T-CELL DEPENDENT AND INDEPENDENT MECHANISMS OF CHLAMYDIAL ERADICATION AND CONTROL

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ABSTRACT

Evidence suggests that Th1 cells and antibody are the primary mediators of chlamydial protection. However, the impact of Th1 polyfunctionality and T-cell independent antibody on host protection against *Chlamydia* has not been fully explored. Using an adoptive transfer approach in the mouse model of *Chlamydia muridarum*, we investigated the role of transgenic *Chlamydia*-specific CD4 T cells and naïve, polyclonal B cells in mediating bacterial clearance and conferring resistance to lethality, respectively. We **hypothesized** that *Chlamydia*-specific Th1 cells would provide enhanced protection against genital infection compared to a polyclonal repertoire, and that B-cells are required for preventing lethality associated with disseminated infection. We found that polyfunctional, transgenic Th1 cells produced the highest levels of IFN-γ, afforded the greatest reduction in bacterial burden from the genital tract during primary infection, and provided equal protection during secondary infection, compared to the polyclonal T cell response. We also found that B cells and IFN-γ synergize to protect against disseminated infection in the absence of Th1 cells, despite mice developing a chronic genital tract infection. Collectively, these data suggest that polyfunctional, *Chlamydia*-specific Th1 cells mediate optimal chlamydial clearance from the genital tract, while the T-independent B-cell response is

primarily involved in limiting extragenital infection to distal organs. Adoptive transfer studies provide a powerful approach for elucidation of protective correlates of immunity against chlamydial infection that can guide development of rational public health vaccine strategies.

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ABBREVIATIONS

Ag	Antigen
APC	Antigen presenting cell
BMDC	Bone marrow-derived dendritic cell
CDC	Centers for Disease Control and Prevention
CM3.1	Plasmid-cured strain of *Chlamydia muridarum*
EB	Elementary body
Gavi	Global Alliance for Vaccines and Immunization
GMFI	Geometric mean fluorescent intensity
HIC	High-income countries
HPV	Human Papillomavirus Virus
ILN	Iliac lymph node
IFU	Inclusion forming units
i.p.	Intraperitoneal
i.v.	Intravenous
LMIC	Low-and middle-income countries
MRI	Magnetic resonance imaging
MOMP	Major Outer Membrane Proteins
NIAID	National Institute of Allergy and infectious Diseases
NHP	Non-human primate
Nigg	Wild-type strain of *C. muridarum*
PAMP	Pathogen-associated molecular patterns

PRR	Pathogen recognition receptor
PBMC	Peripheral blood mononuclear cells
PFU	Plaque forming units
PID	Pelvic Inflammatory Disease
RB	Reticulate Body
TCR	T-cell receptor
TRM	Tissue resident memory
Tg	Transgenic
WHO	World Health Organization

1.0 INTRODUCTION

1.1 CHLAMYDIA AND THE BURDEN OF DISEASE

Chlamydia trachomatis, a gram-negative obligate intracellular bacterium capable of infecting genital tract, ocular, and lung epithelium, is the most common bacterial sexually transmitted infection (STI) globally. Sexually transmitted genital infection and associated disease is caused by *C. trachomatis* serovars D-K. Other serovars cause distinct disease syndromes such as ocular trachoma (serovars A, B, Ba, and C) and lymphogranuloma venereum (serovars L1-L3). The replicative cycle of *C. trachomatis* is made up of two distinct phases. The elementary body (EB) form is responsible for attachment and penetration of the target cell, changing to the metabolically active reticulate body (RB) form, which replicates in a protective intracellular inclusion. After hundreds of progeny are generated, the RBs transform back to infectious EBs and are released from the host cell to be transmitted to neighboring host cells or to contacts. Replication within an intracellular inclusion aids the pathogen's ability to avoid the host immune response and promotes chronic infection.

C. trachomatis is transmitted sexually via vaginal, anal, or oral sex to cause genital, anal, or less commonly, oropharyngeal infection. Infection can also be spread perinatally from an untreated mother to her infant to cause neonatal conjunctivitis or pneumonia. Lower genital tract infection is often asymptomatic, but can manifest as urethritis in males and as urethritis or cervicitis in females. The most serious sequelae of infection result from ascension to the upper

genital tract in women to cause pelvic inflammatory disease (PID), an infection and inflammation of the uterus, fallopian tubes, ovaries and/or pelvic peritoneum. The inflammation and scarring of PID in the fallopian tubes can lead to long-term sequelae including tubal factor infertility, ectopic pregnancy, and chronic pelvic pain. Based on prospective studies, about 10-15% of untreated chlamydia infections lead to clinically diagnosed PID, and about 10-15% of clinical PID cases lead to tubal factor infertility (1-3). Genital infection with *C. trachomatis* may also increase the risk of acquiring HIV infection by 2 to 3-fold (4, 5).

Globally, an estimated 131 million new cases of chlamydial genital infection occur annually (6). Incidence rates are high across all world regions, but the infection disproportionately affects adolescents and young adults under 25 years of age (7). The global burden of chlamydia-associated PID, infertility, and ectopic pregnancy has not been well defined. However, about 68 million chlamydia infections are estimated to occur among women globally each year (6). Given what is known about the natural history of infection, the number of cases of infertility and other adverse outcomes is likely sizable. If all of these infections were left untreated, they could result in close to 1 million new cases of infertility annually. The Global Burden of Disease study (GBD) 2013 estimates that chlamydia results in 647,000 years lived with disability (YLDs) annually (8). The global economic burden of genital chlamydial infection has not been assessed, but annual healthcare costs in the United States are estimated at $517 million (9).

Diagnosis of chlamydia relies on nucleic acid amplification tests (NAATs) of specimens obtained by vaginal or cervical swabs in women or urine collection in men and women. A course of doxycycline or single-dose azithromycin offers effective curative treatment. Because the vast majority of chlamydial infections are asymptomatic but can still lead to adverse sequelae and

ongoing transmission, several high-income countries (HICs) have relied on screening programs to diagnose and treat chlamydia to prevent PID (10-14). In low- and middle-income countries (LMICs), lack of affordable, feasible laboratory tests means most genital chlamydia infections are not diagnosed. However, even in HIC settings with long-standing chlamydia screening recommendations, these programs have been costly and difficult to bring to scale (15, 16). In addition, although screening has likely reduced the incidence of PID, it has not resulted in clear reductions in chlamydia transmission (17, 18). One of the main reasons for ongoing transmission is that management of sexual partners of index cases is logistically difficult and repeat infection rates are high: approximately 10-20% in the months after treatment (19). It has been hypothesized that screening programs might increase the frequency of re-infection through reductions in population-wide protective immunity, or arrested immunity (20). Barrier methods of contraception, including condom use, are effective at preventing chlamydial transmission, however utilization rates are low (21). Shortcomings of current chlamydia control strategies highlight the need for an effective vaccine.

1.2 BIOLOGICAL FEASIBILITY FOR VACCINE DEVELOPMENT

Currently no licensed vaccine exists for *Chlamydia trachomatis*, but evidence from animal models and human studies suggests that a vaccine is feasible. Animal challenge studies, including mouse, guinea pig and non-human primate models, demonstrate that partial and sterilizing natural immunity can develop from a primary infection, however this protection is short-lived and not sufficient to provide long-term immunity (22). In animals, partial immunity can reduce bacterial burden and duration of secondary infection but does not necessarily prevent

upper genital tract pathology. In humans, epidemiologic studies reveal a decreased prevalence of infection and decreased bacterial load with increasing age despite continued exposure (23). In addition, infection concordance between sex partners decreases with increasing age of the partners, and bacterial loads are lower among individuals with a history of infection (24). Furthermore, in a prospective study of 200 women in the US, those whose chlamydial infections cleared spontaneously between testing and treatment were less likely to become re-infected on follow-up (25). The ability of natural infection to induce partial immunity is promising for vaccine development.

The first *C. trachomatis* vaccines, evaluated in the 1960s, were live or formalin-fixed whole bacteria that focused on ocular infection causing trachoma, rather than genital infection (26-28). Multiple studies demonstrated some protection from active (inflammatory) trachoma in vaccinated individuals (29-31). However, these benefits were short-lived, often waning within one to two years (32). Non-human primate studies of these same vaccines showed effective but short-lived protection as in human trials when high doses of organisms were used. However, when low doses were used, more severe disease was observed upon challenge with heterologous serovars (33). Concern for exacerbated disease upon challenge of immunized hosts also arose because of the way data were initially interpreted from live trachoma vaccine studies among Gambian children (27). At the time, trachoma severity scores were reduced when conjunctival scarring was present, as scarring was considered a sign of healing, despite being the undesired sequelae of inflammation. The prevalence of scarring was lower two years post vaccination, suggesting the vaccine reduced longer-term disease sequelae, but the scoring system led to an erroneous conclusion that vaccinated children had enhanced inflammatory disease relative to unvaccinated children. Experts reinterpreting these trials in the context of current trachoma

grading systems and knowledge about disease pathogenesis concluded that concerns about vaccine-induced exacerbation of disease in Gambian children were unfounded (34-36). In addition, ocular inoculation of non-human primates with a live-attenuated trachoma serovar did not worsen disease upon challenge (37).

Overall, the short-term protection observed in human trachoma vaccine trials implies that an effective vaccine for *C. trachomatis* is feasible. Initial concerns about an enhanced pathologic response pushed the field towards development of subunit vaccines to enhance safety. This goal remains because a subunit vaccine would contain only essential antigens for protection and not all the other molecules that make up the chlamydial microbe, reducing the chances of adverse reactions. Induction of complete immunity to infection is the ideal goal, and will require augmentation of protective immune mechanisms at the mucosal site. Recent data indicate mucosal delivery of a chlamydia vaccine may be required to induce resident memory T cells that act as sentinels to protect the mucosa (38). Advances in understanding the immunobiology of *C. trachomatis* infection over the past several decades have markedly increased the likelihood of developing a safe and effective vaccine.

1.3 OBSTACLES TO VACCINE DEVELOPMENT

Chlamydiales have adapted numerous mechanisms to infect the host cell, evade the host immune response, and replicate in a protective, intracellular inclusion. The infectious elementary body (EB) form contains cross-linked protein complexes that render the EB metabolically inactive and resistant to extracellular osmotic stress. The polymorphic, major out member protein (MOMP) is the most abundant component of the bacterium's envelope. MOMP acts as a cytoadhesin and

binds to heparin sulfate proteoglycan receptors on the host cells to invade (39). Additional proteins on the chlamydial outer membrane complex have been shown to be important for cellular invasion. The outer membrane complex B (OmcB) functions as an adhesin with specificity for glycosaminoglycan receptors on host cells (40), and the bacterium has also been shown to utilize the cystic fibrosis transmembrane conductance regulator (CFTR) apical anion channel for internalization (41). Multiple studies have demonstrated that *Chlamydia* can utilize estrogen receptors, particularly the protein disulfide isomerase component, for entry into host cells (42-45). The EphrinA2 receptor has most recently been shown to be an adherence and invasion receptor for infection (46). It is evident that the bacterium has evolved numerous mechanisms to invade the host cell. Additional research is required to identify broadly neutralizing antibodies due to the polymorphic nature of chlamydial binding proteins.

The biphasic developmental cycle of *Chlamydia* creates another obstacle to vaccine development. After host cell entry, the EB avoids the host lysosomal pathway, disulfide bonds are reduced and the bacterium enters a reticulate body (RB) form (47, 48). The RB is metabolically active and capable of generating its own stores of energy and essential compounds, but is also capable of hijacking host cell nutrients for growth and replication from the inside of a protective intracellular vacuole called an inclusion. During stress and in response to host cytokines, *Chlamydia* can enter a delayed growth state characterized by large aberrant bodies that may allow for chronic infections less susceptible to antimicrobial killing (49). The multiple mechanisms utilized by *Chlamydia* to escape the immune system have been well summarized (50). Thus, a vaccine inducing strong cellular immunity is essential to prevent chlamydial infection and disease.

1.4 CHLAMYDIA VACCINOLOGY

Preclinical vaccine development utilizes well-established animal models for candidate testing. Mouse models offer convenient manipulation and research tools for analysis of the immune response, but differ from humans with respect to many facets of infection, disease, and adaptive immune responses. *Chlamydia muridarum* is a mouse-specific strain that shares extensive homology with *C. trachomatis*. However, *C. muridarum* induces a more acute infection with complete resolution compared to the often quiescent, chronic infection of *C. trachomatis* in humans. Further, mechanisms of IFN-γ mediated chlamydial clearance differ in mice and humans. The guinea pig model utilizing *Chlamydia caviae* elicits disease more similar to humans, but the relative lack of immunological reagents detracts from its use for vaccine studies (22). Female minipigs that have a reproductive cycle and genital tract similar to humans are being used for chlamydial vaccine studies but also suffer from reduced availability of reagents (51, 52). Non-human primate (NHP) models are often employed prior to human testing, but infection of the eye or genital tract in NHPs demonstrates a shorter, self-limiting infection compared to humans. Despite this limitation, NHP testing could play an important role in assessing cellular and humoral responses after infection or vaccination to identify correlates of protective immunity. Animal and human studies could provide insight into a protective transcriptional blood signature that might be translated to a biomarker of efficacy for use in human clinical trials (53).

1.4.1 Antigens

Induction of sterilizing immunity against *C. trachomatis* by immunization will require protective antigens targeted to CD4 T cells. Novartis assessed 120 *Chlamydia* proteins for immunogenicity using two parallel high-throughput approaches, and identified 16 MHC class II-specific IFN-γ inducing antigens in mice, and five that induced both humoral and cellular responses. Their assessment in the mouse model identified seven novel antigens conferring partial protection to lung infection (54). A second large-scale antigen discovery approach utilized genome wide screening of human antibodies to over 80% of the expressed *C. trachomatis* proteome (55). Of the 99 infected women studied, only 27 proteins were recognized by at least half of the subjects. The ability of these antigens to induce T cell responses was not analyzed. Another proteomic approach to identify T cell epitopes utilized pulsing of dendritic cells with live *Chlamydia* to identify peptides loaded onto MHC class II molecules (56). Five proteins with MHC Class II epitopes elicited partial protection in the murine model of genital infection. Most recently, the use of ATLAS technology has allowed for the profiling of T cell responses in human subjects in response to *C. trachomatis* infection (57). ATLAS uses a proteomic library with *E. coli* expressing proteins of interest that are fed to APCs, which present the respective antigens to human T cells for high-throughput cytokine detection (58). Examination of CD4 and CD8 IFN-γ responses after *in vitro* exposure to CT antigens in a cohort of 141 subjects led to the identification of 8 CD4 and 18 CD8 antigens associated with spontaneous clearance or resistance to infection. The proteins were varied and included those involved in membrane transport, central metabolism, and secretion pathways. Proteins that were immunodominant were not associated with effective immune responses. Antigens that have been found to induce protection in the mouse model (e.g. MOMP, PmpG, CPAF) were not highly recognized by subjects

identified in this cohort as having effective immune responses. However, recent data in the mouse model using a novel vaccine formulation with a multivalent major outer membrane protein (MOMP) VD4 construct containing a conserved *C. trachomatis* epitope induced robust Th1 responses, broadly neutralizing antibodies, and reduced bacterial burden upon challenge (59). These data illustrate the importance of selecting protective immunogenic epitopes and the appropriate configuration for optimal vaccine-induced immunity. Future work is needed to identify protective antigens and optimally construct them to induce protective cellular and humoral responses in humans.

1.4.2 Live vaccines

A human vaccine capable of activating the cellular and humoral arms of the adaptive response to *Chlamydia* is lacking. The complex physiology of the female genital tract, a paucity of effective mucosal adjuvants, and limited knowledge of protective antigens further complicate vaccine development. An effective vaccine should induce mucosal and systemic immune responses devoid of cross-reactive autoantibodies and pathology. The first *C. trachomatis* vaccines were live vaccines (26). Initial studies focused on ocular, rather than genital infection. Results varied from limited, short-lived protection to substantial protection against infection and pathology. Notably, vaccination of Taiwanese children with formalin-fixed *Chlamydia* exhibited partial protection during three years of follow-up (29). A similar study using two preparations of live *C. trachomatis* in Gambian children also elicited partial protection, similar to the Taiwan study (27). The original interpretation of the Gambian trials indicated vaccine-induced hypersensitivity occurred in some vaccinated children but at the time, scarring was scored as a positive indicator of protection. A reanalysis of the original Gambian trials in the context of current knowledge

about disease pathogenesis found that vaccine-induced hypersensitivity did not occur (34). Live vaccines are advantageous since they contain all antigens in correct conformation; however, large-scale propagation of *Chlamydia* is challenging, requires cold storage, and avirulent strains could potentially revert to infectious wild-type strains (60). These safety concerns resulted in a switch to vaccine studies of inactivated bacteria, but killed organisms led to a suboptimal immune response. Use of attenuated plasmid-deficient *C. muridarum* and *C. trachomatis* strains have been explored as vaccination strategies; however, success in the genital tract mouse model did not translate to NHP (61-63). Yet, ocular inoculation of NHP with a plasmid-deficient strain of *C. trachomatis* elicited partial protection against ocular infection in a subset of macaques (37). This illustrates the potential need for differences in the future formulation of a protective vaccine for ocular or genital *Chlamydia* infection.

1.4.3 Subunit vaccines

Purified antigenic determinants known to elicit immune responses have been explored as a vaccination strategy. These vaccines are safer since they are unable to cause infection and lack virulent components. MOMP is the most well studied vaccine candidate from *C. trachomatis*. Murine immunization with purified-refolded MOMP reconstituted with Freund's adjuvant significantly reduced bacterial burden after genital challenge (64). MOMP immunization combined with saponin-based ISCOM (immune-stimulating complex) elicited Th1 antigen-specific responses and mice cleared vaginal infection within one week (65). Similar results have been achieved with subunit B cholera toxin (CTB-CpG), CPG-2395, and Montanide ISA 720 as adjuvants for MOMP vaccination in mice (66) and CPG-2395 and Montanide ISA 720 formulated vaccines induced robust antibody and T cell responses in non-human primates (66,

67). However, MOMP subunit vaccines present the challenge of extracting, refolding, and purifying protein complexes at a high cost that are not standardized.

1.4.4 Recombinant protein and DNA plasmid vaccines

Molecular cloning has afforded the ability to express an abundance of bacterial proteins. Protein configuration remains challenging in this system. However, the use of plant biotechnology may be useful for large-scale production of antigenic proteins like recombinant MOMP (68). Additional vaccine candidates like recombinant CPAF and an OmcB-CT521 fusion protein have elicited Th1 responses and markedly reduced bacterial burdens in mice (69, 70).

DNA vaccines that inject plasmids carrying the protein(s) of interest confer many advantages. DNA plasmids can be constructed quickly, purified, and can encode multiple epitopes in native three-dimensional structures. A DNA vaccine overcomes the concerns of reversion to virulent forms. While anti-DNA antibodies could be induced, clinical trials have not demonstrated changes in clinical markers of autoimmunity (71). Despite minimal work in this field, some interesting findings have been generated. DNA vaccine delivery encoding MOMP co-administered with the adjuvants GM-CSF and *E. Coli* enterotoxin subunits A and B generated robust protection against *C. trachomatis* genital challenge in pigs (72). A plasmid encoding MOMP epitopes inserted into human papilloma virus (HPV) was protective against vaginal infection and elicited a Th1 response in mice (73). Other studies have shown DNA vaccination to be immunogenic with only modest protection in mice (74, 75). These studies demonstrate the feasibility of a DNA-based vaccine and additional studies may be warranted for development of protective animal vaccines. However, DNA vaccine studies have largely been abandoned in

humans due to the inability to transfer adequate DNA concentrations. The low immunogenicity due to poor uptake has led to efforts to optimize delivery and immune responses (76).

