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Immunopathogenesis of Chlamydial Infections

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Abstract

Chlamydial infections lead to a number of clinically relevant diseases and induce significant morbidity in human populations. It is generally understood that certain components of the host immune response to infection also mediate such disease pathologies. A clear understanding of pathogenic mechanisms will enable us to devise better preventive and/or intervention strategies to mitigate the morbidity caused by these infections. Over the years, numerous studies have been conducted to explore the immunopathogenic mechanisms of *Chlamydia*-induced diseases of the eye, reproductive tract, respiratory tract, and cardiovascular systems. In this article, we provide an overview of the diseases caused by *Chlamydia*, animal models used to study disease pathology, and a historical context to the efforts to understand chlamydial pathogenesis. Furthermore, we discuss recent findings regarding pathogenesis, with an emphasis on the role of the adaptive immune response in the development of chlamydial disease sequelae. Finally, we summarize the key insights obtained from studies of chlamydial pathogenesis and avenues that remain to be explored in order to inform the next steps of vaccine development against chlamydial infections.

1 Introduction

The genus *Chlamydia* consists of various species of *chlamydiae* that are obligate intracellular pathogens and infect humans with a tropism for the epithelial linings of conjunctiva, respiratory tract, and genitourinary tract. The initial infection is typically resolved subsequent to the induction of a robust adaptive immune response, but such immunity only offers partial protection against reinfection (Batteiger et al. 2010). The majority of chlamydial infections tend to be initially asymptomatic and therefore go untreated (Brunham and Rey-Ladino 2005). When the initial infection is symptomatic and identified based on the detection of chlamydial organisms and acute inflammation, treatment with antimicrobial agents is usually efficacious in resolving the infection and inflammation

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(Geisler 2010). However, most infections caused by *Chlamydia* linger for weeks to months in humans, and the chronic low-grade inflammation during this period gives rise to fibrosis and scarring (Paavonen et al. 2008). Another factor contributing to pathology is the high prevalence of repeat infections with the same or different serovars of *Chlamydia* (Bebear and Barbeyrac 2009). Chronic inflammation can occur at the sites of initial infection such as the conjunctiva, respiratory epithelium, and genitourinary epithelium, or at distant sites such as vascular endothelium and synovial membranes (Stephens 2003). Such chronic inflammation leads to clinically important disease sequelae, remnants of the infection which last beyond the clearance of the pathogen, and affects a small subset of all infected people (Oakeshott et al. 2010). These sequelae at the sites of infection may be in the form of blinding trachoma, exacerbation of asthma, pelvic inflammatory disease and infertility in women, and at distant sites in the form of reactive arthritis and exacerbation of atherosclerosis (Stephens 2003; Campbell and Rosenfeld 2015). There is a wide variety of sequelae caused by infection with different species of *Chlamydia*, and these have been investigated using a correspondingly diverse set of conditions and animal models. The vast majority of insights come from studies of trachoma and female reproductive tract disease, and relatively few come from studies of atherosclerosis, asthma, and arthritis. Recently, issues related to immunopathogenesis of trachoma (Taylor et al. 2014; Derrick et al. 2015) and genital tract infections (O'Meara et al. 2014a) have been comprehensively reviewed. We will draw upon literature from multiple *Chlamydia* disease models and discuss the role of immune responses in pathogenesis of *Chlamydia*-induced disease.

2 Diseases Caused by *Chlamydia* in Humans

Infection of *Chlamydia trachomatis* serotypes A to C causes chronic inflammation of the conjunctiva or active trachoma, which is still endemic in many of the poorest or remote countries including Africa, Asia, Australia, and the Middle East (Taylor et al. 2014). The World Health Organization (WHO) classes 53 countries as endemic for blinding trachoma, which is responsible for the visual impairment of about 2.2 million people, including irreversible blindness in 1.2 million people. There are a number of classification systems for the clinical signs of trachoma. Under the WHO-simplified grading system, the presence of five or more follicles on the conjunctival surface of a single eye is classified as trachomatous inflammation follicular (TF). Repeated *C. trachomatis* infection in endemic communities can trigger chronic conjunctival inflammation (trachomatous inflammation intense, TI) in some individuals, causing conjunctival fibrosis (trachomatous scarring, TS). Progressive fibrosis may lead to entropion, inward turning of the lid, or misdirected lashes (trachomatous trichiasis, TT), all of which abrade the corneal surface. This abrasive damage may lead to corneal opacity (CO) and blindness. Not all cases of active trachoma develop conjunctival scarring; the key to trachoma is that repeated episodes of reinfection and inflammation lead to the blinding complications. In this regard, there is accumulating evidence of the importance of lymphoid follicles as local stores of protective T lymphocytes within a mucosal site (Leslie 2016; Mueller and Mackay 2016), and therefore, it is arguable that lymphoid follicles are pathological in context of trachoma.

C. trachomatis serotype D to K infects the endocervical epithelia of women and the urethral epithelia in both sexes and is the leading cause of bacteria sexually transmitted infections in

humans (Stamm and Batteiger 2010). In 2014, more than 1.4 million cases of *Chlamydia* were reported to Centers for Disease Control USA, but an estimated 2.86 million infections occur annually (Centers for Disease Control and Prevention 2014). Each year, there are estimated 357 million new infections with 1 of 4 STIs: *Chlamydia* (131 million), gonorrhea (78 million), syphilis (5.6 million), and trichomoniasis (143 million). The infection rate of *Chlamydia* has been increasing steadily. Individuals with genital *Chlamydia* infection often do not exhibit any symptom (75–90 % of woman; 30–50 % of men); however, they still can transmit infection (Stamm and Holmes 1990). Among untreated women, 20–40 % have involvement of fallopian tubes and pelvic inflammatory disease (PID) (WHO 2006). They also may develop severe sequelae, such as ectopic pregnancy and infertility (Westrom and Mardh 1990; Moore and Cates 1990; Wolner-Hanssen et al. 1990). Neonatal infection also can occur during childbirth. *C. trachomatis* infections in neonates cause conjunctivitis and pneumonitis (Stamm and Batteiger 2010). Chlamydial pneumonitis may have long-lasting effects on respiratory function that last into adulthood and may exacerbate asthma (Hansbro et al. 2004).

Among men with chlamydial urogenital infection, at least 50 % are asymptomatic. Chlamydial urethritis is the primary presentation for men. When left untreated, it can lead to complications such as epididymitis and prostatitis, but neither has been typically associated with long-term sequelae (Berger 1990). It is interesting to note that there are scant reports of prior chlamydial infection resulting in male factor infertility (Adams et al. 2004; Chen and Donovan 2003).

The more invasive serovars L1, L2, L2a, or L3 of *C. trachomatis* primarily infect lymphatics and lymph nodes of human and cause lymphogranuloma venereum (LGV) (Stoner and Cohen 2015). The agent gains entrance through breaks in the skin, or crosses the epithelial cell layer of mucous membranes. LGV is endemic in Africa, Asia, Europe, South America, and the Caribbean, with men affected more commonly than women. As is common to chlamydial infections, chronic LGV causes potentially severe infections with possibly irreversible sequelae (e.g., lymphatic scarring, edema, genital elephantiasis) if adequate treatment is not begun promptly.

Chlamydophila pneumoniae mostly causes upper respiratory tract infections but is also a cause of community-acquired pneumonia (Grayston et al. 1993, 1986). It is very common throughout the world, and it easily spreads in groups of people by inhaling aerosols containing bacteria. It is estimated that about 70 % of *C. pneumoniae* respiratory tract infections are asymptomatic or with minimal symptoms. About 20 % of infected patient are symptomatic upper respiratory tract infections, and the remaining 10 % are pneumonia cases (Choroszy-Krol et al. 2014). As with nearly all chlamydial agents, *C. pneumoniae* also has the ability to induce a chronic inflammatory response. In the respiratory tree, this response has been associated with chronic obstructive pulmonary disease (COPD) and asthma (Hansbro et al. 2004; von Hertzen et al. 1997; Hahn et al. 1991).

