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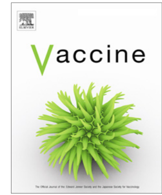

Review

Chlamydia trachomatis: Cell biology, immunology and vaccination

Sam M. Murray, Paul F. McKay *

Department of Infectious Diseases, Imperial College London, Norfolk Place, London W2 1PG, UK

A R T I C L E   I N F O

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A B S T R A C T

Chlamydia trachomatis is the causative agent of a highly prevalent sexually transmitted bacterial disease and is associated with a number of severe disease complications. Current therapy options are successful at treating disease, but patients are left without protective immunity and do not benefit the majority asymptomatic patients who do not seek treatment. As such, there is a clear need for a broad acting, protective vaccine that can prevent transmission and protect against symptomatic disease presentation. There are three key elements that underlie successful vaccine development: 1) *Chlamydia* biology and immune-evasion adaptations, 2) the correlates of protection that prevent disease in natural and experimental infection, 3) reflection upon the evidence provided by previous vaccine attempts. In this review, we give an overview of the unique intracellular biology of *C. trachomatis* and give insight into the dynamic combination of adaptations that allow *Chlamydia* to subvert host immunity and survive within the cell. We explore the current understanding of chlamydial immunity in animal models and in humans and characterise the key immune correlates of protection against infection. We discuss in detail the specific immune interactions involved in protection, with relevance placed on the CD4+ T lymphocyte and B lymphocyte responses that are key to pathogen clearance. Finally, we provide a timeline of *C. trachomatis* vaccine research to date and evaluate the successes and failures in development so far. With insight from these three key elements of research, we suggest potential solutions for chlamydial vaccine development and promising avenues for further exploration.

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Abbreviations: CPAF, Chlamydial protease-like activity factor; Hsp60, Heat shock protein 60; IDO, Indoleamine 2,3-dioxygenase; iNOS, Inducible nitric oxide synthase; MOMP, Major outer membrane protein; Omp, Outer membrane protein; Pmp, Polymorphic membrane protein; RB, Reticular body; saRNA, Self-amplifying RNA; T3SS, Type III Secretion System; Tarp, Translocated actin-recruiting phosphoprotein; VEEV, Venezuelan equine encephalitis virus.

* Corresponding author.
E-mail addresses: smm19@imperial.ac.uk (S.M. Murray), p.mckay@imperial.ac.uk (P.F. McKay).

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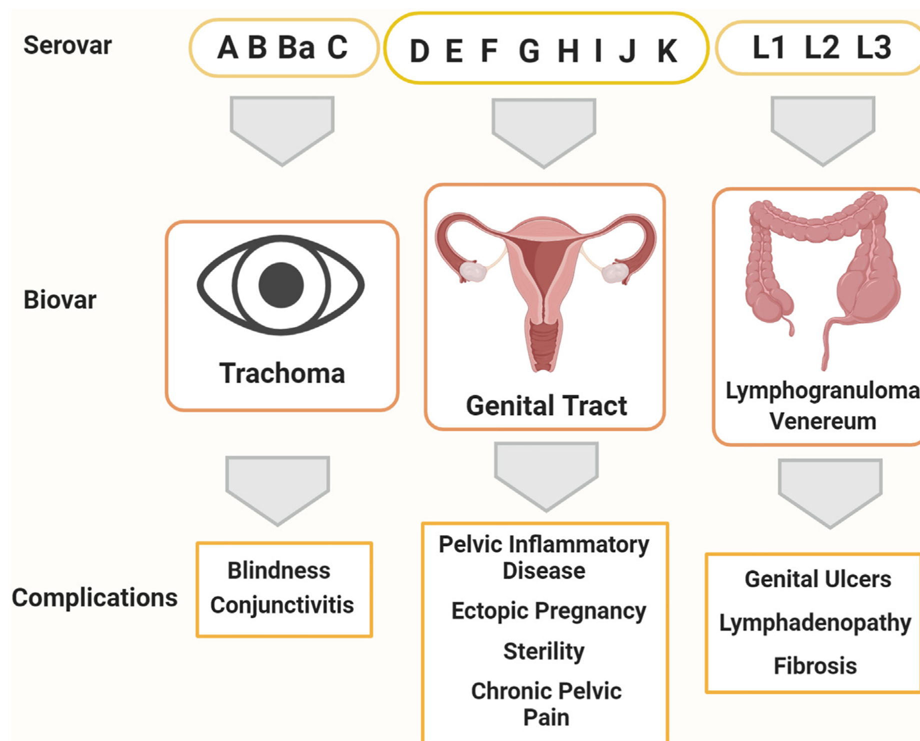

Fig. 1. *Chlamydia trachomatis* and disease. *Chlamydia trachomatis* serovars, their corresponding biovar and associated complications. Created with BioRender.com.

1. Introduction

Chlamydia trachomatis (*Ct*) is the causative agent of a highly prevalent sexually transmitted bacterial disease, with an estimated 127 million acquired infections worldwide in 2016 [1] and as such causes significant economic and social burden. It is an obligate intracellular bacterium comprising of three biovars which in humans exhibit an array of pathological conditions. Biovars of *Ct* are further divided into serovars which are defined by the variable domains of the surface protein major outer membrane protein (MOMP), an important *Ct* antigen (Fig. 1) [2]. Serovars A-C, of the trachoma biovar, is a major cause of blindness and visual impairment, responsible for 0.4- and 1.6-million cases respectively in 2015 [3]. Serovars D-K cause disease in the genital tract and in men are the major cause of non-gonococcal urethritis and epididymitis [4]. In women, genital tract *Ct* causes severe complications including pelvic inflammatory disease which is a leading cause of infertility, ectopic pregnancy and chronic pelvic pain. Genital tract *Ct* is also associated with a significant increase in the rate of HIV contraction and transmission in women [5]. The lymphogranuloma venereum biovar, serovars L1-3, causes invasive urogenital and anorectal infection and has become particularly associated with HIV-infected men who have sex with men – reviewed in detail here [6]. The potential severity of *Ct* pathology highlights its importance as a major public health problem worldwide and as such there is a great need for successful disease control and prophylaxis.