1.4.5 Adjuvants

Due to the safety concerns of attenuated or whole-organism killed vaccines, a subunit vaccine is an attractive approach to vaccine development. Administration of innocuous protein subunits is sub-immunogenic and requires adjuvants. Natural or artificial adjuvants aim to replicate host induction of an immunological response during a live infection. Identifying protective antigens, in combination with an optimal adjuvant, is a crucial goal in vaccine development. An effective vaccine requires an adjuvant that can activate innate effector cells, induce type-1 cytokines, enhance antigen presentation, and induce protective effector cellular and humoral responses. Despite the use of adjuvants in research for many years, there are a limited number of effective adjuvants approved for vaccine usage. Alum hydroxide (Alum), AS04 (monophosphoryl Lipid A with alum), AS03 and MF59 (squalene-based), and liposomes are included in adjuvants currently utilized (77). Binding of the adjuvant and antigen allow for co-stimulation of immune cells, particularly maturation and antigen presentation by dendritic cells and macrophage polarization. Antigen delivery systems with adjuvants can include immune stimulating complexes (ISCOMS), virus-like particles, nanoparticles, emulsions, liposomes, calcium phosphate, tyrosine, and alum (78). When Th1 immunity is required, as in chlamydial protection, there is a dearth of adjuvants. Recently, development of adjuvants derived from plant carbohydrates (e.g. inulin, saponin) has shown the ability to induce protective type-1 responses (79, 80). Other immune potentiators have been explored, usually comprised of purified bacterial or viral components or synthetic mimics. Monophosphoryl Lipid A (MPL), muramyl dipeptide, CpG, lipopeptides, dsRNA, and bacterial

or viral components have been explored as vaccine adjuvants. MPL is a less toxic form of LPS and is licensed for vaccines against HPV and Hepatitis B. The cationic liposome formulation of dimethyldioctadecylammonium (DDA) and trehalose 6,6'-dibehenate (TDB) is currently in human clinical trials for vaccines against tuberculosis and HIV. LTK63, a non-toxic mutant of heat-labile toxin, is the only mucosal adjuvant shown to be safe in human trials (81), and a LTK/CpG formulation generated strong Th1 immune responses against *C. muridarum* in the mouse model (54). There is an unquestioned need to investigate the efficacy of these adjuvants and develop optimal epitope-adjuvant vaccines.

1.4.6 Route of vaccination

An efficacious chlamydial vaccine may need to target and induce protective mucosal immunity. The route of vaccination reflects this necessity and an approach targeting relevant mucosal immune responses at inductive sites of the genital mucosa could be necessary. Mucosal immunization has been shown to be more effective than systemic immunization against mucosal tissue-tropic pathogens (82). *Chlamydia* vaccines should be optimized with appropriate delivery vehicles and adjuvants to induce mucosal immunity, and selection of the appropriate route of vaccination is imperative to generate the most robust mucosal Th1 effector response. Nasal immunization can result in partial genital immunity (38, 64), but recent evidence demonstrates the ability of vaginal immunization to induce robust genital tract and systemic T cell responses (38).

1.5 IMMUNE RESPONSES AND PATHOGENESIS

Inflammatory mediators are capable of inducing tissue destruction and pathogenic immune responses during chlamydial infection (83). Animal models of trachoma and of female genital infection reveal a direct correlation between neutrophil influx and activation and development of tissue damage (84-89) In addition, human transcriptional profiling and genetic studies have determined an association of enhanced innate proinflammatory responses with trachomatous scarring (89-91). Finally, there is in vitro evidence for IL-1, a prominent cytokine released by neutrophils and monocytes, to cause direct oviduct cell damage (92). Since the innate inflammatory response is induced by the interaction of pathogen associated molecular patterns (PAMPs) with pathogen recognition receptors (PRRs) on innate inflammatory cells and host epithelial cells, it should not be surprising that increased bacterial burden leads to enhanced innate inflammation and disease (63, 85, 93). Using the mouse model of genital infection, we demonstrated that repeated abbreviated infections with *Chlamydia muridarum* led to protection from oviduct disease that was associated with a significant reduction in frequency of neutrophils and an increase in the frequency of T cells infiltrating the genital tract upon challenge (94). Furthermore, a single infection with a plasmid-deficient strain of *C. muridarum* protects mice from oviduct disease upon challenge with the fully virulent parental strain (61). This protection is again associated with reduced neutrophil influx and an anamnestic T cell response (94). Thus, avoidance of chlamydial-induced neutrophil influx and neutrophil activation appears essential for disease prevention. A vaccine that promotes adaptive T cell responses that are innocuous for the tissue but potently chlamydiacidal should protect from disease by avoidance of PAMP-induced tissue damaging responses from neutrophils. This is further supported by murine vaccine studies using a variety of antigens and adjuvants that reveal a direct correlation between protection and

the strength of the adaptive IFN-γ-producing CD4 T cell response induced by vaccination (38, 70, 95-98).

Human epidemiological studies demonstrate an increased risk of disease with recurrent infections (99, 100). However, the contribution of pathological effects of the primary infection versus subsequent infections is unknown, and each successful infection would induce an element of tissue-damaging innate responses. Cytokines that have been proposed to play an important role in disease include interleukin-1 (IL-1) (92, 101) and tumor necrosis factor-alpha (TNF-α) (102). Interferon-γ and IL-12 mediate protective T-helper 1 (Th1) responses (103), while T-helper 2 (Th2) responses have been shown to be non-protective and pathogenic (104). The T-helper 17 (Th17) response contributes to generation of Th1 immunity, but is dispensable for both protection and pathogenesis in the mouse model (105, 106). Further experimentation is needed to discern the role of Th17 cells in human chlamydial infection. CD8 T cells have been shown to play a role in pathogenesis in the macaque and mouse models of genital tract infection, possibly through the production of TNF-α (107, 108). Currently, there is no evidence for the role of B cells in tissue pathology during chlamydial infection. Recent technological advances in immune profiling using animal models and human clinical samples provide an opportunity to discern specific components of the immune response that contribute to pathology and provide insight for safe vaccination strategies.

1.6 PROTECTIVE ADAPTIVE RESPONSES

1.6.1 B cells and antibodies

The role of B cells and antibody in the context of anti-chlamydial immunity is not completely understood (109). Mice lacking B cells do not demonstrate an altered course of primary genital infection with *C. muridarum* (110), but are more susceptible to reinfection (111). Immune wild-type mice depleted of CD4 or CD8 T cells clear a secondary challenge; however, B-cell deficient mice are unable to resolve secondary infection after CD4 T cell depletion (112, 113). Passive transfer of immune serum to naïve mice does not provide protection, but antigen-experienced mice with primed CD4 T cells and immune serum are afforded optimal protection (114). Additionally, B cell-deficient mice have a reduced capacity to prime CD4 T cells leading to bacterial dissemination (115). Studies utilizing B-cell deficient mice are an attractive model, but are limited due to the inherent reduction of a significant antigen presenting cell (APC) population and cytokine source, less efficient memory CD4 T cell initiation, possible disruption of lymphoid architecture and sub-capsular sinus macrophages, and enhanced chlamydial dissemination (109).

Antibodies play a role in chlamydial immunity, but their protective effects are likely due to their ability to enhance Th1 activation and cellular effector responses (116). Although early human studies suggested that *Chlamydia*-specific antibodies might play a role in *C. trachomatis* immunity based on *in vitro* neutralization assays (117-119), epidemiological studies indicate that high antibody titers are associated with infertility, and do not correlate with infection resolution or control of ascending infection (120, 121). Murine studies indicate antibody can contribute to pathogen clearance during a secondary infection, but the protective response occurs only if CD4 T cells are present during primary infection (112, 113). The difficulty in developing protective

antibodies relates to identifying epitopes needed for recognition and neutralization. Antibodies specific for chlamydial outer-membrane proteins have shown efficacy in animal models and correlate with protection (122, 123). However, data identifying antigen-specific protective or pathogenic antibodies are limited. Although evidence supports a role for antibody in chlamydial immunity, the protective mechanism seems to be primarily mediated through antibody-augmented cellular immunity.

1.6.2 CD8 T cells

Ample evidence from mice and humans supports the role of CD4 T cells in the resolution of *Chlamydia* infection, but the role of CD8 T cells remains less clear. Mouse models demonstrate that CD8 T cells are not needed for infection clearance; however, antigen-specific CD8 T cell clones can home to the genital tract and enhance clearance through their production of IFN- γ (124, 125). Evidence suggests that up-regulation of PD-L1 in the genital tract following infection may impair CD8 T cell expansion via PD-1 ligation, hampering development of CD8 memory responses (126). This could be a mechanism to avoid cell-mediated uterine pathology, as CD8 T cells can play a role in tissue damage via production of TNF-α (107). Current data suggest that despite the ability of CD8 T cells to contribute to host defense against *Chlamydia*, immunization should seek the induction of Th1 CD4 T cells with the ability to produce high levels of IFN-γ at the site of infection.

1.6.3 CD4 T cells

The critical role of T cells in chlamydial immunity was first demonstrated 30 years ago with the observation that athymic *nude* mice developed a chronic *C. muridarum* infection (127). T cells are detected at the site of infection in mice and humans; antigen-presenting cells can prime T cells in the lymph nodes, where they migrate to inductive sites within the genital tract to clonally expand in response to chlamydial infection. These inductive sites mainly contain CD4 T cells that form perivascular lymphoid clusters (128, 129). CD4 T cells that produce IFN-γ likely mediate protection from *C. muridarum* and *C. trachomatis*. Mice deficient in MHC class II (130), CD4 (113), IL-12 (103), IFN-γ (131), or the IFN-γ receptor (132) have an enhanced susceptibility to infection. Th2 responses correlate with disease progression and pathology during human ocular infection (104). Transfer of chlamydial-specific Th2 clones fails to protect mice from genital infection (133). IFN-γ-mediated control of *in vivo* infection is not fully understood, but IFN-γ controls *in vitro* growth of *C. trachomatis* in human cells by inducing the production of indoleamine-2,3-dioxygenase (IDO) (134). IDO leads to tryptophan degradation and lethality to *C. trachomatis* by starvation of the essential amino acid, but the bacterium can be rescued through the addition of indole (135). IFN-γ producing Th1 cells are essential and sufficient for resolution of infection, but some evidence suggests that a polyfunctional response that includes TNF-α may enhance immunity (136, 137). Additional effector mechanisms include the activation of phagocytic macrophages (138) and CD4 T cell cytotoxicity (139, 140). CD4 T cell recruitment into the female genital tract following infection is dependent on CXCR3 and CCR5, coincident with expression of CCL3, CCL5, and CXCL10 in mucosal tissues (141, 142). T cell recruitment is dependent on the α4β1 integrin-VCAM1 and α4β7-MAdCAM leukocyte adhesion pathways that are upregulated in human genital tract mucosa (143-146). Evidence

suggests that a Th1 CD4 response that generates long-term, sterilizing mucosal immunity will be the optimal goal of immunization.

1.6.4 Resident Memory CD4 T cells

Tissue-resident memory T (TRM) cells have emerged as an important subset of memory T cells in tissue-specific immune responses. These cells reside in epithelial barrier tissues that interface with the environment, such as the gut, lungs, skin, and reproductive tract, and can provide rapid, effective immunity against previously encountered pathogens. TRM are able to respond to a pathogen challenge independent of recruited, circulating systemic T cells (147). The female genital tract mucosa is an important barrier to pathogenic microorganisms. Mouse studies illustrate that HSV infection and vaccination generates accumulation of CD4 TRM cells in the vaginal mucosa that are maintained by a local chemokine gradient and mount a rapid, anamnestic response upon antigenic challenge (148, 149). Mucosal immunization with ultraviolet light (UV)-inactivated *C. trachomatis* complexed with charge switching synthetic adjuvant particles (cSAPs) incorporating the TLR7-agonist resiquimod elicited long-lived protection against chlamydial infection in conventional and humanized mice (38). Vaccination generated mucosal and systemic T cell responses, but optimal clearance required TRM induction in the uterine mucosa. Mucosal CD4 Th1 cells will likely be instrumental to *Chlamydia* vaccine success, as the intensity of mucosal CD4 Th1 cellular responses is an important correlate of immunity (150).

2.0 SPECIFIC AIMS

Chlamydia trachomatis is one of the most common sexually transmitted diseases, with more than 50 million cases occurring worldwide and ~3 million in the United States annually (151). Although infections are frequently asymptomatic, they can lead to the development of serious pathology. In women, untreated cases can result in pelvic inflammatory disease, infertility, chronic pelvic pain, tubal pregnancies, and transmission to uninfected sexual partners. Infection does not induce sterilizing immunity, making the development of an efficacious vaccine necessary. Th1 cells and antibodies play an integral role in the resolution of infection in the mouse model. However, a human vaccine that elicits T cell mediated effector and humoral immunity has been elusive. This shortcoming is compounded by the lack of knowledge concerning immunity in the female genital tract, paucity of data illuminating the mechanisms of protective immune responses to chlamydial antigens, and the inherent difficulty in locating and characterizing low frequencies of *Chlamydia*-specific T cells *in vivo*.

Our long-term goal is to define the protective correlates of immunity to chlamydial genital tract infection in the mouse model and to translate that knowledge to advance a vaccine for humans. Our overall objective is to utilize a T-cell receptor (TCR) transgenic mouse (Tg) approach to characterize the T cell response to infection, and define the role of humoral responses independently of T cells. Our central hypothesis is that a polyfunctional T-helper 1 response is necessary for *in situ* protective immunity, and T-independent B cell responses function to limit bacterial dissemination from the genital tract. The rationale for the proposed research is that, once the contribution of T cell and B cell subsets to infection are characterized, their frequency and phenotype can be manipulated to induce protective responses, resulting in

new and innovative approaches to the prevention of *Chlamydia* infection. We plan to test our hypothesis by pursuing the following three specific aims:

Aim 1: Characterize the phenotype of *Chlamydia*– specific transgenic (Tg) CD4 T cells and their ability to mediate protective immunity. Using an adoptive transfer approach, I will evaluate the ability of Tg CD4 T cells to respond and protect against genital *Chlamydia muridarum* infection *in vivo*. Transgenic cells will be analyzed by flow cytometry and evaluated for activation, proliferation, migration, and Th1 differentiation.

Aim 2: Explore the contribution of T-cell independent B cell responses to protective immunity. To address the role of B cells in immunity to primary chlamydial infection, I will utilize immune-deficient mice and an adoptive transfer approach. Immune deficient mice will be infected with a hypervirulent *C. muridarum* clone (CM001) to determine their ability to survive a disseminated infection in the context of T-cell and B-cell deficiency.

Aim 3: Determine the cognate chlamydial antigen specific for the transgenic TCR. To expand on the adoptive transfer approach, I will utilize biochemical analyses to determine the cognate antigen specific for the Tg TCR. The cytosolic fraction from *C. trachomatis* infected cells and sarkosyl-soluble and insoluble *C. muridarum* EB or RB enriched fractions will be tested for their ability to stimulate Tg T cell proliferation.

3.0 CHAPTER ONE: A *CHLAMYDIA*-SPECIFIC TCR-TRANSGENIC MOUSE DEMONSTRATES TH1 POLYFUNCTIONALITY WITH ENHANCED EFFECTOR FUNCTION

3.1 PREFACE

This chapter is adapted from a published study (Taylor B. Poston[1], Yanyan Qu[2], Jenna Girardi[1], Catherine M. O'Connell[1], Lauren C. Frazer[1], Ali N. Russell[1], McKensie Wall[1], Uma M. Nagarajan[1] and Toni Darville[1]. 2017. Journal of Immunology. pii: ji1700914.)

Work Described in this chapter is in fulfillment of specific aim 1.

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3.2 ABSTRACT

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

3.3 INTRODUCTION

CD4 T cells contribute to cell-mediated immunity through effector functions mediated by the production of cytokines. Polyfunctional T-helper 1 (Th1) cells can sequentially produce IFNγ, IL-2, and TNF in response to T-cell receptor (TCR) stimulation (152). This phenotype has been reported in a variety of infectious disease models, including *Leishmania* (153) tuberculosis (154), HIV (155), *Plasmodium* (156), and *Chlamydia* (38). Polyfunctional Th1 cells demonstrate enhanced protective efficacy in comparison to IFNγ monofunctional cells (154), potentially by producing higher levels of Th1 cytokines (157, 158). Th1 polyfunctionality represents a measure of immunogenicity in vaccine studies (159), and generation of durable polyfunctional Th1 memory will likely be critical for *Chlamydia* vaccine development (38).

Protective immunity against *Chlamydia* is primarily mediated through Th1 cells (103, 160), and the importance of *Chlamydia*-specific CD4 T cells has been demonstrated by adoptive transfer (136, 161) and depletion studies (113). Despite the importance of CD4 T cells in controlling chlamydial infection, little is known about the generation of polyfunctional Th1 cells and how they contribute to cell-mediated immunity. Previous studies showed that a *Chlamydia*-specific IFNγ monofunctional Th1 clone was not protective, whereas a clone producing IFNγ and TNF cleared *C. muridarum* infection in nude mice (162). Vaccine models have shown that antigens and adjuvants generating polyfunctional (IFNγ+ TNF+) Th1 cells were more protective than IFNγ monofunctional Th1 cells (137, 163), and this protection has been observed in immunogenicity studies investigating single (164, 165) or multiple chlamydial antigens (80, 98, 166, 167).

We recently developed the first TCR transgenic (Tg) mouse specific for a conserved antigen in *C. muridarum* and *C. trachomatis* to investigate the polyfunctional Th1 response *in*

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

3.4 MATERIALS AND METHODS

3.4.1 Strains, cell lines, and culture conditions

Chlamydia muridarum Nigg stock (AR Nigg) was obtained from Roger Rank at the University of Arkansas for Medical Sciences, and has been previously described (168). *C. trachomatis* D/UW-3/Cx (169) was obtained from the American Type Culture Collection (Manassas, VA) and plaque purified before use (168). Plaque-purified *C. trachomatis* D/UW-3/Cx, Nigg strain CM001 (170), and plasmid-deficient CM3.1 (61) were propagated in mycoplasma-free L929 cells (171), and titrated by plaque assay or as inclusion-forming units (172), using a fluorescently tagged anti-chlamydial lipopolysaccharide monoclonal antibody (Bio-Rad). UV-inactivated bacteria were prepared, as described (173).

