no. 003584), T-bet-/- (JAX stock no. 004648), RORɣt mutant (JAX stock no. 031393)[3], and IL-12p40-/- (JAX stock no. 002693) mice were purchased at 6–8 weeks old from The Jackson Laboratory (Bar Harbor, ME) and used for experiments at 7–12 weeks old. For many of these strains, breeding colonies were established to supply experiments. Mice were handled and used according to regulations of the Institutional Animal Care and Use Committee at University of California, Davis.

Chlamydia infection

One week prior to infection, mice were given 2.5mg Depo-Provera (medroxyprogesterone acetate, Pfizer) s.c. in a 0.1mL volume. Mice received $1x10^5$ IFU of *Chlamydia muridarum* intravaginally in 5uL SPG buffer. The strain used was derived from ATCC Nigg II stock unless otherwise stated as Nigg, which was kindly provided by the lab of Toni Darville.

Counting *Chlamydia* burden

To monitor vaginal shedding of *Chlamydia muridarum*, vaginal swabs were taken and placed into 2mL microcentrifuge tubes containing 500uL SPG buffer and two glass beads. The tubes were shaken at 1400rpm for 5min at 4°C, and the swab subsequently discarded. Samples were frozen at -80°C until used for the counting protocol. To count the bacteria, samples were diluted in a series used infect a monolayer of HeLa cells in 96-well plates. These were cultured into inclusions overnight, then fixed and stained before counting to calculate IFU per swab.

Flow cytometry

To isolate lymphocytes from lymph nodes, frosted slides were used to break up lymph nodes and washed wish PBS containing 2% fetal bovine serum. To isolate lymphocytes form the female reproductive tract, the FRT was harvested into complete RPMI, then minced into small pieces and incubated with collagenase IV (386mg/L MP Biomedicals) for 1 hour at 37°C. The resulting suspension was filtered (70μm cell strainer, Corning) and lymphocytes isolated on a Percoll gradient (GE Healthcare). For intracellular staining, cells were cultured in stimulating conditions with PMA (0.2 mM, Millipore Sigma) and Ionomycin (1ug/mL, Millipore Sigma) along with Brefeldin A (71.4uM Millipore) for 3.5 hours at 37°C 5% CO_2. A viability stain was performed first using Zombie Yellow (BioLegend), then surface markers were stained, including B220-APC-eF780 (RA3-6B2, eBioscience), CD11b-APC-eF780 (M1/70, eBioscience), CD11c-APC-eF780 (N418, eBioscience), F4/80-APC-eF780 (BMB, eBioscience), CD4-PE (RM4-4, eBioscience), CD8-BV785 (53-6.7, BioLegend), and CD44-APC (IM7, eBioscience). Then intracellular stains IFN-γ-BV785 (XMG1.2, BioLegend), T-bet-PECy7 (4B10, eBioscience), RORγt-BV421 (Q31-378, BD Biosciences), IL-17A-FITC (17B7, eBioscience), and Foxp3-FITC (FJK-16s, eBioscience) were used with the Foxp3 Transcription Factor Staining Kit (eBioscience). Data was acquired on an LSRFortessa (BD) and analyzed using FlowJo (Tree Star, San Carlos, CA). Contour plots are shown with 5% outliers.

Single cell RNA-seq

FRT lymphocytes were isolated as described above. Two FRTs were harvested and processed from each experimental group. Cells were suspended in 10% fetal bovine serum in PBS and filtered with Flowmi 40um cell strainers (Scienceware) to ensure a single cell suspension. Samples were processed for scRNA-seq using the v3.1 of the 10X Genomics platform. 10,000 cells were loaded onto the Chromium Controller per sample. Libraries were prepared according to the v3.1 dual index protocol and submitted for sequencing on.a NovaSeq (Illumina). Approximately 2 billion reads were received from sequencing.