Forty to seventy percent of population has been found to have the presence of specific antibodies against *C. pneumoniae*, and a higher incidence of antibodies to *C. pneumoniae* has observed in patients with ischemic heart disease, prior myocardial infarction, and

atherosclerosis (Player et al. 2014; Sakurai-Komada et al. 2014; Deniset et al. 2010). Interestingly, the organism can be detected in fatty streaks and atherosclerotic plaques, but not in normal vessels (Campbell and Kuo 2002; Campbell et al. 1992; Erkkila et al. 2002). Whereas an association between *C. pneumoniae* and exacerbation of atherosclerosis has clearly been documented based on multiple lines of evidence in human studies (Campbell and Rosenfeld 2015; Player et al. 2014; Honarmand 2013), a cause-and-effect relationship has yet to be conclusively determined. Two large antibiotic treatment trials failed to reduce the risk of secondary coronary events (Honarmand 2013; Campbell and Rosenfeld 2014), but a lead investigator of at least one of those trials, and others, has been careful to point out that the studies did not exclude a role for *C. pneumoniae* in atherosclerosis initiation or progression (Grayston et al. 2015). However, in rabbits or mice fed a hyperlipidemic diet, exacerbation of atherosclerosis can clearly be induced following intravascular or intranasal *C. pneumoniae* infection, respectively (Muhlestein 2000; Saikku et al. 1998). In summary, a definitive role for *C. pneumoniae* in atherosclerosis initiation or progression is worthy of further exploration. If eventually confirmed, this will no doubt add to the growing list of chronic inflammatory and scarring diseases, of chlamydial etiology.

In summary, there are a few common features of chlamydial infections and disease development:

1. Chlamydial infections tend to affect large numbers of individuals globally.
2. Most of such infections are asymptomatic and go untreated.
3. When symptomatic, the acute infection causes inflammation, and at this stage, majority of infections can be identified and treated efficaciously.
4. When the infection remains untreated or with repeated infection episodes, fibrotic scarring and disease sequelae such as blinding trachoma, infertility, and cardiovascular disease ensue in a subset of individuals.

Our review will focus primarily around the last point, and in this context, we will use the terms “pathology,” “pathogenesis,” and “disease” to mean the chronic disease sequelae, not the inflammatory response that accompanies acute infections, although the acute inflammation may culminate in the disease sequelae. Most human chlamydial studies have provided only correlative evidence, as it is understandably difficult to design prospective studies to study chlamydial disease sequelae when treatments are readily available to eradicate known acute infections. Therefore, a variety of animal models have been used to study chlamydial disease development and obtain definitive and mechanistic data. Each model reflects some aspect(s) of human disease, but no model completely mirrors the human infection and disease development.

3 Animal Models of Chlamydial Pathogenesis

Trachoma

Models of trachoma have been developed in non-human primates (NHP; pig-tailed macaque, rhesus, cynomolgus, and owl monkey) using *C. trachomatis* serovars A, B, or clinical isolates and in guinea pigs using *Chlamydia caviae* (guinea pig inclusion conjunctivitis agent

or GPIC) (Taylor 1985; Rank and Whittum-Hudson 1994; Howard et al. 1976). These ocular models in NHP can appropriately mimic the acute aspects of human trachoma infection and ensure that infection exposure and disease are not related to reinfection (Taylor et al. 1981, 1982). In the cynomolgus model, the primary ocular infection leads to an acute, self-limiting conjunctivitis with complete resolution within 10–14 weeks. Monkeys display high levels of bacterial shedding in the initial 4–5 weeks after inoculation, whereas later time periods (6–10 weeks) are characterized by fluctuating periods of culture positivity and negativity. However, the follicular and inflammatory clinical signs remain during this period of low infectious burden. Rechallenged monkeys can show significantly less chlamydial shedding for a much shorter duration and display attenuated clinical signs (Taylor et al. 1984). Resistance to reinfection suggests that monkeys acquire partial immune protection following the primary infection. Similar results have been obtained from the owl monkeys, as well as human volunteers (Mabey et al. 2014), or using the GPIC agent in guinea pigs (Howard et al. 1976; Rank and Barron 1987).

Although scarring per se has not been modeled, the chronic inflammation similar to that leading to scarring in human trachoma can be induced in monkey models using repeated exposure to live *Chlamydia* infection (Taylor et al. 1982). In the cynomolgus monkey model (Taylor et al. 1981), *C. trachomatis* can be detected within one week of a challenge inoculation as also seen after primary inoculation. Even with continuation of weekly inoculations, *C. trachomatis* cannot be identified after a period of time depending on the sensitivity of the test. However, the follicular and inflammatory response persists for as long as inoculations are continued and takes nearly 3 months to resolve once inoculation is stopped. These observations are highly consistent with human trachoma disease. Both have a period in which clinical signs persist and *C. trachomatis* cannot be detected.

Genital tract Chlamydia Infection

Several animal models have been used to model *C. trachomatis* genital tract infections in humans, including mouse, guinea pig, pig, non-human primate, rabbit, and sheep (Rank 1994).

The mouse is still the most commonly used animal in studies of *Chlamydia* genital infection because of several considerations: the ease of handling, small size, relatively low cost, the availability of many defined genetically modified mouse strains, and a plethora of mouse-specific reagents (O'Meara et al. 2014a). More importantly, and as we shall see, the mouse model mimics many aspects of *C. trachomatis* infection in women.

There are two established mouse models: the *C. trachomatis* mouse model and the *C. muridarum* mouse model. Infection in both models is enhanced by pre-treatment mice with long-acting progestins such as medroxyprogesterone acetate (P4), which halts the otherwise rapid estrus cycle and enriches the number of target epithelium cells and thus enhances initial infection (Brunham and Rey-Ladino 2005; Morrison and Caldwell 2002). In the absence of P4 pretreatment, infection is erratic and inconsistent with *C. muridarum*. Without P4 pretreatment, mice are highly resistant to *C. trachomatis* infection.

Chlamydial infection in mice mimics that in humans in many ways. For example, mice can be genitally infected via intravaginal inoculation with either *C. muridarum* or most human *C. trachomatis* serovars. Intravaginal inoculation with *C. trachomatis* typically produces a mild genital tract infection that resolves relatively quickly and is not able to ascend to the upper genital tract with high frequency. Higher doses of infectious EB are required to establish infection with *C. trachomatis* than with *C. muridarum*, but the bacteria shedding from the genital tract are much lower compared to *C. muridarum* infection. There is also less upper genital tract pathology and minimal complications after vaginal *C. trachomatis* inoculation (O'Meara et al. 2014a). However, if high doses of EB are inoculated directly into the mouse uterus, uterine horns, or ovarian bursa, *C. trachomatis* infection can cause severe upper genital sequelae, such as hydrosalpinx and infertility, which is also dependent on the mouse strain (Tuffrey et al. 1986). It should also be noted that careful *C. trachomatis* strain selection can yield an infection that routinely ascends into the upper genital tract to induce chronic inflammatory processes (Sturdevant et al. 2010). Whether these inflammatory changes are permanent (yielding infertility for example) or reversible has not yet been determined. Clearly, this could become an interesting new twist on the mouse model.