While antibiotic therapy (most commonly azithromycin) successfully clears *Ct* infection from individuals with sexually transmitted disease, its use is limited to those that seek clinical treatment or are screened for infection [7]. In trachoma, widespread antibiotic use – as part of the World Health Organisation (WHO) GET2020 campaign to eliminate trachoma globally by 2020 has been largely successful in decreasing prevalence, however there are remain many areas of endemic trachoma worldwide [8]. The majority of *Ct* infection is asymptomatic (~80%) [9] and therefore in areas that are not the target of mass antibiotic campaigns treatment is often not sought, and transmission and prevalence remain high. In high-income countries, the current solution to this issue is widespread screening in targeted populations. Mass screening for urogenital infection however is impractical and expensive (estimated cost of $2.4bn between 2016 and 2021 [10]) and does not prevent disease initiation. In an attempt to control trachoma, the largest infectious disease survey undertaken, the Global Trachoma Mapping Project, was successful at mapping regions of endemic trachoma. This project identified regions to target for introduction of the 'SAFE' intervention, the WHO package to tackle trachoma, precisely: 'surgery for trachoma trachomatous trichiasis', 'antibiotics to clear ocular infection', 'facial cleanliness' and 'environmental improvement' [11]. While SAFE has been successful at decreasing disease in endemic regions, from 0.9 M cases of trachoma induced blindness in 1990 to 0.4 M in 2015 [3], the prevalence of *Ct* as an important urogenital and ocular infection remains. Further complications in the use of antibiotic therapy arise from evidence suggesting that antibiotic therapy for *Ct* may in fact blunt the development of a lasting protective immune response to *Ct,* reducing the capacity of non-human primates (NHP) to produce anti-*Ct* antibody [12] and potentially leaving treated patients more vulnerable to reinfection [13,14]. This is an area of some contention, with evidence that the antibody response to C. *pecorum* following antibiotic treatment in vaccinated koalas was enhanced after treatment [15]. The varied nature of *Ct* infection, from asymptomatic to severe pathological complications, is likely a result of the inherent nuances of an individual's immune response and the extent and background of infection. Nevertheless, it is clear that those that independently mount a successful immune response and 'self-cure' from infection exhibit reduced reinfection and lasting

immune protection [13], and therefore this is a key criteria for success in alternative *Ct* control measures.

Prophylactic vaccination for *Ct* has been promoted as an intervention that addresses the limitations of current therapeutics [16]. Long-term protection against *Ct* may prevent severe sequalae, decrease transmission and inhibit reinfection. Vaccination for *Ct* has long been pursued, with initial candidates explored in the early 1910s by Charles Nicolle at Pasteur Institute in Tunis [17] and later several human vaccine trials in the 1960s looked to induce protection against trachoma [18]. However, several features of chlamydial biology have hampered the discovery of a successful vaccine, in particular, the challenging adaptations *Ct* has developed to evade the immune system which will be further discussed in this review. To successfully approach future vaccine design, it is important to first understand the complexities of *Ct* cell biology and immunity. *Ct* has a biphasic developmental cycle, with a metabolically active phase of division within a protective intracellular vacuole and an infective extracellular phase. Adaptations of the intracellular vacuole, termed the chlamydial 'inclusion', subvert natural intracellular innate immune receptors and confer protection against cellular defence mechanisms [19]. Further alterations between the intra- and extracellular phases of development prevent successful pathogen clearance and enhance chlamydial survival. As a result of these adaptations, immunity to chlamydial infection is complex and has been challenging to characterise. Current understanding highlights the importance of IFNγ production from CD4+ T lymphocytes and neutralising antibody production from B cells [20], however, the precise correlates of protection against infection are still unclear – and the importance of other immune effectors such as CD8+ T cells has been questioned [21].

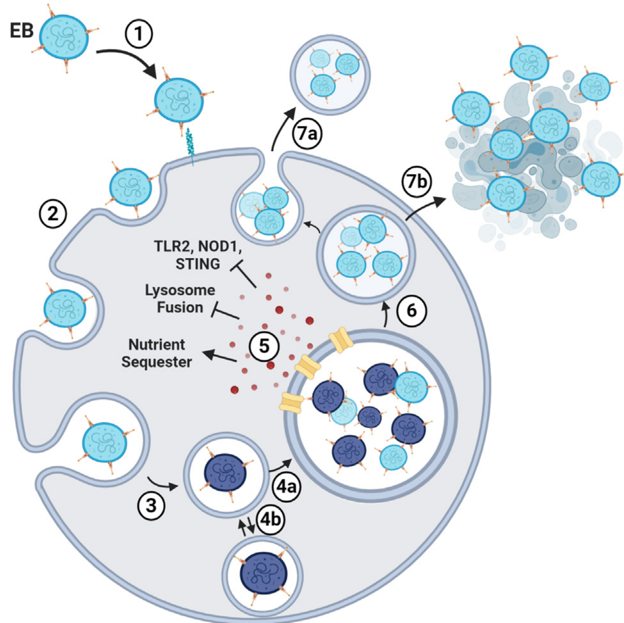

Fig. 2. The *Chlamydia Trachomatis* developmental cycle. 1) The EB (light blue) binds to the surface of a host cell using a two-step mechanism. 2) The Type 3 Secretion system permeates the cell and facilitates the release of proteins that induce actin remodelling for *Chlamydial* uptake to the cell. 3) Within the inclusion, EBs differentiate into RBs (Dark blue), which are metabolically and reproductively active. 4) Depending on cellular conditions, RBs either 4a) replicate within the inclusion or 4b) enter a reversible state of persistence. 5) RBs release Inclusion proteins which act to inhibit cellular defence mechanisms; through inducing nutrient sequestration through the endosomal pathway, inhibiting lysosomal fusion with the inclusion, and inhibiting innate immune sensors such as TLR2, NOD1 and STING pathways. 6) After sufficient division, RBs terminally differentiate back into EBs and exit the cell through exocytosis-like (7a) or induced apoptosis pathways (7b). EB = Elementary Body, RB = Reticular body, TLR = Toll-like receptor, NOD = Nucleotide-binding oligomerisation domain, STING = Stimulator of interferon genes. Image created in Biorender. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. The *C. trachomatis* developmental cycle and its effect on immunity.

