Genital tract $CD4^+$ T cells for vaccination and protection against *Chlamydia trachomatis*

Ellen Marks

GÖTEBORGS UNIVERSITET

Department of Microbiology and Immunology,
Institute of Biomedicine at Sahlgrenska Academy,
The University of Gothenburg,
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To Mum, Dad and G.G.

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Abstract

Vaccination strategies for protection against sexually transmitted diseases are lacking due to an incomplete understanding of genital tract T cell responses. This thesis dissects the generation of T helper subsets, including the recently discovered Th17 subset, during genital tract infection with a common sexually transmitted pathogen, *Chlamydia trachomatis,* and addresses vaccine requirements for the generation of genital tract CD4$^+$ T cell immunity.

Our studies demonstrate the presence of anatomically distinct T helper differentiation patterns in the genital tract. C57BL/6 mice were infected with *C. trachomatis* and the response in the upper genital tract (UGT) was found to be dominated by Th1 cells, whereas the lower genital tract (LGT) hosted Th2 cells in the presence of IL-10-producing DCs. Additionally, Treg and Th17 responses were demonstrated in both the UGT and LGT following infection.

For the generation of T cell-mediated immunity against infection, costimulatory signals through CD28 were critical. We found that T helper differentiation and Treg responses to infection were impaired in both the UGT and LGT of CD28$^{-/-}$ mice. In contrast, in the absence of ICOS-signaling we observed enhanced elimination of bacteria and the development of protective immunity. Here, intense Th1 cell differentiation dominated and we found reduced regulation through both IL-10 and FoxP3$^+$ Tregs. Paradoxically, in mice lacking both CD28 and ICOS molecules (DKO), primary infection with *C. trachomatis* was eliminated more rapidly than in CD28$^{-/-}$ mice. These mice failed to develop protective immunity against reinfection similarly to CD28$^{-/-}$ mice. As in ICOS$^{-/-}$ mice, Th1 differentiation in the LGT was enhanced in DKO mice. This indicated that ICOS costimulation modulates the immune response even in the absence of CD28-signaling, leading to augmented inflammatory immune responses in the genital tract during *C. trachomatis* infection.

The generation of CD4$^+$ T cell immunity is also key to vaccination against other STDs. Because of this we studied intravaginal (i.vag) immunization for priming of CD4$^+$ T cells in the genital tract. We investigated the requirements for progesterone or estradiol for successful immunization. Both intranasal and i.vag delivery of cholera toxin conjugated to ovalbumin peptide (CT-OVA) induced T cell responses in the draining lymph node, however, i.vag immunizations were absolutely required in order to attract T cells to the genital tract mucosa.

In conclusion, the results presented in this thesis provide evidence of anatomically divided T cell immune responses to *C. trachomatis* in the genital tract. Understanding of T cell responses in the genital tract are has important implications for the generation of protective immunity and immunopathology.

Keywords: *Chlamydia*, T cell differentiation, costimulation, Th1, Th2, Th17, Tregs, vaccination.
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Original Papers:

This thesis is based on the following papers, which are referred to in the text by their assigned Roman numeral (I-IV):

I. **IL-10 producing vaginal DC inhibit Th1 responses to *Chlamydia trachomatis* infection.**
Ellen Marks, Miguel Tam, Nils Lycke.
Submitted manuscript.

II. **Differential CD28 and inducible costimulatory molecule signaling requirements for protective $CD4^+$ T-cell-mediated immunity against genital tract *Chlamydia trachomatis* infection.**
Ellen Marks, Martina Verolin, Anneli Stensson, Nils Lycke.
Infect. Immun. 75(9):4638-47 (2007).

III. **Th1 differentiation in the absence of CD28 and ICOS signaling rescues host immune responses to a primary genital tract infection with *Chlamydia trachomatis*.**
Ellen Marks, Anneli Stensson, Woong-Kyung Suh, Nils Lycke.
Manuscript.

IV. **Vaccination of the genital tract for the generation of $CD4^+$ T cell immunity.**
Ellen Marks, Anja Helgeby, Karin Schön, Nils Lycke.
Manuscript.

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Abbreviations

APC	Antigen presenting cell
CT	Cholera toxin
DC	Dendritic cell
EB	Elementary body
FAE	Follicle-associated epithelium
FoxP3	Forkhead box P3
GATA-3	GATA binding protein 3
ICOS	Inducible costimulatory molecule
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
ING	Inguinal lymph node
iNOS	Inducible nitric oxide synthase
i.n.	Intranasal
i.vag	Intravaginal
HSP	Heat shock protein
HSV	Herpes simplex virus
LGT	Lower genital tract
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MOMP	Major outer membrane protein
NK	Natural killer
NOD	Nucleotide-binding oligomerization domain
OVA	Chicken ovalbumin
PALN	Para-aortic lymph node
RB	Reticulate body
ROR	Receptor tyrosine kinase-like orphan receptor
STAT	Signal transducers and activation of transcription
STD	Sexually transmitted disease
T-bet	T-box expressed in T cells
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
Treg	T regulatory cell
UGT	Upper genital tract
WT	Wild-type

INTRODUCTION

Chlamydia trachomatis as a major sexually transmitted pathogen

Chlamydia trachomatis infection in developed countries is best known as a sexually transmitted disease (STD), however in the most impoverished countries it is also the leading cause of preventable blindness (1, 2). The intracellular bacterium, *C. trachomatis* has infected mammals for hundreds of millions of years (3). Two to five million years ago the ocular strains of *Chlamydia* diverged from the genital strains giving rise to the classification we today know of as serovars A-C which infect the ocular mucosa, the genital tract pathogens serovar D-K, and the L1-L3 that initially infect the local epithelium, but which disseminate to the surrounding lymph nodes.

Chlamydia infections of mankind are thought to originate from central Asia with some of the earliest records dating back to 2700B.C., when the emperor Huang Ti Nei Ching underwent surgery for trichiasis (reviewed (4)). Hippocrates was amongst many historical physicians who documented the ocular disease, trachoma, comparing the clinical appearance of the *Chlamydia* infected mucosa to the red and swollen flesh of a ripe fig. Before the development of antibiotics, curious therapies were used for treatment of infection, such as those found in ancient Egyptian Papyrus: “pull hairs and apply mixture of myrrh, lizard’s blood, and bats blood until healed or a mixture of fly’s dirt, red ochre and urine.” (reviewed (4)).

Despite the long history of human affliction with chlamydiae, little progress has been made in eradicating the pathogen. The number of infections are estimated to be in the order of 90 million annually (2). WHO has set 2020 as the target for elimination of ocular chlamydial infections (5), more than 10 000 years after it was first documented. No similar target has been set for eradication of the genital tract pathogen. The incidence of the STD caused by *Chlamydia* infection is steadily rising in many areas of the world, with infection rates reaching 2-5% of the population in many industrialized countries (6, 7). In 2007, the number of cases in Sweden reached record levels, with over 47 000 cases reported (8).

The genital tract infection caused by *C. trachomatis* can have severe long-term complications and if untreated the bacterium may ascend to the fallopian tubes where it can persist for several months or even years (reviewed (9)). Antibiotics are readily available for treatment, however approximately 70-90% of women and 30-50% of men remain asymptomatic during infection (10). Late or absent diagnosis has resulted in *Chlamydia* being the most significant cause of tubal factor infertility (11), driving the growing interest in the development of prophylactic vaccines.

C. trachomatis

Chlamydiae are gram-negative, obligate intracellular bacteria. The genus *Chlamydia* includes the human pathogens *C. pneumoniae* and *C. trachomatis*, which infect the respiratory and oculogenital mucosa, respectively. There are a number of serovariants of *C. trachomatis*, which cause trachoma, sexually transmitted disease, or infect the genital epithelium before disseminating to the lymphatics causing lymphogranuloma venereum.

Common to all chlamydiae is a unique biphasic developmental cycle, whereby small, infectious, but metabolically inert, elementary bodies (EBs) attach to the host epithelium (Fig.1). EBs induce endocytosis into a vacuole, termed an inclusion, and within 2-6 hours after internalization differentiate into metabolically active, but non-infectious, reticulate bodies (RBs). RBs divide exponentially by binary fission before condensing back into EB form, which are then released from the cell by disruption of the host membrane followed by the inclusion membrane, allowing for further propagation of the infection (12).

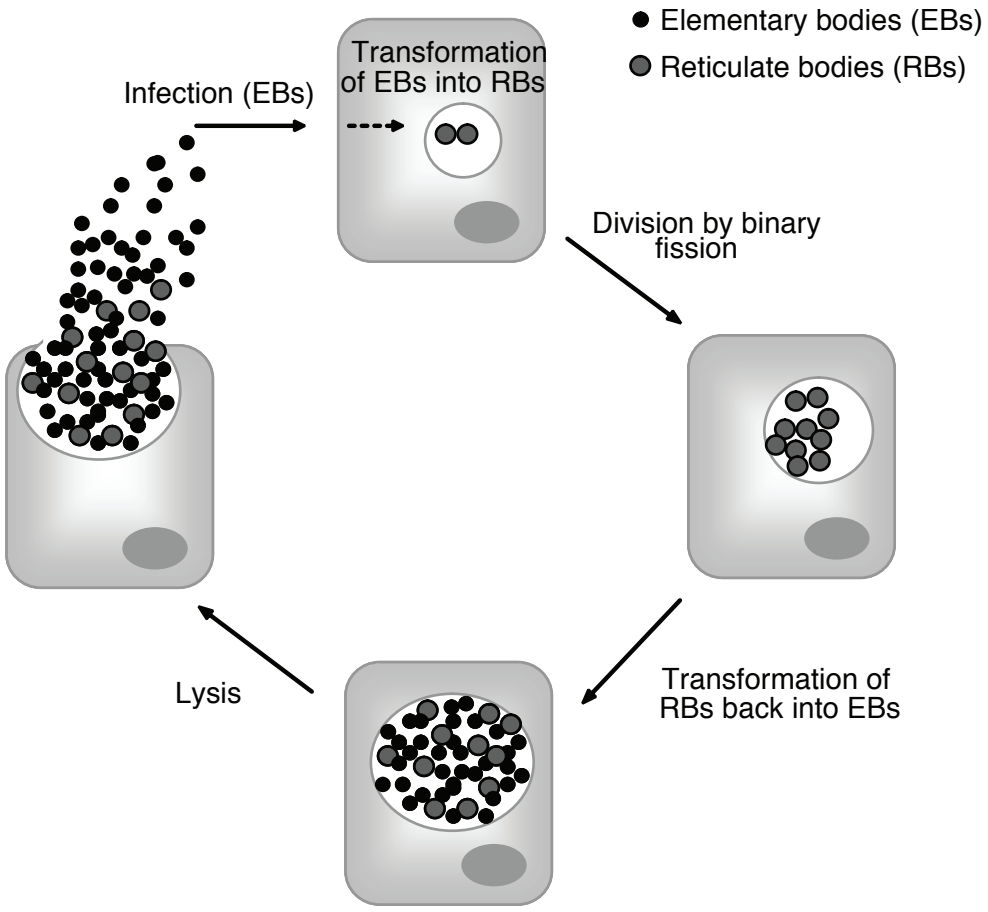


FIGURE 1
The unique biphasic life-cycle of *Chlamydia*. EBs induce their own endocytosis into inclusion bodies inside epithelial cells where they transform into RBs. RBs divide by binary fission and differentiate back into infectious EBs. The EBs are released following lysis of the cell for propagation of infection.

Immunity to *C. trachomatis*

The genital tract is the portal of entry for a number of pathogenic organisms as well as the host to a commensal flora that colonizes the lower genital tract (LGT). Accordingly, there is a full repertoire of immmunocompetent cells at this mucosal site which carry out functions of tolerance against the flora, allogeneic sperm and fetus, as well as protecting against pathogenic organisms. The induction of T and B cell responses in the female genital tract is likely to occur both locally and through recruitment from distant inductive sites. The genital tract is considered to lack local follicle-associated epithelium (FAE) associated with inductive sites, such as the Peyer's patches of the small intestine. However, the human female genital tract contains lymphoid aggregates, consisting of $CD8^+$ T cells with a core of B cells, surrounded by an outer mantle of macrophages, which, upon infection, mature into lymphoid follicles containing germinal centers (13, 14). Another feature that distinguishes the genital tract from other mucosal sites is the influence of sex hormones, which regulate the number and type of cells in the genital tract mucosa at different phases of the menstrual cycle (reviewed (15)). The influence of sex hormones will be discussed in more detail in the section on vaccination of the genital tract.

Innate Immunity

The innate immune response encompasses rapid and non-specific mechanisms of defense against invading pathogens and may have profound implications for the eventual outcome of adaptive immunity generated during infection. Innate defenses include soluble molecules, epithelial barriers, anti-microbial substances, the detection of pathogen-specific molecular structures, as well as the actions of macrophages, neutrophils, dendritic cells (DC), natural killer (NK) cells and NKT cells. An important feature of innate immunity is the production of cytokines which act as antimicrobial substances directly, or which could activate and stimulate adaptive immunity. Chlamydial infection results in the expression of a plethora of innate cytokines such as IL-1β, IL-6, TNF-α, GM-CSF, IL-8, type I IFNs, and IL-12 (16-18).

A fundamental function of the innate immune response is recognition of invading pathogens. Expression of toll-like receptors (TLRs) can be found on many cells of the innate immune system, including epithelial cells, macrophages, and DCs. TLRs are pattern recognition receptors (PRRs) and enable cells to recognize conserved molecules in bacteria, which are distinguishable from host molecules. Expression of TLRs 1-9 have been shown in the genital tract, however, the level of expression is anatomically diverse. For example, TLR4 is weakly expressed or even absent from the LGT, where TLR2 expression dominates (19). Potentially, *Chlamydia* recognition could occur via TLR2 or TLR4, which recognize bacterial lipoproteins, or LPS and heat shock proteins (HSPs), respectively. Evidence, however, suggests that neither TLR2 nor TLR4 are critical for initiation of the immune response to *C. trachomatis*, since $TLR2^{-/-}$ and $TLR4^{-/-}$ mice were unimpaired in their clearance of chlamydiae from the genital tract. However, $TLR2^{-/-}$ mice developed less immunopathology compared to wild-type (WT) mice (16), and this

has also been suggested in human studies (20, 21).

Another family of PRRs is the intracellular nucleotide-binding oligomerization domain (NOD) protein family, including NOD1 and NOD2, which recognize ligands including LPS and peptidoglycans (22, 23). The intracellular character of *Chlamydia* infection makes NOD-receptors a possible mechanism for pathogen recognition. Although peptidoglycan is encoded in the genome of *Chlamydia*, studies have yet to demonstrate conclusively if it is expressed during infection (24). *Chlamydia* infected epithelial cell lines have been shown to upregulate NOD1 expression and increase IL-6 and MIP-2 production, which are strongly impaired in $NOD1^{-/-}$ mice (25), suggesting that NOD receptors may be involved in the innate immune response to *C. trachomatis*.

Following recognition of an invading pathogen, cellular components of the innate immune system are activated to limit infection. As aforementioned, the production of cytokines early in *Chlamydia* infection strongly influences the resistance of the host to infection by activation of phagocytitic cells as well as imprinting effects on the protective adaptive Th1 immune response. NK cells contribute to host protection against *Chlamydia* through direct lysis of infected cells as well as early IFN-γ production (26, 27). This IFN-γ production occurs as early as after 7 days of infection, a time point when the adaptive immune response is not fully established, suggesting that NK cells are the likely source (27, 28). However, in the absence of IFN-γ, bacterial colonization is not enhanced at this early stage of infection, and therefore it is likely that this IFN-γ production is required for polarization of the immune response towards Th1, and consequent down-regulation of the Th2 response, rather than directly impairing bacterial growth (29).

Adaptive immunity

The adaptive immune response develops over a number of weeks following infection and is necessary for clearance of the bacterium as well as for protection against reinfection (30). *Chlamydia* infection can ascend into the fallopian tubes where the effector T and B cells generally eliminate the pathogen from the genital tract. Protection against reinfection with *Chlamydia* is, at best, partial and serovar specific (31). The adaptive immune response to *Chlamydia* contains elements of $CD4^+$ T cell responses, $CD8^+$ T cells responses and B cell responses, although their relative contributions are not equivalent (30, 32, 33).

Antibodies have an important contribution in the protection against STDs, such as human papilloma virus (34, 35). However, in spite of local and systemic *Chlamydia*-specific antibodies produced following infection, the importance of these antibodies for protection is controversial. Mice lacking B cells, and therefore antibodies, are unimpaired in their ability to clear *Chlamydia* infection (36-39). Furthermore, vaccine candidates that elicit only high titers of specific

antibodies and no $CD4^+$ T cells are non-protective (29, 33, 40). Notwithstanding this, recent studies have clearly shown that antibodies may contribute to resistance and dampening of the immunopathogenesis associated with infection (36-40).

