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Chapter 7

Immune Regulation of *Chlamydia trachomatis* Infections of the Female Genital Tract

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Additional information is available at the end of the chapter

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1. Introduction

Tubal infertility and reproductive damage, preeclampsia and preterm births may potentially occur in many of the 50 million women who are annually infected with *Chlamydia trachomatis* [reviewed in 1; 2- 4]. *C.trachomatis* infections of the genital tract are also reported to increase the risk of human immunodeficiency virus type 1(HIV-1) susceptibility and viral shedding in the genital tract either directly by pro-inflammatory signalling pathways or indirectly by their effects on genital epithelial cells [5]. A dramatic increase in the number of HIV-1 chemokine co-receptor CXCR4- and CCR5-positive T cell targets has also been reported in the endocervix of *C. trachomatis*-positive women [6].

Gynecological cancer development is also suggested to be linked with Chlamydial genital tract infections. Epidemiological reports have proposed that *C.trachomatis* may act as a co-factor in the development of human papilloma virus (HPV)-induced squamous cell carcinomas (SCC), with an association between distinct serovariants of *C. trachomatis* (B, D, E, G, I, and J) and SCC being reported [7,8]. It is also likely that *C. trachomatis* co-infections of the lower genital tract (LGT) play a role in carcinogenesis of the female upper genital tract (UGT), particularly epithelial ovarian and type II carcinomas [9,10]. To explain the epidemiological associations of Chlamydial infections cervical and ovarian cancer development it has been hypothesised that the pathogen perhaps triggers epigenetic changes in host chromatin and impairs host DNA repair pathways [11] or that it changes host cell survival pathways by disrupting DNA damage signalling pathways associated with tumorigenesis [12].

The majority (70-80%) of genital tract infections in women are asymptomatic and are not diagnosed or treated and persistent or "silent" infections are reported as occurring years after

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initial acquisition of *Chlamydia* with 50% of women continuing to shed the organism one year after infection is diagnosed [13]. Left untreated the organisms can ascend to the uterus and Fallopian tubes to cause pelvic inflammatory disease (PID). Inflammation of the Fallopian tubes can lead to scarring and fibrosis which can then lead to ectopic pregnancy (EP), subfertility and infertility with repeated or multiple infections increasing the chances of disease sequelae [14-17]. Additionally, infections during pregnancy may result in peripartum transmission of *Chlamydia* to newborns potentially resulting in neonatal conjunctivitis and late-onset pneumonia [18, 19].

Globally it is estimated that over 100 million adults were infected with *C. trachomatis* in 2008 [20]. The annual direct medical cost of sexually transmitted diseases (including *Chlamydia* and those caused by HIV) has been estimated at $US16.9 billion [21]. Despite continued improvements in diagnostic and screening procedures for *Chlamydia* the incidence of infection continues to increase with a significant increase, particularly in the rates of EP [22]. Even though azithromycin is available to treat uncomplicated lower genital tract infections [23], antibiotic therapy will not always be efficacious and one hypothesis is that *Chlamydia* treatments may in fact attenuate protective immunity in some patients [24]. Recent evidence has shown that although spontaneous resolution of infection before treatment occurred in 22% of naturally infected women, following azithromycion treatment, 16.5% of women were re-infected and this rate was higher (19.9%) in those women with persistent infection when compared with the rate (5%) in women who spontaneously cleared the infection [25]. Repeat infections are thought to be one of the primary causes of ongoing transmission of *Chlamydia*. Development of a vaccine that can either prevent infection or disease is therefore of vital importance for *Chlamydia* [26, 27].

1.1. The organism: *C. trachomatis*

*Chlamydia*e are small (0.5μm and genomes of 1.05Mb) gram negative intracellular bacteria and the human pathobiotype, *C. trachomatis* comprises non-invasive biovars that infect squamocolumnar epithelial cells of the female genital tract [28-30]. *C.trachomatis* replicates within non-acidic vacuoles of these genital tract cells, acquiring essential lipids from the host cell during infection to survive and replicate [31]. Based on serologic differences elicited by variable domain (VD) regions of the Chlamydial major outer membrane protein A (OmpA), *C.trachomatis* biovars currently are classified into 19 serovars with serovars D-K causing urogenital infections [32-37].

Urogenital isolates of *C.trachomatis* may also contain a highly conserved 7.5 kB non-integrative plasmid that has a role in the pathobiology of these organisms [38, 39]. The plasmid functions as a significant virulence factor in the murine model of genital tract disease [40] with plasmid loss resulting in failure to induce upper genital tract pathology in this model [41]. Within clinical isolates of *C.trachomatis*, carriage of the 7.5 kB plasmid is almost universal, although a few naturally-occurring, plasmid deficient strains have been reported that are not associated with increased disease severity [42-45]. Recently it was reported that naturally-occurring plasmid-free urogenital isolates of *C.trachomatis* serovar F also had reduced infectivity and virulence in a murine model [46].

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1.2. Developmental cycle

Chlamydia undergoes a unique biphasic developmental cycle that encompasses two forms of reproduction; an intracellular phase of non-infectious metabolically active and replicative forms of reticulate bodies (RBs) and the extracellular phase of infectious elementary bodies (EBs) that attach to and invade epithelial cells [47; reviewed in 48, and 49 and 50].

Chlamydial infection of superficial columnar cervical cells in the FGT is initiated by the attachment and endocytosis of the EB into these mucosal epithelial cells. EB containing endosomes then are internalised within host-derived membranes where the pathogen then hijacks both vesicular and non-vesicular mediated pathways of the host to obtain host-derived membrane lipids, including sphingomyelin (SM) and cholesterol, for intracellular growth and development [reviewed in 51]. Once resources such as sphingomyelin are obtained from the host cell and the vesicle expands, the EBs differentiate into the metabolically active, non-infectious and larger (0.8μm) reticulate bodies (RB) in the normal developmental cycle. Transformation of the metabolically-inactive EBs into the metabolically-active RBs occurs at approximately 3 h post-infection (pi), and replication by binary fission over a 24-48h time period then is initiated [52]. The RB containing endosome expands and produces a microscopically-visible Chlamydial microcolony referred to as an "inclusion" [53]. RBs then use host cell ATP and other metabolites to grow and replicate by binary fission within the Chlamydial inclusion.

Many inducers of persistence (including Interferon gamma (IFN-g), amino acid starvation and antibiotics) will result in deviation from the 'normal developmental cycle', resulting in a viable but non-cultivable growth stage and the formation of large, abnormal developmental forms termed aberrant bodies (ABs) also known as aberrant RB, or persistent bodies [54]. In normal development, RBs will continue to divide undergoing between 8-12 rounds of replication. Later in the infectious process, the RBs asynchronously begin to differentiate back into EBs, there is evidence of intermediate bodies (IBs) at this stage and the EBs accumulate within the inclusion. Depending upon the Chlamydial species, at 30-80h post-infection the infectious EB's that have matured from the RBs are released either by host cell lysis or by a packaged-release mechanism called extrusion, allowing the EBs to infect neighbouring cells for successive rounds of infection.

1.3. Intracellular survival strategies

C. trachomatis is proficient at maintaining enduring relationships with its host by modulating and evading the immune system using a multitude of mechanisms [55]. These include inhibiting IFN-g–inducible major histocompatibility complex (MHC) class II expression [56] that in human endocervical cells is known to be mediated by direct and indirect (soluble) factors [57]. The multiple potential mechanisms used by *Chlamydia* to dampen immune responses recently have been summarized [58].

To modulate host signal transduction cascades and allow their developmental cycles to take place Chlamydiae are known to secrete up to sixteen anti-host (effector) proteins (reviewed in 59; 60). They also recruit proteins from the host and the first identified host proteins to be

recruited into the Chlamydial inclusion include the eukaryotic nuclear protein ZNF23, a pro-apoptotic factor that potentially allows *Chlamydia* to inhibit host cell apoptosis thus enabling bacterial survival in the host [61].

1.4. Chlamydial infections and disease sequelae

1.4.1. Infections in women

Significant reproductive morbidity can occur in women following infection of the LGT with *C. trachomatis*. In the estimated 20% of women with symptoms, *C.trachomatis* infection of the columnar epithelial cells of the endocervix can result in cervicitis [62-64; reviewed in 65] and urethritis [66]. Severe complications of untreated LGT Chlamydial infections in women, whether symptomatic or asymptomatic can result in ascension of the organism from the lower to the UGT. Endometritis represents an early stage in the continuum from LGT infection through to salpingitis, a Fallopian tube disease also confirmed to be associated with *C.trachomatis* infection. Since a critical source of Fallopian tube inflammation is infection with *C.trachomatis,* it has been hypothesized that *C.trachomatis* infection may be involved in chronic tubal inflammation and subsequent fimbrial carcinogenesis and hence could perhaps be the origin of some ovarian cancers [67]. Interestingly, a recent retrospective analysis of 34 cases of serous pelvic carcinomas (which are the most frequent histologic type of ovarian cancers) reported that 70% of cases did in fact involve the Fallopian tubes [68].

