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Preventive strategies and early immune responses in *Chlamydia trachomatis* infection

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Submitted by

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Declaration

The doctoral candidate, Katja Knapp, MSc, conducted her PhD studies at the Department of Dermatology at the Medical University of Vienna under the supervision of Prof. Dr. Georg Stary. This thesis has not been submitted to any other university and was solely written by the author, Katja Knapp.

The majority of the work included in this thesis was carried out at the Department of Dermatology of the Medical University of Vienna (Department Head: Univ.-Prof. Dr. Wolfgang Weninger). The first publication arising from this thesis was performed in close collaboration with Dr. Romana Klasinc and Univ.-Prof. Dr. Hannes Stockinger from the Institute for Hygiene and Applied Immunology of the Medical University of Vienna and the team from Stefan Kubicek at CeMM – Center for Molecular Medicine, Vienna. In the second project, patient recruitment was done by Dr. Marlene Kranawetter and Dr. Christoph Grimm from the Department of Gynecology. Smart-seq2 bulk RNA sequencing was performed in the Biomedical Sequencing Facility of CeMM and flow cytometry experiments were mostly acquired at the Flow Cytometry Facility of the Medical University of Vienna. Additional affiliations of collaborative partners are referred to in the individual manuscripts. This thesis also includes two first or shared first author review articles being referenced in the introduction and attached in the Appendix.

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The author of this thesis did not use any AI tools to generate this thesis nor any AI-assisted methods to write and rephrase sentences, only DeepL was used to translate the abstract from English to German (page vi - vii).

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Abstract (English)

Chlamydia trachomatis (Ct) is a pathogen causing urogenital infection in both men and women and is sexually transmitted. Moreover, it can cause infection of different mucosal regions like the eyes, pharynx and rectum. With a global incidence of around 130 million, it is the most common bacterial sexually transmitted infection (STI). Even though more than 70% of infections remain asymptomatic, they can cause long-term problems like pelvic inflammatory disease, congenital infection during birth and infertility in both men and women. The natural immune response against Ct is insufficient to protect from subsequent infection and vaccines are not available in the clinics so far. Therefore, the aims of this thesis were the following: i) development of novel prophylactic concepts to prevent Ct infection, and ii) identification of mechanisms of early immune response once an Ct infection occurred to address why humans do not develop protective T cells. With a semi-automated screen, a library of 2,000 compounds was assessed, pinpointing pentamidine to effectively protect epithelial cells *in vitro* and mice from Ct infection. Clinically approved as an antiprotozoal drug in humans, we showed that repurposing it as prophylaxis locally applied to the genital tract prevents productive infection in mice. Additionally, it inhibits proliferation of *Neisseria gonorrhoeae*, another common sexually transmitted bacterium. This suggests that pentamidine could be pursued as prophylactic agent against bacterial STIs and we will continue developing a suitable formulation for the anogenital region.

To tackle the question on Ct immune reactions early after infection, we established an *ex vivo* infection model of human cervix uteri explants and assessed antigen presenting cells being involved in Ct uptake and which functional consequences on the induction of an immune response this might have. We could show that $CD11c^+CD14^+$ dendritic cells (DCs) are the most effective subset to take up Ct. Moreover, we observed that DCs which acquire Ct inclusions become immobile despite upregulation of migration marker CCR7. Based on our observations, we propose that reduced mobility of DCs is caused by hijacking of the actin cytoskeleton by the pathogen thereby impairing DC functions. Notably, Ct-exposed DCs can still prime naïve $CD4^+$ T cells in a co-culture model towards a Th1 and Th17 phenotype, suggesting that the ineffective immune response is due to the inability of the DCs to reach naive T cells in the lymph node.

In summary, two important areas of chlamydia research have been addressed in this thesis and could revolutionize our understanding of pathophysiology and treatment of STIs.

Abstract (German)

Chlamydia trachomatis (Ct) ist ein sexuell übertragbarer Erreger, der Schleimhautinfektionen des Urogenitaltrakts sowie der Augen, des Rachens und des Rektums verursachen kann. Mit weltweit rund 130 Millionen Fällen pro Jahr sind Chlamydien die häufigste Ursache für bakterielle sexuell übertragbare Infektionen. Obwohl Ct-Infektionen in mehr als 70% der Fälle asymptomatisch bleiben, können sie sowohl bei Männern als auch bei Frauen langfristige Probleme wie Unfruchtbarkeit, Beckenentzündungen oder Infektionen von Neugeborenen vor oder während der Geburt verursachen. Die natürliche Immunreaktion gegen Ct ist meist unzureichend, um vor einer Reinfektion zu schützen und Impfungen sind bisher nicht zugelassen. Die Ziele dieser Dissertation sind daher einerseits, neue prophylaktische Konzepte zur Vermeidung einer Ct-Infektion zu entwickeln und andererseits, Mechanismen der frühen Ct-Immunantwort zu charakterisieren, um herauszufinden, warum die meisten Menschen keine schützenden T-Zellen aufbauen.

Mit einem halbautomatischen Screen wurden 2.000 chemische Verbindungen in der Zellkultur auf ihre Wirksamkeit gegen Ct untersucht. Wir konnten Pentamidin identifizieren, das beim Menschen bereits als Antiprotozoikum eingesetzt wird. Es verhindert eine Ct-Infektion nicht nur in der Zellkultur, sondern auch *in vivo* bei lokaler Anwendung im Genitaltrakt im Mausmodell. Darüber hinaus hemmt Pentamidin auch *in vitro* das Wachstum von *Neisseria gonorrhoeae*, einem weiteren häufigen sexuell übertragbaren Erreger. Dies deutet darauf hin, dass Pentamidin als prophylaktisches Medikament mit breiterer Wirksamkeit gegen bakterielle Geschlechtskrankheiten eingesetzt werden könnte. Wir planen daher die Weiterentwicklung einer geeigneten Medikamentenformulierung für Anwendung im humanen Anogenitalbereich.

Um die Frage der ungenügenden Immunreaktionen gegen Ct zu klären haben wir ein *ex vivo* Infektionsmodell mit menschlichen Gebärmutterhalsproben etabliert. Das ermöglicht uns, zu untersuchen, welche antigenpräsentierenden Zellen an der Ct-Aufnahme beteiligt sind. Wir konnten zeigen, dass $CD11c^+CD14^+$ dendritische Zellen (DCs) am effektivsten bei der Antigenaufnahme sind. Außerdem wurden DCs, die großen Mengen an Chlamydien aufnahmen, trotz Hochregulation des Migrationsmarkers CCR7, bewegungsunfähig. Wir vermuten, dass dies daran liegen könnte, dass Ct das Aktin-Zytoskeletts für seine Zwecke instrumentalisiert, wodurch normale DC-Funktionen wie die Motilität beeinträchtigt werden. Im Ko-Kultur-Modell mit naive $CD4^+$ T-Zellen konnten Ct-stimulierte DCs dennoch Th1 und Th17 Helferzellen prägen, was darauf hindeutet, dass keine Probleme bei der T-Zellstimulierung per se vorliegen, sondern DCs aufgrund der eingeschränkten Zellmotilität

daran gehindert werden, die T-Zellen im Lymphknoten zu erreichen und dadurch eine Immunantwort gegen Ct hervorzurufen.

Zusammenfassend lässt sich sagen, dass in dieser Dissertation zwei wichtige Bereiche der Chlamydienforschung behandelt werden, was in der Folge dazu beitragen könnte, unser Verständnis über die pathophysiologischen Prozesse sowie die Behandlung von Geschlechtskrankheiten zu reformieren (1).

Publications arising from this thesis

Knapp K, Kopf A, Dingelmaier-Hovorka R, Kranawetter M, Chrapla K, Panday R, Grimm C, Stary G. Impaired migration of dendritic cells limits immune response in human Chlamydia trachomatis infection. *In submission*

Knapp K*, Klasinc R*, Koren A, Siller M, Dingelmaier-Hovorka R, Drach M, … Stary G. Combination of compound screening with an animal model identifies pentamidine to prevent Chlamydia trachomatis infection. Cell Reports Medicine.2024; 5(7):101643.

Ökengin D, Noori T, Alemany A, Bienkowski C, Liegon G, İnkaya AC, Carillo J, Stary G, **Knapp K**, Mitja O, Molina JM. Prevention Strategies for Sexually Transmitted Infections, HIV, and viral hepatitis in Europe Series. The Lancet Regional Health – Europe. 2023; 34:100760.

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Abbreviations

APC	Antigen presenting cell
CCL21	CC motif chemokine ligand 21
CCR7	CC motif chemokine receptor type 7
CD	Cluster of differentiation
CeMM	Center for Molecular Medicine
CFTR	Cystic fibrosis transmembrane conductance regulator
CpG	Cytidine-phosphate-guanosine oligodeoxynucleotides
Ct	Chlamydia trachomatis
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EB	Elementary body
FGFR	Fibroblast growth factor receptors
FRT	Female reproductive tract
GAG	Glycosaminoglycans
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HSV	Herpes simplex virus
IDO1	Indoleamine 2,3-dioxygenase
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
ILC	Innate lymphoid cells
ITGB1	Integrin beta-1
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Langerhans cell

LPS	Lipopolysaccharide
MAIT	Mucosal-associated invariant T
MHC	Major histocompatibility complex
moDC	Monocyte-derived dendritic cell
MRT	Male reproductive tract
MSM	Men who have sex with men
NF-κB	Nuclear factor kappa B
Ng	Neisseria gonorrhea
NK cell	Natural killer cell
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PDGFR	Platelet-derived growth factor receptors
PD-L1	Programmed death-ligand 1
PEP	Post-exposure prophylaxis
PLWH	People living with HIV
Pmps	Polymorphic membrane proteins
Poly(I:C)	Polyinosinic:polycytidylic acid
PrEP	Pre-exposure prophylaxis
PID	Pelvic inflammatory disease
RB	Reticulate body
RNA	Ribonucleic acid
STI	Sexually transmitted infection
Th	T helper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor

Treg	Regulatory T cell
Trm	Tissue resident memory T cell
UV-Ct	UV-inactivated Ct
WHO	World health organization

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1. Introduction

1.1 The genital mucosa

The genital mucosa is the barrier between the reproductive tract and the surrounding world to protect it against damage and pathogens. In the male reproductive tract (MRT), the penis encloses the urethra which connects the glans penis with the urinary bladder and the reproductive system including testis, epididymis, ductus deference and prostate. While the glans penis and the outer foreskin are lined with keratinized stratified squamous epithelium, the inner foreskin and the urethra are comprised of nonkeratinized stratified squamous and columnar epithelial cells, respectively (2,3). As keratinization protects the underlying tissue from infections, the entry ports for sexually transmitted pathogens are mainly the inner foreskin and the urethra which argues for circumcision as prevention against human deficiency virus (HIV) in endemic areas (2). The female reproductive tract (FRT) can be divided into two parts: the lower FRT with vagina and ectocervix and the upper FRT with endocervix, uterus, fallopian tubes and ovaries. In the lower FRT, the vulva lined with skin transits into mucosal tissue at the vagina which consists of multilayered stratified squamous epithelium and additionally lines the ectocervix. The upper FRT is built up with single columnar epithelium in the endocervix and uterus (4,5).

1.1.1 Barrier functions

As a barrier tissue, the epithelium in the reproductive tract has several mechanisms to protect the host against pathogens. Firstly, the physical barrier due to anatomical design of the FRT with the cervix as a gate keeper only allows passage of e.g. sperm or menstrual blood at certain time points, while inhibiting ascending bacteria as a basic defense mechanism (4,5). In the MRT, the structure of the glans penis covered by the foreskin and keratinization offer a fist layer of protection (2). Secondly, mucus is overlaying the epithelium in both the female and male genital tract to lubricate the reproductive tract, thereby preventing physical stress as well as inhibiting attachment of pathogens to the epithelial cells while providing optimal conditions for sperm cells to facilitate fertilization. In the FRT, goblet cells produce mucus consisting of different glycoproteins named mucins depending on the location and menstrual phase. In males, mucus is produced by glands of Littre in the urethra. Additional components like immunoglobulins, complement, cytokines and antimicrobial peptides secreted into the mucus are another layer of protection (3,6). Thirdly, in the FRT the acidic milieu in the vagina with a pH of around 4.5 prevents the proliferation of pathogens but favors the growth of beneficial Lactobacilli forming the microbiome (5). Colonization with different Lactobacilli species is dependent on ethnicity,

reproductive age and hormonal changes as well as lifestyle factors. Lactic acid produced by the bacteria protects from vaginal dysbiosis and STIs (7,8).

1.1.2 The menstrual cycle in the female reproductive tract

The FRT undergoes various changes during the lifetime of a women: on the one hand, developmental changes occur between puberty, fertile age and menopause. On the other hand, the menstrual cycle as well as pregnancy have major impact on the structure and function of different organs in the FRT (4,5). After menstruation, which marks the beginning of a cycle, a new follicle is maturing and estrogen is the dominating hormone, associated with proliferation of the endometrium. Ovulation initiates the luteal phase of the cycle and induces the production of progesterone, thereby preparing the endometrium for potential nidation of the fertilized oocyte. In case this does not occur, levels of both sexual hormones decrease and the cycle restarts (9). The menstrual cycle under the regime of sex hormones does not only influence the growth of the epithelium in the uterus but also impacts immune cell composition and the consistency of the secreted mucus, thereby modulating the susceptibility to genital infection (4,5).

1.1.3 The immune system in the reproductive tract

Despite several levels of protection against infection of the reproductive tract by anatomical and chemical mechanisms, the immune system plays a major role in the defense of pathogens. The main players in tissue immunology are on the one hand myeloid cells, acting as sentinels patrolling the tissue and phagocytosing various antigens they encounter in their tissue environment, and on the other hand lymphoid cells with B and T cells comprising the largest part which are responsible for an adaptive immune response and memory formation. In the FRT, the most important myeloid cell types are macrophages and dendritic cells (DCs) being professional antigen-presenting cells (APCs) (10). Langerhans cells (LCs), a tissue-resident macrophage subset specific for epithelium, are efficient APCs due to their DC-like features and therefore play an important role for the protection against pathogens in the lower FRT (11). After antigen uptake, APCs migrate to the lymph nodes and display antigens via their major histocompatibility complex (MHC) molecules to naïve T cells. T cells are the most abundant lymphoid cells in the FRT (10) and they come in two principal subsets: Cytotoxic T cells and helper T cells. Cytotoxic T cells, characterized by their expression of CD8 and lytic molecules, recognize antigens presented to them via MHC-I molecules and are responsible for killing of cells infected by viruses or intracellular bacteria as well as tumor cells (12). T helper cells, also known as $CD4^+$ T cells, are responsible for production of cytokines and thereby stimulate certain immune cells and activate B cells (12). A specific subset of $CD4^+$ T cells are regulatory T cells (Tregs)

characterized by expression of the transcription factor FoxP3. Their physiological role is to suppress other immune cells to maintain tolerance against harmless antigens and dampen immune responses to prevent tissue damage during infection or autoimmunity (13,14).

In the FRT, the ratio of $CD4^+$ to $CD8^+$ T cells is equally balanced which is in stark contrast to the blood of healthy individuals, where there are around twice as many $CD4^+$ than $CD8^+$ cells (10,15). B cells are relatively sparse in the FRT, accounting for only 0.4% - 5% of immune cells depending on location within the FRT (10,16). Nevertheless, IgA and IgG antibodies are detected throughout the FRT and play a role in protection against infection (4,5,17). Other immune cell subsets in the FRT include innate lymphoid cells (ILCs), natural killer cells (NK cells) and innate-like lymphocytes like γδ T cells and mucosal-associated invariant T (MAIT) cells (4). Of those, NK cells are the most abundant and most studied subset (10). They play important roles in antiviral immune response in herpes simplex virus (HSV) infection or human papilloma virus (HPV) infection by exerting their cytotoxic function independently of APCs (4,5). Additionally, a specific uterine NK cell population is highly abundant in the endometrium during certain phases of the menstrual cycle and essential to facilitate implantation of the embryo and decidualization during pregnancy (18).

In the MRT, the testis is an immune privileged region sealed by the blood-testis barriers resulting in a lack of immune cells in the epithelium and seminiferous tubules to protect spermatogenesis while macrophages, mast cells and especially Tregs are present in the interstitial space to maintain immune homeostasis (19,20). LCs are populating the epithelium of the foreskin but are absent from the urethral mucosa. In this area of the MRT, macrophages are the predominant APC subset (2,3,20). T cells are highly abundant in all penile regions with a balanced ratio of CD4/$CD8^+$ T cells (21). T cells are described to be mainly effector memory T cells and the integrin CD103 is mostly expressed by $CD8^+$ T cells resident in the urethral epithelium (3). B cells, NK cells and NKT cells comprise each around 3% of immune cells in the penile area (21). Most B cells exhibit a memory phenotype and plasma B cells produce mainly IgG and IgA which are secreted to the seminal plasma (21,22). The male reproductive immune system is still not well explored due to limited access to material and focus on the female reproductive tract when studying fertility issues in the past, therefore the immune responses to pathogens in the MRT still needs to be elucidated.

1.1.4 Immunity and tolerance in the female reproductive tract

Depending on the menstrual cycle and the location within the FRT, different immune cell subsets can have various functions in promoting immunity and tolerance and we are only starting to understand the roles of rare immune cell populations in this complex

compartment. More detailed information on antigen uptake by APCs, interaction with T cells and implications for memory formation in homeostasis and infection in barrier tissues like the FRT is covered in a recent review written by the author of this thesis and can be found in the appendix (14).

DCs in the FRT are important to induce systemic adaptive immune responses including IgG production as well as a local response with B cells producing secretory IgA and tissue-resident T cells for achieving a long-lasting cellular mucosal protection against infection (4,14,23). However, they also play an opposing role by inducing tolerance in order to reduce the risk of immunopathology in homeostatic conditions. This dual function of DC is especially important in the FRT mucosa, as the immune system needs to distinguish between pathogens causing sexually-transmitted infections (STIs) and antigens expressed by the fetus and placenta during pregnancy to promote tolerance (14,24–26). To date it is not well established how immune cells of the genital mucosa distinguish between harmful and harmless antigens and which signals need to be induced for a balance of these opposite functions of the immune system. The next section will focus on STIs, how the immune system copes with certain STIs as well as preventive strategies to circumvent an actual infection.

1.2 Sexually transmitted infections

1.2.1 Epidemiology of sexually transmitted infections

STIs burden humanity since time immemorial and are already observed by the ancient Greek physician Hippocrates. Today, there are either preventive or curable treatments available for most STIs. Still, STIs are a major global threat and cause deaths and massive costs to the health care systems. STIs are primarily caused by viral and bacterial agents, but also parasites use this route of infection. In the year 2020, the deadliest viral sexually transmitted diseases with worldwide infection rates of 1.5 million each are human immunodeficiency virus (HIV) and hepatitis B virus (27). Other viral STIs of concern are herpes simplex viruses (HSV) and human papilloma viruses (HPV). Even though there are effective vaccines available against hepatitis B virus and HPV (28), still many patients are affected and eventually die from long-term consequences like liver cirrhosis, liver cancer or cervical cancer (27). For HIV, pre – and postexposure prophylaxis drug regimens effectively prevent infection in individuals at risk (28). Drug interventions are available for hepatitis B and HIV that allow infected patients to live a normal life. However, disease is not curable (27). Being infected with a viral STI is also a risk factor for affected persons becoming susceptible to acquiring an additional STI or transmitting it to sexual partners. Moreover,

the risk to develop cervical cancers upon HPV infection is 6-fold increased in HIV-infected women compared to HIV-negative individuals (29). After the 2022 global outbreak of Mpox virus infections, which are mainly transmitted by sexual contact, Mpox lines up with viral STIs as another disease of concern (30). For Mpox, vaccines are available and show protection in preliminary studies, however, long-term data from bigger cohorts are still lacking (28).

Bacterial STIs with *Chlamydia trachomatis* (Ct), *Neisseria gonorrhea* (Ng) and *Treponema pallidum* (syphilis) and parasitic STIs with *Trichomonas vaginalis* are considered curable with antibiotics. They are very common with 128 million, 82 million, 7 million and 156 million reported acute infections worldwide per year, respectively (27). The problem, as with all STIs, is that they often are asymptomatic and remain therefore undetected and untreated. Apart from syphilis, which can cause serious systemic health problems and neurodegeneration in later stages as well as congenital syphilis during pregnancy, bacterial STIs usually come with local symptoms like genital discharge or urethritis but are not life-threatening in adults. In pregnant women, STIs can induce preterm births and be transmitted to the babies during birth which can cause serious eye or lung infections. Moreover, STIs can cause infertility in both men and women or induce complications in women like ectopic pregnancies even if no symptoms occurred (31).

1.2.2 Chlamydia trachomatis

The infection with Chlamydia trachomatis (Ct) is the most prevalent sexually transmitted bacterial disease with increasing infection rates every year (32). In addition to urogenital infection, Ct can also cause infection of the rectal and pharyngeal mucosa as well as the eye, depending on the serovar. The serovars can be discriminated by their major outer membrane protein (MOMP) which has a unique DNA sequence detectable by PCR. Serovars A - C are associated with ocular infections and trachoma formation, while serovars D - K cause predominately urogenital infections. Serovars L1 - L3 are mainly transmitted via the anogenital route and are responsible for lymphogranuloma venereum, which is characterized by dissemination of the bacteria via the lymph vessels (33). In Austria, serovar E is the most common serovar for urogenital infections (34).

As strictly intracellular bacteria, Ct infects mainly epithelial cells lining mucosal tissues and hijacks the host cell machinery to perform their replication. The infectious, metabolically inactive form of Ct, the so-called elementary bodies (EBs) can attach to host cells by several mechanisms. Firstly, they can bind to glycosaminoglycans (GAGs) like heparan sulfate on the cell surface of host cells (35,36). Then there are host cell receptors described like platelet-derived growth factor receptor (PDGFR), cystic fibrosis transmembrane

conductance regulator (CFTR), fibroblast growth factor receptors (FGFR) or integrin beta-1 (ITGB1) which interact with bacterial surface factors like lipopolysaccharide (LPS), CT017 or polymorphic membrane proteins (Pmps) (37–40). For instance, ITGB1 is even actively upregulated by the Ct-encoded molecule Ctad1 which functions as adhesin and invasin binding to β1 integrin, thereby promoting clustering of β1 integrin on the surface of epithelial cells to increase Ct entry (38). Upregulation of β1 integrin on fallopian tube epithelium cells in Ct infected women predisposes the embryo to stick to the fallopian tube contributing to ectopic pregnancy (41).

Within epithelial cells, Ct undergo two phases of their life cycle: the infectious EBs transform into reticulate bodies (RBs), which are the replicative form (42). After completing the life cycle, RBs transform back into EBs which exit the epithelial cell either via lysis of the host cells or via extrusion, which means that the intact inclusion surrounded by the plasma membrane exits the host cell without inducing cell death (43). Ct infection is dependent on the host cells, as they utilize both the actin cytoskeleton and microtubules for endocytosis and transportation of the inclusion (43). Even though Ct has the machinery for intact glycolysis and fatty acid metabolism, the bacteria manipulate the host metabolisms to obtain nutrients like amino acids, glucose-6-phosphate, iron and lipids like sphingolipids and cholesterol as well as energy in form of ATP from the host cell (44). Additionally, Ct circumvents being disposed by the host by producing anti-apoptotic proteins (44) to prevent host cell death and by inhibiting lysosome fusion (45). Under stress conditions, which can be caused e.g. by nutrient deprivation, antibiotic treatment or an immune response, Ct terminates replication but persists in so-called aberrant bodies instead of conversion of RBs to EBs. They can endure in this form and restart their proliferation once the stressor is removed (42,44). A graphic overview of the Ct life cycle is depicted in Figure 1.

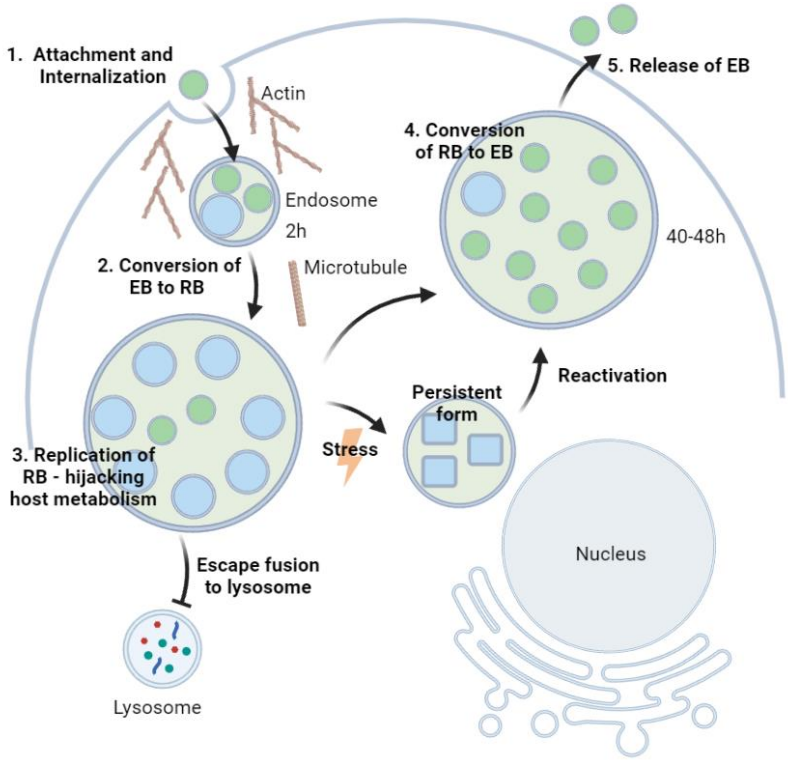


Figure 1. Overview of Chlamydia trachomatis life cycle. Ct EBs attach to the surface of host cells and are internalized utilizing host cell machinery. 2h after infection, EBs converse to RBs within the endosomes and start replication in the chlamydia inclusion. 40-48h after infection, RBs develop into EBs and are released to infect the next host cell. Upon stress, Ct can enter a persistent form and stay dormant until they are reactivated and continue replication. EB, elementary body. RB, reticulate body. Adapted from Gitsels et. al, 2019 (43) with biorender.com.

1.2.3 Infection models of Chlamydia

There are other chlamydia strains which share features with Ct, some of them pathogenic to humans like *C. pneumoniae* infecting the respiratory tract, while others are adapted to various animal species like *C. suis* in pigs, *C. psittaci* in birds, *C. pecorum* in ruminants or *C. abortus* in sheep (46). Additionally, to being a thread to life stock, some strains are reported to cause zoonosis in persons working in close contact with infected animals (47). *C. muridarium* infecting mice is often used as a laboratory model to study chlamydia infection, as it causes a similar phenotype as in humans infected with Ct, including ascending infection in some female animals or hydrosalpinx (48). Additionally, the human Ct strain can also be used in mouse, but hormonal treatment to normalize estrous cycle is necessary as mice are only permissive to Ct in certain phases of estrous cycle. In this transcervical infection model, mice usually resolve infection spontaneously within two weeks (49). In mice infected vaginally, it is detectable for some days, but no efficient colonization occurs (49). Currently available *in vivo* and *in vitro* models to study Ct infection are summarized in a review by the author of this thesis and can be viewed in the appendix (50).

1.2.4 Immune response to Chlamydia

The target cells of Ct are primarily epithelial cells lining the genital mucosal tissues. In males, the primary site of infection is the columnar urethral epithelia (51), while in women the cervix and urethra can be infected with better growth rates in the columnar epithelium of the endocervix (52). Epithelial cells are not only the first cells to encounter Ct, but they are also the initiators of a pro-inflammatory response. Primary ectocervical epithelial cells as well as epithelial cell lines release pro-inflammatory cytokines like IL-6, IL-8 or TNF-α upon Ct infection (53). IL-6 has both beneficial and adverse roles in Ct infection: it is important to kickstart a Th1 response with interferon-gamma (IFN-γ) production but is also described to be involved in chronic inflammation and pathology after Ct infection (54). In female patients with Ct infection, there is a significant increase in IL-6 levels in exfoliated cervical cell samples compared to healthy controls (55). IL-8 is known to attract neutrophils, which can be harmful in Ct infection as they are a main driver of pathology in Ct infection (56,57). Neutrophils were also shown to be paralyzed by Ct, thereby being unable to pursue their normal function like formation of neutrophil extracellular traps and enabling the bacteria to survive within the neutrophils (58). TNF-α is another cytokine with opposing roles in Ct immunopathology. On the one hand, it is described to inhibit the cell metabolism and induce apoptosis in the host cells, thereby withdrawing the foundation for Ct replication (54). On the other hand, TNF-α is not essential for chlamydia clearance in mouse models (59) and absence of it even decreases the immunopathological damage in mouse fallopian tubes (60). In addition to the above discussed cytokines, epithelial cells are a source of IL-12 which is essential for Ct clearance and induction of adaptive immunity (53). IL-12 is also produced by DCs and leads to Th1 T helper cell differentiation.

In early vaccination trials it was observed that vaccinated individuals had exacerbated infections upon challenge with the pathogen (61). In a mouse model of Ct infection, these clinical observations were replicated and revealed that this is mediated by the differential antigen uptake by two distinct subsets of DC (62). When immunizing mice with UV-inactivated Ct (UV-Ct), the antigens are taken up mainly by $CD103^+$ DC, which do not produce IL-12 but IL-10. Subsequently, regulatory T cells (Tregs), inducing a tolerogenic immune response are activated. On the other hand, mucosal exposure to live Ct induces bacteria uptake by $CD103^-$ DC, IL-12 upregulation and presentation to $CD4^+$ T cells which produce IFN-γ and therefore an immunogenic response (62). The release of IFN-γ by Th1 cells is one of the best studies mechanisms of anti-Ct immune response in mice (63). Mice which are deficient for IFN-γ or IFN-γ receptor show an increased bacterial load and cannot clear the infection efficiently (49,64,65), indicating that the DC – IFN-γ-producing T cell axis is essential for anti-chlamydial immune response. A $CD4^+$ T cell response is sufficient to

promote immunity in mice (49). $CD8^+$ T cells are also induced and activated upon Ct infection in mice, but absence of $CD8^+$ T cells does not modulate the immune protection (62), even though they can be an additional source of IFN-γ (63). Essential for a long-lived protective response against subsequent infection is the formation of tissue resident memory T cells (Trm) (62).