Bioinformatics

Preprocessing with HTStream (version 1.3.3) removed any reads mapping to PhiX, trimmed poly-A tails, low quality basepairs, and reads with a post-processing length less than 50bp. Reads were aligned using Cell Ranger (version 6.0.1) [57]. For analysis in Seurat (version 4.1.0) [58], cell barcodes with a minimum of 250 observed genes, and a maximum of 50000 UMIs were retained as cells. Cells with mitochondrial transcripts exceeding eight percent of UMIs were removed from the analysis. After normalization, the effects of cell cycle assignment and percent mitochondrial transcripts were regressed out. Individual cell identities were assigned using SingleR [59] referencing the Immunological Genome Project database [60]. These assignments were double-checked against a list of top 20 features defining clusters and adjusted when needed to define each cluster as a particular cell type. To subset the data on CD4 T cells, we first isolated all T cells based on expression of *Cd3g*, *Cd4*, and *Cd8*, and excluded clusters determined to be NK cells. After reclustering the data, we isolated clusters that were CD4+ and CD8- and did not express NKT cell markers. This subset was then reclustered again for the final selection of CD4 T cells from this data set. Gene Ontology analysis was performed using g:Profiler [61]. Gene Set Enrichment Analysis was performed using clusterProfiler [62] and the Molecular Signatures Database[63, 64]. Code was adapted at various points from DIY.transcriptomics [65].

Statistics

Statistics were performed using GraphPad Prism version 9 (GraphPad Software, LLC). Either t-test or one-way ANOVA were used. * $p<0.05$, ** $p<0.01$, *** $p<0.01$, and **** $p<0.0001$.

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Chapter 5

Conclusion

Chlamydia trachomatis remains the most prevalent bacterial sexually transmitted infection (STI) with serious public health implications [1]. Though antibiotic treatments are effective, repeat infections and rising total infection rates underline the need for a vaccine [2, 3]. The *Chlamydia muridarum* mouse model has provided opportunities to fully interrogate immune responses to female reproductive tract (FRT) infection in order to define targets for the type of immune response a vaccine will need to induce for protective immunity.

Multiple experiments have shown that CD4 T helper cells are the critical component of the adaptive immune response mediating primary clearance of *Chlamydia* in the FRT (Fig. 4.3B) [4]. Classically, the subset of CD4 T cells that control obligate intracellular bacteria like *Chlamydia* are polarized to a Th1 fate, defined by the master transcription factor T-bet and producing the cytokine IFN-γ to activate macrophages to kill the bacteria [5, 6]. However, we have shown that neither T-bet nor IFN-γ have a major role in primary clearance within the FRT (Chapters 2-3). Instead, IFN-γ signaling is vital to control *Chlamydia* replication only after systemic spread to tissues outside the FRT, where *Chlamydia* is likely replicating in non-hematopoietic cells (Chapter 3). Thus, classical Th1 cells are not mediating primary clearance in the FRT.

CD4 T cells have the capacity to polarize to additional fates, each with particular effector modules in combatting other classes of pathogens [7]. We investigated the contribution of the alternate subsets Th2 (effective against helminth infections) and Th17 (effective against extracellular bacteria and fungi) and found that neither were absolutely required (Chapter 3). Surprisingly, wild-type mice exhibited dominance of a Th1-like T-bet^{lo} IFN-γ+ population that were not classical T-bet^{hi} Th1 cells, and T-bet-deficient mice of a Th17 RORγt+ IL-17A+ population, while both were able to clear infection along the same kinetics (Chapters 2-3). Thus, it was