Chlamydial infection of the pelvis can lead to symptomatic pelvic inflammatory disease (PID) that can result in Fallopian tube inflammation [69] and salpingitis is reported to occur in 20-40% of women with untreated or repeated Chlamydial infections [70,71]. A recent model predicts that annual screening would prevent 61% of *C.trachomatis*-related PID in women who became infected in their LGTs with *Chlamydia* [72]. In addition to salpingitis the long-term consequences of PID can also include chronic pelvic pain and ectopic pregnancy (EP). It has been proposed that since *C.trachomatis* infection increases tubal expression of prokineticin receptor (PROKR2) mRNA this expression then predisposes to ectopic implantation in the Fallopian tube [73]. Recent papers have reported that one third of EPs could be attributable to Chlamydial infection [74] and have also reviewed the pathogenesis of *Chlamydia*-induced tubal EP [75].

PID is caused by infection of the female genital tract with many microorganisms including *C. trachomatis* [reviewed in 16] and following symptomatic PID of polymicrobial etiology up to 18% of women may develop tubal factor infertility (TFI), which is amongst the leading causes of infertility accounting for 7-9.8% of all female factor infertilities [76-80]. Testing for serum antibody to the Chlamydial 60kDa Heat shock protein (i.e.cHSP60 antibody) is an accurate means for predicting *Chlamydia*-associated TFI [81] and elevated levels of anti-Chlamydial caseinolytic protease P (ClpP) antibodies were recently identified in 21 TFI patients [82]. Prolonged exposure to *Chlamydia* due to a chronic persistent infection or frequent re-infection has also been associated with chronic inflammation and TFI [83-85]. It has been reported that 9.5% women developed PID over 12 months in women who tested positive for *Chlamydia* at baseline [86].

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1.4.2. Ophthalmia neonatorum and infant pneumonia

The high prevalence of Chlamydial infections of the cervix in women of child-bearing age results in annual exposure of an estimated 100,000 neonates to *Chlamydia* infections [87] and neonatal Chlamydial infections are generally acquired during passage through an infected birth canal. A Chlamydial cause should be considered for all infants aged ≤30 days who have conjunctivitis and, if positively diagnosed, appropriate treatment initiated for the infant, mother and her sex partner(s).

Chlamydia infections of neonates have been reported to be at 18 percent for ophthalmia neonatorum and 16 percent for pneumonia for infants exposed to *C. trachomatis* in vaginal secretions in the birth canal, with up to 60% of these infants showing serological evidence of Chlamydial infection [88]. Using data pooled from studies since 1977 it has been estimated that the incidence of ophthalmia neonatorum caused by exposure of the neonate to *C.trachomatis* at birth is actually around 15 percent and that of pneumonia is around 7 percent [89].

Neonatal infection with *C. trachomatis* involves the mucous membranes of the neonatal eye, oropharynx, urogenital tract, and rectum and when symptomatic can result in a number of diseases in the first few months of life such as inclusion conjunctivitis, pneumonia or both [90,91; reviewed in 92; 93). Ophthalmia neonatorum has been reported as developing from 5-14 days post-delivery in up to 50% of infants of *C.trachomatis* infected mothers [94] and perhaps even as late as 42 days after birth [94] with between 5-30% of perinatally-infected infants also having nasopharyngeal infection [reviewed in 95]. A recent prospective study from Brazil estimated the prevalence of rate of *Chlamydia* among pregnant women (<30 years) in their third trimester as 72.7% and reported that 50% of 16 newborn babies had respiratory symptoms within the first 60 days of life [96].

Afebrile pneumonia may be seen in infants colonised in the respiratory tract with *C.trachomatis* and this may/may not be associated with inclusion conjunctivitis. After three or more weeks following Chlamydial infection of the infants a subacute, afebrile pneumonia with onset at ages 1–3 months develops in approximately seven percent of these cases [97, 92, 19]. Clinical characteristics have been described for infant pneumonia caused by *C.trachomatis* in infants with nasopharyngeal shedding and in around half of the infected infants conjunctivitis was recorded, with middle ear abnormalities recorded in more than half of the *Chlamydia*-positive infants and wheezing an unusual finding in these infants [98]. A more recent study has also highlighted that *C. trachomatis* is an important cause of lower respiratory tract infection in infants in India below six months of age [99].

1.5. Immunopathogenesis and immunoprotection

The host inflammatory response to *C.trachomatis* is involved in immunity but also the pathology that leads to serious morbidities of chronic pelvic pain, EP and tubal infertility following female genital tract infection. Protective responses to *C.trachomatis* infections of the female genital tract are elicited to clear primary infection and to resist re-infection and include both innate and adaptive (particularly CD4+ T cell) cells and immune responses. Cells of both the innate and adaptive immune systems are found in the FGT in Fallopian tubes, uterus, cervix

and vagina [100] and we have recently reviewed the localisation of T lymphocyte populations in the female upper (UGT) and lower (LGT) genital tracts over the menstrual cycle [101].

Effector cells of the innate (non-specific) immune response found in the FGT include epithelial cells, monocytes, macrophages, granulocytes (neutrophils, eosinophils and basophils), dendritic cells (DCs) and Natural Killer (NK) cells conferring protection through chemokines, cytokines and phagocytosis. Innate (non-specific) host defense particularly in the lower genital tract (LGT) is also provided by the vaginal microbiota maintained in a healthy equilibrium (reviewed in [102] as well as by the myriad of antimicrobial peptides (AMPs) and protease inhibitors of cervicovaginal fluid [103] including elafin, lysozyme and cathelicidins, amongst others and reviewed in (Wira and Fahey, 2004). The AMPs and pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs) are key intermediaries of innate immunity in the FGT [104-107]. The innate immune cells (especially neutrophils) elicit inflammation to protect against infections however these inflammatory responses are usually inefficient and repeated Chlamydial infections are common [108].

The rapid innate immune response is the first line of defence against a *C. trachomatis* infection of columnar epithelial cells. PRRs recognise the pathogen and TLRs play a vital role in the host immune system by recognizing pathogenic components (pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs)) and inducing a protective immune response to the pathogen [109]. Data indicate that host genetic factors may contribute up to 40% to the variation in clinical course of *Chlamydia* infection [110]. Host genetic variants in TLRs have been found to influence *Chlamydia* LGT infections in women [111]. For example protection against tubal disease following a *C.trachomatis* infection has been reportedly associated with single nucleotide polymorphisms (SNPs) in the innate immune (TLR) gene, TLR2 [112]. SNPs in TLR4 have also been reported to play a role in making women more prone to subfertility (i.e. no pregnancy after 1 year) as a late complication of *Chlamydia* infection [113] and worldwide estimates are that 10-15% of all couples are subfertile [114]. SNPs in other pathogen recognition receptors including nucleotide-binding oligomerization domain receptors (NODs) have been reported to influence susceptibility and severity of *Chlamydia* infections [115]. Taken together these observations have lead to the recent suggested potential translational and clinical value of adding diagnostic host genetic marker profiles on the basis of infection and inflammation to the current clinical management of subfertility [116].

The more gradual adaptive immune response to Chlamydial infection is mediated through antibodies and/or T cells and is a very specific recognition and response to foreign antigens. Cervical cell production of IFN-g and interleukin-12 (IL-12) in response to stimulation with *C.trachomatis* EBs has a positive correlation with fertility in *C.trachomatis* seropositive patients [117]. Recruitment and activation of B cell (humoral) and T cell (cell-mediated/adaptive) immunity is also coordinated by the release of these factors. The initiation of this adaptive response may lead to spontaneous resolution of natural infection in some populations [25] although chronic infections in women indicate that rarely does this response to *C.trachomatis* result in clearance of the infection; nor does it adequately protect against re-infection. The cellular components of this specific immunity include T lymphocytes expressing $\alpha\beta$- and $\gamma\delta$-T cell receptors (TCR) and immunoglobulin (Ig) producing B lymphocytes [reviewed in 101].

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During active primary genital infections in women, serum and genital mucosal IgA and IgG antibodies to Chlamydial EBs and specific Chlamydial proteins including heat-shock (HSP) and plasmid proteins are usually detected [118]. AntiChlamydial antibodies are not sufficient to protect infected females from re-infections [119].