3.4.2 Generation of a *Chlamydia*-specific T cell transgenic mouse

Two eight-week-old female C57BL/6J mice were intravaginally infected with 3×10^5 inclusion forming units (IFU) of wild-type *Chlamydia muridarum* Nigg. Infected mice were allowed to resolve primary infection, and were re-challenged two months later. The spleen and lymph nodes were collected one-week post-secondary challenge, and single-cell suspensions were stimulated *ex vivo* with reticulate body (RB)-enriched Nigg (1μg/mL) for 5 days prior to fusion with murine BW5147 T cell lymphoma cells (174) in 50% PEG solution. Fused cells were cultured in HAT medium for an additional 7 to 9 days. Hybridomas were screened and sorted based on CD3, CD4, CD8, and TCRβ expression. Sorted CD4 T cell hybridomas underwent limiting dilution and were co-cultured with irradiated syngeneic splenocytes in the presence of Nigg elementary bodies (EB) or RB (1μg/mL) for 24-48 hours at 37°C. Harvested supernatants were tested for IL-2 and IFNγ levels by enzyme-linked immunosorbent assay (ELISA) from R&D Systems. The CD4 T cell clone with the highest co-production of IL-2 and IFNγ in the presence of Nigg EB was harvested and cultured for cloning of TCRα and TCRβ cDNA. RNA from the CD4 T cell clone was made using the Qiagen RNAeasy method, and TCRα and TCRβ cDNA was obtained using the SuperTCRExpress™ Mouse TCR Vα/ Vβ Repertoire Clone Screening Assay Kit (BioMed Immunotech), which contains 5' RACE primers for all TCR Vα/Vβ. The cDNAs were cloned into the TOPO vector (Promega), sequenced, and identified as Vα6 and Vβ10. Genomic sequences corresponding to the mRNA sequences were used to map the variable, joining, and constant regions in the sequence. Primers with flanking XmaI site and NotI site, GATCCCGGGCAGAGCTGCAGCCTTCCCAAGGCTC and CATGCGGCCGCAGTGCTAGGAAGGGCGGCCTGGAC were generated for amplifying the variable region of Vα6 from genomic DNA. Primers with flanking XhoI site and SacII site,

TCCGCTCGAGCCTTGACCCAACTATGGGCTGT and ATTCCCGCGGCTGGTCTACTCCAAACTACTCCAGG were generated to amplify the variable region of Vβ10. Vα6 amplicon was cloned into the pTαcass and Vβ10 amplicon into pTβcass vectors (175), which contain the respective promoters for Vα and Vβ expression and provided the joining and constant region, as a genomic clone. DNA constructs were sequenced for confirmation, linearized at SalI (Vα6) and KpnI (Vβ10) sites, respectively, purified and injected into the pronuclei of (C57BL/6J x SJL/J) F2 fertilized eggs.

3.4.3 Animals

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

3.4.4 Generation of bone marrow-derived DCs

Dendritic cells were generated from the tibias/femurs of C57BL/6J mice as previously described (176). Briefly, erythrocytes were lysed with ACK lysis buffer, and bone marrow precursors were cultured for 7 days in complete media (RPMI containing 10% fetal bovine serum, 2 mM glutamine, 10 mM HEPES, pH 7.4, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and 50 μg/ml gentamicin) supplemented with 1000 U/mL recombinant murine GM-CSF and 1000 U/mL recombinant murine IL-4 (both from Peprotech). CD11c+ DCs were isolated using specific beads (Miltenyi Biotech), according to the manufacturer's protocol.

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

The spleens of littermate or TCR transgenic mice were processed into a single cell suspension, as described previously (177). Splenocytes (1×10^5 cells/well) were seeded in a 96-well flat-bottomed tissue culture plate in complete media with 5 μg/ml *C. muridarum* AR Nigg, plasmid-deficient CM 3.1, *C. trachomatis* D/UW-3/Cx, or recombinant ovalbumin (Sigma) for 6 days. Splenocytes were treated with 20 U/mL murine IL-2 (Peprotech) over the final 48 hours. Cells were treated with 20 μl of Alamar Blue (Biosource) 6 hours before the end of the 6-day culture, and proliferation was measured at 530-nm excitation/590-nm emission with a Biotek fluorescence microplate reader.

Alternatively, transgenic or polyclonal CD4+ T cells were isolated from the spleens of naïve TCR transgenic or wild-type C57BL/6J mice by negative magnetic selection (EasySep™ Mouse CD4 T cell). Isolated CD4+ T cells were co-cultured at a 1:5 ratio with bone marrow-derived dendritic cells (BMDCs) for 3 days in the presence or absence of *C. muridarum* AR

Nigg (5μg/mL). Expression of CD69 and Ki67 was determined by FACS surface and nuclear staining respectively, as described previously (178). Supernatants from dendritic-cell stimulated CD4+ T cells were collected and IL-2 concentrations determined by ELISA.

3.4.6 Murine *Chlamydia* infection and monitoring

For genital tract infection, female mice at least 8 weeks old were s.c. injected with 2.5 mg medroxyprogesterone (Depo-Provera®; Upjohn) 5–7 days prior to infection to induce a state of anestrous (179). Mice were intravaginally inoculated with 3×10^5 inclusion-forming units (IFU) CM001 diluted in 30 μl sucrose-sodium phosphate-glutamic acid buffer. Mice were monitored for cervicovaginal shedding via endocervical swabs (180), and IFUs were calculated, as described previously (85). Prior to reinfection, mice were treated intraperitoneally with 0.3 mg doxycycline in 100 μl phosphate buffered saline for 5 days (94). Animal welfare was monitored daily. Genital tract gross pathology, including presence of hydrosalpinx, was examined and recorded at sacrifice.

3.4.7 Lymphocyte isolation and flow cytometry

Spleen, iliac lymph nodes, oviducts, uterine horns, and cervical tissues were isolated from sacrificed mice. Cervical tissue and uterine horns were minced separately and incubated with 1 mL of collagenase I (Sigma) for 20 minutes at 37°C before neutralization with EDTA (10 μM). Single-cell suspensions were prepared by dispersing tissues through a 70-micron tissue strainer (Falcon). Cell suspensions were treated with erythrocyte lysis buffer (VitaLyse®; BioE), incubated in Fc block (5 μg/ml) for 10 minutes, and stained with LIVE/DEAD Fixable Yellow (Life Technologies) plus various combinations of the following fluorochrome-labeled antibodies:

anti-CD3 (clone 17A2) anti-CD3e (145-C211), anti-CD4 (GK1.5, RM4-5, H129.19), anti-CD8a (53-6.7), anti-TCRVβ10 (V21.5), anti-TCRβ (H57-597), anti-CD45 (30-F11) anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD44 (IM7), anti-CD62L (MEL-14), and anti-CD69 (H1.2F3), from BD Biosciences. The samples were analyzed on a CyAN ADP (Beckman Coulter) or LSR II flow cytometer (BD Bioscience), and data were analyzed with FlowJo software.

3.4.8 CFSE-labeling and adoptive transfer

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

3.4.9 Intracellular cytokine detection

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

following fluorochrome-labeled antibodies: anti-IL-2 (JES6-5H4), anti-TNFα (MP6-XT22), and anti-IFNγ (XMG1.2) from BD Bioscience.

3.4.10 Statistical analysis

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

3.5 RESULTS

3.5.1 Generation of *Chlamydia*-specific TCR transgenic mouse

Previous studies demonstrate that the frequency of IFNγ-producing CD4 Th1 cells correlate with enhanced chlamydial clearance from the genital tract (103, 136, 160, 181). To directly monitor CD4 T cell responses during murine infection, we generated a Tg mouse strain with a TCR specific for *C. muridarum,* which demonstrated cross-reactivity with *C. trachomatis* (Fig. 1A). The TCR genes were isolated from a hybridoma expressing Vα6 and Vβ10 chains specific for *C. muridarum* elementary and reticulate bodies. These genes were cloned into an expression vector used to generate germline Tg mice. Founder Tg mice almost uniformly express Vβ10 on the surface of autologous CD4 T cells (Fig. 1B), and were backcrossed to C57BL/6J mice for over ten generations. Tg mouse splenocytes demonstrated a 6-8-fold increase in *Chlamydia*-specific proliferation compared to littermates, with minimal proliferation induced by ovalbumin (Fig.

1A). *C. muridarum* Nigg plasmid-competent and -deficient (CM3.1) strains stimulated TCR Tg splenocytes equally. To further confirm the specificity of Tg CD4 T cells, we analyzed their ability to activate and proliferate in comparison to wild-type polyclonal CD4 T cells *in vitro*. Transgenic CD4 T cells demonstrated the ability to co-express high levels of CD69 and Ki67 when cultured with BMDC and *C. muridarum* elementary bodies, with over 40% being double-positive and ~50% expressing CD69 (Fig. 1C). In contrast, minimal activation was observed with polyclonal CD4 T cells. This enhanced proliferation was associated with significantly increased levels of IL-2 in the supernatants of stimulated TCR Tg CD4 T cells that were 29 times higher than polyclonal CD4 T cells (Fig. 1D). We next examined the ability of Tg CD4 T cells to become activated and proliferate *in vivo*.

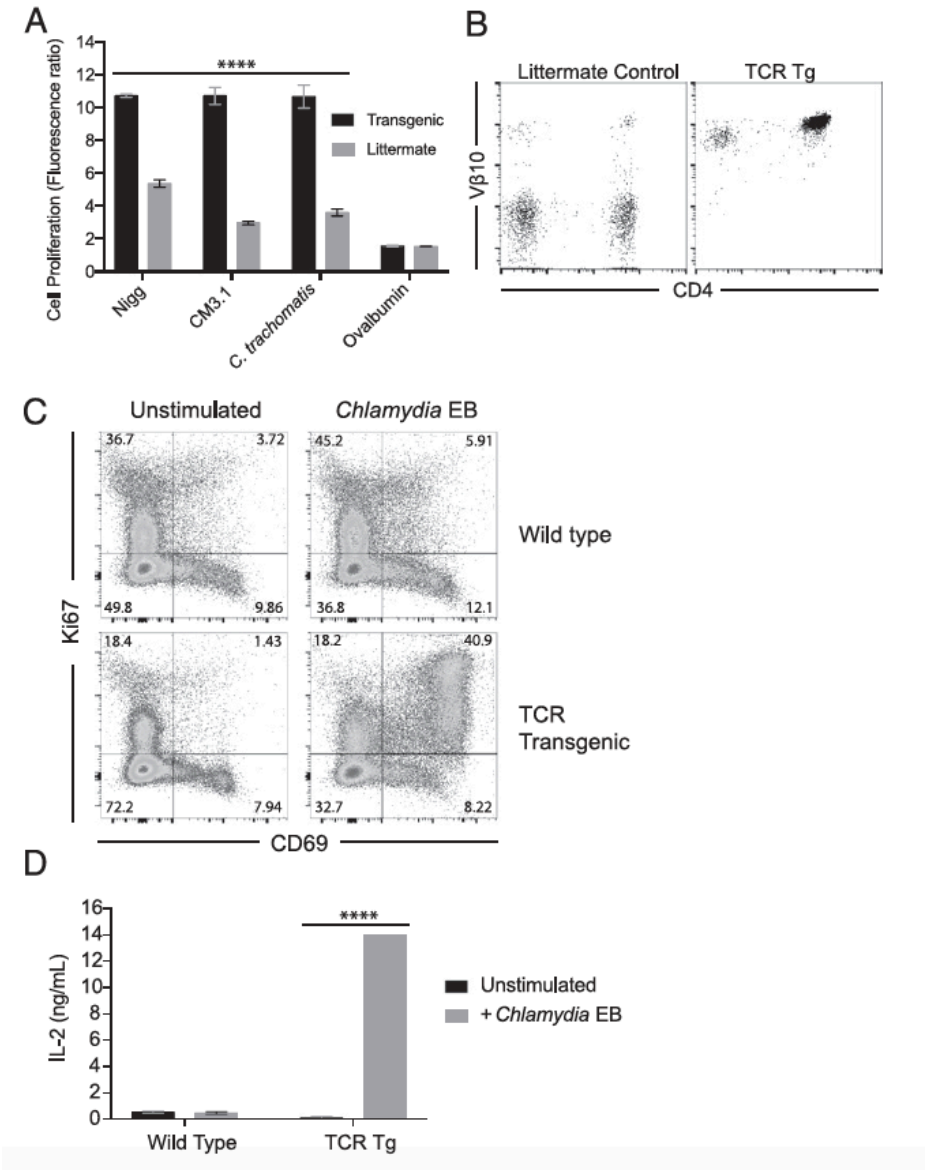

Figure 1. Generation of a *Chlamydia*-specific TCR transgenic mouse.
(A) TCR Tg or littermate splenocytes were stimulated with 5 μg/ml *C. trachomatis, C. muridarum* AR Nigg, plasmid-deficient Nigg (CM 3.1), or recombinant ovalbumin, as indicated. Splenocytes were stimulated for 4 days, followed by 2 additional days in the presence of 20 U/mL IL-2. Change in proliferation was determined by the ratio of Alamar Blue fluorescent intensity compared to unstimulated controls (**** $P < 0.0001$ determined by two-way ANOVA). (B) Peripheral blood from C57BL/6J backcross progeny of Tg founder mice or littermate controls was stained with antibodies against CD3, CD4, and Vβ10. The right dot plot is representative of the Vβ10 expression on CD3+CD4+ T cells from Tg mice. (C) CD4+ T cells from Tg mice or wild type mice were incubated for 3 days with BMDC pulsed with and without 5 μg/ml *C. muridarum*. The right dot plots show CD69 and Ki67 expression from Tg and polyclonal CD4 T cells after stimulation. (D) Supernatants from dendritic cell-stimulated CD4 T cells were analyzed for IL-2 by ELISA (**** $P < 0.0001$ determined by two-way ANOVA).

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

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

$CD44^{hi}$ expression is used as a marker of T cell activation and Th1 memory (182, 183), and this memory phenotype was significantly increased among TCR Tg CD4 T cells in the iliac

nodes throughout the course of primary infection, and on day 13 of secondary infection, compared to endogenous cells. Similar CD44 expression was observed for splenic TCR Tg cells. On day 13 post-secondary challenge, 85-98% of splenic Tg T cells were $CD44^{hi}$, and these Tg cells comprised ~7% of the total splenic $CD44^{hi}$ CD4 T cell pool (Fig. 2F). The kinetics reflect the enhanced ability of Tg cells to adopt an activated effector and/or effector memory phenotype in the lymphoid tissues throughout infection compared to the endogenous T cell repertoire, and by day 8 in the infected peripheral tissues. These data collectively support other studies demonstrating *Chlamydia*-specific CD4 T cell priming and proliferation in the ILN (115, 184) and the presence of activated cells in the genital tract one-week post-infection (185).

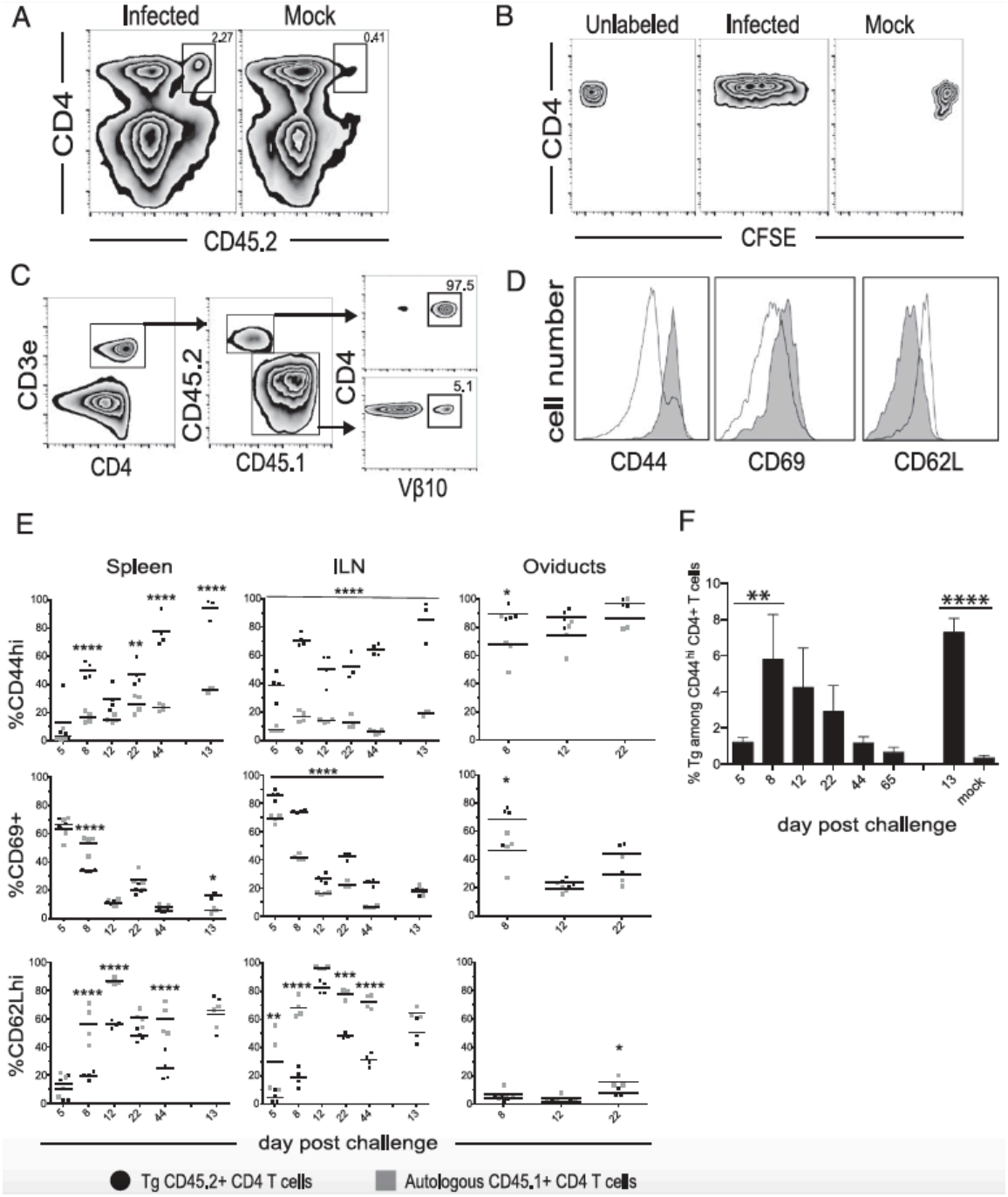

Figure 2. Proliferation and activation kinetics of TCR Tg CD4 T cells during *C. muridarum* genital tract infection.

One million CFSE-labeled Tg T cells were transferred into CD45.1+ female recipients, which were mock infected or infected with CM001. (A) Iliac lymph nodes from infected (top left) or mock-infected (top right) mice were examined for the presence of Tg T cells, and (B) Tg cells were examined for CFSE fluorescence. (C) Diagram of the flow cytometric gating strategy used to analyze CD62L, CD69, and CD44 expression by CD45.2+ Tg and CD45.1+ endogenous, polyclonal CD4 T cells. (D) Representative histograms comparing surface marker expression

between Tg (grey) and endogenous (white) CD4 T cells during early infection. (E) Expression of CD62L, CD69, and CD44 on donor Tg and endogenous host CD4 T cells in the spleen, iliac lymph node, and oviducts on the indicated days post primary and secondary infection. Data points are representative of individual mice. Horizontal bars indicate the mean of 3-4 mice per group. Statistical significance was noted relative to autologous CD45.1+ CD4 T cells and indicated by asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ by two-way ANOVA. (F) Percentage of transgenic cells comprising the $CD44^{hi}$ CD4 T cell population from the spleen on the indicated days post-infection (** $P < 0.01$, **** $P < 0.0001$ by RM one-way ANOVA).

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

After demonstrating that TCR Tg CD4 T cells become activated, proliferate, and migrate to the infected genital tract, we investigated whether they would provide protection and produce IFNγ upon challenge. Prior data from our lab had revealed the plaque-purified strain of *C. muridarum* Nigg, CM001, resulted in disseminated lethal infection in *Rag1*$^{-/-}$ mice after intravaginal inoculation (170). *Rag1*$^{-/-}$ mice received adoptive transfers of 10^3 Tg or 10^3, 10^4, and 10^6 polyclonal CD4 T cells 5 days prior to infection (Fig. 3A). A precursor frequency of 10^6 polyclonal CD4 T cells was used as a positive control, based on previous observations that this dose conferred protection (data not shown). Mice receiving 10^3 Tg CD4 T cells survived infection, whereas 10^3 and 10^4 polyclonal CD4 T cells were not protective. TCR Tg CD4 T cells demonstrated a recall response characterized by the production of inflammatory cytokines. Transgenic CD4 T cells isolated from the uterine horns and oviducts on day 7 post-secondary challenge produced IFNγ and TNF in response to *in vitro* re-stimulation with UV-irradiated *C. muridarum* (Fig. 3B). These data indicate that TCR Tg CD4 T cells prevent death from disseminating infection, migrate to infected tissues, and acquire Th1 effector functions post-infection.