reasonable to expect that both populations were being regulated by the same upstream signals that were driving expression of a common effector mechanism. Indeed, we found that IL-12p40 is a critical factor determining the ability of the immune system to clear primary FRT infection (Chapter 4). Here, using scRNA-seq we showed that mice lacking IL-12p40 were unable to upregulate a common set of genes in both the Th1-like cells in wild-type mice and the Th17 cells in T-bet-deficient mice (Table 4.4, Fig. 4.9). Further experiments will be necessary to determine which of these genes are most critical to clearing *Chlamydia* and through what mechanisms (Fig. 4.9). IL-12p40 forms both IL-12 and IL-23 in complex with other IL-12 family cytokine subunits, which respectively promote Th1 and Th17 responses [8-10]. Considering that IL-12 and IL-23 signaling both involve STAT4-mediated signal transduction, it will be informative to interrogate how STAT4 regulates the genes used for bacterial clearance in both the Th1-like cells in wild-type mice and the Th17 cells in T-bet-deficient mice (Fig. 4.9). An alternative explanation for the delay in clearance in IL-12p40-deficient mice centers around the approximate doubling of regulatory T cells in these mice relative to wild-type (Fig. 4.6-7, 9). While the population remained minor (less than 10%), it will be important to determine if this shift is sufficient to exert suppressive functions over the remaining responding Th1-like cells.

Interestingly, the severe clearance delay phenotype in IL-12p40-deficient mice was dependent on using a more virulent strain of *Chlamydia muridarum* and did not occur when a less virulent strain was used (Fig. 4.3A). Considering how similar wild-type Th1-like and IL-12p40-deficient Th1-like CD4 T cells were in the scRNA-seq experiment, with relatively small fold-changes for most differentially expressed genes (Fig. 4.6A, Supplemental Table 4.1), the effector mechanism mediating clearance may be the same in both cases. IL-12p40 is likely required for fully activating particular genes in CD4 T cells over a threshold that is necessary to control the strain with increased virulence, while this is not required to control the less virulent strain. The implications for human infections with *Chlamydia trachomatis* are that more virulent strains in the human population may require a similar IL-12p40-dependent activation in order to establish

protective immunity. The mechanism that IL-12p40-deficient mice use to mediate late-stage FRT clearance may also be effective to elicit as a vaccination strategy. The further infiltration of B cells and NK cells at this late time point may point to antibody and NK-mediated cytotoxic killing of infected epithelial cells as other effective mechanisms for clearance. Work from Naglak et al. has indeed indicated that antibody plays a critical role in secondary clearance, though in this case it is in cooperation with neutrophils [11].

Overall, this IL-12p40-dependent CD4 T cell response to intracellular bacteria in the epithelial cells of the FRT does not fit neatly into the existing framework of CD4 T cell subsets. Even though *Chlamydia* is an obligate intracellular pathogen, we have shown that classical Th1 cells that target macrophage activation are not required for clearance. Others have proposed that CD4 T cell subsets be reframed not by the profiles of markers and effector cytokines produced, but by the type of help provided [12]. Generally, type 1 would comprise activation of mononuclear phagocytes (monocytes, macrophages, and dendritic cells), type 2 would support polymorphonuclear granulocytes (mast cells, basophils, and eosinophils), and type 3 would protect barrier functions by acting on epithelial and stromal cells. In this framework, *Chlamydia* infection of the mucosal epithelial layer in the FRT fits under a type 3 category, though it appears to be driven by IL-12p40 and likely STAT4 signaling normally ascribed to Th1 and Th17 responses. IL-12p40 is thus a novel driver of a mechanism that can operate in cells that express either Th1 or Th17 markers but is effective at targeting bacteria within mucosal epithelial cells. Fully characterizing the markers of these CD4 cells and the mechanism used against *Chlamydia* infection will provide accurate targets for vaccine development to establish sterilizing immunity.

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Supplemental Table 4.1: Differentially expressed genes between IL-12p40-/- CD4 T cells at day 14 and wild-type CD4 T cells in order most downregulated to upregulated.