Once *Chlamydia* has established intracellular infection, cells of the adaptive immune system, and particularly T helper 1 (Th1) type CD4+ T cells secreting IFN-g are required for clearance of primary infection and to protect from re-infection [120]. It has been observed from human data that MHC Class II-restricted CD4+T cells of the Th1 phenotype are critical for the recovery from primary Chlamydial infection and also have a role in protecting from disease sequelae [121,122]. Other major lineages of activated CD4+ T cells that play critical roles in Chlamydial infections include the Th2 cells, Th17 cells (producing IL-17 and IL-23) and Tregulatory (Treg) cells [123,124]. Additional T cells involved in adaptive immunity include intraepithelial lymphocytes (γδ T cells) and cytotoxic T lymphocytes (CD8+ T cells) that are known to induce apoptosis of infected Chlamydial cells [125].

Disease outcomes are dependent upon the complex interactions between virulence factors of, and evasion strategies used by, *C.trachomatis* and host immune responses including variants in genetic markers associated with infection and inflammation. Host factors and cellular immune responses associated with susceptibility to, or protection from Chlamydial infection and/or diseases have recently been reviewed [126].

2. Chlamydial genital tract infection and immune regulation by sex hormones

The FGT comprises several immune compartments found in the UGT (endocervix, ovaries, Fallopian tubes and uterus) and LGT (ectocervix, and vagina). The UGT is lined with a single layer of columnar epithelium and the LGT with is lined with stratified squamous epithelium [100]. The transitional zone where squamous epithelium changes to columnar epithelium is the most immunologically active site of the FGT [127].

The human FGT contains components of the innate and adaptive mucosal immune responses that are found in various distributions at different sites throughout the tract. In the FGT the major lymphocyte components are Natural Killer (NK) cells and T lymphocytes including CD3+ T lymphocytes that are present in all tissues of the tract. In the LGT the CD8+ and CD4+ are dispersed throughout the stroma whilst lymphoid aggregates of these cells are formed in the uterus [128]; granulocytes are present and these are principally located in the Fallopian tubes. Finally, relative to T lymphocytes, smaller numbers of monocytes and B lymphocytes are found throughout all tissues of the FGT [129].

Host protection of the FGT is afforded by two arms of defense comprising innate (non-specific) and acquired (specific) immunity. The cellular components of the specific immune responses at this site are T lymphocytes expressing αβ- and γδ-T cell receptors (TCR) and immunoglobulins (Ig) producing B lymphocytes [130]. Effectors of non-specific responses include

epithelial cells, monocytes, macrophages, granulocytes (neutrophils, eosinophils and basophils), dendritic cells (DCs) and Natural Killer (NK) cells that confer protection due to chemokines, cytokines and phagocytosis. Cells of both the innate and adaptive immune systems are found in Fallopian tubes, uterus, cervix and vagina [131].

Both innate and adaptive mucosal immunity in the FGT are regulated by the female sex hormones estrogen (E2) and progesterone (pregn-4-ene3,20-dione) or (P4) and have been reviewed [130, 132, reviewed in 101]. Hormones regulate the transport of Igs, levels of cytokines, expression of Toll-like receptor (TLR) genes [133,134] and the distribution of immune cells and antigen presentation in the genital tissues during the reproductive cycle. Estradiol regulates the structure and function of the FGT via the classic model of steroid action ('i.e. genomic regulation) which is mediated by activated nuclear receptors including estrogen receptors (ER)-α and ER-β [131,135]. Estradiol can also exert rapid "non-classic" steroid effects on diverse signalling pathways and second messenger systems that occur independently of transcriptional or classic genomic regulation [136]. These rapid responses are often referred to as 'non-genomic' steroid effects and they can affect genital tract cells and innate and humoral immunity at this site which may alter the pathogens ability to infect the genital tract [137].

The recruitment and function of immune components of the FGT are precisely regulated by hormonal changes during the menstrual cycle by the sex steroids that co-ordinate cell trafficking and immune activation at this mucosal site. Female sex hormones E2, progestins and androgens are produced by ovarian cells with levels of estradiol (17β-estradiol) and P4 varying in accordance with fluctuations in the menstrual cycle [130]. In the menstrual/ovarian cycle of humans the endometrium develops and follicles grow until ovulation in the Proliferative/ Follicular phase. The Secretory/Luteal phase is characterised by high levels of progesterone from the corpus luteum to maintain the endometrium. Finally, corpus luteum regression and menstruation occurs during the menstrual phase At menstruation, serum estradiol levels measure typically <50pg/ml. Gradually increasing amounts of estradiol are found in the follicular (proliferative) phase of the adult female menstrual cycle with serum levels at a pre-ovulatory stage (day 14) ranging from 110-410 pg/ml and dropping briefly at ovulation. Levels of estradiol (20-160pg/mol) and P4(>5ng/ml) are present during the luteal (secretory) phase from days 14-28 of the menstrual cycle typically peaking at day 21 of the cycle. At the end of the secretory phase estradiol levels return to their menstrual levels.

The innate response is triggered by PRRs (including TLRs and non-TLRs such as NOD-like receptors) expressed predominantly by innate immune effector cells such as macrophages, neutrophils and DCs that are found in the genital mucosa. Human oviduct epithelial cells (ECs) express Toll-like receptor 3 and E2 has been reported to suppress the TLR-3-induced cytokine and chemokine production in human endometrial epithelial cells [138].The effects of E2 on the differentiation and function of antigen-presenting cells (APCs) *in vitro* has been reviewed (see [139; 132]. Much of the current evidence shows that E2 can activate dendritic cells and differentially regulate adaptive immune responses through direct effects on DC functions [140] via E2 receptor (ER) ligands [141]. These include TLR4 and its co-receptor Cluster of Differentiation (CD) 14 (Chlamydial LPS and Chlamydial HSP60) [142; 143; 144] TLR2 (Chlamydial HSP60) [145,146] and NOD protein, Nod2 (rudimentary

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proteoglycan motif produced by *C. trachomatis* and *C.muridarum*) [147]. Expression of TLR genes 2,3,4,5,6,9 and 10 is reportedly significantly higher in human endometrial tissue during the secretory phase (when P4 is the predominant hormone) compared to all other phases of the menstrual cycle [148].

Changes in the populations of DC and macrophage cells in the murine FGT occur due to varying levels of ovarian steroid hormones at this site in the model [149], and in women, changes in these cell populations occur due to the varying stages of the menstrual cycle, noting that the frequencies of macrophages and immature DCs are higher in the menstrual phase when compared to all other phases of the cycle [150, 151]. CD16-expressing macrophages and monocytes are also key components of the innate immune system of the FGT and are cells that are capable of producing cytokines and chemokines upon activation. These cells are also known to be profoundly affected by E2 [reviewed in 152]. Studies have shown that E2 uses both estrogen receptor (ER) alpha (ERα) and ER beta (ERβ) to decrease CD16 expression on monocytes and hence to alter monocytic cytokine release following CD16 receptor activation [153].

T lymphocytes present in the FGT, specifically CD4+ and CD8+ T cells, and immune mediators of cell-mediated immunity are found distributed within distinct regions of the female LGT. The normal vaginal mucosa contains T cells and APCs with the few immune cells present consisting primarily of CD1a+ DCs and CD8+ intraepithelial lymphocytes (IELs) [154]. The tissues in the UGT have been found to express a greater number Th1-associated chemokines than the tissues in the LGT comprising the cervical-vaginal region [153]. The menstrual cycle and menopause have been reported to have no apparent effect on cellular localization or abundance of T lymphocyte subsets and APCs in any of the lower genital tract tissues [127]. In humans, expansion of human CD4+CD25+ regulatory T cells (Tregs) has been reported to occur in the late follicular (proliferative) phases of the menstrual cycles of fertile women [124].

Levels of IgG and IgA antibodies in FGT fluids are also known to be modulated by the stage of the menstrual cycle in women. E2 up-regulates the *in vitro* expression in the FGT of secretory component (SC) increasing transport of IgA into the lumen [154] and transport of polymeric IgA (pIgA) into FGT tissues is significantly decreased by ovariectomy, although this decline can be reversed by addition of E2 but not P4 [155]. The effects of ovarian hormones on immunoglobulin secreting cells (ISC) function in healthy women were also investigated and it was reported that ISC frequency in peripheral blood mononuclear cells (PMBC) was highest during the peri-ovulatory stage of the menstrual cycle [156].