Human monocyte-derived DCs (moDCs) mature upon Ct-L2 infection, but also bystander cells upregulate HLA-DR, CD83 and produce IL-12 (66). Chlamydia can survive inside myeloid cells like moDCs and macrophages (66–69) and some report that Ct replicates within myeloid cells (70). However, it is still under debate if Ct can hijack immune cells like macrophages to be transported to and even infect cells at distant locations (67). In human cervical Ct infection, myeloid DC are recruited to the endocervix and upregulate the co-stimulatory molecules CD80, CD83 and CD86 (71). Moreover, both neutrophils and especially T cells are recruited to the endocervix of infected individuals and numbers decrease again one month after antibiotic treatment (72). Both $CD4^+$ and $CD8^+$ T cells derived from peripheral blood mononuclear cell (PBMC) of infected patients stimulated with Ct EBs produce mainly IL-4 during acute phase of infection, whereas an IFN-γ response is only detected one month after infection (73). Additionally, $CD4^+$ T cells expressing the transcription factor GATA3 accumulate in the endometrium of infected women (73). Together, these findings suggest that the immediate anti-Ct immune response in humans is skewed to a Th2 response, an IFN-γ response develops later but apparently does not sufficiently protect from subsequent infection. Also, another study shows that Ct-specific IFN-γ-producing T cells are mainly induced at follow-up visits 3 and 6 months after initiation of treatment and are generally much lower in patients which present with reinfection (74). IFN-γ induces indoleamine 2,3-dioxygenase (IDO1), which is in turn an essential player in Ct clearance as it starves the bacteria from the amino acid tryptophan but can also cause persistent growth due to stress induced by nutrient deprivation (75). IDO1 levels increase in vaginal swabs from Ct infected patients and are highest after antibiotic treatment and in patients with repeated infections (76). Ct specific $CD8^+$ cell frequencies are low in patients with ascending infection, whereas $CD4^+$ T cell frequencies are reduced in patients which were infected at follow-up (77). This indicates that $CD4^+$ and $CD8^+$ T cells may play different roles in human Ct immunity: $CD8^+$ cells prevent ascending of Ct while $CD4^+$ cells protect from re-infection. A summary of the most important immune mechanisms in Ct infection can be found in Figure 2.

Ct has an arsenal of mechanisms by which it can modulate the function of host cells in their own interest to evade recognition and clearance by the immune system. Firstly, it inhibits apoptosis of host epithelial cells to stay below the radar of the immune system which would

detect inflammation and infection. Then, the pathogen can alter the immune compartment like cytokines to prevent formation of a protective immune response but induce low grad chronic inflammation. Thirdly, if stress conditions occur, the pathogen can persist in both epithelial cells and immune cells, reported e.g. in macrophages, and can lead to re-infections from other body niches or dissemination to distant organs. Therefore, ideally Ct infections should be prevented before they are established in the host.

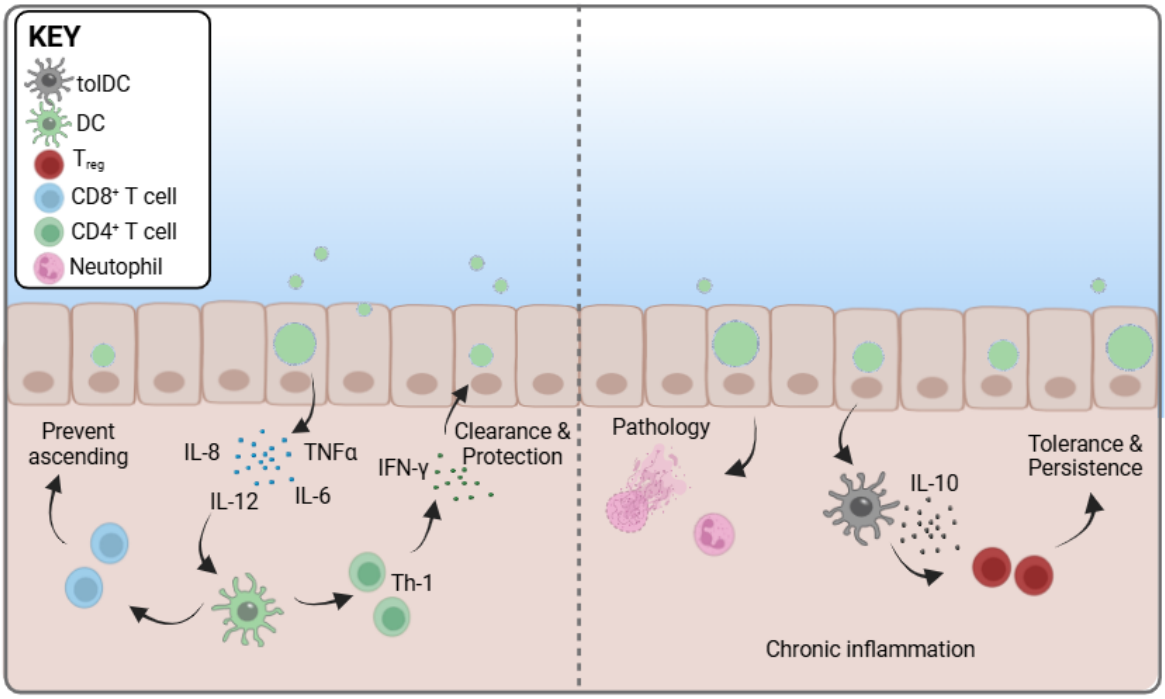


Figure 2. Overview of anti-Ct immune response. Created with biorender.com.

1.2.5 Prevention and treatment

Even though infections with Ct are easily treatable with antibiotics like doxycycline (doxy), the major problem is that they remain asymptomatic in more than 70% of cases and therefore untreated (78). This is not only critical as undiagnosed people transmit the disease to their sexual partners, but despite no symptoms, low grade chronic infection is associated with several severe adverse effects. Most prominently it can ascend from the vagina to the upper FRT, where it can cause pelvic inflammatory disease (PID) and subsequently female infertility by scarring of the tubes (79). Infections with Ct are the most common cause for non-inherent infertility (80,81) and thereby generating enormous costs for the health care systems. Ct infections can also ascend in males potentially causing epididymitis (82). In both males and females, Ct infection can cause urethritis. Current prevention strategies include use of condoms, regular testing and treatment in case of infection as well as doxycycline post-exposure prophylaxis (doxy-PEP) in patients with high-risk behavior. To date, there is no vaccine on the market to protect against bacterial STIs,

but phase I trials with the vaccine candidate CTH522 showed promising results and will possibly advance to a phase II study (83,84).

1.3 Aims of this thesis

As efficient preventive measures or vaccines against bacterial STIs and especially Ct are not yet available, this doctoral thesis aims to identify novel approaches to inhibit chlamydia growth or prevent infection in the first place. To do so, we focused on the one hand on the chlamydia replication in epithelial cells to find novel targets of chlamydia life cycle. On the other hand, we aimed to investigate the function of immune cells and especially antigen-presenting cells in the female reproductive tract upon early genital Ct infection. More precisely, the following three aims will be addressed within the framework of this thesis:

- Aim 1: Identification of novel compounds reducing infectivity of Ct in epithelial cells
- Aim 2: Development of a preventive strategy to reduce Ct infection rates
- Aim 3: Assessment of early anti-chlamydial immune responses in human by phenotyping antigen-presenting cells to support vaccine development strategies

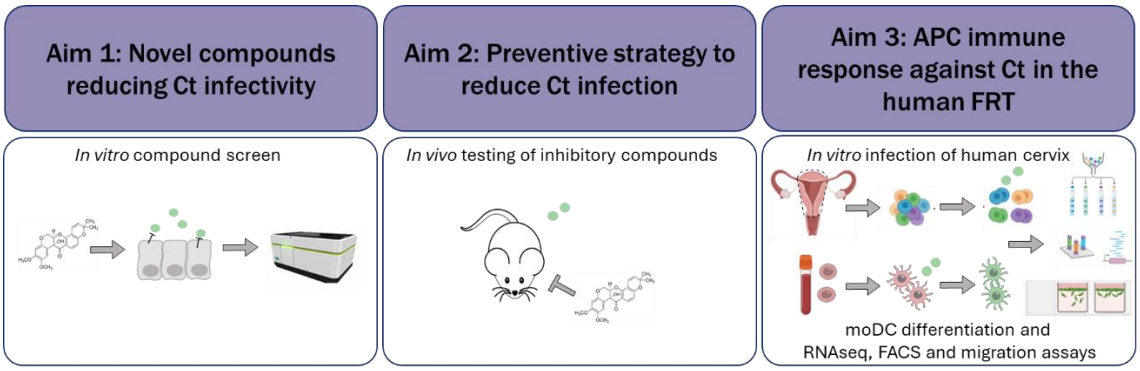


Figure 3. Graphical summary of the 3 aims of this doctoral thesis.

2. Results

2.1 Prologue

Even though many host mechanisms are already known by which Ct enter host cells and hijack the host cell metabolism for their own purposes, none of them is targeted to actually prevent infection. We decided to screen a library of compounds to potentially identify more potent inhibitors of Ct replication. As our results appeared promising, we kick-started the project of applying these compounds for prophylactic usage against Ct infection. Eventually, we identified pentamidine as a potent drug to inhibit Ct growth *in vitro* and *in vivo* and would be an interesting candidate for drug repurposing. The author of this thesis contributed to the following publication by developing the methodology for screening compounds in a semiautomated matter together with a team at the chemical screening facility of CeMM. The mouse model of Ct infection as well as read-out of mouse experiments was also established in the lab by the author of this thesis. Additionally, she spearheaded and performed *in vitro* experiments to identify the mechanism of action of pentamidine in Ct infection and established cultures of *Neisseria gonorrhea* and Lactobacilli in the lab. With input of the project team, she drafted the manuscript and even filed a patent application together with MedUni Wien for the prophylactic treatment with pentamidine.

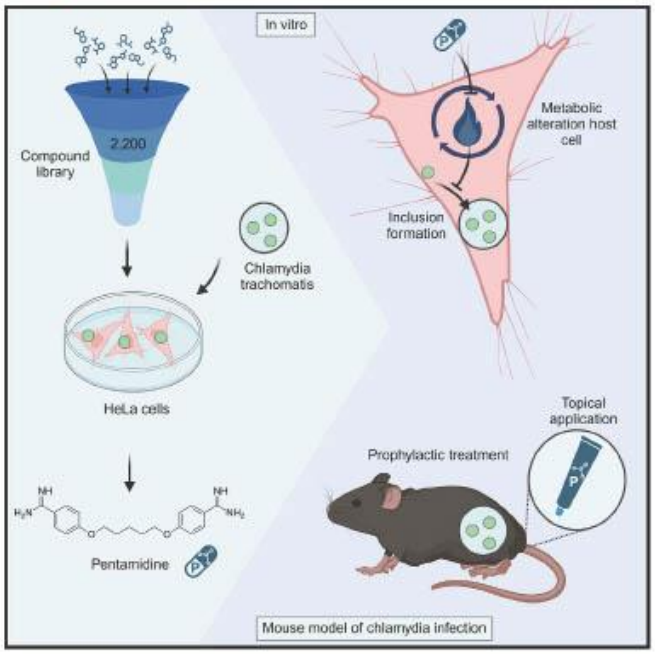
2.2 PDF of first paper - Combination of compound screening with an animal model identifies pentamidine to prevent Chlamydia trachomatis infection

Cell Reports
Medicine

Article

Combination of compound screening with an animal model identifies pentamidine to prevent *Chlamydia trachomatis* infection

Graphical abstract



Highlights

- A compound screen identifies 28 non-antibiotics inhibiting *Chlamydia trachomatis*
- Pentamidine inhibits chlamydia replication indirectly via the host cells
- Systemic and intrauterine pentamidine treatment decreases chlamydia burden in mice
- Pentamidine is a promising candidate for prophylaxis against bacterial STIs

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In brief

As the numbers of sexually transmitted infections are rising, innovative prophylactic measures are needed. Knapp et al. performed a medium-throughput compound screen to identify new drugs inhibiting *Chlamydia trachomatis* growth in cell lines. The top hits were tested in a *Chlamydia trachomatis* mouse model for their ability to prevent infection.

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Combination of compound screening with an animal model identifies pentamidine to prevent *Chlamydia trachomatis* infection

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SUMMARY

Chlamydia trachomatis (*Ct*) is the most common cause for bacterial sexually transmitted infections (STIs) worldwide with a tremendous impact on public health. With the aim to unravel novel targets of the chlamydia life cycle, we screen a compound library and identify 28 agents to significantly reduce *Ct* growth. The known anti-infective agent pentamidine—one of the top candidates of the screen—shows anti-chlamydia activity in low concentrations by changing the metabolism of host cells impairing chlamydia growth. Furthermore, it effectively decreases the *Ct* burden upon local or systemic application in mice. Pentamidine also inhibits the growth of *Neisseria gonorrhea* (*Ng*), which is a common co-infection of *Ct*. The conducted compound screen is powerful in exploring antimicrobial compounds against *Ct* in a medium-throughput format. Following thorough *in vitro* and *in vivo* assessments, pentamidine emerges as a promising agent for topical prophylaxis or treatment against *Ct* and possibly other bacterial STIs.

INTRODUCTION

The risk of death or serious health restraints from infectious diseases has decreased in developed countries based on the availability of efficient antimicrobial measures, including prophylaxis and treatment.[1,2] However, many sexually transmitted infections (STIs) are currently increasing in North America and Europe,[3,4] especially among individuals engaging in high-risk sex practices, including unprotected sex with multiple partners.[5] This trend was even intensified by the COVID-19 pandemic due to reduced screening and access to health care facilities.[6,7]

The most common bacterial STIs are *Chlamydia trachomatis* (*Ct*) infections with an estimated prevalence of around 4% in the Americas and around 2% in Europe with highest infection rates in the population between 15 and 24 years.[8,9] There are different *Ct* serovars characterized by their major outer membrane proteins: serovars A–C primarily cause ocular infection, while serovars D–K preferentially infect the urogenital tract, and serovars L1–L3 can lead to lymphogranuloma venereum.[10] *Ct* is an intracellular pathogen utilizing the host cell machinery for replication.[11] Briefly, *Ct* elementary bodies (EBs) can interact with various surface receptors on epithelial cells, which facilitate the uptake of the pathogen.[11] Using cell cytoskeletal proteins, EBs form an inclusion within the endocytic vacuoles where they replicate in the form of reticulate bodies (RBs) utilizing nutrients provided by the host cell. After finishing their replication cycle, RBs transform back into EBs and leave the cell by cell lysis or extrusion.[11,12] Since the *Ct* developmental cycle is strictly dependent on the host cell, treatment approaches do not need to solely rely on antibiotics inhibiting, e.g., bacterial protein synthesis but may also consider alternative mechanisms by interfering with cell entry or modulating host cell structures.[13] Alternative strategies involve, among others, inhibiting type III secretion systems of *Ct*, blocking chlamydial attachment by destroying the bacterial membrane or binding certain membrane structures, and enhancing host cell defense mechanisms by cytokines or blocking of metabolic processes utilized by *Chlamydia* spp.[13,14] Even though human chlamydial infections are well treatable with antibiotics, there are studies reporting tetracycline resistance by the presence of a Tet(C)-island in the genome in the species-specific strain *Chlamydia suis* infecting pigs.[13,15]

Another STI of concern with high rates of antibiotic resistance is *Neisseria gonorrhea* (*Ng*). A large portion of clinical *Ng* isolates are resistant to early developed antibiotics like penicillin,

sulfonamides, and tetracyclines, and additional resistance to extended-spectrum cephalosporins and macrolides, which are currently recommended for treatment, is on the rise.[16,17] Moreover, co-infections of *Ng* with various STIs are common,[18] as there exists an increased susceptibility of already infected individuals to other STIs.[19,20] A significant problem with both *Ct* and *Ng* infections is posed by the high rates of asymptomatic cases that can still cause severe long-term sequelae in females like pelvic inflammatory disease resulting in infertility or ectopic pregnancy.[21]

Preventive measurements include screening programs for individuals at high risk. Nevertheless, screening is expensive for the health care system, and a cost-effectiveness assessment is often not available.[22,23] The ultimate goal would be to develop a vaccine as preventive measure, which was not successful so far despite 60 years of research with only one vaccine candidate currently being in a clinical trial.[24–27] Other preventive strategies involve pre- and post-exposure prophylaxis (PrEP/PEP) with doxycycline (doxy) in patient groups with high-risk behavior for the acquisition of chlamydial infections and syphilis that are frequently using human immunodeficiency virus (HIV)-PrEP.[28–30] However, the concern of antimicrobial resistance of *Ng* and also other sexually transmitted pathogens as well as commensals greatly limits the doxy-PEP. In a recent study from Luetkemeyer et al., the development of resistance in *Ng* and *Staphylococcus aureus* isolates was increased in the doxy-PEP group.[30] Therefore, alternatives to doxy to prevent and treat STIs are of growing importance.

In this project, we report a systematic screen for molecules that reduce *Ct* growth *in vitro*. We identified pentamidine as a candidate compound that was also effective in reducing *Ct* burden (by topical or systemic treatment) in an *in vivo* mouse model of genital *Ct* infection. In addition, we demonstrated that already low concentrations modulate the host metabolism and inhibit the growth of *Ct* and *Ng*, making it an ideal agent for PrEP or PEP of STIs.

RESULTS

Identification of reagents inhibiting chlamydia growth in a medium-throughput compound screen

To identify novel classes of pharmacological agents inhibiting chlamydia growth, we chose a library of ~2,200 compounds, which include approved drugs and well-described experimental molecules. As *Ct*-infected cells are not dividing, we defined the optimal ratio of 2,000 HeLa cells per 384-well infected with *Ct*-L2-GFP at a multiplicity of infection (MOI) of 2.5 (Figures 1A and 1B). To determine the number of nuclei and inclusions in each condition, we fixed the cells with 4% paraformaldehyde/1% methanol, which allowed for the detection of the endogenous GFP signal of *Ct* inclusions and nuclear staining with DAPI and cell staining with Evans blue for HeLa cells (Figure 1C). A decrease in the number of cell nuclei per field of view to less than 100 was considered as cytotoxic effect of a given compound and excluded from analysis (Figure 1D). To test the efficiency of the screening compounds to reduce *Ct* growth, we set the cutoff to 50% in relation to the negative control (DMSO) (percentage of control, POC < 50) and the positive control (azithromycin 50 nM) (Figure 1E). In the initial screening approach, we identified 88 compounds having the potential to control chlamydia infection. We only identified 26 compounds that are known antibiotics. These compounds were excluded from subsequent analysis as the aim was to identify novel inhibitors. The screen revealed the following main substance classes as effective: (1) antineoplastics, (2) antivirals, and (3) psychoactive drugs (Figure 1F; Table S1). In a validation experiment, dose responses of the remaining 62 reagents in 3-fold dilutions revealed 28 reagents that showed a reduction of chlamydia growth by more than 40% (POC < 60) in two or more concentrations (Figure 2A; Table S2). These 28 compounds were further evaluated in various conditions to select the most effective candidates for chlamydia inhibition.

Validation reveals candidate drugs being effective against different genital chlamydia serovars

Ct-L2 causing lymphogranuloma venereum is more invasive and grows faster *in vitro* than genital serovars *Ct*-E and *Ct*-F.[31] Therefore, we investigated if the compounds validated from the primary screen are reactive against different genital *Ct* serovars in various concentrations (Figure 2A; Table S3). The most efficient compounds identified are avapritinib, CAY10571, CAY10574, dolutegravir, EKI-785, metergoline, methotrexate, pentamidine, and pixantrone. Most drug candidates showed a similar trend in all three *Ct* serovars (Figure 2B). By using the CellTiter-Glo, we demonstrated that compounds used in the validation screen are well tolerated by the cells as viability was above 90% for all compounds (Figure S1). In addition to the experimental layout with the compound treatment before *Ct* infection (Figure 1A), we assessed the effectivity of drugs if added 1 h after infection (Figure S2; Table S4). This time point of infection was chosen to identify drugs that have an effect during early chlamydia life cycle. We observed a slight decrease for dolutegravir and pentamidine if compounds are added after infection (Figure S2). This suggests that those compounds might play a role during early events in chlamydia replication or might take some time until they are fully active within the cells. We selected pentamidine, dolutegravir, and metergoline to test in a mouse model for genital chlamydia infection as (1) these compounds significantly block *Ct* growth in various conditions, (2) their targets in the chlamydia life cycle have not yet been described, and (3) they are approved drugs that allow for easier translation to patients (Figure 2C).

Pentamidine is effective in a mouse model for genital chlamydia infection

To investigate if the compounds inhibiting *Ct* growth in HeLa cells also work *in vivo* during female genital tract infection, we established a mouse model of *Ct* infection for prophylactic compound treatment. Mice received drug doses every 24 h starting 1 day before infection, resembling continuous schemata for pre-exposure prophylaxis.[29] At the peak of chlamydia burden 4 days after genital *Ct* infection,[32] uteri were harvested to quantify chlamydia burden (Figure 3A). Mice receiving a combination of systemic and local treatment with doxy or pentamidine had a significantly lower chlamydia burden compared with DMSO-treated mice (Figure 3B). Treatment with either dolutegravir or metergoline did not protect mice from chlamydia replication within their uteri (Figure 3B).

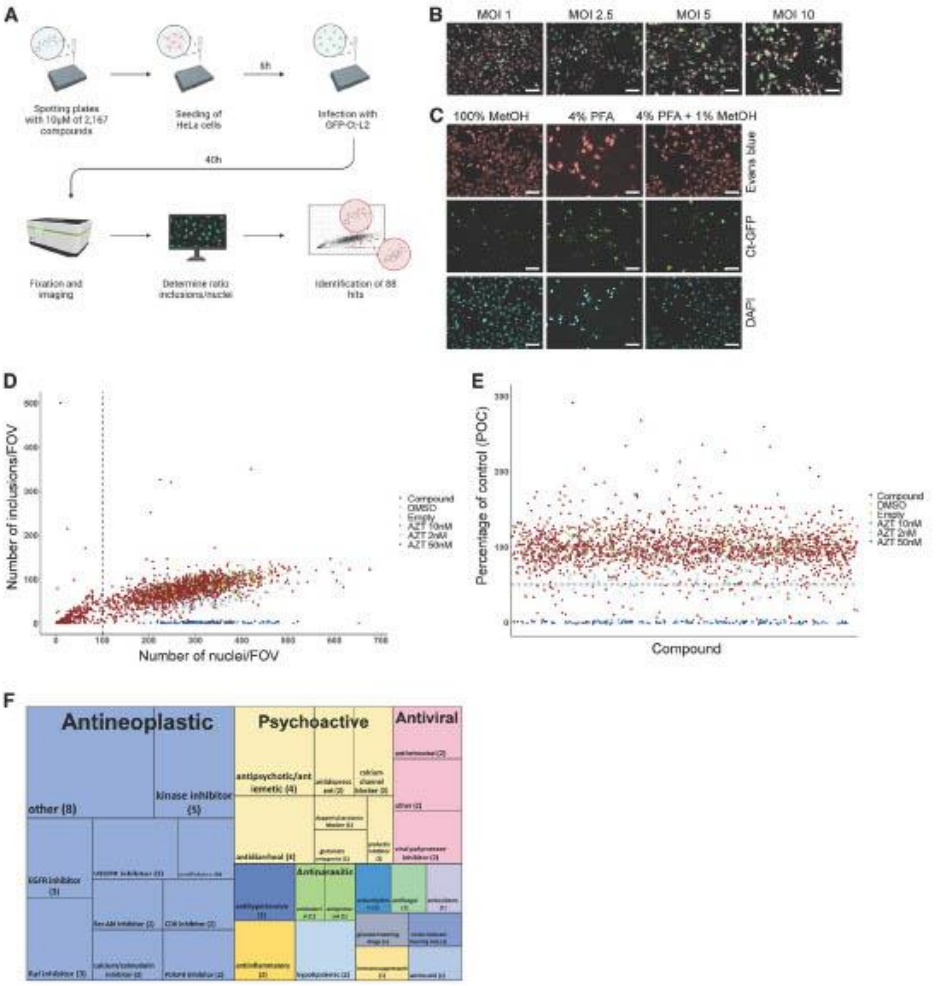

Figure 1. Medium-throughput screen reveals compounds inhibiting chlamydia growth

(A) Schematic of the experimental setup of the medium-throughput screen. HeLa cells were seeded in 384-well plates containing 10 µM of compounds. *Ct*-containing media were added after 6 h on top. Plates were incubated for 40 h.

(B and C) Overview of test conditions.

(B) Overlay of fluorescent images of DAPI (blue), Evans blue (red), and *Ct* inclusions (green) infected with different MOI (PerkinElmer Operetta high-content automated confocal microscope, 20× long field WD); representative images; scale bars, 100 µm.

(C) One representative field of view (FOV) per well is depicted after fixation with either 100% methanol (MetOH), 4% paraformaldehyde (PFA), or 4% PFA containing 1% methanol.

(D) Plot showing number of nuclei vs. number of inclusions of initial screen. Each dot represents one test compound (Cpd). DMSO-treated wells as negative controls (DMSO), empty wells (Empty), and azithromycin-treated wells as positive controls (AZT 10 nM, AZT 2 nM, AZT 50 nM). The cutoff for cytotoxic drugs was 100 nuclei per FOV.

(E) Percentage of control vs. compound ID for compounds being not cytotoxic. Hits are compounds that reduce chlamydia growth to less than 50% of control.

(F) Tree map plot of the drug classes and subclasses identified to inhibit *Ct* growth.

Next, we explored the effect of local application of pentamidine in the mouse model for genital *Ct* infection starting 1 day prior infection. Local pentamidine treatment significantly reduces the chlamydia burden in mice (Figure 3C). In comparison to the systemic pentamidine treatment where all mice presented with bradykinesia, we did not observe these therapy-related adverse events in mice with the local treatment regime. Histopathological assessment of uteri of locally treated mice revealed

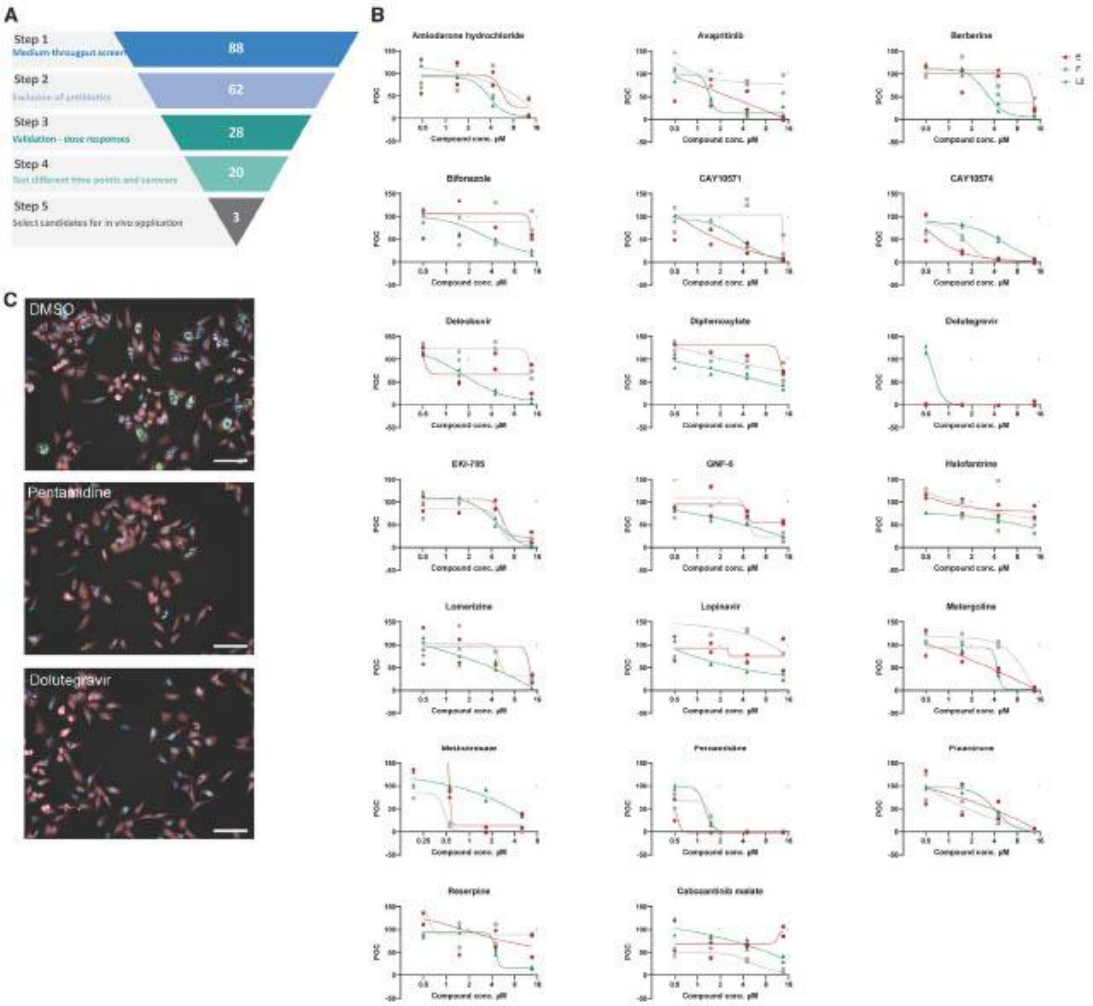

Figure 2. Validation experiments reveal inhibition of *Ct* growth across various serovars

(A) Schematic of the compound sets used in this study and the sequential screening steps applied to obtain a selection of a final set of 3 drugs for *in vivo* experiments.

(B) Dose-response curves for the 20 compounds remaining after the validation conditions. 3-fold dilutions in technical duplicates starting from 13.5 μM for *Ct* serovars E, F, and L2 were assessed. POC, percentage of DMSO-treated control.

(C) Representative images of indicated compounds in 13.5 μM concentration (PerkinElmer Operetta high-content automated confocal microscope, 20× long field WD); scale bars, 100 μm.

similar grading of inflammation between pentamidine-treated and -untreated *Ct*-infected mice (Figure 3D). Additionally, three mice received only one local dose of pentamidine to assess pathology (Figure 3D) and absorption (Figure 3E) of pentamidine upon transcervical drug inoculation. Singular drug doses do not cause any pathological changes in the uterus, liver, or kidney of these mice, and the highest drug levels could be detected within the uterus (Figure 3E). Therefore, pentamidine represents an interesting compound for prophylaxis against *Ct* infection in a mouse model, having potential as a local microbicide preventing human *Ct* infection.

Lack of effectiveness of dolutegravir against *Ct* infection in people with HIV

For dolutegravir, we analyzed a cohort of people living with HIV receiving dolutegravir as part of their antiretroviral therapy.