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

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

We also investigated if TCR Tg CD4 T cells would contribute to a recall response upon secondary challenge. Immune mice that had received 10^3 or 10^6 TCR Tg CD4 T cells prior to primary infection exhibited a 4.5- and 3-log reduction in shedding, respectively, on day 3 post-challenge compared to primary infection (Figs. 3C, 3D). Mice that received 10^3 Tg CD4 T cells prior to primary infection were more resistant to challenge when compared to mice that received 10^6 Tg CD4 T cells (Fig. 3D). This was consistent with findings in other TCR transgenic models, where lower numbers of adoptively transferred naïve Tg CD4 T cells induce better memory development. It is possible that decreased interclonal competition for antigen leads to enhanced differentiation of the fittest effectors into memory cells (186). In contrast, infectious burden during secondary infection was significantly lowered in mice receiving 10^6 but not 10^3 polyclonal CD4 T cells. In this instance, a broad array of antigen-specific cells avoids interclonal competition for peptide-MHC class II stimulation.

Although mice that were re-infected without prior receipt of adoptive T cells failed to exhibit any decline in infectious burden up to two weeks post inoculation, on day 3 post-

challenge, their infectious burden was 2-log lower than that observed during primary infection. This transient protection may be a result of circulating T-cell independent antibody or memory γδ T cells that are not capable of clearing infection independent of conventional CD4 T cells.

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

The TCR transgenic mouse was developed using a TCR that induced a Th1 response after chlamydial stimulation *in vitro*. We hypothesized that TCR Tg CD4 T cells would differentiate into polyfunctional Th1 cells *in vivo* since adoptive transfer of these cells led to enhanced chlamydial clearance during primary infection (Fig. 3C). We detected significantly increased percentages of IFNγ+TNF+ double-positive cells in the spleen and genital tissues of *Tcra*$^{-/-}$ mice receiving Tg CD4 T cells, on day 13 post-infection compared to mice receiving polyclonal CD4 T cells (Fig. 4A, 4B). Additionally, transgenic polyfunctional IFNγ+TNF+ CD4 T cells expressed significantly higher amounts of IFNγ compared to polyfunctional, polyclonal populations. TCR Tg polyfunctionality for TNF and IFNγ was associated with increased IFNγ production compared to cells singly positive for IFNγ (Fig. 4C). These data indicate that TCR Tg CD4 T cells preferentially adopt a polyfunctional phenotype characterized by high levels of IFNγ production. Furthermore, the percentage of triple-positive (IFNγ+TNF+IL-2+) CD4 T cells in the spleen was increased among the Tg CD4 T cell population when compared to polyclonal CD4 T cells on day 48 post-infection (Fig. 4D, 4E). Triple positive transgenic cells expressed significantly higher levels of IFNγ per cell compared with single- or double-positive cells (Fig. 4F), as previously described for pathogen-specific polyfunctional T cells in other models of infection (155, 158, 187). Triple positive cells also expressed significantly higher levels of TNF per cell compared to TNF monofunctional cells, but at a reduced magnitude (60% increase), and

no differences were observed in IL-2 GMFI (data not shown). These data collectively show that Tg CD4 T cells have superior functional capacity with enhanced cytokine production, and this polyfunctional effector response is associated with enhanced bacterial clearance.

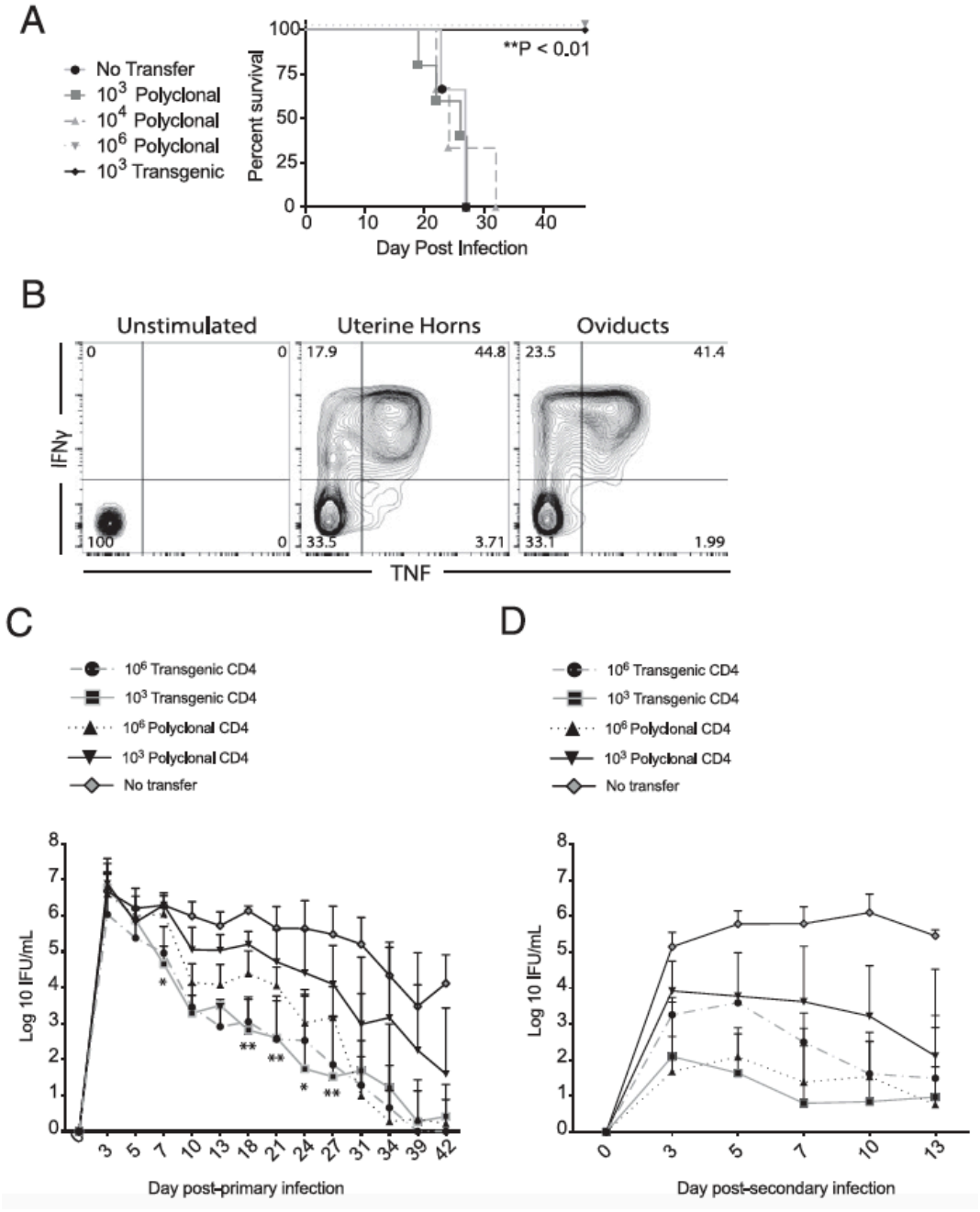

Figure 3. TCR Tg CD4 T cells mediate protection and demonstrate a recall response following challenge.

Rag1$^{-/-}$ mice (4-5 per group) were mock treated or intravenously injected with indicated numbers of CD4 T cells isolated from the spleens of naïve C57BL/6J wild type or Tg mice, and intravaginally inoculated with CM001 5 days later. Survival was monitored daily, and an exact log rank test was used to analyze survival differences between polyclonal and Tg groups (** $P <$ 0.01). (B) Recall response of CD4+Vβ10+ Tg CD4 T cells to secondary infection. Following primary infection, Tg mice were treated with doxycycline, rested for 9 weeks, and re-challenged with CM001. On day 7 post-challenge, genital tract CD4 T cells were harvested, stimulated with 5 μg/ml *C. muridarum*, and analyzed for IFNγ and TNF production by intracellular cytokine staining. (C) Indicated numbers of naïve Tg or polyclonal CD4 T cells were adoptively transferred to *Tcra*$^{-/-}$ mice 5 days prior to intravaginal infection with CM001, and the course of primary infection was monitored by culture of lower genital tract swabs. Significance was determined by two-way RM ANOVA with a post-hoc Tukey test. Data represent the mean ± SEM of 10 mice per group. Comparison of individual days for 10^3 Tg versus 10^6 polyclonal: * P < 0.05, ** P < 0.01. Comparison of primary infection course between groups: P=NS for 10^6 Tg versus 10^3 Tg. P =0.0001 for 10^3 Tg versus 10^6 Polyclonal. P <0.0001 for all remaining group comparisons. (D) Immune mice were treated with doxycycline on days 52-56 post-infection, rested for 5 weeks, re-challenged with CM001, and infection monitored by culture of vaginal swabs. Significance was determined by two-way RM ANOVA with a post-hoc Tukey test. Data represent the mean ± SEM of 4-5 mice per group. Comparison of groups over primary infection course: P=NS for 10^3 Tg versus 10^6 polyclonal, 10^6 Tg versus 10^3 or 10^6 polyclonal. P < 0.05 for 10^3 Tg versus 10^6 Tg. P <0.0001 for all remaining group comparisons.

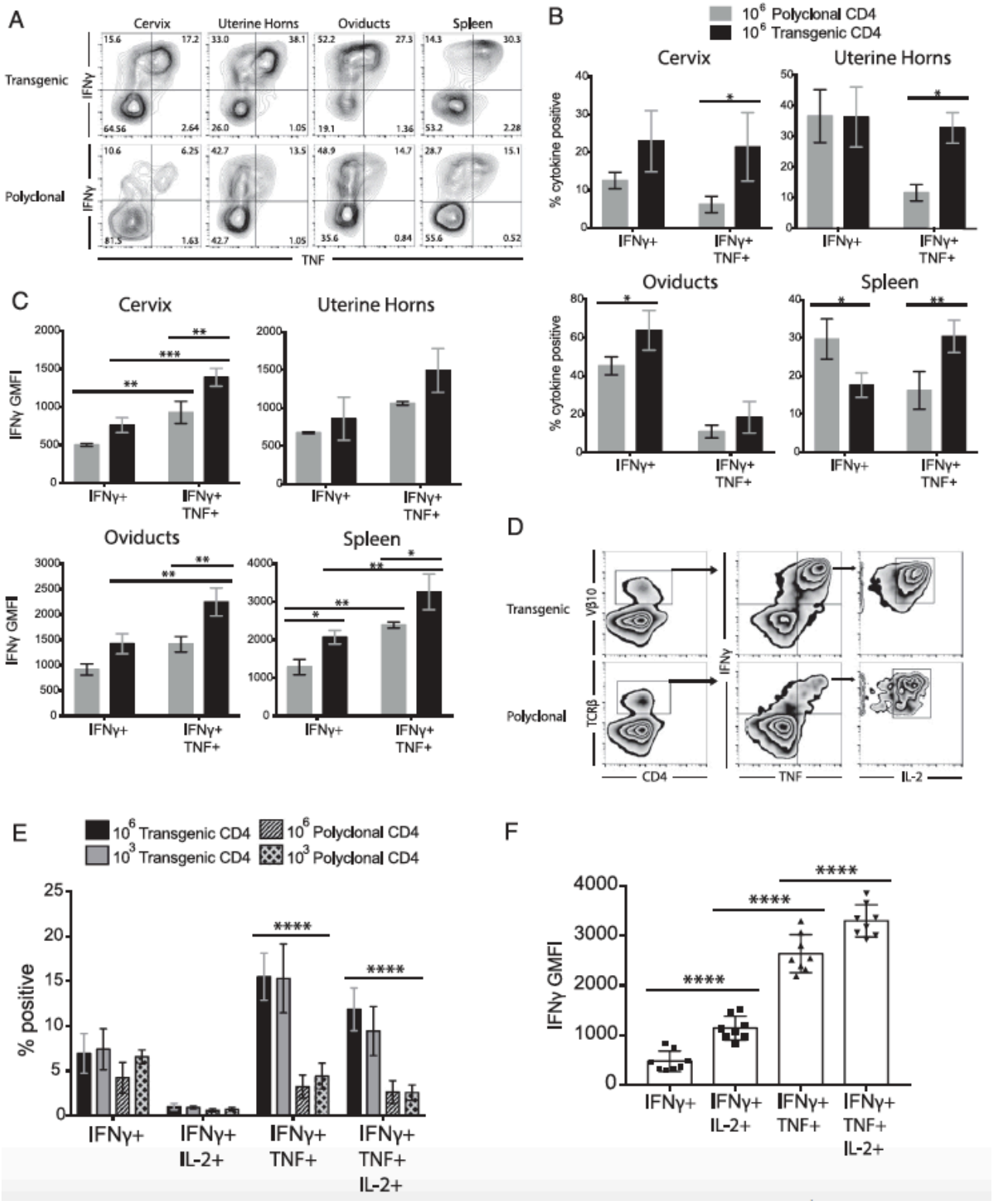

Figure 4. Naïve TCR Tg CD4 T cells differentiate into polyfunctional Th1 cells with increased IFNγ production.

Tcra$^{-/-}$ mice receiving polyclonal or Tg CD4 T cells were analyzed for polyfunctional Th1 responses on Day 13 (A-B) and Day 48 (D-E) post-infection. (A) CD4 T cells isolated from

indicated tissues were stimulated with 5 μg/ml *C. muridarum* and evaluated for intracellular cytokine production. Contour plots show representative IFNγ and TNF co-production by Tg CD3e+CD4+Vβ10+ cells (top) and polyclonal CD3e+CD4+TCRβ+ cells (bottom). (B) Comparison of the percentage of cytokine positive cells between single positive (IFNγ+) and double-positive (IFNγ+ TNF+) polyclonal and Tg CD4 T cells (C) Associated IFNγ geometric mean fluorescent intensities (GMFI). Data represent the mean ± SD of 3 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ determined by two-way ANOVA. (D) Diagram of the flow cytometric gating strategy used to analyze CD4 T cell polyfunctionality on day 48 post-infection. (E) The frequency of single positive (IFNγ+), double positive (IFNγ+ TNF+), and triple positive (IFNγ+ TNF+IL-2+) Tg or polyclonal CD4 T cells on day 48 post-infection. Data represent the mean ± SD of 4-5 mice per group (**** $P < 0.0001$). (F) Comparison of the IFNγ GMFI between spleen-isolated Tg CD4 T cell single, double, and triple-positive populations. Data represent the mean ± SD of 8 mice receiving 10^3 and 10^6 Tg cells. **** $P < 0.0001$ determined by two-way RM ANOVA.

3.6 DISCUSSION

Pathogen-specific TCR Tg mice have been utilized in a variety of infectious disease models (188-195), including NR1 mice that recognize *C. trachomatis* (185). Adoptive transfer of naïve TCR Tg cells is a superior approach to transfer of *in vitro* maintained T cell lines, since naïve TCR Tg cells allow analysis of the initial antigen encounter and phenotypic differences between *in vivo* derived effector and memory populations. We developed a TCR Tg mouse that recognizes a conserved antigen between *C. trachomatis* and *C. muridarum*. The TCR Tg cells of this mouse react to *C. trachomatis* serovars D, F, H, and L2 (data not shown), and preliminary biochemical analyses reveal that they recognize a soluble, secreted protein enriched in reticulate bodies (data not shown). We have excluded commonly studied immunogenic antigens such as MOMP, OmcB, HSP60, and PmpG. This report demonstrates the first TCR to protect against *C. muridarum* genital infection, and allowed us to analyze enhanced effector function afforded by Th1 polyfunctionality at a level that had not been previously attainable. Generation of this mouse has allowed for the unique ability to adoptively transfer TCR Tg cells for investigation of

antigen-specific T cell responses to both mouse and human chlamydial strains in the murine model of genital tract infection.

Development of the *Chlamydia*-specific TCR Tg mouse was based on selection of a Th1 clone specific for both *C. muridarum* elementary bodies and reticulate bodies. We used a non-biased approach, by analyzing T cell clones demonstrating the strongest IFNγ and IL-2 production, which has been shown to be effective in TCR Tg mouse development. Selecting clones reactive against whole organism or crude antigen preparations has resulted in Tg CD4 T cells with the capacity to mount robust effector and memory responses following infection and vaccination (194), compared to model antigens (196). Our studies reveal that the Tg CD4 T cells possess a TCR, which confers protection against intravaginal *C. muridarum* infection.

These Tg CD4 T cells become activated, proliferate extensively, and produce high levels of IL-2 when stimulated with *C. muridarum*. Naïve and memory CD4 T cells require TCR stimulation in combination with IL-2 signaling to proliferate (197), and TCR engagement upregulates IL-2R subunits (198). The strength of IL-2 signaling also correlates with the magnitude of proliferation in Th1 cells (199), and IFNγ expression increases with successive cell divisions (200). Furthermore, IL-2 signaling during priming enhances differentiation of the effector pool into memory (201).

Based on the ability of these cells to recognize chlamydia *in vitro*, we used an adoptive transfer approach to analyze the proliferation and activation kinetics *in vivo*. Similar to the *C. trachomatis* model, our approach revealed that *C. muridarum* infection induced significant TCR Tg CD4 T cell activation and expansion in the iliac lymph nodes by day 5 and Tg cells expressed an activated phenotype ($CD44^{hi}CD69+CD62L^{lo}$). The $CD44^{hi}$ $CD62L^{lo}$ phenotype was also observed in the infected oviducts. Expression of CD69 on Tg cells in the spleen and oviducts on

day 8, in our model, is likely due to the ability of CM001 to quickly disseminate to the distal organs and rapidly ascend the genital tract. Increased CD69 expression on CD4 T cells early in CM001 infection may be a result of local priming events, prior to tissue infiltration of activated T cells primed in the ILN. At later time points, CD44 expression in SLOs steadily increased, particularly during re-infection, consistent with the formation of memory T cells. These kinetics are similar to other infectious disease models of CD4 T cell activation and memory (185, 190, 195, 202). After priming in the ILN, Tg cells made up ~7% of all $CD44^{hi}$ CD4 T cells in the spleen, which consistently decreased through infection, until mice received a secondary challenge. This is consistent with other systems demonstrating that peak CD4 T cell expansion typically occurs after one week (203), and is followed by CD4 T cell contraction over 1-2 weeks, where 90-95% of the expanded population undergoes cell death (204, 205). Late in the course of *C. muridarum* infection and during reinfection, a majority of Tg cells expressed high levels of the memory marker CD44. Additional phenotyping experiments are required to determine the proportions of Tg T cells in the terminal effector, effector memory, and central memory pools.

TCR Tg cells prevented death in immunocompromised mice infected with CM001 and upon secondary challenge, these cells were recalled to the infected tissues and produced IFNγ and TNF. These results parallel other infectious disease models demonstrating that adoptive transfer of antigen-specific naïve CD4 T cells can protect against lethality (195). Adoptive transfer to T- and B-cell deficient *Rag1*$^{-/-}$ hosts illustrates the CD4 T helper-independent protective function of TCR Tg cells, likely mediated through their production of IFNγ (206). Furthermore, transfer of these cells into αβ TCR-deficient mice led to enhanced protection against primary infection and equivalent protection against a secondary challenge compared to the polyclonal response. Comparable levels of oviduct gross pathology were observed between

the TCR Tg and polyclonal groups (100% and 95% hydrosalpinx, respectively), which was not surprising given the ability of CM001 to induce severe pathology in wild-type mice (207). Future studies utilizing vaccination or adoptive transfer of *in vitro* primed TCR Tg cells should help reveal their capacity to protect against oviduct pathology.