2.1. Sex hormones and chlamydial infection

It is becoming increasingly well documented that sex hormones E2 and P4 regulate host susceptibility, as well as innate and adaptive immune responses to the sexually transmitted pathogen *Chlamydia* infecting the mucosal surfaces of the FGT [reviewed in 101].

Data from several animal models of genital tract Chlamydial infection have shown that Chlamydial infection may be influenced by timing of exposure and the steroid status of the mucosal epithelium. In the murine model of *C.muridarum* infection pre-exposure of the animals

to P4 is required to achieve maximal infection outcomes in the animals [157]. However, treatment of female guinea pigs with E2 resulted in *Chlamydia caviae* infections of greater intensity and longer duration [157-160] an effect that it also observed following oral contraceptive administration to these animals [161]. In the female reproductive tract rat model it was reported that no Chlamydial inclusions were detected in either the uterus or vagina in rats that had been infected with *C. trachomatis* (mouse pneumonitis strain MoPn) at estrus and diestrus without prior P4 priming [162]. In rat studies it has also been reported that when ovariectomized animals were administered estradiol, progesterone, or a combination of both and infected with *Chlamydia trachomatis* via the intrauterine route no Chlamydiae were found vaginal secretions of E2-treated animals nor were there any signs of uterine or vaginal inflammation in these animals [163].

Additionally, the use of *in vitro* cell lines to investigate *C. trachomatis* infection have produced data showing that the hormonal status of the epithelium can influence the outcome of Chlamydial infections in these models. It was shown that E2 treatment of HeLa cells pre-infection with *C.trachomatis* (serovar K or L1) enhanced both of adherence of EBs to the cells as well as Chlamydial inclusion formation; post-infection, the presence of E2 was required for the enhancement of inclusion formation [164]. Using a hormone responsive human endometrial cell line an association between repeated Chlamydial infections in women and high levels of E2 has been reported [165]. More recently it has been reported that the sex hormones E2 and P4 can directly modulate Chlamydial genes associated with persistence (the viable but non-culturable state in the Chlamydial developmental cycle) in the host. It is likely, however, that the major effects of these hormones on Chlamydial infection are indirect via the host cells [166]. The outer membrane complex B (*omcB)* and tryptophan B (*trpB*) genes are two of several reliable markers for Chlamydial persistence [167,168]. It was reported that in E2-supplemented cultures of *C.trachomatis* serovar D infections of a hormone-responsive human endometrial cell line, an up-regulation of the *omcB* and *trpB* genes was observed suggesting a stress response indicative of Chlamydial persistence; morphological changes (aberrant, enlarged RBs) were also observed in these cultures consistent with a persistence response [166]. P4-supplemented cultures resulted in a general up-regulation of genes encoding amino acid and carbohydrate metabolism pathways [166].

Chlamydia trachomatis infection of the female LGT stimulates innate immune cells by activation of TLR2/TLR4 [reviewed in 169]. TLRs 2 and 4 have been found at the highest expression levels in endometrium and Fallopian tube tissue [170] with the predominant expression of TLR4 and its co-receptor CD14 in the Fallopian tubes proposed to play an important role in the innate host defence mechanism against ascending *C. trachomatis* infections [169,171]. In a recent study *C.trachomatis*-infected women have been reported to express higher TLR2, TLR4 and inducible nitric oxide synthase (iNOS) in their cervical monocytes compared to controls. It is thought that TLR4 initiates the innate immune response to Chlamydial infection, activation of TLR2 leads to expression of inflammatory cytokines whilst iNOS contributes to Chlamydial clearance from this mucosal site [172]. Recently it has been reported that E2 reduces TLR4 expression of human monocyte derived Dendritic cells and suggested that this may increase host susceptibility to *C. trachomatis* infections [173].

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Studies of *C.trachomatis* infections of the FGT have reported many effects of hormones on infection and immunity to the pathogen at this mucosal site. For example significant increases in the sensitivities of cervical epithelial cells to infection with *C.trachomatis* occur in the later stages of the female menstrual cycle [174]. It has been reported that Chlamydial infection occurring in the early E2-dominant phases of the cycle was a significant predictor for development of salpingitis [175]. Clinical findings have also reported on the enhancing effects of E2 on Chlamydial infection and disease sequelae in the infected female genital tract. It has been reported that women are more susceptible to Chlamydial infection under the influence of E2 [165]. In women with fertility disorders (n= 115) and in women with *C.trachomatis* mucopurulent cervicitis (n=86) a significantly positive correlation was recorded between Chlamydial load and E2 levels [117].

Chemokine receptors direct T lymphocytes to the site of mucosal infection, including infection of genital tract epithelial cells with *C.trachomatis* and these receptors can be modulated by E2. In a study examining samples from human patients it was reported that CCR5 (chemokine (C-C motif) receptor 5) expression increased followed *C. trachomatis* genital infections [125].

Reproductive hormones may potentially act with cytokines to regulate immune responses in the FGT to Chlamydial genital tract infections *in vivo*. Significant negative correlation has been reported between E2 levels in women with primary Chlamydial genital infections and cervical wash concentrations of IL-10, IL-1beta and IL-6 cytokines. Significant negative correlations were also recorded between IL1-beta cytokines and P4 levels in women with recurrent Chlamydial infections [176]. Thus it would appear that Chlamydial infection of the human FGT is modulated in part by the combined actions of cytokines with E2 and P4. More recent investigations reveal that levels of E2 were significantly higher in *Chlamydia*-positive women with fertility disorders when compared to fertile women suggesting that this sex hormone contributes to development of disease sequelae to *C.trachomatis* infection of the FGT[165].

E2 has been reported to exacerbate Chlamydial infections in women following administration of oral contraceptives [177] an observation also noted for female guinea pigs [161]. A prospective study on oral contraceptive pills or depo medroxyprogesterone acetate and sexually transmitted disease acquisition reported that women using oral contraceptive pills were at increased risk for acquisition of *Chlamydia* [178]. Hormonal contraceptive use in women, particularly depo medroxyprogesterone (DMPA), has also been reported in observational studies to be associated with a four-fold increase in Chlamydial genital tract infections [179].

3. Immunopathogenesis — chlamydial infections, tubal factor infertility, ectopic pregnancy and pid

A primary Chlamydial infection of the FGT can elicit host immune responses that are involved both in immunity to infection and in pathology of disease. Innate immune responses constitute the first line of defence against pathogens and include TLRs that are expressed in the FGT. Further, although the host immune response affords protection against infection *via* means of

rapid and efficient bacterial destruction, resultant inflammatory responses may lead to tissue damage and/or persistent infection.

In reaction to Chlamydial entry in host cells, innate immune responses occur at the infected mucosal site 1-2 days post infection [180]. Intense inflammation and predominant mucosal infiltration of phagocytes, neutrophils and macrophages ingest and enzymatically destroy the bacteria. In conjunction with the production and subsequent activation of phagocytic cells, others, crucial to infection resolution are also activated [181]. Natural killer (NK) cells are activated in direct response to *C. trachomatis* infection or indirectly by macrophagic stimulation of IL-12, a potent NK cell-triggering cytokine. Once activated, NK cells produce IFN-g which stimulate macrophages, further promoting phagocytosis. Innate immunity may impede infection, however, the inaccessibility of *Chlamydia* to circulating antibodies whose sole purpose is elimination necessitates adaptive immune effector mechanisms of both humoral and cell-mediated immunity.

Activation of cell-mediated immunity post infection is demonstrated by the proliferative response ratio of peripheral blood lymphocytes to Chlamydial EBs. T cells, which functionally differentiate into Th1 or Th2 subtypes based upon cytokine secretion profiles, also amass at the infection site [182]. Further, interaction of *C. trachomatis* with the cytokine network is a key component for resolution of infection and one in which a cytokine response is elicited either through direct infection of epithelial cells or by host cell interaction. In concert with the inflammatory process, tissue repair, comprising the removal of dead cells and the ingrowth of fibroblasts, leads to scar formation and potential impairment of Fallopian tube function [183]. Moreover, all three tubal layers i.e. interstitial, muscular and serosal may be implicated in scar development. Infection of the serosal layer elicits an inflammatory exudate, present on the peritoneal surface, thereby adhering all adjacent surfaces and structures. Although the innate and adaptive immune responses systematically co-ordinate their efforts to ultimately resolve infection, the immune response to Chlamydial infection can cause deciliation of tubal epithelium, intralumineal adhesions, tubal occlusion and peritubal adhesions. As a result, such gross anatomical and pathological changes may lead to infertility, EP and possible spontaneous abortion [184].