Gene ID	Average log2 fold change	Percent of IL-12p40-/- cells expressing	Percent of wild-type cells expressing	Percent difference	Adjusted p value
Furin	-1.2091538	0.574	0.813	-0.239	4.26E-88
Ifng	-0.9353567	0.374	0.603	-0.229	1.26E-37
Il18rap	-0.7766194	0.396	0.626	-0.23	1.23E-49
Plac8	-0.7707934	0.493	0.664	-0.171	7.74E-28
Fes	-0.6710372	0.161	0.395	-0.234	4.78E-39
Serpinb1a	-0.6430074	0.122	0.236	-0.114	1.70E-11
Man2a2	-0.6404552	0.356	0.509	-0.153	9.39E-20
Il18r1	-0.5585903	0.584	0.739	-0.155	4.16E-30
Ctla2a	-0.5089463	0.664	0.799	-0.135	6.61E-17
AA467197	-0.4798912	0.31	0.421	-0.111	5.90E-09
Dgat1	-0.4547447	0.7	0.76	-0.06	1.02E-14
Dusp2	-0.4200476	0.643	0.711	-0.068	1.54E-10
Il12rb2	-0.4064506	0.388	0.508	-0.12	3.27E-12
Bcl2a1b	-0.4054464	0.773	0.807	-0.034	8.71E-12
Nkg7	-0.3994897	0.519	0.647	-0.128	5.25E-11
Pde4b	-0.3987906	0.66	0.697	-0.037	6.88E-10
Gm47283	-0.3964356	0.364	0.468	-0.104	3.72E-10
Ccr5	-0.3883738	0.271	0.381	-0.11	2.13E-08
Cxcr6	-0.3779551	0.591	0.706	-0.115	1.05E-12
Dennd4a	-0.377339	0.827	0.875	-0.048	5.81E-13
Cpm	-0.3757409	0.208	0.315	-0.107	1.25E-08
Nabp1	-0.3732431	0.662	0.714	-0.052	2.01E-10
Ckb	-0.368863	0.227	0.321	-0.094	6.82E-06
Arap2	-0.3675036	0.771	0.816	-0.045	4.09E-08
Unc45a	-0.3666161	0.348	0.411	-0.063	0.00167706
Btg1	-0.3496816	0.999	0.996	0.003	4.85E-25
Irf4	-0.3485301	0.23	0.31	-0.08	0.00051985
Ctsw	-0.3476078	0.508	0.617	-0.109	2.40E-08
P2rx7	-0.3433718	0.2	0.304	-0.104	8.26E-09
Ttc39b	-0.3416257	0.619	0.669	-0.05	1.82E-06
Picalm	-0.3396111	0.752	0.794	-0.042	8.84E-10
AW112010	-0.3247905	0.963	0.975	-0.012	3.37E-16
Wipf1	-0.3227072	0.615	0.675	-0.06	4.92E-08

Egr1	-0.3226239	0.495	0.518	-0.023	1
Anxa2	-0.3216591	0.459	0.513	-0.054	0.01198215
Hectd1	-0.3210137	0.769	0.809	-0.04	1.55E-10
Themis	-0.3202505	0.582	0.663	-0.081	6.66E-09
Pttg1	-0.3094491	0.153	0.282	-0.129	7.71E-13
Ttc39c	-0.308281	0.492	0.56	-0.068	0.00142823
Rora	-0.3070598	0.895	0.917	-0.022	1.48E-10
Apaf1	-0.3031928	0.37	0.434	-0.064	0.0046502
Pgk1	-0.3017655	0.659	0.663	-0.004	1
Gpr171	-0.2964015	0.367	0.454	-0.087	2.82E-05
Hmgb2	-0.2962946	0.918	0.931	-0.013	9.76E-11
Btg2	-0.2962218	0.788	0.792	-0.004	0.0256032
Bhlhe40	-0.2952185	0.872	0.904	-0.032	9.42E-09
Jmjd1c	-0.291165	0.795	0.823	-0.028	5.51E-05
Gzmk	-0.2888856	0.029	0.123	-0.094	6.51E-13
Gnas	-0.2863072	0.934	0.927	0.007	7.46E-10
Dgkh	-0.2854284	0.166	0.251	-0.085	0.00015732
Atp2b4	-0.2845818	0.616	0.669	-0.053	0.00024089
Rbm47	-0.2839945	0.123	0.21	-0.087	2.54E-06
Hexim1	-0.2831368	0.63	0.659	-0.029	0.00266686
Zfp36l2	-0.2809259	0.912	0.924	-0.012	0.00733064
Bcl2l11	-0.2790179	0.467	0.511	-0.044	0.05588599
Gramd3	-0.2781843	0.792	0.84	-0.048	8.86E-09
Itgal	-0.2780633	0.816	0.852	-0.036	5.29E-10
Pgam1	-0.2777962	0.446	0.49	-0.044	0.39984236
Runx1	-0.2774393	0.574	0.647	-0.073	2.04E-05
Icos	-0.2742645	0.893	0.909	-0.016	4.54E-07
Ltb	-0.2715712	0.851	0.884	-0.033	2.04E-10
Stk17b	-0.2710432	0.956	0.952	0.004	7.88E-15
Hopx	-0.2696149	0.528	0.609	-0.081	1.20E-06
Tnf	-0.2674443	0.182	0.232	-0.05	1
Arid5a	-0.2628036	0.713	0.756	-0.043	6.48E-06
Gtf2i	-0.261596	0.446	0.513	-0.067	0.00055865
Cd28	-0.2602453	0.877	0.887	-0.01	1.73E-06
Gmfg	-0.2572358	0.88	0.889	-0.009	2.44E-10
Icam1	-0.2551051	0.289	0.336	-0.047	1
Golga4	-0.2550047	0.401	0.442	-0.041	0.86697813
Ctsd	-0.2547826	0.727	0.745	-0.018	6.03E-05
Gng2	-0.2517086	0.709	0.737	-0.028	0.00090123