Reduced bacterial burden mediated by TCR Tg cells was associated with increased frequencies of double- and triple-positive Th1 populations producing higher levels of IFNγ compared to polyclonal CD4 T cells. IFNγ is a critical effector molecule for controlling chlamydial replication (131, 136, 208-210), and enhanced frequencies of polyfunctional Tg cells producing IL-2 could allow for enhanced Th1 effector proliferation. Our TCR Tg cells clearly recognize an antigen that drives a favorable response that leads to enhanced bacterial clearance and resistance to challenge infection. Persistent antigen and antigen depots reduce the memory pool leading to non-protective responses from terminally differentiated, exhausted T cells. Removal of antigen drives T cell transition to memory (211), and these cells remain plastic and heterogeneous (212, 213). Thus, triple-positive Th1 Tg cells could be a consequence of improved effector function leading to lower bacterial load (214, 215). In addition, Tg cells may also demonstrate greater functional avidity, which has been linked with improved disease outcomes (216) and expression of decreased levels of inhibitory receptors (217). High avidity T cells are less susceptible to activation-induced cell death (218) and demonstrate increased polyfunctionality (219, 220).

Our analyses were limited to the study of CD4 T cells and focused on profiling three major Th1 cytokines. A comprehensive analysis of TCR Tg cell production of cytokines, chemokines, and cytotoxic effectors, as well as their helper function for antibody production by B cells is needed to fully delineate their protective, or pathological, mechanisms. Alternative

effectors (140) and antibody (111, 221) have been shown to play a significant role in mediating chlamydial clearance. Additional analysis of the recall response is needed to determine the mechanisms whereby equivalent protection from reinfection occurred in *Rag1*$^{-/-}$ mice that had received polyclonal T cells or monoclonal TCR Tg T cells. Potentially, polyclonal, polyfunctional T cells were maintained and IFNγ monofunctional cells culled, or monofunctional cells responding to a variety of antigens elicit similar protection to polyfunctional TCR Tg cells recognizing a single antigen. The finding that a single immunogenic antigen that elicits polyfunctional T cells can successfully induce a protective response is encouraging from a subunit vaccinology perspective. Viral models demonstrate that primary and secondary effectors share organ-specific expression patterns, but secondary effectors are more polyfunctional (triple positive); polyfunctional cells also express higher levels of genes associated with survival and migration (222). Whether CD4 T cell polyfunctionality can predict memory generation and subsequent, enhanced secondary effector functions is an important area to be addressed. Once we have identified the antigen recognized by the TCR Tg T cells, we can determine if vaccination with this antigen drives induction of a protective polyfunctional response, and whether adoptive transfer of *in vitro* antigen-primed Tg T cells can protect from infection and disease.

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

4.0 CHAPTER TWO: T-CELL INDEPENDENT INTERFERON GAMMA AND B CELLS SYNERGIZE TO PREVENT MORTALITY ASSOCIATED WITH DISSEMINATED *CHLAMYDIA MURIDARUM* GENITAL TRACT INFECTION

4.1 PREFACE

This chapter is adapted from a manuscript in preparation (Taylor Poston[1], Catherine O'Connell[1], Amy Scurlock[3], Jenna Girardi[1], Tony Marinov[2], Jeannie Sullivan[1], Uma Nagarajan[1], and Toni Darville[1]). Work described in this chapter is in fulfillment of specific aim 2.

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4.2 ABSTRACT

CD4 T-cells and antibody are required for optimal acquired immunity to *C. muridarum* genital tract infection, and conventional T-cell mediated IFN-γ production is required to survive and clear hypervirulent *C. muridarum* infection in the absence of humoral immunity. However, the role of protective T-cell independent responses against primary *C. muridarum* infection remain unclear. We addressed this problem by inoculating wild-type and immune-deficient mice with a hypervirulent strain (CM001) isolated from *C. muridarum* Nigg stock. Genital CM001 inoculation resulted in transient dissemination to the lungs and spleen in immunocompetent mice prior to clearance. However, CM001 infection induced lethality in *Rag1*$^{-/-}$, *STAT1*$^{-/-}$, and *IFNG*$^{-/-}$ mice, while muMT, nude, and *Tcra*$^{-/-}$ mice survived. Adoptive transfer of convalescent immune sera or naïve B cells to *Rag1*$^{-/-}$ mice protected against CM001 lethality. B cell protection was associated with a significant reduction in the lung chlamydial burden of genitally infected mice. These data reveal a T-cell independent role for B cells and IFN-γ in control of disseminating *C. muridarum* infection and could be an important mechanism of extragenital host defense.

4.3 INTRODUCTION

Chlamydia trachomatis is the most prevalent sexually transmitted bacterial infection and a significant cause of female reproductive tract morbidity. The development of a vaccine remains a top global health priority (223). Pre-clinical *C. trachomatis* vaccine development includes utilization of the murine genital infection model for determination of protective immune responses against *Chlamydia muridarum*. Genital infection of mice with specific immune deficiencies has provided a method for determining the protective contribution afforded by humoral and cell-mediated immunity.

Previous studies revealed a central role for CD4 T cells in protection against primary intravaginal *C. muridarum* infection, and wild-type mice demonstrate comparable clearance to B-cell deficient mice (110). It was further revealed that infection of B-cell deficient mice results in a transient disseminated infection that is likely cleared through enhanced systemic CD4 T cell responses (115). Multiple experiments have demonstrated a clear role for IFN-γ producing Th1 cells in primary and acquired immunity against chlamydial infection (103, 113, 136, 160, 224). Furthermore, recent evidence suggests that antibody and CD4 T-cell derived IFN-γ optimally cooperate to protect against infection through neutrophil activation and subsequent chlamydial killing (208, 225). Thus, the requirement for Th1 cells and T-cell dependent antibody during protective adaptive responses is well accepted (114).

Earlier studies demonstrate that T-cell deficient athymic nude mice, and severe combined immune deficient (SCID) mice, which lack functional T and B lymphocytes because of impaired VDJ rearrangement, uniformly fail to resolve genital infection with the *C. muridarum* Weiss and Nigg strains, respectively (127, 226). SCID mice demonstrate high levels of dissemination, while IFN-γ deficient mice exhibit enhanced chlamydial dissemination, and a portion fail to resolve

genital tract infection (226). Furthermore, T and B-cell deficient *Rag1*$^{-/-}$ mice that fail to express functional Rag1 proteins required for somatic recombination develop a lethal systemic infection after intravaginal infection with the *C. muridarum* Weiss strain (227). Taken together, these data suggest an important, less characterized T-cell independent IFN-γ and B cell co-requirement for primary infection protection.

We recently identified a hypervirulent clonal isolate (CM001) from *C. muridarum* Nigg stock (224, 228). Identification of CM001 allowed us to explore mechanisms of protection against a disseminated primary intravaginal infection. Wild-type, B-cell deficient, and T-cell deficient mice survived CM001 infection. However, mice lacking IFN-γ signaling and *Rag1*$^{-/-}$ mice succumbed. Adoptive transfer of convalescent immune sera or naïve B cells protected *Rag1*$^{-/-}$ mice from CM001 lethality. B cell adoptive transfer to *Rag1*$^{-/-}$ hosts reduced disseminated lung chlamydial burden to comparable levels found in T-cell deficient mice. These are the first studies to demonstrate a T-cell independent co-requirement for B cells and IFN-γ in controlling extragenital chlamydial dissemination and associated lethality.

4.4 MATERIALS AND METHODS

4.4.1 Strains, cell lines, and culture conditions

Chlamydia muridarum Nigg stock (AR Nigg) was obtained from Roger Rank at the University of Arkansas for Medical Sciences, and has been previously described (168). Plaque-purified *C. muridarum* strains CM001, CM002, CM005, CM006, CM012 (170), and plasmid-deficient CM3.1 (61) were propagated in *Mycoplasma*-free L929 cells (171), and titrated as inclusion-

forming units (172), using a fluorescently tagged anti-chlamydial lipopolysaccharide monoclonal antibody (Bio-Rad).

4.4.2 Animals

6-8-week-old, female C57BL/6J (Stock No: 000664), B6.129S2-*Ighm*^{tm1Cgn}/J (muMT^{-}, Stock No: 002288), B6.129S(Cg)-*Stat1*^{tm1Dlv}/J (*Stat1*$^{-/-}$, Stock No: 012606), B6.129S7-*Ifng*^{tm1Ts}/J (*Ifng*$^{-/-}$, Stock No: 002287), B6.129S7-Rag1^{tm1Mom}/J (Rag1 KO; Stock No: 002216), B6.129S2-Tcra^{tm1Mom}/J (*Tcra*$^{-/-}$; Stock No: 002116), and B6.Cg-*Foxn1*^{nu}/J (C57BL/6 nude, Stock No: 000819) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were given food and water ad libitum in an environmentally controlled pathogen-free room with a cycle of 12 h of light and 12 h of darkness. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and University of North Carolina.

4.4.3 Murine *Chlamydia* infection and monitoring

Female mice at least 8 weeks old were s.c. injected with 2.5 mg medroxyprogesterone (Depo-Provera®; Upjohn) 5–7 days prior to infection to induce a state of anestrous (179). Mice were intravaginally inoculated with 5×10^5 inclusion-forming units (IFU) of Nigg or *C. muridarum* clones diluted in 30 μl sucrose-sodium phosphate-glutamic acid buffer. Mice were monitored for cervicovaginal shedding via endocervical swabs (180), and IFUs were calculated, as described previously (85). Ascending and disseminated infection was confirmed by determining the bacterial load in homogenized oviduct tissues, spleen, and lungs, from sacrificed mice, as

previously described (61). Animal welfare was monitored daily and immune deficient mice were euthanized after reaching a body conditioning score of 2- (229).

4.4.4 B cell isolation and adoptive transfer

B cells were isolated from the spleens of naïve *Tcra*$^{-/-}$ mice by negative magnetic selection (Miltenyi Biotech), according to the manufacturer's protocol. A sample of isolated cells was analyzed by flow cytometry to confirm 98% CD19+ B cell purity using LIVE/DEAD Fixable Yellow (Life Technologies) plus the following fluorochrome-labeled antibodies: anti-CD45 (clone 30-F11), anti-CD5 (53-7.3), and anti-CD19 (1D3), from BD Biosciences. The samples were analyzed on a CyAN ADP (Beckman Coulter), and data were analyzed with FlowJo software. 3×10^6 spleen-derived B cells were injected i.v. into Depo-Provera®-treated *Rag1*$^{-/-}$ mice 5 days prior to intravaginal infection.

Convalescent immune serum was generated after intravaginal *C. muridarum* Nigg infection of C57BL/6J mice. Mice were bled at sacrifice following infection resolution, and serum was collected after high speed centrifugation. Sera was pooled and complement-inactivated at 56°C for 30 minutes. Naïve-mouse derived IgM (Rockland Immunochemicals, Inc.) was dialyzed overnight prior to i.p. injection. Naïve Depo-Provera®-treated *Rag1*$^{-/-}$ mice received i.p. injections of PBS, 0.5 mL of immune serum, or 200 μg IgM on days -1, 0, 1, 3, 7, 10, and 13 of infection.

4.4.5 Statistical analysis

Differences between the means of experimental groups after infection were calculated using Mann Whitney U Test or two-way repeated measures (RM) ANOVA. Comparisons of animal

survival were performed by an exact log rank test. Prism software (GraphPad Software) was utilized for statistical analyses, and values of $P \leq 0.05$ were considered significant.

4.5 RESULTS

4.5.1 C. *muridarum* clonal isolates reveal a variant with enhanced virulence

Previous studies revealed that *C. muridarum* 'Weiss' and 'Nigg' stocks contain clonal isolates with genotypic and phenotypic differences (230). We hypothesized that plaque-purified isolates would reveal a variant with the capacity to cause enhanced burden and disease. We explored this possibility by inoculating wild-type mice with plaque-purified clonal isolates from a polyclonal population of *C. muridarum* Nigg (AR Nigg). Groups of mice were intravaginally inoculated with Nigg and five different clones. Clone CM001 demonstrated a significantly increased cervicovaginal burden, while clone CM012 demonstrated a reduced cervicovaginal burden compared to Nigg (Fig. 5A). Due to the differential cervicovaginal burden levels between CM001 and Nigg, we next examined the ability of these clones to ascend to the oviduct and disseminate to the lungs and spleen. Mice intravaginally infected with CM001 demonstrated a significantly higher chlamydial burden in the lungs on day 8, and in the spleen on days 8 and 10 post-infection, compared to Nigg (Fig. 5B). However, there was no significant difference in oviduct burden between CM001 and Nigg on any day examined. Both Nigg and CM001 were undetectable in the lungs and spleen by day 14. These data indicate that although ascension to the oviducts is equivalent for Nigg and CM001, CM001 exhibits enhanced dissemination to the lungs and spleen, although transient, in wild-type mice.

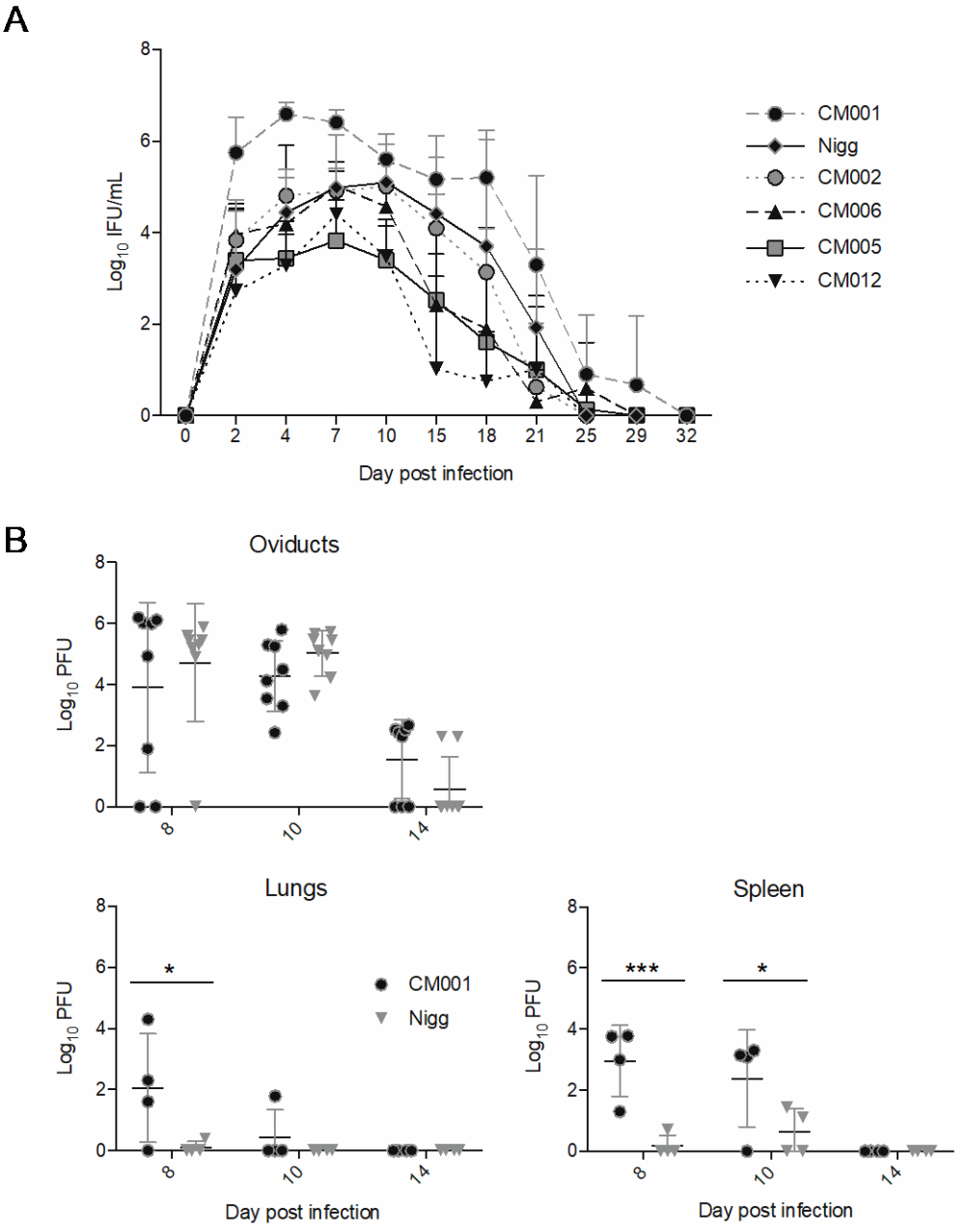

Figure 5. *C. muridarum* CM001 intravaginal infection disseminates to distal organs in wild type mice and is rapidly cleared.
(A) C57BL/6J mice were intravaginally infected with Nigg or *C. muridarum* clones and the course of primary infection was monitored with lower genital tract swabs. Significance was determined by two-way RM ANOVA with a post-hoc Tukey test. Data represent the mean + SD of swabs from infected mice or swabs from infected and uninfected mice (N = 4 mice per group). Comparison of groups over primary infection course: *P*=0.02 for Nigg versus CM001, *P*=0.05 for Nigg versus CM012. (B) CM001 and Nigg lung, spleen, and oviduct burdens were compared

by PFU on the indicated days post-infection. Statistical significance indicated by asterisks: * $P < 0.05$, *** $P < 0.001$ by two-way ANOVA.

4.5.2 CM001-mediated lethality and dissemination is plasmid-independent

Prior data from our lab revealed that *Rag1*$^{-/-}$ mice intravaginally infected with plaque-purified *C. muridarum* (now designated CM001) succumbed to infection (228). Since *C. muridarum* virulence has been directly linked to the presence of its plasmid (61, 231), we further investigated if lethality and dissemination to the lung and spleen was plasmid-dependent. *Rag1*$^{-/-}$ mice infected with CM001 or plasmid-cured CM001 (CM3.1) displayed a significantly increased bacterial burden, compared to Nigg-infected controls (Fig. 6A). However, the infection course between CM001 and CM3.1 was not significantly different, and both CM3.1 and CM001 infection resulted in lethality, while Nigg and CM012-infected mice survived (Fig. 6B). Mortality was associated with high chlamydial burdens in the oviducts, lungs, and spleens of CM001 and CM3.1 infected mice (Fig. 6C). Mice infected with CM012 demonstrated a significantly increased chlamydial burden in the oviducts, compared to Nigg, while both strains demonstrated a low burden in the lungs and spleens at the time of sacrifice on day 40. These data collectively show that CM001 lethality in *Rag*1$^{-/-}$ mice is plasmid-independent and is associated with high burdens in distal organs at the time of death.