Primary Chlamydial infections of the FGT thus induce both innate and adaptive host immune responses that contribute to clearance of the infections. However, results from a 5-year study of natural genital Chlamydial infections in women strongly suggested that *C. trachomatis* infection was cleared from the cervix epithelium in only 54% of the women at 12 months [185]. This low clearance rate is due, in part, to the organism's ability to utilise various mechanisms with which to evade the host immune system. In fact rarely do host immune responses to *Chlamydia* induce a total clearance of genital infections. Indeed, the women remaining infected in the genital tract with *C. trachomatis* are unable to mount strong adaptive immune responses to clear the pathogen hence they become asymptomatic and remain undiagnosed with a persistent Chlamydial infection. Chronic inflammation resultant from a persistent *C. trachomatis* infection can lead to a wide spectrum of disease manifestations. In persistently-infected *Chlamydia*l host cells, inflammatory cytokines are released in response to the latent infection to aid in the eradication of the pathogen. Unfortunately, these cytokines, which act as immu-

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nomodulators may in fact, induce and sustain tissue damage and host inflammatory responses. Compounded with repeated infections, the continued production of cytokines may cause serious disease sequelae such as PID, TFI, chronic pelvic pain or EP. Chlamydial Hsp60 (cHsp60), a known potent inducer of host inflammatory responses, may protect against Chlamydial infections [186]; paradoxically, however, cHsp60 is also able to induce pro-inflammatory responses, which can lead to tissue damage in women infected with *C. trachomatis* [187-189].

Recent data from a prospective study of 30 women diagnosed with a ruptured EP demonstrated that anti-cHsp60 immunity confers a higher risk of EP [190]. Furthermore, in those women who were positive for anti-cHsp60 antibodies and suffered an EP, all had significantly lower IL-1β levels than Chlamydial sero-negative EP cases [190]. Importantly, high serum concentrations of IL-1β in the Fallopian tubes correlated with successful uterine implantation of embryos [191-192]. Data obtained from a prospective cohort of women at high risk for *C. trachomatis* infection demonstrated that a Th1 response i.e. the release of IFN-g to cHSP60 was associated with protection against the bacterial infection [186]. In contrast, it was also shown that an IL-10 response to the same Chlamydial antigen increased infection risk. In a study that investigated cytokine responses of cervical mononuclear cells obtained from 153 women all serologically positive for *C. trachomatis*, it was found that exposure to cHsp60 and cHsp10 may drastically affect the host's mucosal immune function by increasing the production and subsequent release of IFN-g, IL-10 and tumour necrosis factor-α (TNF-α) [193] thereby potentiating further tissue damage and disease sequelae.

TLRs are responsible for initiation of the adaptive immune system, thereby leading to the ultimate destruction of foreign microbes. Moreover, since they recognise structurally conserved molecules from various bacteria, they play a vital role in host defence. Chlamydial-induced PID was investigated and it was reported that among women clinically diagnosed with PID, those who carried variants in TLR 1 and TLR 4 genes had significantly increased odds of *C. trachomatis* infection [191]. In a more recent study, it was concluded that racial variation in TLR variants amongst women with PID may alter signalling pathways subsequent to microbial recognition, thus causing an increase in associated inflammation [194]. An investigation of pregnant women positive for *C. trachomatis* showed that those with serological evidence of prenatal *C. trachomatis* infections were more likely to develop pre-eclampsia [192].

Polymorphic membrane proteins (Pmps) represent 13.6% of *C. trachomatis'* entire coding genome [195], therefore inferring a potential role in Chlamydial biology and virulence. In a group of 40 *C. trachomatis*-infected women with clinically diagnosed mild to moderate PID, antibody levels of PmpA, PmpD and PmpI were evaluated. Interestingly, those women with PmpA antibodies were less likely to fall pregnant ($p=0.042$) and achieve a live birth ($p=0.005$) compared with those sero-negative for PmpA [196] suggesting that the immunopathological damage may indeed be a result of PmpA virulence. Further, it was also reported that women who were sero-positive for PmpI antibodies had a higher incidence of upper genital tract infection ($p=0.026$). However, antibodies to PmpD and PmpI did not alter the risk of FGT inflammation or sequelae.

One of the most important and common long-term complications of Chlamydial PID is TFI. Numerous studies have demonstrated serological evidence of prior *C. trachomatis* infection is associated with TFI [197-201]. An investigation conducted by the World Health Organisation (1995) of TFI rates as a consequence of *C. trachomatis* or *N. gonorrhoeae*-induced PID showed that *C. trachomatis*-induced PID was associated with a higher incidence of TFI when compared to PID caused by *N. gonorrhoeae*. A retrospective study of 1844 women all laparascopically diagnosed with PID due to *C. trachomatis* showed that 209 (16%) failed to conceive [202]. Of these, confirmed TFI was established in 141 patients with PID. Importantly, the rate of infertility was directly associated with the number and severity of PID infections. Specifically, every subsequent episode of PID approximately doubled the rate of TFI i.e. 8% upon one *C. trachomatis* infection, to 19.5% from two exposures resulting in infection and an increase to 40% resultant from three or more exposures.

In a more recent study that evaluated cHsp60-specific antibody and cell-mediated responses as predictors for TFI [201] it was reported that *C. trachomatis*-specific IgG antibodies were more common in TFI patients (43.2%) when compared to the control group (13.5%), thus suggesting *C. trachomatis* may play a significant pathogenic role in the development of TFI. To elucidate the clinical variability of TFI caused by *C. trachomatis*, functional polymorphisms in various cytokines were assessed [203]. The study showed that allelic variation in IL-10 and TNF-α increased the risk of severe tubal damage in women diagnosed with infertility caused by *C. trachomatis*. Similarly, a more recent study demonstrated that variation in the IL-12B gene was associated with increased susceptibility and TFI severity [204]. Interestingly, polymorphic changes in IL-10 and IFN-g appear to be linked with a more intensive lymphocytic proliferation in response to *C. trachomatis* antigens [205]. Antibody production following cHSP60 exposure was found to be significantly higher in those women (n=21) clinically diagnosed with TFI [206]. Furthermore, elevated antibody levels to caseinolytic protease P, a proteolytic subunit of the ATP-dependent Clp protease complex involved in the degradation of aberrant proteins, was also demonstrated in TFI patients. Serum levels of IgG1 and IgG3 antibodies against chlamydial major outer membrane protein (MOMP) and cHSP60 were shown to be elevated in women (n=70) with TFI compared with the control group (n=92; normal Fallopian tubes and sero-negative for *Chlamydia*) (p=<0.001) [207]. A study that evaluated the potential association of anti-Chlamydial IgM antibodies and TFI showed that of the 50 women assessed, 60% were sero-positive [208]. Of these, 52% presented with bilateral tubal blockage, which was more commonly detected in the ampullary portion (36%) of the Fallopian tube.

4. Immune responses, protection and pathology of chlamydial genital tract infections

Chlamydia (serovars D-K) infects epithelial cells lining the FGT with primary infection establishing in the endocervix [209]. If not controlled, primary infection can ascend the reproductive tract leading to the establishment of infection within the endometrium and Fallopian tubes. The kinetics of ascension of *Chlamydia* to the URT is unclear. Although *Chlamydia* can be transported via attachment onto sperm, the demonstration that small particles (the size of

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sperm) when deposited into the vaginal vault, rapidly ascend up into the uterus within 2 mins suggests as the general flow of fluids within the FGT may facilitate ascending infection [210, 211. For most women natural immunity appears to take a long time to acquire - if at all. As previously noted in a study by Molano and colleagues, it was demonstrated that 50% of women continue to shed the organism one year after infection is first documented, and 5 to 10% continue shedding even 3 years later [13]. There is limited evidence to suggest that the generation of natural immunity occurs in some individuals. A number of studies have reported that in around 20% of cases, spontaneous resolution of infection occurs [212,213]. As mentioned previously, Giesler and colleagues showed that women with spontaneously resolved genital infections were less likely to be re-infected within twelve months [25]. The host's immune factors associated with the spontaneous resolution of infection are yet to be elucidated. Unfortunately, any natural immunity induced following primary infection appears to be short-lived and serovar-specific resulting in many re-infections [214-217]. Animal models of Chlamydial genital tract infection have shown that the generation of Th1 cell mediated immunity is characterized by a strong IFNγ and TNFα cytokine production and that this strong response is essential for clearance of primary Chlamydial genital infections [218-220]. The observation that B cell deficient mice can resolve a primary infection suggests that antibodies play a non-critical role in protection [100]. However, B cell responses are important in resolving secondary infections. The generation of *Chlamydia* specific antibodies that are able to neutralize bacteria preventing infection have been reported in various cell lines *in vitro* [221-223] and *in vivo* [224]. $CD4^+$ T helper cells induced during primary infection mediate affinity maturation of antibodies, Ig class switching, and B cell memory responses during subsequent infection. Therefore, B cell memory and antibody production in the presence or absence of $CD4^+$ cells is critical for the prevention of *Chlamydia* reinfection [225-227] In addition, results from murine studies show the infiltration of immune cells, including CD8+ T cells, B cells, neutrophils and dendritic cells is associated with clearance both of intracellular and of extracellular Chlamydial bodies [228, 117, 229,230]. Using cytobrush sampling of women, an increase in neutrophils, dendritic cells and lymphocytes (CD4+ and CD8+) was reported during *Chlamydia* infections in these woemn [125,231].

