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Diversity in the T cell response to *Chlamydia*-sum are better than one

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Abstract

Chlamydia trachomatis is responsible for an increasing number of sexually transmitted infections in the United States and is a common cause of serious pathology in the female reproductive tract (FRT). Given the impact and incidence of these infections, the production of an effective *Chlamydia* vaccine is a public health priority. Mouse models of *Chlamydia* infection have been utilized to develop a detailed and mechanistic understanding of protective immunity in the FRT. These studies reveal that MHC class-II restricted *Chlamydia*-specific CD4 T cells are critical for primary bacterial clearance and provide effective protection against secondary infection in the FRT. Despite the clear importance of IFN- γ produced by CD4 Th1 cells, there are also suggestions of wider functional heterogeneity in the CD4 T cell response to *Chlamydia* infection. Understanding the role of this diversity in the CD4 T helper cell response in the FRT should allow a more nuanced view of CD4 T cell biology in the context of *Chlamydia* infection and may be critical for vaccine development. Here, we summarize our current understanding of CD4 T helper subsets in the clearance of *Chlamydia* and discuss some areas where knowledge needs to be further extended by additional experimentation.

Introduction

The *Chlamydiaceae* family consists of 11 different species of *Chlamydia*. *Chlamydia trachomatis* (*Ct*) and *muridarum* (*Cm*) infect human and mouse reproductive tracts, respectively, and will be highlighted in this review [1]. *Chlamydiae* are gram-negative, obligate intracellular bacteria [1]. Their typical life cycle is bi-phasic, consisting of elementary (EBs) and reticulate bodies (RBs). The spore-like elementary bodies are built to withstand the noxious extracellular environment, while reticulate bodies acquire nutrients and replicate inside a host cell vacuole known as an inclusion [2]. After replication, bacteria are released from the host cell by one of two mechanisms: lysis or extrusion. During lysis, permeabilization of the inclusion, and nuclear and plasma membranes all lead to rupture of the host cell and release of EBs [3]. Extrusion occurs when EBs exit the cell by budding off

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from the plasma membrane, leaving the host cell uncompromised [3]. After exiting the initial target cell, *Chlamydia* initiate the replication cycle again in a neighboring host cell.

The incidence of *Chlamydia* infection is over 100 million worldwide cases [4], and a study of women in the UK estimates that 5% of 16–24-year-old women are infected [5]. Furthermore, *Ct* infections are responsible for 35% of incidents of pelvic inflammatory disease (PID) in 16–24 year olds and 29% of tubal factor infertility cases (TFI), making this pathogen a substantial threat to the reproductive health of young women [5, [6]. Due to the asymptomatic nature of this infection, patients run the risk of developing severe complications prior to seeking medical attention. Efforts to regularly screen patients and treat them with antibiotics have been implemented to address this problem [7]. While employment of this strategy has coincided with reduced incidence of PID, the incidence of *Ct* infections is still rising [7, [8]. Indeed, antibiotic use may be limiting acquired immunity to infection and thus contributing to the rising incidence of infection [9, [10]. Therefore, an effective vaccine would be the preferred method of diminishing the frequency of *Ct* infections and associated pathology in the population. Clinical reports of *Ct* infections suggest that primary infection can be resolved naturally in some women, as evidenced by swab collections at clinical follow-up visits that are *Ct* negative [11, [12]. Mouse studies support a model in which adaptive immunity, particularly CD4 T cells, are required to clear primary *Chlamydia* infection from the female reproductive tract (FRT). These data suggest that a vaccine targeting adaptive CD4 T cells will be most promising in protecting patients from *Ct* infection.

Protective immunity in clinical infection

Precisely defining the factors contributing to *Chlamydia* immunity in humans is a daunting task for researchers evaluating clinical studies. Indeed, many studies investigating the duration of the infection and the host factors that influence the resolution of infection are confounding [13]. However, these studies point to some important characteristics about natural human *Chlamydia* infection, including the simple fact that some women can naturally resolve the infection. A 5-year study of a cohort of Colombian women showed that approximately 50% of women cleared *Ct* without any reported treatment after 1 year, and 94% were able to clear infection after 4 years [14]. These clinical observations indicate that many women naturally generate adequate protective responses to *Chlamydia*, although the long delay in this immune development may predispose some of these individuals to severe complications associated with prolonged infection [15]. Exactly how a subset of women is able to spontaneously clear or resist primary *Chlamydia* infection is poorly understood.