Antibody secreting plasma cells are present in the lamina propria of the endocervix but are scarce in the vagina, however, serum-derived IgG may also contribute to the antibody repertoire in the genital tract. Unique amongst other mucosal surfaces of the body, the dominant antibody isotype in the female genital tract is IgG, while IgA is present in significant amounts only in the cervical mucosa and fallopian tubes (reviewed (41)). The antibody-dependent mechanisms of protection against reinfection with *Chlamydia* are unknown. However, antibodies in general have many immune effector functions. For example, antibodies may act as opsonins, coating the EBs for complement-mediated elimination. Binding of antibodies to the surface of the pathogen can stimulate effector cells which express the Fc-receptor (FcR), leading to killing of the bacteria. Indeed, studies in $FcR^{-/-}$ mice showed a somewhat reduced resistance against infection, due to decreased antibody-dependent cell-mediated cytotoxicity (ADCC) and reduced antigen presentation for the generation of protective Th1 responses (38).

Specific antibodies may also neutralize infection by preventing bacterial up-take or host cell invasion. Immunization with antigenic proteins from *Chlamydia*, such as the major outer membrane protein (MOMP), results in high antibody titers in both serum and mucosal secretions, which display strong neutralizing properties of infectious particles *in vitro* (42). Despite this, these antibodies are not sufficient to protect against infection in non-human primates (42). The neutralizing efficiency of *Chlamydia*-specific antibodies is likely to be affected by titers, since between 10^3 and 10^4 *Chlamydia*-specific monoclonal Abs are required for 50% neutralization of a single infectious *Chlamydia* EB particle (43). This large number of binding sites on the surface of *Chlamydia* EBs (44) far exceeds the number of antibodies required to neutralize some viral particles (45). However, antibody-mediated immunity against *Chlamydia* infection is likely to be more complicated than simply insufficient titers; passive transfer of immune serum does not protect against a primary infection, only against reinfection, a fact which argues against a neutralizing or complement activating mechanism (36). Examples of the role of antibodies in protection against other STDs, where protection is also modulated largely by $CD4^+$ T cell immunity, have been reported. In the mouse model of genital herpes simplex virus-2 (HSV-2), priming of the host with an attenuated thymidine kinase mutant HSV-2 via the intravaginal (i.vag) route provides life-long protection against challenge with virulent WT HSV-2 (46). Iijima *et al.* demonstrated that depletion of DC or B cells alone did not affect the immunity provided by vaccination (47). However, mice that were depleted of both DC and B cells showed that both populations are required for maximal Th1 memory responses *in vivo* (47). The precise mechanism of this partial protection mediated by B cells is unclear.

The generation of appropriate effector and memory responses to *Chlamydia* is dependent primarily upon $CD4^+$ T cells (29, 30, 33, 48). In the absence of $CD4^+$ T cells infection is not controlled (49). Mice deficient in IFN-γ, MHC-II, or IL-12 or WT mice depleted of $CD4^+$ T cells, are severely impaired in clearance of infection (29, 48-50). The protective effects elicited by $CD4^+$ T cells are partially mediated through the production of IFN-γ and are of the T helper 1 (Th1) subset of effector T cells. Various studies have shown that Th1-dominated immunity is protective, and that Th2 cells are associated with delayed clearance (51, 52). Furthermore, mice which have impaired $CD8^+$ T cell responses (30) or mice lacking $CD8^+$ T cells, but hosting $CD4^+$ T cells, such as the β2-microglobulin deficient mice, effectively eliminate a genital tract infection (49). However, both *Chlamydia*-specific $CD4^+$ and $CD8^+$ T cells are generated as part of the adaptive immune response and cytolytic activity of $CD8^+$ CTLs has been demonstrated during *Chlamydia* infection (53).

The generation of $CD4^+$ T cell-mediated immunity

The immune response to an invading pathogen is orchestrated by a complex sequence of events that gives rise to activation and differentiation of T cells into distinct T helper cell (Th) subsets with vastly diverse effector functions. This process begins with antigen uptake by APCs at the site of infection, and migration of the APCs to the draining lymph node, where antigen is presented to naïve $CD4^+$ T cells by mature APCs. In parallel to the antigen-specific interactions between the T cell and APC, costimulatory molecules on the T cell interact with their ligands on the mature APC resulting in activation of the T cell. Activated T cells then undergo intrinsic changes during their differentiation into specific T effector subsets, namely, Th1, Th2, Th17 and T regulatory cells (Tregs), as well as memory cells. These memory subsets are capable of rapid reactivation and expansion for protection against reinfection (Fig. 2).

The recruitment and differentiation of distinct T helper subsets which are best suited to eliminate a particular pathogen is partially determined by the cytokine environment and the costimulation provided by the APC (54-57). Binding of cytokines to their receptors stimulates a signaling pathway which begins with the activation of receptors-associated Janus family tyrosine kinases (JAKs). JAKs are then able to phosphorylate and activate transcription factors called signal transducers and activators of transcription (STATs). STATs regulate a wide variety of genes, including those involved in T cell differentiation (Fig. 3). Different members of the STAT family control networks of subset-specific gene-expression, which ultimately silences gene transcription of the opposing T helper subsets. The dominant T helper subset generated during an immune response can have important implications for elimination of the pathogen, as well as contribute to the extent of immunopathology.

Th1 cell differentiation is promoted by IFN-γ, which activates STAT1, and IL-27 which signals through STAT1 and STAT4, resulting in upregulation of the expression of the transcription

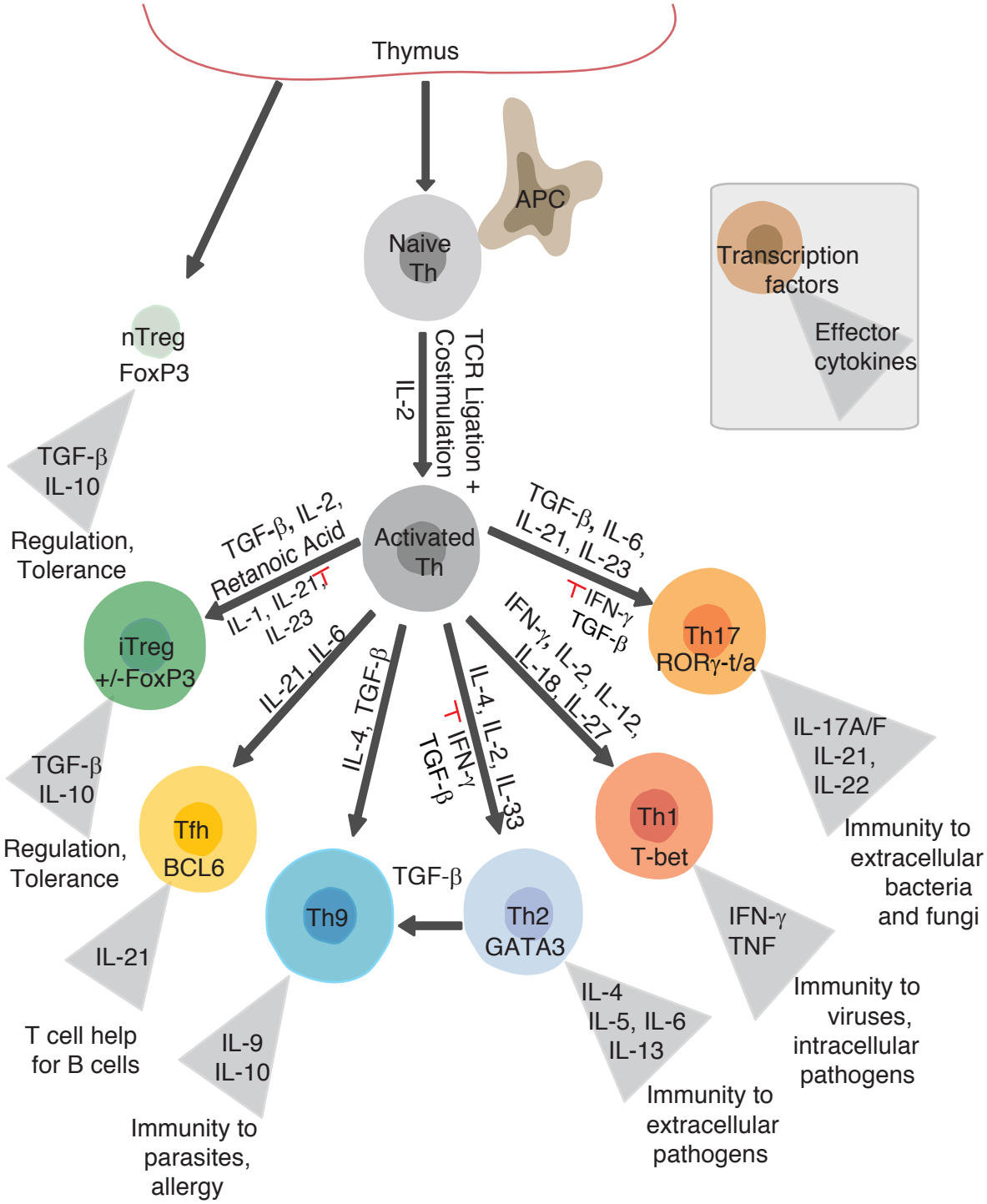


FIGURE 2
Differentiation of naïve T cells into unique T helper subsets. Binding of cytokines to their receptors stimulates signaling pathways that control expression of subset-specific gene-expression.

factor, T-bet (58-60). T-bet drives further transcription of IFN-γ gene, and silences genes encoding transcription factors of opposing T helper subsets, such as GATA-3 (61). Simultaneously, the IL-12-receptor is upregulated on the T cell, which binds IL-12 produced by APCs and mediates the activation of STAT4, which also acts to drive IFN-γ production and thereby reinforces Th1 commitment (62). The effector functions of Th1 cells, such as IFN-γ production, are critical in the defense against a number of pathogens, including *Salmonella enterica,* HSV-2 and critically protects against *C. trachomatis* infection in the genital tract (29, 63-65).

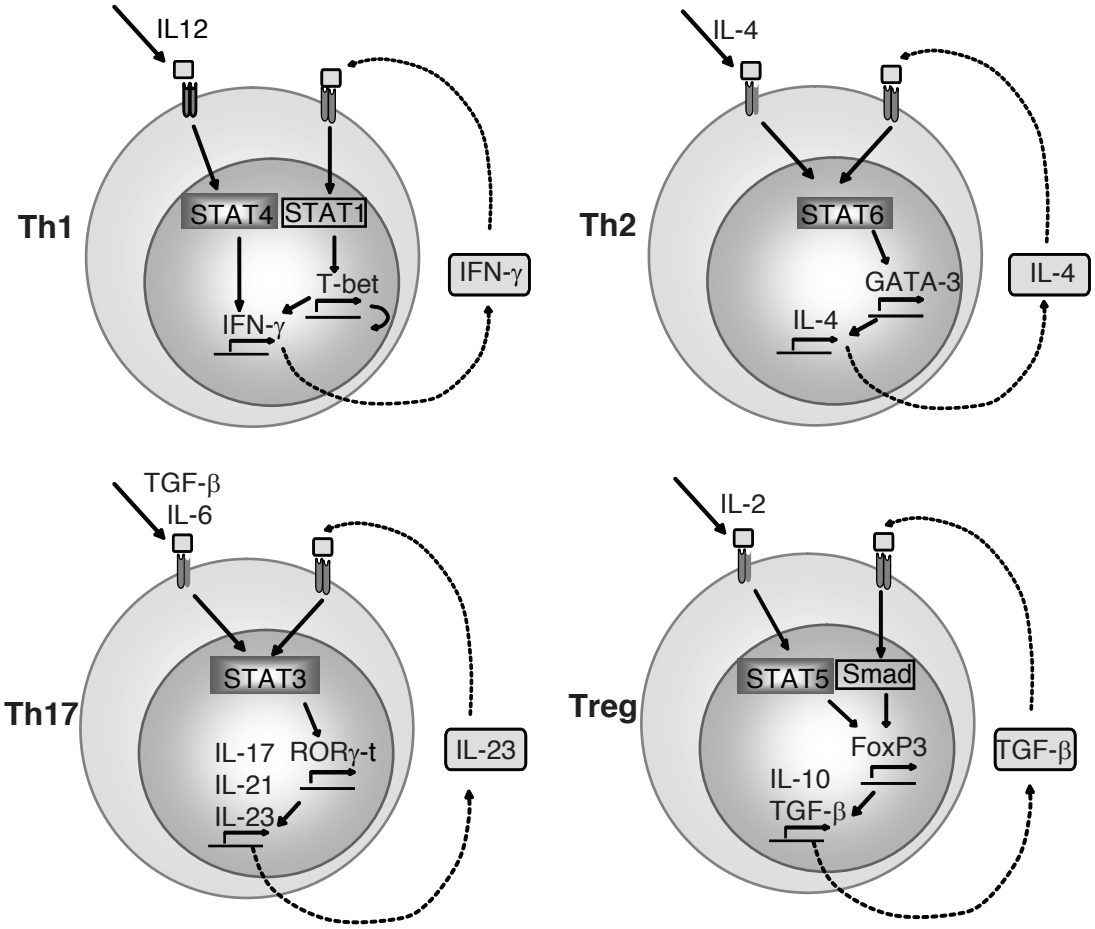


FIGURE 3
STAT-mediated pathways of T helper differentiation. Naïve T cells differentiate towards T helper subsets in the presence of certain cytokines. Cytokine binding to receptors initiates JAK/STAT signaling in the T cell which results in lineage-determining transcription factor expression.

In contrast to Th1, the Th2 subset develops following IL-4-mediated activation of STAT6, which stimulates the expression of the transcription factor GATA-3 (66, 67). The Th2 subset is characterized by a signature cytokine profile which includes the production of IL-4, IL-5 and IL-13. These cytokines are effective in the immune defense against helminths and other parasites, and also contribute to the pathogenesis seen in allergy and autoimmune diseases.

Many studies have attributed immune responses to either Th1 or Th2 polarization. However, the recognition of an additional T helper subset, Th17 cells, helped to explain inconsistencies in the Th1-Th2 paradigm. Th17 cells arise following the stimulation by TGF-β, IL-6, and IL-21 or IL-23, which activate STAT3 and subsequently induces expression of the transcription factor RORγ-t or RORγ-α (68). Characteristic for Th17 cell is the production of inflammatory cytokines including IL-17A, IL-17F, IL-21 and IL-23 (reviewed (69)). The existence of the Th17 subset was established in the experimental allergic encephalitis model and has since been attributed a role in the immunopathology seen in autoimmune disease (70). Moreover, Th17 cells are known to influence neutrophil migration and macrophage activation. Hence, neutralization of the Th17 signature cytokine, IL-23, results in impaired clearance of *Mycoplasma pneumoniae* infection and IL-17-deficient mice infected with *Salmonella enterica* carry higher bacterial loads than control mice (71, 72). In addition to enhancing protection against infection, Th17 cells also exhibit exacerbated inflammation resulting in severe immunopathology. Chronic gastric inflammation in *Helicobacter pylori* infected patients show increased IL-17 levels in the gastric mucosa (73). Although the Th17 subset is a major source of IL-17 production, IL-17 can also be produced by RORγ-t$^+$ γδ$^+$ T cells (71, 74). T cells expressing the γδ$^+$ TCR are innate-like T cells that are enriched at mucosal surfaces. Unlike the αβ$^+$ TCR, the murine γδ$^+$ TCR interacts with the MHC-I-related proteins T10/T22 (75) and have been shown to be critical for the maintenance of the Th2 bias in the genital tract during pregnancy (76). Taken together, this demonstrates that Th17 cells and IL-17 production contribute not only to host defense against invading pathogens, but also contribute to immunopathology. To date, the role of IL-17 and Th17 in immunity against *C. trachomatis* is poorly understood.

Recent studies have revealed the presence of a possible fourth helper subset, Th9, which produce IL-9 in large quantities and contributes to immune responses during allergy and parasitic infections (77). Previously, IL-9 production has been attributed to Th2 cells, however, *in vivo* studies have revealed that IL-9 producing T cells do not express any of the previously described transcription factors, T-bet, GATA-3 or RORγ-t, which can be used to identify Th1, Th2, and Th17 subsets respectively (78), however, the existence of a unique transcription factor in Th9 cells has yet to be found. It is unclear if Th9 cells truly represent a unique subset, or if they arise from reprogramming of Th2 cells.

IL-9 production in response to *C. trachomatis* infection is not well documented. He *et al*., have suggested that superior immunity seen in IL-10 deficient mice is mediated through inhibition of LEK1-related cytoskeletal events, that are related to APC maturation, antigen processing and presentation (79). In this study, He *et al*. showed that LEK1- knockdown DCs respond to *C. trachomatis* antigens with enhanced IL-9 production but not IL-4 or IL-10 production (79). If this represents the emergence of the Th9 subset in the absence of IL-10-mediated effects is unclear, however, further investigations into this subset are warranted in order to determine if

Th9 cells play a role in protection against *C. trachomatis.*

Effector cell functions are kept in check by regulatory elements in order to limit aberrant inflammation and for the maintenance of self-tolerance. A hallmark of Treg activity is suppression of the proliferative responses of $CD4^+$ T cells. The exact mechanisms responsible for inhibition are still unclear. There are two main categories of Tregs; those which occur naturally and are thymically-derived (nTreg), and those that are induced in the periphery from naïve $CD4^+$ T cells following antigenic stimulation under certain conditions (iTreg). Several subsets of Tregs can be characterized on the basis of expression of the transcription factor FoxP3, i.e. nTregs and the TGF-β-induced iTregs which arise in the periphery (reviewed (80)).