C. muridarum, a natural pathogen of the mouse, was originally isolated from the lung of mice (Barron et al. 1981). While it has never been isolated from any animal but laboratory mice and hamsters (Zhang et al. 1993), there is evidence of rodent chlamydial infection in nature (Ramsey et al. 2016). Intravaginal inoculation with *C. muridarum* resembles an acute genital *C. trachomatis* infection in women. The primary infected cell is the cervical epithelium, but under the influence of P4, the vaginal epithelium can also be infected (O'Meara et al. 2014a). The infection ascends quickly to the upper genital tract (uterine horns by day 4–7 and oviducts by 10–14). The infection resolves within 30 days in most, and this is frequently followed by the development of hydrosalpinx, fibrosis, and infertility, 30–90 days post-infection, which in at least some ways mirrors common sequelae in woman after infection. The observation that mice pretreated with P4 and infected with *C. muridarum* develop disease sequelae after primary infection is a significant difference from the development of such sequelae in women. Women rarely develop these sequelae after a single primary infection but most often after repeated episodes of natural infection (Darville and Hiltke 2010). That said, the pathology in mice worsens with repeated infections similar to that in natural genital chlamydial infections in humans (Igiertseme et al. 2009; Murthy et al. 2011a). Due to these reasons, the mouse model of *C. muridarum* infection lends itself well for the studies of chlamydial disease development in the genital tract. Using many well-defined inbred and knockout mouse models, the role of immune cells, cytokines, and pathology after *Chlamydia* infection is fully studied. Many of the insights into innate and adaptive immunity against chlamydial infection have resulted from mouse model of *C. muridarum* infection and have, in some cases, guided or corroborated similar assessments and corollaries in humans (O'Meara et al. 2014a).

Another model for chlamydial genital infections is the guinea pig infected with the *Chlamydia caviae* GPIC. This guinea pig genital tract infection closely resembles a genital infection with *C. trachomatis* in human, and it also frequently develops the pathology in the endometrium and oviduct (Rank et al. 1982). An important advantage of this model includes the ability to study chlamydial sexual transmission (Rank et al. 2003). Additionally, the

guinea pig is a good model for hormonal research and its influence on chlamydial infection since humans and guinea pigs have comparable estrous cycles (19 days in guinea pigs), as opposed to the much shorter 4–5 day cycling in mice. Immunity to reinfection that occurs in the guinea pigs is short lasting, which is also analogous to humans. Therefore, this model may be suitable to evaluate potential vaccine candidates. Unfortunately, studying immune responses in detail represents a greater challenge in this model where few genetic knockout or transgenic strains exist and immunological reagents are limited.

The pig as a large animal model has also been used for chlamydial research. They are not only physiologically and genetically more closely related to humans, but also the immune system between pigs and human is more similar than between mice and humans. Pigs are naturally infected with *Chlamydophila abortus* and *Chlamydia suis*, but *Chlamydophila psittaci*, *Chlamydophila pecorum*, and *C. trachomatis* are also able to infect pigs. The pig model has been used successfully to investigate recombinant protein-based and DNA-based vaccine candidates (Harkinezhad et al. 2009; Schautteet et al. 2012). However, the natural pathogen of pigs *C. suis* does not induce tubal infertility and PID. Therefore, the pig model may be more appropriate for investigating putative vaccine candidates and less so for studies of human urogenital pathology. Additionally, pigs are more expensive and complicated than using rodents in the laboratory.

Several non-human primates have been used as potential models to study genital *C. trachomatis* infections, including the marmoset, grivet and baboon, and pigtailed macaque. The pigtailed macaque model was initially developed by Patton and colleagues; it has been the most frequently used primate model for genital *Chlamydia* research (Ripa et al. 1979; Bell et al. 2011; De et al. 2013). *C. trachomatis* can naturally infect this macaque species without hormonal pretreatment, and the infected macaques develop cervicitis and salpingitis, which are highly similar to genital tract inflammation in humans. Repeated infection of macaques leads to cause extensive tubal scarring, chronic salpingitis, and distal tubal obstruction, like the development of PID in women and what is seen in the *C. muridarum* mouse model. Therefore, this model is ideal to study the histopathology and immunopathology of *C. trachomatis*-induced salpingitis. Certainly, macaques are also very good model for vaccine and immune response studies. However, the high-cost, limited resources, the need for extensive adequate facilities, and rarely available expertise limit their use.

Pneumonia and chronic disease

The mouse, rat, hamster, rabbit, and monkey have been used to study *C. pneumoniae* infection (including lung infection), *C. pneumoniae*-induced atherosclerosis, and cardiovascular infection (Campbell and Kuo 2002; Saikku et al. 1998). Recently, *C. pneumoniae* has been identified as a zoonotic pathogen, and it can infect a wide range of host species, such as the koala, frogs, and reptiles (Roulis et al. 2013; Waugh et al. 2015). The mouse model is used extensively in the investigation of a possible causal link between respiratory *Chlamydia pneumoniae* infection and asthma, COPD, and atherosclerosis and for the evaluation of immunopathogenic mechanisms (Campbell et al. 2005a; Campbell and

Kuo 1999; Chen et al. 2010a; Zafiratos et al. 2015). It also has been used to screen vaccine candidates (Vaughn et al. 2015).

***C. muridarum* infections in mice**

At this juncture, it is pertinent to make a special mention of the *C. muridarum* model of chlamydial infection. Significant advances have been made in our understanding of protective immunity and pathogenesis using this mouse model. *C. muridarum* infects the columnar epithelial cells in the genital tracts of female and male mice, and lower tract infection closely mimics acute genital tract infection in men and women (O'Meara et al. 2014a). *C. muridarum* also infects respiratory tract and causes pharyngitis, bronchitis, and pneumonitis in mice (Barron et al. 1981). Therefore, this mouse chlamydial pathogen has been used as a model organism for the study of human *C. trachomatis* urogenital and respiratory tract infections. Following intravaginal infection, the infected mice shed high numbers of bacteria from the vagina during the first week and progressively clear the infection by a month after challenge. Beginning as soon as 28 days and up to 50 days after inoculation, mice begin to display sequelae in the upper genital tract and develop hydrosalpinx (fluid-filled oviducts). A significant number of female mice are rendered infertile by one or more infections of *C. muridarum*, and hydrosalpinx has been used as a surrogate marker of infertility (O'Meara et al. 2014a). There is strong correlation between scarred oviducts and the formation of hydrosalpinx (Shah et al. 2005). Although some strains of mice are more resistant to hydrosalpinx than others (Chen et al. 2014), the overall finding of hydrosalpinx in women is not thought to be as common in women as in mice. Although the basis for this difference is not completely understood, the use of P4 in the mouse model may offer a partial explanation. P4 is often used in the mouse model to make the female mice more susceptible to infection and pathology; mice infected without this treatment display considerable variation in infectivity and shedding patterns (Vasilevsky et al. 2014). Conversely, women in general have lower frequency of hydrosalpinx, but those who use P4 have higher incidence of infection and PID (Darville and Hiltke 2010). Additionally, following the identification of different downstream mechanisms of IFN- γ mediated clearance of *C. trachomatis* and *C. muridarum* in their respective natural hosts (Nelson et al. 2005), there has been cautious uncertainty of the relevance of data acquired using this model to human infections. Despite these differences, in the sections below, we will discuss how the bulk of available evidence suggests that mechanistic findings made using the *C. muridarum* mouse model have largely been supported by correlative evidence from human studies and other animal models, dispelling undue fears about the usage of this model.

4 Chlamydial Immunopathogenesis

A number of host and pathogen factors appear to determine the disease outcomes following chlamydial infections, and these have been discussed in context of trachoma, infertility, and atherosclerosis in detail in recent review articles (Campbell and Rosenfeld 2015; Taylor et al. 2014; Derrick et al. 2015; O'Meara et al. 2014a; Menon et al. 2015; Sessa et al. 2014). We will focus our discussion to emphasize the literature pertaining to the role of adaptive immunity in immunopathogenesis of chlamydial infections.

A discussion regarding immunopathogenesis of chlamydial infections has centered around two hypotheses: the “immunological hypothesis” implicating adaptive immune cells in causing pathology (Brunham and Rey-Ladino 2005) and the “cellular hypothesis” implicating the infected cell as a key inducer and regulator of pathology (Stephens 2003). The historical perspective on how the immunological and cellular hypothesis was proposed was elegantly discussed in 2003 by Stephens (2003), the original proponent of the cellular hypothesis. To lay a platform for our discussion, we briefly restate that historical perspective here.