There are several genetic and environmental factors linked to resistance or susceptibility to *C. trachomatis* (*Ct*) infection in women. The HLA class II variant DQB1*06 is reported to be associated with *Chlamydia* infection in North American adolescents [16], pointing to a major role for CD4 T cells in *Chlamydia* immunity. Interestingly, HIV-infected women that lack healthy CD4 T cells have an increased risk of developing chlamydial PID [17], suggesting that CD4 T cells are required for clearing infection and/or regulating pathology. Peripheral blood mononuclear cell (pbmc) secretion of IFN- γ or IL-13, cytokines that are produced by T helper cells, has been associated with resistance to *Ct* infection in a cohort of

female sex workers in Kenya [18]. Detection of these cytokines may indicate that CD4 T cell differentiation is heterogeneous in the FRT. Women lacking an IL-10 variant produced higher levels of this cytokine after infection, and this correlated with increased susceptibility to re-infection [19]. Thus, the development of an appropriate effector CD4 T cell response appears to be required for resolution of *Ct* infection and counter regulation of these responses may allow reinfection.

CD4 T cells are essential for protection in animal models

Animal models of *Chlamydia* infection have been useful tools for dissecting the specific types of immune responses important for chlamydial immunity. Nude mice lacking all T cells, as well as TCR $\alpha^{-/-}$ and TCR $\beta^{-/-}$ knockout mice are unable to clear *Chlamydia* from the FRT [20, [21, [22]. Furthermore, infection of MHC class-II-deficient mice, which lack the ability to activate most CD4 T cells, results in non-resolving infection and perpetual shedding of *Cm* from the FRT [23]. Moreover, multiple studies have reported CD4 T cells are essential for optimal resolution of primary and secondary *Chlamydia* infection in mice [24, [25, [26, [27]. Genetic knockout of CD4 T cells during primary *Cm* infection causes delayed resolution of infection by about 10 days [23], whereas CD4 depletion prior to infection results in 100,000-fold higher bacterial burdens after 30 days of infection [28, [29, [30]. The discrepancy between the CD4 knockout and depletion experiments could be due to MHC class II-restricted CD4(-) T cells that arise in CD4 $^{-/-}$ mice and that may contribute to clearing the infection [31]. The persistent high bacterial shedding observed in mice infected after CD4 depletion closely resembles infection of MHC class II-deficient mice, which cannot resolve primary infection [23]. Thus, the phenotype observed after antibody depletion of CD4 T cells is probably a more accurate reflection of the importance of CD4 T cells in clearing *Chlamydia*. The high FRT bacterial burdens observed in the MHC class II knockout and CD4-depleted mice contrast with studies using IFN- γ -deficient mice in which the mice shed low levels of bacteria from the FRT for long periods of time and have increased bacterial loads peripheral tissues [27]. These studies suggest the involvement of other MHC class II-restricted CD4 T helper subsets that are required to eradicate bacteria using other protective mechanisms.

Adoptive transfer of memory CD4 T cells into naïve mice provides modest protection against secondary challenge [25]; however, the magnitude of this protective effect is lower than the protective response in a previously infected mouse [26]. It is possible that the artificial process of T cell adoptive transfer reduces the protective capacity of the CD4 T cells *in vivo*, but alternative techniques such as parabiosis may be more appropriate for teasing apart the arms of the immune response contributing to protective immunity. More likely, the protective capacity of CD4 T cells works more efficiently in concert with other aspects of immune memory, including *Chlamydia*-specific B cell responses.