Chlamydia infection of endocervical cells results in an increase in pro-inflammatory cytokine and chemokine production such as IL-1, IL-8, IL-12, IL-6[232, 233, 209]. Live *C. trachomatis* is innately recognized by pattern recognition receptors (PRRs) including Toll-like receptors and nucleotide-binding oligomerization domain (NOD)-like receptors. Both TLR2 and TLR4 are highly expressed in female genital tissues and have shown to be activated by live *Chlamydia* [234]. The myeloid differentiation primary response gene (MyD)88 is essential for nuclear factor–κ-β (NFκβ) signalling and transcription of pro-inflammatory cytokines and along with TLR2, MyD88 has been shown to co-localize within the intracellular Chlamydial inclusion during infection [235]. Mouse TLR2 knockout animals produce lower TNFα and macrophage inflammatory protein-2 (MIP-2) and report a significant decrease on oviduct pathology and hydrosaphinx, suggesting a major role for TLR2 in pathogenesis [236]. The direct activation of another PPR NOD-1 by *Chlamydia* results in the production of IL-8, which is produced at high concentrations during infection and recruit neutrophils to the genital tract [237, 238]. Overall, cytokine production during infection mediates the recruitment of innate immune cells that

secrete IFNγ and TNFα. These include natural killer (NK), Dendritic cells and neutrophils [239, 229]. The recruitment of innate immune cells and cytokine production is linked to upper reproductive tract pathology and scaring leading to the development of tubal infertility and ectopic pregnancy. In particular, Hvid and colleagues reported that during a *C.trachomatis* infection the production of IL-1 from Fallopian tubes biopsies led to the destruction ciliated epithelium. Furthermore IL-1 production additionally induced IL-8 production from Fallopian tube epithelial cells thereby potentially meditating the recruitment of neutrophils to this site [232]. The development of a chronic or persistent *Chlamydia* infection would induce continued production of innate immune mediators perpetuating cellular recruitment leading to epithelial damage, scaring and disease. The innate immune cell generation of immunopathogenesis and disease is termed the cellular paradigm [182].

Following clearance of a primary *Chlamydia* infection, re-infections are common in women; moreover, re-infections are strongly associated with development of pathology in these women. Results from animal models of Chlamydial infection have shown that during re-infection T cells are present within the URT at a higher magnitude than in primary infection [240]. *Chlamydia* has shown to act as both an immunizing and sensitizing infection. Non-human primate vaccine trials have shown that immunization with whole cell organisms led to hypersensitivity and an increase immunopathogical response leading to greater scaring in an ocular model. This suggests a specific role for adaptive responses in pathology and this is termed the immunological paradigm. Although the specific mechanism or causative antigen in unknown, it is hypothesized that during re-exposure pathogenesis is associated with either delayed-type hypersentitivity (DTH) or molecular mimicry causing autoimmunity [241]. DTH is hypothesized to be associated with persistent Chlamydial infection. During latent infections, low level antigen specific immune stimulation is believed to contribute to chronic inflammatory cell infiltration [242]. A role for antigenic sensitivity associated with DTH has been observed in animal models. Guinea pigs sensitized with Triton-X-100 soluble EBs and monkeys sensitized through immunization with a whole Chlamydial vaccines both reported the generation greater inflammation and DTH during re-exposure compared to un-sensitized controls [243,244]. In additon to DTH, autoimmunity may play a role in pathogenesis through the mechanism of molecular mimicry. Chlamydial HSP-60 (cHSP60) shares similar homology to self HSP-60 with recent evidence showing that C. trachomatis HSP-60 contains four T-cell epitopes that display identity with human HSP-60 [245, 246].T cell stimulation by self HSP-60 when pre-immunized with cHSP60 is characterized by the production of pro-inflammatory IFN-g. In contrast, pre-immunization and and re-exposure with self HSP60 results in the production of the anti-inflammatory cytokine IL-10 [247] although previous studies have shown that T cells isolated from patients with PID and TFI respond to cHSP-60 stimulation, and the presence of cHSP-60 specific antibodies correlate with the severity of PID and TFI pathology [248,188]. A recent study by Ness *et al.*, showed that increased cHSP60 antibody titres are not associated with the development of PID [249]. Further the presence of strong cHSP60 T cell IFN-g has more recently been shown to predict protection rather than pathology during reinfection [250]. The conflicting association of cHSP60 with protection or pathology is indicative of a balance between specific cell mediated responses leading to protection or pathogy.

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Recent evidence has shown that the Chlamydial plasmid may play a role in generating pathology during infection. As mentioned earlier, almost all *C. trachomatis* isolates contain a 7.5 kB cryptic plasmid although plasmid-free isolates have been shown in genital serotypes L2, D and E [43-45]. During infection of mice with plasmid-cured *C. muridarum* strains a strong Th-1 cell response is induced however immune pathology and tissue damge is not observed [41] Further, plasmid cured *C. muridarum or C. trachomatis* strains do not stimulate the production of cytokines though TLR2-dependent activation *in vivo* and *in vitro* respectively [251]. In contrast, Frazer *et al* showed that in the guinea pig model, a plasmid cured *C. caviae* strain both signals through TLR-2 and induced post infection pathology suggesting that the association of the *Chlamydia*l plasmid with virulence is not universally conserved among Chlamydial species [252]. Importantly, murine studies have shown that previous infection with plasmid-free is protective against the development of immunopathology with subsequent infection with a virulent strain [41]. This has lead to the use of plasmid-cured strains as attenuated Chlamydial vaccines in mouse and primate models of infection [41, 253].

Overall, the natural history of Chlamydial infections in women varies in which some untreated individuals with untreated infections persist for long periods asymptomatically and progress to cause pathology and disease, while others spontaneously resolve infection. Although the mechanisms are yet to be fully elucidated, there are currently two proposed mechanisms for the development of pathology in the female reproductive tract; the cellular and the immunological paridigms that are associated with innate and adaptive responses respectively. Further, these mechanisms are not mutually exclusive and may both play a role in the development of upper reproductive tract pathology and disease during primary and subsequent infections. The recent identification of plasmid-free strains associated with induced protection against immunopathology may provide a mechanism for immune stimulation leading to pathology and disease.

5. Past and present treatments, how to improve local immunity and future vaccine developments

5.1. Antibiotic treatment

Chlamydia infection of the FGT is largely asymptomatic with approximately 80% of women observing no reported symptoms [254]. Whilst there are no specific genital symptoms associated with Chlamydial cervical infection, 37% of women who develop cervicitis observe mucopurulent discharge and in 19%, hypertrophic ectopy (Distinctive oedema of the columnar epithelium in the female endocervix) [29, 62, 255]. Due to the asymptomatic nature of infection in the reproductive tract many infections remain undetected and therefore, untreated. Chlamydial infections, if symptomatic, can be treated through the administration of antibiotics. The current recommended antibiotic for treatment are either a single dose (1g) azithromycin or 7 day treatment with doxycycline (2x100mg per day)[256]. Other drug treatment programs used to treat *Chlamydia* infections in humans include erythromycin base, (4x500mg per day for 7 days), or erythromycin ethylsuccinate (4x800mg per day for seven days).

Erythromycin is effective against *Chlamydia* infection in the FRT however its use is associated side effects that may limit compliance [256]. Likewise, Levofloxacin (1x500mg per day for 7 days) and ofloxacin (2x300mg per day for 7 days) are effective for treating *Chlamydia* infections but are expensive compared to other alternatives [256]. Following antibiotic treatment it is recommended that treated persons abstain from sexual intercourse for 7 days to prevent spreading the infection to partners which may lead to subsequent re-infections [41]. Partner notification and partner treatment are also important targets to halt re-infections.