2.1. Developmental cycle

The developmental cycle of *Ct* occupies a unique niche of intracellular bacterial development [19]. It is biphasic, consisting of two morphologically distinct forms, the extra-cellular elementary body (EB) and the intra-cellular reticular body (RB). In short, the life cycle follows the transmission of EBs from person to person through sexual contact, through neonatal transmission and through contact secretions from the eye [22]. Upon contact with host epithelial cells, EBs gain entry to the cell using a collection of machinery including the important Type III Secretion system (T3SS) complex. Following entry, they differentiate into the intracellular RB within a parisitophorous vacuole termed the inclusion. Within the inclusion RBs hi-jack cellular machinery to promote bacterial replication, or if cellular conditions are not optimal, they enter a state of persistence within the inclusion. After replicating RBs differentiate back into EBs and exit the cell (Fig. 2).

EBs are metabolically inert and adapted for survival in extracellular environments and for host cell adhesion and entry. Unusually, EBs maintain structural integrity through a web of disulphide linked outer membrane proteins (Omp), relying on this rather than peptidoglycan as with most other gram-negative bacteria [23]. Arranged within the Omp matrix of EBs are needle-like projections, identified as the chlamydial T3SS, which facilitates inclusion ligand release and likely plays a role in cell adhesion and entry [24]. Upon host cell contact, binding is a two-step process. The first is a reversible, low-affinity electrostatic interaction of EB membrane proteins with heparan sulphate containing glycosaminoglycans on the host cell membrane [25]. This is followed by irreversible high affinity binding of host cell receptors – such as MOMP binding to mannose receptor and lipopolysaccharide (LPS) binding to cystic fibrosis transmembrane conductance regulator [26]. On binding, the T3SS penetrates the host cell membrane and releases translocated actin-recruiting phosphoprotein (Tarp) which recruits and remodels cellular actin and initiates rapid internalisation of the EB into the inclusion [27]. The inclusion is an early-endosomal pathway type vacuole that forms an intracellular niche for pathogen replication; it is non-lysosomal and determines interactions with the host cell that benefit *Ct* survival [28].

Within the inclusion, EBs undergo differentiation into the larger, transcriptomically and metabolically active RB. This process is marked by a shift in the transcriptional profile of *Ct*, resulting in the expression of bacterial metabolic proteins [29]. The RB, within the inclusion, is adapted in several ways to promote survival and replication. 1) selective fusion of vesicles - inhibition of lysosome fusion and promotion of nutrient rich exocytic vesicles [30]; 2) acquisition of essential nutrients, including eukaryotic membrane lipids such as cholesterol and phosphatidylcholine, through complex sequestration mechanisms; 3) modulation of host cell innate immune and cellular survival pathways [19]. Depending on host cell conditions the RB undergoes cellular division or, when deficient of metabolites, enters a state of persistence within the inclusion.

In nutrient limited conditions RBs enter the 'persistence' phase where replication and many transcriptional and metabolic processes are halted [31]. A well-studied example of the conditions inducing the persistence phase is in the presence of the cytokine interferon-gamma (IFNγ), which, while cytotoxic in high concen-

tration, has been shown to induce persistence when exposed in low-moderate concentration to chlamydial infected cell cultures *in vitro* [32]. IFNγ is thought to act indirectly on chlamydial cells, by limiting the tryptophan required for chlamydial reproduction by activating indoleamine 2,3-dioxygenase (IDO) to deplete intracellular tryptophan pools [33]. Tryptophan is necessary for chlamydial replication within the inclusion. Interestingly, the presence of tryptophan synthase genes may play an important role in determining the tropism of *Ct* serovars; where serovars effecting the genital tract have functioning tryptophan synthase, they may be able to evade the IFNγ response to infection and persist to long-term infection (reviewed [34]). Other mechanisms have been identified for inducing persistence including therapy with penicillin, and starvation from lipids and micronutrients such as Iron [31,35]. While there is strong evidence for the capacity of the RB to enter a state of persistence *in vitro*, direct knowledge of *in vivo Ct* persistence is less clear. Though, evidence of enlarged RBs, indicative of interrupted RB to EB differentiation in persistence, in human cervical *Ct* infection [36] and recent work demonstrating the presence of a maintained single strain of genital tract *Ct* over several years [37] in humans provides promising insight into the *in vivo* presence of persistence.

Upon normalisation of physiological conditions RB growth is no longer inhibited and they become metabolically active within the inclusion. In this phase they express and secrete large amounts of inclusion protein and undergo several rounds of division by binary fission [38]. Following rapid division, RBs undergo terminal differentiation back into the infectious EB in an asynchronous and to date largely unknown manner. Recent evidence suggests that differentiation is dependent on RB size, as shown by serial block-face scanning electron microscopy [39], and is prompted by the expression of late-cycle gene expression [40]. Nevertheless, once differentiated back into an EB, *Ct* moves to exit the host cell through either induced host cell lysis, or inclusion extrusion. Host cell lysis involves protease dependent lysis of the inclusion, followed by calcium-cathepsin dependent plasma membrane lysis and subsequent host-cell rupturing [41]. Inclusion extrusion is mediated through an exocytosis-like mechanism, with budding and pinching from the cell membrane – leaving the host-cell intact and a membranous compartment for chlamydial survival [41]. Lytic release of *Ct* likely increases the release of apoptotic damage-associated molecular patterns (DAMPS), increasing the immunogenicity of the site of release [42]. Extrusion release, on the other hand, may contribute to chlamydial persistence through retention of *Ct* within the cell after release [43], improved *Ct* survival extracellularly and within macrophage [44], and alter the dendritic cell (DC) cytokine response and induce DC apoptosis [45].