B cells and antibody are involved in combating both primary and secondary *Chlamydia* infection respectively, and this role for humoral immunity has been recently summarized [32]. After vaginal infection, mice lacking B cells show similar FRT bacterial shedding as wild type mice, implying that B cells are not involved in resolution of primary infection [24]. However, a more detailed analysis of this process revealed that B cell-deficient mice

develop systemic disease that causes ascites formation and induces a larger systemic T cell response to infection [33]. Investigation is underway to determine whether this requirement for B cells necessitates antibody secretion or simply B cell antigen presentation to CD4 T cells. The transfer of immune serum alone provides some protection against secondary challenge in guinea pigs; however, the absence of immune controls in the study makes it difficult to determine whether this represents complete protection in this animal model [34]. The transfer of immune serum into B cell-deficient mice provides incomplete protection to primary or secondary *Cm* challenge, suggesting distinct roles for B cells and antibody. Ongoing studies will carefully delineate the role of B cells versus antibody in the protective immune response to *Chlamydia*.

CD4 T cell subsets in *Chlamydia* immunity

Despite a clear requirement for CD4 T cells in protective immunity to *Chlamydia*, the precise role of CD4 T helper cell subsets remains incompletely defined. Our laboratory previously developed multiple MHC class-II tetramers that allow *ex vivo* detection of expanded *Chlamydia*-specific CD4 T cells during infection [33]. This technical approach allows identification of CD4 T cell responses without predicting effector capacity. Using tetramers, we detected expansion of Th1, Th17 and Tregs, but virtually no GATA3⁺ Th2 cells, in the draining lymph nodes and reproductive tract of mice infected with intravaginal *Cm* [33]. This finding is somewhat at odds with reports in mice and humans regarding the presence of IL-13 or IL-13-producing CD4 T cells [17, [35]. There are multiple possible explanations for this discrepancy. Perhaps the IL-13-producing Th cells identified in mice are producing this cytokine in a GATA3-independent manner [36]. Indeed, other transcription factors such as E4BP4 have been shown to regulate IL-13 production in T helper cells [37]. Another possibility for the presence of IL-13-producing CD4 T cells in the mouse study by Johnson et al. is the technique used to identify these cells [35]. The authors cultured these cells *in vitro* for many passages in order to create cell lines [35]. It is well established that cell lines do not often reflect the *in vivo* phenotype of the cells originally responding to the infection [38]. In the next sections, we will present our view of what is known regarding the heterogeneity of T helper cell subsets responding to *Chlamydia* infection.

Th1 cells

T helper type 1 cells characteristically facilitate the clearance of intracellular bacterial pathogens [39], and are essential for the resolution of *Salmonella*, *Mycobacteria*, and *Leishmania* infections in mice [40]. Th1 cells secrete the key cytokine IFN- γ , which allows infected macrophages to eliminate intra-phagosomal bacteria by inducing production of toxic radicals, including nitric oxide, which can directly destroy the pathogen [41].

IFN- γ is also important for the resolution of *Chlamydia* infection. IFN- γ -mediated destruction of *Chlamydia* can occur in multiple cells *in vitro* including macrophages and epithelial cells [42]. Additionally, IFN- γ up-regulates IDO in host cells, resulting in tryptophan degradation which is known to starve *Chlamydia* and severely inhibit its growth [43]. Consistent with this *in vitro* activity, Th1 responses can facilitate protection against *Chlamydia* infection *in vivo* [27, [44, [45]. Th1 cells are initially polarized by IL-12,

secreted by dendritic cells, while IFN- γ production by natural killer, CD8, or Th1 cells maintains this type I polarization. Mice lacking IL-12 display prolonged periods of vaginal *Chlamydia* shedding, but can eventually resolve infection [22]. In marked contrast, mice lacking IFN- γ display lower bacterial burdens in the FRT but can develop systemic disease [27, [45]. These observations indicate distinct roles for Th1 cells in the local reproductive tract versus systemic organs. The phenotype of IL-12-deficient mice resembles CD4-depleted mice in that they shed high numbers of *Cm* bacteria at the FRT mucosa [27, [45], suggesting they are deficient in Th1 responses at the tissue site. In contrast IFN- γ -deficient mice shed low levels of bacteria from the FRT, while B cell-deficient mice clear *Cm* from the FRT completely [22] [33]. Both mice, however, develop severe concurrent systemic infection, indicating that IFN- γ and humoral responses may be necessary for containing disseminating infection [33, [46, [47]. CD4 T cells are likely the main source of IFN- γ in these scenarios since NK or CD8 T cell depletion during primary or secondary *Cm* infection causes only mildly increased bacterial burdens [24, [29], [48]. This might suggest that CD4 T cell production of IFN- γ to direct antibody class-switching is critical to preventing disseminated primary infection, and this notion is supported by a recent study [47]. The source of this protective IFN- γ is likely to be Tfh cells in the draining lymph node, a CD4 subset that has not been carefully studied in the context of *Chlamydia* infection. Indeed, in a study utilizing a *Salmonella* vaccine, repeated antigen exposure generated robust germinal center formation that was dependent on IFN- γ the primary source of which was Tfh cells [49]. The antibody response generated from these germinal centers enhanced pathogen clearance. It is possible that the humoral immune response to *Chlamydia* functions through a similar mechanism, where IFN- γ -producing T follicular helper cells direct the generation of protective antibody.