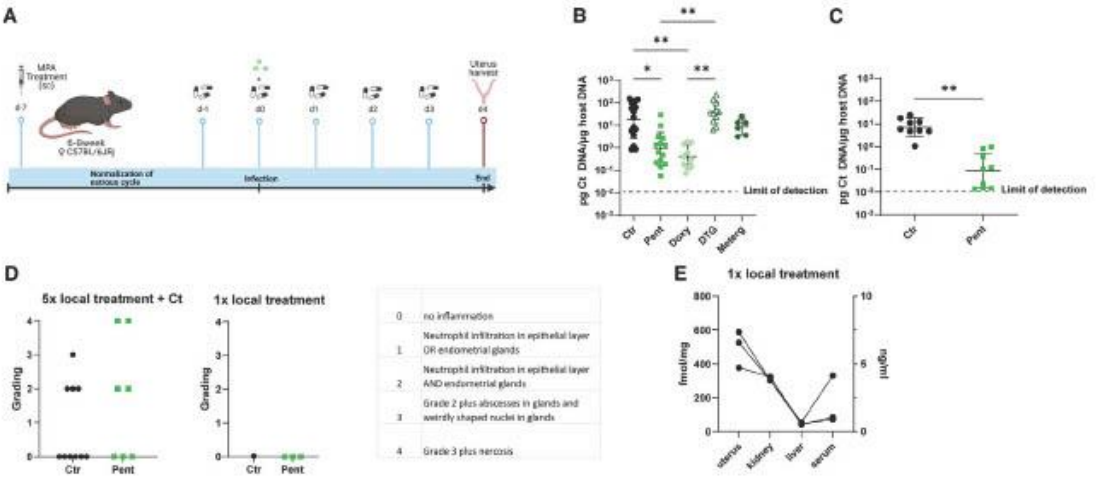

Figure 3. Pentamidine prevents *Ct* infection *in vivo*

(A) Schematic of setup of animal experiments. Created with BioRender.

(B) Bacterial burden in mouse uteri at the end of experiment in different groups under systemic plus local treatment: mock-treated mice (Ctr), pentamidine (Pent), doxy, dolutegravir (DTG), or metergoline (Meterg)-treated mice. Bacterial burden is indicated as pg *Ct* DNA per µg host DNA. One-way ANOVA and Tukey's multiple comparisons tests were performed to find differences between treatment groups (*$p < 0.05$, **$p < 0.01$). Data are presented as the mean of biological replicates ± SD.

(C) Bacterial burden in mouse uteri after local Pent or mock (Ctr) treatment was analyzed by unpaired t test (**$p < 0.01$). Data are presented as the mean of biological replicates ± SD.

(D) Histopathological grading of uteri. Uteri from mice receiving 5× local treatment and *Ct* infection (same mice as shown in C, $n = 7–10$) and mice treated once locally ($n = 3$) were stained with H&E and assessed by a pathologist 24 h after last treatment.

(E) Pentamidine absorption 24 h after 1× local treatment in different organs (fmol/mg) and serum (ng/µL).

Individuals receiving dolutegravir are as likely to acquire a *Ct* infection as patients without dolutegravir intake (Figure S3). Therefore, our results suggest that in the mouse model for genital chlamydia infection and in humans, dolutegravir does not reach inhibitory levels in tissue to prevent *Ct* infection.

Pentamidine impairs *Ct* rapidly and permanently

The mode of action for pentamidine in protozoan infection is not well described. Pentamidine is reported as an inhibitor of (1) DNA, RNA, or protein biosynthesis[33] and topoisomerase,[34,35] (2) polyamine synthesis,[36] (3) folate metabolism,[37,38] and (4) membrane integrity.[39] To learn if pentamidine has a direct effect on *Ct* or an indirect effect by inhibiting host cell metabolic processes, we first tested if pentamidine acts directly on *Ct* before they enter host cells rather than acting indirectly via affecting the host cell metabolism. Upon treatment of *Ct* with different concentrations of pentamidine 30 min or 2 h before adding *Ct* to the untreated cells, we did not observe a *Ct* growth reduction in HeLa cells (Figure 4J). Next, we investigated if pentamidine interferes with *Ct* uptake or with later steps during replication. 2 h after infection, the number of bacterial particles taken up in both conditions was comparable (Figures 4A, 4B, and 4E). After 24 h, however, *Ct* inclusions only occur in control cells, whereas single *Ct* particles are present in pentamidine-treated cells (Figures 4A–4E). To determine if *Ct* particles taken up in pentamidine-treated cells are showing hallmarks of bacterial growth, we used N-[7-(4-Nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl-D-erythro-sphingosine (C6-NBD-ceramide) labeling. Upon uptake in the cells, C6-NBD-ceramide is modified to sphingomyelin at the Golgi apparatus, resulting in the transfer and retention of sphingomyelin in *Ct* inclusions upon bacterial growth.[40,41] We performed confocal imaging of infected cells treated with C6-NBD-ceramide to assess the recruitment of C6-NBD-sphingomyelin to the inclusions. C6-NBD-sphingomyelin accumulates in the Golgi apparatus, and our findings demonstrate a clear co-localization of C6-NBD-sphingomyelin with *Ct*-lipopolysaccharide (LPS)-positive vesicles and inclusions in both controls and pentamidine-treated samples. Notably, in the pentamidine-treated cells, there was a halt in the development of the inclusions (Figure S4). This suggests that the uptake and early steps in the *Ct* life cycle are not impaired by pentamidine, but only later stages, including bacterial replication, are affected by pentamidine.

To elaborate on the function of pentamidine in *Ct* infection, we investigated the kinetics of clearance of chlamydia upon *in vitro* treatment. Therefore, HeLa cells were pre-treated with compounds in a similar setup as in our initial screen. Next, compounds were washed out 24 or 48 h after infection, respectively (Figures 4F–4I). While *Ct* persists and still proliferates in azithromycin- and doxy-treated samples if washout occurs already 24 h after infection, *Ct* growth is significantly impaired after the 24 h washout in pentamidine-treated samples (Figures 4F, 4G, and 4I). A comparable effect to pentamidine is achieved for azithromycin and doxy if they are present for at least 48 h (Figure 4H).

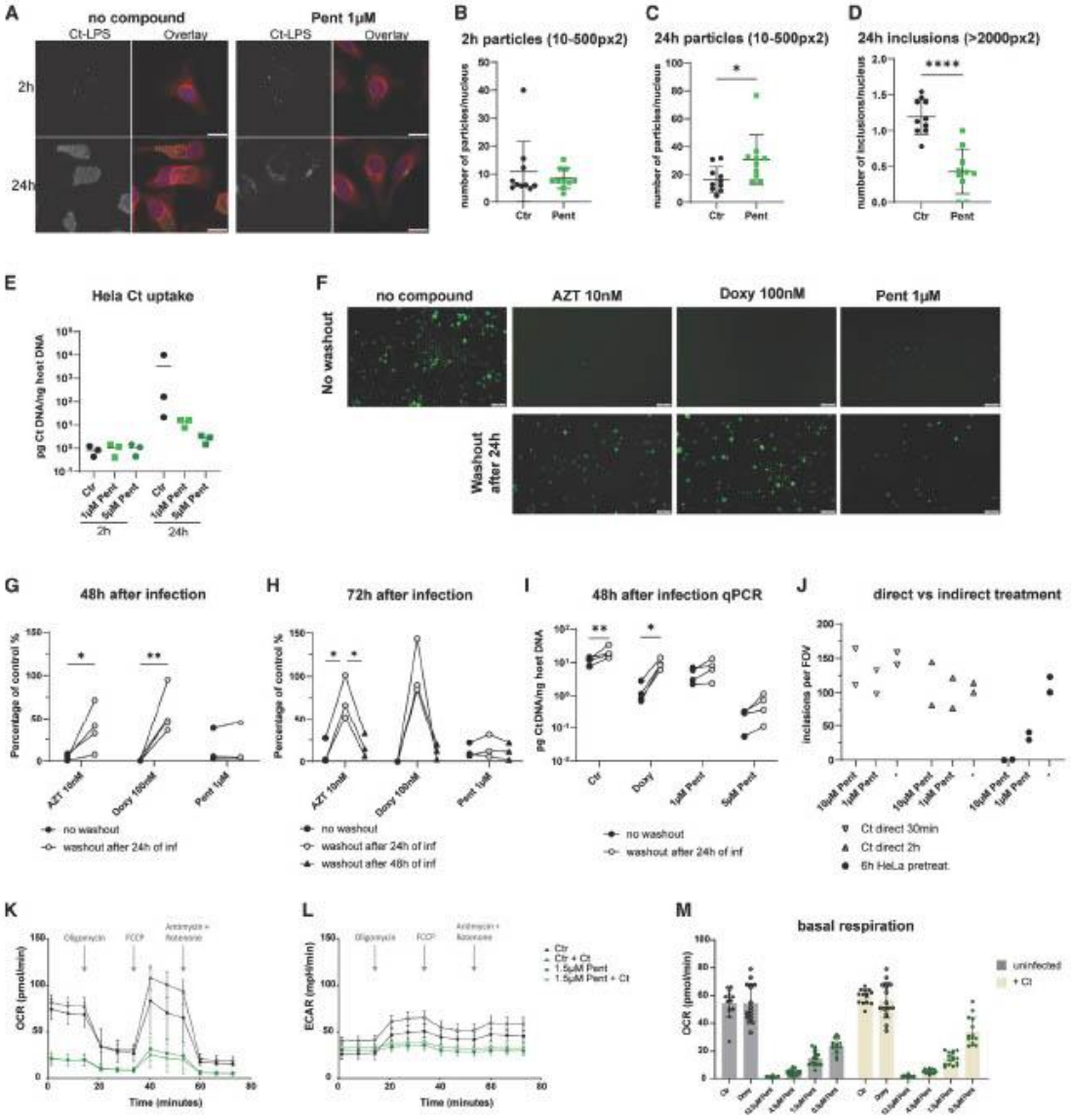

Figure 4. Pentamidine acts more rapidly against chlamydia than antibiotics by an indirect effect via host cells

(A–D) HeLa cells pre-treated with 1 µM Pent for 6 h were infected with *Ct*-L2 (no GFP) at MOI 100. *Ct* uptake was quantified after washing 2× with PBS to remove unbound *Ct*, PFA fixation and antibody staining for *Ct*-LPS in 10 FOV per condition (Olympus IX53, LUCPlanFL N, 40×). Significant differences were tested by unpaired t tests ($^{*}p < 0.05$, $^{****}p < 0.0001$). Data are presented as the mean of 10 FOV ± SD. (A) Representative images of chlamydia uptake and inclusion formation 2 and 24 h after infection. Scale bars, 20 µm; Ct-LPS (green), Evans blue (red), DAPI (blue). (B) Ratio of chlamydia particles (size = 10–500 px^2) and nuclei per FOV 2 h after infection. (C) Ratio of chlamydia particles (size = 10–500 px^2) and nuclei per FOV 24 h after infection. (D) Ratio of chlamydia inclusions (size > 2,000 px^2) and nuclei per FOV 24 h after infection.

(E) Quantification of *Ct* uptake with qPCR. HeLa cells were treated with 1 or 5 µM Pent and infected with Ct-L2 (no GFP, MOI 100). HeLa cells were harvested 2 or 24 h after infection and ratios of *Ct* DNA/host DNA were determined ($n = 3$ independent experiments).

(F–I) Medium containing 10 nM azithromycin (AZT), 100 nM doxy, and 1 µM or 5 µM Pent was replaced every 24 h supplemented with freshly prepared compounds or just medium. Significant differences were tested by two-way ANOVA with to Šídák's multiple comparisons test ($^{*}p < 0.05$, $^{**}p < 0.01$). (F)

(legend continued on next page)

This suggests that pentamidine permanently inhibits *Ct* growth at an early time point and that it does not only suppress bacterial translation, as it occurs in the presence of macrolides and tetracyclines.

In summary, we show that *Ct* is taken up into pentamidine-treated cells and early steps of inclusion formation occur. However, replication of *Ct* is significantly impaired, and after washout of pentamidine, the inhibitory effect persists.

Impairment of *Ct* growth by modulation of host cell metabolism

As we did not observe direct disruption of *Ct* by pentamidine, we assessed other mechanisms of pentamidine inhibiting *Ct* growth indirectly via host cells. Solute carriers (SLCs) might be essential for *Ct* growth by providing necessary factors of the bacterial life cycle. The reduced folate carrier SLC19A1 was shown to be transporting antimetabolites like methotrexate and pentamidine.[37] Treatment of SLC19A1$^{-/-}$ cells with methotrexate resulted in a loss of anti-chlamydia effects, whereas treatment with pentamidine led to a similar reduction of *Ct* growth as observed in Renilla$^{-/-}$ control cells (Figure S6). We therefore conclude that pentamidine and the antifolate methotrexate have different modes of action in their activity against *Ct*.

We tested the possibility that the host metabolism might be influenced by pentamidine in a way that prevents further Ct growth in infected cells. We performed Seahorse analysis, using the mitotic stress test kits on HeLa cells treated with pentamidine and infected with *Ct*. When assessing the oxygen consumption rate, we noticed that pentamidine-treated cells have reduced basal respiration and that their spare capacity for mitochondrial respiration is reduced (Figure 4K). Interestingly, infected control cells perform more oxidative phosphorylation at baseline. This difference is not observed in pentamidine-treated samples. Similarly, pentamidine-treated cells perform less glycolysis as shown by the extracellular acidification rate and increased glycolysis in infected control cells, indicating that *Ct* proliferation is reflected by high energy demand by the host cells (Figure 4L). This effect of reduced metabolic activity in pentamidine-treated cells is dose dependent, while doxy-treated cells show similar basal respiration as control cells (Figure 4M). These data illustrate that, by reducing the basal metabolism in host cells via pentamidine, growth of *Ct* within the host cells is inhibited. We next investigated if the metabolic effect of pentamidine results in reduced viability and proliferation of the host cells. By performing lactate dehydrogenase (LDH) assays and fluorescence-activated cell sorting-based viability assays (7-Aminoactinomycin (7-AAD) staining and CellTrace Violet staining), we observed that HeLa cell viability and proliferation is not affected by pentamidine (Figures S5A–S5C). As HeLa cells are robust cells, we additionally assessed the viability of primary cervical epithelial cells upon pentamidine treatment, which validated our observations from HeLa cells in this more physiologically relevant cell type (Figures S5D and S5E). These data show that the inhibitory effect on bacterial replication observed in pentamidine-treated cells is probably due to metabolic changes within the host cells. However, the drug doses used in our study do not impact the viability and proliferation of HeLa cells and primary cervical epithelial cells as host cells of *Ct*.

Pentamidine inhibits the growth of *Ng*, while commensals of the physiologic vaginal flora retain replication capacity

As *Ct* and *Ng* infections often coincide, PrEP or PEP strategies are usually designed to be effective against *Ct* and *Ng* infection.[29,30] We therefore assessed the antimicrobial potential of pentamidine against *Ng* as it was reported to have some inhibitory effect on other gram-negative bacteria such as *E. coli*.[42] By treating liquid cultures of *Ng* with pentamidine, we identified the minimal inhibitory concentration between 3.12 and 6.25 μM (Figures 5A and 5B). By spreading the liquid cultures after 24 h of pentamidine treatment on untreated plates, we determined that the minimal inhibitory concentration corresponded to the minimal bactericidal concentration, as regrowth of *Ng* was absent (Figure 5C).

Vaginal dysbiosis is a common side effect of antibiotic treatment.[43] As prophylactic treatment of STIs should not permanently affect the urogenital microbiome, we next assessed the effect of pentamidine on *Lactobacillus acidophilus* as a representative species of the female vaginal flora. We observed reduced growth of the bacteria in the presence of pentamidine; however, the drug did not have a bactericidal effect on *L. acidophilus* in any concentration tested as bacteria grew back after withdrawal of the drug (Figures 5D and 5E). Our data indicate that the bactericidal pentamidine concentrations do not have a lasting detrimental effect on the human female genital tract microbiome.

In summary, we successfully applied a medium-throughput screen to *Ct* infection and validated the most promising compounds in a mouse model for genital *Ct* infection. Pentamidine emerged as the lead candidate demonstrating efficacy against *Ct in vivo* and inhibiting *Ng* growth *in vitro*, all while preserving *L. acidophilus*, a key component of the natural vaginal flora. Furthermore, when applied topically in the *Ct* mouse model,

Representative images of washout experiments 72 h after infection (Olympus IX53, CPlan N, 10×). Scale bars, 100 μm. (G) HeLa cells treated with indicated compounds, analysis 48 h after infection ($n = 4$ independent experiments, 10 FOV per experiment were analyzed, Olympus IX53, LCAch N, 20×). (H) HeLa cells treated with indicated compounds, analysis 72 h after infection ($n = 3$ independent experiments, 10 FOV per experiment were analyzed, Olympus IX53, LCAch N, 20×). (I) Quantification of *Ct* burden with qPCR. HeLa cells were harvested 48 h after infection and ratios of *Ct* DNA/host DNA were determined ($n = 4$ independent experiments). (J) *Ct* pretreated with pentamidine before infection. HeLa cells were infected with pre-treated *Ct* (for 30 min or 2 h; indicated pentamidine concentration during *Ct*-pretreatment) or HeLa cells were pre-treated with pentamidine 6 h before infection (final concentration of 10 or 1 μM concentration). Quantification of inclusions per FOV (Olympus IX53, LCAch N, 20×) normalized to inclusions in untreated control samples ($n = 2$ independent experiments, expressed as mean of 10 FOV per experiment).

(K–M) Seahorse analysis using the mitotic stress test kit, performed on 8,000 HeLa cells treated with compounds (Ctr = DMSO, 3-fold dilutions of Pent starting at 13.5 μM or 100 nM doxy) and/or Ct-L2-GFP (MOI 2.5) for 48 h. Data are pooled replicates from two independent runs ($n = 14$–16), mean ± SD. (normalized to cell number). (K) Oxygen consumption rate (OCR) in pmol/min. (L) Extracellular acidification rate (ECAR) in mPH/min. (M) Basal respiration of all treatment conditions (OCR).

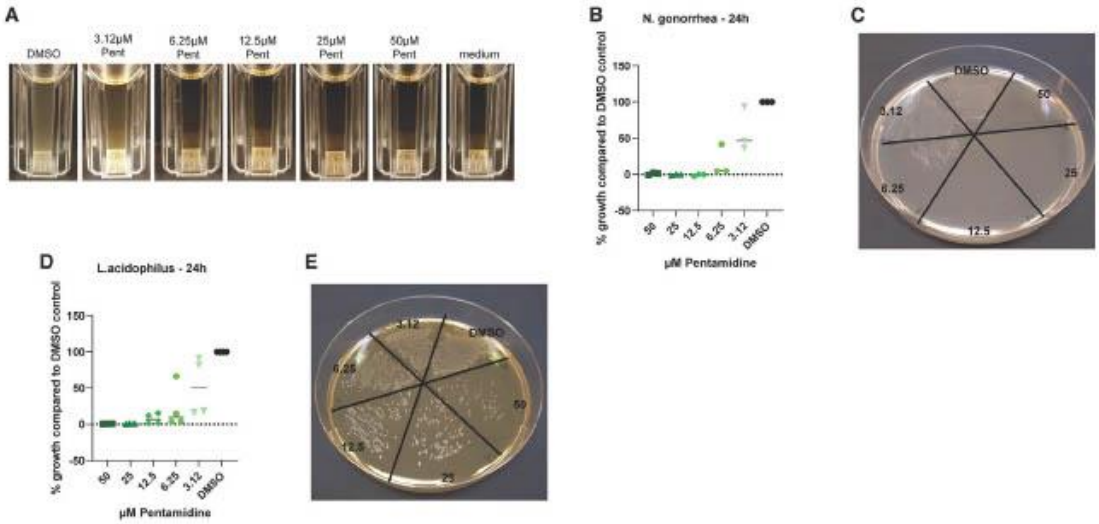

Figure 5. Pentamidine inhibits *Ng* growth *in vitro* but does not stop the growth of *Lactobacilli*

(A) Liquid 24 h cultures of *Ng* in the presence of 2-fold serial dilutions of Pent (starting at 50 µM).

(B) Quantification of growth of *Ng* in Pent-treated cultures in comparison to DMSO control cultures. Data are presented as the means of 3 independent experiments.

(C) Representative image of *Ng* bacterial smears derived from Pent-treated 24 h liquid cultures.

(D) Quantification of growth of *L. acidophilus* in Pent-treated cultures in comparison to control cultures. Data are presented as the means of 4 independent experiments.

(E) Representative image of *L. acidophilus* bacterial smears derived from Pent-treated 24 h liquid cultures.

pentamidine successfully prevented *Ct* infection. However, its mode of action appears to be mediated through the host cells, because direct incubation with pentamidine does not affect *Ct*. Instead, pentamidine modifies the metabolic activity within the host cells. In comparison to azithromycin and doxy, the effect of pentamidine is rapid and long lasting, making it an ideal candidate as topical agent against bacterial STIs in a therapeutic or even prophylactic setting.

DISCUSSION

In this study, we utilized a medium-throughput discovery screen to evaluate the potential of approximately 2,200 compounds in inhibiting the intracellular growth of *Ct*. As a result, we successfully identified and validated 28 non-antibiotic compounds that demonstrated the ability to reduce *Ct* inclusion formation. Notably, we found that pentamidine, when topically or systemically applied, effectively prevented *Ct* infection *in vivo*.

In their global health sector strategy on STIs, the World Health Organization aims to reduce the global cases of bacterial STIs from 374 million in 2020 to below 150 million until 2030. The main pillars will focus on prevention, screening programs for priority populations, and innovative approaches to treatment and vaccines.[44] Considering the absence of available vaccines against any of the most common bacterial STIs, namely *Ct*, *Ng*, and *Mycoplasma genitalium*, coupled with the escalating antibiotic resistance observed among these pathogens, it is crucial to investigate novel strategies for STI prevention.

In a similar setup as described in this study, Mojica et al. tested 339 Australian natural products with an mCherry-expressing *Ct* strain.[45] They identified mainly tetrahydroanthraquinone and thiaplakortone compounds as hits, which had been classified as antiparasitic agents against malaria or trypanosomes.[45] We tested our top candidates—pentamidine, dolutegravir, and metergoline—for their efficacy in a mouse model of female genital tract *Ct* infection. Pentamidine was the only compound of the three drugs that significantly reduced *Ct* burden in the mice comparable to antibiotic treatment upon systemic and local treatment. In comparison to the antibacterial activity of metergoline against the intracellular bacterium *Salmonella typhimurium* in a murine *Salmonella* infection model,[46] we did not see a *Ct* growth reduction upon metergoline application *in vivo*. For the intraperitoneal application, we used the same dosage, but the frequency of application was every 24 h, in contrast to the mouse model for *Salmonella* infections where application followed a 12 h interval. Explanations for the absence of effect in our mouse model could be that (1) the agent was not sufficiently concentrated in the female genital epithelium, (2) the dose is not high enough for *Ct* inhibition *in vivo*, or (3) more dosages would be required due to the half-life of metergoline. Dolutegravir was the most effective drug against *Ct in vitro* but did not show an effect in the mouse model for *Ct* infection. Even though the usual application route

is oral, we chose systemic treatment by injection and determined the optimal/maximal dosage based on previous literature using either oral application[47] or injection with dolutegravir equivalents.[48] Dolutegravir was shown to modify the folate metabolism, which might be its potential mode of action during *Ct* infection,[49,50] similarly to methotrexate.[51] Dolutegravir leads to the downregulation of SLC19A1 in placental cells, thereby reducing the uptake of both methotrexate and folic acid.[50] Only 5%–7% of plasma concentration of dolutegravir reaches the cervical tissue, which could be one reason for the lack of activity against *Ct*, as only very low concentrations are needed for its activity against HIV.[52,53] Follow-up experiments could assess if long-acting dolutegravir derivatives can reach a dosage sufficient for chlamydia growth inhibition *in vivo*.[48,54] Recently, the dolutegravir derivative 7-methoxy-4-methyl-6,8-dioxo-N-(3-(1-(2-(trifluoromethyl)phenyl)-1H-1,2,3-triazol-4-yl) phenyl)-3,4,6,8,12,12a-hexahydro-2H-pyrido[1′,2′:4,5]pyrazino[2,1-b][1,3]oxazine-9-carboxamide (DTHP) was shown to inhibit cancer cell growth in a murine xenograft model after intraperitoneal application.[55] Recognizing the importance of refining the delivery method as well as the dosage for *in vivo* application, our research specifically emphasized pentamidine as a promising prophylactic agent against *Ct*.

The concept of PrEP and PEP proved efficient against STI such as HIV.[56] Recent findings suggest doxy prophylaxis as PEP against bacterial STIs in certain individuals at high risk for STI acquisition.[28–30] A potential caveat with doxy prophylaxis is the development of antibiotic resistance of the pathogens and also commensals. In *C. suis*, which is related to the human-adapted pathogen *Ct*, tetracycline resistance is common. *C. suis* can also infect other hosts than pigs, as infection is documented in humans working in life stock facilities,[57,58] where selective pressure occurs due to the enormous use of antibiotics.[59] In co-infected individuals with *C. suis* and *Ct*, horizontal transfer of the tet(C) gene might occur, which was demonstrated *in vitro* between *C. suis* and other chlamydia strains.[60,61] For *Ng*, antibiotic resistance is very common and even multidrug-resistant strains are frequently observed in some countries. Doxy-PEP reduced *Ng* cases depending on the resistance frequency in the respective study area.[28,30]

We propose pentamidine identified in this study as a promising alternative agent for STI prophylaxis. In a PrEP setting in mice *in vivo*, pentamidine reduced the chlamydial burden similarly to doxy. We observed inhibition of *Ng* growth in the presence of >6.25 μM pentamidine. However, minimum inhibitory concentration (MIC) breakpoints and pharmacokinetic or pharmacodynamic models for pentamidine and *Ng* are not established so far. Pentamidine is reported to have antibacterial properties by targeting the bacterial membrane.[42,62] Gram-negative bacteria can become more susceptible to antibiotics by disrupting their membrane and making it permeable for other drugs.[39,62] This synergistic effect of combining pentamidine with antibiotics was shown for *Acinetobacter baumannii* in a mouse model,[39] a finding that could be translated to multidrug-resistant bacteria strains, thereby increasing the treatment options and delaying the development of resistance.[62–66] With regard to potential side effects associated with pentamidine, ongoing advances are being made in the development of pentamidine analogs that exhibit improved performance in disrupting bacterial membranes while being less cytotoxic to the host organism, thereby increasing their potential as adjuvants for antimicrobial therapies.[67–69] It remains to be investigated if these analogs are also effective against *Ct* as the mechanism of action is probably more complex than just disrupting the membrane of the pathogen.

In the clinics, pentamidine is already used as prophylaxis against pneumocystis pneumonia with either systemic or topical (inhalation) application once every 3–4 weeks. During systemic treatment, pentamidine can have side effects like ventricular arrhythmia, hypotension, nephrotoxicity, or hepatic dysfunction.[70] During local application by inhalation with a nebulizer, it can cause cough, chest pain, difficulty breathing, or skin rash.[70] Nevertheless, also doxy can cause symptoms like headache, nausea, vomiting, diarrhea, skin rash, or bacterial vaginitis. In the mouse model for genital *Ct* infection, we observed bradykinesia and dizziness after systemic treatment with pentamidine, while mice treated only locally did not present with obvious adverse events. *In vitro*, pentamidine has an immediate and long-lasting effect, as *Ct* inclusions do not grow back as observed for the bacteriostatic compounds azithromycin and doxy if treatment is stopped after 24 h. Thus, the bactericidal compound pentamidine might facilitate a long-lasting effect or even better clearance *in vivo*, which would make the application more convenient and less error-prone than daily intake as required for doxy-PrEP or intake after every risk contact as recommended for doxy-PEP. This enhanced effectiveness may be attributed to the ability of pentamidine to enhance the fitness of host cells. Through the utilization of various incubation strategies, encompassing both *Ct* alone and in combination with host cells, we observed that pentamidine may not directly affect *Ct* but rather bolsters the resilience of host cells in combatting this pathogen by reducing the metabolic activity in host cells to a level that does not kill the host but significantly impairs the pathogen. This mechanism holds promise in reducing both the mutation pressure exerted on *Ct* and the development of resistance.

Pentamidine was shown to be effective in a mouse model of *Leishmania* infection after topical application on the skin as a cream formulation.[71] It remains to be tested if pentamidine is suitable for being applied locally to the cervix in form of an ointment, as we dissolved it in 0.9% saline solution and applied it directly into the murine cervix before *Ct* infection. Previous publications describe the development of vaginal microbicides like salicylidene acylhydrazides restricting iron in *Ct* and *Ng* infection or the LPS-binding molecule alkylpolyamine DS-96 blocking *Ct* attachment.[72,73] Approved for other clinical applications, long-term clinical experience is an advantage for our approach to pentamidine treatment. Histopathological assessment revealed that single local treatments do not cause any irritation or organ toxicity in the female reproductive tract. The effect on the local microbiome was assessed by susceptibility testing of *L. acidophilus*, a commensal of the female genital tract, which revealed to survive *Ct* and *Ng*-bactericidal pentamidine dosages. In addition, there is evidence for synergistic effects of pentamidine in combination with antibiotics, which could contribute substantially to reducing total antibiotic load and increase the variability of different antibiotic classes that can be used to treat certain pathogens.

In conclusion, we identified 28 highly effective compounds from diverse substance classes that demonstrated significant efficacy against *Ct in vitro*. Some of these compounds belong to classes that were previously shown to be associated with pathways involved in chlamydia replication. Furthermore, our study provides the first evidence of pentamidine's efficacy in a mouse model of *Ct* infection. Based on these findings, we propose that pentamidine holds potential as an alternative topically or systemically applied agent for prophylactic strategies (PrEP and PEP) not only against *Ct* but also against other bacterial STIs, such as *Ng*, without disrupting the genital flora. Given the substantial increase in *Ng* antibiotic resistance, pentamidine could serve as an adjuvant in combination with antibiotics, offering a backup plan for multi-drug-resistant strains of *Ng* and other bacteria like *Mycoplasma genitalium*. Future endeavors should focus on developing a formulation of pentamidine or one of its analogs suitable for local application in humans or as a low-dosage systemic antibiotic adjuvant. One emphasis will be the development of a pentamidine derivative that exhibits bactericidal properties while minimizing severe side effects linked to the original compound. Additionally, it is crucial to investigate whether lower concentrations of the drug remain effective. It is imperative to investigate the inhibitory effect of pentamidine on other STIs besides *Ct in vivo* and explore the development of resistance to pentamidine.