The development of iTregs from conventional naïve $CD4^+$ T cells in the periphery is influenced by the context in which antigen exposure occurs. The presence of IL-10 can result in the induction of $FoxP3^-$ iTregs, which are also named Tr1 cells, while the presence of TGF-β generates iTregs, also termed Th3 cells. These iTregs are thought to partially exert their suppressive functions through the production of IL-10 and/or TGF-β (81, 82). Several factors are highly influential on iTreg development in the periphery, including the antigen itself, cytokines released, and the type of APC. There are several types of DC that induce iTreg formation. For example, plasmacytoid DC (pDC) have this ability (83), as well as tolerogenic myeloid DC (84), gut-associated DC (85) and DCs found in tumors (84). Conversely, iTreg-mediated production of TGF-β or IL-10 can imprint DC with tolerogenic properties for further induction of iTregs. Not all DCs display this ability, for example splenic DCs are unable to induce Foxp3 expression in naïve T cells, whereas DCs of the small intestine can readily stimulate Tregs (85).

The mechanisms of suppression employed by Tregs are not fully understood. However, studies have revealed roles for inhibitory cytokines such as IL-10, TGF-β or IL-35 or via granzyme A/B-mediated cytolysis (86-89). IL-2 deprivation is also thought to be an effector mechanism of Tregs, and represents a form of metabolic disruption which results in apoptosis of the conventional $CD4^+$ T cell (90). Programmed cell death-1 (PD-1) has been shown to be one likely mechanism of IL-2 deprivation. PD-1 was found to accumulate intracellularly in Tregs, compared to membrane expression in activated T cells (91). Intracellular PD-1 controls Treg proliferation by limiting STAT-5 phosphorylation that is caused by the capture of high amounts of paracrine IL-2 (92). In this way Tregs can suppress T effector cells by deprivation of IL-2 for proliferation. Additionally, Tregs are capable of augmenting the ability of APCs to stimulate conventional $CD4^+$ T cells through costimulatory molecules, such as the inhibitory signals delivered via interactions between CD80/86 and CTLA-4 (93).

The presence of Tregs alters the clearance of invading pathogenic organisms. It is thought that inhibition of inflammation reduces clearance of the pathogen and can result in persistent

infection. Furthermore, depletion of Tregs in the model of *Leishmania major* infection results in sterilizing immunity (94). However, Treg functions are also vital to avoid tissue damage created during clearance of a pathogen. While the role of Tregs in a variety of diseases has been investigated, the role of regulatory cells in the genital tract during *C. trachomatis* infection has yet to be addressed. Furthermore, the genital tract mucosa is host to a unique repertoire of T cells, housing both thymically-derived and extra-thymically derived T cells. The majority of $CD4^+$ T cells express the αβ TCR, while 20% express γδ TCR (95). Additionally, Johansson *et al*. identified a population of $CD3^+CD4^-CD25^+$ extrathymically-derived T cells, expressing the αβ TCR, in the uterine mucosa of mice, which appear to have regulatory properties (96). The significance of the distinct T cell populations in the genital tract for protection against pathogens is still to be elucidated.

Costimulation

Early studies of specific immune responses led to the discovery of the TCR, however it was shown that TCR-mediated signals were not alone in activation of naïve T cells, and that a second signal was necessary to avoid anergy. This second signal results from interactions of costimulatory molecules on the APC and their ligands on T cells. Costimulatory signals are delivered by a vast constellation of surface molecules with differential functions and complex interactions. These signals influence not only T cell development and functions, but also act on other cell types including DC and B cells.

A large number of molecules belonging to the category of costimulatory molecules. For example, CD28, is expressed constitutively on naïve T cells, and interacts with CD80 and CD86 on APC. CD80 expression is present on immature DC, however, the expression of both CD80 and CD86 is upregulated following maturation of the DC. CD28-mediated signaling is largely stimulatory, resulting in proliferation of T cells, IL-2 production (97), enhanced survival (98), and cytokine production (99). CD28-costimulation also promotes the development of Tregs (100). nTregs are selected in the thymus following antigen presentation by thymic APCs, a process, which like its conventional T cell counterparts, relies on costimulation. Indeed, CD28-deficient mice have reduced numbers of nTregs in the spleen and lymph nodes, and those nTregs which do arise in these mice have reduced suppressive capacity (101). Moreover, nTregs rely on paracrine IL-2 for survival and function, and therefore CD28-signalling mediates conventional T cell production of IL-2. By contrast, CD28 is dispensable for iTreg induction (102).

In contrast to CD28, cytotoxic T lymphocyte associated antigen-4 (CTLA-4), whose expression is induced on activated T cells, mediates an inhibitory costimulatory signal. CTLA-4 acts as competitor to CD28 for CD80/CD86 binding, thereby limiting T cell activation. Also, CTLA-4 and has been found to be crucial in Treg-suppressive functions (103). Other inhibitory costimulators include programmed cell death-1 (PD-1) and the B and T lymphocyte attenuator

(BTLA) molecules. PD-1 and its ligands are strongly associated with the induction and maintenance of tolerance (104), while data on the inhibitory functions of other molecules including BTLA, B7-H3, TLT-2, B7-H4, B7S3 and BTNL2 is still limited.

Some costimulatory molecules have mixed stimulatory and inhibitory modes of action (Fig. 4). The inducible T-cell costimulatory (ICOS) necessitates T cell effector functions, which include T-dependent B-cell responses (105). As the name suggests, ICOS expression is induced on T cells following activation and interacts with its ligand ICOS-L, which is expressed on the surface of a variety of cells such as DC, macrophages and B cells (106).

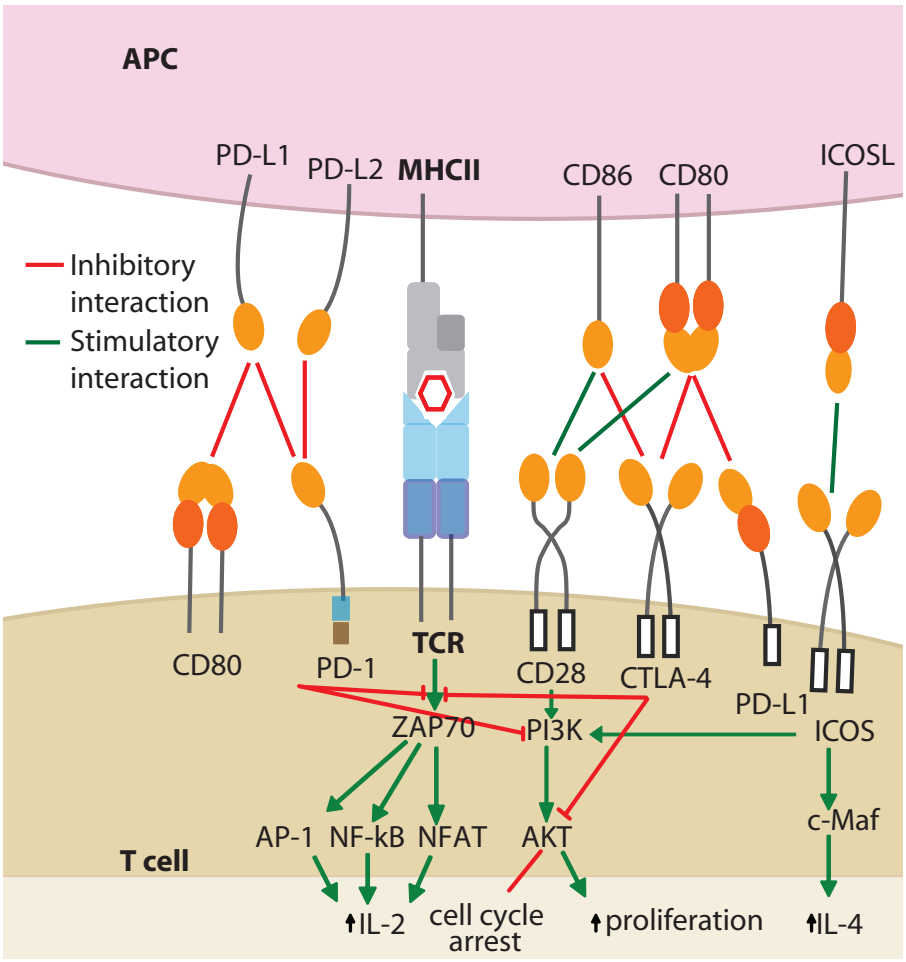


FIGURE 4
An overview of several major costimulatory pathways. Activation of naïve T cells requires both TCR-MHC interactions as well as interactions of costimulatory molecules on the T cell with their ligands on the APC. Costimulatory-signaling can have both stimulatiory and inhibitory actions on cell function.

CD28 and ICOS have similar functions in early T cell activation, however, it has been shown that ICOS also uniquely augments late events such as effector responses. ICOS-mediated regulation of the immune response was at first thought to affect predominantly Th2 responses since expression of ICOS is higher on Th2 cells. In addition, stimulation of ICOS signaling often results in increased levels of IL-4, IL-5 and IL-10 production (97, 98, 107). Host resistance against pathogens that require protective Th2 responses, such as *L. monocytogenes*, show increased susceptibility to infection in the absence of ICOS signaling (108). However, recent studies of *S. typhimurium* infections have showed delayed adaptive immunity in $ICOS^{-/-}$ mice which were a result of poor $CD8^{+}$, Th1 and antibody responses in these mice (109). Furthermore, ICOS signaling has been implicated in the expansion of Th17 cells, since $ICOS^{-/-}$ mice have reduced levels of IL-17 (110). Thus, ICOS may, indeed, be implicated in the differentiation and function of Th1, Th2 or Th17 cells.

Like CD28, ICOS could influence the development of peripheral tolerance and Treg function. Noteworthy, Treg and T memory cells express ICOS (111, 112). ICOS-deficiency leads to impaired IL-10 production in both humans and mice, but, little is known about how ICOS contributes to IL-10-mediated regulation of the immune response.

Molecular mechanisms of IFN-γ-mediated immunity

IFN-γ is a cytokine produced by a number of activated immune cells including both $CD4^{+}$ and $CD8^{+}$ T cells, NK cells, NKT cells and macrophages (113). It has a wide range of effector functions. IFN-γ signaling is complex and involves both STAT1 and non-STAT1 pathways following the binding of two IFN-γ surface receptors (IFN-γR) (114). IFN-γ-mediated effector mechanisms are central to immunity to a number of viral and bacterial pathogens, including HSV-2 and *S. typhimurium* (115, 116).

Following *Chlamydia* infection, IFN-γ is produced in a biphasic pattern, with an early peak after 1 week, which is thought to originate from NK and NKT cells, and after 3 weeks which corresponds to infiltration of *Chlamydia*-specific $CD4^{+}$ and $CD8^{+}$ effectors cells (28). Clearly IFN-γ is a critical immune mediator of protection against *Chlamydia*, since both $IFN\text{-}\gamma^{-/-}$ and $IFN\text{-}\gamma R^{-/-}$ mice are unable to resist infection (29, 50). It is known that high doses of IFN-γ may confer protection against *Chlamydia* infection by blocking chlamydial growth, albeit low doses of IFN-γ appears to rather promote persistent infection by increasing production of aberrant RBs (117). The critical role of IFN-γ in the polarization of the adaptive immune response towards Th1 during *C. trachomatis* infection has been well documented (48, 50). However, IFN-γ is also capable of mediating protection to pathogens both indirectly through enhanced phagocytosis by macrophages and by increasing MHC-II expression. It also acts directly by inducing antimicrobial defenses in infected cells. One such defense mechanism is IFN-γ-mediated activation of the enzyme indoleamine 2,3-dioxygenase (IDO), which degrades

tryptophan to kynurenine, thereby causing inhibition of bacterial replication and growth by deprivation of the intracellular pools of the essential amino acid, tryptophan. In addition, IFN-γ is capable of stimulating activity of the enzyme iNOS in activated phagocytes, which catalyses the production of immunoregulatory and antimicrobial reactive nitrogen species, including nitric oxide (NO). Another antimicrobial effect of IFN-γ is the induction of GTPase-binding proteins (118), which are thought to regulate maturation of pathogen containing vacuoles, vesiculation, entry of pathogens into an autophagic pathway and decreased lipid trafficking to the vacuole from the Golgi (reviewed (119)).

However, *C. trachomatis* and *Chlamydia muridarium* have both evolved methods of escaping IFN-γ-mediated protective mechanisms in their native target host, humans and mice respectively. As a result *C. trachomatis* and *C. muridarium* are differentially susceptible to IFN-γ-mediated inhibition depending on the strain of host cell and the infection conditions. Considering firstly the IFN-γ-mediated induction of IDO as a mechanism of anti-chlamydial defense; IFN-γ treatment of human HeLa cells induces IDO expression, resulting in depletion of intracellular tryptophan pools and inhibition of chlamydial growth (120). Interestingly, human strains of *C. trachomatis* are able to evade IDO-mediated growth restriction due to the expression of tryptophan synthase, which is lacking in the mouse-native *C. muridarium* (121). Tryptophan synthase is capable of synthesizing tryptophan from indole, which is released by the metabolic processes of bacterial flora in the genital tract. In contrast, IDO expression is not induced in murine cell lines following IFN-γ treatment, and therefore it is not likely to contribute to IFN-γ mediated protection in the mouse model of infection with either *C. muridarium* or *C. trachomatis* (120). Accordingly, $IDO^{-/-}$ mice infected with *C. trachomatis* or *C. muridarium* are equally capable to WT mice in clearing infection from the genital tract (120).

Likewise, the iNOS pathway does not appear to significantly contribute to host immunity against *Chlamydia*. IFN-γ treatment of human cell lines does not result in increased expression of iNOS or its reaction product, NO, following *Chlamydia* infection (120). In contrast, IFN-γ treatment of murine cell lines results in increased iNOS and NO expression. However, irrespective of these differences, growth of *C. muridarium* and *C. trachomatis* is not impaired (120). In addition, $iNOS^{-/-}$ mice are unimpaired in the resolution of *C. trachomatis* and *C. muridarium* infections. Nonetheless, iNOS may contribute to the imunopathology observed, since $NOS^{-/-}$ mice demonstrate greater inflammation and more dissemination of infection (122, 123).

Recently, immunity-related GTPases (IRGs) have been strongly implicated in IFN-γ-mediated immunity against both *C. muridarium* and *C. trachomatis*. In infected murine cells, IFN-γ induces the upregulation of GTPase genes, which results in enhanced GTPase production localized to the chlamydial inclusion, and inhibits bacterial growth (124). Interestingly, *C. muridarium*, but not *C. trachomatis*, contains TC438, a cysteine protease with homology to the YopT virulence

factor of *Yersinia*. YopT modifies Rho-GTPAses, releasing the GTPase from the membrane and thereby inhibiting its antimicrobial actions (125). In a study by Nelson *et al*., the target of TC438 was proposed to be Irga6 (120). However, in a conflicting study, Coers *et al*. claim that Irga6 is not involved in inhibition of growth, and since it is the only GTPase with a C terminal sequence that likely could be cleaved by a YopT homologue, it is unlikely to be the mediator of GTPase evasion (124). Instead, Coers *et al*., show that the GTPases Irgb10, Irgm1 and Irgm3 are required for host protection against *C. trachomatis* and that evasion of these GTPases by *C. muridarium* is probably mediated by another factor secreted into the cytosol (124).

Immune regulation by IL-10

IL-10 was first described as a cytokine synthesis inhibitory factor (CSIF) due to its ability to inhibit activation and cytokine production by Th1 cells (126). IL-10 is known to inhibit a range of immune functions and is produced by a broad spectrum of cells. T cell sources of IL-10 include primarily Th2 cells and Tr1 cells, but in addition the Th1 and Th17 subsets. Non-T cell sources include DC, macrophages, B cells, monocytes, eosinophils, epithelial cells and mast cells (reviewed (127)).

The receptor for IL-10 consists of 2 subunits; IL-10R1 and IL-10R2. Binding of IL-10 to its receptors activates the JAK/STAT3 intracellular signaling pathway. STAT3 binds to the IL-10 promoter in the nucleus, and stimulates IL-10 gene transcription. The inhibitory effects of IL-10 are also mediated by STAT3, primarily through the ability of STAT3 to also activate the suppressor of cytokine signaling 3 (SOCS3), but also by inhibiting NF-KB activation, translocation and DNA binding (128).

Other than its impact on T cells, the immunoregulatory influence of IL-10 on the APC system is substantial. IL10$^{-/-}$ mice display accelerated clearance of chlamydial infection from both genital and respiratory tracts (129, 130). The basis for IL-10-mediated immunosuppressive functions is through the influence on the APCs. In the absence of IL-10, APCs more effectively stimulate strong Th1-medaited immune protection (131). As an example, transfer of *Chlamydia*-pulsed IL-10$^{-/-}$ DCs confered superior protection against infection than WT pulsed DCs. This effect was ameliorated if IL-10$^{-/-}$ DCs were transferred into IFN-γR$^{-/-}$ mice, indicating that IL-10$^{-/-}$ APCs were protective due to the ability to stimulate IFN-γ production (131). Moreover, the phenotype of IL-10$^{-/-}$ mice during *Chlamydia* infection was linked to APC function. IL-10$^{-/-}$ APC rapidly activated WT *Chlamydia*-specific T cells to produce IFN-γ, whereas IL10$^{-/-}$ T cells with WT APC were poor stimulators of IFN-γ production (131).