The immunological paradigm of chlamydial pathogenesis was initially proposed based on several observations. Scarring trachoma occurs only after repeated episodes of infection, and in trachoma vaccine trials in monkeys, reinfection often produced more severe clinical disease (Mabey et al. 2014). Since repeat infection can occur with the same or different serovars, adaptive immune response to a chlamydial antigen shared between different serovars of *Chlamydia* was considered an important player in pathogenesis (Grayston et al. 1985). T lymphocytes were implicated because guinea pigs which had resolved an infection demonstrated delayed-type hypersensitivity (DTH) reaction following either a challenge with high dose of chlamydial organisms or with a triton X-100 extract, not with the triton X-100 buffer alone (Watkins et al. 1986). Subsequent experiments in cynomolgus monkeys supported that DTH could be induced in the eyes of previously infected, not naïve, monkeys with the triton X-100 extract (Taylor et al. 1987). The triton X-100 buffer itself induced inflammation after 3 days of instillation leading to the speculation whether the disease was induced by the buffer itself. However, inflammation was observed as early as 24 h after instillation of chlamydial extract, not buffer, and the lack of similar responses to buffer or extract in naïve monkeys suggested that a chlamydial component was involved (Taylor et al. 1987). Additionally, these studies suggested that the pathogenic chlamydial component was not expressed on the chlamydial surface and was not LPS, but probably a subsurface component of *Chlamydia*. Subsequent studies indicated that the potential offender could be a soluble 57,000 m.w protein (Morrison et al. 1989a), which later was concluded to be the chlamydial heat-shock protein 60 (Hsp60) based on its ease of extraction by detergents (Bavoil et al. 1990; Morrison et al. 1989b). A subsequent study used cloned chlamydial Hsp60 to subcutaneously prime guinea pigs and subjected those animals to ocular challenge with GPIC, but did not find enhancements in disease severity (Rank et al. 1995). This further raised the speculation whether the triton X-100 buffer induced inflammation was a confounding factor in the guinea pig and monkey experiments conducted previously (Stephens 2003). However, studies using detergent-free preparations have not been pursued further in animal models. Correlative clinical studies in humans using anti-Hsp60 responses based on serology in trachoma, salpingitis, and *C. pneumoniae* cardiovascular disease yielded supportive evidence initially (Stephens 2003; Brunham et al. 1985), but some recent large studies have failed to show an association (Lu et al. 2012a; Ness et al. 2008). Given that the guinea pig studies by Rank et al. (1995) had not only failed to show exacerbation of disease, but demonstrated the induction of protection against the challenge infection, the correlations of anti-Hsp60 antibody response to disease in individuals with repeated infection may be ascribed to the increased exposure of this population to the infection. Thus,

the role of Hsp60 in DTH and chlamydial pathogenesis has yet to be conclusively determined.

An alternative hypothesis involving autoimmunity was also proposed because chlamydial Hsp60 and human Hsp60 share at least some antibody-binding epitopes (Yi et al. 1993), and a subset of *Chlamydia* seropositive individuals display such antibodies (Domeika et al. 1998). T cells reactive to chlamydial Hsp60 have been found in mice and humans, and these cells predominantly produce IL-10 and presumably down regulate Th1 responses, but were not tested for cross-reactivity against human Hsp60 (Beatty and Stephens 1992; Witkin et al. 1994; Kinnunen et al. 2002). Thus, the bulk of evidence implicating autoimmunity involving chlamydial Hsp60 as a mechanism of chlamydial pathogenesis is correlative. In summary, conclusive evidence was not available from studies of chlamydial Hsp60 to support the immunological hypothesis as a mechanism of chlamydial pathogenesis, and there seemed to be no breakthroughs to suggest a route forward in that direction.

Based on evidence in vitro wherein chlamydial infection of cell cultures induces a variety of inflammatory mediators (Rasmussen et al. 1997), Stephens proposed the cellular hypothesis in 2003 (Stephens 2003) as an alternate way of approaching the problem of chlamydial pathogenesis. The essence of this hypothesis is that:

- A.** infection of non-immune cells such as epithelial and vascular endothelial cells is the primary driver of *Chlamydia*-induced inflammation;
- B.** the infection induces production of chemokines, cytokines, and growth factors, and much of that response is dependent upon chlamydial growth or persistence in the affected cell;
- C.** this inflammatory milieu would further recruit immune cells, including T cells, which also would produce inflammatory mediators; and
- D.** the collective inflammatory environment thus lays the framework for a persisting inflammatory response and/or repeated bouts of inflammation, which is eventually responsible for chlamydial disease manifested as chronic inflammatory infiltrates and, as often seen, scarring in affected tissues.

Thus, the cellular hypothesis allowed for a pathogenic role for T cells, albeit secondary to the infection itself. Additionally, it was hypothesized that during repeat infection, the adaptive response is robust and even if short-lived could induce significant inflammatory damage leading to long-term disease sequelae. Therefore, the cellular and immunological hypotheses are not mutually exclusive, but primarily differ in the nature and extent of involvement of adaptive immune responses in chlamydial pathogenesis. Since the proposal of this hypothesis, numerous studies have been conducted and found a number of pro-acute or innate inflammatory mediators that correlate with or contribute to chlamydial pathogenesis and support the cellular hypothesis.

5 Role of Components of Innate Immune System in Chlamydial Pathogenesis

The contribution of a number of innate immune mediators has been evaluated in the context of chlamydial pathogenesis. These have been recently reviewed in detail (Derrick et al. 2015; O'Meara et al. 2014a; Menon et al. 2015).

The role of pattern recognition receptors (PRR) including Toll-like receptors (TLR) 2 and TLR 4, and cytosolic PRRs including nucleotide oligomerization domain 1 (NoD1) in chlamydial pathogenesis has been evaluated. It was reported initially that Toll-like receptor (TLR) 2-deficient mice display comparable kinetics of chlamydial shedding, but significantly reduced oviduct pathology following *C. muridarum* infection (Darville et al. 2003). However, a later study demonstrated that while TLR2-deficient mice may display a trend toward a reduction in oviduct pathology at day 35 after infection which represents acute/ongoing inflammation, chronic sequelae measured at day 60–80 after challenge in these mice do not show any significant differences in hydrosalpinx (Dong et al. 2014). These results were supported by the observation that mice deficient in MyD88, a critical adaptor molecule for TLR2, displayed more severe infection, compared to wild-type animals (Nagarajan et al. 2005). Additionally, another study in MyD88-deficient mice reported the induction of a TH2-dominant response and severe pathology in the upper genital tract following *C. muridarum* infection (Chen et al. 2010b). In summary, the predominant evidence suggests against a significant role for TLR2/MyD88 pathway in induction of chlamydial pathologies.