Limitations of the study

We provide a proof of concept for the use of pentamidine as a locally active prophylactic agent against *Ct* infection. However, the precise mode of action remains unclear, even though extensive *in vitro* infection experiments revealed that pentamidine most likely acts via metabolic alterations in host cells, thereby limiting the proliferation capacity of bacteria. It remains to be elucidated if long-term treatment and related metabolic alterations lead to toxic side effects in epithelial tissue. Another challenge with pentamidine to prevent STIs is the effect on the local microbiome, which needs to be carefully assessed with other species than *L. acidophilus* and with *in vivo* studies. The next step will be the translation of topical drug application in humans. While pentamidine application is reasonably feasible with creams and suppositories at the penis and anal region, respectively, topical preventative considerations for the female lower genital tract and especially the cervix are more challenging. Possible options include vaginal application by cream, suppository, or sprays that might allow dissemination of pentamidine to the cervix. Developing pentamidine derivatives that are suitable for easy systemic distribution or innovative topical administration are options for future studies in the context of topical pentamidine prophylaxis. Pentamidine as a lead compound could help to identify pentamidine analogs or structurally related compounds using structure-activity relationship studies for similarly active compounds with fewer side effects on host cells and the microbiome.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2024.101643.

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AUTHOR CONTRIBUTIONS

K.K. planned and conducted the experiments, analyzed the data, and drafted the figures and manuscript. R.K. planned and conducted the experiments and analyzed the data. A.K. conducted screening experiments and analyzed the data. M.S. conducted and analyzed the Seahorse experiments. R.D.-H. conducted the experiments. M.D. performed the histopathological grading. J.S. performed the mass spectrometry experiments. D.C. collected and analyzed patient data of the dolutegravir cohort. M.K. and C.G. recruited patients to obtain primary material and planned experiments. A.B., S.K., H.S., and G.S. provided reagents and revised the manuscript. H.S. and G.S. supervised the project.

DECLARATION OF INTERESTS

K.K., R.K., A.K., S.K., H.S., and G.S. are inventors on a patent application entitled "Pentamidine in the treatment of genital infections and/or STIs" (EU application number EP 24 177 796.0) filed by the Medical University of Vienna that relates to the use of pentamidine against genital infections.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Ct LPS FITC, clone B410F	Invitrogen	Cat # MA1-7339; RRID: AB_1016792
Ct LPS, clone 512F	Invitrogen	Cat # MA5-16287; RRID: AB_2537804
Goat anti-mouse IgG AF680	Invitrogen	Cat # A-21058; RRID: AB_2535724
Bacterial and virus strains		
CTL2P-pGFP:pSW2	Chlamydia Biobank	CT401
Ct serovars L2	DSMZ-German Collection of Microorganisms and Cell Culture GmBH	DSM 19102
Ct serovars E	DSMZ-German Collection of Microorganisms and Cell Culture GmBH	DSM 19131
Ct serovars F	DSMZ-German Collection of Microorganisms and Cell Culture GmBH	DSM 19410
Neisseria gonorrhea	ATCC	ATCC 49226
Lactobacillus acidophilus	ATCC	ATCC 4356
Biological samples		
Healthy adult cervical tissue	Medical University of Vienna	N/A
Chemicals, peptides, and recombinant proteins		
Azithromycin	Synovo	N/A
C6-NBD Ceramide	Focus Biomolecules/Biotrend	Cat# 10-5496-1mg
Doxycycline Vibravenös	Pfizer	N/A
Dolutegravir	MedChemExpress	Cat# HY-13238
Cycloheximide	Sigma	Cat# 01810
Gastrografin	Bayer	Cat# 80375310
Medroxyprogesterone acetate	TCA	Cat# M1964
Metergoline	Sigma	Cat# M3668-500MG
Penicillin	Sigma	Cat# P3032-1MU
Pentamidine isethionate	Thermo Fisher Scientific	Cat# 461860010
Critical commercial assays		
CellTrace Violet	Thermo Fisher Scientific	Cat# C34557
7-AAD	BD	Cat# 559925
LDH-Cytox™ Assay Kit	Biolegend	Cat# 426401
QIAamp DNA mini kit	Qiagen	Cat# 51306
Phalloidin AF594	Invitrogen	Cat# A12381
Luna® Universal Probe qPCR Master Mix	New England Biolabs	Cat# M3004X
Seahorse XF Cell Mito Stress Test	Agilent	Cat# 103015-100
Experimental models: Cell lines		
HeLa cells	ATCC	ATCC® CCL-2™; RRID:CVCL_0030
McCoy cells	ATCC	ATCC® CRL-1696™; RRID:CVCL_3742
HCT116-SLC19A1-KO-c4	RESOLUTE	CE0540-U; RRID:CVCL_D4IH
HCT116-Renilla-KO-c1	RESOLUTE	CE04CG-T

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mouse: C57BL/6JRj	Janvier labs/Charles River	RRID: IMSR_RJ:C57BL-6JRJ
Oligonucleotides		
Primer Ct 16S DNA	IDT	F: 5′-GGA GGC TGC AGT CGA GAA TCT-3′ R: 5′-TTA CAA CCC TAG AGC CTT CAT CAC A-3′ Probe: 5′-[6-FAM]-TCG TCA GAC TTC CGT CCA TTG CGA-[TAMRA]-3′
Rodent GAPDH VIC	Applied Biosystems	Cat# 4308313
Human GAPDH VIC	Thermo Fisher Scientific	Cat# 4448489
Software and algorithms		
FlowJo Version 10.8.1	FlowJo	https://www.flowjo.com/
Olympus FV31S-SW software	Olympus	https://www.olympus-lifescience.com/en/support/downloads/
Prism Version 8 & 9	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
BioRender	BioRender	https://www.biorender.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Georg Stary (georg.stary@meduniwien.ac.at).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bacterial strains and cell culture

Ct serovar CTL2P-pGFPpSW2 (referred Ct-L2-GFP) was purchased from the Chlamydia Biobank (University of Southampton).[74] *Ct* serovars L2 (DSM 19102), E (DSM 19131) and F (DSM 19410) were purchased from the DSMZ-German Collection of Microorganisms and Cell Culture GmBH. Serovar Ct-L2-GFP was propagated in McCoy cell monolayers in DMEM supplemented with 10% FBS (Biowest, Nuaille, France), 1x non-essential amino acids (MEM NEAA 100X, Gibco, Thermo Fisher Scientific) in the presence of 10 U ml^{-1} penicillin (Sigma, St. Louis, USA) to select for GFP expressing bacteria. Strains L2, E and F were propagated in HeLa cells and 1 μg mL^{-1} cycloheximide (Sigma, Burlington, USA) was added to the medium as described previously. All strains were purified using gastrografin (Bayer, Germany) gradient centrifugation and titers were determined as described previously.[75–77] Purified stocks were stored in sucrose-phosphate-glutamate buffer at −80°C and thawed immediately before use (220 mM sucrose, 8.6 mM Na2HPO4, 3.8 mM KH2PO4 and 5 mM L-glutamic acid).

HeLa cells (ATCC CCL-2) and McCoy cells (ATCC CRL-1696) were maintained in DMEM with 10% FBS. During infection, all cells were cultured in DMEM with 10% FBS (supplemented with 10 U ml^{-1} penicillin if Ct-L2-GFP was used). HeLa cells were authenticated using highly polymorphic short tandem repeat loci (STRs).

CRISPR/Cas9 knock out lines of HCT116 cells transduced with sgRNA targeting either SLC19A1 (CE0540-U) or Renilla luciferase (CE04CG-T) cDNA were obtained from RESOLUTE[37] and cultured in RPMI containing 10% FBS. During infection, all cells were cultured in DMEM with 10% FBS (supplemented with 10 U ml^{-1} penicillin).

Primary cervical epithelial cells were derived from hysterectomy samples from premenopausal healthy donors which were recruited at the University Hospital, Medical University of Vienna, Austria after obtaining appropriate fully informed written

consent. Cervical biopsies were digested overnight at 4°C with dispase II (2U ml^{-1}). Epithelial layer is scraped off with tweezers and cultivated in Keratinocyte Growth Medium-2 (Lonza) and CnT-IsoBoost until the first split (CnT-ISO-50, CELLnTEC). The study was approved by the local ethics committee, Medical University of Vienna (ECS 1503/2020).

For experiments with *Neisseria gonorrhea*, the fully antibiotics susceptible strain ATCC 49226 was used. *Ng* were cultured on homemade agar plates with gonococcal base medium supplemented with Kellogg's supplement I and II.[78] For experiments with *Lactobacillus acidophilus*, the ATCC 4356 strain was cultured on MRS plates.

Mice

All mouse experiments were approved by the Institutional Review Board of the Austrian Ministry of Sciences (BMBWF 2020–0.380.439). Female 6–8-week-old C57BL/6JRj mice were purchased from Janvier labs/Charles River and maintained under specific pathogen free conditions in Biosafety Level 2 (BSL-2) facilities at the Medical University of Vienna. Mice were housed in standard cages in a temperature and humidity-controlled room with a 12 h light/dark cycle.

METHOD DETAILS

Medium-throughput compound screening

The compound screening was performed in collaboration with the CeMM Molecular Discovery Platform using a customized library of 2167 compounds. The compounds derived from the NIH clinical collection, CeMM library of unique drugs CLOUD, and collections of anti-cancer agents, natural products, epigenetic compounds, metabolites and kinase inhibitors were spotted on 384-well assay plates at concentrations of typically 10 μM (ranging from 10 to 50 μM) in 0.1% DMSO. As positive control, azithromycin at concentrations of 2, 10 and 50 nM was used. 2,000 HeLa cells were seeded per well in 25 μL and incubated for 6 h to ensure adherence of cells. Then, Ct-L2-GFP at MOI 2.5 were added on top in 25 μL suspension and incubated for 42 h. Cells were fixed with 3.7% paraformaldehyde with 1% methanol and stained with 1 μg mL^{-1} DAPI and 0.002% Evans blue. Number of nuclei and chlamydia inclusions were counted for each well. Z-factors were calculated using negative controls (DMSO) and positive controls (azithromycin 50 nM) from each plate individually.[79] All plates passed the quality control with Z'>0. By linear regression, the percentage of control (POC) was calculated, setting the mean of negative controls to 100% and the mean signal of positive controls to 0% separately for each plate. Hits were defined as compounds with POC <50 and number of nuclei >100. Compounds with less than 100 nuclei were considered toxic (306 in total).

For the dose response validation, all hits except known antibiotics were tested in an 8-point dose-response in duplicates in 3-fold dilutions typically starting at 13.5 μM. Azithromycin was used at an assay concentration of 67.5 nM. Top hits must fulfill the criteria of POC <60 and the number of nuclei must be >150. The 20 top candidates from the validation screen were tested also in serovar E and F. As for these serovars, a centrifugation step is crucial to obtain sufficient infectivity, the plates were centrifuged for 30 min at 600 g before incubation for 46 h. In experiments with serovars not expressing GFP, cells were permeabilized with a 0.1% saponin/PBS solution for 20 min before immunofluorescence staining with FITC-conjugated anti-*Ct* LPS monoclonal antibody (B410F, Invitrogen, 1:100) in 0.1% Saponin in PBS +2% BSA for 30 min.

Compounds for *in vitro* tests

For other assays than medium-throughput screening, the following compounds and suppliers were used: azithromycin (Synovo, Tübingen, Germany), doxycycline (Vibravenös, Pfizer, Pocé-sur-Cisse, France), dolutegravir (MedChemExpress, Monmouth Junction, USA), metergoline (Sigma, St. Louis, USA), pentamidine isethionate (Thermo Fisher Scientific, Germany). Compounds were dissolved in DMSO and diluted in 0.9% NaCl to final concentrations indicated in the respective experiments (final DMSO concentration in the cultures <1%).

In vitro experiments with cell lines

For *in vitro* uptake and washout experiments, HeLa cells were seeded in the presence of pentamidine and infected with Ct-L2 (MOI 100 for uptake, MOI 2.5 for washout) after 6 h. For microscopy, cells were fixed with 4% paraformaldehyde and stained as described for the *Ct* screen. Acquisition was performed with an Olympus IX53 microscope (LUCPlanFL N, 40x). For qPCR, DNA was isolated with Qiagen DNA mini kit. For viability assays with HeLa cells and primary epithelial cells either calorimetric assays (CellTiter-Glo and LDH-assays) or flow cytometry-based assays (staining with CellTrace Violet and 7-AAD) were used. Cells were cultured in the presence of pentamidine for 48 h before viability was assessed. For CellTrace Violet labeling, 1 million HeLa cells were incubated in 1 mL PBS containing 1 μM CellTrace Violet for 15 min. Cells are washed with 5 mL medium before plating. After harvest, cells were stained with 2.5 μg mL^{-1} 7-AAD in PBS for 10 min and directly acquired with a Cytek Aurora flow cytometer. FACS data were analyzed using FlowJo (Version 10.8.1).

For C6-NBD-sphingomyelin staining and confocal microscopy, HeLa cells were treated with 1 μM pentamidine or DMSO in controls for 6 h before infection with Ct-L2 MOI 2.5 (no GFP-expressing). At indicated time points (2 h, 6 h or 18 h after infection), C6-NBD-Ceramide was added to the medium (final concentration 5 μM C6-NBD-Ceramide in 0.05% BSA) and incubated for 30 min at 37°C. Cells were washed 2x with PBS and cells were incubated with fresh DMEM +10% FCS for 60 min at 37°C to allow

back-exchange. Cells are fixed with 4% PFA solution for 20 min at 37°C. Subsequently, cells are stained with anti-Ct-LPS antibody (Clone 512F, Invitrogen, 1.3 μg mL^{-1} in 0.1% Saponin in 2% BSA/PBS) for 30 min. Secondary antibody staining with anti-mouse-IgG (AF680, Invitrogen, 5 μg mL^{-1} in 0.1% Saponin in 2% BSA/PBS) was combined with Phalloidin staining (AF594, Invitrogen, 1:100) for 30 min at RT. After washing with PBS, counterstaining with DAPI for 5 min, coverslips were mounted onto slides. Samples were acquired at a confocal laser scanning microscope (Olympus, FLUOVIEW-FV 3000, equipped with OBIS lasers: 405, 488, 561, 640 nm and ×60 UPlanXApo objectives and Olympus FV31S-SW software).

For assessment of cellular metabolic activity, we performed a Seahorse XF Cell Mito Stress Test (Agilent). 8,000 HeLa cells were seeded per well in 80 μL DMEM supplemented with 10% FCS in the presence of pentamidine. Cells were incubated for 6 h before adding 80 μL medium containing Ct-L2-GFP (MOI 2.5). Cells are incubated for 40 h at 37°C, 5% CO2 before exchanging the culture medium with assay medium (Agilent Seahorse DMEM, 1 mM pyruvate, 2 mM glutamine and 10 mM glucose). Plates were incubated for 1 h at 37°C without CO2 before loading them into the Agilent XFe96 Extracellular Flux Analyzer. The assay was performed using 1 μM oligomycin, 1 μM FCCP and 0.5 μM rotenone/antimycin A.

Mouse model of *Ct* infection

Mice were treated with 2.5 mg medroxyprogesterone acetate (MPA; TCA, Tokyo, Japan) in 100 μL PBS subcutaneously 7 days before infection to normalize the estrous cycle. Prophylactic treatment with compounds was started one day before infection and was repeated every 24 h until the end of experiment. The doses chosen for *in vivo* treatment were according to the highest still tolerated systemic doses reported in the literature[46,47,80] and suitable for dilution in small volume for transcervical application. Doses were calculated for an average mouse weight of 20 g in our study. For systemic treatment, doses were 0.8 mg pentamidine, 0.45 mg dolutegravir, 0.25 mg doxy or 0.1 mg metergoline in 100 μL 0.9% NaCl containing 10% DMSO administered intraperitoneally. For local treatment, mice received half of the systemic dosage in 15 μL 0.9% NaCl with 10% DMSO alone in control conditions or containing 0.4 mg pentamidine, 0.22 mg dolutegravir, 0.125 mg doxy or 0.05 mg metergoline by intrauterine application using an NSET device. For intrauterine infection, 1x 10^6 IFU of Ct-L2-GFP in sucrose-phosphate-glutamate buffer were added to the local treatment dose and administered to the uterus using an NSET device as previously described.[32] On day 4 after infection, uteri were minced and snap frozen.

For absorption studies, mice were treated once transcervically with 15 μL 0.9% NaCl with 0.4 mg pentamidine and organs and serum were harvested 24 h after treatment. Organs were either snap frozen in liquid nitrogen for targeted MS/MS or paraffin embedded for histopathological assessment.

qPCR to assess *Ct* burden

To assess the chlamydia burden in the murine uteri or in HeLa cells, DNA was isolated using the QIAamp DNA mini kit (Qiagen) and host GAPDH DNA and chlamydia 16S DNA were quantified by qPCR using Luna Universal Probe qPCR Master Mix (New England Biolabs) on a StepOnePlus Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific) in a multiplexed manner as previously described.[32,81] Using standard curves from known amounts of *Ct* and host DNA, the amount of chlamydia DNA (in pg) per unit weight of host DNA (in μg) allowed to calculate the ratio of pathogen DNA/host DNA.

Antimicrobial susceptibility testing

For susceptibility testing of Ng, liquid gonococcal base medium containing Kellogg's supplement I and II and $NaHCO_3$ was prepared as previously described.[78] Pentamidine was added at a concentration of 3.12, 6.25, 12.5, 25 and 50 μM and samples were inoculated with a 0.5 McFarland bacterial suspension in NaCl 1:100.

For susceptibility testing of L. acidophilus, inoculum was prepared by dissolving single colonies in 0.9% NaCl solution at a McFarland standard of 0.5 and using bacterial solution 1:500 in MRS broth containing various pentamidine concentrations.

OD600 was measured after 24 h and growth rate was calculated comparing to optical density of medium only and untreated cultures. To assess bactericidal effect of pentamidine, 5 μL of 24 h liquid cultures treated with various drug concentrations were spread out on fresh agar plates and incubated for 48 h.

Chlamydia incidence in HIV cohort receiving DTG

For this analysis, all Ct-positive results from 04/2014-11/2020 from males visiting the HIV- and STI-clinic of the Medical University of Vienna were retrospectively evaluated, and patients' characteristics (HIV status and DTG exposure) were retrieved from the medical records. GraphPad Prism 8 was used to perform the statistical analyses. Nominal variables were plotted as number and percentage of patients with a specific feature. To calculate the incidence rate of infections and the respective 95% confidence interval (95% CI), the person-time method was used. Reinfections during the observational period were analyzed using a Kaplan-Meier curve and a log rank test was used to compare the incidence of Ct-reinfections by HIV-status. The presented analysis complies with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. The Local Ethics Committee of the Medical University of Vienna provided the ethical approval (2175/2020). Due to the retrospective design, the need for an informed consent had been waived.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis of medium-throughput screen was done in R calculating Z-factors and linear regression models. All other statistical analyses were done in GraphPad Prism version 9.5.0. If not stated differently, data are expressed as mean ± standard deviation (s.d.). For analysis of mouse experiments, outliers were excluded using Grubbs' method with alpha = 0.05. One-way ANOVA and Tukey's multiple comparisons were performed to find differences between treatment groups. For comparison between only two groups, unpaired t-tests were performed. For statistical analysis of *in vitro* experiments, two-way ANOVA with matching across row and multiple comparison testing according to Šídák's (comparing 2 means) or Tukey's (more than 2 means) was used.

2.3 Interlude

Another strategy to prevent infection is the development of a successful vaccine. To date, there is no vaccine available against Ct infection. Mostly, candidates fail to induce a long-lasting immune response in the mucosa and some early trials even led to tolerance induction to the pathogen. The early immune response includes antigen uptake and processing by DCs, their migration to lymph nodes, where they prime naïve T cells as prerequisite to successfully kickstart immunity. We therefore focused on exploring these mechanisms in the FRT upon Ct infection. The author of this thesis developed methods for isolation and *in vitro* infection of patient cervix samples and implemented the Ct infection model with moDCs. Together with a team from the lab she performed experiments to unravel the role of moDCs during Ct infection and spearheaded drafting a manuscript. This manuscript is currently under submission.

2.4 PDF of second manuscript - Impaired migration of dendritic cells limits the immune response in human Chlamydia trachomatis infection

Impaired migration of dendritic cells limits the immune response in human *Chlamydia trachomatis* infection

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Abstract

Urogenital infections caused by *Chlamydia trachomatis* (Ct) represent the most common bacterial sexually transmitted infection worldwide. The majority of these infections are asymptomatic and consequently remain untreated, yet they can induce low-grade inflammation with long-term complications such as infertility. While some infected individuals exhibit signs of immunity, protective immune responses are generally weak and no vaccine candidate has yet advanced to clinical use in humans.

In this study we investigated the potential contribution of antigen-presenting cells to control Ct infection in humans. Using an *ex vivo* cervical cell infection model, we identified CD14+ CD11c+ dendritic cells (DCs) as the primary subset involved in Ct uptake. Monocyte-derived dendritic cells (moDCs) served as a model to further examine antigen presentation capacities upon Ct infection. Although Ct-stimulated moDCs are highly activated as they express co-stimulatory molecules and upregulate the chemokine receptor CCR7, they exhibited reduced migration properties. Despite this, *in vitro* T cell priming remained functional, with a predominant induction of Th1 and Th17 cells following Ct infection.

In conclusion, our study reveals a migration defect in otherwise functionally competent Ct-exposed DCs. This may limit the ability of DCs to reach lymph nodes and effectively induce an adaptive immune response as an immune evasion strategy of Ct, thereby contributing to the lack of robust protective immunity.

Keywords

Chlamydia trachomatis, dendritic cells, antigen-presenting cells, antibacterial immune response, sexually transmitted infection, T cell response, cellular migration

Introduction

Chlamydia trachomatis (Ct) is the leading cause of bacterial sexually transmitted infection worldwide (1). It is an intracellular bacterium relying on the host cell metabolism for its replication and primarily infects epithelial cells of mucosal surfaces such as the anogenital tract (2). Even though infections with Ct are readily treatable with antibiotics, they often remain asymptomatic and undiagnosed yet can still cause serious long-term sequalae such as pelvic inflammatory disease, infertility, and ectopic pregnancies (3). Ct strains are classified into serovars based on differences in their major outer membrane proteins (MOMP). These serovars differ in their tissue tropism and clinical outcomes: serovars A – C cause ocular infection, serovars D – K are commonly found in urogenital infection and serovars L1 – L3 typically infect anogenital mucosa, leading to lymphogranuloma venereum (4). Interestingly, some individuals naturally clear the infection and exhibit partial, though transient, immunity (5). Upon repeated infections, resistance to reinfection can occur (6–8). However, the immune mechanisms underlying protective immunity against Ct in humans remain poorly understood.

Antigen-presenting cells (APCs) serve as crucial link between innate and adaptive immunity. These cells constantly patrol epithelial barriers, sampling the environment for self- and non-self-antigens (9). Upon capturing antigens, APCs migrate to draining lymph nodes where they present processed peptides to naïve T cells, initiating adaptive immune responses. In murine models, immunization with live Ct leads to antigen uptake by immunogenic dendritic cells (DCs), which then stimulate effector T cell responses and confer protection. In contrast, UV-inactivated Ct is taken up by tolerogenic DCs, promoting tolerance by activation of regulatory T cells (10). Both in mice and humans, clearance of Ct has been associated with IFNγ-producing $CD4^+$ T cells, whose presence correlates with Ct clearance and reduced risk of reinfection (11–14). The cervix serves as the primary site for Ct infection within the female reproductive tract (FRT), which is populated by several APC subsets including macrophages, myeloid DCs, plasmacytoid DCs and Langerhans cells (LCs) (9,15). Clinical studies have shown that Ct-infected patients exhibit an increased number of activated myeloid DCs in the endocervix (16). However, it remains unclear which specific APC subsets are responsible for antigen uptake, migration to lymph nodes, and initiation of protective T cell responses. While murine studies have implicated $CD103^-$ DCs in trafficking to lymph nodes and priming tissue-resident memory T cells, the corresponding dynamics in humans are not yet fully elucidated (10).

A key feature of DC maturation is the upregulation of CC chemokine receptor 7 (CCR7), which directs migration toward secondary lymphoid organs via gradients of its ligands,

CCL19 and CCL21. These chemokines are produced by endothelial cells and reticular fibroblasts, guiding DCs to afferent lymphatic vessels via a chemokine gradient and ultimately to lymph nodes, thymus and spleen (17–19). Notably, several pathogens have evolved mechanisms to interfere with APC migration to evade immune detection. For example, *Escherichia coli* (*E.coli*) strains causing urogenital tract infections express certain pili which overstimulate DCs, leading to integrin overexpression and impaired migratory capacity (20). Conversely, pathogens like *Toxoplasma gondii* hijack APCs, inducing a hypermotile phenotype in infected DCs that facilitate pathogen dissemination (21).

In this study, we aimed to assess the role of human DC subsets during Ct infection, focusing particularly on their ability to migrate following Ct antigen uptake. Understanding the mechanisms that impair APC function and trafficking may shed light on why protective immunity to Ct is rarely achieved and why repeated reinfections are so common.

Results

CD11c$^+$CD14$^+$ DCs of the human cervix predominantly take up Ct

To identify APC subsets responsible for Ct uptake in the human cervix, we established an *ex vivo* cervical cell infection model: cervical biopsies from healthy donors undergoing hysterectomy were digested and subsequently exposed to GFP-expressing Ct-L2 (GFP-Ct-L2). After 24-hours of incubation with GFP-Ct-L2, we subjected the single cell suspension to flow cytometry and gated on CD45$^+$ immune cells, which mainly comprised CD3$^+$ T cells. APCs defined as HLA-DR$^+$CD3$^-$ represented approximately 20% of the immune cell population (Fig. 1A). We further characterized APCs based on the expression of the pattern recognition receptor CD14 and the integrin alpha X (CD11c). Upon infection with Ct, a clear shift of GFP-Ct-L2-positive cells was observed in the HLA-DR$^+$CD3$^-$ cell fraction (Fig. 1B), indicating bacterial uptake by APCs while CD3$^+$ T cells only rarely appeared infected by Ct (Fig. 1BC). Quantification revealed that both live and UV-inactivated Ct are taken up by APCs compared to uninfected controls (Fig. 1D). Among APCs, CD11c$^+$CD14$^+$ DCs took up the largest proportion of Ct (Fig. 1D).

The proportions of immune cells remained stable during the 24-hour culture compared to freshly digested tissue (Suppl. Fig. 1A). Immunofluorescence analysis of cervical tissue sections showed that APC subsets were present in both the epithelial layer and the stromal compartment of the cervix. While CD11c$^-$CD14$^+$ macrophages are the most abundant APC subset in the cervix, CD11c$^+$CD14$^+$ DCs were primarily located at the epithelial-stromal interface, suggesting their involvement in Ct antigen uptake *in vivo* (Fig. 1E-G). Langerin (CD207)-expressing cells are abundant in the cervical epithelium and around half of them co-express CD11c (Suppl. Fig. 1B-D).

In summary, human APCs from cervical tissue are proficient to take up Ct antigens in an ex vivo cervical cell infection model, with CD11c$^+$CD14$^+$ DCs being the subset with the highest propensity for bacterial uptake.

Ct induce maturation and CCR7 upregulation in human DCs

To investigate the functional consequences of Ct uptake by human DCs, we used monocyte-derived DCs (moDCs) as model cells, given their phenotypic similarity to cervix-derived CD11c$^+$CD14$^+$ DCs. Bulk RNA sequencing of uninfected and Ct-L2-infected moDCs revealed a strong inflammatory signature upon infection, with enrichment of KEGG pathways involved in infectious disease response (e.g., legionellosis, measles, influenza A, Epstein-Barr virus infection, TNF and NF-kappa B signaling) as well as innate immune recognition (cytosolic DNA sensing, RIG-I-like, NOD-like and Toll-like receptor signaling

pathways) (Fig. 2A). Genes related to proteasomal activity were also upregulated (Fig. 2A), indicating enhanced antigen processing for presentation on MHC molecules. In contrast, pathways involved in DNA replication, the cell cycle and biosynthesis were downregulated (Fig. 2B).

We further detected an upregulation of maturation markers and pro-inflammatory cytokines in moDCs upon Ct infection (Fig. 2C,D). Genes involved in phagocytosis (e.g., CD209, MRC1) were downregulated, while co-stimulatory molecules (CD80, CD83, CD40, CD274) and the migration marker CCR7 were significantly upregulated (Fig. 2C). Key pro-inflammatory cytokines such as IL-6, IL-1β, IL-23 (via the subunits IL-23A and IL-12B), and IDO1 were induced (Fig. 2D). Notably, IL-12A, required for functional IL-12 formation, remained low, indicating a bias toward Th17 rather than Th1 responses, contrasting data from murine Ct immune responses (10,22). IL-10 was not upregulated in moDCs upon Ct infection, indicating a non-tolerogenic profile (Fig. 2D). No major differences in gene expression were observed between moDC infected with live or UV-inactivated Ct, indicating that immune activation is independent of Ct replication (Suppl. Fig. 2A-C).

Flow cytometry analysis revealed that monocytes indeed differentiated into moDCs and not macrophages (Suppl. Fig. 3A.B). We validated our findings by assessing protein expression of these maturation markers by flow cytometry on moDCs stimulated with the lymphogranuloma venereum serovar Ct-L2, the urogenital serovar Ct-E, LPS derived from *E.coli* as positive control and *Lactobacilli* (Lb) as a commensal of the female genital tract. Despite only limited bacterial replication in moDCs, Ct-L2 led to higher intracellular bacterial burden than Ct-E (Fig. 2E, Suppl. Fig. 3C). The pattern recognition receptor CD209 expression decreased with moDC maturation, especially in Ct-L2 and LPS conditions (Fig. 2F). Flow cytometry revealed robust CCR7 surface expression following Ct-L2 and LPS stimulation, while Ct-E and Lb induced only moderate CCR7 levels (Fig. 2G,H). Co-stimulatory molecules (CD80, CD83, CD86, PD-L1, PD-L2) were strongly upregulated, particularly with LPS and Ct stimulation (Fig. 2H, Suppl. Fig. 3D).

Together, these results demonstrate that Ct-infected moDCs undergo activation and maturation, characterized by upregulation of co-stimulatory molecules, CCR7, and pro-inflammatory cytokines important for T cell priming.