2.2. How the developmental cycle impacts immunity

The developmental cycle of *Ct* impacts the immune response to infection in multiple ways. 1) Morphological adaptation: The EBs have reduced levels of LPS on their surface, which is structurally distinct and less immunogenic than other bacteria [46]. This is in part due to the enhanced structural integrity afforded to them by the di-sulphide bond cross-linked MOMP [47]. 2) The inclusion formation process is adapted to subvert the immune system through the release of inclusion proteins that interrupt usual cellular recognition receptors [48]. Proteins released can alter the intracellular pathogen detection mechanisms (TLR2, NOD1, cGAS/STING) of the cell, blocking the production of protective inflammatory cytokines and subsequent systemic immune response [48]. The chlamydial protease-like activity factor (CPAF) protein has been shown to directly suppress the release of pro-inflammatory cytokines, such as CXCL10 [49], and innate immune effectors such as the NFκB subunit, p65 [50]. 3) *Ct* inhibits cell apoptosis and alters survival signals within the cell to maintain an optimal reproductive niche through factors such as CPAF, HIF1α and Pgp3 [51]. It induces the degradation of the tumour suppressor p53, which acts as a DNA damage sensor and plays a central role in inducing cellular apoptosis [52]. Additionally, *Ct* has been shown to inhibit apoptosis by blocking caspase mediated cellular apoptosis through release of the inclusion protein CPAF [53]. Together, these adaptations subvert immune recognition of intracellular *Ct*, allowing it to grow within the inclusion in a relatively secure environment.

3. The immune response to *Chlamydia trachomatis*

3.1. Intracellular innate immunity

Multiple pattern recognition receptors (PRRs) have been associated with the detection of *Ct* PAMPs [54]. The first, and best understood, is toll-like receptor 2 (TLR2), whose activation has been associated with several chlamydial proteins including MOMP and HSP60 [55,56]. The exact *C. trachomatis* derived ligand of TLR2 is unclear, but investigation of plasmid-cured *Ct* suggests that the ligand is either plasmid encoded or edited in a plasmid dependent manner [57]. Macrophage from TLR2 knockout (KO) mice produced significantly less IL-6 and TNF-α in response to infection *in vitro*, and *in vivo* TLR-2 KO mice produce less TNF-α and exhibit muted immunopathology later in disease [58]. In human embryonic kidney 293 (HEK293) cells, TLR2 and its adapter protein MyD88 were required for IL-8 production and found to localise at the inclusion [59]. Interestingly, IL-8 production was abrogated in cells inoculated with UV-inactivated *Ct,* suggesting TLR2 activation is dependent on productive infection and differentiation to RB [59].

Ct is also recognised by stimulator of interferon genes (STING) which cause the production of type 1 IFNs. STING is activated by dimerization upon recognition of cytosolic dsDNA or by recognition of bacterial cyclic di-AMP or di-GMP [60]. Cyclic di-AMP is a nucleic acid metabolite produced by *Ct,* which has been shown to activate STING and cause the production of IFN-β in infected cells [61]. IFNγ has been shown to play a central role in clearance and protection against *Ct* [54], however, type-I IFNs seem to play a negative role in chlamydial infection and indeed may exacerbate disease. Initial evidence in IFNα receptor deficient (IFNAR$^{-/-}$) mice, lacking the ability to detect type-I IFNs, *Chlamydia muridarum* genital infection caused less chronic oviduct pathology, decreased chlamydial shedding and reduced duration of infection in a manner corresponding with increased CD4+ T cell activation [62]. This is supported by association studies in women with cervical and endometrial *C. trachomatis* infection where evaluation of cervical secretion cytokines suggested that type-I IFNs are associated with increased susceptibility to infection and increased risk of endometrial ascension – and therefore PID and infertility [63]. Earlier models investigating IFN-β in combination with TNF in *Ct* infection of human cells however demonstrated a role of IFN-β in *Ct* growth inhibition through increased tryptophan degradation [64]. Due to the apparent increase in CD4+ T cell activation in IFNAR$^{-/-}$ mice, it may therefore be suggested that type-I IFNs play a variable role in *Ct* control intracellularly and when acting on immune effector cells. Unfortunately, it is likely that complete knockout of IFNAR across a mouse is likely to induce several changes on expected immune responses due to the diverse effect type-1 IFNs have in many cell types [65]. To clarify this role, effort must be placed on defining the regulation and function of type-I IFNs in more accurate models of *Ct* infection beyond association studies or in C. *muridarum (Cm).* Models utilising conditional and cell specific knockout will be valuable in concluding the *in vivo* effect of type-1 IFNs in host cells. An elegant *in vitro* model of bi-allelic IRF5 knockout in macrophage derived from human induced pluripotent stem cells

led to a 45% increase in *Ct* bacterial load, highlighting both the use of novel humanised infection models and the role of IFNs in *Ct* clearance. As is seen in infection with Listeria monocytogenes [66] it is likely that the timing and expression level of IFNs will be vital in the control of infection, but clarity is needed on the precise mechanisms of type-I IFNs in *Ct* clearance before its role as a protective/suppressive immune regulator is clear.