The development of *Chlamydia*-specific tools to track T cell responses has allowed more precise measurement of T helper cell subset development in response to chlamydial antigens. Two independent TCR transgenic lines, one responding to *C. trachomatis* (NR1) [50] and the other specific for *C. trachomatis* and *C. muridarum* [21] have been generated. When used as part of an adoptive transfer system, both of these TCR transgenic populations develop into Th1 effector cells in response to challenge with their respective chlamydial species. The latter TCR transgenic line was protective against *C. muridarum* and exhibited polyfunctionality, secreting both TNF- α and IL-2 in conjunction with IFN- γ , which lends support to previous evidence documenting polyfunctional CD4 T cell responses to *Chlamydia* [18, [33, [51]. An extensive and elegant vaccine study using the NR1 mouse showed that NR1 cells differentiate into Th1 cells upon challenge with UV-*Chlamydia trachomatis* (UV-*Ct*) conjugated to an adjuvant linked to a charge-switching particle (UV-*Ct*-cSAP). These *Chlamydia*-specific Th1 cells were protective against *Ct* challenge in immunized mice. Interestingly, these protective Th1 cells were primed mainly by CD103-DCs in the uterus or iliac lymph node as opposed to non-protective UV-*Ct* specific T cell responses, which were primed by tolerogenic CD103+ DCs. Th1 cells in UV-*Ct*-cSAP immunized mice reduced *Chlamydia* bacterial burdens in the reproductive tract by 10-fold compared to UV-*Ct* immunized mice. However this protection was equivalent to that conferred by live *Ct* infection, characterized by an initial bacterial load of 100,000 IFU [52]. This level of protection may be sub-optimal, as it allows a significant amount of bacteria to enter the tissue, greatly contrasting the robust protection observed after *C. muridarum*

infection that consists of approximately 10 IFU of bacteria in the FRT upon secondary challenge [24].

Although it is clear that Th1 cells make a significant contribution to *Chlamydia* immunity, the phenotypes of mice lacking Th1 immunity are not synonymous with the those of mice possessing a deficiency in MHC class-II, which display high levels of bacterial shedding and nonresolving infections [23]. It seems possible therefore that other cytokines produced by polyfunctional Th1 cells, such as TNF- α and IL-2 [51], serve to restrain bacterial infection in the absence of IFN- γ . Alternatively, there may well be contributions from additional CD4 T helper cell subsets within the female reproductive tract that control *Chlamydia* infection, but remain poorly characterized.

Th2 cells

The prominent functions of Th2 cells are in assisting mast cells during allergy and hypersensitivity reactions as well as in immunity to helminth infections [39]. They can also coordinate tissue repair responses [53, [54]. A study of human *Chlamydia* infection revealed there are significantly more IL-4 producing antigen-specific CD4 T cells in pbmcs from *Chlamydia*-infected female patients than non-infected control subjects [55]. Robust cellular production of this cytokine was detected at enrollment, and at 1 and 4-month follow-up visits. The author's suggest that this finding "implies that Type 2 immunity was evolutionarily selected to control genital *C. trachomatis* infection" [55]. While Th2 cells may be detected during active *Chlamydia* infection, it is less clear that Th2 cells are actually protective. Because women were treated with antibiotics prior to follow-up visits, associations of Th2 responses with the ability to control infection, or reduced pathology could not be measured in the study [55].