Among pro-inflammatory cytokines, IL-1 β has been shown to play a modest role in chlamydial clearance, but contributes significantly to oviduct pathology in mice (Prantner et al. 2009). Caspase-1 cleaves pro-IL-1 β to the biologically active form, and mice deficient in caspase-1 also display comparable kinetics of infection but significant reduction in oviduct pathology (Cheng et al. 2008). The process by which pro-caspase-1 is converted to mature and biologically active caspase-1 is known to involve Nod-1 or the formation of the inflammasome involving NoD-like receptor P3 (NLRP3) and the adaptor molecule apoptosis-associated speck-like (ASC) protein. Mice deficient in either NLRP3, NLRC4, or ASC do not display reductions in chlamydial oviduct pathology (Nagarajan et al. 2012). On the other hand, it has been demonstrated that the cytosolic PRR NoD1, not the NLRP3/ASC inflammasome, contributes to IL-1 β secretion in human trophoblasts after sensing *C. trachomatis* (Kavathas et al. 2013). NoD1 also has been shown to induce IL-8, an important chemokine responsible for the recruitment of inflammatory cells, during *C. trachomatis* infection (Buchholz and Stephens 2008), and the IL-8 production was shown to occur independently of TLR2/MyD88 pathway. Collectively, in vitro evidence suggests that NoD1 is important in the activation of pro-caspase-1 to caspase-1 and subsequent induction of IL-1 β and IL-8, both of which contribute to pathogenesis. NoD1 signaling also has been shown to induce Type 1 IFNs, especially IFN- β during chlamydial infections which has been shown to contribute significantly to oviduct pathology following *C. muridarum* infection (Nagarajan et al. 2008). NoD1 functional polymorphisms have been shown to correlate with *C. trachomatis* infection/tubal factor infertility (Brankovic et al. 2015), as well as *C.*

pneumoniae infection/stroke (Tiszlavicz et al. 2009). However, mice deficient in NoD1 do not display differences in cytokine induction, infection course, or pathology following *C. muridarum* infection when compared to wild-type animals (Welter-Stahl et al. 2006), suggesting possible compensation from other signaling pathways.

Notwithstanding the upstream mechanisms, it is clear that caspase-1 is a key player in chlamydial pathologies. Caspase-3, along with caspase-1, and presumably other caspases were recently shown to be involved in the development of chlamydial pathologies (Igietseme et al. 2013). In this study, caspases were shown to degrade Dicer, a fertility-promoting ribonuclease II enzyme, and lead to alteration in critical miRNAs that regulate growth, differentiation, and development including mir-21 (Igietseme et al. 2013). Interestingly, uninfected mice deficient in Dicer display oviduct abnormalities, ectopic pregnancy, and infertility (Luense et al. 2009), which are strikingly similar to chlamydial pathologies in human. A more recent study by this group further demonstrated caspase-mediated alteration of a number of microRNAs involved in epithelial–mesenchyme transition, fibrosis, and fertility-related epithelial dysfunction (Igietseme et al. 2015a).

Tumor necrosis factor- α (TNF- α) is another pro-inflammatory acute phase cytokine implicated in chlamydial pathogenesis. We have demonstrated the mice deficient in TNF- α , TNF receptor 1 and/or TNF receptor 2, display minimal oviduct pathology following *C. muridarum* infection (Manam et al. 2015). The contribution of TNFR1 to pathogenesis of *C. muridarum*/oviduct pathology (Dong et al. 2014) and *C. pneumoniae*/atherosclerosis (Campbell et al. 2005b) in mice has been demonstrated by independent groups. Igietseme et al. (2015a) also suggested recently that TNF- α would lead to downstream activation of caspases and inactivation of Dicer leading to chlamydial pathologies. This proposed connection between TNF- α and caspases in chlamydial pathogenesis is a distinct possibility but one that remains to be definitively proven.

Aside from epithelial cells which get infected by *Chlamydiae* and participate in pathogenesis via the mechanisms described above, leukocytes including macrophages and neutrophils are capable, perhaps more so than epithelial cells, in producing the plethora of inflammatory mediators implicated in pathogenesis. In this regard, we have previously provided multiple lines of evidence implicating neutrophils in chlamydial immunopathologies. Specifically, mice deficient in CXC chemokine receptor 2 (CXCR2), matrix metalloprotease-9 (MMP-9), or phagocyte oxidase display significantly reduced chlamydial UGT pathology (Lee et al. 2010a; Imtiaz et al. 2007; Ramsey et al. 2001). CXCR2 is involved in chemotaxis inflammatory cells, MMP-9 induced degradation of extracellular matrix and inflammation, and phagocyte oxidase is a membrane-bound complex that is used to engulf microorganisms, and all are important components of neutrophil function. Additionally, depletion of neutrophils using anti-Ly6 depleting antibodies induces partial reduction of chlamydial UGT pathology (Lee et al. 2010b). The full extent of neutrophil involvement could not be studied using depleting antibodies because upon antibody-mediated depletion, band cells with partial neutrophil function populate peripheral tissues including the genital tract (Frazer et al. 2011). It is important to note many of the mediators described above also can be produced by macrophages, suggesting a possible pathogenic role for this cell type. However, definitive evidence to this end has yet to be attained.

Collectively, a number of components of the innate immune system have been described as key players in chlamydial pathogenesis. It is to be noted that much of the definitive evidences have come from the mouse model of *C. muridarum* infection. Additionally, the available evidence suggests a complex meshwork of events, rather than individual mediators/pathways acting in isolation, in causation of the chronic disease sequelae following chlamydial infections.

6 Role of Components of Adaptive Immune System in Chlamydial Pathogenesis

Whereas accumulating evidence suggests a plethora of innate immunological mediators may contribute to chlamydial disease, the immunological hypothesis is important primarily for two reasons: (A) the exacerbation of ocular disease in vaccinated monkeys of and in the human trachoma vaccine trials suggested the importance of avoiding a deleterious antigen-specific response, if one were to exist (and to be sure, we do not have firm data that it does) (Mabey et al. 2014), and (B) antigen-specific immune responses would make better biomarkers of impending disease and also better targets for the prevention/treatment of disease when compared to innate immune responses. New evidence continues to support a prominent role for adaptive immune responses in the disease induction process. For example, O'Meara et al. (2014b) demonstrated that even when upper genital tract chlamydial infection was not detectable, oviduct pathology occurred at wild-type levels. Johnson et al. (2012) have demonstrated that unmanipulated splenocytes from *Chlamydia*-immune mice can activate specific protective clones CD4⁺ T cells, and such antigen-presenting cell effects were enhanced at 6 months compared to three week following infection. A similar process could be envisioned for ongoing stimulation or targeting of pathogenic T cell responses to sites of sequelae well after clearance of the infection. Support for this line of thought also comes from a recent trachoma study where scarring and clinical inflammation continued to progress in the absence of detectable infection over a 2-year period, and there was an association with clinical inflammation, not scarring progression, of multiple innate immune markers such as S100A7, IL1B, IL17A, CXCL5, CTGF, CEACAM5, MMP7, CD83, and reduced SPARCL1 (Burton et al. 2015). These results support the possibility of a *Chlamydia*-specific adaptive immune response in chlamydial pathogenesis.

The role of specific components of the adaptive immune response has been evaluated for their contribution to chlamydial pathogenesis.

6.1 B Cells

Although a Th2 cytokine profile has been associated with a robust antibody response, including mucosal immunoglobulin A, as well as chlamydial pathologies, B cells and antibody themselves have for the most part been associated with protective immunity, particularly to secondary or “challenge” infections rather than pathogenesis (Farris et al. 2010; Su et al. 1997; Moore et al. 2002; Yang and Brunham 1998), and we have reported that the absence of B cells enhances oviduct pathology (Murthy et al. 2009). A recent study further demonstrated the importance of B cells in preventing dissemination of *Chlamydia*

into the peritoneal cavity, thus supporting a protective role for these mediators in chlamydial infections (Li and McSorley 2013).