3.2. Cellular innate immunity

Antigen presenting cells, such as DCs, play a critical role in priming the adaptive immune response to establish immune memory and thus vaccine success and are necessary for priming of both CD4+ and CD8+ T cell responses to *Ct* [67]. TLR2, STING and NLRs are activated upon *Ct* uptake within the DC, leading to the production of pro-inflammatory cytokines such as IL-6, TNF-α, CCR7, CXCL10, IL-1α and IL-12 which are key in inducing the maturation and optimal presentation of antigen in DCs [68]. The preferential production of IL-12 from DC upon antigen uptake drives the activation and differentiation of naïve CD4+ T lymphocytes into primarily the Th1 subset, which provides primary protection against *Ct* infection [69]. In models of infection, the importance of DCs is evident. DCs pulsed with UV inactivated EBs provoke a lower protective immune response than those with live EBs [70], clarifying the importance of effective antigen processing in the presentation of *Ct* antigen for protective immunity. DCs adoptively transferred with recombinant MOMP and CPAF were able to elicit protective immunity in murine challenge studies [71]. Exploration of extrusion-released versus free *Ct* in DCs highlighted the importance of the mechanism of *Ct* host-cell exit on DC inflammatory cytokine activation, indicating an increase in IFN-β, IL-12p40, IL-10 and PD-L1 transcription in extrusion-containing DCs [45]. In a murine model, DCs were seen to harbour long-time surviving infectious *Cm* but were still able to present antigen to T cells [72]. The role of DCs as a link between innate and adaptive immunity in the control of chlamydial infection is therefore likely a highly nuanced one, relying on the context of chlamydial uptake and subsequent survival. It is clear that DCs have the capacity to induce protection when adoptively transferred in murine challenge studies, however it is interesting to see the upregulation of conventionally immune suppressing (IL-10, PD-L1) regulators when DCs uptake extrusion-released *Ct.* Similarly, increase in production of IFN-β by DCs, a suggested immuno-suppressive actor in *Ct* development, highlights an interesting role for this cytokine in *Ct* clearance. Exploration of the diverse serovars of *Ct* will likely further cloud this intricate role, as growing evidence demonstrates their varied responses to differing cytokines [73].

In addition to DCs, other innate immune effector cells are important in clearing *Ct* infection, including macrophage and natural killer cells (reviewed in detail here [20]).

3.3. Adaptive immunity: T cells

The importance of T lymphocytes in clearance of *Ct* infection was first demonstrated in athymic mice which were shown to establish chronic *Cm* infection, opposed to the self-limiting infection of wild-type mice [74]. In humans, CD4+ and CD8+ T lymphocytes are clearly recruited to the site of infection during disease and are upregulated during *Ct* infection [75]. Initial evidence from murine models lacking MHC II indicated that CD4+ T cells are particularly important in clearing disease [76] and they, rather than CD8+ T cells, are necessary for resolution of disease and reduction of bacterial burden and shedding [77]. However, subsequent findings highlight an important role for both CD4+ and CD8+ T cells in both protection and in driving pathology [78].

3.3.1. CD8+ T cells in Ct infection

In studies of MHC I KO mice lacking a CD8+ T cell response, mice were still able to resolve *Ct* infection, despite exhibiting increased bacterial burden and mortality [79]. While these data from early murine models of *Ct* infection highlighted the importance of CD4 + T cells rather than CD8+, it is important to note that *Ct* is not a natural pathogen of mice and therefore conclusions made from these studies are limited. Indeed, subsequent study of *Ct* infection in NHP has since found evidence of the importance of CD8+ T cells in protection. Olivares-Zavaleta *et al.*, in investigating the live attenuated vaccine, A2497P-, found that not only was the proliferative response of CD8+ T cells to secreted *Ct* virulence factors (CPAF, Pgp3) significantly stronger than that of CD4+ T cells, but depletion of CD8+ T cells from macaques abrogated protection induced by vaccination [21]. A2497P- differs from WT *Ct* as it is plasmid deficient – therefore lacking virulence factors responsible for pathogenesis [80]. A potential conclusion that can be made from this data therefore is that *Ct* avoids CD8+ T cell clearance in natural infection through plasmid-derived effectors. Nevertheless, this evidence for the role of CD8+ T cells highlights a need for further investigation of T cell responses particularly in NHP and humans.

3.3.2. CD4+ T cells in Ct infection

The role of CD4+ T cells in infection is better characterised than that of CD8+. Evidence from DC studies, cytokine profiling and T cell activation studies highlight the particular importance of Th1-type CD4+ lymphocytes in *Ct* clearance [81]. However, distinguishing the specific effector functions of Th1 in response to *Ct* infection has proven complex and is multifaceted (Fig. 3). IFNγ has consistently been found to be important in controlling chlamydial replication and host susceptibility through induction mechanisms such as IDO and inducible nitric oxide synthase (iNOS). Induction of IDO by IFNγ causes the degradation cellular tryptophan pools, which are necessary for chlamydial growth and can inhibit growth, leading to *Ct* death or persistence [82]. In addition to IDO, IFNγ induces the upregulation of inducible nitric oxide synthase (iNOS) which synthesises nitric oxide (NO). NO is a well-defined anti-bacterial factor which damages bacterial DNA and is cytotoxic, [83] however its role in chlamydial clearance is unclear. Ramsey *et al.* describe exacerbated pathological outcome of genital tract *Ct* infection in mice deficient of iNOS, and that *Ct* growth in murine lung fibroblasts was decreased in iNOS KO/inhibited cells [84]. However, further exploration of IFNγ in human and mice cells finds differing bactericidal responses in each [85] – potentially limiting our ability to extrapolate data found in different species models. It is likely therefore that further mechanistic evidence of its action will need to come from more humanised models. Nevertheless, in both mouse genital tract infection with *Cm* [86] and *in vitro* human cellular infection with C*t* [87] the role of iNOS and NO is evident.