Ct-infected moDCs exhibit limited migration properties despite high CCR7 expression

Given the profound activation phenotype of moDCs upon Ct-stimulation, we next assessed the adhesion and migration behavior of Ct-stimulated DCs to evaluate the functional

consequences of maturation. Mature DCs stimulated with LPS or Ct became adherent in culture, while uninfected, immature moDCs show a tendency to remain in suspension (Fig. 3A, Suppl. Fig. 4A). However, both - adherent and non-adherent moDCs - displayed similar activation profiles, migration behavior, and Ct uptake (Suppl. Fig. 4B-F). While some integrins such as β-2 (CD18), β-1 (CD29), and α4β7 were more prominent in adherent moDCs (Suppl. Fig. 4D), their expression did not differ significantly between the treatment conditions (Suppl. Fig. 4D,E). Given that CCR7 is essential for DC migration towards lymph nodes via a CCL19 chemokine gradient, we assessed the capacity of moDCs to migrate in response to CCL19 using a 2D transwell assay. Despite high CCR7 expression, Ct-L2-stimulated cells migrated at rates comparable to uninfected moDCs, whereas LPS-stimulated moDCs showed robust migration (Fig. 3B). A similar trend was observed in a 3D collagen matrix invasion assay, where Ct-stimulated moDCs migrated shorter distances than LPS-treated cells (Fig. 3C,D). Additionally, Ct-infected cells in the collagen matrix appeared shorter in length, indicating impaired cell polarization (Fig. 3E). Due to high biological variability in human moDCs in morphology and response to chemokine gradients, we assessed murine bone marrow-derived DCs (BMDCs) as an alternative model to monitor migration of DCs after Ct infection. The migration and polarization defects were also observed in Ct-stimulated BMDCs, suggesting that high Ct load within APCs interferes with DC migratory capacity across species (Suppl. Fig. 4G).

As we observed impaired cell polarization in of Ct-stimulated moDCs in CCL19 gradients, we were wondering whether other actin-mediated processes were likewise affected upon Ct infection. We therefore assessed phagocytic capacity by incubating Ct-stimulated moDCs with uncoated fluorescent latex beads. Interestingly, only Ct-L2-infected moDCs showed substantially impaired phagocytosis of fluorescent latex beads (Fig. 3F,G), possibly due to the high intracellular bacterial burden (Fig. 2E) restricting normal cellular function potentially by hijacking the actin machinery.

Collectively, these results suggest that Ct fails to stimulate moDC migration despite inducing high CCR7 expression and limiting additional antigen uptake, thereby potentially hindering efficient T cell priming in lymph nodes *in vivo*.

Ct-infected moDCs can induce a $CD4^+$ T cell response

To assess the T cell-priming capacity of Ct-infected moDCs, we co-cultured stimulated moDCs with autologous naïve $CD4^+$ T cells for 5 days. T cell polarization was analyzed by flow cytometry based on lineage-specific surface marker for various T helper (Th) cell subsets (Fig. 4A, Suppl. Fig. 5A).

While CD69 expression indicated comparable activation across LPS, Ct-L2, and Ct-E conditions, differences in polarization of T helper subsets were evident (Fig. 4B, Suppl. Fig. 5A,B). Although the majority of differentiated T cells were classified as Th0/Th2 ($CCR7^+$ $CD45\text{-}RA^{lo}$ lacking the other chemokine receptors (CCR4, CCR6, CCR10, CXCR5, CXCR3 as subset markers), the proportion of these cells was reduced in Ct-L2-stimulated conditions (Fig. 4B). Instead, a trend of higher frequencies of Th17 cells were observed (Fig. 4B). This is consistent with our transcriptomic findings, where moDCs upregulated IL-6 and IL-23 upon Ct-L2 infection (Fig. 2A). Regulatory T cells (Tregs) were also increased in all stimulated conditions (Fig. 4B). Th1 cells ($CXCR3^+$) were similarly induced by both LPS or Ct-infected moDCs to a very low extent (Fig. 4B). To validate these phenotypes, we performed intracellular cytokine staining following PMA/Ionomycin restimulation. IFNγ and TNFα (Th1 cytokines) were primarily detected in T cells primed with LPS or Ct-stimulated moDCs (Fig. 4C,D). Th2 cytokine expression was consistent across all conditions (Fig. 4D), supporting our results from the surface marker expression. IL-10 and IL-17 levels were generally low and only marginally higher in T cells after moDC stimulation compared to unstimulated controls (Fig. 4C,D).

These findings demonstrate that Ct-infected moDCs are capable of priming naïve $CD4^+$ T cells and favor differentiation toward Th1 and Th17 lineages but not Th2 cells. While migration deficits may impair DC trafficking *in vivo*, the capacity to activate T cells remains intact if DC-T cell contact is established.

Discussion

APCs in the human genital mucosa are the first cells encountering pathogens upon sexually transmitted infection to kick-start an immune response. However, anti-chlamydia immune responses are insufficient to protect humans from subsequent infection. Even though we clearly show that human cervical DCs take up chlamydial antigens in an *ex vivo* cervical cell infection model, further investigation of DC functions reveals a migration defect in Ct-infected human DCs despite strong induction of activation and maturation markers. Interestingly, T cell priming capacity remains unaffected *in vitro*, suggesting that lymph node homing of human DCs is hampered upon Ct-stimulation and therefore DCs cannot encounter T cells for mounting an adaptive immune response, even though T cell priming itself would be functional.

Although macrophages are the most abundant APCs in the cervix compromising around 30% of $CD45^+$ immune cells (15), our data show that $CD14^+CD11c^+$ DCs are significantly more efficient in chlamydial antigen uptake. DCs appear to be more resilient to tissue digestion and *in vitro* culture conditions than macrophages, which may explain their dominance in our *ex vivo* cell infection model. Interestingly, $CD14^-CD11c^+$ DCs were markedly less efficient in taking up Ct-LPS compared to $CD14^+CD11c^+$ DCs, suggesting a role of CD14 in facilitation Ct-LPS recognition via the toll-like receptor 4 complex (23). This is consistent with findings that $CD14^+CD11c^+$ DCs are also highly efficient in HIV uptake and transmission to $CD4^+$ T cells (24). We found $CD14^+CD11c^+$ within both the epithelia and stromal compartments of the ectocervix, contrasting with prior reports that primarily locate them in the stroma (15). Nevertheless, their proximity to the epithelium – the primary site of Ct infection – suggests they are well-positioned to capture Ct antigens. A fraction of these cells also expresses CD207 which is typically associated with Langerhans cells occurring in the skin epithelium (25). In the mucosa, however, real Langerhans cells presenting with Birbeck granules are rare, while so called epidermal or vaginal epithelial DC expressing CD11c and CD207 are the predominant APC type (26) and are also the primary target in HIV infection (27). Whether $CD14^+CD11c^+$ DC are the key APC subset mediating antigen uptake and presentation during *in situ* Ct infection of the cervix and if these are also expressing CD207, remains unresolved. Studies of cervical tissues of Ct-positive women reveal an increase in $CD14^+$ cells compared to uninfected controls, though further phenotype characterization is lacking (16). Myeloid DCs from Ct-infected patients consistently express increased levels of co-stimulatory molecules, such as CD80, CD86 and CD83, regardless of clinical symptoms (16).
In our model, stimulation with Ct-L2 induced moDCs maturation and upregulation of co-stimulatory markers to a degree comparable to LPS-treated conditions. In contrast,

stimulation with the urogenital serovar E resulted in less robust activation, likely due to lower uptake and bacterial load. Previous studies have demonstrated that Ct can actively replicate within immune cells like DCs (28–31), with lymphogranuloma venereum serovars such as Ct-L2 being more efficient in exploiting DCs for intracellular replication than urogenital serovars like Ct-E (30,31). Gorvel *et al*. demonstrated that while extracellular bacteria like *E.coli* trigger strong co-stimulatory molecule expression and T cell activation, intracellular pathogens, including Ct, actively suppress DC maturation to evade immune detection (32). This aligns with the clinical observation that Ct infection rarely confers protective immunity. Indeed, live Ct appears to actively prevent lysosome fusion, as evidenced by the absence of Lamp-1 co-localization with Ct inclusions (29). In contrast, UV-inactivated Ct localize to lysosomes, indicating that only viable Ct interfere with lysosomal trafficking to enable replication (29). In our model, we observed minimal differences between moDCs infected with live versus UV-inactivated Ct and therefore focused on the immunological effects of live Ct infection on DC function relative to LPS stimulation. Consistent with previous reports, Ct-infected moDCs produced IL-6, IL-1B, IL-18, and IDO1, though IL-12 induction remained modest (29–31).

Beyond their maturation, DCs must migrate to lymphoid organs to initiate T cell priming. Various pathogens have evolved strategies to impair DC motility as a means of immune evasion. For instance, uropathogenic *E. coli* triggers β2 integrin overactivation, effectively anchoring DCs and reducing their migratory capacity (20). Key integrins for DC adhesion and trafficking include α4β7, which interacts with VCAM, and αxβ2, and binds to ICAM and collagen I (33). Integrin dysregulation has also been implicated in Ct pathology. For instance, Ct-induced expression of β1 integrin on fallopian tube epithelium contributes to ectopic pregnancy via enhanced bacterial adhesion mediated by the Ct-encoded adhesin Ctad1 (34,35). Integrins have primarily been studied in the context of T cell recruitment to the genital tract during Ct infection, where α4β1 is essential for trafficking of Ct-specific $CD4^+$ T cells (36). In our model, LPS-stimulated moDCs exhibited increased adherence, consistent with other reports (37,38). Notably, adherent DCs displayed a more mature phenotype and superior antigen uptake and T cell stimulation capacity than their non-adherent counterparts (37,38). Interestingly, Toxoplasmosis infection promotes the opposite phenotype, increasing non-adherent moDCs (21), an effect dependent on integrins β1 (CD29), β2 (CD18), and αM (CD11b) (21). However, in our transwell and 3D collagen migration assays, Ct-infected moDCs exhibited low migration properties, even in environments where integrins are not essential, such as 3D matrices (39). We detected slightly elevated expression of β1, β2 and α4β7 integrins in adherent versus non-adherent moDCs but no striking differences across maturation conditions. Transcriptomic analysis

revealed only β7 upregulation in Ct-L2-stimulated non-adherent moDCs. These results suggest that integrin expression is more reflective of the maturation status than the specific activation stimulus. As even immature moDCs display basal activation, the proportion of cells responding to a given stimulus may ultimately determine integrin expression patterns and migratory capacity.

The actin cytoskeleton is another key regulator of DC migration and morphology. Immature DCs form podosomes that facilitate extracellular matrix degradation and antigen uptake (40,41). Upon maturation (e.g., upon LPS stimulation), podosomes are lost and replaced by structures supporting directed migration, such as filopodia and lamellipodia (40,41). Several actin-binding proteins like WASP, Arp2/3 and the WAVE complex govern these essential DC functions (40). Ct manipulates these pathways to support its intracellular lifecycle (2). For example, Tarp (Translocated actin recruiting phosphoprotein) is one of the first proteins secreted by Ct to induce actin polymerization for membrane remodeling and thereby facilitate Ct entry (2,42). Additionally, actin is also used to stabilize the inclusion membrane (43) and control Ct exit (2). Toxoplasma induces similar changes via the WAVE complex-Interacting Protein (TgWIP), promoting the formation of filopodia and stress fibers to enhance cell motility (44). In DCs, antigen capture and migration are typically mutually exclusive. Migration speed is regulated by the positioning of myosin II, which supports antigen uptake when located anteriorly and migration when relocated to the cell rear (45). The actin distribution thus determines cell motility, and Ct may hijack actin networks to stabilize their inclusion, preventing DCs from migrating despite inducing high CCR7 expression. Interestingly, Li *et al* showed that CCR7-deficient mice exhibit enhanced clearance of *Chlamydia muridarum* infection, possibly due to an increased baseline presence of T cells in the female reproductive tract and the formation of tertiary lymphoid structures, which enable local T cell priming directly at the site of infection (46). In contrast, when only DCs exhibit impaired lymph node homing capacities, such as the lack of CCR7 or other migratory defects, they may fail to reach draining lymph nodes, resulting in suboptimal activation of naïve T cells and compromised adaptive immunity.

$CD4^+$ T cells are essential for Ct clearance and the establishment of protective immunity (14), whereas $CD8^+$ T cells appear to play a limited role, likely due to impaired antigen cross-presentation in Ct-infected DCs (47). In murine Ct infection models, Th1 responses and IFNγ production are critical for bacterial control (13,22). In contrast, human studies suggest a skewing toward Th2 responses: GATA-3^+ Th2 cells have been detected in the endometrial tissue of infected women (48), and infected individuals show an increased frequency of Th2 and Th17 effector memory T cells in peripheral blood (8). Moreover, T cells from infected individuals exhibit sustained IL-4 production over time, while IFNγ

secretion declines (48). However, a Th2-biased immune response fails to confer protection in mice, as demonstrated in a vaccination study with MOMP (49). To achieve durable immunity, robust Th1 and Th17 responses are desirable. These are effectively induced by the promising vaccine candidate CTH522/CAF®01which elicits protective responses in both mice and humans (50). Notably, cytokines also contribute to Ct-associated pathology: Elevated IL-17A levels correlate with endometrial infection in humans, suggesting that Th17 responses may facilitate pathogen ascension (51). Moreover, TNFα, particularly in combination with IFNγ, is suspected to promote tissue destruction (52,53).

In our human moDC model, we observed baseline Th2 polarization across all conditions, with negligible Th17 response. In contrast, Ct infection induced a predominant Th1 polarization characterized by robust TNFα and IFNγ production. T cell activation was comparable between DCs stimulated with Ct and those treated with LPS, indicating that when DCs and T cells are in close proximity, effective antigen presentation and T cell priming can still occur.

Conclusion

Our data suggest that Ct-L2 replicates more efficiently in DCs than urogenital serovars like Ct-E or Ct-D, consistent with previous findings (54). The higher bacterial load associated with Ct-L2 may impair DC migration by disrupting cytoskeletal integrity. Supporting this, we found lower uptake and replication of Ct-E correlating with reduced DC activation and milder migratory impairment when compared to Ct-L2. In Ct-L2-infected DCs, cytoskeletal disruption – likely due to actin sequestration by bacterial inclusions – may hinder chemotactic migration despite intact CCR7 expression. Nevertheless, we show this is a non-cell autonomous effect, because if infected DCs encounter T cells, they remain capable of inducing Th cell subsets. These findings suggest that excessive bacterial replication may impede DC migration and thereby compromise immune activation, potentially contributing to the lack of protective immunity in Ct-infected individuals.

Methods

Patient cohort

Female, adult patients (n=16, mean age 45, range 30 – 68) undergoing hysterectomy for benign reasons like uterine fibroids or uterine adenomyosis were recruited at the Division of General Gynecology and Gynecologic Oncology, Medical University Vienna. Exclusion criteria were chronic infections (HIV, Hepatitis C), autoimmune diseases, systemic skin and mucosal diseases and menstruation at the time of sampling. Furthermore, we collected data from a questionnaire to get information on potential previous genital infections. We obtained biopsies from uterine cervix, PBMCs and a genital swab. All patients were tested Chlamydia trachomatis negative at the time of sampling. The study protocol involving patient material had been approved by the local ethics committee of the Medical University of Vienna, Austria (EK number: 1503/2020).

Bacterial strains

Ct serovar CTL2P-pGFP::pSW2 (referred GFP-Ct-L2) was purchased from the Chlamydia Biobank (University of Southampton) (55). Ct serovars L2 (DSM 19102) and E (DSM 19131) were ordered from the DSMZ-German Collection of Microorganisms and Cell Culture GmBH and all strains were propagated and purified as described previously (56). Ct strains were isolated in collaboration with Romana Klasinc and Hannes Stockinger at the Institute for Hygiene and Applied Immunology. *Lactobacillus gasseri* strain ATCC 33323 was provided by Konrad Domig from University of Natural Resources and Life Sciences, Vienna, and cultured as previously described (56).

In vitro differentiation of moDCs and infection

Monocytes were isolated from healthy donor PBMCs derived from heparin tubes by Lymphoprep (Stemcell) density gradient centrifugation and differentiated into monocyte-derived DC as described (57). Briefly, $CD14^+$ monocytes were obtained using $CD14^+$ MicroBeads (Miltenyi). Monocytes were seeded at a density of 0.6 x 10^6/ml in RPMI-1640 medium (Gibco) containing 10% FCS (Gibco), 1% Glutamax (Gibco), 1% Sodium pyruvate (Gibco) (moDC medium) and supplemented with 110ng/ml rh-GM-CSF (ImmunoTools) and 20ng/ml rh-IL4 (Biolegend). 48h after isolation, fresh medium containing 2x cytokines was added. On day 5, non-adherent immature moDCs were replated and stimulated with Ct-L2 or Ct-E at MOI 10, Lb at MOI 10 or 20ng/ml *E.coli* LPS for 24h.

Flow cytometric analysis

Cells were harvested by washing them off the plate with cold PBS containing 2mM EDTA. Cells were transferred to FACS tubes and all centrifugation steps were performed at 4°C at 150g. Pellets were washed once with FACS buffer (PBS, 2mM EDTA, 1% BSA) and stained with antibody mixes in FACS buffer as listed in Table 1 for 20min. Samples were fixed with fixation buffer (Biolegend) for 30min. For intracellular cytokine staining, cells were washed once with Perm buffer (Biolegend) and stained with antibody mix diluted in Perm buffer for 30min. Samples were acquired on an Aurora spectral analyzer (Cytek) using SpectroFlo software. Data were analyzed using FlowJo software (version 10.8.1).

Migration assays

For transwell migration assays, 3µm clear PET cell culture inserts (CellQART) were applied to 24-well plates and 5x10^4 immature moDCs were plated in upper chamber in 200µl moDC medium and directly stimulated with Ct-L2 or Ct-E at MOI 10 or 20ng/ml LPS for 24h. On the next day, the lower chamber was filled with 600µl moDC medium supplemented with 0.625µg/ml CCL19 (R&D). After 4h of incubation at 37°C, inserts were removed and cells in the lower chamber were harvested and quantified using flow cytometry.

For collagen invasion assays, self-made migration chambers (Suppl. Fig. 6A,B) using glass slides (Epredia Superfrost Plus Slides) were prepared by sticking 22 x 22mm coverslips on top using paraplast X-tra (Sigma) with a 1-2mm distance and sterilized under UV-light for 30min. 100µl collagen polymerization solution (30µl 10x MEM (Gibco), 15µl sodium bicarbonate (Sigma), 225µl PureCol bovine (Advanced BioMatrix)) was mixed with 50µl moDC medium and supplemented with 0.625µg/ml CCL19, applied to migration chambers and polymerized at 37°C for 30min. 1x 10^5 stimulated moDCs were placed on top in 60µl moDC medium and chambers were sealed with paraplast. Images were acquired 24h-48h later with an Olympus IX53 microscope (4x) and migration distance and cell shape analyzed with Image J.

Phagocytosis assay

5x10^4 immature moDCs were seeded in a 96-well plate in 200µl medium and stimulated with Ct-L2 or Ct-E at MOI 10 or 20ng/ml LPS for 24h. Then 0.5µm latex beads (Sigma, fluorescent red) were added to the cultures at a 1:200 dilution and cells were incubated at 37°C for 2h. Cells were harvested by scraping off, washed with PBS and fixed with fixation buffer (Biolegend). Uptake of fluorescent particles was quantified using Aurora flow cytometer.

moDC – T cell co-cultures

For co-cultures with autologous T cells, the $CD14^-$ population was kept in culture in ImmunoCult™-XF T Cell Expansion Medium (Stemcell) for 5 days until moDCs are differentiated. Immediately before the start of co-culture, naïve $CD4^+$ T cells were isolated by negative selection using the Naive $CD4^+$ T Cell Isolation Kit II (Miltenyi). 5x 10^4 moDCs which were stimulated for 24h were co-cultured with 200.000 naïve T cells in RPMI containing 10% FCS and 1% PenStrep for 5 days with media exchange on day 3. On day 5, surface flow cytometry staining for markers assessing Th subset polarization was performed. For cytokine staining, T cells were kept in culture for 14-16 days in presence of 10U/ml IL-2 after day 5. Cells were rested overnight in medium without IL-2 before re-stimulation of T cells with Cell activation cocktail with Brefeldin A (Biolegend) for 4h. Subsequently, surface and intracellular cytokine staining was performed.

***Ex vivo* cervical cell infection model**

Tissue samples were minced with a scalpel into 1 mm^3 cubicles. Subsequently, single-cell suspensions were generated by digestion with the whole skin dissociation kit (Miltenyi) for 3h at 37°C using gentleMACS tubes. The cell suspensions were pressed through 70µm cell strainers and resuspended at a density of 1 x 10^6/ml in RPMI-1640 medium (Gibco) containing 10% FCS (Gibco), 1% Glutamax (Gibco), 1% Sodium pyruvate (Gibco) and 10U/ml Penicillin (Sigma). Single-cell suspensions were infected with GFP-Ct-L2 (MOI 5). After 24h, cells were stained with antibodies for surface staining, fixed and permeabilized (Biolegend), intracellularly stained for Ct-LPS and acquired at the Aurora spectral analyzer (Cytek). FACS data was analyzed using FlowJo (Version 10.8.1).

qPCR to assess Ct burden

DNA was isolated using Qiagen DNA Mini kit. Chlamydia uptake by moDCs was quantified by qPCR as described previously (56).

Tissue microscopy

Multicolor immunofluorescence staining procedures were performed on 5µm sections of frozen cervical mucosa embedded in Tissue-Tek OCT Compound and fixed in ice cold acetone. After blocking the slides with 2% goat serum in 2%BSA/PBS, slides were stained with directly conjugated antibodies against CD11c (PE, clone S-HCL-3, BD), HLA-DR (APC, clone L243, Biolegend), CD14 (FITC, clone TÜK4, Dako) and CD45 (AF750, clone 2D1, R&D) for 2h at room temperature. Secondary antibody staining with goat-anti FITC antibody (Life technologies) was performed for 1h followed by counterstaining with DAPI

for 5min. DC in human cervix were identified as $CD45^+$ and $HLA\text{-}DR^+$ either expressing or lacking CD14 and CD11c.

Smart-seq2 bulk RNA sequencing

We sorted 100 live $CD45^+CD11c^+HLA\text{-}DR^+$ moDCs of 3 donors which were infected with Ct-L2, UV-Ct L2 or remained uninfected into 4µl lysis buffer using a FACS Aria III cell sorter (BD Biosciences). cDNA synthesis and enrichment will be done according to the Smart-seq2 protocol and library preparation was done with an Illumina Nextera XT library preparation kit (58). Sequencing was performed on the Illumina HiSeq 4000 platform in 50-bp (base pair) single read configuration.

Bioinformatic analysis of RNA-seq samples

Single end reads were trimmed for low-quality bases and low-quality reads using ReadTools were removed (version 1.0.0) (59). Trimmed reads were mapped to the Homo sapiens genome (GRCh38.92 assembly) using STAR (v2.5.3a) mapper (60). The reads mapped in multiple genomic locations were eliminated by using SAMtools (61). Read counts for features (exons) were generated using the featureCounts function from the Subread package (v 1.22.1) (62). For bulk RNA-seq data analysis, genes with fewer than 10 reads were eliminated from further analysis in all samples. Gene expression analysis was performed with DESeq2 (version 3.22.3) using the Ensembl Known Gene models (version GRCh38.92) as reference annotations. For differential gene expression analyses, genes were considered differentially expressed if they show a P value (FDR) < 0.05, adjusted for multiple hypothesis correction, and an absolute log2 (fold change) > 1. Normalized count (log2) by DESeq2 was used to perform principal components analysis, t-Distributed stochastic neighbor embedding (t-SNE), and hierarchical clustering, and to perform KEGG pathway enrichment analysis and to create heatmaps and scatterplots.

Statistical analysis

Statistical analyses were done in GraphPad Prism version 10. If not stated differently, individual data points were plotted as well as mean ±standard deviation (s.d.). Mixed-model analysis and Tukey's multiple comparisons were performed to find differences between treatment groups.

Acknowledgements

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Figure legends

Fig. 1: Human cervical DCs take up Ct.

A) Distribution of immune cells ($CD45^+$) after 24h explant culture analyzed by flow cytometry according to their expression of CD3, CD11c and CD14 (n=8). B) Gating on $CD45^+$ cells according to expression of CD11c and CD14. Representative plots of $CD11c^+CD14^+$ cells cultured for 24h in the presence of Ct-L2 (MOI 10) or uninfected controls and stained with anti-Ct-LPS-FITC. C) Violine plots quantifying Ct-uptake in all immune cell subsets stimulated with live Ct-L2 (n=8). D) Quantification of Ct-uptake in different APC cell subsets after cultivation for 24h with Ct-L2 or UV-Ct-L2 (n= 5-8 donors). Significant differences were tested by mixed model analysis and Tukey's multiple comparison test between individual groups (*$p < 0.05$). E) Representative immunofluorescence images of cervix tissue stained for CD45, HLA-DR, CD11c, CD14 and DAPI. F) Quantification of APC subsets in epithelium and stroma in cervix biopsies within $CD45^+$ immune cells (n=7 donors, mean + SD of the mean). G) Quantification of total number of APCs per mm2 (n=7 donors, mean + SD of the mean).

Fig. 2: Human moDCs stimulated with Ct mature and upregulate CCR7.

A) KEGG pathway enrichment analysis of genes upregulated in Ct vs uninfected moDCs. B) KEGG pathway enrichment analysis of genes downregulated in Ct vs uninfected moDCs. C) Heatmap of selected surface markers from bulk RNA-seq data derived from uninfected and Ct-L2 infected moDCs after 24h (n=3, *$p < 0.01$, **$p < 0.0001$, ***$p < 1e\text{-}10$). D) Heatmap of selected cytokines and chemokines from bulk RNA-seq data. E) Quantification of Ct burden by qPCR in moDCs infected with Ct-L2 or E after 24h, paired t-test show significant difference (*$p < 0.05$). F-H) Flow cytometry analysis of moDCs 24h after stimulation with Ct-L2, Ct-E, LPS or uninfected cells (n= 5-7 donors). Significant differences were tested by mixed model analysis and Tukey's multiple comparison test between individual groups (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$). F) Mean fluorescence intensity (MFI) of CD209 surface expression. G) Representative histogram plots of CCR7 surface expression. H) Percentage of CCR7, CD83, PD-L1 and CD80 positive cells of total moDC.

Fig. 3: Ct-primed moDCs show a migration defect.

A) Ratio of floating/adherent moDCs in 24h cultures after stimulation with LPS, Ct-L2, Ct E. B) Number of cells migrated through transwells to a CCL19 gradient normalized to uninfected samples (n=4-8 donors). Significant differences were tested by mixed model analysis and Tukey's multiple comparison test between individual groups (*$p < 0.05$). C)

Representative images of collagen invasion chambers in a CCL19 gradient 24h after embedding. Scale bar 200µm. D) Quantification of migration front in collagen gels towards CCL19 (n=5-7 donors). Significant differences were tested by mixed model analysis and Tukey's multiple comparison test between individual groups (*p < 0.05). E) Quantification of cell length within collagen matrix (n=5-7 donors). 10 cells were measured per condition (n=5-9 donors). Significant differences were tested by mixed model analysis and Tukey's multiple comparison test between individual groups (***p < 0.0005, ****p < 0.0001). F) Representative histogram plots of latex bead uptake by flow cytometry. G) Percentage of moDC which take up no, 1, 2, 3 or 4 and more latex beads within 2h (n=3 donors).

Fig. 4: T cell activation of moDCs stimulated with Ct.

A) Scheme of the experimental setup of T cell priming experiments. B) Differentiation of naïve T cells into different Th cell subsets upon a 5d co-culture with stimulated moDCs assessed by flow cytometry analysis for surface markers (n=5 donors). C) Representative flow cytometry plots of intracellular cytokine staining from T cells re-stimulated with PMA/Ionomycin + Brefeldin A after 2-weeks co-culture with stimulated moDCs. D) Quantification of T cells producing TNFα, IFNγ, IL-4, IL-17 or IL-10.

Fig. 1: Human cervical DCs take up Ct.

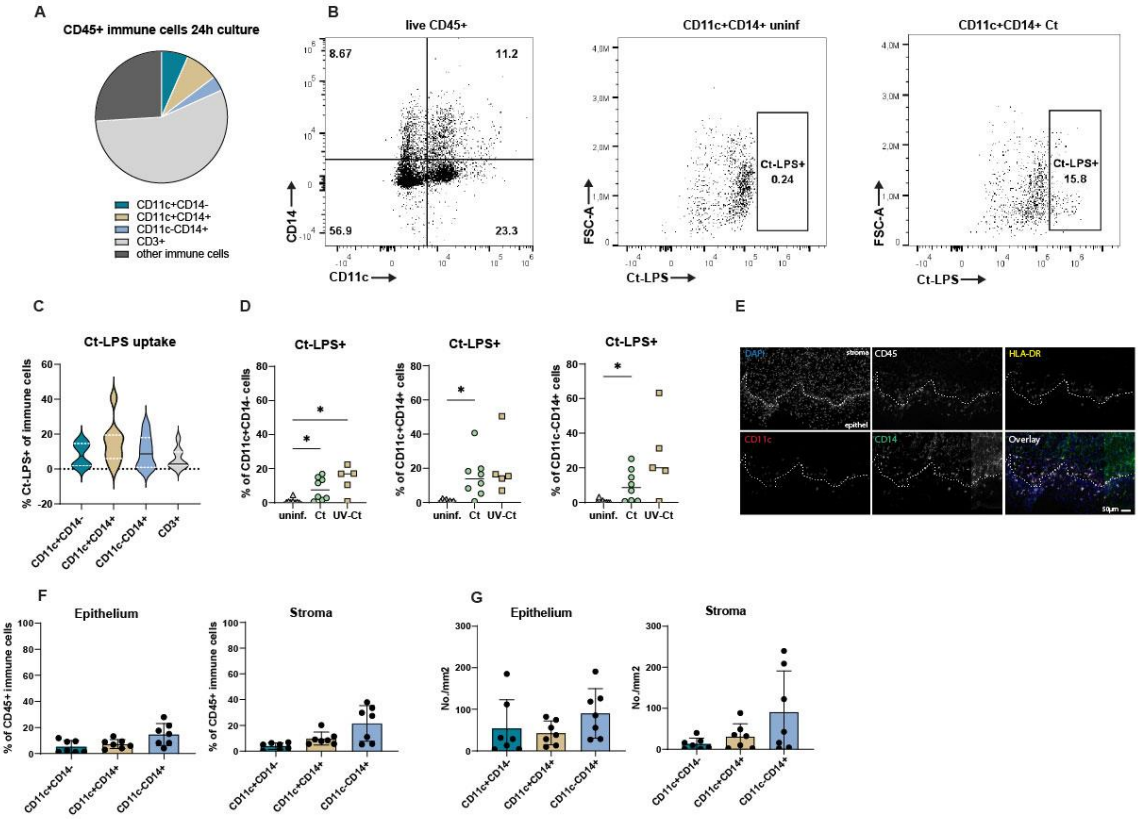


Fig. 2: Human moDCs stimulated with Ct mature and upregulate CCR7.