6.2 T Helper Cells

In context of CD4⁺ T cells, the bulk of evidence from the mouse model of *C. muridarum* and *C. trachomatis* infections and data from human studies including those in HIV-seropositive women suggest a role for Th1-type CD4⁺ T cells in protection against infection and reduction of chronic disease (O'Meara et al. 2014a; Kimani et al. 1996). To this end, supportive evidence for the immunological hypothesis comes from the observations in humans and animal models of trachoma, salpingitis, lung infection, and reactive arthritis wherein a Th1 cytokine profile (IL-2, IL-12, and IFN- γ) correlates with protection against chlamydial infection and disease, whereas a Th2 cytokine profile (IL-4, IL-5, IL-10, IL-13) correlates with persistence and disease (Stephens 2003). Persistence of *Chlamydia* is a viable but non-cultivable state of the organism brought about due to a submicrobicidal level of various stressors such as downstream IFN- γ effectors, hypoxia, and antimicrobials, which do not kill the organism but drive it into an aberrant resting state (Bavoil 2014). Upon removal of the stressor, there is evidence that the organism may return to its replicating state. For the most part, evidence has suggested that a potent Th1 cytokine environment is essential to curtail the infection (Derrick et al. 2015; O'Meara et al. 2014a; Menon et al. 2015). Mice deficient in interleukin-10, a Th2 cytokine displayed superior clearance of lung *C. trachomatis* infection and a robust Th1-type DTH response and these changes could be reversed by local delivery of recombinant IL-10 (Yang et al. 1999). Additionally, IL-10 knockout mice displayed accelerated clearance of *C. pneumoniae* respiratory infection and enhanced splenic antigen-specific cell proliferation as well as pulmonary pro-inflammatory cytokine production (Penttila et al. 2008). Although the lung infection model in either study only addressed inflammation and antigen-specific response during acute infection, not chronic pathogenesis, these results were interpreted to mean that a balance between Th1 and Th2 cytokine profiles directly affects bacterial clearance or persistence; thus, pathogenesis was linked to the duration of bacterial presence and resulting prolonged inflammation. One could envision that during a reduction or even absence of an otherwise protective Th1 response, prolonged infection or persistence would occur with concomitant prolonged inflammation, a fibrogenic tissue remodeling response, and this would lead to enhanced disease. This argument appeared to further support only a secondary/indirect role for adaptive immunity in chlamydial pathogenesis.

6.3 Regulatory T Cells

Whereas an IL-10 response was considered a Th2 cytokine profile in earlier studies, it later began to be associated with the then newly characterized regulatory T cell subset (CD4⁺ CD25⁺ FoxP3⁺ Tregs). This cell subset, capable of producing IL-10 and TGF- β , was reported in several disease models to be involved in the induction of immunological tolerance (Sakaguchi et al. 2010). Human studies in infertile women had demonstrated the presence of T cells that were reactive to chlamydial Hsp60 and produced IL-10 (Kinnunen et al. 2002), suggesting a pathogenic role of Treg cells. Conjunctival IL-10 and Foxp3 transcripts were reported to be elevated in patients with conjunctival chlamydial infection, with or without disease, and the Foxp3 transcript was elevated in patients where clinical

signs of disease were present without detectable infection (Faal et al. 2006). One study using a human genital tract isolate of *C. trachomatis* serovar D displayed a protective role of Tregs against immunopathology using mice deficient in inducible costimulatory molecule (ICOS), a molecule expressed on a subset of Tregs that depend on IL-10 production for the suppression of Th1 responses (Marks et al. 2007). ICOS KO mice displayed greatly augmented Th1 effector cells and impaired Treg responses, along with sterilizing immunity against reinfection, but moderately enhanced immunopathology. However, the study directly addressed only the role of ICOS, not that of Tregs. Nevertheless, the study somewhat supported the conclusion that in the absence of IL-10, a robust Th1 response enhances chlamydial clearance and reduces pathology. An effector role of IL-10 in inducing chlamydial pathogenesis in the female upper reproductive tract came under further question with the demonstration that the lower genital tract is dominated by an IL-10-producing dendritic cells and poor Th1 cell response, whereas the upper genital tract is dominated by a Th1 CD4⁺ T cell response following *C. trachomatis* serovar D infection (Marks et al. 2010).

While a plethora of damaging innate immune effectors had been identified at this stage, the research field had yet to find evidence of offending adaptive immune response component(s) that overtly caused pathology. Again, we feel it prudent to emphasize that the lung infection model and the *C. trachomatis* genital infection model are reasonable models to study acute chlamydial infections and protective immunity, not well-suited for mechanistic investigation of chronic pathogenesis. Where they were used for that purpose early on, the main interpretation that could be made was that pathology correlated with a longer duration of infection or inflammation.

Subsequently, the mouse model of genital *C. muridarum* infection model began to be used widely (Barron et al. 1981). Not widely embraced at first, this model grew from the seminal work of Rank and colleagues, exploring mechanisms of protective immunity in the 1980s and 1990s. These studies showed that immunity in the *C. muridarum* model is primarily T cell-mediated. While the model was primarily used to assess factors that influence lower genital tract shedding during primary infection, it should be noted, however, that these investigations laid the framework for mechanistic studies of pathogenesis pertaining to the long-term disease sequelae such as hydrosalpinx and infertility. Many of the initially identified mediators were those elicited via the innate immune response as discussed above and eventually others in the adaptive immune response.

In this model, Moniz et al. demonstrated that depletion of pDC from mice resulted in significantly reduced oviduct dilatation and fibrosis scores (Moniz et al. 2010). In addition, they found that reduction in pathology correlated with a modest reduction in the ratio of splenic Treg to Th1 cells, as well as significant (fourfold to sixfold) enhancements in IFN- γ -producing T cells. This underscored a role for pDC in chlamydial pathogenesis and indicated a potential pathogenic role of Tregs. Direct evidence came more recently in a study from Moore-Connors et al. (2013), wherein depletion of Tregs using anti-CD25 treatment prior to *C. muridarum* genital infection resulted in significantly attenuated inflammation, neutrophil recruitment, and oviduct pathology. An interesting finding of this study was that Tregs were shown to induce conversion of naïve CD4⁺ T cells, as well as themselves, into

Th17 T cells. Additionally, depletion of Tregs resulted in significant reduction of Th17, not Th1, CD4⁺ T cell response (Moore-Connors et al. 2013). These results provided strong support for a role of Tregs in chlamydial pathogenesis. In a recent study addressing mechanisms of protective immunity against genital *C. trachomatis* challenge, it was found that an immunization regimen successful in inducing early resolution of challenge infection and reducing chronic pathology activated immunogenic CD103-dendritic cells and a Th1 CD4⁺ T cell response (Stary et al. 2015). Conversely in the same study, an immunization regimen which did not efficaciously reduce chlamydial burden or oviduct pathology upregulated expression of anti-inflammatory markers including PD-L2 and IL-10 on a tolerogenic CD103⁺ dendritic cell subset. Although Tregs were not evaluated directly, these results support a pathogenic role for Tregs in chlamydial infection. On the other hand, a regulatory subset of CD8⁺ T cells (CD8⁺ CXCR5⁺) expressing Treg markers Foxp3 and CD25 has been reported to reduce the severity of oviduct dilatation following genital *C. muridarum* infection when adoptively transferred into CXCR5 knockout mice (Jiang et al. 2013). In summary, the role of Tregs in pathogenesis may be complex and involve multiple subsets of T cells, but the effector mechanism by which these cells contribute to pathogenesis has yet to be determined.