3.3.3. T cells in human Ct infection

In human infection, accumulation of HLA-DR + CD4+ and CD8+ T cells primarily of a memory phenotype is seen in the endocervix at the time of chlamydial infection, suggesting increased T cell accumulation and activity [88]. This is supported by flow cytometric analysis of cervical secretions in infertile women with ongoing *C. trachomatis* that also indicate increased T cell presence [75]. The expression of IFNγ is also increased in endocervical secretions of women infected with *Ct* [89] as well as the cytokine IL-12p70 which is associated with Th1 differentiation, and CX3CL1, a T cell chemoattractant [90]. Patients with *C. trachomatis* infection were found to have higher levels of T cell recruiting cytokines than four other common sexually transmitted infections, a factor associated with higher risk of HIV coinfection [91]. In the first immunoepidemiological prospective cohort study of *Ct* infection in commer-

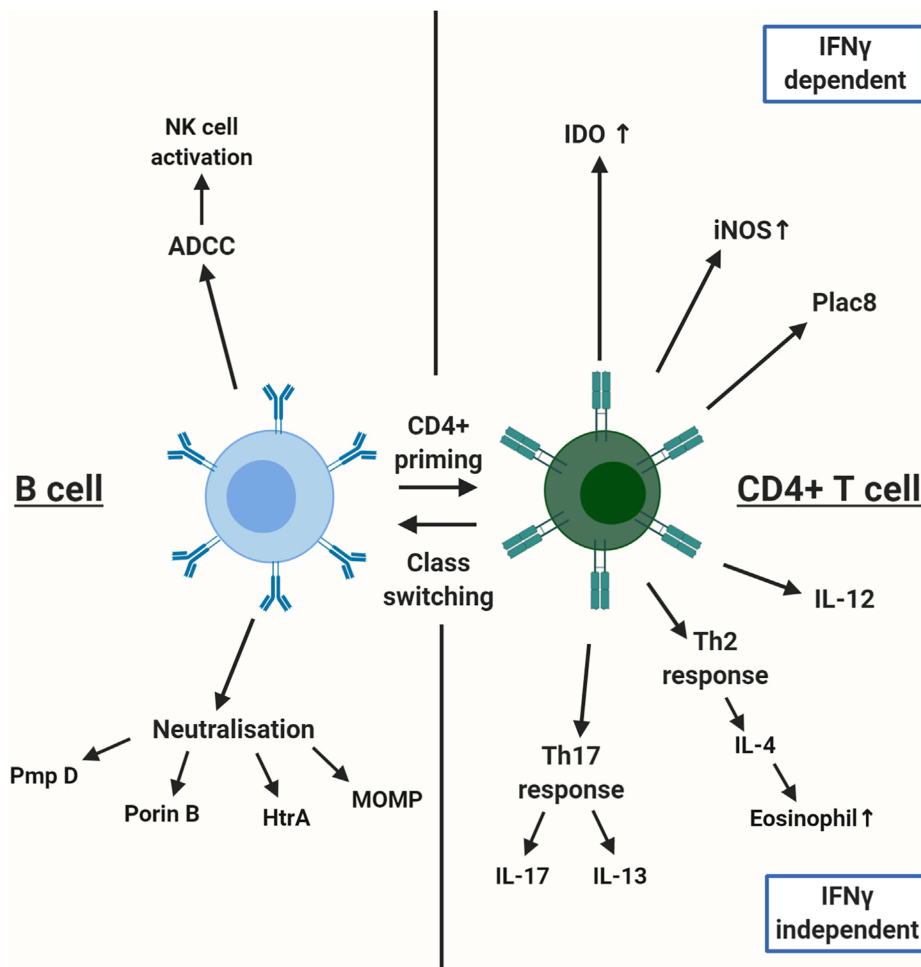

Fig. 3. Adaptive immunity to *C. trachomatis.* B cells have 3 primary roles in clearance of *C. trachomatis*: ADCC, which causes foreign antigen clearance by antibody tagging for NK cell clearance, antibody binding and neutralisation against listed antigens and CD4+ T cell priming through antigen presentation. CD4+ T cells are also key in clearing infection doing so through B cell class switching and IFNγ dependent and independent mechanisms. ADCC = Antibody-dependent cell mediated cytotoxicity, MOMP = Major outer membrane protein, Pmp = Polymorphic membrane protein, NK = Natural killer, IDO = Indoleamine 2,3-dioxygenase, iNOS = inducible nitric oxide synthase. Created with BioRender.com.

cial sex workers, it was demonstrated that *ex vivo* PBMC production of IFNγ in response to HSP60 (but not whole EB) was associated with protection against infection [92]. IL-10 production, conversely, increased risk of infection. Similarly, a CD4+ IFNγ response was associated with decreased risk of reinfection after azithromycin treatment for *Ct* infection, promoting the importance of CD4+ Th1 type cytokine production in protection against infection [93]. Taken together, data from animal models and human study suggest an important role for IFNγ producing CD4+ T cells in protection against infection. Despite this, the complexity of the cytokine response to *Ct*, compounded by the variable nature of the immune response to *Ct* variants and their diverse resistance to IFNγ control, means that still IFNγ is an inconsistent biomarker of protection in infection. Perhaps future vaccine studies, where detailed immunophenotyping of infection can be done in tandem with evidence of protection against *Ct* infection will provide the necessary information to confirm the role of IFNγ and CD4+ T cells, as well as other factors, as correlates of protection against *Ct*.

3.4. Adaptive immunity: B cells

The role of humoral immunity in chlamydial protective immunity is less apparent than that of the CD4+ T lymphocyte. A series of seminal studies by Morrison and Morrison unveiled the importance of B lymphocytes in clearance of secondary infection. Initially, they explored the synergistic interplay of B cells and CD4+ T lymphocytes in protection against secondary infection of *Ct* [77]. In B cell KO mice, depletion of CD4+ T cells by monoclonal antibody resulted in significantly delayed resolution of disease. A follow up study found that mice with a properly formed B cell response were able to resolve secondary infection even in the absence of CD4+ or CD8+ T cells [94]. Since, it has been made clear that several *Ct* proteins promote antibody production and many immunodominant peptides have been identified using ELISA-based antigenic profiling and proteomic microarrays [95,96]. Interestingly, Follmann *et al.* demonstrated through analysis of 116 proteins in 40 patients with confirmed genital *Ct* that B cell antigens are compartmentally biased, being primarily found on the surface of EBs rather than intracellularly – this is unlike T cell antigens, which are not biased by location [97]. Through exploration of cellular and humoral responses to *Ct* proteins, they found 5 T cell, 5B cell and two T&B cell (CT443 and CT110) immunogens. Using CELLO to predict the subcellular location of the antigens, they found that significantly more B cell antigens were predicted to be found on the *Ct* outer membrane than intracellular compartments. The evidence suggested here may be useful in shaping future vaccine design, as it provides initial evidence of the potential importance of subcellular location in antigen recognition.