The requirement for DCs

DCs are known for their ability to stimulate T cell activation, differentiation and expansion. In peripheral tissues, DC capture antigen and migrate to the T cell areas of the draining lymph nodes. These DC undergo a maturation process, which results in increased expression of costimulatory molecules and cytokine production. However, DCs are also known for their ability to stimulate suppressive T cells. Studies of DC migration into local tissues have shown that DC recruited early to the sight of aseptic inflammation have potent T cell stimulating properties, whereas those that are recruited to the tissue in the chronic phase of infection can exert a tolerogenic effect (132). Furthermore, DC from the gut associated lymphoid tissue (GALT) have been reported to mediate suppression by production of IL-10 and the stimulation of antigen-specific regulatory T cells (133).

APC of the genital tract, under homeostatic conditions, host both Langerhans cells within the epithelium and DC in the submucosa. Following infection of the genital tract, monocyte-derived DCs and pDCs are recruited into the mucosa (134). Antigen processing can be carried out by both DCs and Langerhans cells of the vagina. DC of the submucosa consist of several subsets which include $CD11b^+$ DCs (murine), $MHC\text{-}II^+F4/80^+$ cells (murine), DC $sign^+$ (humans and macaques) and $CD123^+$ DC (macaques) (135-137). Studies of DC and macrophages of the genital tract are complicated by the lack of sufficiently unique surface markers to distinguish DC populations. Furthermore, during infection, macrophages are capable of upregulating MHC-II expression in response to IFN-γ (138). Whereas, DCs are central to the induction of protective immunity against *C. trachomatis*, the mechanism by which submucosal DCs in the genital tract induce mucosal immunity is still unclear.

Vaccination for protection against genital tract infections

For successful vaccination of the genital tract several considerations must be made. Along with the lack of FAE in the genital tract, vaccination regimens need to consider the influence of sex hormones (139-143). Both the cell type and cell numbers change throughout the menstrual cycle, which can have important implications for effective antigen uptake. In addition, the production of local antigen-specific IgA in genital secretions fluctuates with hormone levels, peaking during the late secretory phase of the menstrual cycle, when progesterone levels are high (144). Furthermore, when progesterone levels are high, during diestrus, the epithelial layer protecting the vagina is thin, consisting of 2-3 epithelial cells layers, compared to 15-20 layers under the influence of estrogen. Changes in epithelial layer thickness can alter not only the ability of antigens to cross the epithelial barrier for induction of immunity following immunization, but also increases the susceptibility of the vagina to infection by invading pathogens. Progesterone treatment in animal models of vaginal infection is often necessary to facilitate infection (145).

Immunization at a remote site has been considered a possibility to circumvent the influence of

sex hormones and lack of local immune inductive sites in the female genital tract. Also, it is known that systemic vaccination may fail in the generation of vaginal immunity. Subcutaneous immunization against *Trichomonas vaginalis* is capable of producing protective immunity against a challenge infection (146). In contrast, though, systemic immunization has been shown to be inferior for the generation of protection against most other pathogens of the genital tract, including *C. trachomatis* infections (147). Intranasal immunization has been shown to be an alternative method of generating genital tract immunity. In fact, many studies have shown strong IgA production in the vagina following intranasal immunization, irrespective of the hormonal cycle (148-150). However, the generation of strong innate or humoral responses is of little consequence for resistance against many STDs, which instead are effectively eliminated through strong $CD4^+$ T cell mediated immunity. Nevertheless, little is known about the requirements for effective vaccination that can generate strong protective $CD4^+$ T cell-mediated immunity in the genital tract.

Vaccination for protection against *C. trachomatis*

The first *Chlamydia* vaccine to enter clinical trials was based upon knowledge derived from the successful vaccination against other pathogens using whole cell preparations. However, not only was the immunity produced following vaccination with inactivated *Chlamydia* EBs short-lived, but severe hyper-reactivity to natural infection occurred in some vaccinated individuals, highlighting the need for the identification of safe and immunogenic chlamydial-antigens to be used in subunit vaccines (reviewed (151)).

A wide variety of candidate chlamydial-antigens have since been identified, and most interest and efforts have been focused on the major outer membrane of *Chlamydia* (MOMP). MOMP accounts for 60% of the outer membrane of *C. trachomatis* and it is composed of 4 variable regions, which differ between serovars, and 5 constant regions. MOMP contains both neutralizing epitopes (152) and several MHC-II helper T cell-epitopes, but few have been shown to effectively stimulate immunity in mice (153). Vaccination using whole MOMP has yielded promising results, however, cross-serovar protection appears to be poor (31). Although the different serovars of *Chlamydia* share 84-97% homology of the MOMP, the most immunogenic sequences are also those which vary most between the different serovars (154). Furthermore, immunization with MOMP stimulates a range of immune protection depending on the strain of mice, route of administration, as well as the choice of adjuvant and delivery system (155-159).

Experiments showed that mice immunized with DCs pulsed with whole EBs developed protection superior to that observed with DCs pulsed with MOMP alone (52). This suggests that inclusion of additional antigenic components of chlamydiae provides a means to increase the level of protection. Following sequencing of the genome of chlamydiae, several alternative antigens have been identified, including the outer membrane proteins (Omp-1, Omp-2, and

Omp-3) which are more highly conserved and contain both $CD4^+$ and $CD8^+$ T cell epitopes (160, 161), polymorphic outer membrane proteins (pmp) (162), conserved PorB membrane proteins (163), an ADP/ATP translocase (Npt1) (164), a plasmid protein (pgp3) (165), the proteasome/protease-like activity factor (CPAF) (166), toxins (167) and members of the type III secretory machinery (168). Many of these antigens have yet to be thoroughly tested experimentally. New techniques for delivery of the antigens and novel adjuvant strategies are likely to enhance immunogenicity. Mice immunized with bacterial ghosts from *Vibrio cholerae,* expressing both MOMP and OMP2 resulted in significant protection against infection (169). Similar results were demonstrated with MOMP expressed in an attenuated influenza A virus strain (170). Other immunogenic delivery systems include lipophilic immune stimulating complexes (ISCOMs), which are negatively charged cage-like assemblies, composed of saponin Quil A, cholesterol, and phospholipids. ISCOMs and are often used to incorporate antigens for immunization. Its is thought that the immunogenic properties of ISCOMs arise from the inflammatory nature of the Quil A component, complex formation with membrane cholesterol, and stimulation of B and T cells following accumulation in secondary lymphoid tissues (reviewed (171)). Moreover, MOMP incorporated into ISCOMs have proven effective for immunization through the induction of strong local Th1-mediated immunity (156, 157, 172-174). Taken together, these examples suggest that novel means of antigen delivery could be critical for progress in vaccine design against *C. trachomatis*.

Central to mucosal vaccination is the choice of adjuvant. To date vaccines have been difficult to develop as a consequence of the few effective mucosal adjuvants available. Some of the most potent substances with strong adjuvant function belong to the family of ADP-ribosylating bacterial enterotoxins, cholera toxin (CT) and the related *E.coli* heat-labile toxin (LT) (155, 157, 172). These toxins bind GM1-ganglioside membrane receptors and gain access to the cytoplasm, where they stimulate ADP-ribosylation. This leads to dramatic increases in cAMP. However, due to the promiscuous GM1-binding to all nucleated cells, the holotoxins have been found to be too toxic to be used in clinical practice (175, 176). As a means to avoid the toxicity, mutant toxins with less or no ADP-ribosylating activity have been developed (177, 178). An example of an alternative strategy is CTA1-DD, which contains the enzymatic activity of CTA1 subunit genetically fused to a dimer of the D-fragment of *Staphylococcus aureus* protein A (179, 180). The CTA1-DD adjuvant targets antigen-presenting cells and B cells, in particular. The CTA1-DD adjuvant was recently used together with MOMP in mice and was found to stimulate strong neutralising antibodies in both serum and vaginal lavage, associated with enhanced protective immunity against a live challenge infection with *C. muridarium* (181). This and other promising results demonstrate that prospects have improved for the development of a mucosal vaccine against STDs in general and *Chlamydia* infections in particular.

Aims of this thesis:

The general aim of this thesis was to dissect the generation of genital tract $CD4^+$ T cell-mediated immunity during vaccination and infection.

Specifically:

- To investigate $CD4^+$ T cell subset differentiation in the mouse model of *C. trachomatis* genital tract infection
- To examine the contributions of costimulation through CD28 and ICOS in T helper subset polarization during *Chlamydia* infection
- To define the most effective vaccine regimens for the generation of genital tract $CD4^+$ T cell immunity
- To asses the effectiveness of a novel adjuvant vector, CTA1-DD/ISCOMs, for vaginal immunizations

Materials and Methods

The following section describes the main experimental procedures used and the rationale behind their use. Further details can be found in the Materials and Methods section in the individual articles.

Mice

Limitations of the mouse model include the use of hormone treatments to facilitate infection, which is not required in other models, such as the Guinea pig (182). However, *Chlamydia* infection in the mouse model is somewhat similar to a clinical infection in humans, in that the infection is ascending and self-limiting. The availability of knockout mice and the extensive literature on infection is a major advantage of the mouse model. In this thesis, 6-8 week old female mice were used in all experiments. For papers I-III, mice on C57BL/6 background were used and in paper IV all mice used were on BALB/c background. Mice were bred and kept under specific pathogen-free conditions at the Department of Experimental Biomedicine, The University of Gothenburg, Sweden.

Bacteria and infection protocols

The use of progesterone treatment prior to inoculation with *Chlamydia* is necessary to facilitate infection in mice. The effects of progesterone treatment are vast and dramatic and include thinning of the epithelial layer, reduced antigen presentation, and decreased IgA and IgG antibody levels (183). In our model, mice received 2.5mg medroxyprogesterone acetate subcutaneously 7 days prior to intravaginal infection. Infection was achieved by vaginal innoculation with approximately 1 x 10^6 inclusion forming units (IFU) of a human genital tract clinical isolate of *C. trachomatis* serovar D (papers I-III) or *C. muridarium* (strain Nigg) (paper IV). Human isolates of *C. trachomatis* differ from *C. muridarium* in several ways, particularly in their susceptibility to IFN-γ-mediated growth restriction, as discussed earlier in the Introduction section. However, infection with either strain results in an ascending self-limiting infection. Moreover, there is controversy surrounding the inoculating dose of *Chlamydia,* because of a lack of knowledge of the natural infectious dose in humans. In a study by Rank and coworkers, it was estimated that the transmission dose, in mating guinea pigs was as low as 10^2 IFU of *Chlamydia caviae* (185). Noteworthy, the use of a higher dose, such as the dose used in this paper, results in rapid ascension of the infection into the oviducts of the mouse. Lower doses are more prone to infect the cervico-vaginal region (186). In our model, we used 1 x 10^6 IFU to achieve 100% infectivity, which is necessary for the evaluation of protective immunity. Reinfection was always carried out 4 weeks after clearance of the primary infection.

There are several diagnostic methods used for assessment of chlamydial shedding. These include the culture method, the enzyme immunoassay, and a PCR assay. Each method of

detection has points of criticism and advantages. The culture method of detection, previously considered to be the gold standard, has a sensitivity ranging from 50 to 85% (187-189). The necessity for sensitive and accurate methods of detection of clinical infection have resulted in the development of PCR based assays. Currently available commercial *C. trachomatis* DNA amplification tests include PCR (Roche Molecular Systems), the ligase chain reaction (LCR; Abbott Laboratories), and strand displacement amplification (SDA; Becton Dickinson). In this thesis we have used the MicroTrak II Chlamydia EIA kit, according to the manufacturer's instructions (Trinity Biotech plc.). Samples with an absorbance greater than the provided cut-off value were considered positive for chlamydial shedding. The EIA method is based on the enzymatic detection of chlamydial LPS. Detection of LPS does not require that the bacteria in the genital tract is viable, however, the method is cost effective and rapid. Importantly, studies have shown reasonable correlation between samples that test positive by EIA and by the culture method (40, 190).

Immunohistochemisty

Investigations into the immune response in the reproductive mucosa of mice are hindered by the lack of available methods. Immunohistochemisty is often used in place of isolation of genital tract lymphocytes, and allows for *in situ* localization of target cells. In papers I-III, sections were incubated with rat anti-CD4-biotin antibodies (BD PharMingen) followed by anti-rat IgG antibodies. Peroxidase-conjugated avidin (DAKO Cytomation) and a commercial peroxidase AEC substrate (Sigma-Aldrich) were used to develop the sections before counterstaining with HTX. In paper IV, sections were labeled with biotinylated anti-B220, Pacfic Blue-conjugated anti-B220 (BD PharMingen), FITC-conjugated anti-peanut agglutinin (PNA, Sigma), rabbit anti-OVA, FITC-conjugated anti-OVA, hamster anti-mouseCD11c (Serotec). For secondary antibody labeling, sections were washed and stained for 30 minutes with FITC- (BD PharMingen) or TxRd-labeled anti-rabbit Ig (Southern Biotech), streptavidin-conjugated TxRd (Vector), or donkey anti-armenian hamster Cy3 (Jackson Immunoresearch). Ovalbumin-specific transgenic T cells were detected using the FITC-conjugated clonotypic KJ1-26 MAb produced from the original hybridoma (191). Counterstaining was achieved by incubating sections for 10 minutes with To-Pro-3 (Invitrogen). For light microscopy sections were visualized using a Leica LSC microscope. The development of imaging technology also permitted the use of confocal microscopy, which was performed at the Centre for Cellular Imaging (CCI) using the Zeiss LSM 510 META system.

RT-PCR assays for studies of T cells in the genital tract

Studies of the $CD4^+$ T cells in the genital tract mucosa are hindered by the relative scarcity of cells when compared to e.g. the gut mucosa. A typical $CD4^+$ T cell isolation using collagenase digestion yields approximately $1x\ 10^6$ cells/mouse (192). Therefore, studies of the genital tract immune system have relied heavily on the use of immunohistochemistry and isolation of cells

from the draining lymph nodes. Whereas studies of pathogen-specific T cells in other infections have been greatly enhanced by the development of transgenic mice, the *Chlamydia* research field has not provided such models. Recently, a *Chlamydia*-specific TCR T cell transgenic mouse was developed. Little is known about when and where *Chlamydia*-specific $CD4^+$ T cells respond to infection and the extent to which they migrate into infected tissues. It is also unknown when effector cytokines are produced by *Chlamydia*-specific T cells and where the effector functions are deployed. Advantageously, cells from transgenic animals can be labeled for tracking after transfer into recipient mice, enabling efficient monitoring of pathogen-specific T cells during infection. *Chlamydia*-specific T cells were shown to produce IFN-γ following transfer into infected mice and demonstrated the ability to migrate to the genital tract mucosa. However, studies of *Chlamydia* TCR-transgenic T cells are preliminary and access to these mice is limited. Therefore, alternative methods that allow for better analysis of the build-up of $CD4^+$ T cells in the genital tract are much warranted.

To overcome the difficulties with low frequencies of genital tract T cells, we developed quantitative RT-PCR assays using the Roche Applied Bioscience Universal ProbeLibrary Assay Design Center (http:www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP0 30 000) in combination with the LightCycler System and relative quantification software (Roche Diagnostics, GmbH). Transcription factor mRNA expression can be used to identify unique subsets of T helper cells in the genital tract tissue. However, using whole tissue preparations, quantification of mRNA levels may also reflect activity in other cells types. Hence, results were expressed as normalized ratios of the target mRNA to the housekeeping mRNA (HPRT) (Paper I, III) for this reason. In paper II, expression levels were normalized to CD3-γ to better define the target population. Following serial analysis of gene expression (SAGE) of multiple T cell populations, the gamma chain of CD3 is the most stable subunit of the CD3 molecule, and therefore the most reliable marker to use as an internal standard. Noteworthy, though, we did not observe any significant difference in expression kinetics between HPRT and CD3-γ normalized values. Therefore, we consider both methods to be valid.

Adoptive transfer and immunization protocols

Despite advances in the area of *Chlamydia* genomics, the development of vaccines and studies of T cells in the genital tract require the availability of *Chlamydia*-specific TCR-transgenic mice as well and protective antigens. To circumvent this, we used a model of adoptive transfer of $CD4^+$ T cells from mice specific for the $cOVA_{peptide323-339}/I\text{-}A^d$-specific DO11.10 T cell receptor transgenic mice. TCR transgenic T cells were detected using the clonotypic anti-TCR KJ1-26 mAb. One week prior to immunization, female BALB/c aged 6-8 weeks were injected subcutaneously with 2.5mg of DepoProvera (medroxyprogesterone, Pharmacia Sverige AB) or 0.1mg of beta-estradiol valerate (Sigma). One day after adoptive transfer, mice were immunized with a single dose of OVA (chicken ovalbumin, Sigma-Aldrich), 5μg CT + 20μg OVA, 5ug CT

+ 20µg Tetanus toxoid, 5µg CT-OVA conjugate, 5µg CTA1-OVA-DD/ISCOM, or 5µg CTA1-DD/ISCOM + 20µg OVA given intranasally or vaginally. Control mice received PBS only.