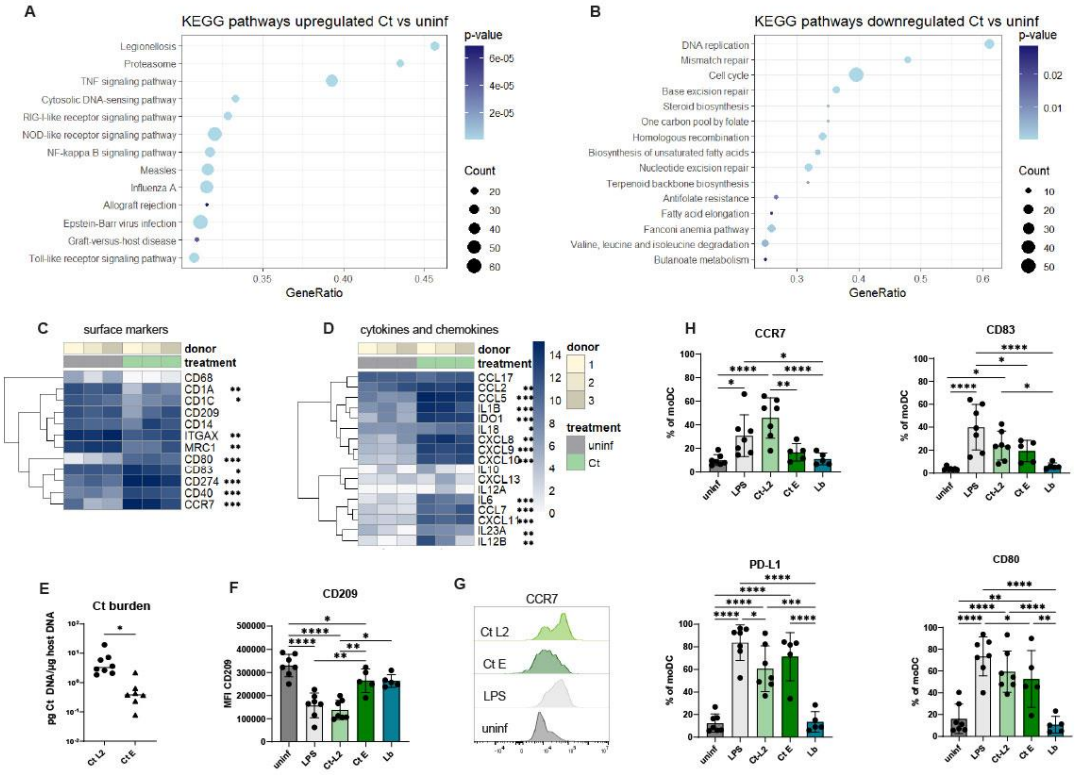


Fig. 3: Ct primed moDCs show a migration defect.

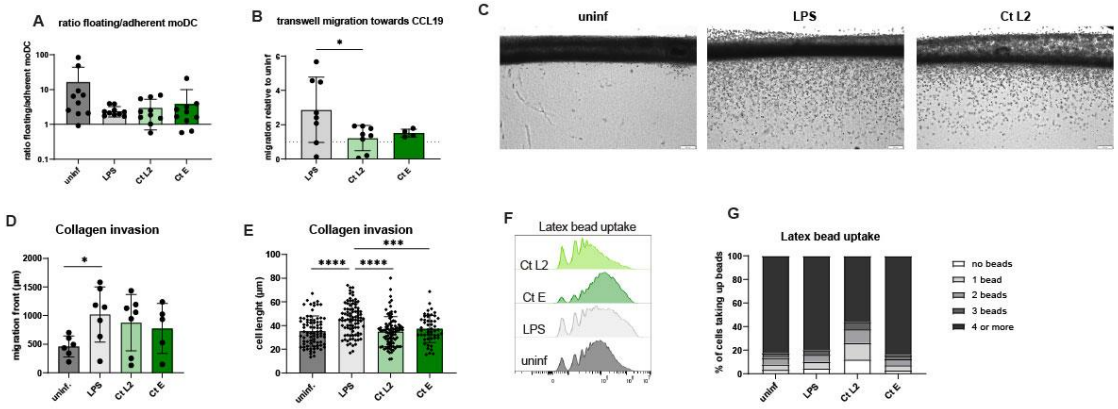
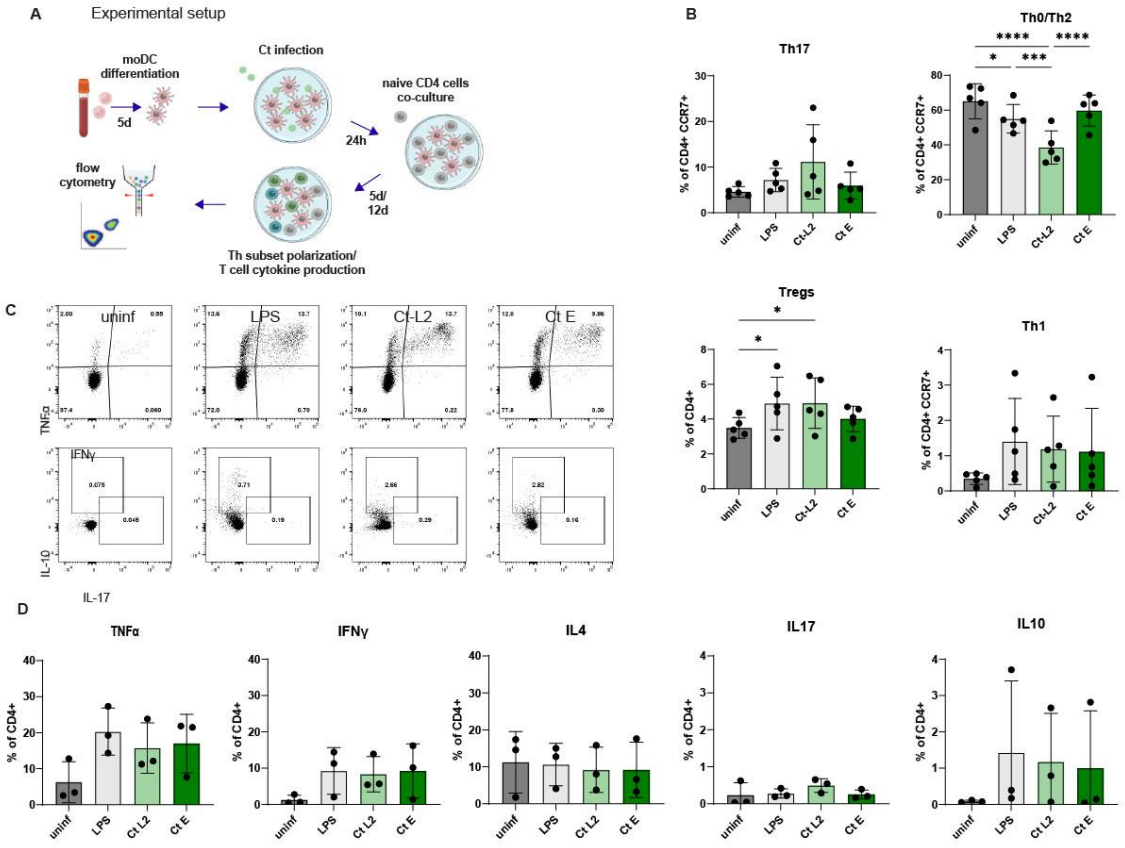
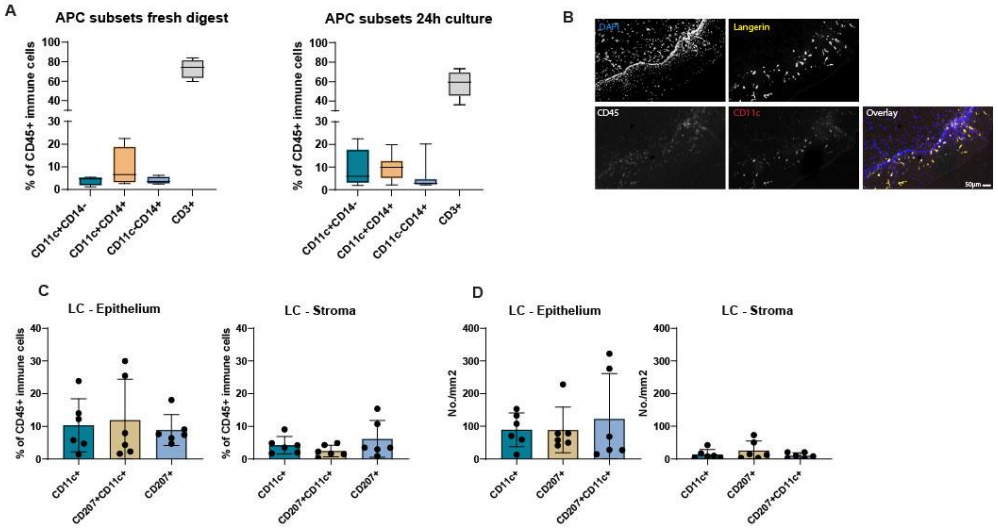


Fig. 4: T cell activation of moDCs stimulated with Ct.

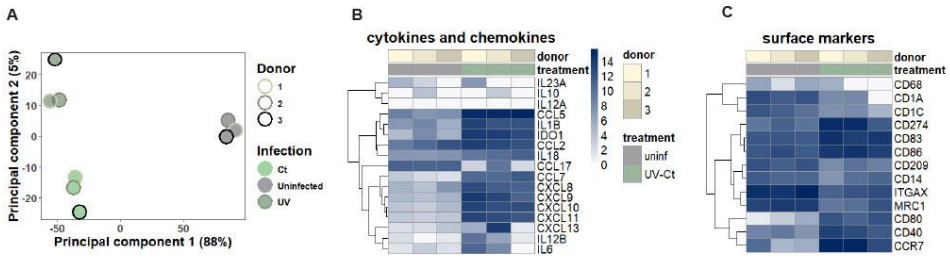


Supplementary Fig. 1: Human cervical DC take up Ct.



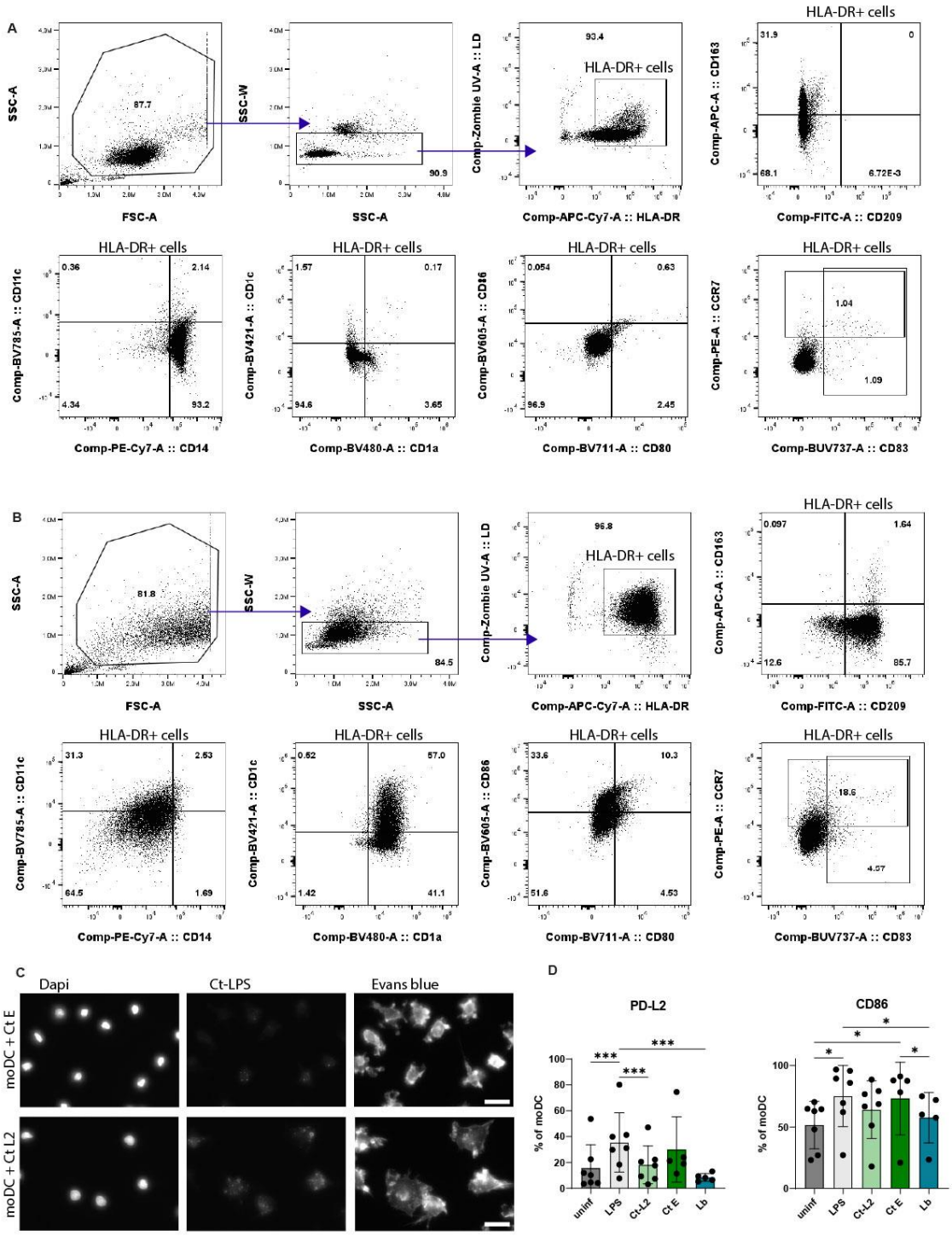
Supplementary Fig. 1: A) Comparison of APCs from fresh cervix digest (n = 4) and APCs after 24h culture (n = 8) gated on CD45+ immune cells. B) Representative images of LC staining. For IF stainings, the following antibodies were used: CD11c (PE, clone S-HCL-3, BD), CD45 (AF750, clone 2D1, R&D), CD207 (unlab., clone DCGM4, Beckman; 2nd anti-mouse IgG-AF647, Invitrogen). C) Quantification of LCs in epithelium and stroma in cervix biopsies within CD45+ immune cells (n=6 donors, mean + SD). D) Quantification of total number of APCs per mm2 (n=6 donors, mean + SD).

Supplementary Fig. 2: Live Ct vs UV-inactivated Ct-stimulated moDC.



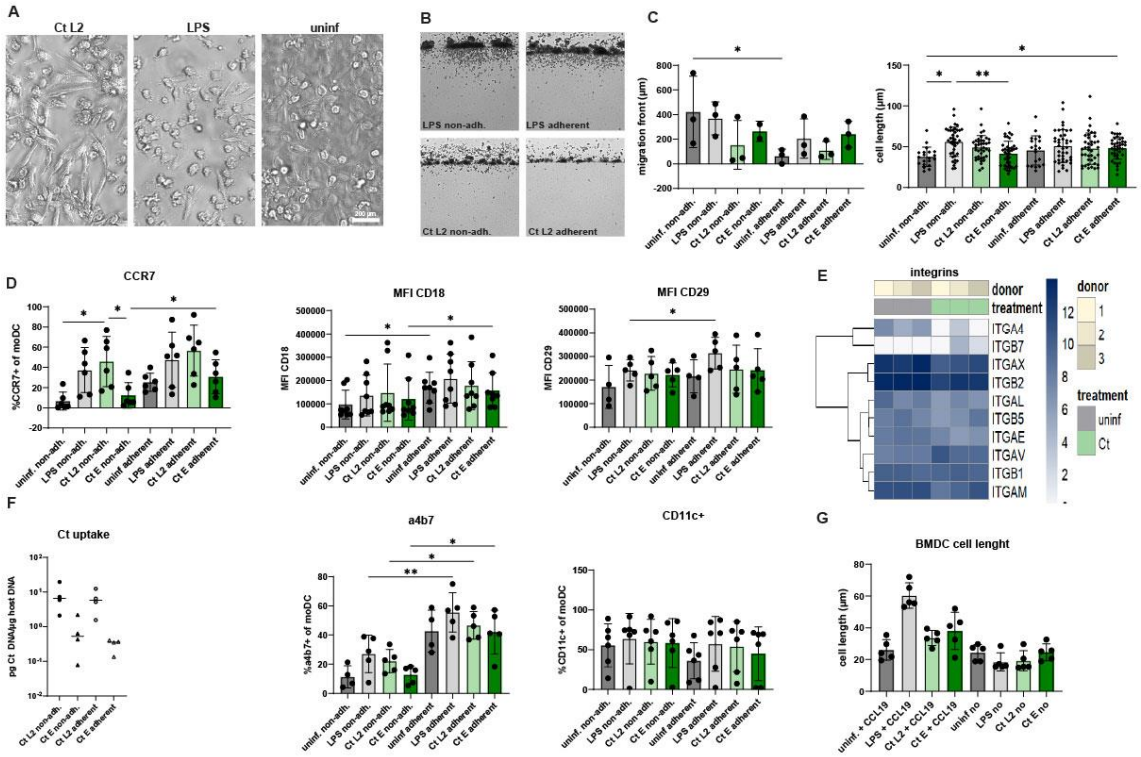
Supplementary Fig. 2: A) PCA plot of bulkRNA seq data from Ct-L2, UV-Ct infected and uninfected moDCs after 24h (n=3). B) Heatmap of cytokines and chemokines derived from bulkRNA seq data from Ct-L2 and UV-Ct infected moDCs after 24h (n=3). C) Heatmap of surface markers derived from bulkRNA seq data (n=3).

Supplementary Fig. 3: Gating of human moDC and surface marker expression after stimulation.



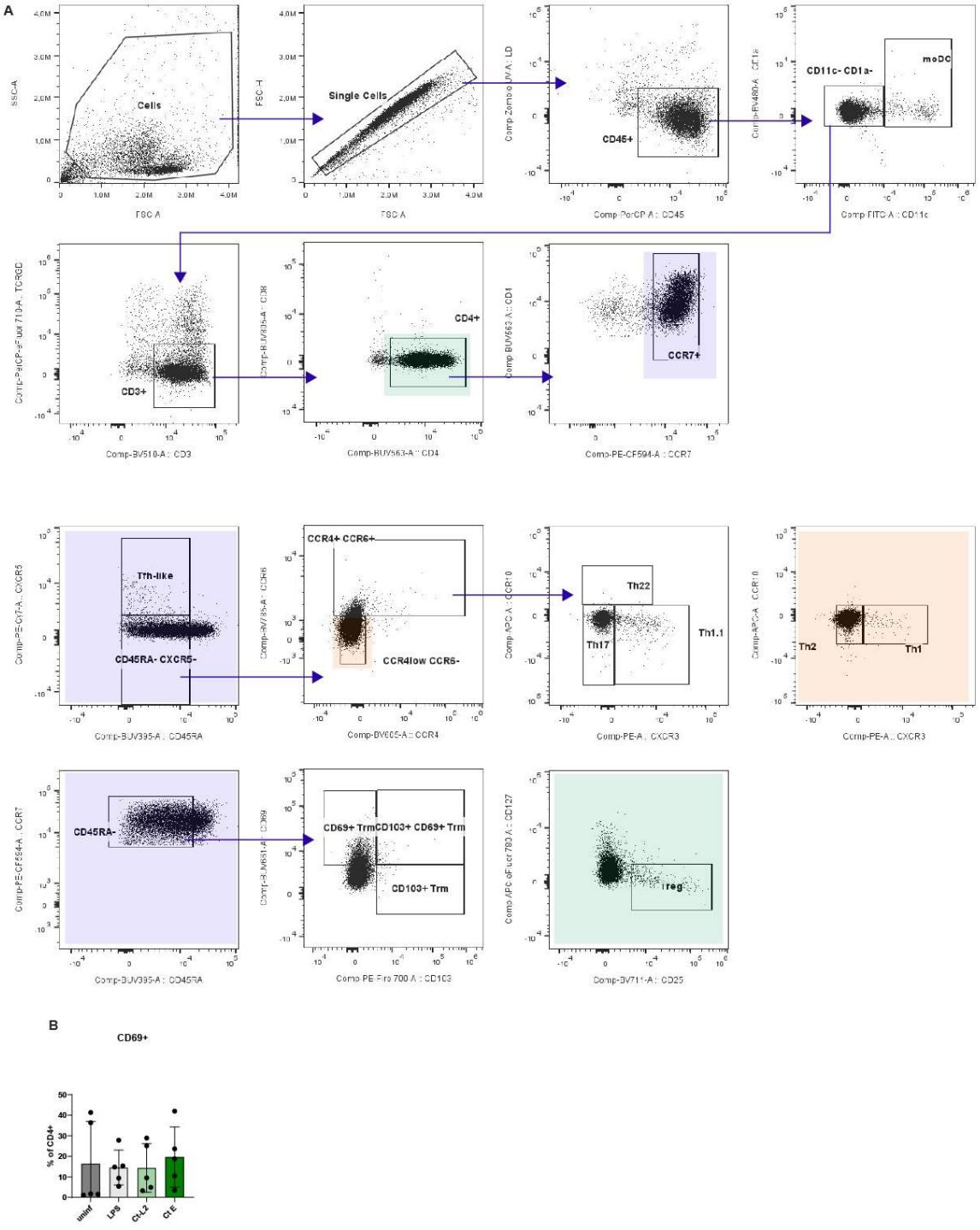
Supplementary Fig. 3: A) Gating strategy of CD14+ monocytes. B) Gating strategy of differentiated immature moDC. C) Ct burden in moDC visualized by IF 24h after infection. Scale bar = 20µm. D) Percentage of PD-L2 and CD86 positive cells of total moDC between individual groups (*p < 0.05, ***p < 0.0005).

Supplementary Fig.4: Characterization of sticky moDC - integrin activation upon different stimuli



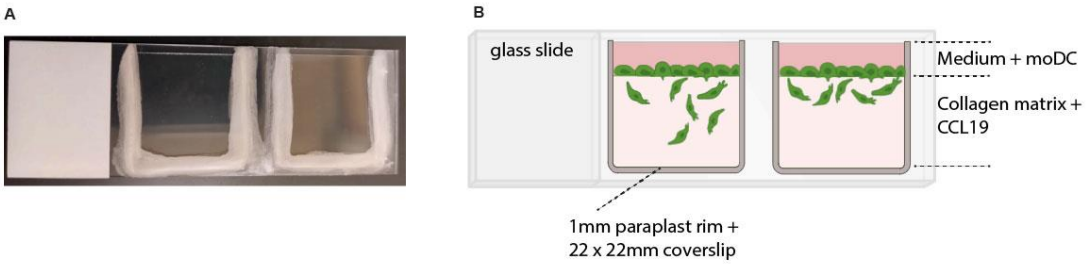
Supplementary Fig. 4: A) Representative images of adherent moDC stimulated with LPS or Ct-L2. B) Representative images of non-adherent (non-adh.) and adherent fraction of moDCs in collagen invasion chambers containing a CCL19 gradient (24h after embedding). C) Quantification of migration front and cell polarization of non-adherent and adherent moDCs in collagen gels towards CCL19 (n = 3 donors). 10 cells were measured per condition for lenght. Significant differences were tested by mixed model analysis and Tukey's multiple comparison test between individual groups (*p < 0.05, **p < 0.005). D) Flow cytometry analysis of non-adherent and adherent moDC 24h after stimulation with Ct-L2, Ct E, LPS or uninfected cells (n= 4-5 donors). Significant differences were tested by mixed model analysis and Tukey's multiple comparison test between individual groups (*p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001). E) Heatmap of integrins derived from bulkRNA seq data from uninfected and Ct-L2 infected moDC after 24h (n=3). F) Quantification of Ct burden by qPCR in non-adherent and adherent moDC. G) Cell polarization of individual BMDC within a collagen gel with or wihtout CCL19 for 24h. BMDCs were derived from C57BL/6j mice, differentiated for 6d with GM-CSF, then stimulated with 200ng/ml LPS, Ct-L2 and Ct-E (MOI10) for 24h before applying them to invasion chambers.

Supplementary Fig. 5: Gating strategy for Th cell polarization upon moDC co-culture.



Supplementary Fig. 5: A) Gating strategy for Th subsets according to surface marker expression. B) Surface staining for CD69 on T cells co-cultured with moDC for 5 days.

Supplementary Fig. 6: Invasion assays setup.



Supplementary Fig. 6: A) Photo of home-made migration chamber. B) Scheme for migration chambers.

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3. Discussion

3.1 General discussion

The objectives and questions addressed in this thesis highlight two complementary strategies to tackle chlamydia infection in the future: first, the identification of novel targets to prevent initial infection; and second, a deeper understanding of immune responses against the pathogen to develop more efficient vaccines. In the following chapter, our findings are contextualized with current advances in STI treatment and prevention, helping to identify priorities and direction for future STI research.

3.1.1 STI prevention with vaccines vs. chemical prophylaxis

While Ct infection is asymptomatic in 70 – 95% of women and around 50% of men, it can still be accompanied by unspecific symptoms like pain, dysuria, and discharge. Most infected individuals clear chlamydia infections spontaneously within 4 years, but the remaining population can experience long-term problems like pelvic inflammatory disease associated with infertility, ectopic pregnancy or serious infections of newborns. Therefore, screening is especially important for pregnant women, but also for people who had risks contacts. For therapy, doxy is recommended as first-line treatment or azithromycin in pregnant women (85,86). Recently, the novel concept of oral doxy-PEP against bacterial STI was tested in clinical trials (87,88). The idea is to take a doxy pill within maximal 72h after unprotected sexual intercourse. Both studies included only men who have sex with men (MSM) or transgender women at high-risk defined by taking HIV pre-exposure prophylaxis (PrEP) or people living with HIV (PLWH). Doxy-PEP effectively reduced the cases of the major bacterial STIs including chlamydia infection, gonorrhea and syphilis. One big concern using frequently prescribed antibiotics like doxy just for preventive measures against STIs is antibiotic resistance. Especially Ng are susceptible to resistance which is reflected by a constant race of the bacteria against antibiotics since their introduction in the 1930s (89). Therefore, antibiotics like sulfonamides, penicillin, tetracyclines and quinolones can no longer be used to treat Ng (90). The current European treatment regimen of gonorrhea involve treatment with high-dose third-generation cephalosporin like ceftriaxone, while azithromycin should not be added anymore due to increased macrolide resistances (91,92). However, the world health organization (WHO) reports already resistance to third-generation cephalosporins in some countries (93). Differences in antimicrobial resistance profiles between countries will also influence the effectiveness of doxy-PEP. While the ANRS IPERGAY trial performed in France did not show a positive effect of doxy on the number of detected gonorrhea cases, the DoxyPEP

trial in the US reported significantly reduced infection with Ng upon doxy intake (87,88). Alternatives to doxy-PEP will be necessary as resistance is likely increasing if doxy is more widely used. While the acquisition of tetracycline resistance still needs to be assessed in follow-up studies, a current study observed an increased expression of tetracycline antimicrobial resistance genes in the gut microbiome of patients after a six-months period of doxy-PEP. However, the diversity of the microbiome and therefore the colonization remains unchanged (94). Both antibiotics doxy and azithromycin, which are usually prescribed as treatment against Chlamydia infection, are generally well tolerated and seem not to impact the composition of commensals like Lactobacilli 6 weeks after end of treatment (95).

The first female doxy-PEP study conducted on cisgender women taking HIV PrEP in Kenya did not show STI protection in the doxy-PEP group compared to the standard-care group (96,97). Low adherence is suggested as an explanation for the failed success, as in half of the women of the treatment group, no doxy was detected in their hair samples. Additionally, relatively high tetracycline resistance of Ng in Kenya could contribute to the lack of efficacy of doxy-PEP (97). Considering the current level of knowledge, use of doxy-PEP is recommended according to STI guidelines by health authorities like the US Centers for Disease Control and Prevention (CDC) or the International Union against STI (IUSTI) Europe only in MSM or transgender women who presented with a bacterial STI in the previous year on a case-to-case basis (98,99). Prevention strategies for women who bear the highest burden from STI complications are still lacking. In comparison to the doxy-PEP studies, where mainly MSM were the target group of the drug interventions, we used female mice and transcervical Ct infection to test local pentamidine efficacy. There are some publications that used rectal infection of mice with *C. muridarum*, the murine strain of chlamydia (100,101). However, this model is not very common in the chlamydia field and has not been tested yet with human Ct strains. We are aware that testing the drug in a mouse model, in which the bacteria are directly injected into the upper genital tract instead of a natural infection starting in the lower genital tract and naturally ascending during the cause of infection is not ideal.

A chemical prophylaxis with pentamidine which is applied topically either before or shortly after exposure is a promising alternative or could complement antibiotics like doxy to prevent Ct infections. By targeting the infection sites directly, this approach could minimize systemic side effects and potentially complement oral antibiotics like doxy. While oral doxy offers the advantage of systemic protection across multiple anatomical sites, such as the urethra, pharynx and rectum (87), topical prophylaxis requires correct and consistent

application to the relevant mucosal surfaces. Accordingly, we are currently developing formulations tailored to the female and male anogenital tract.

For local formulations, sufficient tissue adherence at the respective tissue is a key requirement to provide a film allowing intracellular uptake of the active substance. In designing these, pH compatibility is critical, as local pH varies significantly across body sites. While a typically measured pH value on the skin is around 5 (102), in the rectum the pH is around 7.9 (103) and in the vaginal tract of healthy women of reproductive age it is below 4.5 (7,8). Importantly, changes of the pH balance can be associated with infection. Therefore, it is essential to restore the physiological pH with a prophylactic approach while ensuring optimal pentamidine bioavailability. In mice the vaginal pH is neutral and - in contrast to humans - does not change upon hormonal treatment (104), despite estrous cycle-dependent microbiome changes (105). This highlights species differences that limit direct extrapolation to humans. The predominant commensals in the human vaginal tract are *Lactobacillus* species, which were shown to have a beneficial effect in Ct infection by preventing Ct infection of epithelial cells (106). *In vitro*, we observed that pentamidine temporarily inhibits *Lactobacillus* growth, though this effect is reversible upon washout. This finding is important for considering pentamidine as prophylaxis, as it is essential to preserve the microbiome with a prophylactic strategy. Interestingly, not all *Lactobacillus* species offer the same protective outcome for Ct infection: While *Lactobacillus fermentum* and *Lactobacillus crispatus* are associated with decreased risk for Ct infection, women with *Lactobacillus iners* colonization are more susceptible to infection (107). This opens possibilities for future formulations combining pentamidine with beneficial *Lactobacillus* strains to merge the anti-chlamydial benefits of both elements. Another option to optimize a vaginal drug formulation could be combining it with pH-modulating gels. A clinical trial using the spermicide EVO100, containing L-lactic acid, citric acid, and potassium bitartrate, demonstrated vaginal pH maintenance and a significantly reduced incidence of Ct and Ng infection in women at high risk (108). Pentamidine does not only prevent Ct infection effectively in a mouse model but additionally has antibacterial potential against Ng, a pathogen increasingly difficult to treat due to rising antibiotic resistance as mentioned above. Therefore, synergistic combinations of pentamidine with other agents enhancing STI resistance may further strengthen its potential as a broad-spectrum prophylactic strategy.