Verification of the location of these proteins will be valuable in confirming these predictions, and further evidence of the extracellular accessibility of the epitopes would validate these findings and provide insight into the effect of subcellular location on the immune response.

With evidence that B cells play an important role in disease, what specific mechanisms do they use to fulfil this role (Fig. 3)? The involvement of antibody-mediated neutralisation in ocular *Ct* infection was explored as early as 1974, where anti-*Ct* immunoglobulin in the eye secretions of trachoma patients were found to reduce infectivity on co-transfer to NHPs, but were not protective in either [98]. Since, exploration of neutralising antibodies through *in vitro* infectivity assays has been a useful tool in the discovery of antibodies that are sufficient to block the function of a pathogen. One study investigated the effect of the chlamydial surface serine protease, HtrA, when expressed in outer membrane vesicles (OMVs) of E. *coli* and used to immunise mice [99]. HtrA bearing OMVs induced the production of neutralising antibodies and blocked infectivity, indicating the ability of neutralising antibodies to limit infection. It also demonstrates the capacity of an uncommonly used vaccine platform to induce antibodies and protection against disease. In another vaccine study, the protective capacity of the recombinant multivalent MOMP vaccine candidate Hirep1 (heterologous immune-repeat 1) was explored. Hirep1 induced neutralising antibodies were shown to induce protection against *Ct* when passively transferred to Rag1 KO mice, lacking T and B cells [100]. Further evidence of this capacity is seen in studies exploring the chlamydial proteins Porin B and polymorphic membrane protein D [101,102].

The importance of B cells in chlamydial clearance is also seen in their ability to help T cell differentiation and activity. In murine genital infection studies, *Cm* was used to determine the local response of CD4+ T cells in genitalia of mice with and without B cells [103]. Mice that lacked B cells demonstrated markedly decreased activation and clonal expansion of CD4+ T cells in response to *Cm*, and had coinciding systemic dissemination of infection. Interestingly however, bacterial shedding was not affected by the absence of B cells suggesting that CD4+ T cells without B cells are sufficient to stop shedding. A follow-up study recently sought to determine the means of B cell mediated protection in this model [104]. Here, Lin-Xi *et al.* described the importance of antibody in reducing systemic dissemination, showing that passive sera transfer rescues systemic infection but antigen presentation by B cells was unnecessary for protection. Earlier work by Johnson *et al.*, however, suggests that B cell antigen presentation to induce protective T memory cells was capable of completely preventing immunopathology when adoptively transferred to mice [105]. These results provide evidence of a potentially complex and location specific role for B cell antigen presentation in Chlamydial clearance of infection. It may be that the function of B cells in induced memory lymphocyte clusters, as in the work by Johnson *et al.*, provide a distinct antigen-presenting role that is accessory to the importance of antibody prevention of systemic disease.

4. Vaccine development for *C. trachomatis*

Following the early trials of Nicolle, initial human vaccine trials focused on ocular inoculation of whole inactivated bacteria, primarily for the treatment of trachoma rather than genital tract infection (Fig. 4) [17,106-138]. These early trials showed some success in induction of low-level immunogenicity, providing initial evidence that vaccination to *C. trachomatis* was possible [111]. The proof of concept provided by these trials was marred however by the significant discovery of inflammatory disease exacerbation by vaccination in trial participants and in NHP studies where increased inflammation after vaccination and reinfection was observed [139]. With the benefit of modern chlamydial understanding, reinterpretation of data gathered in these trials suggests that disease may not in fact be exacerbated, but that initial fears were due to inaccurate assessment of disease markers and a focus on identification of active disease, rather than induced protection against scarring [16,18,111]. Thus, while the data from these initial trials provided evidence that vaccination against *Ct* was possible, they also prompted a move away from whole cell vaccination toward subunit vaccination strategies.

Subunit vaccines exist in multiple forms, making use of many individual antigen expressed through several differing vectors. MOMP is the most highly expressed surface antigen of *C. trachomatis* and is the most prominently explored vaccine antigen to date. Early exploration of purified MOMP as an oral vaccine against *Ct* in NHPs showed a limited immunogenic capacity with little protection against ocular challenge [113]. Since however, its capacity to protect against genital infection in mice has been demonstrated [140], and it has become recognised as having several potentially immunodominant peptides capable of stimulating cellular and humoral immunity [95,141]. Indeed, in 2019 a multivalent vaccine