Although antibiotics are an effective treatment option, they have been shown to be less effective in cases of well-established chronic Chlamydial infections. Early reports determined that the current treatment of azithromycin and doxycycline are more that 95% effective at clearing *Chlamydia* genital tract infection [257, 259]. However, recent strong evidence suggests that antibiotic treatment failure could be greater than the 5% previously reported when accounting for re-infection [260]. A major hurdle in measuring the rates of antibiotic treatment failure is the inability to distinguish between recurrent infections and re-infection from infected untreated or new sexual partners [261]. Discrepancies in the proportion of treatment failures have been directly associated with testing methods used. Most of the published work investigating treatment failure involved tissue culture as a means to detect infection, which has now been predominately replaced with nucleic acid amplification testing (NAAT). Tissue culture is less sensitive and has shown to be less effective at identifying small numbers of persistent bacteria leading to an over estimation of successful *Chlamydia* eradication following treatment [257,262]. In a recent study by Batteiger *et al* (2010) investigating post treatment infection in adolescent girls, it was observed that 7.9% of those sampled developed recurrent infections following azithromycin treatment which was not attributed to reinfection [263]. Similarly, Goldern et al (2005) study in women without the risk for re-infection showed a recurring infection rate of 8% following azithromycin at 3-19 week follow-up representing treatment failure.[264]. Current studies argue that previous rates of antibiotic treatment failure of less than 5% were underestimated due to the available information at the time and that a true rate of treatment failure of may be greater than 8%. One major concern associated with increasing antibiotic failure is the development of antibiotic resistance. However, unlike other organisms such as *Staphylococcus*, to date there is no evidence of natural and stable antibiotic homotypic resistance (genetically inherited) *in vivo* in Chlamydial strains collected from human genital tract infections [265].

Contributing to antibiotic treatment failure in *Chlamydia* infections is the phenomenon called heterotrophic antimicrobial resistance. Heterotrophic resistance refers to the replication of heterogeneous population containing both resistant and susceptible bacteria from a subculture of a single resistant organism propagated on antimicrobial-containing medium, that is not genetically inherited [266]. It is hypothesized that at high bacterial loads a small population of *Chlamydia* organisms survive, potentially due to an innate ability to establish a latent/persistent infection [266]. Importantly these isolates at low loads do not show altered antibiotic susceptibility or increased resistance due to genetic changes [267]. Latent, non-replicating, non-infectious aberrant RBs associated with persistence can survive within host cells for extended periods of time at which infectious organism can not be isolated or able to be cultured.

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However, latent bodies can revert to infectious organisms leading to a recurrent infection. *In vivo* persistent infections in women are supported by the observation that some positive samples obtained using *Chlamydia*-specific DNA or antigen specific testing do not report the presence live infectious EBs via cell culture [268-270]. Furthermore, alternating infectious and persistent phases of Chlamydial growth correlate with acute and chronic infections *in vivo* [271]. During Chlamydial infection in women, a large variation in bacterial load has been observed in the genital tract and antibiotic failure due to heterotrophic resistance is associated with high bacterial loads [272, 273]. West *et al* (2005) showed that 91% of individuals with a low load at baseline observed no infection at follow up (2 months), whereas 74% percent of individuals with high bacterial load reported no follow up infection [274]. The development of persistent bodies *in vivo* during high bacterial load may be an important mechanism driving antibiotic failure, as these slow growing aberrant forms of *Chlamydia* may be less susceptible to antibiotic therapy. *In vitro* studies have shown that antibiotic treatment directly induces the presence of non-cultivable *Chlamydia* that can be reactivated with the withdrawal of treatment. *In vivo* the re-emergence of the active infectious form from persistence may take a number of weeks or months in which previous short term (<3 weeks) follow up testing via culture methods would provide a false negative result. Likewise false positive results may arise post-treatment due to persistent DNA detected through NAAT testing [275]. As such, one recommendation is that follow up screening > 5weeks post treatment is implemented for screening programs to determine treatment failure [259]. Estimating a true rate of treatment failures is complicated as many re-current infections remain unreported due to the asymptomatic nature of infections coupled with a lack of follow up testing in many screening and treatment programs.

5.2. Are screening and treatment programs leading to arrested *Chlamydia* immunity?

In many western countries, screening and antibiotic treatment programs for early detection of *Chlamydia* have been implemented over the past ten years. The goal of these programs is to reduce *Chlamydia* transmission leading to the long-term prevention of disease sequelae, including PID and infertility. *Chlamydia* infections are most common among young people accounting for approximately 80% of all infections. The United States Center for Disease Control (US CDC) estimates that in the United States alone, one in 15 sexually active females aged 14-19 years has *Chlamydia*. Due to this high burden, the US CDC recommends annual screening for all sexually-active women under 26 years of age [276]. As such, most programs have been specifically targeted towards young women with a focus to identify and treat asymptomatic infections. In addition, these programs seek to promote partner notification and treatment to prevent subsequent re-infection. Although screening and treatment programs coupled with aggressive education and health programs have been implemented in many countries for over a decade, *Chlamydia* rates have continued to increase. For example, the US CDC reported an increase of 9.7% of *Chlamydia*l infections between 2007 and 2008 [277]. This increasing trend has also be reported in many countries where long term screening and treatment programs have been in place including Sweden, Canada, the United Kingdom and Australia [278-281. Interpreting this increased prevalence or incidence of *Chlamydia* in the population requires caution and some argue that increases are directly associated with

increased testing rates and detection associate with easy home sampling and the development of the more sensitive nuclear acid amplification test in the 1990's.

However, it has been hypothesized that an increase in *Chlamydia* prevalence worldwide at a time of aggressive screening and testing programs is associated with 'arrested immune hypothesis' [24]. The 'arrested immune hypothesis' states that early detection and treatment of *Chlamydia* actually impairs the development of natural immunity in these antibiotic treated individuals. A recent study by Giesler *et al* (2013) supports the concept of antibiotic treatment may attenuate protective immunity against reinfection. This work showed that women with spontaneous resolution of *Chlamydia* infection were four times less likely to be re-infected compared to women with infections cleared by azithromycin treatment [25]. The potential hampering of protective immunity development through antibiotic treatment is believed to be the associated an increased *Chlamydia* re-infections. In the province of British Columbia, Canada, *Chlamydia* screening and treatment programs have been in place since 1991. Here they have reported a 5% increase in the re-infection rate over the course of the program [282]. Moreover, re-infection rates were reported to be greatest in young women and potential increased risk of development Chlamydial disease sequelae. The emergence of antibiotic resistant organisms, development of persistence, poor patient compliance and high re-infection rates has highlighted the need for an efficacious vaccine to halt the spread of infection throughout the population.

5.3. Chlamydia vaccine development

The development of an efficacious vaccine is the greatest potential to prevent infection and the subsequent development of disease. According to the World Health Organization a vaccine for the common STI "would have a significant impact on the spread of the disease". An ideal prophylactic Chlamydial vaccine would target immune responses to each of the two stages of the Chlamydial life cycle; (1) Antibodies directed against infectious extracellular elementary bodies preventing cell-adhesion; and (2) T cell mediated immune responses targeting infection cells during early to mid Chlamydial replication. Both responses require the activation of CD4-T cells, which have been shown through mouse models to be essential for clearing *Chlamydia* in the genital tract [283]. A number of considerations are required when designing vaccines; choice of antigen and adjuvant, administration route, the potential target of the vaccine (who to vaccinate?) and the purpose of the vaccine, prophylactic or therapeutic.

Early attempts in the 1950's and 1960's to develop a Chlamydial vaccine were based on the Pasteurian principle of isolate, inactivate and inject [284].However, although this has been a useful principle in the development of vaccine for veterinary field leading to a commercial vaccine for feline Chlamydial infections [285] a human whole-cell vaccine has been unsuccessful. Although crude whole cell vaccine trials in against ocular Trachoma in humans had observed short-term protection in 70% of vaccinated individuals, the development of strong pro-inflammatory immune responses to bacterial components led to adverse side effects in vaccinated individuals. Furthermore, vaccine trials using primates reported the generation of hypersensitivity leading to increased inflammation and extensive ocular scarring in vaccine recipients therefore whole *Chlamdyia* vaccine trials in humans subsequently were abandoned

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[286]. This work suggested that specific antigens within the *Chlamydia* proteome are associated with protection while others mediate immunopathology that may be shared between Chlamydial strains. The recent development of genomic and proteomic technology has provided an unbiased approach to antigen discovery for the generation of a sub-unit *Chlamydia* vaccine [287]. Antigen discovery approaches have included; (1) 2D gel elctrophoresis combined with immunoblotting or radio-immunprecipitation for the discovery of antibody specific antigens [288-290]; (2) Genome-wide protein expression to discover both antibody and T cell antigens [291]; (3) Antigen discovery using of T cell clone lines [292] and; (4) Immunoproteomic approach to identify peptides presented to CD4+ T cells on class II MHC molecules [293].