Vaccination of the genital tract

Intranasal immunization bypasses a lack of inductive sites as well as a distinct dependence on hormonal influences. In this way, the immune response could be elicited and later followed in the genital tract. It has previously been demonstrated that intranasal immunization with live chlamydiae induces superior protective immunity compared to parental immunization (193). In fact, intranasal, intravaginal, oral and rectal immunization can all induce specific-IgA and IgG in the genital tract, but intranasal and intravaginal immunization are by far most effective (193). Accordingly, we compared the local immune response following CT-OVA administration intravaginally or intranasally.

Novel delivery systems and adjuvants are necessary for progress in STD vaccine design. Bacterial ghosts from *Vibrio cholerae* (169), attenuated influenza A virus strain (170), and ISCOMs are among the promising delivery systems which have been tested in mice (156, 157, 172-174). ISCOMs can be used to incorporate antigens for immunization and stimulation of B and T cells (reviewed (171)). In this thesis the novele vaccines CTA1-OVA-DD or CTA1-DD were incorporated into ISCOMs. ISCOMs were prepared by mixing 1.0mg of CTA1-OVA-DD with 1.0mg of cholesterol (Sigma), 1.0mg of phosphatidylcholine of egg-origin (Lipoid Gmb), 5.0mg of quillaja saponins (Spikoside, Isconova AB) and 2% Mega-10 (Bachem). The formation of ISCOMs was confirmed by electron microscopy (194).

A fundamental component of any vaccine is the use of a powerful adjuvant that is both safe and effective. To date there are few adjuvants that enhance mucosal immunizations. Importantly, ineffective stimulation of an immune response at mucosal sites may, in fact, increase the risk of development of antigen-specific immune tolerance rather than promote mucosal IgA immunity (195, 196). Some of the most effective substances that exert strong adjuvant function belong to the family of ADP-ribosylating bacterial enterotoxins; cholera toxin (CT) or the closely related *E. coli* heat-labile enterotoxin (LT) (155, 157, 172). These toxins bind to GM1-gangliosides on the cell membrane of most mammalian cells. Because of the promiscuous binding to all nucleated cells the clinical use of the holotoxins are precluded (175, 176). An alternative approach to circumvent toxicity is to design of an adjuvant that combines the full enzymatic activity of CTA1 with a synthetic dimer of the D-fragment of *Staphylococcus aureus* protein A (179, 180). The resulting CTA1-DD adjuvant, is devoid of the GM1-ganglioside binding B-subunit, and has been found to be completely non-toxic (179, 197).

For production of the adjuvants, *E.coli* DH5 cells were transformed with the expression vector for the CTA1-OVAp-DD fusion protein, containing one copy of $OVA_{323-339}$ peptide inserted

between the CTA1 and DD moieties. *E.coli* were grown in 500 ml cultures over night in SYPPG medium with 100 μg/ml carbenicillin, at 37 °C. The cells were harvested by centrifugation and inclusion bodies were washed before extraction by treatment with 8M urea. After refolding the proteins by slow dilution in Tris–HCl pH 7.4 at +4 °C, the fusion proteins were purified in two steps, by ion exchange and size exclusion chromatography. After concentration and sterile filtration the purified fusion proteins were stored in PBS at −80 °C until use. Fusion proteins were routinely tested for the presence of endotoxin, by end-point chromogenic limulus amebocyte lysate methods (LAL Endochrome™). Endotoxin-levels were below 100 endotoxin units/mg (EU/mg). The enzymatic activity was measured using NAD:agmatine assay as described earlier (28-30). Samples of 10μg of CT, CT-OVA, CTA1-OVAp-DD fusion protein were diluted 2-fold and the enzymatic activity was assessed.

Results and comments

T cell differentiation in the genital tract during *C. trachomatis* infection

$CD4^+$ T cells are of central importance to the clearance of *C. trachomatis* from the genital tract and for protection against reinfection (30). On the other hand, cell-mediated immune responses generated during infection may also be causal to the development of immunopathology. To gain a better understanding of protective immunity against *Chlamydia* we investigated the generation of T helper subsets during infection.

$CD4^+$ T cell effector functions are carried out by distinct subsets of T helper cells, and it has been shown that clearance of *C. trachomatis* from the genital tract requires Th1 effector cells and their characteristic IFN-γ production (198). After 10 days of infection, the normally quiescent UGT was populated by large numbers of $CD4^+$ T cells. This was concurrent with T-bet expression and IFN-γ production (Fig. 5). This Th1 bias was not reflected in the LGT, where instead increased mRNA expression of the Th2 specific transcription factor, GATA-3, was prominent (Fig. 5). Moreover, to reconcile the lack of the Th17 subset during early infection with a slight increase in IL-17A production late in infection in the LGT, we hypothesized that there may be an alternate source of IL17A. Our results demonstrated that the immune response to *Chlamydia* was highly compartmentalized, in that the UGT and the LGT displayed remarkably different cytokine and T cell profiles.

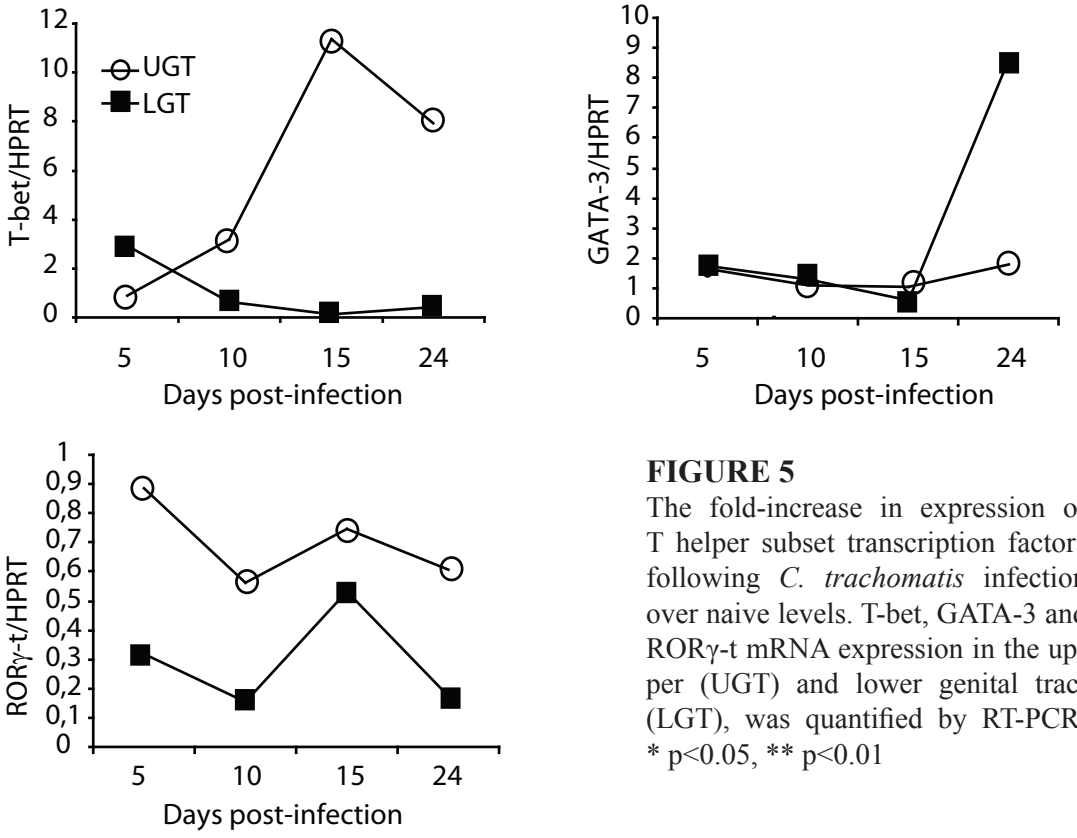


FIGURE 5
The fold-increase in expression of T helper subset transcription factors following *C. trachomatis* infection over naive levels. T-bet, GATA-3 and RORγ-t mRNA expression in the upper (UGT) and lower genital tract (LGT), was quantified by RT-PCR. * $p<0.05$, ** $p<0.01$

Regulation of the immune response to *C. trachomatis*

We found a dramatic increase in IL-10 in the LGT, which was localized to $CD11b^{+}CD11c^{+}$ $MHC\text{-}II^{+}$ cells rather than the $CD4^{+}$ Tregs (Fig. 6). Transcription factor expression in $IL\text{-}10^{-/-}$ mice revealed that IL-10 in the LGT is likely to strongly inhibit Th1 differentiation, since T-bet expression in these mice greatly exceeded WT expression 5 x 10^{7}–fold, without affecting the development of the other T helper subsets.

Whereas IL-10 is an important factor produced by Tregs, FoxP3 expression and TGF-β could also be associated with regulatory control of immune responses in peripheral tissues. Both TGF-β and FoxP3 mRNA expression increased in the mucosa of the UGT and LGT after infection, however, FoxP3 expression increased as late as on day 24, which was later than TGF-β mRNA expression (Fig. 7). Thus, regulatory elements of the immune response are induced during *Chlamydia* infection, and may have important implications for both protective immunity and persistence of infection.

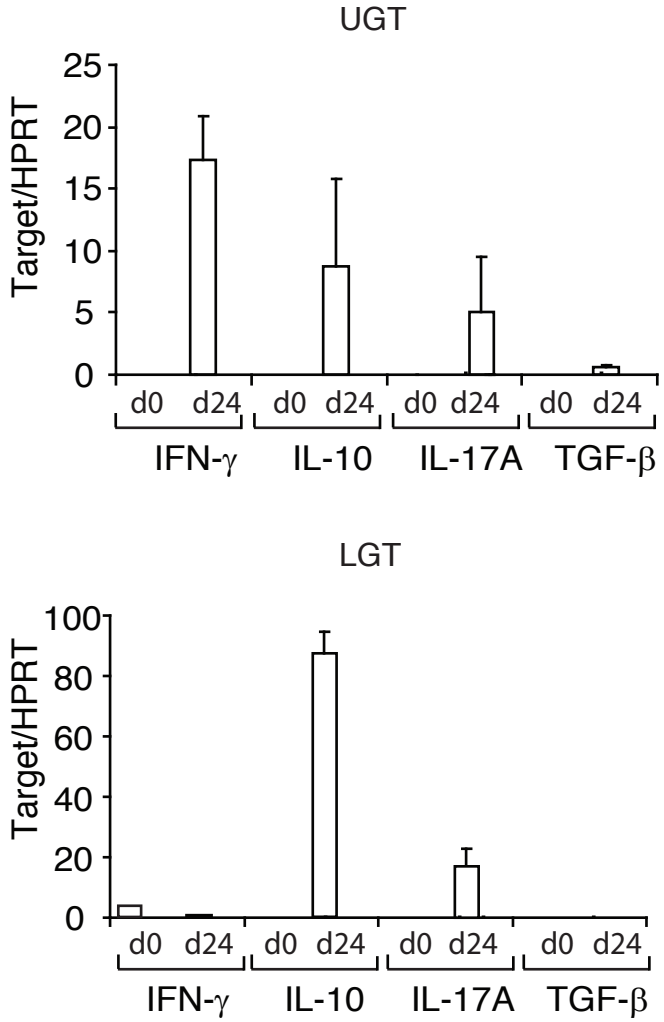


FIGURE 7
The expression of cytokines following *C. trachomatis* infection. RT-PCR was used to quantify the mRNA expression levels of FoxP3, IFN-γ and TGF-β in the upper (UGT) and lower genital tract (LGT) of naïve mice and after 24 days of infection.

Costimulation provides critical contributions to cytokine production, T cell differentiation and clearance of *C. trachomatis*

The polarization of the immune response to distinct T helper subset profiles is influenced by costimulatory signals and the resulting cytokine environment. Originally CD28 was thought to contribute to the overall development of T cell-mediated immunity, since in its absence, reports have documented T cell anergy, reduced T cell survival and poor cytokine production (97, 98, 100, 199-201). In contrast, ICOS-signaling has been predominantly associated with Th2 differentiation during many infections, and greatly augmented generation of antibody mediated immunity. However, ICOS-signaling has also been demonstrated to affect Th1 differentiation (56, 107, 109, 202-204). Further, it has been speculated that ICOS signaling is required for T effector cell responses while CD28 is required for initial activation. Of note, CD28-mediated activation of T cells induces ICOS expression. In spite of this, we found that ICOS-signaling has distinct and independent functions. Using mice deficient in the costimulatory molecules CD28 or ICOS, we were able to address several important issues surrounding correlates of immunity against *Chlamydia*;

Firstly, are the immune responses in the absence of costimulation effective for clearance of the bacteria and for protection against reinfection?

Our data showed that CD28 was crucial for both the immune response to a primary infection as well as the development of protective immunity against reinfection (Fig. 8). In direct contrast, $ICOS^{-/-}$ mice effectively cleared the bacteria and developed sterilizing immunity against reinfection. Over 65% of $CD28^{-/-}$ mice were still infected on day 32 of infection, compared to less than 10% of WT and no $ICOS^{-/-}$ mice by that time point ($p<0.05$). More strikingly, $ICOS^{-/-}$ mice were more highly protected than WT mice against reinfection, with all $ICOS^{-/-}$ mice shedding negative by day 8, compared to day 16 for WT mice. Remarkably, $CD28^{-/-}ICOS^{-/-}$ (DKO) mice displayed partially restored bacterial clearance during a primary infection however the immune response was not protective against reinfection. This suggests that removal of ICOS signaling enhances clearance of the bacteria from the genital tract, however, CD28-signaling is required for the development of memory and protective immunity against reinfection with *Chlamydia.*

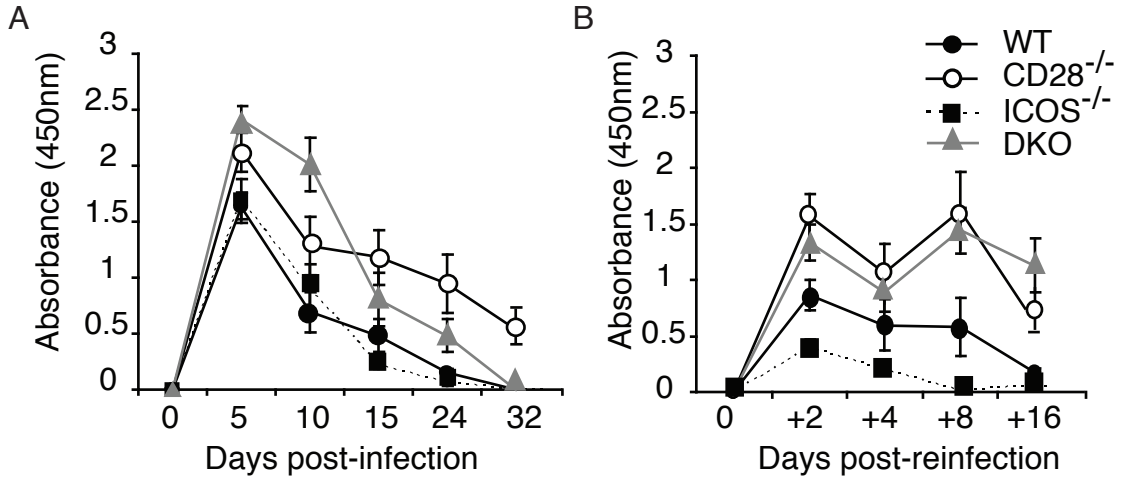


FIGURE 8
Costimlulation augments the immune response to *Chlamydia*. (A) WT, $CD28^{-/-}$, $ICOS^{-/-}$, and $CD28^{-}/ICOS^{-}$ (DKO) mice were infected with *C. trachomatis* and bacterial shedding determined by an EIA. (B) Following clearance, mice were reinfected to asses the development of protective immunity (right panel).

Secondly, we dissected which T helper profiles correlated with different outcomes of infection in the absence of costimulatory molecules

Mice lacking CD28 and/or ICOS developed strikingly different degrees of protective immunity. To gain an insight into the correlates of immunity to *Chlamydia* we characterized the T helper subset profiles and cytokine responses that emerged during infection in these mice. In poorly protected $CD28^{-/-}$ mice we found decreased $CD4^{+}$ T cell infiltration, as well as reduced IFN-γ and IL-17A mRNA expression in both the UGT and LGT. In turn, these mice displayed impaired transcription factor expression for all T helper subsets compared to that observed in WT mice (Fig. 9). Hence, costimulation through CD28 is critical for stimulation of T cell responses during infection with *C. trachomatis*.