The potential role of Th2 cells during *Chlamydia* infection has not been extensively characterized. As noted above, very few GATA3+ CD4 T cells were detected during active infection in a study using MHC Class-II tetramers to identify endogenous polyclonal responses [33]. Furthermore, genetic deletion of IL-4 or or IL-4R α did not affect Chlamydia bacterial burdens relative to wildtype mice [33, [56]. Additionally, the protective capacity of an *in vitro* generated Th2 clone specific to *Chlamydia muridarum* has been examined. When transferred into nude mice, this Th2 clone provided no protection against *Cm* compared to mice lacking T cells. Using the same approach, transfer of Th1 clone provided robust protection against *Cm*, and infection was cleared after 30 days [57]. Likewise, adoptive transfer of NR1 TCR transgenic T cells that had previously been skewed toward a Th2 phenotype resulted in higher bacterial burdens compared to mice receiving Th1 cells. Intriguingly, transferred Th2 cells responding to infection eventually skewed towards a Th1 phenotype [44]. Collectively, these results suggest that few Th2 cells are generated in the mouse model of infection and that artificially generated Th2 cells lack the capacity to clear *Chlamydia* infection *in vivo*. Thus, if further data confirms that Th2 cells are involved in human *Chlamydia* immunity, alternative animal models may be required to study Th2mediated protection.

Although few classical Th2 cells are elicited during murine infection, eosinophils are recruited to the FRT during *C. trachomatis* infection in mice [56]. A reduction in eosinophil

frequency in IL-4-deficient mice correlated with increased severity of upper reproductive tract pathology [56]. Recent data suggest that multifunctional CD4 T cells producing both IFN- γ and IL-13 may be protective against reproductive tract pathology [35, [58]. Since few Th2 cells are detected in the FRT during *C. muridarum* infection, it is possible that IL-13 production from this unusual population orchestrates the recruitment and/or retention of reparative eosinophils [59, [60]. While these type 2 immune responses may play an important role in tissue protection and repair following damage of the FRT, it seems unlikely that these responses effectively reduce the bacterial burden of *Chlamydia* [56]. Future clinical studies should clarify which cells produce IL-13 following stimulation of human pbmcs [18].

Th17 cells

T helper 17 cells typically orchestrate the clearance of extracellular bacteria through the recruitment of neutrophils [39]. Additionally, they contribute to the maintenance of barrier function at mucosal surfaces and Th17 responses have been implicated in pathology associated with autoimmune conditions [61, [62]. Recent studies suggest that Th17 cells play a role in the development of pathology during *Chlamydia* infection. For example, antibody neutralization of IFN- γ causes CD4 T helper cells to divert towards a Th17 phenotype that corresponds to worsened reproductive tract pathology, and only slightly increased bacterial burden [63]. The exacerbated pathology may be due to the greater influx of neutrophils and monocytes into the tissue mediated by enhanced Th17 cell production [63]. BALB/c mice deficient in IL-17, a canonical Th17 cytokine, exhibit significantly reduced FRT pathology after *Chlamydia* infection when compared to wildtype controls [64]. IL-17-deficient mice also displayed a corresponding reduction in neutrophil and monocyte infiltration, which could explain the differences in pathology. Interestingly, bacterial shedding was decreased in IL-17-deficient mice compared to WT controls [64]. It is unclear whether CD4 T cells were directed toward a more robust Th1 phenotype in *Chlamydia*-infected IL17-deficient mice, but this could explain the observed reduction in bacterial loads. The effect of IL-17-deficiency in mice on a C57BL/6 background differed markedly from the findings in BALB/c mice. C57BL/6 IL-17-deficient mice displayed diminished Th1 responses and neutrophil recruitment. This effect of IL-17 deficiency on Th1 development is supported by studies using other bacterial infection models [62]. Interestingly there were no significant changes in bacterial clearance or FRT pathology in C57BL/6 IL-17-deficient mice compared to WT mice [65]. The opposing phenotypes observed in BALB/c and C57BL/6 backgrounds are likely due to strain-specific differences in CD4 T cell differentiation. However, a pathological role for Th17 cells has been observed in many other studies, supporting a similar function for these cells in *Chlamydia* infection [66, [67, [68].