6.4 Th 17 Cells

One of the intriguing findings by Moore-Connor et al. study (2013) was the role of Tregs in activation of Th17 CD4⁺ T cell responses. Th17 cells produce IL-17 that recruits neutrophils and possibly other pro-inflammatory cells to sites of infection; therefore, this response is thought of as a bridge between innate and adaptive immune response (Khader et al. 2009). The role of Th17 CD4⁺ T cells in chlamydial pathogenesis has been addressed by several studies (O'Meara et al. 2014a), and an association between IL-17 and chlamydial disease has been suggested in studies from animal models as well as humans (Masson et al. 2015). Early studies of *C. muridarum* respiratory infection demonstrated a role for IL-17A in the recruitment of inflammatory cells to sites of infection and protection against mortality from infection (Zhou et al. 2009). Additionally, it was observed that in vivo neutralization of IL-17A during respiratory *C. muridarum* infection significantly reduced Th1 response and enhanced Th2 responses (Bai et al. 2009). However, neither of these studies evaluated the chronic disease sequelae. Using IL-17A knockout mice on a C57BL/6 wild-type background, IL-17A was found to be pro-atherogenic in a high-fat diet-induced *C. pneumoniae*-accelerated atherosclerosis in mice (Chen et al. 2010a), providing evidence for a pathogenic role of Th 17 cells in chronic disease sequelae. This was supported by evidence from studies using the genital infection model wherein IL-17A knockout mice on either a BALB/c or C57BL/6 wild-type background also displayed reduced infection clearance as well as reduced oviduct pathology (Andrew et al. 2013; Arkatkar et al. 2015). Additionally, mice immunized intranasally live *C. muridarum* elementary bodies (EB) induced robust protection against both chlamydial burden and oviduct pathology following genital *C. muridarum* challenge, and this correlated with high IFN- γ and low IL-17 production from T cells (Lu et al. 2012b). In the same study, mice immunized with UV-inactivated chlamydial EB induced a low IFN- γ and high IL-17 response from T cells and did not protect against oviduct pathology. These studies suggested a pathogenic role for IL-17 in chlamydial infections. Furthermore, O'meara et al. (2014b) found that among two vaccination regimens

they were using, MOMP with cholera toxin and CpG as adjuvants prevented ascent of *Chlamydia* to upper genital tract, but not oviduct pathology; on the other hand, MOMP with CTA1-DD as adjuvant reduced oviduct pathology, not chlamydial shedding. They explored the IL-17 receptor signaling pathways and found that the regimen that protected against infection, not oviduct pathology, displayed an upregulation of IL-17 receptor signaling, whereas protection against oviduct pathology, not infection, correlated with downregulation of IL-17 receptor signaling. Based on this, the authors suggested that IL-17 signaling may play a role in both protection and pathogenesis based on the balance of downstream receptors that are activated and that protection against chronic chlamydial disease involved reduced induction of IL-17. IL-17 receptor A knockout mice displayed reduced neutrophil recruitment and Th1 response, but the course of infection and oviduct pathology remained unaltered (Scurlock et al. 2011). A compensatory influx of macrophages and TNF- α production was found in the IL-17RA KO mice, and this was ascribed to compensate for the deficient IL-17 signaling and neutrophil recruitment toward induction of pathology in these animals. However, mice deficient in IL-23, required for activation of IL-22 and Th17 CD4⁺ T cell response, also displayed reduced Th17 but unaltered Th1 CD4⁺ T cell response, and comparable resolution of infection and only a trend toward reduction of oviduct pathology when compared to wild-type animals (Frazer et al. 2013). Overall, the role of Th17 responses in chlamydial pathogenesis appears to be complex and continues to be explored, with strong suggestions that they contribute to pathogenesis.

6.5 CD8⁺ T Cells

CD8⁺ T cells are the other important T cell subsets have been studied extensively in context of protective immunity against chlamydial infections. CD8⁺ T cells are recruited in considerable numbers to sites of chlamydial infection, but evidence for a role in protective immunity for this cell type has been sparse. Some clones of CD8⁺ T cells have been shown to induce protective immunity against chlamydial infection, but they do so based on their ability to secrete IFN- γ , not cytotoxicity (Starnbach et al. 1994; Igietseme et al. 1994). It has been considered counterintuitive that CD8⁺ T cells, known for their ability to use cytolytic mechanisms and protect against intracellular viral infection, seem to have a relatively minimal role in protection against a primary or secondary chlamydial infection. In this regard, *Chlamydia* is a vacuolar pathogen and so far it seems relatively few chlamydial components access the host cytosol, which may partly explain the relatively small role for CD8⁺ T cells in cytotoxicity. Chlamydial infection also has been demonstrated to induce downregulation of MHC I on infected cells, providing another possible mechanism of evading CD8⁺ T cell-mediated cytotoxicity (Zhong et al. 2000; Ibana et al. 2011). It has been shown that a polyclonal CD8⁺ T cell response against chlamydial antigens primarily is induced against soluble chlamydial antigens secreted out of the bacterium, not to integral antigens in the chlamydial EB or RB (Johnson et al. 2014). It is also possible that chlamydial proteins that reach the host cytosol in substantial quantities typically activate a CD8⁺ T cell response very late in the intracellular developmental cycle and cannot prevent the reticulate body to elementary body reversion and therefore cannot contain infection. Conversely, antigen-specific CD8⁺ T cells that recognize the chlamydial protein CrpA, which is an inclusion membrane protein and enters the MHC I pathway, have been demonstrated to respond to genital *C. trachomatis* infection (Fling et al. 2001; Starnbach et

al. 2003). A T cell receptor transgenic mouse in which all CD8⁺ T cells recognize CrpA was shown to display partial protective immunity against an intravenous *C. trachomatis* challenge, although the relevance of this model of infection is arguable (Starnbach et al. 2003). Additionally, one recent study in an ocular infection monkey model demonstrated that monkeys prevaccinated with plasmid-deficient *Chlamydiae* and determined to be solidly protected against infectious challenge with virulent *Chlamydia* were unable to control a subsequent challenge infection when CD8⁺ T cells were depleted using neutralizing antibodies (Olivares-Zavaleta et al. 2014). This may suggest that multiple vaccinations may generate high enough numbers of particular antigen-specific clones of CD8⁺ T cells and/or populate them at sites of infection as tissue-resident memory cells (Johnson and Brunham 2016). Alternatively, chlamydial infection has been shown to upregulate molecules such as PD-L1 at sites of infection which dampen the activation of antigen-specific CD8⁺ T cell response. Deletion or inhibition of PD-L1 has been shown to allow activation and enhance the clearance of *C. trachomatis* infection by CD8⁺ T cells (Fankhauser and Starnbach 2014). It has been proposed that immunization with a plasmid-deficient *Chlamydia* perhaps lacks the ability to induce such dampening response and leads to activation of protective CD8⁺ T cell responses (Porcella et al. 2015). Although a subject of high interest for several years, the role of CD8⁺ T cells in protective immunity against chlamydial infections remains to be clarified.

On the other hand, multiple lines of evidence from various chlamydial infection models implicate CD8⁺ T cells in chlamydial pathogenesis. A study of 302 sex workers in Kenya indicated that HLA-A31 was significantly associated with *C. trachomatis* pelvic inflammatory disease (Kimani et al. 1996). In a Gambian study of trachoma, the MHC I allele HLA-A28 was found to be associated with scarring trachoma, whereas the MHC class II genes, HLA-DRB1 or HLA-DQB1, were not (Conway et al. 1996). In other studies involving Tanzanian and Gambian populations, other MHC class I genes including HLA-B*07 or HLA-B*08 were associated with TS (Abbas et al. 2009; Roberts et al. 2014). The Tanzanian study also indicated an association of HLA-DRB1*11 (an MHC class II gene) in women with less TS (Abbas et al. 2009). Following repeated *C. trachomatis* infection, fallopian tubes of female macaques also displayed CD8⁺ T cells as the majority (nearly 62 %) of T cells infiltrating sites of infection and these cells produced Th1-type cytokines (Van Voorhis et al. 1996, 1997). Igietsme et al. demonstrated a significant protection against infertility in CD8 knockout mice compared to WT animals, following four episodes of vaginal *C. muridarum* infection (Igietsme et al. 2009). We have demonstrated that following primary *C. muridarum* infection, a deficiency of CD8⁺ T cells (in three different models of deficiency) resulted in unaltered kinetics of vaginal chlamydial shedding, but significantly reduced oviduct pathology (Murthy et al. 2011b). Reconstitution of CD8 KO mice with wild-type CD8⁺ T cells at the time of infection restored pathology to wild-type levels. We also found that mice deficient in TNF- α or perforin displayed the same phenotype. Reconstitution of CD8 KO mice with wild-type or perforin KO, not TNF- α KO, CD8⁺ T cells restored pathology to wild-type levels (Murthy et al. 2011b). A recent study using the genital *C. trachomatis* serovar D infection induces infertility in wild type, not TNF- α KO female mice (Igietsme et al. 2015b). In that study, reconstitution of TNF- α KO mice with wild-type T cells restored infertility to wild-type levels following genital *C.*

trachomatis infection. These results suggest that CD8⁺ T cells cause genital chlamydial pathogenesis and implicate TNF- α production as a pathogenic mechanism. We also demonstrated recently that high-fat diet-fed CD8 KO mice displayed significant reduction in atherosclerosis development following intranasal *C. pneumoniae* infection (Zafiratos et al. 2015). Additionally, repletion of CD8 KO mice with wild-type CD8⁺ T cells restored the development of atherosclerotic pathology to wild-type levels. These results from a different infection model using a human pathogen *C. pneumoniae* further support the pathogenic role of CD8⁺ T cells; however, whether or not CD8⁺ T cells induce diseases in different organ systems using similar mechanisms remains to be determined.