The T cell response to infection in $ICOS^{-/-}$ mice was strikingly different to that seen in both WT and $CD28^{-/-}$ mice. Superior resistance to infection correlated with intense Th1 responses and increased levels of IFN-γ mRNA. In fact, Th1 responses in $ICOS^{-/-}$ represented 11x 10^{5} and 9 x 10^{12}-fold increases ($p<0.05$), respectively, over expression in naïve $ICOS^{-/-}$ UGT and LGT. Th1-polarized immunity was concurrent with silenced Th2 differentiation in the LGT (Fig. 9). Additionally, $CD4^{+}$ T cell numbers infiltrating the genital tract, as well as *in vitro* re-call responses to chlamydial antigens, were greater in $ICOS^{-/-}$ than WT mice. Of note, in comparison

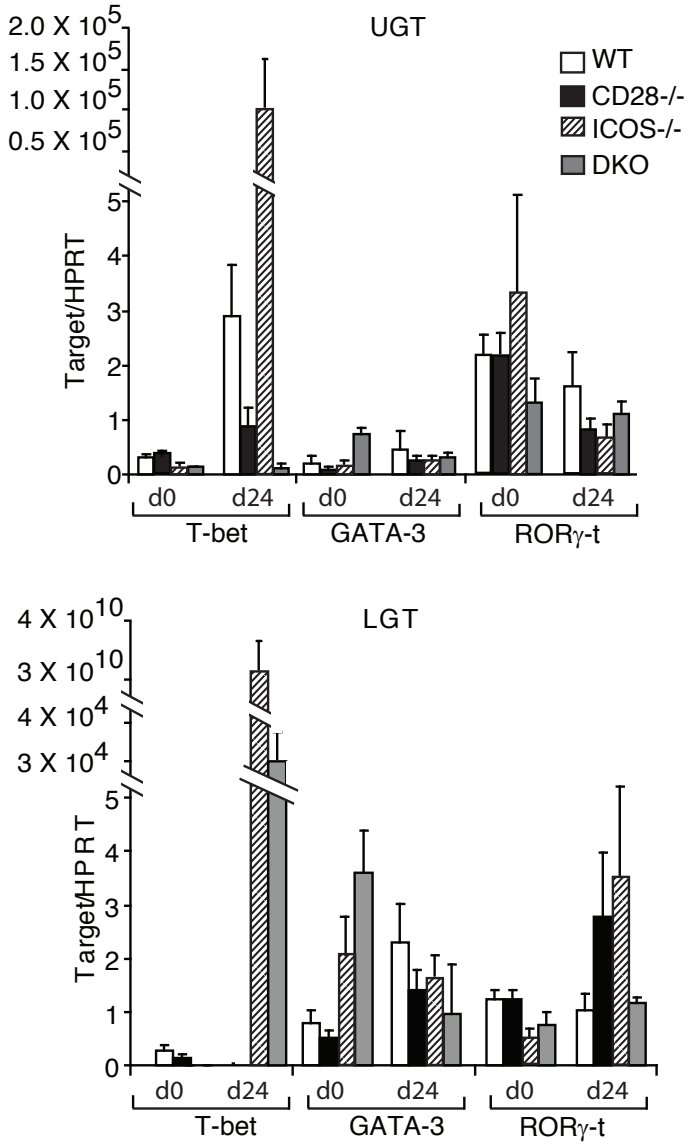


FIGURE 9
Mice lacking both ICOS and CD28 are unimpaired in Th1 differentiation for clearance of the bacteria in the lower genital tract (LGT). Transcription factor expression in before and after 24 days of infection in $CD28^{-/-}$, $ICOS^{-/-}$, $CD28^{-}/ICOS^{-}$ (DKO) and WT mice.

to WT mice, the LGT of $ICOS^{-/-}$ mice also displayed higher levels of RORγ-t mRNA expression, indicating expansion of the Th17 subset.

The mechanisms through which DKO mice clear bacteria may be attributed to upregulation of Th1 in the LGT following infection (Fig. 9). In DKO mice, IFN-γ expression in the UGT was partially restored from levels observed in $CD28^{-/-}$ mice. Thus, rather than synergistic defects of a combined CD28 and ICOS-deficiency, the immune response was partially restored in DKO mice. Importantly, though, this was not sufficient to correct for CD28-mediated defects in the generation of $CD4^{+}$ memory T cells and protection against a reinfection.

Thirdly, we addressed the contribution of costimulation to the development of regulatory elements of the immune response to Chlamydia

Knowledge of the qualities of the immune response that correlate with protective immunity

could be critical for the development of a vaccine against *Chlamydia*. However, for a vaccine, it is critical that protective immunity does not occur at the expense of immunopathology. Thus, a more thorough understanding of the contributions of regulatory elements is required. Since costimulation has been implicated in the development and functions of Treg, we investigated expression of the regulatory elements IL-10, TGF-β and of the Treg transcription factor, FoxP3, in the genital tract of WT, CD28$^{-/-}$, ICOS$^{-/-}$ and DKO mice during *C. trachomatis* infection.

In agreement with previous studies (100, 199), CD28$^{-/-}$ mice were impaired in the development of Tregs as well as the production of the anti-inflammatory cytokines IL-10 and TGF-β (Fig. 10). This was likely the result of overall impairment of T cell priming in these mice.

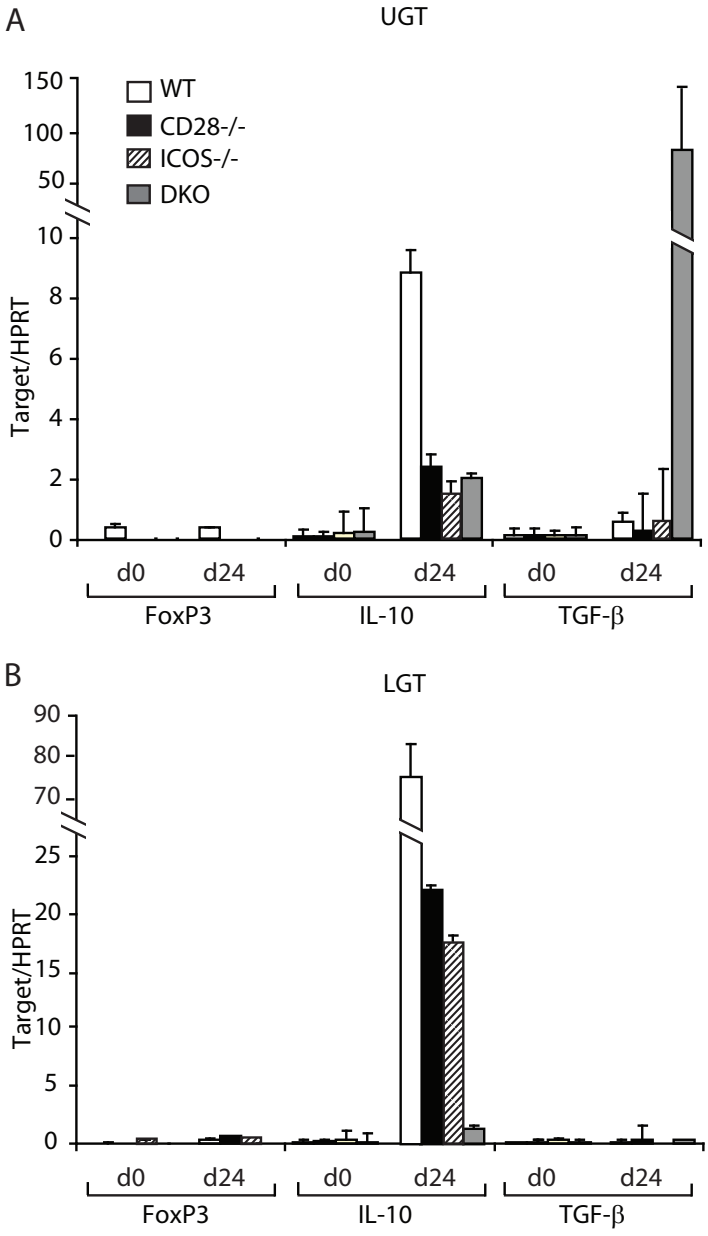


FIGURE 10
Costimulation is required for regulatory immune responses to *C. trachomatis* infection. Transcripts for the regulatory factors FoxP3, IL-10 and TGF-β in CD28$^{-/-}$, ICOS$^{-/-}$, CD28^{-}/ICOS^{-} (DKO) and WT at the indicated time points. Expression was determined before infection and after 24 days of infection in the upper (UGT) and lower (LGT) genital tract.

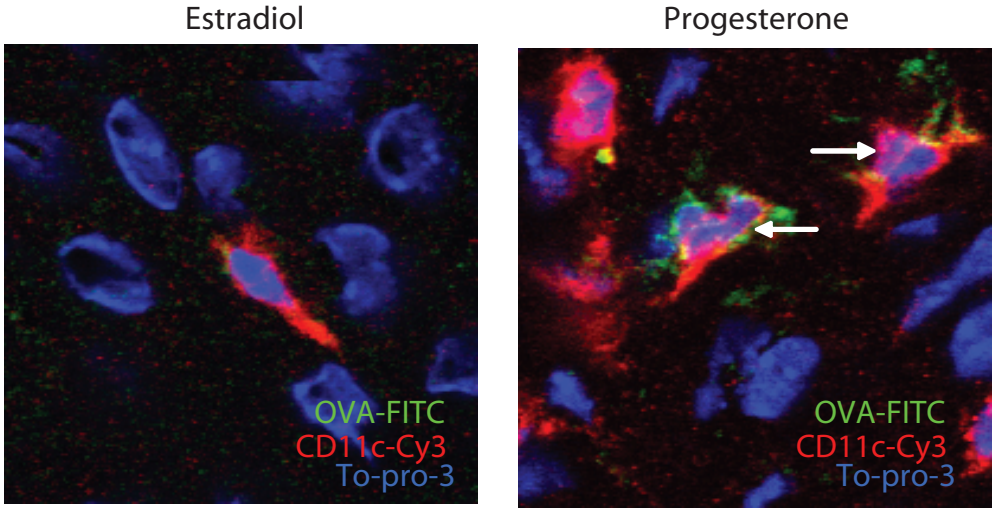
DKO mice were also impaired in IL-10 production and FoxP3 expression in both the UGT and LGT. Interestingly, in the UGT of DKO mice, TGF-β mRNA expression was strongly upregulated, representing an 800-fold increase in expression over naive DKO levels. In contrast, however, aggressive Th1 responses to *C. trachomatis* in ICOS$^{-/-}$ mice, coupled with poor Treg differentiation and impaired IL-10 and TGF-β mRNA expression led to, even macroscopic, signs of severe tubal edema and inflammation. This immunopathology was striking especially in the light of the fact that human serovars of *C. trachomatis* do not normally result in severe sequelae in mice. Thus, impaired regulatory responses in the genital tract, as seen in ICOS$^{-/-}$ mice, may assist elimination of the bacteria, but could also cause severe immunopathology.

Vaccination for the generation of T cell-mediated immunity in the genital tract

Since protection against STDs such as *C. trachomatis* is clearly a CD4^{+} T cell-based phenomenon we sought to define the criteria required for the generation CD4^{+} T cell responses in the genital tract following immunization. For local vaccination of the genital tract the influence of sex hormones must be better understood. To address this, we used an adoptive transfer model, whereby OVA-specific TCR expressing CD4^{+} T cells from DO11.10 mice, were transferred into BALB/c mice that had received estradiol, progesterone or PBS.

Advantageous to this model is that these transgenic cells can be tracked using an anti-TCR mAb, KJ1-26. Treatment of mice with progesterone, but not estradiol, prior to immunization with CT-OVA allows for efficient antigen uptake across the epithelial barrier, where antigen colocalized to CD11c^{+} cells in the submucosa (Fig. 11). Estradiol treatment failed to facilitate T cell responses, whereas following progesterone treatment, OVA could be detected in the draining lymph node as early as 30 minutes and up to 4 days. Proliferative responses were superior in progesterone treated mice, as measured by proliferative responses to recall antigen *in vitro* and by *in vivo* dilution of CFSE-labeled in CD4^{+} T cells following transfer (Fig. 11).

A



B

	Untreated	Estradiol	Progesterone
PBS	-	-	+
CT-OVA i.vag	-	-	+++

FIGURE 11
Progesterone facilitates effective immunization of the genital tract mucosa. (A) Mice were immunized i.vag with CT-OVA, and the deposition of OVA in the vaginal tissues detected using FITC-anti-OVA, CD11c-Cy3 and To-Pro. Illustrations are show the vaginal mucosa after 30 min. (B) $CD4^+$ OVA transgenic T cells were transferred to naive, estradiol, or progesterone treated mice and immunized with PBS or CT-OVA i.vag. Results show relative proliferative response 4 days after immunization as analysed by CFSE dilution.

Local antigen delivery is required for effective $CD4^+$ T cell-mediated immunity

Although progesterone treatment allows for local antigen uptake in the vagina, intranasal immunization has been suggested to be an alternative approach to avoid the influence of hormones. Intranasal administration of CT-OVA resulted in fewer KJ1-26^+ T cells in the genital tract lymph node. Intranasal priming followed by intranasal boost was insufficient to draw KJ1-26^+ T cells to the genital tract, however, intranasal prime followed by vaginal boost, or vaginal prime and vaginal boost attracted KJ1-26^+ T cells to the local vaginal mucosa (Table I).

Priming	Booster	Genital KJ1-26$^+$ T cells
i.n.	/	-
i.n.	i.n.	-
i.n.	i.vag	++
i.vag	/	-
i.vag	i.vag	++++
i.vag	i.n.	-

TABLE I
Local antigen delivery is necessary for attraction of T cells to the mucosa. CD4$^+$ OVA transgenic T cells were transferred to progesterone treated mice before immunization with the indicated prime/boost combinations of CT-OVA. Tissue sections were scored for the number of KJ1.26$^+$ T cells.

By contrast, the uterine mucosa was devoid of KJ1-26$^+$ T cells regardless of the route of immunization. We hypothesized that local inflammatory stimulation may be required to induce migration of T cells to the tissue. However, even in the presence of a highly inflammatory *C. trachomatis* infection, KJ1-26$^+$ T cells were absent from the uterus. In contrast, KJ1-26$^+$ T cells were attracted to the vaginal tissue following infection. Importantly, these findings suggest that neither inflammation nor vaginal immunization are effective for the generation of T cell responses in the UGT.

CTA1-DD/ISCOMs is an effective mucosal adjuvant vector for the generation of T cell responses

CT is a powerful adjuvant in mice, however too toxic for use in humans. The development of novel mucosal adjuvants and delivery systems is crucial for the development of effective vaccines. CTA1-DD is a non-toxic adjuvant that combines the enzymatic activity of the A subunit of cholera toxin, to a dimer of the D portion of *S. aureus* protein A. Incorporation of CTA1-OVA-DD into ISCOMs provides a powerful tool for antigen and adjuvant delivery into the genital tract. Intravaginal immunization with the CTA1-OVA-DD/ISCOMs vector stimulated T cell proliferation and accumulation of KJ1-26$^+$ T cells in the draining lymph node equivalent to CT-OVA immunization (Fig. 12). Studies have identified a cell-surface sialylated glycan, GL7, specifically expressed on activated germinal center B cells (205). Therefore, we used the anti-GL7 antibody as a means of detecting germinal centers following immunization. CTA1-OVA-DD/ISCOMs stimulated the generation of GL7$^+$ B cells in the draining lymph node. The combined CTA1-DD/ISCOMs vector represents a promising candidate for the generation of genital tract T cell-mediated immunity.

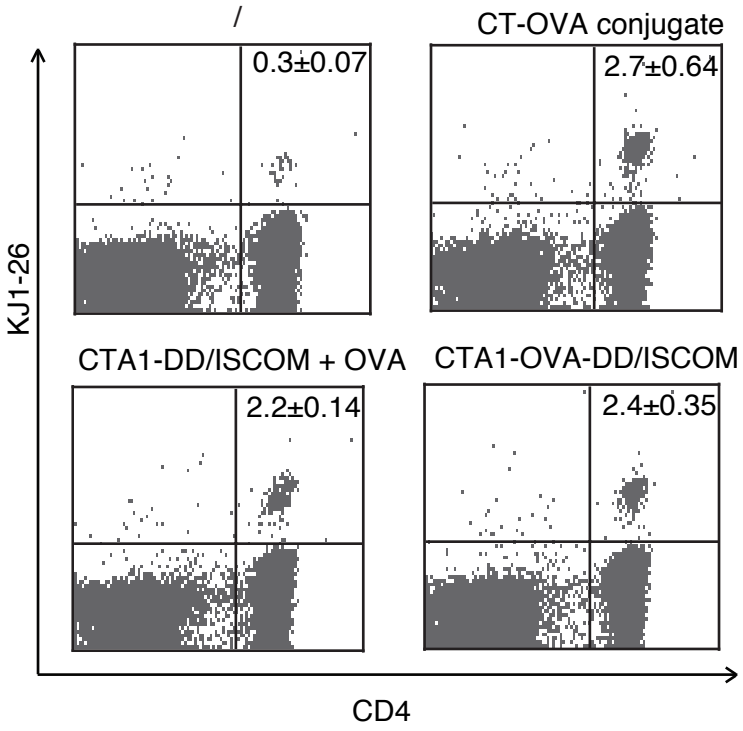


FIGURE 12
The CTA1-OVA-DD/ISCOMs vector is a strong mucosal adjuvant. $CD4^+$ OVA transgenic T cells were transferred to progesterone treated mice before immunization with the indicated adjuvant/antigen combinations. 4 days later, the number of KJ1-26^+ cells in the PALN were determined by FACs.