Lars2	-0.2510845	0.969	0.963	0.006	2.14E-12
Clec4d	0.25064285	0.078	0.035	0.043	0.00065946
Il1rn	0.25109575	0.11	0.048	0.062	2.52E-07
Capg	0.25278394	0.434	0.341	0.093	0.00170552
Dnaja1	0.25282127	0.932	0.89	0.042	0.00016575
Hsp90ab1	0.25285084	0.99	0.992	-0.002	6.67E-10
Rpl36	0.25650683	1	0.996	0.004	1.57E-25
Tox	0.2580807	0.186	0.099	0.087	8.49E-09
Rtp4	0.25857412	0.263	0.146	0.117	3.18E-12
Hcar2	0.25958635	0.079	0.031	0.048	3.49E-06
Xaf1	0.26057237	0.279	0.17	0.109	3.49E-09
Fabp5	0.26279668	0.289	0.201	0.088	0.00137128
Paxbp1	0.26370163	0.672	0.57	0.102	0.00024879
Rpl36a	0.26384562	0.955	0.924	0.031	8.28E-14
Sell	0.26553867	0.128	0.065	0.063	5.83E-06
mt-Atp6	0.266264	1	0.999	0.001	5.22E-33
Tmem176a	0.26765382	0.139	0.097	0.042	1
Ets2	0.26926094	0.313	0.199	0.114	2.77E-08
Cd14	0.2711538	0.108	0.067	0.041	0.7446008
Rps21	0.27276653	0.999	0.998	0.001	4.26E-33
Nt5e	0.27331173	0.308	0.18	0.128	2.39E-12
Timp2	0.27375826	0.329	0.217	0.112	1.63E-08
Ar	0.27694739	0.184	0.102	0.082	9.05E-08
Rpl38	0.27899191	0.998	0.999	-0.001	2.13E-35
S100a6	0.2797115	0.908	0.869	0.039	0.01132682
Prkca	0.28280951	0.543	0.379	0.164	8.20E-14
Rps29	0.28374341	1	1	0	4.48E-40
Foxp3	0.28774395	0.078	0.027	0.051	3.19E-08
Rpl35	0.28839609	0.973	0.949	0.024	1.04E-19
Tyrobp	0.29586765	0.126	0.102	0.024	1
Itgav	0.29630841	0.327	0.242	0.085	0.00146334
Ptms	0.29645464	0.451	0.363	0.088	0.00137289
Ifit3	0.29706757	0.106	0.057	0.049	0.00454355
Cd27	0.29960848	0.356	0.212	0.144	1.36E-13
Slc15a3	0.29962374	0.224	0.122	0.102	1.70E-10
Hspd1	0.30252862	0.593	0.492	0.101	0.00054592
Rgs2	0.30319951	0.675	0.599	0.076	0.00091972
Irf7	0.3078092	0.355	0.22	0.135	3.29E-11
Rpl39	0.31061876	1	0.998	0.002	2.10E-41