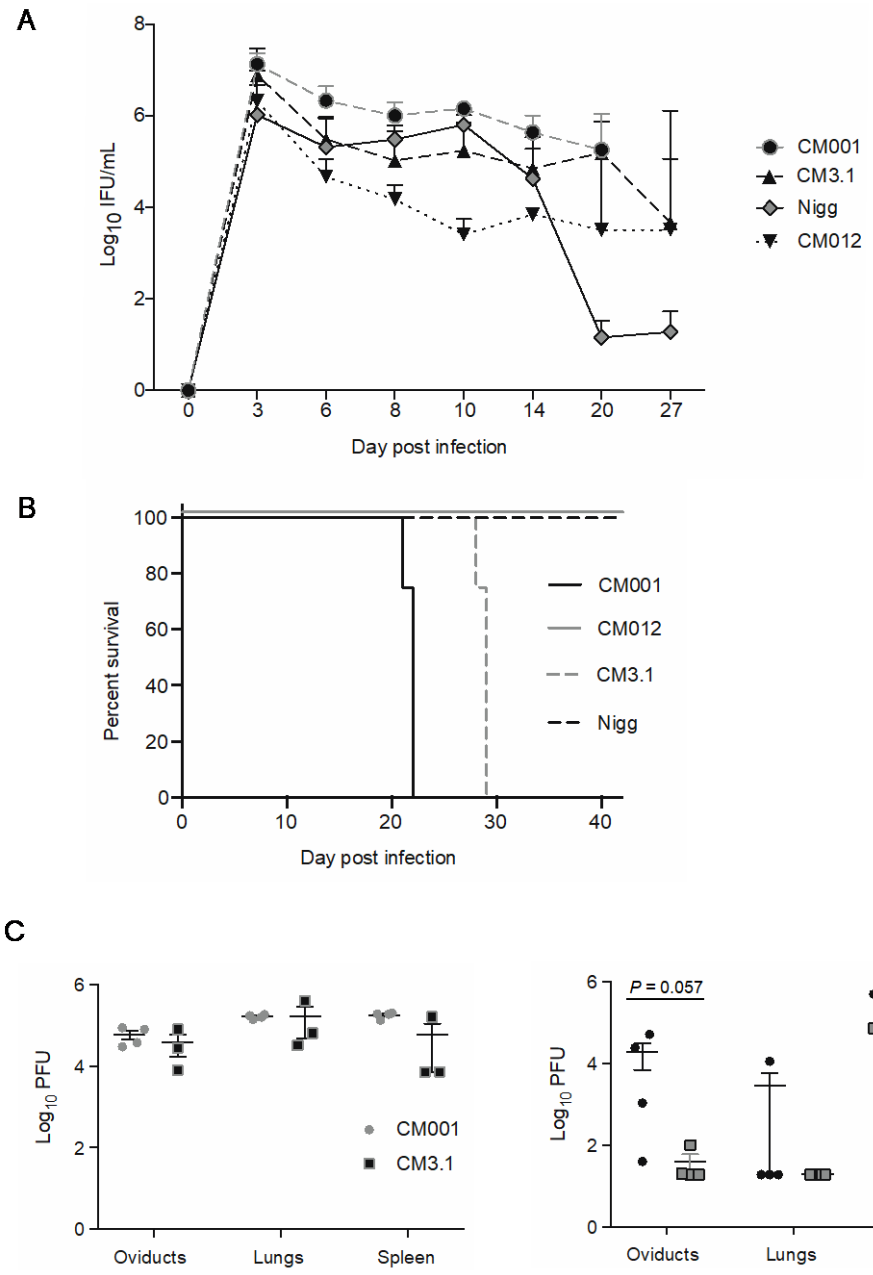

Figure 6. *C. muridarum* CM001 intravaginal infection in *Rag1*$^{-/-}$ mice is associated with fatal dissemination to distal organs.
(A) *Rag1*$^{-/-}$ mice were intravaginally infected with Nigg, CM001, CM012, or plasmid-deficient CM3.1, and the course of primary infection was monitored with lower genital tract swabs. Significance was determined by two-way RM ANOVA with a post-hoc Tukey test. Data represent the mean + SD of 4 mice per group. Comparison of groups over primary infection course: P=0.01 for Nigg versus CM001, P < 0.001 for Nigg versus CM3.1, P=0.01 for CM012 versus CM001, P=0.001 for CM012 versus CM3.1, P=NS for remaining group comparisons. (B) Mice were euthanized after reaching a body condition score of 2-, and an exact log rank test was used to analyze survival differences between Nigg and CM001 or CM3.1 groups (P<0.01). (C)

Chlamydial loads in the lungs, spleen, and both oviducts were determined by plaque assay (PFU) at time of euthanasia (left) or on day 40 (right). Statistical significance determined by Mann-Whitney U test.

4.5.3 IFN-γ signaling is required for protection against CM001 lethality

B-cell deficient mice have been shown to clear a disseminated infection with *C. muridarum* Nigg (115). Furthermore, *IFNG*$^{-/-}$ mice demonstrate extragenital dissemination, and a portion of mice succumb to infection (226). Thus, we next determined if CM001 infection of these immune deficient mice would yield similar results. Wild-type and B-cell deficient mice survived infection with CM001 and demonstrated a similar course of infection and clearance (Fig. 7A), but infection of *STAT1*$^{-/-}$ mice, which are deficient in IFN-γ signaling, and *IFNG*$^{-/-}$ mice with CM001 resulted in complete lethality, while Nigg-infected mice survived (Fig. 7B). Based on these results, we extended our analysis to determine if Th1 cells were dispensable for protection against CM001. Nude mice have been shown to develop a chronic chlamydial infection (127), and with the previous observation that CM001 is lethal in *Rag1*$^{-/-}$ mice (224), we examined if T-cell deficient mice would succumb to CM001 intravaginal infection. *Tcra*$^{-/-}$ and nude mice survived intravaginal CM001 infection, while *Rag1*$^{-/-}$ mice were not protected (Fig. 7C). These data collectively indicate that IFN-γ prevents lethal CM001 infection in the absence of T cells and suggests a B cell co-requirement for protection.

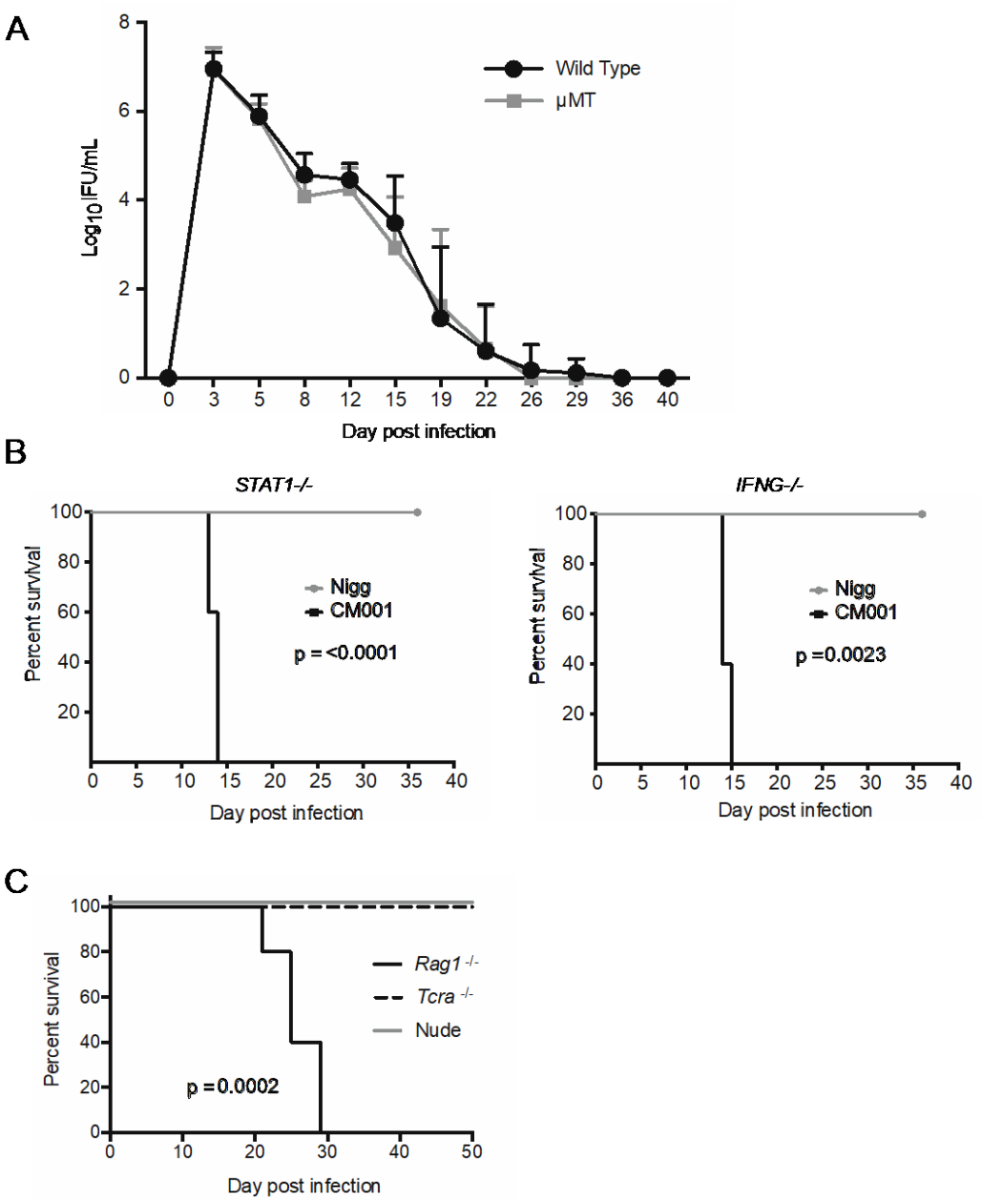

Figure 7. Genetic ablation of IFNγ signaling results in CM001-mediated lethality.
(A) C57BL/6J (N=24) and muMt (N=14) deficient mice were intravaginally infected with CM001 and the course of primary infection was monitored with lower genital tract swabs. Data represent the mean + SD. Significance was determined by two-way RM ANOVA with a post-hoc Tukey test (*P*=NS). (B) *STAT1*$^{-/-}$ (Nigg group N=10, CM001 group N=5) and *IFNG*$^{-/-}$ (5 mice per group) mice were intravaginally infected with Nigg or CM001. Animal welfare was monitored daily, and an exact log rank test was used to analyze survival differences between Nigg and CM001 in *STAT1*$^{-/-}$ ($P < 0.0001$) and *IFNG*$^{-/-}$ ($P = 0.0023$) mice. (C) Nude, *Rag1*$^{-/-}$, and

Tcra$^{-/-}$ mice were infected with CM001 and monitored daily (P=0.0002 for nude versus both *Rag1*$^{-/-}$ and *Tcra*$^{-/-}$).

4.5.4 B cells synergize with IFN-γ to protect against CM001 lethality in the absence of T cells

A recent study revealed that convalescent immunoglobulin synergizes with T-cell derived IFN-γ for neutrophil activation and chlamydial clearance (225). Based on our previous finding that *Rag1*$^{-/-}$ mice were susceptible to CM001 lethality, while T-cell deficient mice survived, we hypothesized that adoptive transfer of immune sera from convalescent wild-type mice to *Rag1*$^{-/-}$ mice would provide transient protection. Adoptive transfer of immune sera significantly increased survival time compared to mice receiving naive IgM or untreated controls (Fig. 8A). We next investigated if *Rag1*$^{-/-}$ mice could be completely rescued by the adoptive transfer of naïve B cells and recapitulate the survival phenotype of T-cell deficient mice. Adoptive transfer of B cells derived from the spleens of T-cell deficient rescued an otherwise lethal CM001 infection in *Rag1*$^{-/-}$ mice (Fig. 8B). T-cell deficient mice and B-cell reconstituted *Rag1*$^{-/-}$ mice demonstrated a respective 1.2 and 1.5 log reduction in lung chlamydial burden on day 19 post-infection, compared to *Rag1*$^{-/-}$ controls (Fig. 8C). The chlamydial burden in the genital tract was not significantly different between groups over the course of primary infection (Fig. 8D). These data collectively demonstrate the ability of B cells to mediate protection against chlamydial dissemination in the absence of T-cell helper and effector functions.

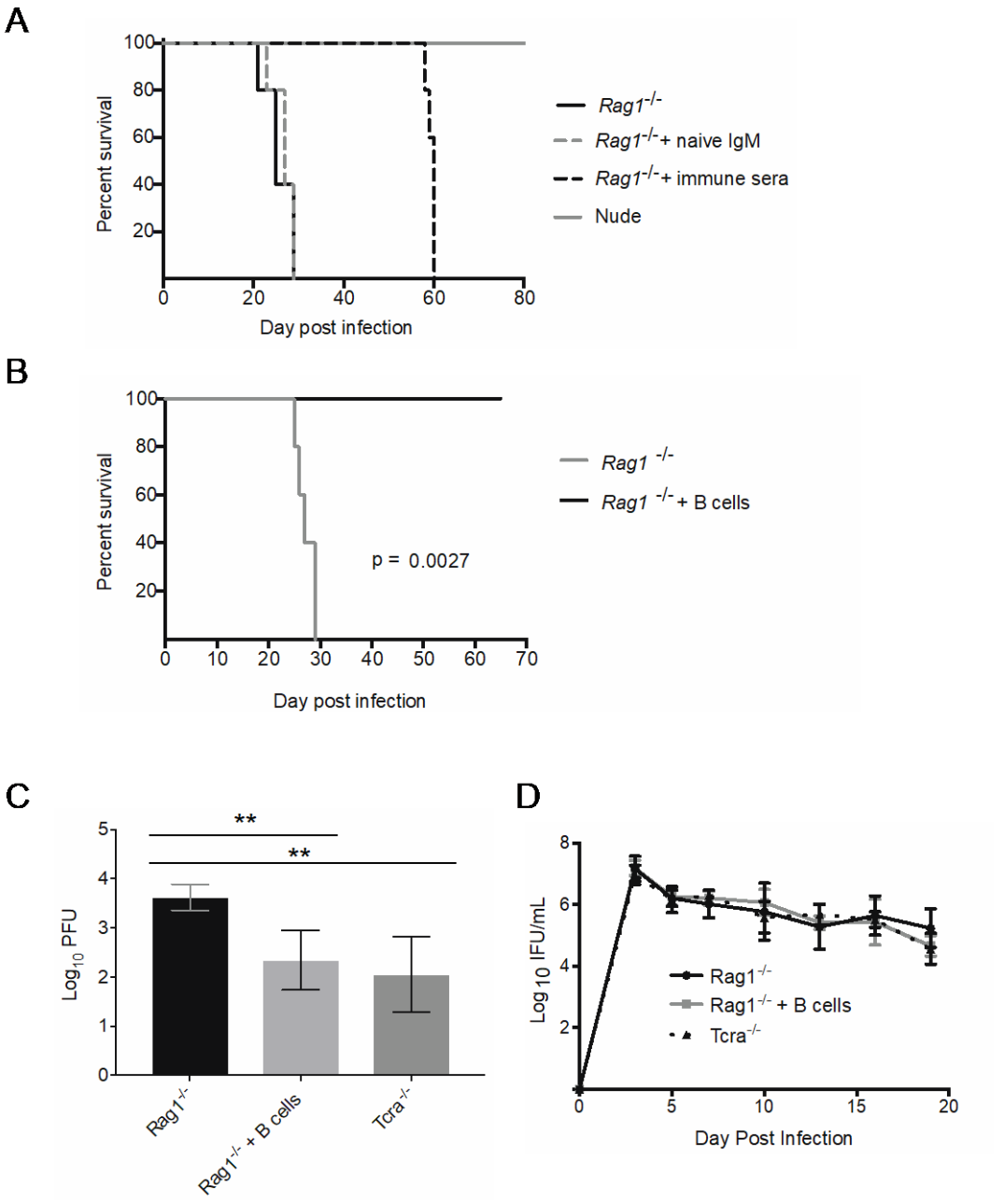

Figure 8. B cells and IFNγ synergize to prevent CM001 lethality independently of T cells. (A) Nude mice, and *Rag1*$^{-/-}$ mice receiving intravenous transfer of naïve mouse IgM, convalescent immune sera, or PBS (N=5 per group) were infected with CM001 and monitored daily. An exact log rank test was used to analyze survival differences in nude mice ($p<0.0001$) and *Rag1*$^{-/-}$ mice receiving immune sera ($P = 0.0026$). (B) *Rag1*$^{-/-}$ mice were mock treated or intravenously injected with 3 x 10^5 B cells (N=5 per group) prior to CM001 infection, and were monitored daily ($P = 0.0027$). (C) *Tcra*$^{-/-}$ (N=3), *Rag1*$^{-/-}$ (N=5), and B-cell reconstituted *Rag1*$^{-/-}$ (N=5) mice were infected with CM001 and sacrificed on Day 19 post infection. Chlamydial load

in the lungs was determined by plaque assay (PFU). Statistical significance indicated by asterisks: ** $P< 0.01$ by one-way ANOVA (D) Course of primary infection was monitored with lower genital tract swabs (P =NS). Significance was determined by two-way RM ANOVA with a post-hoc Tukey test.

Table 1. Outcome of CM001 infection and immune status of mouse strains.

Mouse Strain	Mortality	Clearance	IFN-γ Signaling	Antibody	αβ T cells
Wild Type	No	Yes	Yes	Yes	Yes
Rag1-/-	Yes	No	Yes	No	No
Stat1-/-	Yes	No	No	Yes	Yes
Ifng-/-	Yes	No	No	Yes	Yes
muMT	No	Yes	Yes	No	Yes
Tcra-/-	No	No	Yes	Yes	No

4.6 DISCUSSION

Low levels of extragenital dissemination have been described in murine chlamydial infections previously, however CM001 intravaginal inoculation results in significant dissemination to distal organs. Based on this enhanced ability to disseminate in wild type mice, we explored CM001's hypervirulent potential in immune deficient mice. High chlamydial burdens were found in the lungs and spleens of underconditioned *Rag1*$^{-/-}$ mice that were euthanized after intravaginal infection. This phenotype allowed us to further explore determinants for preventing fatal dissemination. B-cell deficient mice were protected and cleared CM001 infection (Table 1), similar to previous experiments with the Nigg strain (115). However, CM001 intravaginal infection of mice lacking IFN-γ signaling resulted in universal lethality and supports previous studies demonstrating a protective role for IFN-γ in control of dissemination (226, 232, 233). In our experiments, in the absence of IFN-γ, dissemination of CM001 from the genital tract to the

lungs likely leads to lethality. Furthermore, the observation of complete clearance of CM001 from the genital tract of wild-type or B-cell deficient mice indicates an *in situ* requirement for Th1 cells. Taken together, T-cell independent IFN-γ is capable of controlling CM001 dissemination and preventing lethality, while Th1 cells are necessary for clearing infection from the genital tract.

T-cell deficient mice with intact IFN-γ signaling survived intravaginal CM001 infection. However, *Rag1*$^{-/-}$ mice lacking both T and B cells succumbed to CM001 infection despite the presence of IFN-γ (Table 1). *Rag1*$^{-/-}$ mice were transiently rescued by the adoptive transfer of convalescent immune sera from wild-type mice. This result demonstrated that antibody generated in the presence of T-cell help can afford protection against disseminated chlamydial infection, without assistance from the adaptive cellular arm of the immune response. Furthermore, transfer of naïve B cells taken from T-cell deficient mice completely rescued *Rag1*$^{-/-}$ mice from lethality, and this protection was associated with decreased chlamydial burden in the lungs of genitally infected mice. These experiments reveal a previously undescribed T cell-independent role for B cells in concert with IFN-γ for protection against *C. muridarum* disseminated disease.

Our analyses were limited to delineating general requisites for protection against disseminated extragenital infection. While IFN-γ and B cells were shown to play a pivotal role in protection, the cellular source of IFN-γ and mechanism of B-cell protection was not investigated. However, previous experiments have demonstrated that NK cells are a critical source of IFN-γ in the absence of T cells (234), and B-cell protection is likely antibody-dependent, based on the observation that antibody-deficient (AID$^{-/-}$μS$^{-/-}$) B cells are unable to control dissemination from the genital tract (235). A role for antibody is further suggested by the successful rescue of *Rag1*$^{-/-}$

mice from CM001 lethality with administration of convalescent serum. However, we cannot rule out a contribution of antibody-independent mechanisms of protection, such as cytokine production (236). Recent investigation suggests that chlamydial-specific IgG and IFN-γ are necessary for neutrophil activation prior to chlamydial killing (225); however, only T-cell dependent antibody production was investigated.