Discussion

Prevention is the best form of protection. The sexually transmitted disease caused by *C. trachomatis* is entirely preventable, and despite organized education efforts and pathogen screening, the incidence of this infection is increasing world-wide. Vaccination is the most effective means of reducing transmission of infectious pathogens, however this requires a thorough understanding of the local immune response to infection and effective methods of immunization. This thesis addresses one of the crucial gaps in knowledge, namely the generation of $CD4^+$ T cell-mediated immunity in the genital tract, in response to infection and vaccination.

Studies of *Chlamydia*-specific T cells have been hindered due to the lack of efficient techniques that allow for tracking of the T cells in the local mucosa. Isolation of T cells from the genital tract is cumbersome and results in poor yields of cell numbers. Recently, Starnbach and colleagues were successful in the generation of a *Chlamydia*-specific TCR T cell transgenic mouse (206). However, due to limited access to these mice, alternative strategies for the study of T cells are important for progress in the field. To this end we have developed RT-PCR assays for the study of T cell transcription factors in the local genital tract and the draining lymph node. The addition of the transcription factor RORγ-t, specific for Th17 cells, allows for analysis of a population that has not previously been described in *C. trachomatis* infections.

Normal T cell differentiation during *C. trachomatis* infection

Protective immunity against *C. trachomatis* relies on the effector functions of $CD4^+$ T cells. This thesis provides a detailed analysis of the kinetics and dynamics of T helper subset differentiation, including the recently discovered Th17 subset, during infection of the murine genital tract (Fig. 13). The UGT and LGT are vastly different in many ways which reflects their distinctive functions. The LGT is host to a large number of bacterial species that make up the common flora, while the UGT is largely sterile and is adapted to tolerate allogeneic fetus and sperm. In healthy females these functions are carried out without the generation of pathology. By contrast, immune responses such as those seen during *Chlamydia* infections can cause imbalance of effector and regulatory functions which may result in permanent tissue damage. T cells are central to both the pathology and the development of protective immunity against *Chlamydia* infection. Hence, the types of T cells generated during the immune response are of critical importance for host resistance against infection.

The normally quiescent UGT is infiltrated by $CD4^+$ T cells during *Chlamydia* infection. Studies by Rank and co-workers have described peak numbers of $CD4^+$ T cells 3 weeks following inoculation with the bacterium (207), which correlates to our finding of peak T helper transcription factor expression and associated cytokine production. $CD4^+$ T cells in this tissue were predominantly of the Th1-subset and produced large amounts of IFN-γ, although Th17

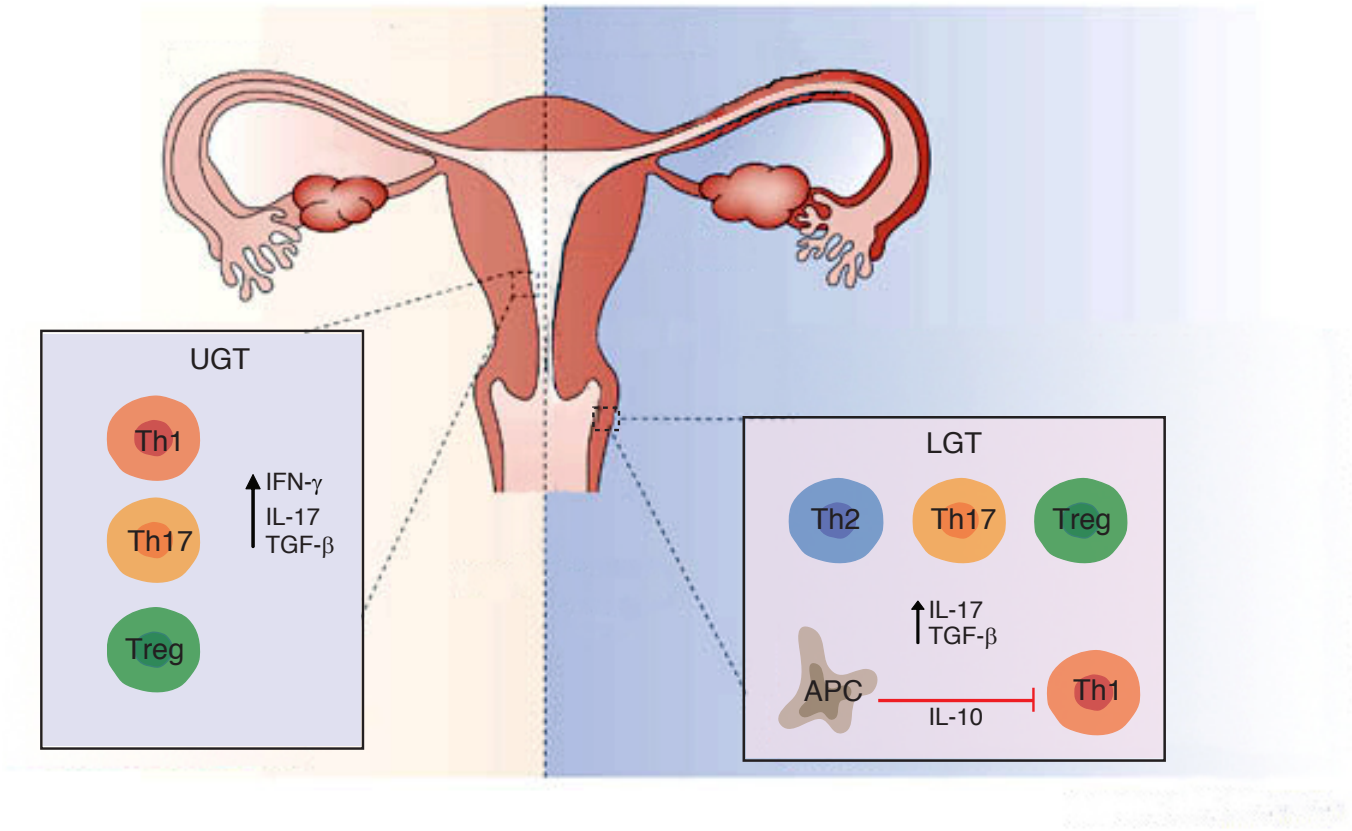


FIGURE 13
An overview of T cell immunity in the upper (UGT) and lower (LGT) genital tract following *C. trachomatis* infection. The UGT is dominated by Th1 responses, while the LGT is host to IL-10-producing DCs which inhibit Th1 expansion, resulting in Th2 dominance.

differentiation also occurred at this location. Th17 cells have not been ascribed a role during *Chlamydia* genital tract infection, however, during infection of the lung with the closely related *C. muridarium* the Th17 signature cytokine, IL-17, was produced as part of the innate immune response at 48 hours following infection (208). IL-17 enhanced proinflammatory innate-type cytokines such as IL-6 and MIP-2, but did not directly inhibit chlamydial growth as IFN-γ does, but did contribute to survival of the animals, since neutralizing IL-17 resulted in higher mortality (208). The early nature of this cytokine production suggested that it was of non-$\alpha\beta^+$T cell origin, and may instead be produced by other cell types such as $\gamma\delta^+$ T cells (209), NKT cells (210) or neutrophils (211). Innate production of IL-17 can contribute to the attraction of other inflammatory, innate-like, cells, such as neutrophils, and IL-17 production is crucial for early $\gamma\delta^+$ T cell-mediated protection against infections such as *L. monocytogenes* (209). Our study suggests that IL-17A production is part of the adaptive immune response, since peak expression is 24 days after infection, at a time when $CD4^+$ T cell numbers are at their highest levels. This data suggests that Th17 responses are able to function simultaneously with Th1 responses, and are not subject to the same feedback inhibition as is observed with Th1-mediated silencing

of Th2-responses. Given that IL-17 is not always protective, IL-17-mediated inflammatory response may even increase susceptibility to pathology. Thus further investigation of the role of Th17 in UGT immunopathology is warranted.

Immune-mediated control of the response to *Chlamydia* infection in both the UGT and LGT was carried out by FoxP3$^+$ Tregs. These cells may well be the source of the TGF-β production we observed. FoxP3 mRNA expression increased in parallel to Th1 responses indicating that the Th1-based immune response could be dampened by FoxP3$^+$ Tregs. IL-10 expression was also upregulated in the UGT, although only moderately and, thus, while it may contribute to regulation of the immune response, the high levels of expression in the LGT suggests that IL-10-mediated functions are of greater significance in this location.

Contrasting with the UGT, the LGT was dominated by Th2 responses and IL-10 production. Th1 responses in this tissue were inhibited by IL-10 production. This is in agreement with studies of IL-10 knockout mice, which have been shown to display enhanced bacterial clearance as a result of enhanced antigen presentation and Th1-bias (79, 129, 131). The mechanisms of enhanced immunity to *Chlamydia* in IL-10$^{-/-}$ mice was proposed by Iijima *et al*. to be the result of enhanced antigen presentation in the lymph node. IL-10$^{-/-}$ DC, but not IL-10$^{-/-}$ T cells from infected mice were capable of mediating enhanced immunity. Our data support these results, and show that CD11b$^+$CD11c$^+$MHC-II$^+$ cells in the local genital tract mucosa are the origin of IL-10 production. The CD11b$^+$CD11c$^+$MHC-II$^+$ phenotype of these cells resembles the phenotype of classical DC. When these cDC were deficient in IL-10 expression, they were strikingly strongly proinflammatory. Plasmacytoid DC (pDC) have also been demonstrated to be a source of IL-10 following restimulation *in vitro* (134). Kelly and coworkers have shown that pDC differentiated from bone marrow precursors produce IL-10 when restimulated with *C. muridarium in vitro*. In our studies we did not find IL-10 in the pDC population of the genital tract, rather IL-10 was located to CD11b$^+$CD11c$^+$MHC-II$^+$ DCs isolated directly from the mucosa during infection.

The function of IL-10 producing DCs late in infection is perplexing. It is estimated that 25% of monocytes that are recruited from the blood differentiate to DCs in the peripheral tissue (212). From here, DCs are capable of antigen uptake and migration to the lymph node for presentation to naïve T cells. However, DCs at the site of inflammation can also recruit inflammatory cells and promote or suppress T cell effector functions. The induction of suppressive DCs has been shown to be directed by a number or cells types including epithelial cells and NKT cells (213, 214). The physiological significance of DC accumulation late during the immune response is unclear, however, study by Vasilijic *et al*. showed that DCs that accumulated early during the immune response to sterile implants were inflammatory, while those recruited later had tolerogenic properties (132). These DCs may function to capture and process self antigens from damaged tissue. Another possibility is that DCs are involved in the resolution and regulation of

inflammation through the induction of Tregs (215). DCs which appear late during the immune response have been shown to induce proliferation of Tregs during hepatitis infection (216) and may be involved in the establishment of chronic infection. DCs-mediated IL-10 production has also been associated with T helper polarization. DCs of the Peyer's patch preferentially secrete IL-10 and generate Th2 responses, whereas non-mucosal spleen DC produce IL-12 and favor Th1 generation (217). Thus IL-10 production in the LGT may have a number of functions including T helper polarization and establishment of Treg populations.

As aforementioned the LGT and UGT have vastly different functions, and we have shown that T helper differentiation during infection results in the expansion of functionally opposing subsets. Different homing patterns of different types of $CD4^+$ T cells may contribute to this. Vascular addressins, used for entry of circulating lymphocytes, are anatomically distinct in the reproductive tract. A study by Kelly *et al*. showed that in the *Chlamydia* infected murine UGT VCAM-1 is more highly expressed than in the LGT (207). Futher, expression of chemokines that are known to attract Th1 cells, such as interferon gamma inducible protein-10 (IP-10) and monokine induced by interferon gamma (MIG) were elevated only in the UGT (18).

The columnar epithelial cells of the cervix, which we have defined as belonging to the LGT, are the initial target of infection for chlamydiae. The dominance of Th2 responses and IL-10 production in this area may contribute to delayed clearance of infection and ascension of the infection into the oviducts. In a study of female patients suspected of suffering PID conducted by Kiviat *et al*., in over 50% of cases chlamydiae could be found in the cervix but not in the upper genital tract (218). In mice, infection of the cervix and UGT are cleared simultaneously (207), however, the data presented by Kiviat *et al*., indicates that there may be delayed cervical clearance of the bacterium in humans (218). The striking differences between the LGT and the UGT as indicated by our study and others, indicates that the presence non-protective T helper subsets may exacerbate both infection and pathology following *Chlamydia* infection.

The role of CD28-mediated costimulation

Costimulation is an important event involved in development and regulation of the T cell response. In the absence of CD28, T cells enter a state of anergy (201). Thus, our finding that $CD28^{-/-}$ mice are impaired in the generation of appropriate immune responses for clearance of chlamydiae from the genital tract and protection against reinfection, was expected. However, a recent study of *C. muridarium* infection in $CD28^{-/-}$ mice reported that clearance of the bacterium was unaffected by the lack of costimulation, although the mice were less prone to immunopathology (219). The most striking difference between the two studies is the inoculating dose and the strain of bacteria, whereby, we infected with a 1×10^6 IFU of human serovar of *C. trachomatis*, while Chen *et al.* used 1×10^4 IFU of *C. muridarium*. Defense against some infections is entirely dependent on CD28-signalling (220), while costimulation appears

dispensable for others (97). For example, *T. gondii* does not require CD28 for elimination of the pathogen, however, memory generation is highly CD28-dependent (221).

The level of CD28-dependency is partially determined by the strength of the TCR signal, that is, strong ligation of TCR may bypass the need for CD28 costimulation (222). Several studies have investigated differences in virulence between *Chlamydia* serovars, whereby the most virulent strains have faster rates of replication and, therefore, are more competent at establishing infection (223, 224). Indeed, *C. muridarium* appears to be more virulent than *C. trachomatis* serovar D, since *C. muridarium* EBs replicate faster and are significantly more cytotoxic to McCoy cells than serovar D (225). Another striking difference is the severe UGT pathology following infection with *C. muridarium* after inoculation with as few as 100 IFU. In contrast, serovar D and other human strains are less likely to ascend to the upper genital tract and cause pathology even at 10^4-fold high doses (226-228). An alternative hypothesis to explain the differences in the outcome of infection, could be that compensatory costimulatory molecules operate in the absence of CD28 for elimination of *C. muridarium*. Regardless of the mechanisms which operate to protect against *C. muridarium* infection, what is clear is that T cell activation appeared not to be impaired since neither bacterial clearance nor cytokine production was affected in $CD28^{-/-}$ mice (219). In contrast, in our hands, CD28-deficiency resulted in strongly reduced T cell differentiation, impaired cytokine production and failure to develop memory responses. Such defects have also been reported in several other models of infection in $CD28^{-/-}$ mice (229-231).

A role for ICOS-signaling in the development of $CD4^+$ T cell regulatory or helper functions

A striking finding in this thesis is that $ICOS^{-/-}$ mice displayed enhanced bacterial clearance and superior protective immunity compared to WT mice. ICOS and CD28 cooperated during early T cell activation but ICOS also had important functions in augmenting effector cell functions. In this model, CD28-signalling was likely to be sufficient for activation of T cells, however in the absence of ICOS, regulatory factors such as IL-10 and $Foxp3^+$ Tregs were inhibited leading to uncontrolled Th1 responses and immunopathology. Interestingly, in the absence of ICOS-signaling the immune response remarkably resembled that of $IL\text{-}10^{-/-}$ mice. Since we found that IL-10 production was of DC-origin, it is possible that the lack of ICOS/ICOS-L interaction affects the ability of DC to produce IL-10. In a paper by Moniz *et al*., pDC were suggested to be a source of IL-10 in the genital tract. These pDC expressed ICOS-L, in contrast to cDC, strengthening the correlation between ICOS expression and IL-10 production (134). This raises the interesting possibility that the IL-10-producing $CD11b^+CD11c^+MHC\text{-}II^+$ cells we described in WT mice, may be a population affected by the lack of ICOS.