We have further characterized that both TNF receptor 1 and TNF receptor 2 are required for *Chlamydia*-induced oviduct pathology, not chlamydial clearance. Moreover, TNF receptor 2, not TNF receptor 1, on CD8⁺ T cells was important for the activation of *Chlamydia*-specific CD8⁺ T cells and causation of oviduct pathology (Manam et al. 2015). TNFR 1 is expressed on all nucleated cells of the body, whereas TNF receptor 2 has a more limited distribution of hematopoietic cells. Therefore, our results collectively suggest that pathogenic TNF⁺, TNFR2⁺, CD8⁺ T cells, and TNFR1⁺ non-CD8 cells mediate chlamydial pathogenesis. While CD8⁺ T cells are an adaptive immune cell type, there has been evidence for roles of non-antigen-specific CD8⁺ T cells in other disease processes (Chen et al. 2005; Sobottka et al. 2009). When we characterized genital *C. muridarum* infection in OT-1 transgenic mice, wherein 100 % of CD8⁺ T cells recognize an irrelevant ovalbumin peptide and therefore do not recognize chlamydial antigens, we found that vaginal chlamydial shedding remained unaltered, whereas oviduct pathology was significantly reduced (Manam et al. 2013). Repletion of OT-1 mice with wild-type CD8⁺ T cells restored the ability of these mice to generate *Chlamydia*-specific CD8⁺ T cell response and to induce oviduct pathology at wild-type levels (Vlcek et al. 2016). In summary, we have demonstrated compelling evidence in the mouse model using *C. muridarum*/oviduct pathology and *C. pneumoniae*/ atherosclerosis for a pathogenic role of CD8⁺ T cells as well as the antigen-specific nature of this phenomenon.

In the recent ocular challenge study with virulent *C. trachomatis* in monkeys prevaccinated with plasmid-deficient *C. trachomatis*, depletion of CD8⁺ T cells abrogated the protective immunity and promoted high chlamydial conjunctival burden following challenge (Olivares-Zavaleta et al. 2014). At the outset, it may seem to argue against the role of CD8⁺ T cells in chronic chlamydial pathogenesis; however, as also discussed by the authors, scarring sequelae were not evaluated in this study and two of the three monkeys depleted of CD8⁺ T cells displayed minimal inflammatory changes despite the high ocular chlamydial load. Collectively, multiple lines of evidence strongly suggest a role for CD8⁺ T cells in pathogenesis.

7 Summary

Since the proposal of the cellular hypothesis of chlamydial pathogenesis, numerous studies have unearthed a wealth of information and insights into mechanisms of chlamydial disease sequelae. There are a few key insights that we have gained from these results:

1. **The mouse model of chlamydial infection is well-suited for mechanistic studies of chlamydial pathogenesis.** Much progress has been made using the genital *C. muridarum* and oviduct pathology or *C. pneumoniae* and atherosclerosis/asthma model in mice. For one, it allows the study of a true chronic disease process, rather than extrapolating results of acute inflammation during infection to manifestations of chronic disease. Additionally, the availability of various whole body or conditional gene knockout or knock-in mice including as well as transgenic mice allows studies of pathogenic mechanisms to an extent that is practically impossible in other animal models or humans. Other animal models including the guinea pig and monkeys are available, but are highly restrictive with respect to reagents, access, and cost. In the last decade, over a dozen immunological mediators of chlamydial pathogenesis have been identified and characterized using the mouse model, and to a large extent, these findings have been corroborated in human studies.
2. **Protection against infection may involve different mechanism(s) compared to protection against pathology.** At the outset, it would seem that if infection was prevented, pathology is automatically avoided. That is true; however, reinfections are common and thus even a natural chlamydial infection does not provide 100 % resistance to reinfection. Therefore, absolute resistance to infection may not be a realistic goal for an anti-*Chlamydia* vaccine. If we can only achieve partial protection against infection, there would still be room for a shortened infection to activate a sequence of immunopathogenesis. On the contrary, multiple lines of evidence have demonstrated that reduction in pathology can be achieved by altering certain immune components with minimal or no changes in kinetics of clearance of infection. Therefore, it could theoretically be possible to design a vaccine that directly targets the avoidance of a pathogenic immune response following infection, rather than enhanced clearance of the bacterium. We (Murthy et al. 2007) and others (O'Meara et al. 2014b) have described vaccine regimens that prevent pathology with only a moderate effect on reduction of infection.
3. **Multiple adaptive immune mechanisms play a role in chlamydial pathogenesis.** From the days of Th1/Th2 balance, this research field has come a long way and now identified the roles of various T cell populations including regulatory T cells, Th17 cells, and CD8⁺ T cells in chlamydial pathogenesis. A key question that remains to be answered conclusively is to whether adaptive immune responses contribute in an antigen-specific fashion to pathogenesis? The role of *Chlamydia*-specific CD8⁺ T cells in pathogenesis supports this line of thought.
4. **Mechanisms of chlamydial immunopathogenesis appear to work in series.** Multiple immunological knockout mice including mice deficient in caspase-1, neutrophils, metalloproteinases, TNF- α , TNF receptor 1, TNF receptor 2, and CD8⁺ T cells display comparable bacterial shedding to wild-type mice, with significantly reduced oviduct pathology. This suggests that multiple innate and adaptive components work in series, not parallel, toward induction of chlamydial

disease sequelae. Although a number of innate immune mediators have been shown to play roles, a strong interaction of innate and adaptive immune response components in chlamydial pathogenesis is emerging.

5. **Adaptive immunopathogenic mechanisms appear to modulate pro-inflammatory mediators.** Traditionally, it is thought that an infection induces an innate immune response followed by adaptive immune response. A number of innate immune factors including neutrophils and TNF- α have found to play a role in chlamydial pathogenesis. That said, studies have also shown these innate immune factors to be induced/modulated by components of the adaptive immune response. For example, Th17 cells induce neutrophil recruitment, and CD8⁺ T cells induce pathology via TNF- α production while mediating chlamydial pathogenesis. Therefore, components of innate immune system may be induced directly by infection and/or by the adaptive immune response and may contribute to pathogenesis in both capacities.

8 Outlook for Future

Based on the state of the art, we are now poised with important issues pertaining to mechanisms of chlamydial immunopathogenesis. One important question is “how do multiple components of the immune system interface with each other to culminate in disease pathology?” In this context, we propose that a “*Chlamydia* immunopathogenesis sequence/network” would be a more reasonable hypothesis than classical and somewhat more restrictive “immunological” or “cellular” hypothesis. Another important question: “Is a pathogenic response determined by particular chlamydial antigens or is it based on skewing of a polyclonal response toward a pathogenic phenotype?” The answer to this question will have a direct bearing on anti-chlamydial vaccine development.

The last decade has brought abundant and important insights into the significant contribution of adaptive immune responses to chlamydial pathogenesis. Given the inherent advantages of targeting an antigen-specific response for biomarker or therapy discovery, or for developing safe vaccines, future studies should focus on bringing clarity to this arena.

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