Significant T-cell independent immunity has been described for *Ehrlichia muris* (237), *Salmonella* (238), and *Streptococcus pneumoniae* (239). Our analyses implicate a potential role for T-cell independent B cell responses in prevention of lethal extragenital chlamydial infection. Interestingly, spleen-derived B cells have been shown to adopt a predominately marginal zone phenotype *in vivo* after adoptive transfer to lymphopenic hosts (240), and neutrophil-derived cytokines can directly stimulate marginal zone B cells for T-independent IgG production (241). T-independent IgG could activate neutrophils to phagocytose bacteria (239, 242), which would be enhanced in the presence of IFN-γ (225, 243). Detectable, low levels of IgG have been observed in nude mice intravaginally infected with *C. muridarum* (127), but even low levels of T-independent IgG can afford sufficient protection against infection (244).

In conclusion, we have demonstrated that IFN-γ and B cells cooperate to provide protection against a lethal disseminated chlamydial infection independently of T cells. Examination of T-cell independent responses may further reveal detailed mechanisms of extragenital host defense against *Chlamydia*. Increased understanding of T-independent mechanisms of anti-chlamydial host defense may also inform methods to augment T cell responses generated with targeted vaccines.

5.0 CHAPTER THREE: THE *CHLAMYDIA*-SPECIFIC TRANSGENIC T CELL RECEPTOR REACTS WITH A SOLUBLE, SECRETED PROTEIN

5.1 PREFACE

This chapter is in fulfillment of specific aim 3 and data herein will be incorporated into a separate manuscript for future publication.

5.2 INTRODUCTION AND RESULTS

CD4-T cell immunity is a critical component of immune responses against chlamydial infection, and an efficacious vaccine will require induction of T-cell memory specific for protective antigens. Many studies have characterized antigenicity based on antibody responses (55, 245), but few have determined the ability of chlamydial antigens to directly stimulate memory T cell responses (54, 57, 246). The induction of robust effector and memory T cell responses is mediated by dendritic cells (38, 247), and previous studies have illustrated the ability of dendritic cells to present conserved overlapping MHC class II peptides from chlamydial species (248). We recently identified the first Tg TCR cross-reactive with *C. muridarum* and *C. trachomatis* (224); as evidenced by the ability of *C. muridarum* and *C. trachomatis* live or UV-irradiated bacteria to stimulate proliferation of our TCR Tg CD4 T cells. We have further characterized additional determinants sufficient for their stimulation. Specifically, we evaluated the ability of different *C. trachomatis* serovars, cytosolic fractions from infected cells, and soluble or insoluble fractions

from *C. muridarum* elementary and reticulate bodies to induce proliferation of our TCR Tg T cells.

5.2.1 The transgenic TCR demonstrates reactivity with *C. muridarum* and multiple *C. trachomatis* serovars.

We determined that splenocytes from our Tg mice expressing the TCR originally cloned from CD4 T cells harvested from *C. muridarum*-infected mice proliferated in response to stimulation with *C. trachomatis* serovar D (224). Thus, we investigated the ability of additional *C. trachomatis* serovars to stimulate cellular proliferation (Fig. 9). Tg mouse splenocytes demonstrated marked and similar levels of proliferation in response to stimulation with serovars D, F, H, and L2, but minimal proliferation was induced by culture with recombinant chlamydial major outer membrane protein (MOMP). We also determined that TCR Tg splenocytes also proliferated in response to stimulation with plasmid-deficient *C. muridarum* Nigg (227). These data reveal that the transgenic TCR is specific for a conserved antigen present in both *C. muridarum* and *C. trachomatis,* and that the antigen is not encoded by the virulence plasmid or by conserved plasmid-regulated chromosomal loci (171, 249) whose expression is significantly downregulated in the absence of the plasmid-borne regulator Pgp4 (250).

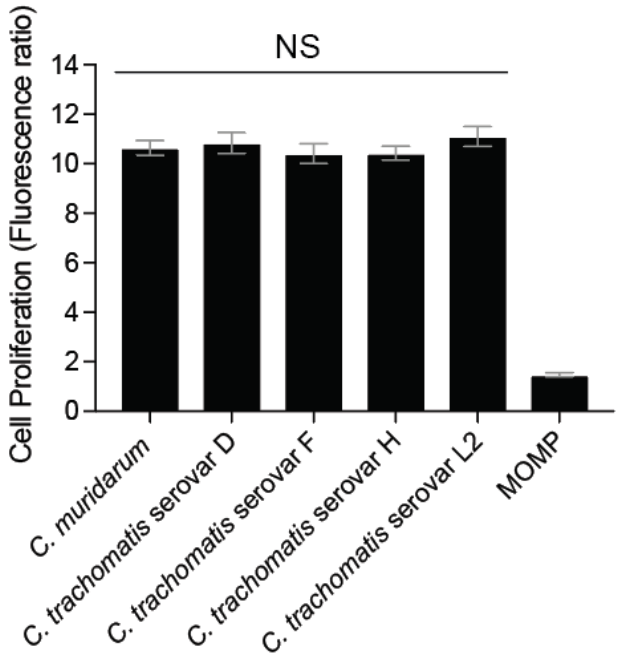

Figure 9. The transgenic TCR demonstrates reactivity with *C. muridarum* and multiple *C. trachomatis* serovars.
TCR Tg splenocytes were stimulated with 5 μg/ml of the indicated serovars of live *C. trachomatis*, *C. muridarum* AR Nigg, or recombinant MOMP. Splenocytes were cultured for 4 days with the indicated chlamydial strain or MOMP, after which 20 U/mL IL-2 was added for 2 days. Cellular proliferation was determined by the ratio of Alamar Blue fluorescence intensity compared to unstimulated controls ($P < 0.0001$ for all chlamydial strains versus MOMP, P=NS between all chlamydial strains, determined by one-way ANOVA).

5.2.2 TCR transgenic splenocytes proliferate after stimulation with the cytosolic fraction of *C. trachomatis*-L2 infected cells.

Previous studies have shown that secreted chlamydial antigens can induce robust T cell responses (69, 80, 248, 251). Based on these observations and the ability of *C. trachomatis* serovar L2 to induce TCR Tg cellular proliferation, we investigated if a cytosolic fraction from L2-infected cells was stimulatory (252) and found that it induced marked proliferation comparable to live *C. muridarum* Nigg and *C. trachomatis* serovar D (Fig. 10). These data

indicate that the Tg TCR is likely specific for a chlamydial antigen that is secreted during infection.

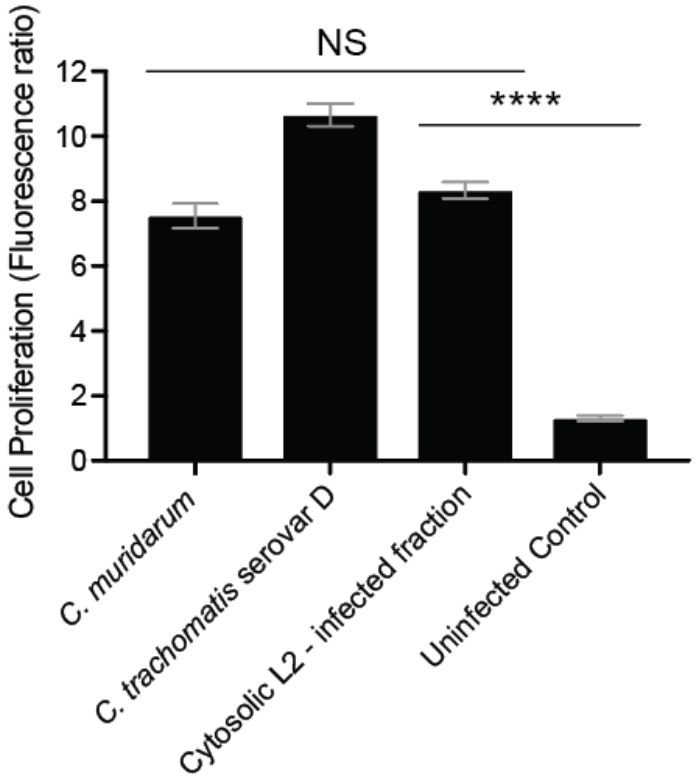

Figure 10. TCR transgenic splenocytes proliferate after stimulation with the cystosolic fraction of *C. trachomatis*-L2 infected L929 cells.
TCR Tg splenocytes were stimulated with 5 μg/ml of *C. muridarum* AR Nigg, *C. trachomatis* serovar D, L2-infected L929 cytosolic fraction, or the mock-infected host cytosolic fraction. Splenocytes were stimulated for 4 days, followed by 2 additional days in the presence of 20 U/mL IL-2. Proliferation was determined by the ratio of Alamar Blue fluorescence intensity compared to unstimulated controls (****$P < 0.0001$ for L2-infected fraction versus uninfected control, P=NS for *C. muridarum* versus L2-infected fraction determined by one-way ANOVA).

5.2.3 The transgenic TCR preferentially responds to the sarkosyl-soluble fraction from *C. muridarum* reticulate bodies.

Chlamydial antigens have been shown to be infection-dependent and independent (55). Thus, some antigens are found in the infectious EB, while some are only present within the infected cell. Antigens can also be selectively or predominantly expressed by infectious EBs or

replicating RBs. Thus, we investigated the ability of purified EB and RB to activate TCR Tg T-cell hybridoma cells. Live *C. muridarum* fractions enriched for either EB or RB induced production of IL-2 and IFN-γ by TCR transgenic hybridoma cells after 24 hours (Fig.11A), and EB stimulation elicited significantly increased cytokine production compared to RB. Based on the reactivity of the cytosolic fraction and the possibility that soluble proteins still associate with the chlamydial membrane (253), we next determined the ability of EB and RB sarkosyl-soluble or insoluble fractions to induce TCR Tg splenocyte proliferation. Membrane and soluble fractions from both EB and RB preparations stimulated cellular proliferation, but the strongest response was elicited by the soluble RB fraction that induced proliferation equivalent to irradiated *C. muridarum* mixed EB and RB (Fig. 11B). Furthermore, this soluble RB fraction induced significant proliferation in TCR Tg splenocytes compared to splenocytes from littermate controls (Fig. 11C). Due to the ability of the soluble RB fraction and infected cell cytosolic fractions to stimulate proliferation, we next examined if convalescent mouse sera could identify reactive proteins of equal size in both fractions. Immunoblotting using immune mouse sera detected a reactive 27-kDa protein (Fig. 11D). These data suggest that the antigen is a 27-kDa soluble, secreted protein enriched in RBs that may associate with the chlamydial membrane.

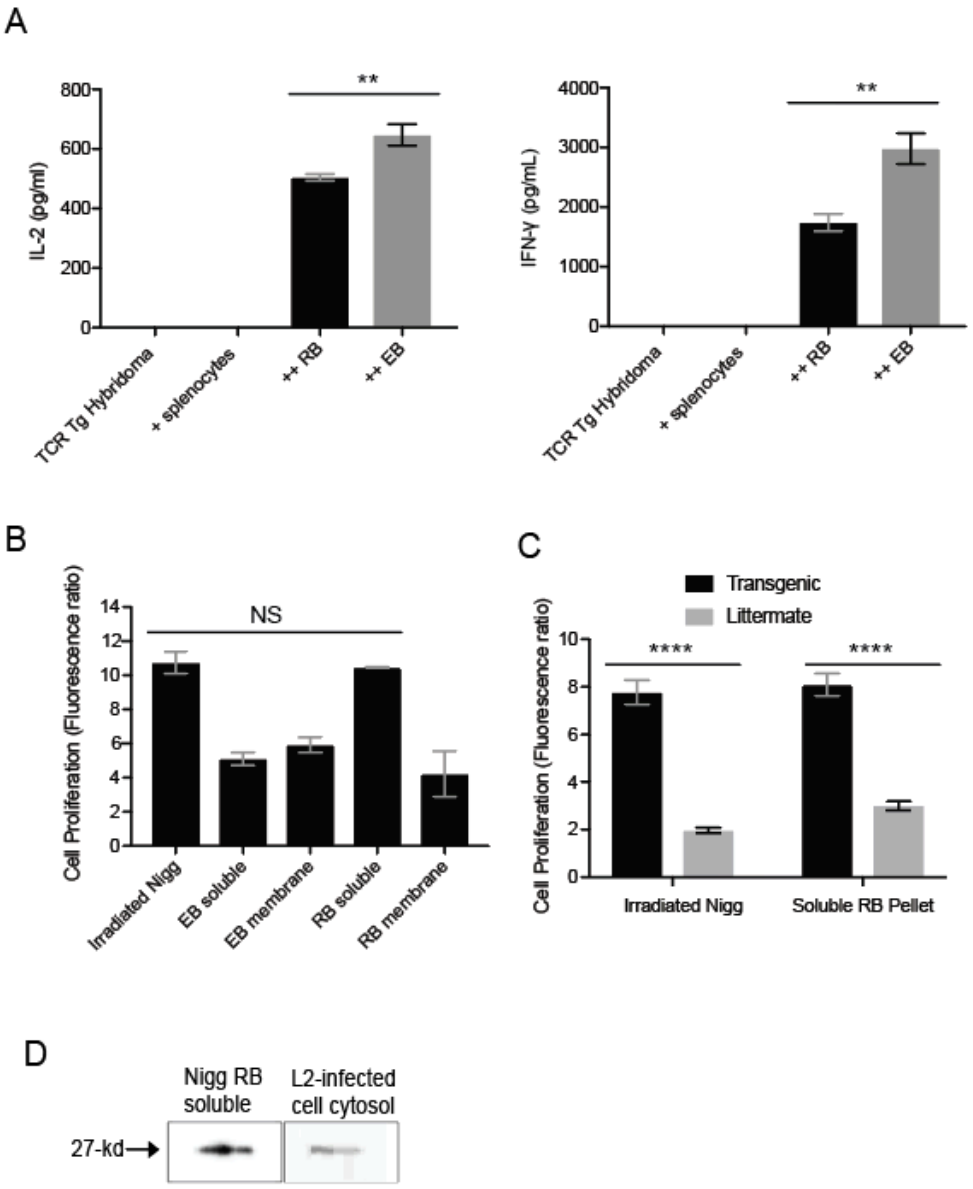

Figure 11. The transgenic TCR preferentially responds to the sarkosyl-soluble fraction from *C. muridarum* reticulate bodies.

(A) TCR Tg hybridoma cells were cultured with irradiated syngeneic splenocytes at a 1:1 ratio in the presence of 5 μg/ml purified live *C. muridarum* EB or RB. Supernatants were harvested after 24 hours and analyzed for IL-2 and IFN-γ by ELISA. (**$P < 0.01$ by one-way ANOVA). (B) TCR Tg splenocytes were stimulated with 5 μg/ml irradiated *C. muridarum* AR Nigg, or 5 μg/ml each of the sarkosyl-soluble and –insoluble fractions from *C. muridarum* elementary or reticulate bodies. (C) TCR Tg splenocytes preferentially proliferate in response to the ammonium-sulfate precipitated sarkosyl-soluble RB fraction compared to splenocytes from littermate controls. (****$P < 0.0001$ by Student's t-test). (D) A 27-kDa protein present in sarkosyl-soluble RB and L2-infected host cytosolic fractions is immunoreactive with convalescent mouse serum.

5.3 MATERIALS AND METHODS

5.3.1 Strains, cell lines, and culture conditions

Chlamydia muridarum Nigg stock (AR Nigg) was obtained from Roger Rank at the University of Arkansas for Medical Sciences (168). and plasmid-deficient CM3.1 has been previously described (61). *C. trachomatis* D/UW-3/Cx was obtained from the American Type Culture Collection (Manassas, VA); *C. trachomatis* F/IC-Cal-3 and H/UW-43/Cx were obtained from Guangming Zhong (254). *C. trachomatis* L2/434/Bu has been previously described (255, 256). All serovars were plaque purified before use and propagated in mycoplasma-free L929 cells (171).

5.3.2 *Chlamydia*-specific cellular proliferation

The spleens of littermate or TCR transgenic mice were processed into a single cell suspension, as described previously (177). Splenocytes (1×10^5 cells/well) were seeded in a 96-well flat-bottomed tissue culture plate in complete media with 5 μg/ml irradiated *C. muridarum* EB/RB preparations (257), live *C. muridarum* AR Nigg, indicated live *C. trachomatis* serovars, or recombinant MOMP (258) for 6 days. Splenocytes were treated with 20 U/mL murine IL-2 (Peprotech) over the final 48 hours. Cells were treated with 20 μl of Alamar Blue (Biosource) 4-6 hours before the end of the 6-day culture, and proliferation was measured at 530-nm excitation/590-nm emission with a Biotek fluorescence microplate reader. Alternatively, TCR transgenic hybridomas were cultured at a 1:1 ratio with irradiated syngeneic splenocytes in the presence or absence of purified *C. muridarum* AR Nigg EB or RBs (5μg/mL) for 24 hours. Supernatants from stimulated Tg hybridoma cells were analyzed for IL-2 and IFN-γ by ELISA. All experiments were performed in triplicate.

5.3.3 Generation of soluble *C. trachomatis* L2 antigen

Preparation of soluble chlamydial antigen from *C. trachomatis* L2-infected cells has been described previously (252). Briefly, L929 infected cells were incubated for 48 hours at 37°C. Infected monolayers were washed with HBSS, removed by scraping, and disrupted by sonication. Host cell debris was removed by centrifugation at 500 × g. Supernatants were collected and centrifuged at 22,000 × g for 30 minutes at 4°C to pellet chlamydial organisms. Clarified supernatant was centrifuged at 100,000 × g for 1 hour at 4°C. The supernatant was collected and concentrated using an Amicon Ultracell-10K (Millipore).

5.3.4 Sarkosyl fractionation of EB and RB

C. muridarum Nigg EBs and RBs were isolated at the appropriate stage in the developmental cycle (259) and subjected to sarkosyl fractionation, as previously described (253, 260). Briefly, 5 mg of density gradient purified EB or RB were suspended in PBS (pH 7.4) containing 2% Sarkosyl and 1.5 mM EDTA. These suspensions were sonicated for 2 minutes and incubated at 37°C for 1 hour. The sarkosyl-insoluble (membrane) fraction was pelleted by centrifugation at 12,000 × g for 10 minutes, and the soluble fraction was removed from the pellet. The insoluble pellets were washed twice with PBS, and then suspended in DNase I and RNase A at 37°C for 2 hours. This suspension was centrifuged, and the insoluble pellet was washed twice with PBS. The soluble and insoluble fractions were dialyzed for 2 hours and overnight at 4°C to remove excess detergent. A portion of the soluble fraction was precipitated with 40% ammonium sulfate, as previously detailed (261).

5.3.5 Immunoblot assay

Convalescent mouse sera from *Tcra*$^{-/-}$ deficient mice that had received an adoptive transfer of 1x10^{6} TCR Tg CD4 T cells (224) was used to detect reactive proteins in the *C. muridarum* RB sarkosyl-soluble fraction and cytosolic fraction of L2 infected cells. Primary antibody binding was probed with HRP (horse radish peroxidase)-conjugated goat-anti-mouse IgG + IgM secondary antibody (Jackson ImmunoResearch).

5.3.6 Statistical analysis

Significant differences were determined by one-way ANOVA and Student's t-test. Prism software (GraphPad Software) was utilized for statistical analyses, and values of $P \leq 0.05$ were considered significant.

5.4 DISCUSSION

Identification of protective T cell antigens is a critical component of vaccine development, since CD4 T cells are a major determinant of acquired immunity against *Chlamydia*. These studies investigated chlamydial factors sufficient for activation of our recently identified *Chlamydia*-specific transgenic TCR. We determined that the Tg TCR is stimulated by a conserved, chromosome-derived antigen expressed by the major developmental forms of all strains of *C. trachomatis* and *C. muridarum* tested. We further determined that the antigen is enriched in soluble RB fractions and is possibly derived from a 27-kDa secreted protein.