Plek	0.31536873	0.26	0.21	0.05	1
Tmem176b	0.31724995	0.139	0.091	0.048	0.1558208
Ier5l	0.31789879	0.241	0.141	0.1	5.19E-09
Neb	0.31880312	0.143	0.063	0.08	1.38E-10
S1pr1	0.32286504	0.406	0.323	0.083	0.01668776
Areg	0.32414656	0.123	0.081	0.042	1
Ccnd2	0.32647326	0.746	0.627	0.119	4.12E-11
Hspa1b	0.33042966	0.034	0.031	0.003	1
Rps28	0.33130078	0.997	0.996	0.001	1.70E-40
Hsph1	0.3336728	0.403	0.277	0.126	1.02E-09
mt-Atp8	0.34006236	0.766	0.603	0.163	3.50E-19
Hnrnpa0	0.34446162	0.837	0.692	0.145	7.25E-22
Tnfsf8	0.3492576	0.259	0.164	0.095	1.88E-06
Mbd2	0.35256423	0.788	0.654	0.134	8.17E-20
mt-Nd4l	0.36049643	0.82	0.694	0.126	1.19E-15
Ifitm2	0.36121115	0.454	0.413	0.041	1
Ide	0.36540776	0.433	0.229	0.204	6.49E-29
mt-Cytb	0.36753467	0.999	0.993	0.006	2.25E-36
Iigp1	0.38017812	0.243	0.147	0.096	7.64E-08
Hspa1a	0.3810761	0.043	0.03	0.013	1
Hdc	0.38200527	0.116	0.056	0.06	8.58E-06
Pmepa1	0.3879595	0.543	0.383	0.16	6.27E-15
Slfn5	0.3895808	0.223	0.116	0.107	1.55E-12
mt-Nd2	0.40558434	0.997	0.99	0.007	3.26E-45
Cxcl10	0.41131022	0.154	0.117	0.037	1
Rnf213	0.41184059	0.495	0.367	0.128	6.52E-09
Nrgn	0.4182974	0.053	0.026	0.027	0.93166341
Gm10076	0.43277081	0.902	0.764	0.138	5.46E-37
Eea1	0.43358763	0.446	0.294	0.152	9.96E-15
Cebpb	0.43844086	0.598	0.457	0.141	4.41E-10
Tnfrsf9	0.44967825	0.356	0.266	0.09	0.00058974
mt-Nd3	0.47178356	0.926	0.854	0.072	3.78E-39
Bst2	0.47675507	0.683	0.499	0.184	7.97E-22
Ccl4	0.49947874	0.149	0.109	0.04	1
Isg15	0.5029609	0.457	0.312	0.145	1.48E-12
Ifit1	0.50660034	0.222	0.107	0.115	5.44E-15
Acod1	0.51860452	0.179	0.078	0.101	4.40E-14
Slfn4	0.55348831	0.183	0.05	0.133	1.07E-34
Ikzf2	0.59725	0.376	0.236	0.14	2.59E-14

Gzmb	0.61401517	0.279	0.2	0.079	0.00344282
Rsad2	0.64115332	0.178	0.057	0.121	2.39E-26
Hbb-bt	0.66601611	0.008	0.016	-0.008	1
S100a8	0.69468976	0.766	0.516	0.25	4.53E-37
S100a9	0.85224577	0.8	0.557	0.243	7.86E-37
Hba-a2	0.86698936	0.017	0.025	-0.008	1
Ifi27l2a	0.96600496	0.938	0.825	0.113	2.62E-62
Il17a	1.02297115	0.091	0.023	0.068	2.36E-16
Hba-a1	1.07362745	0.015	0.018	-0.003	1
Hbb-bs	1.70403838	0.042	0.057	-0.015	1