Tregs

Induced regulatory T cells dampen pro-inflammatory T cell responses through direct cell-cell contact mechanisms as well as through the secretion of immune-suppressive cytokines [69]. However, during *Chlamydia* infection Treg-induced immunosuppression has deleterious outcomes for the host in controlling infection. In a clinical trial of inactivated *Ct* immunization, a subset of vaccinated subjects experienced worse symptoms of ocular trachoma than control subjects [70]. This deleterious clinical outcome was also observed in

mice immunized with UV treated *Ct*, since they experienced higher bacterial burdens than unimmunized controls after infection [52]. When this same UV-Ct was physically linked to an adjuvant, CD4 T cell responses were directed away from Treg development and toward a protective Th1 response. This lineage choice was influenced by the DC subset priming the naïve T cells during the primary response to vaccination. Some reports indicate that Treg formation is dependent on CD11b⁻ plasmacytoid DCs while other studies pinpointed CD103⁺ CD11b⁻ cDCs in the priming of Treg responses [52, [71]. In contrast, protective Th1 responses appear to be primed by CD103⁻ CD11b⁺ DCs [52]. In addition to Treg differentiation driving the chlamydial T cell responses away from a protective Th1 lineage, the induction of Tregs may assist the development of other pro-inflammatory responses that encourage pathology. For instance, co-culturing of Tregs with conventional T cells (Tconv) resulted in increased production of IL-17A in the supernatant [72]. Additionally, Treg-depleted mice had diminished Th17 responses, which correlated with reduced oviduct pathology, but not inhibited bacterial clearance [72]. The available data therefore point to T regulatory cells having an overall harmful influence on the host during *Chlamydia* infection.

Conclusion

The *Chlamydia* community has repeatedly demonstrated in animal models the importance of Th1 immunity in fighting infection. Clearly, canonical Th1 cytokines IL-12 and IFN- γ are required for optimal anti-chlamydial immune responses. However, an area that has remained relatively unexplored is the examination of transcription factors in the regulation of *Chlamydia*-specific CD4 T cell lineages. In preliminary studies, our laboratory has observed that T-bet, the transcription factor that classically defines Th1 cells, is not required for *Chlamydia* clearance from the reproductive tract. This finding actually fits well with previous observations showing a marked discrepancy between mice lacking MHC class-II and those lacking IFN- γ [23, [73]. These observations highlight a need to understand cytokines relevant to protection against *Chlamydia* and how they are transcriptionally controlled, especially if through non-canonical pathways. Together, these studies suggest a more nuanced understanding of how CD4 T cells regulate bacterial growth in the reproductive tract.

Other investigators have suggested that Tissue Resident memory development may be a more comprehensive model to understand protection than Th1 development. However, while vaccine-induced TRMs can protect against *Chlamydia*, the role of this population has not been clearly defined in *C muridarum* infection. Another MHC class II-restricted immune response that is often overlooked is the development of T follicular helper cell response to *Chlamydia*. The importance of B cells and antibody during *Chlamydia* infection suggests a requirement for this subset that will demand greater investigation. While Th2, Th17, and Treg responses appear to play roles in regulating pathology, there is less evidence that they contribute to bacterial clearance. Thus, there remains the potential that a non-classical CD4 effector response plays a major role in bacterial clearance from the FRT during *Chlamydia* infection. Future studies are certainly required to examine this possibility more carefully. In summary, a heterogeneous CD4 T cell response is induced during *Chlamydia* infection and multiple populations likely contribute to protection and pathology. The development of an effective *Chlamydia* vaccine will depend on the ability of immunologists to define these

responses in more details and devise immunization approaches that induce similar responses in naive individuals.

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Highlights

- Overview of literature on clinical data supporting investigation of CD4 T responses to *Chlamydia* infection
- Summary of animal studies supporting investigation of CD4 T responses to *Chlamydia* infection
- Discussion of studies examining different T helper type responses to *Chlamydia* infection