These data demonstrate that the first TCR Tg mouse cross-reactive with both human and mouse chlamydial species is likely specific for a secreted antigen. This unknown antigen is

sarkosyl-soluble; however, insoluble chlamydial protein fractions shown to contain outer membrane complexes (253) also stimulated TCR Tg splenocyte proliferation. This suggests the antigen may be loosely associated with the chlamydial membrane and identifies this specific antigen as a potential secreted substrate. This furthers implies that the antigen is unlikely to be involved in central metabolism or protein synthesis (246), since these proteins are more likely to be in the bacterial cytosol. Future studies are required to confirm if the secreted antigen localizes solely to the infected cell cytoplasm, chlamydial inclusion space, or both compartments. However, this approach is limited due to the low intracellular concentrations of secreted proteins and difficulty in preparing uncontaminated subcellular fractions. Additional experiments will investigate the reactivity of conserved *C. muridarum* and *C. trachomatis* MHC Class II epitopes (248) due to the cross-reactivity of Tg TCR, and the Tg CD4 T cells will be screened against a cell-free *in vitro* chlamydial protein expression library.

Chlamydia-specific T cell immunogenicity is potentially dictated by antigenic characteristics such as expression kinetics, cellular location, abundance, and similarity to host proteins. These data are encouraging from an immunological perspective if future experiments identify the antigen as a secreted effector molecule. This would allow for novel analysis of T-cell immunity against a chlamydial effector and help determine the contribution of this specific T-cell response to overall acquired adaptive immunity. TCR Tg mouse models have demonstrated utility in characterizing the immune response against effector molecules from *Salmonella*, *Listeria*, and *Mycobacterium* (192, 193, 262, 263). It is suggested that the chlamydial inclusion restricts MHC class II presentation of antigenic proteins. The identification of secreted, immunogenic antigens expressed on multiple MHC alleles would be a significant advancement. This could potentially allow for development of a chlamydial subunit vaccine that selectively

activates robust dendritic cell-mediated CD4 T cell responses against Class II epitopes expressed on the infected mucosal epithelium. Furthermore, T-cell epitopes could be conjugated with potential B-cell antigens for induction of protective antibody responses.

In conclusion, we have demonstrated that the Tg TCR is specific for a chlamydial antigen present in *C. muridarum* and multiple *C. trachomatis* serovars. This antigen is a soluble protein enriched in RBs and potentially secreted during infection. Better understanding of how CD4 T-cell immunity against chlamydial effectors is generated and sustained will help instruct successful vaccine development.

6.0 OVERALL DISCUSSION AND PUBLIC HEALTH SIGNIFICANCE

Critical advances have been made in the field of chlamydial immunology that include identification of the basic correlates of protective immunity and capacity of vaccination to induce resident-memory T cells. However, there are many challenges and questions that need to be addressed regarding the adaptive response to infection, in order to develop an efficacious chlamydial vaccine. Use of the mouse model is limited because T-cell mediated clearance operates through different mechanisms compared to humans. Additionally, the role of antibody and capacity to induce broadly neutralizing antibodies to multiple serovars remains unresolved; however, the recent ability to induce broadly neutralizing antibodies to the MOMP VD4 region shows promise. The mechanism by which *C. trachomatis* induces genital tract pathology in humans is also unclear, and we lack understanding of why some patients remain asymptomatic with no pathology and others develop pelvic inflammatory disease. Additionally, there is a basic requirement to characterize the correlates of immunity that allow for chronic infection versus spontaneous clearance. Exacerbating this problem is the paucity of data reflecting protective responses in humans, and the NHP model for vaccine testing has demonstrated more success for trachoma than genital infection. There is a need for more basic testing of the NHP response to infection and vaccination and exploration of human responses to infection. Furthermore, identification of protective human antigens is in its infancy (54, 57). Current research is focused on delineating protective versus pathogenic antigen-specific responses.

A better understanding of mucosal immunity is necessary to address proper adjuvants and vaccine delivery methods. To enhance and guide the proper immune response to vaccination, current mucosal adjuvants will require further testing and new adjuvants may need to be

developed. Additionally, a vaccine may require reformulation to protect against ocular or genital tract infection, if a single vaccine does not prove efficacious against both routes of transmission. A mucosal vaccine may not be necessary if systemic immunity is capable of preventing upper genital tract infection and pathology. Sterilizing immunity is the ultimate goal; however, a partially protective vaccine that could be boosted by a live infection may be more pragmatic, particularly if it's able to prevent disease, transmission, and seed the genital mucosa with tissue-resident memory T cells (TRM). Ideally, a chlamydial vaccine would be combined with other vaccinations delivered during childhood or adolescence to enhance vaccine uptake, improve marketability, and avoid multiple immunizations. Economic analysis suggests a vaccine that provides partial immunity would be cost-effective compared to current screening and treatment strategies (264). A partially protective vaccine would reduce the prevalence of genital infection (265), and vaccination of both sexes could synergize to impart sterilizing immunity against sexual transmission (266). Current research must continue to focus on identifying correlates of protective immunity versus pathogenic responses and delineate adjuvants and antigens that can enhance protective T cell responses.

A genital chlamydia vaccine would ideally target adolescents before sexual debut to maximize immunity during the period of highest transmission risk. As most of the adverse sequelae of chlamydial infection occur among females, an argument could be made for vaccinating adolescent girls only, as has been done with chlamydia screening and for HPV vaccination in many settings (267, 268). However, mathematical modelling and cost considerations can inform whether a vaccine should target both adolescent males and females to optimize reductions in population transmission. The vaccine should ideally be combined with other adolescent vaccines to improve uptake and marketability. The market profile for a

Chlamydia vaccine might emulate currently licensed HPV vaccines. Complete immunity to infection is the best-case scenario for *Chlamydia* vaccine development, but may be difficult to achieve. However, even a partially protective vaccine that inhibits upper genital tract infection and damage or reduces ongoing transmission could have significant impact and provide individual-level or population-level benefits (265). Mathematical modelling demonstrates that a partially protective vaccine added to current screening and treatment efforts could be cost-effective compared to screening and treatment alone (9).

An effective chlamydial vaccine would have public health benefits in both high-income countries (HICs) and low-middle income countries (LMICs). However, a chlamydial vaccine would probably provide the greatest benefits in LMIC settings, where lack of medical infrastructure and resources preclude chlamydia screening programs and the burden of chlamydia-associated sequelae is likely greatest. In LMICs, up to 186 million couples report being unable to have a child over 5 years (269). Although infertility is a global problem, the proportion that is tubal factor, and thus primarily caused by scarring from genital infection such as chlamydia, varies widely by population. In the United States, the proportion of infertility that is tubal factor ranges from 10–40% (270, 271). However, in sub-Saharan Africa, tubal infertility is the dominant cause for women, present in up to 65-85% of infertility cases (272, 273). In addition, the consequences of chlamydial sequelae such as ectopic pregnancy can be life-threatening in resource-poor settings. In African developing countries, ectopic pregnancy has case fatality rates that are 10 times higher than those reported in high-income countries (274). Additional, updated, and more precise data on the attributable fraction of chlamydia to PID and longer-term sequelae in LMICs will be essential for better defining the potential impact of a chlamydial vaccine in these settings. Given the link between chlamydial infection and

acquisition of HIV infection, a chlamydia vaccine could also have added benefits in areas of high HIV prevalence (5). In addition, what is learned from chlamydial vaccine studies targeted to prevention of genital infection can be used to inform vaccine development for prevention of trachoma, which would expand the benefits for LMIC settings.

The critical role of T cells in chlamydial immunity was first demonstrated 30 years ago (127). CD4 T cell IFN-γ production likely confers protection against *C. trachomatis*. Control of *in vivo* infection is not fully understood, since IFN-γ induces the expression of over 200 different genes in target cells (275). However, *in vitro* studies indicate it controls chlamydial growth in part by inducing indoleamine-2, 3-dioxygenase (IDO) production (134). IDO prompts tryptophan degradation and ultimately microbial starvation. IFN-γ producing Th1 cells are essential and sufficient for resolution of infection, but a polyfunctional response including IL-2 and TNF-α may be optimal for clearance (136, 137). Tissue-resident memory T (TRM) cells have emerged as an important T cell subset in mucosal immunity. TRM are long-lived non-circulating memory cells able to respond to infection independent of systemic T cells. After vaginal HSV-2 infection, CD4 TRM cells are maintained in the vaginal mucosa by a chemokine network facilitated by local macrophages (148). Mucosal Th1 cells could be instrumental to vaccine success, as the intensity of mucosal CD4 T cell responses is a correlate of protective immunity (150). Antibodies boost chlamydial protection, but the mechanism remains unclear and may be multifaceted, including enhancement of Th1 effector responses (116). Only recently has there been convincing data on the effect of neutralizing antibodies. Immunization with an extended major outer membrane protein (MOMP) VD4 region containing the conserved LNPTIAG region elicited neutralizing antibodies in mice (59). This protection was attributed to chlamydial

neutralization and CD4-T cell mediated immunity. Studies demonstrating protective adaptive immune responses to *Chlamydia* have recently been reviewed (276).

Preclinical vaccine development utilizes well-established animal models for candidate testing. Mouse models offer convenient manipulation and research tools for analysis of the immune response, but differ from humans with respect to many facets of infection, disease, and adaptive immune responses. *Chlamydia muridarum* is a mouse-specific strain that shares extensive homology with *C. trachomatis*. However, *C. muridarum* induces a more acute infection with complete resolution compared to the often quiescent, chronic infection of *C. trachomatis* in humans. Further, mechanisms of IFN-γ mediated chlamydial clearance differ in mice and humans. The guinea pig model utilizing *Chlamydia caviae* elicits disease more similar to humans, but the relative lack of immunological reagents detracts from its use for vaccine studies (22). Female minipigs that have a reproductive cycle and genital tract similar to humans are being used for chlamydial vaccine studies but also suffer from reduced availability of reagents (51, 52). Non-human primate (NHP) models are often employed prior to human testing, but infection of the eye or genital tract in NHPs demonstrates a shorter, self-limiting infection compared to humans. Despite this limitation, NHP testing could play an important role in assessing cellular and humoral responses after infection or vaccination to identify correlates of protective immunity. Animal and human studies could provide insight into a protective transcriptional blood signature that might be translated to a biomarker of efficacy for use in human clinical trials (53).

The ultimate goal of a chlamydia vaccine is to reduce the burden of upper genital tract sequelae in women. However, the use of disease as a clinical endpoint in vaccine trials is influenced by several considerations, including the natural history and timing of clinical events

such as infertility following infection, the measurement of PID, the proportion of PID associated with *C. trachomatis*, and factors related to trial design. The clinical diagnosis of PID is notably insensitive and nonspecific, and the previous gold standard laparoscopic diagnosis is invasive, not widely available, and no longer routinely performed. In addition, PID is a clinical syndrome that has multiple causes. Typically, *C. trachomatis* is involved in about one third of cases; however, attribution of PID to a particular pathogen may be difficult. More precise, feasible, non-invasive measures of chlamydia-specific upper genital tract inflammation and damage are a critical priority for the design of practical and informative clinical studies. Additional studies are required to define the role of radiologic techniques such as MRI and power Doppler in PID diagnosis (277). In women, endometrial biopsies via minimally invasive sterile endometrial sampler have been increasingly used to yield data on ascension of infection and presence of upper tract inflammation (278, 279). Current efforts are focused on identification of a blood biomarker for less invasive sampling (280). Cervical bacterial burden may also be an appropriate surrogate for upper tract ascension (281).

Use of *C. trachomatis* infection as a clinical endpoint is relatively straightforward and would involve interval *C. trachomatis* NAAT testing via urine samples for men and vaginal or cervical swabs for women in placebo-treated versus vaccinated subjects. Reflex quantitative NAAT could be used to evaluate bacterial load. However, given that complete immunity may be hard to achieve, it will be important to build consensus around the most appropriate primary and secondary vaccine trial endpoints as *C. trachomatis* vaccine development moves forward. Including disease endpoints will be most valuable if only partial immunity is achieved, since a vaccine might still limit ascension and protect from PID. Choice of endpoints also has implications for trial design, such as sample size considerations and whether frequency of

follow-up testing and treatment affects assessment of PID outcomes. Discussion will be aided by better measures of upper genital tract infection and damage, predictors of ascension, and a package of evidence to confirm a vaccine would not increase tubal immunopathology on breakthrough infection.

Vaccine development for *C. trachomatis* has been in the preclinical phase of testing for many years, but the first Phase I trials of chlamydial vaccine candidates are underway, and scientific advances hold promise for additional candidates to enter clinical evaluation in the coming years. Current strategies hinge on a variety of different platforms and are supported by academic, government, and corporate institutions.

A major focus is development of vaccines prepared with *C. trachomatis* MOMP. MOMP vaccination utilizing cationic liposomes (CAF01) induced robust antibody responses, type-1 immunity, and partial protection from infection in minipigs, and significant protection from upper tract disease in mice (59, 282). A second MOMP formulation prepared with a novel oil-in-water nanoemulsion (Nanostat™) and delivered intranasally purportedly decreased oviduct pathology in mice by 80 percent (283). Protection was associated with high levels of serum and vaginal antibodies and robust IL-17/IFN-γ responses. An immunoproteomics approach identified *Chlamydia* polymorphic membrane proteins (PMPs) preferentially loading MHC Class II, and vaccination with three MOMP and four PMP alleles emulsified with DDA/MPL adjuvant significantly reduced bacterial shedding in a transcervical *C. trachomatis* mouse model (248). Current investigation is centered on development of an outer membrane protein based vaccine for Phase I testing.

The ability to generate vaccine-induced resident memory T cells in the mouse genital mucosa is a major advancement in the field (38). Mucosal immunization with ultraviolet light

(UV)-inactivated *C. trachomatis* complexed with novel, charge switching synthetic adjuvant particles (cSAPs) incorporating the TLR7-agonist resiquimod conferred significant protection against chlamydial infection in mice. Uterine vaccination induced mucosal resident and systemic T cell responses that induced optimal chlamydial clearance compared to intranasal and intramuscular vaccine delivery.

Another major advancement is the use of high-throughput technology for determination of T cell–specific epitopes. Examination of T-cell IFN-γ responses in a cohort of 141 subjects led to identification of eight CD4 and eighteen CD8 antigens associated with clearance or resistance to infection (57). Another group assessed 120 *Chlamydia* proteins and identified seven novel antigens that conferred partial protection in mice (54). Recent analysis demonstrated chlamydial proteins recognized by highly exposed women that limit or resist genital tract infection (281). These proteins were primarily involved in protein synthesis, central metabolism, and type III secretion. Ongoing research is focused on *in vitro* screening of PBMC responses from previously infected subjects to chlamydial proteins. These efforts will help identify protective antigens broadly expressed by human leukocyte antigen (HLA) haplotypes to better guide an effective vaccine strategy.

A Vaxonella® platform for chlamydia immunization is being investigated for immunogenicity and efficacy in animal models. The oral delivery system utilizes an attenuated *Salmonella enterica* vector that has passed Phase II trials as the Typhella® vaccine and allows for insertion of chlamydial antigenic gene sequences. The bacteria are ingested and transverse M cells in the gut where they mount an immune response within Peyer's patches. *Salmonella* act as an immunostimulator bypassing the necessity of additional adjuvants. The vector is constructed

with technology designed to generate stable attenuation and is formulated to exclude toxic bile salts during ingestion for optimal delivery (284, 285).

Finally, work related to vaccine development for *C. trachomatis* ocular infection might shed light on vaccine development for the genital tract. Ocular inoculation of NHPs with attenuated, plasmid-deficient *C. trachomatis* ocular serovar A elicited partial protection against a virulent strain in a subset of cynomolgus macaques that appeared to correlate with MHC Class II haplotype (37) and CD8+ T cell responses (252). This strategy is currently in preclinical development with the National Institute of Allergy and Infectious Diseases (NIAID). Murine genital inoculation with plasmid-deficient *C. muridarum* conferred protection against upper genital tract pathology (61). These results were not replicated in the NHP model of genital infection; however, pathology was minimal in monkeys inoculated with wild-type *C. trachomatis* oculogenital serovar D (63). This illustrates the need for delineating protective immune mechanisms and optimal vaccine formulations in ocular versus genital tract infections (286).

The likelihood for financing a chlamydial vaccine by multilateral agencies is currently unknown, but vaccine development is likely to depend on its applicability to both HIC and LMIC markets. Gavi (Global Alliance for Vaccines) support of HPV vaccination provides a model for prioritization of an STI vaccine to prevent adverse reproductive health outcomes, as well as an adolescent platform for vaccine delivery in LMICs. The possibility that chlamydia prevention could lower HIV transmission may have a positive impact for other funding agencies. Developing a strong public health investment case, by obtaining better data on chlamydia-associated PID and outcomes like infertility, ectopic pregnancy, and chronic pelvic pain and their costs in LMICs, will be crucial for encouraging investment in and financing of a future *C.*

trachomatis vaccine. Gaining consensus on clinical endpoints and developing more feasible and reliable measures of upper genital tract disease for clinical evaluation will also “de-risk” chlamydial vaccine development for industry and funders (287). The global roadmap for STI vaccine development, generated jointly by WHO, NIAID, and a wide range of technical partners, outlines critical next steps to address barriers to development and encourage investment in these important vaccines for global sexual and reproductive health (288, 289).

7.0 CONCLUSIONS AND FUTURE DIRECTIONS

These studies demonstrate that future vaccines should focus on development of durable polyfunctional Th1 responses capable of producing high levels of IFN-γ. Protective Th1 cells may further enhance humoral immunity through antibody production, which would be critical for acquired immunity against re-infection and control of extragenital dissemination. *Chlamydia*-specific antibody would further augment Th1 activation by antigen-presenting cells and may additionally encourage development of higher affinity B cells. Future work will focus on identifying the Tg TCR cognate antigen. This discovery would abet additional studies identifying the protective capacity of subunit vaccination, and encourage strategies investigating recombinant chimeric antigens consisting of T and B cell epitopes. We will also determine if TCR Tg CD4 T cells develop a resident-memory phenotype that can be induced through vaccination.

8.0 RELEVANT PUBLICATIONS

1. Qu Y, Frazer LC, O'Connell CM, Tarantal AF, Andrews CW, Jr., O'Connor SL, Russell AN, Sullivana JE, **Poston TB**, Vallejo AN, and Darville T. Comparable Genital Tract Infection, Pathology, and Immunity in Rhesus Macaques Inoculated with Wild-Type or Plasmid-Deficient *Chlamydia trachomatis* Serovar D. Infect Immun. 2015;83:4056-67.
2. **Poston TB**, Darville T. *Chlamydia trachomatis*: Protective Adaptive Responses and Prospects for a Vaccine. Curr Top Microbiol Immunol. 2016. *(Introduction and Discussion)*
3. **Poston TB**, Gottlieb SL, Darville T. Status of vaccine research and development of vaccines for *Chlamydia trachomatis* infection. Vaccine (2017), http://dx.doi.org/10.1016/j.vaccine.2017.01.023 *(Introduction and Discussion)*
4. **Poston TB**, Qu Y, Girardi J, O'Connell CM, Frazer LC, Russell AN, Wall M, Nagarajan UM, and Darville T. A Chlamydia-specific TCR-Transgenic Mouse Demonstrates Th1 Polyfunctionality with Enhanced Effector Function. Journal of Immunology. 2017. pii: ji1700914. *(Chapter One)*

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