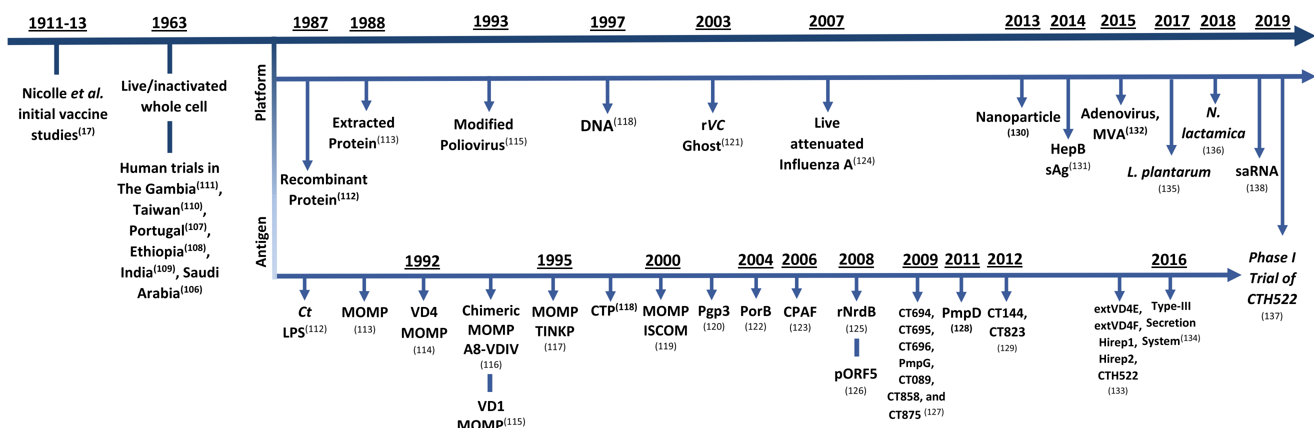

Fig. 4. Timeline of *C. trachomatis* vaccination. Timeline to depict the first use of vaccine platforms and antigens for *Chlamydia trachomatis.* Ct = *Chlamydia trachomatis*, LPS = Lipopolysaccharide, MOMP = Major outer membrane protein, VD = Variable domain, CTP = Cytosine triphosphate, ISCOM = Immune stimulating complex, rVC = Recombinant *Vibrio cholerae.* PorB = Porin B, CPAF = Chlamydial protease-like activity factor, Pmp = Polymorphic membrane protein, HepB sAg = Hepatitis B Surface Antigen, MVA = Modified vaccinia Ankara, saRNA = Self-amplifying RNA.

incorporating MOMP proteins, termed CTH522, became the first vaccine candidate trialled in humans since the 1970s [137]. The data gathered in this Phase I safety and efficacy trial demonstrated vaccine-induced immunogenicity: significantly increasing the titre of antigen-specific mucosal IgG and IgA, antibody neutralisation and increasing antigen-specific cellular IFNγ production. These data provide promising insight into the functionality of MOMP as a vaccine candidate and demonstrate its capacity to induce antigen-specific immune responses in humans. Further in-human trials will be necessary to not only advance immunogen characterisation, but also to provide evidence of protection against infection and importantly to develop tangible, quantifiable, evidence of immune correlates of protection in humans. In addition to MOMP, various other candidate immunogens have been explored for immunogenicity in subunit vaccine pre-clinical models [142].

Development of vaccine delivery platforms that tailor the immune response have been a key area of advance in chlamydial vaccine development. Combination of adjuvant technologies and specific molecular design allow for orchestrated immune responses that are potent but with reduced off-target effects. The most common method used to date for subunit vaccination is recombinant protein, including fusion proteins with multiple *Ct* epitopes have shown success in many studies [137,141]. Other vaccine modalities such as DNA plasmid, mRNA, viral, nanoparticle and extracellular vesicle vectors have also been also utilised to explore more cost-effective, scalable and immunogenic alternatives for vaccine delivery. Difficulties in cheaply producing recombinant MOMP in its native form has prompted design of vaccines fusing or integrating *Ct* specific MOMP epitopes with stable vectors including Hepatitis B core antigen [143] and *Neisseria lactamica* porin B [144]. Nucleic acid vaccines by-pass the need to synthesise protein *in vitro,* are cheap to produce, highly adaptable and prompt strong Th1 skewed immune responses, making them highly appropriate for *Ct* vaccination. While initial attempts to utilise DNA plasmid vaccination for *Ct* were disappointing and did not impart protection in murine models [145], recent DNA vaccines incorporating additional immunogenic epitopes have shown some capacity for inducing protection against *Ct* in mice [146]. Their capacity to be optimised for the presentation of effective CD8, CD4 or B cell epitopes highlights them as interesting candidates for future vaccine work [147]. Self-adjuvanting nanoparticle encapsulated MOMP peptide has been shown in murine models of *Cm* infection to significantly decrease bacterial load upon challenge [148] and promote a novel way for adjuvant delivery. mRNA vaccines are a little explored modality for *Ct* vaccination that have shown great capacity to induce IFNγ and neutralising antibody responses to other intracellular pathogens [149]. In complex with cationic adjuvant formulations, self-amplifying RNA incorporating MOMP (Venezuelan equine encephalitis virus backbone with MOMP insert) induced a self-adjuvanted MOMP-specific IFNγ dominated cellular and humoral immune response in mice [138]. It is likely that implementation of increasingly intricate delivery platforms that orchestrate an appropriate immune response through incorporation of adjuvants and through molecular design will be essential in advancing the development of successful vaccines for *Ct* infection [138].

5. Challenges and future avenues for *Chlamydia trachomatis* vaccines

In spite of the numerous successes of vaccine research thus far, there remain several issues that impact our ability to translate immunological and biological understanding into vaccine advances. More work must be done to identify novel immunogen candidates and platforms for delivery that are safe, sufficiently immunogenic and induce appropriate protective immunity. While the greatly explored MOMP has been effective at demonstrating some immunogenicity in models and in human trials – discovery of alternative candidates that are pan-serovar neutralising and cheap to produce will be key in providing a broadly effective vaccine. PmpD is a potential example of one such candidate [102], which may benefit from the alternative vaccine design strategies highlighted above. It will be important in the future to explore new and robust delivery modalities that tailor immunogenicity, cost-effectiveness, environmental sustainability and scalability and overcome issues of native protein folding [150]. The combination of these approaches will be critical in the effort to establish protection against chlamydial infection in a global setting and in orchestrating a suitable immune response that effectively prevents disease transmission and pathology.

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Authors' contributions

P.M and S.M conceived the original idea for the review. SM completed the first draft of the manuscript and subsequent edits. PM provided critical feedback and edits on the work throughout. Both authors approved the final edit of the manuscript as submitted.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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