Sub-unit vaccines provide safer alternatives to whole cell preparations and a number of *Chlamydia* antigen candidates have been investigated [294]. A number of antigens have been used unsuccessfully in animal trials that include cHsp60 and Chlamydial lipopolysaccharide (cLPS). Chlamydial Hsp60 is an important pathogenicity factor for the development of human Chlamydial infection-associated disease sequelae [295]. Although *Chlamydia* specific immunity is generated, cHsp60 as an antigen was associated with the induction of inflammation limiting the potential as a vaccine antigen. Chlamydial LPS was a prime antigen candidate due to its localization to the outer cell membrane. Unfortunately, immunization with LPS does not induce the production either of post-immunization or of post-infection antibodies and does not confer protection following live bacterial challenge in primate ocular infection models [296]. Immunization with surface exposed membrane proteins have proved more successful in animal models. These include the Chlamydial major outer membrane protein (MOMP) and the polymorphic membrane protein family (Pmps). The 40kDa MOMP constitutes 50-60% of the outer membrane of the bacterium [297-299]. To date, Chlamydial MOMP has been the leading vaccine antigen candidate investigated in animal models, including non-human primate models. Within the many serovars of the *C. trachomatis* species a large amount of primary structural homology is observed, however, antigenic properties of MOMP are related to the serovar specific surface exposed variable domain (VD) regions. For example, serological studies have shown cross reactivity between the Chlamydial L2 and D serovars, but the G, H and I serovars differ significantly [300]. Therefore the use of Chlamydial MOMP from one serovar results in only serovar-specific immunity and this alone is not sufficient for an effective, protective, multi-serovar vaccine. Combining multiple MOMP proteins from specific serovars in a multi sub-unit vaccine may potentially protect against the most common human genital tract infections. Recently with the aid of immunproteomics Brunham and colleagues identified the expression of several Pmps which when used in mouse *C. muridarum* infection model led to the production of neutralizing antibodies *in vitro* and partial protective immunity *in vivo* [301, 302]. Pmp antigens have also been reported to be immunogenic in human infections [303]. In contrast to EB membrane-bound antigens, Chlamydial secreted proteins have been used as antigen targets. During intracellular *Chlamydia* replication, proteins are secreted from the inclusion body into the host cytosol and may therefore be available for processing and packaging on MHC molecules, thus specifically inducing T cell immunity. The secreted proteins known as Inclusion proteins (Inc) and Chlamydial proteasome/protease-like activity factor (CPAF) have been shown to be potential vaccine targets in mouse models [304, 305]. During Chlamydial infection of humans, both CPAF and Inc antigen specific responses are

observed supporting their potential as antigens in a human vaccine [305, 306] [305]. A number of Chlamydial antigens trialled as vaccine candidates have recently been reviewed by Hafner et *al.* [27].

Although sub-unit vaccines provide safer alternatives to the use of live or attenuated organisms, they are poorly immunogenic. Adjuvants are required to direct the immune response to a co-administrated antigen. For *Chlamydia*, a balance of Th2 driven neutralizing antibodies and Th1 cytokine production characterized by strong IFN-g within the FGT mucosae is required. A number of adjuvants have been developed for use experimentally in trials for Chlamydial vaccines. These include live viral vectors [307], immunostimulating complexes such as DDA and ISCOMs [308, 309, liposomes [310], detergent/surfactant-based adjuvants [311, Lipid formulations [312,313, *Vibrio cholerae* ghosts [314, unmethylated 1.7.4.1CPG-ODN bacterial motifs [308,222] and cytokines [315]. In addition to antigen and adjuvant combinations, the route of immunization is an important component of vaccination strategies. Historically most vaccines are delivered through injection that is either sub-cutaneous (S.C) or intra-muscular (I.M). Although effective in generating strong systemic immunity, the generation of mucosal immunity has shown to be varied and limited when using systemic immunization routes. However, these methods have shown in animal models to generate partial protection against *Chlamydia* infections. A recent study by Eko et al. (2011) using I.M. vaccination of *Chlamydia* antigens expressed by Vibrio Cholerae Ghosts (VVG) has shown to be effective in reducing the duration of infection in mice; however, sterilizing immunity was not observed in this trial [316]. Another approach is targeting the mucosal immune system directly with needle free mucosal routes. Not only are mucosal immunization regimes able to generate immunity and multiple mucosal surfaces, the needle free nature of delivery has the advantage of being safe and inexpensive to deliver. Immunization with candidate Chlamydial vaccines via mucosal routes such as oral [312], intranasal [223,317], intravaginal [318 and transcutaneous [222,223,313] have shown to target protective immunity in the female reproductive tract; again, however sterilizing immunity has not yet been achieved using these vaccine approaches.

Over the past 60 years since the first crude whole cell Chlamydial vaccines were investigated, a number of technologies such as gemomic sequencing have enhanced the forward movement of the development of a protective Chlamydial vaccine. In light of the continued increased infection and re-infection rates, even with aggressive screening and treatment programs, the need for an efficacious Chlamydial vaccine is essential. Mathematical modeling that simulates transmission of *Chlamydia* in a heterosexual population has been developed to determine the impact of an efficacious vaccine regime [319]. The model tracks the infection time course, disease progression, and dynamic infectiousness of infected individuals and the transmission to others. This model determined that a fully protective vaccine administered prior to sexual debut could eradicate Chlamydial epidemics within 20 years. Furthermore, the specific targeting of women (100% vaccine coverage) would have an increased impact in reducing epidemiology than vaccination 50% of the male and female population together. However, currently no vaccine has been shown to generate sterilizing immunity. Modelling reports that in the absence of a sterilizing vaccine, a *Chlamydia* vaccine effective for at least 10 years would provide order to lead to population-level eradication. Furthermore, a non-sterilizing vaccine

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could protect individuals by raising the infectiousness threshold and secondary reduce the peak load and the duration of the infection in vaccinated individuals. Animal models have shown that some experimental vaccines, although not producing sterilizing immunity in these models, may reduce the development of pathology e.g. hydrosalphinx in the murine model. This suggests that a therapeutic vaccine could potentially be given to women a past or even current infection to prevent the development of pathology. Although little work has been undertaken in this area, Carey et al. reported that in the mouse *C. muridarum* model vaccination during or after infection reduction in the strength of the immune response was observed. This work demonstrates that a therapeutic vaccine could be used to limit an uncontrolled host response leading to immunopathology during re-infection [320]. In light of the fact that there currently is no vaccine to prevent Chlamydial genital tract infections in women we believe that a vaccine designed to reduce bacterial burden and prevent disease may be a more rational target for future research.

6. Summary/conclusions

In this Chapter we have presented current knowledge of immune regulation during infections of the female genital tract caused by the intracellular bacterial pathogen, *Chlamydia trachomatis*. Our review has highlighted the following information regarding Chlamydial infections of the FGT:

- Tubal infertility and reproductive damage, preeclampsia and preterm births may potentially occur in many of the 50 million women who are annually infected with *Chlamydia trachomatis*
- Gynecological cancer development is also suggested to be linked with Chlamydial genital tract infections.
- The majority (70-80%) of genital tract infections in women are asymptomatic
- The annual direct medical cost of sexually transmitted diseases (including *Chlamydia* and those caused by HIV) has been estimated at $US16.9 billion
- Urogenital isolates of *C.trachomatis* may also contain a highly conserved 7.5kB non-integrative plasmid that has a role in the pathobiology of these organisms
- *Chlamydia* infections of neonates have been reported to be at 18 percent for ophthalmia neonatorum and 16 percent for pneumonia for infants exposed to *C. trachomatis* in vaginal secretions in the birth canal
- The host inflammatory response to *C.trachomatis* is involved in immunity but also the pathology that leads to serious morbidities of chronic pelvic pain, EP and tubal infertility following female genital tract infection
- Disease outcomes are dependent upon the complex interactions between virulence factors of, and evasion strategies used by, *C.trachomatis* and host immune responses including variants in genetic markers associated with infection and inflammation

- It is becoming increasingly well documented that sex hormones E2 and P4 regulate host susceptibility, as well as innate and adaptive immune responses to the sexually transmitted pathogen *Chlamydia* infecting the mucosal surfaces of the FGT
- In light of the continued increase in infection and re-infection rates even with aggressive screening and treatment programs, a need for an efficacious vaccine is essential.
- Finally, since there currently is no vaccine to prevent Chlamydial genital tract infections we consider that a vaccine designed to reduce bacterial burden and prevent disease pathology may be a rational target for future Chlamydial research.

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