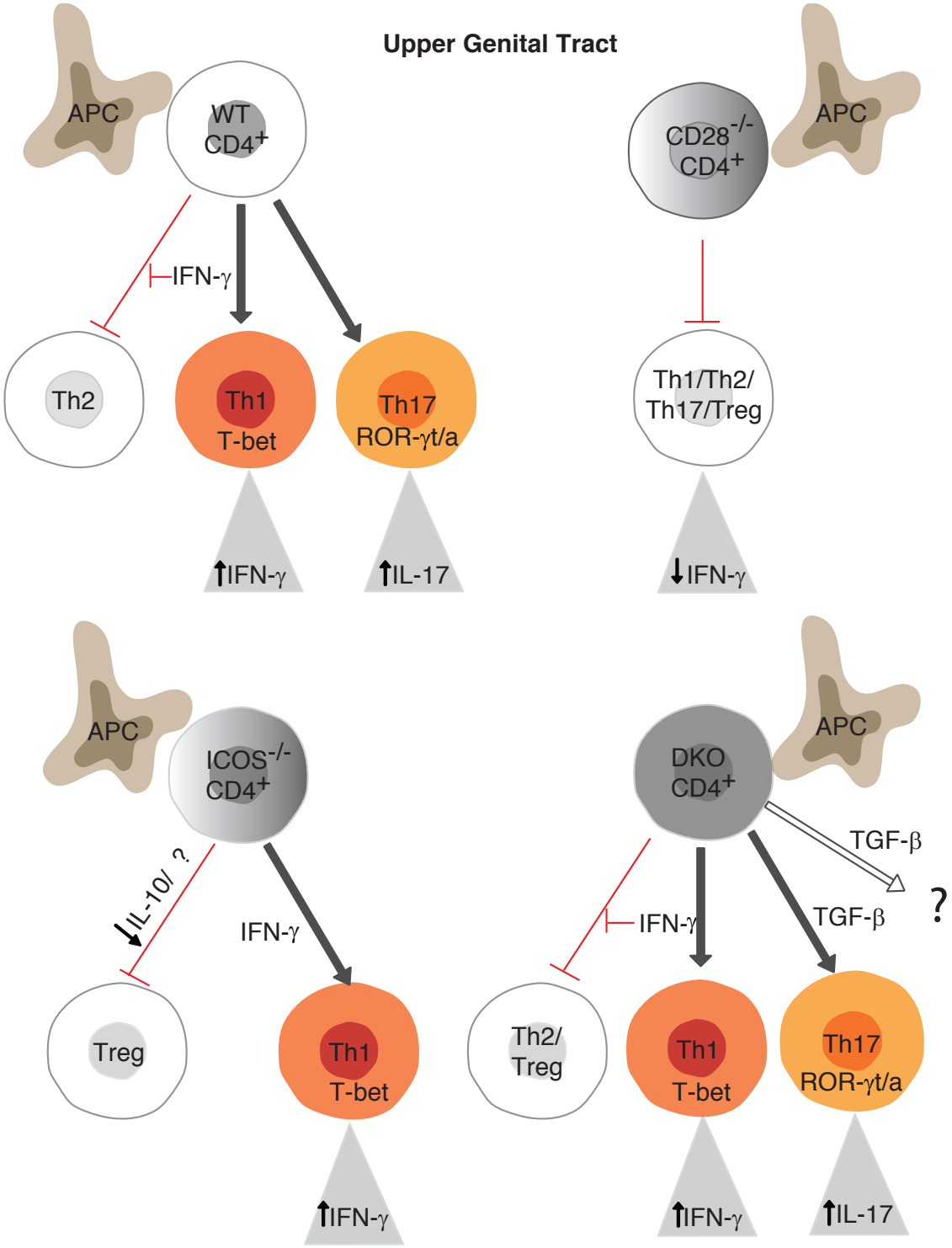


FIGURE 14
T cell differentiation and regulation in the upper genital tract (UGT) of CD28$^{-/-}$, ICOS$^{-/-}$, CD28^{-}/ICOS^{-} (DKO) and WT mice following infection with *Chlamydia*.

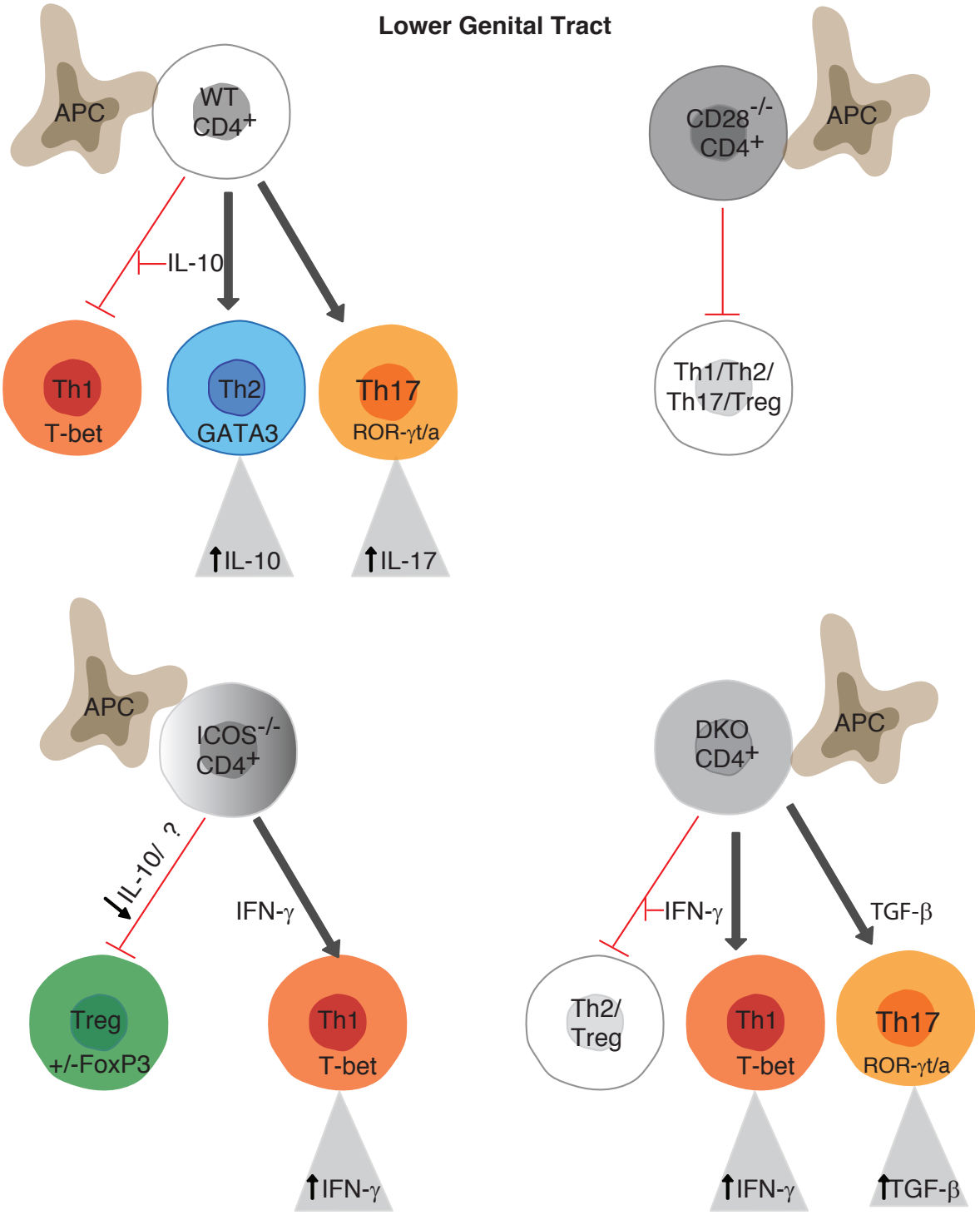


FIGURE 15
T cell differentiation and regulation in the lower genital tract (LGT) of CD28$^{-/-}$, ICOS$^{-/-}$, CD28^{-}/ICOS^{-} (DKO) and WT mice following infection with *Chlamydia*.

ICOS is a costimulator in its own right

Initial activation of T cells involving CD28 signaling results in expression of ICOS. Therefore, ICOS has been considered secondary to CD28 in the hierarchy of costimulation. Our data though, suggests that ICOS is a costimulatory factor much in its own right, since our DKO mice exhibited a phenotype unique from that of $CD28^{-/-}$ mice (Fig 14-15). However, augmented immune responses that occurred as a result of the ICOS-deficiency alone were not sufficient for clearance of *Chlamydia* infection.

There are three obvious possibilities to explain why successful clearance of bacteria takes place in these DKO mice. Firstly, increase in Th1 cells in the LGT is sufficient to clear the bacteria. But, in the simultaneous absence of CD28 and ICOS, memory development is impaired. Secondly, compensatory costimulatory pathways have been activated in DKO mice. Candidate compensatory mechanisms could include 4-1BB. This molecule is an inducible costimulator expressed on activated T cells and DCs which interacts with its ligand, 4-1BBL, on activated APC (233). 4-1BB has direct stimulatory effects on both $CD4^+$ and $CD8^+$ T cells (234, 235). In fact, 4-1BB has been shown to act in cooperation with ICOS in $CD28^{-/-}$ mice to deliver costimulatory signals that allow T cells to produce IL-2 and survive (236). Anti-4-1BB alone is capable of inducing division of T cells even in the absence of a signal through ICOS (237)

Thirdly, the innate immune system operating in DKO mice could be superior to that of WT mice and therefore promoted non-specific clearance of the bacteria from the genital tract. In light of previous publications, all data suggest that $CD4^+$ T cells are required for clearance of the bacteria (29, 30). Hence, unless the lack of costimulation results in significantly enhanced innate immunity, this is unlikely. Further, the cells of the innate immune system that are capable of producing IFN-γ, which is only partially inhibited in DKO mice, include NK and NKT cells. The influence of NKT cells on Th1 compared to Th2 responses is highly dependent on the invading pathogen. For example, NKT cell activity during lung infection with *C. pneumoniae* is protective, while the role of NKT cells during infection with the closely related *C. muridarium* appears to be counter-productive (238). Vaginal epithelial cells produce IL-12 and IL-15 following NKT-cell mediated CD1d ligation, and, thus, NKT cells could contribute to Th1 polarization of the immune response to *C. trachomatis* in the genital tract (239). Despite this, we have found that $CD1d^{-/-}$ mice are unimpaired in clearance of *C. trachomatis* from the genital tract (Marks *et al*., unpublished data). Moreover, NKT cell function and survival is dependent on ICOS- and CD28-signalling, and in their absence activation of NKT cells has been reported to be completely inhibited (240, 241). Perhaps NKT cells are unlikely to explain the clearance of a primary infection in DKO mice.

On the other hand, NK cells are able to produce IFN-γ following direct stimulation by cytokines independently of TCR recognition. Such a mechanism has been implicated in the early resistance

to murine *Leishmania major* infection by upregulating Th1 activity (242). While CD28 is required for NK cytotoxicity, it may not be necessary for NK cell activation (243). In a study by Rank and coworkers, early IFN-γ production following *C. muridarium* infection was thought to be of NK-origin, since depletion of $CD3^+$ T cells did not alter IFN-γ production (27). Following depletion of NK cells, the immune response was shifted towards Th2, with delayed clearance of bacteria (27). Thus, NK cell activity may be involved in clearance of bacteria from the genital tract. However, since IFN-γ production in DKO mice was somewhat reduced, NK cells could only partially explain the clearance of bacteria.

Paradoxically DKO mice expressed high levels of TGF-β mRNA, a cytokine that is normally associated with tissue repair following menstruation and facilitation of pregnancy (reviewed (244)). Given that this cytokine exerts anti-inflammatory effects this finding was unexpected in DKO mice. However, TGF-β has a variety of effector functions which include regulatory, inflammatory and wound healing activities. The promotion of inflammatory responses has been reported through TGF-β-mediated production of the proinflammatory leukemia inhibitory factor (LIF) from endometrial epithelial cells (245). Moreover, activin A and TGF-β are also abundant in the pre-menstrual endometrium at a time when there is increased immune cell infiltration and other inflammatory events (246). In combination with IL-6, TGF-β is necessary for the differentiation of Th17 cells (247). The expression of the Th17 transcription factor RORγ-t in the UGT of DKO mice was increased compared to that in $ICOS^{-/-}$ mice, but this was still below expression levels observed in WT mice. Additionally, regulatory activities of TGF-β could be exerted through Th3 regulatory type cells. These cells have been reported to express FoxP3, and given that we did not observe a corresponding increase of FoxP3 in the UGT of DKO this explanation is unlikely (248, 249). Further studies are required to determine if TGF-β in the UGT of DKO mice is exerting regulatory, inflammatory or wound healing actions.

Another interesting cell population worth considering as a source of TGF-β is $\gamma\delta^+$ T cells, which have a pivotal regulatory role during gestation but are also potentially cytotoxic and NK cell activating (reviewed (244)). $\gamma\delta^+$T cells represent a large portion of T cells in the reproductive tract which are capable of providing early IFN-γ for the polarization of T helper responses in the HSV-2 infection model (250). However, like conventional $\alpha\beta^+$ T cells, $\gamma\delta^+$ T cells are also reliant on CD28-signalling for proliferation and cytokine production and, thus, $\gamma\delta^+$ T cells may not contribute to protective immunity in DKO mice (251). A recent study showed that TGF-β was able to induce a novel population of $\gamma\delta^+$ $FoxP3^+$ Tregs that were themselves able to exert regulatory functions and produce large amounts of TGF-β. These cells were induced following anti-CD3/anti-CD28 stimulation (252). If this population of Tregs operates in the absence of costimulation and are the origin of TGF-β in the uterus of DKO mice remains to be investigated. Nevertheless, these unique cells in the genital tract may provide insights into both immunity and immunopathology during *Chlamydia* infection.

Vaccination of the genital tract for $CD4^+$ T cell responses

Currently the only licensed STD vaccines are based upon attachment inhibition of the human papilloma virus (HPV) through antibody-mediated immunity (34, 35, 253, 254). This approach is viable for preventing HPV infection but for protection against other STDs like *Chlamydia* it is likely that T cell-mediated immunity is required. The second part of this thesis addressed the requirements for the generation of $CD4^+$ T cell immunity in the genital tract following immunization. We used an adoptive transfer model where DO11.10 T cells are injected into BALB/c mice. This allowed us to follow the development of OVA-specific immune responses in the genital tract following hormone treatment and after different vaccination regiments.

Development of strong immunity in the genital tract is suboptimal following systemic immunization. Thus, intravaginal delivery of a vaccine is an alternative approach for boosting local immune responses. Importantly, we found that progesterone treatment was necessary for uptake of antigen in the vaginal mucosa. One striking difference in the genital tract of estradiol treated mice compared to progesterone treated mice was the thickness of the epithelium. Progesterone treatment reduced the epithelial layers lining the vagina from 15-20 cell in thickness, to just 2-3 cells in the vagina. In a similar manner, estradiol increased the thickness of mucus. In the absence of the mucus layer, antigen uptake was restored (255). However, it has not been demonstrated if the uptake of antigen following progesterone treatment is simply due to decreased mucus thickness. Furthermore, the influence of hormone treatment also has other effects. Estradiol affects regulatory factors such as $FoxP3^+$ Tregs (256) and TGF-β production, which could effectively suppress APC maturation and function (257).

Regardless of the hormonal treatment or adjuvant preparation, at no time did we detect OVA-specific T cells in the uterus or uterine horns of immunized mice. This was surprising since studies in humans have shown that uterine peristaltic waves radio-opaque dye entered the uterine lumen as early as 2 minutes of placement in the vagina of women, independent of the phase of the menstrual cycle (258). We hypothesized that in the mouse model, local inflammatory responses to the CT adjuvant may have been partially responsible for the appearance of OVA-specific T cells in the vaginal mucosa. Hence, inflammatory responses in the upper genital tract may be required in order to attract T cells to the uterine mucosa. Surprisingly, even in the presence of an ascending chlamydial infection no $KJ1\text{-}26^+$ T cells could be observed in the uterus. By contrast, local inflammation in the lower genital tract was sufficient to attract T cells to the vaginal mucosa. Thus, UGT T cell responses are likely to require the delivery of antigen to the uterine mucosa, either by ascending transport of antigens or intrauterine immunization.

The physiological differences between mice and humans are significant. CT is a powerful adjuvant in mice, however, the toxic effects in humans are far too severe for clinical use. Currently there is a deficit in safe and effective mucosal adjuvants. The CTA1-DD/ISCOMs

combined vector has also previously been shown to be effective when administered orally, sub-cutaneously and intranasally (194, 259). This vector combines two powerful mechanisms of immune modulation. The particulate nature of ISCOMs facilitates APC uptake, while it is thought that the DD fragment of the CTA1-DD targets this molecule to B cells. The combination of these two molecules results in uptake by both DC and B cells, with the enzymatic activity of CTA1-DD mediating enhanced DC maturation and function for antigen presentation to T cells (259). We found that the CTA1-DD/ISCOMs vector is an effective adjuvant and delivery system for the stimulation of both B and T cell responses in the genital tract following intravaginal immunization.

Conclusions

Our studies demonstrate the presence of two anatomically distinct microenvironments in the genital tract. The response in the UGT was suited for elimination of pathogens, at the same time effectively silenced T helper functions which are non-protective. In contrast, immunologically privileged, IL-10-producing, DC in the LGT directed Th2 differentiation. CD28 was necessary for the development of both protective and regulatory $CD4^+$ T cell responses, while the absence of ICOS signaling removed the dampening effects of IL-10 in the LGT, allowing for enhanced Th1 responses and clearance of bacteria at the expense of immunopathology. ICOS-deficiency in the simultaneous absence of CD28, restored IFN-γ production in the UGT and promoted Th1 expansion in the LGT for clearance of the bacteria, but did not impact on the development of memory.

This thesis work highlights the need for a better understanding of $CD4^+$ T cell-mediated immunity in the genital tract for protection against STDs. Strategies for vaccine based generation of $CD4^+$ T cell-immune responses require progesterone treatment for antigen uptake and local delivery of the antigen to attract T cells to the mucosal tissue of the vagina. We also show that CTA1-DD/ISCOMs is a promising mucosal adjuvant vector for stimulation of genital tract immune responses.

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