No vaccines are currently approved against Ct, Ng or *Treponema pallidum* (28). The reasons for that are manifold: While it was not possible to propagate *Treponema pallidum* at all *in vitro* for a long time (109,110), Ng lacks practical animal models to study infection and effects of protective immunity and there is high variability of gonococcal antigens (90).

Currently, there are several ongoing trials with serogroup B meningococcal vaccines as there are indications for partial cross-protection against the related Ng bacteria (28). In the chlamydia field, the problem so far was not lack of models but rather insufficient induction of immunity or occurrence of adverse events that led to development of tolerance instead of immunity upon immunization with inactivated bacteria (111). While mice immunized with UV-inactivated Ct respond with activation of Treg and increased Ct burden after challenge (62), pigs immunized with UV-inactivated *C. suis* obtained higher numbers of IFN-γ-producing $CD4^+$ T cells and lower chlamydia burden (112). As the human immune system and mechanisms of IFN-γ response are more alike in pigs than in mice, pigs are a valuable complementary model for studying immune response against chlamydia and Ct vaccines but are not so popular due to more complex animal keeping facilities and higher costs (50,112). Another problem with mouse models is that mice clear Ct and *C. muridarum* infection naturally and develop immunity, which cannot be observed in humans. Explanations for low immunogenicity in human Ct infection could be low-grade infection and therefore hiding from DCs or impaired migration as observed in our Ct-infected moDCs which received high Ct doses.

One current promising vaccine candidate against Ct is the recombinant protein CTH522. It is a modified version of MOMP, which induces a strong systemic and mucosal antibody response if adjuvanted with CAF01 liposomes (83). While this phase-I vaccine trial focused mainly on antibody responses, IFN-γ production was also observed upon restimulation of PBMCs of immunized subjects (83). In a follow-up study, the authors could show that the CTH522:CAF01 immunization elicits a strong IFN-γ and IL-17 response in mice and in humans, indicating expansion of Th1 and Th17 cells as well as production of neutralizing antibodies (113). They also report that the immune response is long-lived, an important prerequisite considering teenagers should be vaccinated before their first sexual contacts and remain protected (113). The CTH522:CAF01 seems to not only induce protective immunity against urogenital infection but might also evoke ocular IgA titers against both genital and ocular Chlamydia strains which suggests a benefit of the vaccine also for trachoma prevention (84). Also, Lactobacilli could be used as a promising adjuvant strategy to boost the immune response against mucosal subunit vaccines by increasing antibody titers especially in the vaginal mucosa (114).

3.1.2 DC activation in Ct infection

DCs are the immune sentinels patrolling the body and constantly capture antigens of self and non-self. In case of an infection or vaccination, they are activated, mature and migrate to the lymph nodes to present antigens to lymphocytes like $CD4^+$ and $CD8^+$ T cells (14). In both human and murine infection of the female genital tract, DCs upregulate co-stimulatory markers like CD80, CD86 or CD83 that are essential for T cell activation (62,71). We and others could show that *in vitro* Ct stimulation of human DCs results in production of mainly pro-inflammatory cytokines IL-6, IL-8 and IL-1B (68,70). These cytokines are important for the recruitment of immune cells like neutrophils and T cells during inflammation. Secretion of IL-12 was shown to be essential in murine DCs to prime a Th1 response (62). However, the subunit IL12A forming a heterodimer with IL12B to build IL-12 was not induced in our human moDC infection setting. While also other studies reported IL-12 secretion below standard in human DCs (70), high IL-12 secretion was observed by others, even after stimulation with inactivated Ct (66,68). In mouse models for chlamydia infection, *in vivo* immunization with live and dead bacteria leads to opposed outcomes: Live Ct-L2 induced an IL-12 response by DCs and subsequently effector memory cells, while UV-Ct-L2 responded with IL-10 production and Treg proliferation (62). A similar phenomenon was observed upon *in vitro* infection of mouse bone marrow derived DCs with *C. muridarum*. Even though uptake of bacteria is independent of bacteria viability, DCs produce more IL-12 and TNF-α if stimulated with live Ct (115). Interestingly, the immunosuppressive features of UV-inactivated Chlamydia could be overcome using adjuvants like Cytidine-phosphate-guanosine oligodeoxynucleotides (CpG) (115) or charge-switching synthetic adjuvant particles (62). Yu *et al.* immunized mice nasally with live or dead *C. muridarum* before vaginal challenge. They could show that only live *C. muridarum* immunization resulted in a majority of $CD4^+$ T cells being positive for IFN-γ, TNF-α and IL-2, while dead *C. muridarum* immunization leads to reduced induction of IFN-γ and TNF-α double positive cells. This is indicative of the necessity of Th1 cells for protection against Ct also after nasal immunization (116). Zaharik *et al.* assessed several cytokines and chemokines in murine DCs infected with live or UV-inactivated *C. muridarum* and compared it to LPS stimulation. Live EBs induced much higher levels of IL-6 and IL-12 than both UV-EBs or LPS. However, the neutrophil attractants CXCL1 and CXCL2 were similarly induced with live EBs and LPS stimulation. This led to neutrophil infiltration to infected tissues only in mice infected with live EBs (117). Even though it seems to be essential that Ct is viable as surface expression of Ct proteins is not sufficient to induce DC maturation for a productive immune response, Ct survival is limited and they can only to some extend proliferate within DCs. 6 or 9 days after infection, only a minimal number infectious Ct can be recovered from DCs, reflecting

non-optimal growth conditions for Ct in immune cells (118). Vice-versa DCs also suffer from Ct infection as viability is reduced to 50% after 2 days of infection (118). However, in our moDC model we could still observe viable DCs after co-culture with naïve $CD4^+$ T cells and despite only low expression of IL-12, Th1 cells producing IFN-γ, TNF-α and IL-2 were efficiently induced. Moreover, while moDCs stimulated with live Ct had a clearly distinct RNA expression profile from UV-Ct stimulated ones, the main immune pathways described by others to play a role in Ct immunity were similarly regulated. Cell death of DCs was observed especially if they digested extrusions from infected epithelial cells in comparison to free EBs (69). However, extrusion-stimulated DCs respond with increased expression of IL-12, IL-10 and PD-L1 than DCs stimulated with sonicated Ct (69). Together, these findings suggest that for an optimal start of an adaptive immune response, not only the bacterial dose makes a difference, but also viability and packaging of the bacteria. However, also tissue context seems to play a role as direct infection with live or inactivated Ct did not drastically alter the immune response of DCs as it was described in mouse models.

One of the most striking phenotypes we observed in moDCs stimulated with Ct is the migration defect despite high levels of CCR7. Possible explanations and advantages for the pathogen are intensely discussed in the second manuscript. We hypothesize that due to the hijacking of the actin cytoskeleton by the bacteria, which is essential for their proliferation, DCs cannot perform their normal function anymore. Actin is one of the main players responsible for cell shape and motility. We found changes in adhesion of moDCs once they were activated as they become more adhesive. Furthermore, moDCs had an elongated, spindle-like shape upon LPS-stimulation but not upon contact with Ct. There is a direct link between the cell shape, actin cytoskeleton and CCR7 expression: if immature DCs are confined in 3µm spaces, they upregulate CCR7 in an Arp2/3 dependent manner and increase their migration speed (119). Interestingly, it was reported that in mice lacking CCR7 constitutively, *C. muridarum* can be cleared from the FRT much faster. This could be explained by T cell accumulation in the FRT in tertiary lymphoid structures, so T cells are readily available in the infected tissue without functional lymph node homing (120). CCR7 upregulation is not only beneficial to trigger an adaptive immune response in the lymph nodes but is also responsible for *C. muridarum* dissemination as $CD11c^+$ DCs carry the bacteria to distant organs like the spleen and further to the gastrointestinal tract like the cecum (121). Lucas *et al.* could show that PD-L1 is upregulated in dermal DCs upon innate stimulus with poly(I:C) or LPS. Knock-out of PD-L1 results in reduced migration towards CCL21 despite high CCR7 expression by impacting actin polymerization (122). In our Ct-L2 stimulated moDCs we observed lower PD-L1 induction than in LPS-stimulated cells, which could partially explain the migration defect.

3.1.3 DC – T cell axis in Ct infection

For efficient T cell priming, it is not only essential that APCs like DCs arrive in the lymph nodes to present antigens to T cells but that APCs also express the essential markers to activate the correct T cell subset. In murine lung infection with *C. muridarum*, chemokine receptor CCR2 is upregulated in infected tissue and is essential for the recruitment of myeloid cells. CCR2 KO mice showed differentiation of Th2 cells instead of Th1 (123). The necessity of CCR2 for an efficient IFN-y response was also shown in an HSV-2 infection mouse models, where CCR2 is needed to attract monocytes to infected tissue to restimulate an IFN-y response from Th1 cells (124). CD14 functions as a receptor for LPS also in Ct infection. However, Yang *et al.* report that TLR4/MD-2 dimerization and endocytosis is impaired, possibly due to unique structure of Ct-LPS which is penta-acetylated instead of hexa-acetylated resulting in inefficient inflammasome activation (125). However, a similar set of experiments in THP-1 cells reveal TLR4/CD14/MD2 dependent NF-κB activation only in presence of Ct-L2-LPS but not Ct-E-LPS (126), indicating that there is a difference between serovars but also variation in uptake-mechanisms depending on if intact Ct or Ct-LPS is used for stimulation. KEGG pathway analysis of our bulk RNAseq dataset revealed that NF-κB signaling is one of the top upregulated pathways upon Ct-L2 infection in moDCs. Interestingly, Arya *et al.* showed that LPS stimulation of moDCs increases the total protein synthesis by roughly 50% (127). While endocytosis and cytokine signaling proteins are induced in the first 6h after stimulation, later pathways involved in antigen-presentation were predominant (127). For our experiments, we focused on the 24h timepoint and observed already a downregulation of endocytosis markers (CD209, MRC1) on both RNA and protein level while cytokines and co-stimulatory proteins were upregulated upon *Escherichia coli* LPS or Ct-strains stimulation. We compared it to data from moDCs stimulated with LPS or gram-positive bacteria causing hypersensitivity pneumonitis using a similar protocol, which also resulted in about 10% Th1 cells producing the TNF-α and IFN-γ (128). In our *ex vivo* stimulation model, Th cell subset polarization of naïve $CD4^+$ T cells was similar in LPS or Ct-stimulated wells, only Th17 cells were slightly higher upon Ct-L2 stimulation. Numbers of T cells producing cytokines upon re-stimulation with PMA/Ionomycin for 4h was between 10 – 20% for IFN-γ and TNF-α and below 1% for IL-17. Grandclaudon *et al.* performed a comprehensive analysis of moDCs activated with 82 different stimuli including TLR ligands like LPS, zymosan or poly(I:C) or whole heat-killed pathogens like *Staphylococcus aureus* or influenza virus (129). This allows to make predictions of Th cell subset response in presence of a distinct profile of DC communication markers and signals. CD80 signaling from DCs is linked to production of IL-17F and IL-17A and, surprisingly, IL-9, while signaling of CD86 and CD83 is more related to a Th2 response

including IL-13, IL-31, IL-5 and IL-4 (129). CD86 is similarly expressed on moDCs in all conditions, accompanied by equal priming of Th2 cells and IL-4 production.

As mainly IFN-γ producing $CD4^+$ T cells are indispensable for Ct clearance and anti-Ct immune response, we focused on the priming of $CD4^+$ T cells in our moDC-co-culture model. $CD8^+$ T cells only play a marginal role in protection against Ct infection despite Ct being an intracellular bacterium. The main function of $CD8^+$ T cells in anti-Ct immune response is being another source of IFN-γ supporting Th1 cells (63). It is believed that $CD8^+$ T cells are not efficiently primed in Ct infection due to impaired degradation processes which affect recycling and transport of MHC-I molecules to the surface of Ct infected DCs, thereby limiting cross-presentation to $CD8^+$ T cells (130). Blocking immunoinhibitory proteins like PD-L1 increases the IFN-γ response of $CD8^+$ T cells and improves bacterial clearance but can also become a double-edged sword as several studies describe $CD8^+$ cells being involved in uterine and oviduct pathology (63). A study assessed LC and T cell abundance and activation in the cervix of women infected with HPV, Ct or both STIs. While infection with Ct alone did not alter LC activation and T cell numbers, HPV infection leads to reduced numbers of LCs and CD4/$CD8^+$ T cells but increased Treg frequency. In women affected by both HPV and Ct infection, this pattern was even aggravated, suggesting that having a co-infection with Ct can inhibit HPV clearance and thereby causing persistent infection (131). Together, moDCs seem to be a valid model to assess DC function and T cell priming *in vitro,* and many features observed by others are recapitulated by our data. $CD4^+$ T cells and, more specifically, Th1 cells are vital for an effective immune response against Ct, which we corroborate in our study.

3.2 Conclusion & future prospects

Pentamidine as topical prophylaxis seems to be a promising approach as it efficiently prevents female infection in the mouse model of Ct infection. However, many unanswered questions still remain regarding i) a formulation suitable for application in different areas of the human urogenital tract, ii) an exact dosing and time point of application, iii) regulatory aspects, iv) potential adverse effects and, finally, iv) clinical testing of its effectiveness to prevent Ct infection. We are currently evaluating scientific, technical, regulatory, and commercial aspects of developing a topical prophylaxis with pentamidine in a follow-up project to develop a formulation and treatment regimen that could be suggested for a clinical trial in humans. It is also conceivable to develop pentamidine analogues for STI prophylaxis or use it in a combination with other anti-infective agents or antibiotics. We also already filed a patent application for pentamidine as prophylaxis. The development and low-threshold access of a local STI prophylaxis would be a breakthrough in the prevention of STIs and would allow to finally halt the constantly rising numbers of cases of bacterial STI. In addition, this approach could reduce the numbers of STI diagnostic tests, lower short-term and long-term treatment costs resulting from recurring STI and have major implications on public health as stigmatization accompanied by having a STI, especially for certain minority groups, will be eliminated.

On the other hand, promising advances were made for vaccine development against Ct as the CTH522:CAF01 candidate is the first ever Ct vaccine which successfully completed the phase I clinical trial (84,113). Understanding how immune responses are initiated – and why they fail – in the female genital tract is critical for development of effective vaccines against Ct and other STIs. Gaining deeper knowledge of how the actin cytoskeleton regulates DC function and how Ct infection disrupts this system, could have broader implications beyond Ct, potentially informing therapeutic strategies against other intracellular pathogens, including bacteria and viruses.

4. Material and Methods

Materials and methods are listed and described in detail in the manuscripts inserted in the results section.

The antibodies used for the experiments in the second manuscript are listed in Table 1.

Table 1. Antibodies

Antibody	Fluorophore	Clone	Source	Cat. No
cervical DC - Ct uptake				
Viability dye	eflour506			
CD1c	BV421	L161	Biolegend	331526
CD11c	BV4510	3.9	Biolegend	301628
CD45	BV570	HI30	Biolegend	304033
CD86	BV605	BU63	Biolegend	374213
CCR7	BV711	G043H7	Biolegend	353228
PD-L1	BV785	29E.2A3	Biolegend	329736
Ct-LPS	FITC	B410F	Invitrogen	MA1-7339
CD3	PerCp	SK7	BD	345766
CD19	PerCp-Cy5.5	H1B19	Biolegend	302229
CD11b	PE	D12	BD	333142
CD103	PE-Dazzle	Ber-ACT8	Biolegend	350223
CD14	PE-Cy7	HCD14	Biolegend	325618
CD83	APC	HB15e	Biolegend	305311
EpCAM	AF647	9C4	Biolegend	324212
HLA-DR	APC-Cy7	L243	Biolegend	307618
moDC surface markers				
Viability dye	Zombie UV		Biolegend	423107
PD-L2	BUV615	MIH18	BD	751456
CD11c	BV421	3.9	Biolegend	301628
CD86	BV605	BU63	Biolegend	374213
CD80	BV711	2D10	Biolegend	305236
PD-L1	BV785	29E.2A3	Biolegend	329736
CD209	FITC	9E9A8	Biolegend	330104
CCR7	BV711	G043H7	Biolegend	353228
CD14	PE-Cy7	HCD14	Biolegend	325618
CD83	APC	HB15e	Biolegend	305311
HLA-DR	APC-Cy7	L243	Biolegend	307618
moDC integrins				
Viability dye	Zombie UV		Biolegend	423107
CD103	BUV805	Ber-ACT	BD	748501
CD11c	BV421	3.9	Biolegend	301628
CD11a	BV650	HI111	Biolegend	301239
CD80	BV711	2D10	Biolegend	305236
CD11b	BV785	ICRF44	Biolegend	301346

a4b7	AF488	Hu117	R&D	FAB10078G
CD14	PerCp-Cy5.5	HCD14	Biolegend	325622
CCR7	PE	G043H7	Biolegend	353204
CD29	PE-Dazzle	TS2/16	Biolegend	303031
CD18	Pe-Cy7	CBR LFA-1/2	Biolegend	366309
CD51	APC	NKI-M9	Biolegend	327913
HLA-DR	APC-Cy7	L243	Biolegend	307618

moDC - T cell co-culture

Viability dye	Zombie UV		Biolegend	423107
CD45RA	BUV395	5H9	BD	740315
CD4	BUV563	OKT4	BD	750979
CD69	BUV661	FN50	BD	750213
CD8	BUV805	SK1	BD	612889
CD1a	BV480	HI194	BD	566147
CD3	BV510	SK7	Biolegend	344828
CCR4	BV605	L291H4	Biolegend	359418
CD25	BV711	2A3	BD	563159
CCR6	BV785	G034	Biolegend	353422
CD11c	FITC	Bu15	Biolegend	337214
CD45	PerCp	HI30	Biolegend	304026
TCRgd	PerCp-eFluor 710	B1.1	invitrogen	46-9959-42
CXCR3	PE	1C6	BD	557185
CCR7	PE-CF594	150503	BD	562381
CD103	PE/Fire700	Ber-ACT8	Biolegend	350240
CXCR5	PE-Cy7	J252D4	Biolegend	356924
CCR10	APC	314305	R&D	FAB3478A-100
HLA-DR	APC-R700	G46-6	BD	565127
CD127	APC-eF780	RDR5	Invitrogen	47-1278-42

T cell cytokines

Viability dye	Zombie UV		Biolegend	423107
CD4	BUV563	OKT4	BD	750979
CD69	BUV661	FN50	BD	750213
IL-9	BV421	MH9A3	Biolegend	507707
CD3	BV510	SK7	Biolegend	344828
TNF-a	BV605	Mab11	Biolegend	502936
CD25	BV711	2A3	BD	563159
IFN-y	FITC	25723.11	BD	340449
IL-10	PerCP-Cy5.5	JES3-9D7	Biolegend	501418
IL-2	PE	5344.111	BD	340450
IL-4	PE-Cy7	MP4-25D2	Biolegend	500824
IL-17	APC	eBio64DEC17	eBioscience	17-7179-42

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Appendix

Review: (Not) home alone: Antigen presenting cell – T Cell communication in barrier tissues

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(Not) Home alone: Antigen presenting cell – T Cell communication in barrier tissues

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Priming of T cells by antigen presenting cells (APCs) is essential for T cell fate decisions, enabling T cells to migrate to specific tissues to exert their effector functions. Previously, these interactions were mainly explored using blood-derived cells or animal models. With great advances in single cell RNA-sequencing techniques enabling analysis of tissue-derived cells, it has become clear that subsets of APCs are responsible for priming and modulating heterogeneous T cell effector responses in different tissues. This composition of APCs and T cells in tissues is essential for maintaining homeostasis and is known to be skewed in infection and inflammation, leading to pathological T cell responses. This review highlights the commonalities and differences of T cell priming and subsequent effector function in multiple barrier tissues such as the skin, intestine and female reproductive tract. Further, we provide an overview of how this process is altered during tissue-specific infections which are known to cause chronic inflammation and how this knowledge could be harnessed to modify T cell responses in barrier tissue.

KEYWORDS

skin, T cells, antigen-presenting cells, female reproductive tract, tissue-resident T cells, intestine, barrier tissue

Introduction

T cells are highly specialized executors of immune responses against pathogens and play important roles in maintaining tissue homeostasis. During infection or acute and chronic inflammatory responses, effector T cells (T_{eff}) can infiltrate from the periphery and establish residency and subsequent memory, involving a switch in transcriptional program using different transcription factors and signaling hubs (1–6). This explains why the majority of the T cell population found in tissues are memory T cells

(7), subdivided into central memory T (T_{cm}), effector memory T (T_{em}), and resident memory T (T_{rm}) cells. T_{em} and T_{cm} were first identified in the peripheral blood (8). T_{em} were found to be the predominant subset in non-lymphoid tissue while their T_{cm} counterparts are mainly found in secondary lymphoid organs (9–17). Later, a long-lived memory population with little to no recirculatory capacity was identified and termed T_{rm} (12–18). Another prevalent T cell subset in tissues are regulatory T cells (T_{regs}), particularly important for maintaining a tolerogenic tissue environment, preventing excessive immune responses to harmless antigens often found at barrier tissues [reviewed in (19, 20)]. T_{regs} usually refer to $CD4^+$ T cells with the unique ability to suppress pro-inflammatory effector functions in other T cells as well as contribute to tissue homeostasis (21, 22). Tissue T_{regs} can also be subdivided by the central and effector memory cell classification based on the expression of CD44 and CD62L (23–25), with central T_{regs} being able to recirculate through secondary lymphoid tissues, while effector T_{regs} exhibit a more resident phenotype, representing the predominant T_{reg} population in nonlymphoid tissues (23). Non-conventional T cells can also be found in barrier tissues. An example of this are γδT cells, which are mainly found in epithelial tissues and are particularly abundant in the intestine (26). In homeostatic conditions, γδT cells have been described to exhibit a pre-activated memory phenotype (27), being able to exert direct cytotoxic functions (28, 29). As for other T cell subsets in tissues, roles in wound healing and tissue homeostasis have also been attributed to γδT cells (30, 31). A broad overview of T cell subsets found in tissues and surface markers most commonly associated with each subset is depicted in Figure 1. It should be noted that these markers are not absolute and their expression is often changed in different tissues. However, these figures aim to give a broad overview over the most common and widely distributed markers of each subset and highlight commonalities and differences between mice and humans.

Priming by antigen presenting cells (APCs) is crucial for T cells to exert their correct functions and home to tissues. For example, the presence and function of T_{regs} in tissue has been directly linked to the presence of dendritic cells (DCs) (32). Tissue-patrolling DCs are of an immature phenotype and internalize antigens by endocytosis or phagocytosis, which are

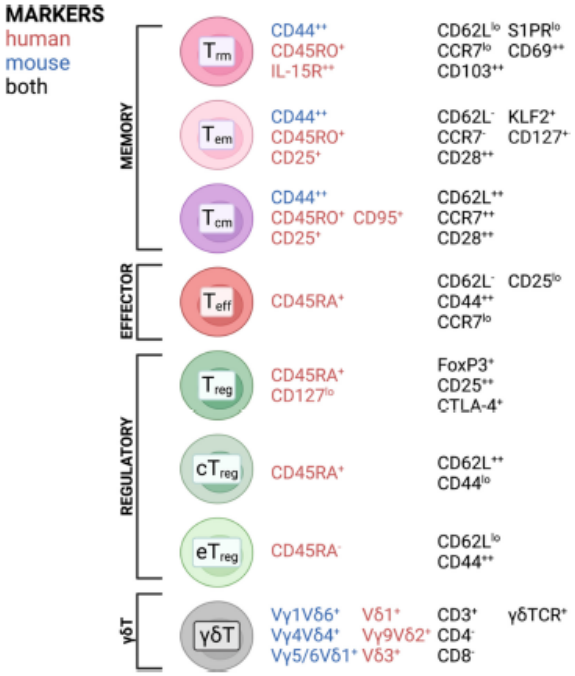

FIGURE 1
T cell subsets and commonly associated markers in mice and humans found in barrier tissues discussed in this review. Created with BioRender.com.

loaded to major histocompatibility complex class II (MHC-II) for CD4 T cell presentation *via* endosomal pathways (33). However, DCs are also efficient in cross-presenting extracellular antigens *via* MHC-I to CD8 T cells, by which exact mechanism is still under debate (33, 34). Apart from antigen uptake, DCs need to receive additional stimuli in order to mature and upregulate CCR7, by which they interact with the ligands CCL19 and CCL21 guiding them to the lymph nodes to meet naïve T cells (35, 36). Under homeostatic conditions, DCs mainly collect non-hazardous antigens from food or commensal bacteria in the intestinal tract and skin or paternal antigens of fetal cells within the female genital tract during pregnancy (37–39). On the other hand, DCs are highly sensitive against pathogen-associated molecular patterns (PAMPs), which they detect *via* their toll-like receptors or C-type lectin receptors and they sense cytokines produced by other cell types during infection (33, 40). Mature DCs upregulate molecules necessary for co-stimulation of T cells like CD86 and CD80 (41).

Classically, DCs are divided into several subclasses: conventional DCs (cDCs), monocyte-derived DCs (mo-DCs) and plasmacytoid DCs (pDCs) (42). Langerhans cells were previously also classified as DC population; however, they developmentally originate from yolk sac progenitors, which identifies them as member of the tissue-resident macrophage family. In contrast to macrophages, they can efficiently present antigens and possess a migration potential to the lymph node (43). Therefore, they are often mentioned along with other DC subsets inducing T cell responses. Conventional DCs are subdivided into type 1 classical DC (cDC1), which are known to cross-present antigens *via* MHC-I to CD8 T cells but also polarize CD4 T cells towards T_h1, while type 2 classical DCs (cDC2) mainly present antigens *via* MHC-II to CD4 T cells. The cDC1 subset in mice is $CD11b^{lo}$ and shows CD8a and CD103 on their surface, while human cDC1 express and XCR1 and CD141 (33). cDC2 express CD172a and depending on murine or human origin they highly express CD11b or CD1c, respectively (33). Especially cDC2 comprises a very heterogenous immune cell population which can acquire quite contrary immune functions depending on the context. For examples, in human there exists a cDC2 subset which expresses at the same time monocyte-related genes like CD163 and CD14, which was termed DC3 (44, 45). LCs are a population patrolling the epidermis as well as the epithelial layer of the vagina and cervix and are characterized by expression of a specific lectin receptor, langerin (CD207) and CD1a (46). Monocytes express CD14 and can be differentiated *in vitro* to monocyte-derived DCs (mo-DCs) by addition of GM-CSF and IL-4 and are a widely used model for priming T cells *in vitro* (47). However, the existence of mo-DCs *in vivo* remains under debate, but several mouse (48, 49) and human (50) studies observed that monocytes can differentiate into DC-like cells, especially under inflammatory conditions (45, 51). With the evolving of single-cell sequencing technology, more and more DC subsets are discovered and it now appears that the discrimination between DC and monocyte subsets is not that black and white, with mo-DCs in comparison to DC3 being just one example (44, 45, 52, 53). APC subset composition varies widely throughout tissues and we are still far from understanding which subset contributes to immunity and tolerance under certain conditions (54–57). DCs are in general $CD45^+$ cells, expressing HLA-DR and lacking other linage markers, such as CD3 or CD19 (52). In Figure 2, a simplified overview of the most important DC subsets in human can be found with the markers for those respective subsets in mice included.

In this review, we discuss the different subsets of T cells and APCs present in the skin, intestine and female reproductive tract (FRT) and how their interplay contributes to maintaining a homeostatic tissue environment as well as how this composition shifts during chronic inflammatory diseases and infection. While the term "immune homeostasis" is widely used, we refer to "homeostasis" as the balance between immune activation and suppression in tissues and organs in contribution to maintaining a healthy state of an organism under normal physiological conditions. This review aims to focus on the human system wherever possible; however, some insightful mechanistic studies in different animal models are included as these contribute greatly to our understanding of tissue immunity where human studies are not yet possible. To give a more comprehensive view of already described mechanistic studies not yet discovered in humans we also included animal studies when appropriate. Therefore, unless otherwise stated, findings summarized were done in humans and deviation to animal models is indicated.

Skin

The skin is one of the largest organs in the human body and essential for protection against external injury and pathogens. Next to its role in physical protection, the skin also houses a vast landscape of resident and recirculating immune cells which are poised locally to respond to tissue damage and infection. The skin is comprised of three layers: the outermost epidermis, an intermediate layer termed dermis, and the innermost layer called hypodermis (Figure 3). The epidermis is mainly comprised by structural cells such as keratinocytes, as well as melanocytes. The main immune cells found in this layer are $CD8^+$ T cells and LCs, skin-resident macrophages which originate from the fetal liver and the yolk sac, and exhibit DC-like characteristics (58). Next to structural fibroblasts, the dermis contains the majority of immune cells, including DCs, macrophages, natural killer (NK) cells, innate lymphoid cells (ILCs), as well as $CD4^+$ and $CD8^+$ T cells. Further, this layer is also supplied with lymphatic and blood vessels which allow immune cell trafficking in and out of the tissue. The lowest layer, the hypodermis, is mainly comprised of adipocytes responsible for thermoregulation (59, 60).

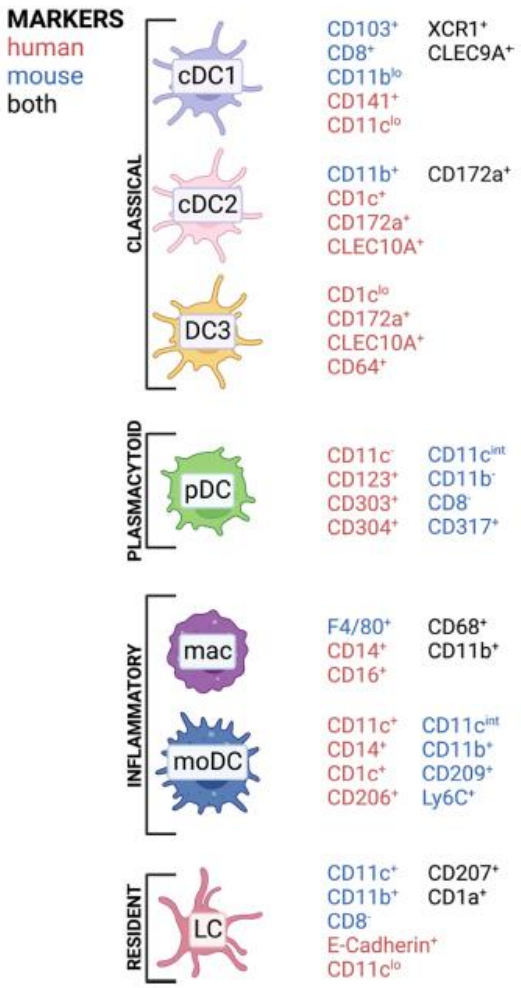

FIGURE 2
APC subsets and commonly associated markers in mice and humans found in barrier tissues discussed in this review. Created with BioRender.com.

However, recently an immunological role has been attributed to adipose tissue as it has been shown to house multiple types of immune cells (61–66). Additionally, structures such as hair follicles and nerve endings are major players in regulating immune responses in the skin. Hair follicles represent unique structures in the skin, as many studies in mice have shown that they are the primary site for T_{reg} maintenance, which are in turn essential for establishing the stem cell niche at the hair follicle (67–69). In human skin, the hair follicle is also the major site of T_{reg} localization (70). Further, the hair follicle is also of importance for DC function in the skin of mice (68, 71). Besides the hair follicle, nerve endings in the skin have been

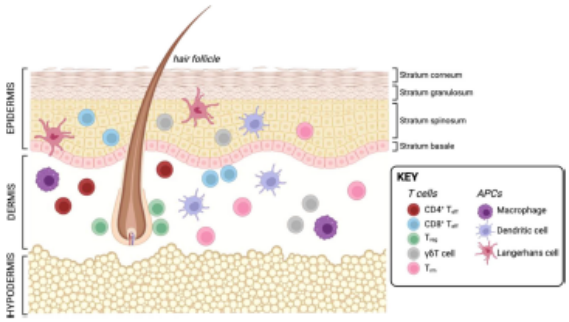

FIGURE 3
Resident T cells and APCs in the human skin. Created with BioRender.com.

shown to play an important role for $CD8^+$ T cell mediated immunity (72) as well as create a special environment for specific macrophage subsets (73) as demonstrated in mouse models.

Upon encountering pathogens or injury to the epidermal layer, LCs are the first to initiate an immune response. These cells constitute approximately 2-4% of all cells in the epidermis and are specialized macrophages with DC characteristics, expressing the surface markers CD1a and Langerin/CD207 (46), whose dendrites can extend through the stratum corneum to sample antigen without disturbing the epithelial barrier (74, 75). LCs preferentially recognize mannosylated ligands on surfaces of pathogens *via* C-type lectins and pattern recognition receptors (PRRs) (76). Binding of these receptors leads to receptor-mediated endocytosis thereby activating the LC (77). Like their conventional DC counterpart, LCs have been found to be able to traffic to the skin-draining lymph nodes (LNs) and activate naïve T cells (78–80) as well as activate skin-resident T_{regs} (81). LCs have also been described to be highly efficient at inducing a neutralizing IgG response against *S. aureus* from B cells (82). While LCs have their primary role in immune surveillance of the skin, macrophages are mainly responsible for initiating inflammatory responses in response to infection or injury as well as to tissue regeneration (83–85).

Apart from the acute role of innate immune cells in clearing infection, APCs also play a major role in activating an adaptive immune response. As in other tissues, dermal APCs expressing CD1c (86, 87) can be divided into multiple subsets. In healthy human skin, the main subsets at steady-state are $CD1a^{++}CD207^{+}$ $^{+}$ LCs, $CD1a^{+}CD1c^{+}$ DCs, $CD141^{++}CD14^{-}$ DCs, as well as two populations of macrophages that can be, in part, distinguished by their autofluorescence (AF) created by their high scatter properties: $CD14^{+}AF^{-}$ monocyte-derived macrophages (mo-Mac), and $FXIIIA^{+}CD14^{+}AF^{++}$ macrophages (88). Upon antigen encounter in the skin, dermal DCs (DDCs) become migratory and act as APCs in the lymph node where they activate and polarize different adaptive immune cells, such as naïve T cells (88, 89). It was shown in mice that the constant travel of skin APCs to the LN during homeostasis is only dependent on the CCR7 ligand CCL21, whereas CCL19 presence is dispensable for the trafficking (90, 91). However, CCL19 deficient mice exhibit lower T cell numbers due to decreased cell survival (91). However, DCs in the skin have also been shown to locally activate memory T cells within the skin, bypassing the need for tissue egress (81) and thereby enabling a rapid adaptive immune response locally.

Specifically, T cells play a major role in the cutaneous immune system, with a large tissue-resident population being found throughout the dermis and epidermis. In healthy skin, this population can comprise up to $2x10^{10}$ cells, which is nearly two times as many as found in circulation (17). Differences in T cell composition between murine and human skin have made studies using mouse models difficult. In mice, the majority of resident T cells are γδT cells with a limited T cell receptor (TCR) repertoire (92), while in human skin most resident T cells are αβT cells with a much greater TCR diversity (17). Overall, T cells in the epidermis are less proliferative but have increased capacity to produce cytokines such as IFN-γ and TNF-α (93). While αβT cells rely on antigen presentation *via* MHC molecules, γδT cells have a restricted TCR repertoire, with their receptors recognizing unconventional antigens such as phosphoantigens, stress molecules, as well as non-peptide metabolites (94–96). Human γδT cells express the Vδ1, Vδ2, and Vδ3 chains, with each subtype having a preferential distribution across the body (97). A murine-specific γδT cell subset, called dendritic epidermal T cells (DETCs), have also been shown to significantly contribute to immune homeostasis in mouse skin (98), but don't have a human counterpart. How different T cells subsets contribute to maintaining homeostasis

and how this paradigm is shifted during the inflammatory response and infection will be discussed below.

DC-T cell composition in homeostasis

Memory T cells

While the T cell subsets above mainly describe different effector states of activated T cells, a central part of T cell function is the capacity to develop long-lived immunological memory. T_{eff} cells primed in the lymph nodes by an APC are maintained in the skin as memory T cells, whose survival is supported by keratinocytes producing growth factors as well as other tissue resident (immune) cells (99, 100). These resident memory cells are crucial for maintaining tissue homeostasis as they contribute to immune surveillance and supply a rapid, specific response when re-encountering pathogens. As with all other immune cell subsets, memory T cells in the skin can be divided into two major groups: resident and recirculating. Using a human skin xenograft model with nude NSG mice, four distinct memory populations in the skin have been identified using the resident vs. recirculating paradigm. In human and mouse skin, the primarily resident subsets are T_{em} and T_{rm}. Recirculating subsets can further be subdivided into migratory memory (T_{mm}) and T_{cm} (8, 93, 101). Cutaneous lymphocyte antigen (CLA) is a marker that specifically distinguishes memory T cells originating from the cutaneous immune system as well as skin-infiltrating T cells. CLA binds to chemokine receptors, E-selectin which together with Very late antigen 1 (VLA-1)/Vascular cell adhesion protein 1 (VCAM-1) and Lymphocyte function-associated antigen 1 (LFA-1)/Intercellular adhesion molecule 1 (ICAM-1) enables skin tropism of these cells (102–105).

T_{em} are thought to be the first responders, expressing high levels of CD44 but lacking migratory and homing receptors such as L-selectin and CCR7 (8, 106, 107), making them incapable of recirculating. As their name suggests, they provide immediate effector function, which is underscored by their production of IFN-γ as well as other pro-inflammatory cytokines (93). While T_{em} are crucial for immediate adaptive responses, this population undergoes significant contraction after an infection is resolved and their niche has been found to be replaced by T_{cm} which enter from the circulation over the course of an acute inflammatory response (13). T_{cm} express high levels of homing receptors that are lacking on T_{em} (CCR7, LCA, CCR4) (17, 108, 109). Contrary to T_{em}, their reactivation primarily takes place in the local LNs. There, they undergo extensive proliferation and adopt a T_{cm}-like phenotype (8, 110). The other circulating subset, T_{mm}, was described by Rei et al. (93) and shows a population of cells high in skin-homing receptors such as CLA and CCR7 but are defined by the absence of L-selectin. This lack of L-selectin has raised suspicion that these cells are able to remain in the skin after infection, where they contribute to immune homeostasis as these cells are not high producers of pro-inflammatory cytokines (93). Another, more recently discovered, family member are T_{rm} which express high levels of the integrin CD103 as well as the glycoprotein CD69. While their overall phenotype is similar to that of T_{em}, they have been shown to be maintained long-term even after an infection, as well as being significantly more potent in their effector response while also being limited in their proliferative capacity (13, 111). An essential tool in understanding the migratory behavior of T_{rm} is two-photon intravital microscopy. Multiple studies in mice have revealed that, in the skin, these cells are relatively stationary and confined in and close to the epidermis where they surveil their environment and are responsible for regulating secondary recall responses after primary challenge (112–114). Together, these memory subsets contribute to long-lasting immune memory and surveillance in the skin.

Effector T cells

While T cells in the skin at steady-state are mostly memory T cells, effector T cells (T_{eff}) can also be found. These are activated by APCs in the skin-draining lymph nodes and traffic to the skin, where they further encounter cutaneous APCs presenting their cognate antigen, which leads to T cell activation and production of effector cytokines (115, 116). Most studies on T_{eff} cells have described essential roles for $CD8^+$ T_{eff} cells in maintaining tissue homeostasis in the skin. $CD8^+$ T cells can be found in both the dermis as well as the epidermis. $CD8^+$ T cells have been shown to have increased migratory capacity within different skin compartments, albeit with slower kinetics than migration in the lymph node (117). In a sophisticated *ex vivo* imaging system of whole skin to observe T cell migration, Dijkgraaf et al., could demonstrate that human $CD8^+$ skin-resident T_{rm} in the epidermis migrate along the stratum basale, close to the basement membrane and preferentially localize below aggregations of stationary LCs. In contrast, $CD8^+$ T cells in the papillary dermis were observed to accumulate in collagen I rich regions as well as collagen I-poor dermal vessels. These observed migration dynamics highlight an important function of $CD8^+$ skin-resident T cells in tissue patrol, possibly enabling immediate cytotoxic response to antigen presentation by co-localized LCs at the epidermal-dermal junction (118). While $CD8^+$ T cell co-localization with LCs at the epidermis-dermis interface may hint at increased priming capacity by local epidermis-patrolling APCs, observed changes in morphology of $CD8^+$ T_{rm} to a more dendrite-like shape (7, 117, 119, 120) could also suggest that these memory cells can act, at least in part, independently of APCs when confronted with their respective antigen, which has been described to be the case in mice (121–123). However, it is known that specialized $CD141^+CD103^+$ DCs are especially effective at cross-presentation for $CD8^+$ T cells in the skin (124, 125).

Regulatory T cells

Similar to other immune cell populations, T_{regs} can reside in non-lymphoid tissue (NLT) such as the skin. Specific residency transcriptional programs in these organs have been described, mediating T_{reg} adaptability to different tissues in mice (126). In human skin, T_{regs} represent between 5% and 20% of all resident T cells under homeostatic conditions (127, 128), where they are known to interact with LCs and fibroblasts (81, 127). Most circulating T_{regs} found in peripheral blood express skin-homing markers which indicates that these cells are constitutively recruited to the skin over other organs (129). Similar to their effector memory counterparts, T_{regs} from the skin are also able to elicit a memory response and have been shown to persist in the skin and induce tolerance to autoantigens in a mouse model (130). In human skin, the function of skin-resident T_{regs} remains elusive, with few studies investigating their function under homeostatic conditions. Other than the canonical transcription factor FoxP3, skin T_{regs} express CLA, as well as the chemokine receptors CCR6, high levels of CCR4, a skin homing marker, high levels of L-selectin and HLA-DR. Similar to their blood counterparts, they express GITR and high levels of intracellular CTLA-4. Contrary to other skin-resident T_{eff} cells, skin T_{regs} tend to express much lower CD103 (127). Seneschal et al. demonstrated that the function of skin-resident T_{regs} is highly dependent upon the context under which they are activated by local LCs. Under steady-state, LCs appear to preferentially activate and expand $CD4^+CD25^+FoxP3^+CD127^-$ T_{regs}, which were functionally competent in suppressing autologous skin resident T_{em} cells. Further, it was suggested that this effect is MHC-restricted, showing that under steady-state conditions, LCs act in concert with T_{regs} to induce and maintain tissue homeostasis (81). While reports of antigen-specific responses by T_{regs} do exist, it is well-established that skin T_{regs} have a high proliferative capacity in response to non-antigen dependent stimuli, such as contact with dermal fibroblasts in combination with IL-15 (127). Other than their immediate immunological function, cutaneous T_{regs} are known to be involved in wound healing (131, 132), where their primary role lies in inhibiting IFN-γ production by other T cells and inflammatory macrophages (132), as well as and modulating hair follicle stem cells (133).

γδT cells

In human skin, 1-10% of all resident T cells are estimated to be γδT cells (134), with the majority expressing the Vδ1 TCR (135, 136). One known ligand for this TCR is CD1d which is able to present lipid antigens on DCs (137). CCR6 on γδT cells is thought to be an important receptor mediating recruitment of activated γδT cells *via* CCL20 expression by keratinocytes, DCs as well as endothelial cells (138). CCL20 secretion by keratinocytes is especially upregulated during acute injury, suggesting an important role for γδT cells in response to injury (139). Cytokines important in αβT cell maintenance in the skin have also been found to play key roles for γδT cell maintenance and development in this organ. IL-7R signaling, for example, has been shown to induce rearrangement and transcription of the TCR γ-chain, and IL-15 is also involved in the expansion of γδ epidermal T cell precursors as well as their survival, while IL-4 signaling has been shown to promote growth of the epidermal γδT cell compartment (140–142). The skin residency marker CD103 has also been implicated to play a role in establishing γδT cells in the murine epidermis, with CD103-deficient mice showing significant reduction in γδT cell numbers in the skin as well as abrogated morphology in the γδT cells present (143). Further, murine $CD103^-$ DETCs share a competitive niche in the epidermis with $CD103^+$ T_{rm}, indicating that CD103 is an important determinant in establishing tissue residency in the murine epidermis (113). If CD103 expression by γδT cells is also vital in human skin remains to be uncovered. Co-stimulation for γδT cells is less understood than for their αβ counterparts. However, in mice CD27 has been shown to contribute to the function of Vγ2Vδ2 T cell activation and promote IFN-γ production by these cells (144). Further CD2 and ICAM-1 have been suggested as costimulatory molecules for Vδ1 T cells (145–147). Specific functions of γδT cells in human skin are known to include regulation of keratinocyte proliferation and homeostasis through the production of insulin-growth factor 1 (IGF-1) and other keratinocyte growth factors (98, 148). Further, γδT cells are also able to contribute to skin homeostasis by recognizing damaged cells and exhibit cytotoxic activity *via* the NKG2D receptor (149), as well as perforin secretion and Fas-mediated cell lysis (150).

DC-T cell composition in infection and inflammation

Chronic inflammatory diseases

A skewed composition in terms of T cell numbers and function of skin-resident T cells has been described in a plethora of chronic inflammatory skin diseases. Accordingly, the populations of APCs in inflamed skin also shift, with the dominant subsets being $FcER1^+CD1a^{lo}$ (inflammatory dendritic epidermal) DCs, $CD1c^+CD14^{+/-}$ DC (inflammatory), $TNF\text{-}\alpha^+INOS^+CD14^-CD11c^+CD1c^-$ (TNF-α and iNOS producing) DCs, and $CD123^+$ pDCs depending on the nature of the disease (88). Further, in a mouse model of skin inflammation, Chow et al. demonstrated that specifically usually resting T_{regs} become highly motile during both adaptive and innate inflammation, highlighting the importance of these cells to control local inflammation (151).

One prominent example of such a disease is psoriasis, which affects 2-3% of the population (152). Skin lesions in psoriasis are thought to be caused by dysregulated cross-talk between APCs and T cells, which leads to an increased production of pro-inflammatory cytokines such as IL-17, IL-12, IFN-γ, TNF-α, and IL-23 (153, 154). This creates a positive feedback loop by recruiting more lymphocytes, neutrophils and myeloid cells to the lesion ultimately causing chronic cutaneous inflammation and epidermal hyperplasia (155). Blocking of TNF-α significantly reduced expression of the DC migration marker CCR7 and its ligand CCL19, thereby supporting clinical remission of patients (156). Dermal $CD3^+$ T cells in these skin lesions are often increased by up to 15%. The composition of αβ and γδT cells in psoriasis also shifts, with some studies observing more than 40% of $CD3^+$ T cells also expressing γδ TCRs and secreting the pathogenic cytokines IL-17 and IL-23 (157). Other studies have observed CLA^+ Vγ2Vδ2 T cells homing to the skin to be increased in patients with psoriasis (158). Further, LCs have been described to preferentially utilize the MAPK-p38α signaling pathway, which has been linked to psoriasis susceptibility in humans (159). This has been shown to specifically promote production of IL-17 in $CD4^+$ T cells by promoting the expression of IL-23 and IL-6, both of which are essential for T_h17 differentiation and known to drive psoriasis pathogenesis (160). Additionally, LCs are able to induce a peripheral T cell response by priming immature $CD4^+$ T cells in the lymph node to produce IL-22 which then acts on epithelial cells, further promoting tissue inflammation *via* alarmins such as the antimicrobial peptide HBD3 (161).

While many chronic inflammatory diseases are of unknown etiology, some have been correlated to dysbiosis of the skin microbiota. An example of this is atopic dermatitis (AD), a chronic T_h2-dominated disease characterized by eczematous lesion and severe pruritus caused by immune cell infiltration of inflammatory DCs, macrophages and eosinophils (162). Further, AD is often found to be associated with transepidermal water loss due to a mutation in the filaggrin gene which leads to enhanced susceptibility to overgrowth of pathogenic *S. aureus* (163, 164). Further, patients with acute flares of the disease have been found to have an acute expansion of the cutaneous *S. aureus* population and significant loss of diversity in the cutaneous microbiome. Conversely, resolution of lesions has been association with a more diverse microbiome composition and contraction of the *S. aureus* population (165). Chronic inflammatory skin disorders still represent a major subset of disease with little mechanistic understanding of how T cell responses are shifted to cause disease.

Infection

It is becoming clear that the capacity of LCs in activating T cells in human skin is highly context dependent with their homeostatic role being more regulatory rather than activating T_{eff} cells. However, it has been demonstrated that LCs are indeed able to activate skin-resident T_{em} in the context of *C. albicans* infection, driving them to produce effector cytokines such as IFN-γ and IL-17 (81).

As the skin is constantly exposed to pathogens, the pool of T_{rm} in this and other organs is thought to reflect previous infections and exposures. In humans, many $CD69^+$ T_{rm} have been shown to recognize prevalent viruses such as influenza A (166, 167), and respiratory syncytial virus (RSV) (168) in the lung. Further, viruses that cause latent and re-activating infections such as herpesvirus (HSV)-1 and -2 (72, 169, 170), Eppstein-Barr virus (171–173), and cytomegalovirus (174) are also known to elicit a strong T_{rm} response. This is further corroborated by the correlation between presence of virus-specific T_{rm} and increased immune protection and ability to control infections, which was shown to be the case for RSV (168), hepatitis B virus (175), and HSV-2 (170) infection. Specifically, in HSV infections, $CD8^+$ T_{rm} seem to play a crucial role in resolution and protection. HSV-specific $CD8^+$ T_{rm} have been found at the dermal-epidermal junction, close to sensory nerve endings which connect the latently infected ganglia to the skin as well as the genital mucosa (72, 170, 176). These cells have been shown to rapidly produce perforin and pro-inflammatory cytokines upon asymptomatic HSV-2 shedding. Further, cluster formation around virally infected epithelial cells and recruitment of $CD8^+$ T cells from the dermis (170) emphasize that $CD8^+$ T_{rm} are at the forefront of the immune response against acute and latent HSV. While it is now possible to also study T_{rm} in humans, it is worth mentioning that the great majority of current knowledge of T_{rm} behaviour during infection was acquired using murine models of HSV infection which greatly contributed to our understanding of these cells in mucosal tissues (11, 123, 177–180).

Intestine

Similar to the skin, the intestine is constantly exposed to exogenous triggers such as food or microbiota-derived antigens. These antigens are prevented from triggering a pathogenic immune response by cellular barriers. Physically, the intestine is protected by a layer of mucus and glycocalyx which coats the epithelial layer (181) and contains high concentration of secreted IgA (182, 183). In the small intestine, this is composed of a single unattached layer, while the large intestine has two layers of protective mucus, respectively relating to the bacterial burden in each location (184). The intestine is also home to intraepithelial lymphocytes (IELs), other immune cells resident in the lamina propria (LP) and gut-associated lymphoid tissue (GALT), comprising Payer's patches (PP), cecal patches, and colonic patches distributed along the small and large intestine (185). There are differences in immune cell composition between the

small and large intestine which have been extensively reviewed elsewhere (186, 187). A simplified overview of the architecture of the small and large intestine including resident T cells and APCs is shown in Figure 4.

At the bottom of the intestinal crypts, Paneth cells are the main producers of antimicrobial products such as defensins (188) and lysozyme (189), which are secreted into the mucus at the opening of the crypt. Goblet cells, responsible for the production of intestinal mucus, have the ability to take up antigen from the intestinal lumen and deliver these antigens to DCs in the LP *via* a process called goblet cell-associated antigen passage (GAP) (190). Antigens delivered *via* this process have been shown to be taken up by $CD103^+CD11c^+$ DCs which preferentially present to T_{regs}, suggesting that this way of antigen delivery significantly contributes to induction of oral tolerance (191). While this mechanism is not well-understood yet, the more accepted route of antigen delivery from the lumen to the epithelium is *via* M cells on lymphoid follicles (e.g. on Payer's Patches), which can transport whole bacteria (192, 193) that can then be taken up by DCs in the epithelium. This continued sampling of the microbiota by the immune system is crucial to maintaining homeostasis and resistance to pathogens. For example, expression of the chemokine receptor CX3CR1 in mice is essential for APCs to extend their dendrites between epithelial cells and take up intestinal bacteria from the lumen (194) which are then transported to the mesenteric LNs, where production of secretory IgA by plasma cells is induced (195–197). While originally being described as DCs due to their functional properties (194), CX3CR1+ APCs were classified as macrophages by others as they also express the macrophage markers CD64 and F4/80 and derive from monocytes (198, 199). Specifically, DCs in the intestine have the major responsibility in establishing tolerance to oral and microbiota-derived antigens. The gut-draining LNs as well as the GALT are the primary sites of T cell priming by intestinal DCs. As in other tissues, many DC subsets have been identified in the human intestine, with specific subsets more prevalent at specific anatomic locations. In humans, intestinal cDCs are divided into subgroups based on the expression of CD103 and SIRPα (200, 201), with $CD103^-$ $SIRPα^+$ cDC2 further subcategorized based on the expression of the chemokine receptor CCR2 (202).

Intestinal cDCs are the only DCs expressing the enzyme RALDH2, which is required for metabolizing Vitamin A to all-trans retinoic acid (RA) (203). This metabolite is required for imprinting gut-homing receptors on T cells, namely α4β7 and CCR9 (204–207). Both $CD103^+$ and $CD103^-$ cDCs in humans have been found to express RALDH2 (208), which is reinforced by expression of RA by stromal cells in the mesenteric LNs (209, 210). In humans, the majority of IELs are T cells, with the highest proportion of non-T immune cells in the colon (211). The highest number of IELs are found in the proximal small intestine, decreasing in the distal small intestine, and lowest numbers in the colon (212). In the adult jejunum, the majority of IELs are $CD8^+$ αβT cells with a tissue-resident T_{em} phenotype [reviewed in (213)], while the ileum and colon have higher numbers of $CD4^+$ αβT cells, with a minor population of γδT cells (212). In the LP, $CD4^+$ T cells dominate over $CD8^+$ T cells, with the majority of cells exhibiting T_{reg}-like or T_{em} phenotypes (214–217). IL-17 producing $CD4^+$ T cells are most common in the LP of the colon and ileum, with lowest numbers in the jejunum (216), which is inverse to the distribution of T_{reg}:non-T_{reg} T cells observed in mice (215, 217).

DC-T cell composition in homeostasis

Memory T cells

In contrast to skin, sustained CD69 expression is not necessary for T_{rm} formation in the small intestine (7). Further, in the human intestine CD103 is also not necessary for T_{rm}

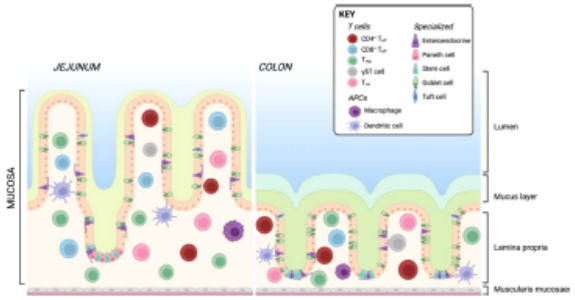

FIGURE 4
Resident T cells and APCs in the human small and large intestine. Created with BioRender.com.

persistence (218, 219), and is higher expressed on $CD8^+$ T_{rm} than $CD4^+$ T_{rm} (216, 220, 221). Human intestinal T_{rm} specifically express CD161, a C-type lectin-like receptor (222, 223), and they share the classic T_{rm} phenotype of downregulating LN homing receptors CD62L and CCR7 as well as the upregulation of adhesion molecules CRTAM and chemokine receptors CXCR6 and low expression of CX3CR1 (224). In the human small intestine, both $CD4^+$ and $CD8^+$ T_{rm} have been described to survive years, with $CD4^+$ T_{rm} exhibiting a T_h1 phenotype upon reactivation (218, 225). In the gut, it has yet to be elucidated if T_{rm} are continuously replenished from circulating T_{cm} under homeostatic conditions or whether the local population proliferates *in situ*, which has so far not been described. The TCR repertoire of $CD8^+$ $CD103^+$ vs. $CD103^-$ T_{rm} has been described to have low clonal overlap, however overlap between $CD103^-$ $CD8^+$ T_{rm} was shown to be similar to that of T cells from the peripheral blood, indicating that $CD103^-$ T_{rm} are recruited from the periphery and represent an intermediate state between circulatory and resident T cells (218). A study utilizing two-photon laser scanning microscopy revealed that intestinal T_{rm} have restricted mobility (226), indicating that intestinal T_{rm} are able to remain at the site of primary infection.

In mice, memory precursor cells expressing low levels of KLRG1 have been identified as a T_{rm} precursor, whose development is accelerated by DC-derived TGF-β (227). Inflammatory monocytes expressing IL-12 and TNF-β have been shown to suppress TGF-β-induced CD103 expression, leading to an increased population of $CD103^-$ LP T_{rm} (228). Additionally, intraepithelial $CD103^-$ T_{rm} appear to preferentially develop from $KLRG1^+$ T cells over T cells that never express KLRG1 (229). Lastly, while IL-15 is critical for T_{cm} and T_{em} maintenance, this cytokine is not necessary for T_{rm} retention in the intestine (230).

Overall, T_{rm} biology and contribution of antigen presenting cells to T_{rm} generation and maintenance in the human intestine still have many open questions. More detailed reviews on intestinal T_{rm} can be found elsewhere (231, 232).

Effector T cells

While at steady-state, DCs in the gut preferentially induce T_{regs}, with T_{eff} cells being primarily induced during infection or inflammation, which has mostly been studied in mice. Intestinal cDCs "escaping" regulatory conditioning in the gut at homeostasis have, however, been shown to induce tonic protective T_{eff} responses. This escape has been proposed to be mediated by early exposure to TLR ligands and pro-inflammatory cytokines, reducing residency time of cDCs and pDCs in the epithelium and thereby limiting exposure to regulatory-inducing factors (233, 234). Another example of this is p38-MAPK signaling in mouse $CD103^+$ DCs, which has been shown to regulate fate-decision between T_{reg} and T_h1 cells from infiltrating naïve T cells by influencing RALDH2 expression required for T_{reg} induction (235). Further, specific TLR5 signaling activating $CD103^+CD11b^+$ cDCs induces IL-6 and IL-23 production which promotes T_h17 development and antimicrobial peptide production (200, 236).

The local microbiota is also essential in inducing T cell subset differentiation and polarization in the gut. In mice, it has been shown that monocolonization with segmented filamentous bacteria (SFB), which are members of the order Clostridiales, can induce the development of LP-resident $CD4^+$ T_h17 cells (237). This selective T_h17 induction is MHC class II-dependent and requires presentation of SFB antigens by resident intestinal $CD11c^+$ DCs (238). The relationship between SFB and T_h17 has further been demonstrated in mice engineered to express the human antimicrobial peptide HBD5. These mice exhibited loss of SFB which subsequently correlated to a lower percentage of T_h17 cells in the lamina propria (239).

Regulatory T cells

T_{regs} are central components of establishing tolerance in the intestine and crucial for maintaining homeostasis. Specifically in the gut, T_{regs} are necessary for controlling pro-inflammatory responses to commensal pathogens as well as establish tolerance to food antigens (240–242). Both thymus-derived (t)T_{regs} and periphery-induced (p)T_{regs} have been described in the gut, with pT_{regs} being thought to play the main role in establishing oral tolerance (243, 244), having been shown to control dysregulated T_h1 responses to food antigens (245). In the colon, the predominant subset of pT_{regs} expresses the T_h17 master transcription factor ROR-γt, the expression of which is dependent on the microbiota (245–248). The ROR-γt^- pT_{regs} conversely are critical for homeostasis maintenance in the small intestine (245). In mice, $Helios^+$ tT_{regs} in the gut express GATA3 and exhibit a tissue-repair phenotype (246, 249, 250). This $GATA3^+$ T_{reg} subset has not, however, been described in humans so far.

TGF-β is an essential cytokine for pT_{reg} differentiation and is, unsurprisingly, present at high concentrations in the intestine (251). DC-derived TGF-β in the gut is essential for local T_{reg} differentiation, which has been demonstrated in mice by ablating expression of the integrin responsible for activation of latent TGF-β ($\alpha_v\beta_8$) on DCs which lead to impaired induction of T_{regs} in the mesenteric LNs (252). Contrarily, deletion of the TGF-βR1 on T_{regs} resulted in normal T_{reg} numbers in the gut (253). However, the authors did not analyze T_{reg} subsets in this study, therefore it cannot be excluded that compensatory T_{reg} expansion was the underlying cause for this observation. Other than cytokines, the metabolite RA is an important contributor to T_{reg} differentiation in the gut. Together with TGF-β, RA has been shown to induce pT_{reg} characterized by upregulation of CCR9 and $\alpha_4\beta_7$ (254–256). Particularly $CD103^+$ DCs are crucial for this induction, as they show a high expression of RALDH2, the enzyme metabolizing vitamin A to RA (257, 258). Particularly development of ROR-γt^+ pT_{regs} is dependent on DC-derived RA (247, 259), further emphasizing that local T_{reg}

induction is crucial to intestinal homeostasis. Other than RA, DCs play a role in T_{reg} induction *via* TLR signaling in the gut. For example, TLR2-mediated recognition of polysaccharide A on the commensal *Bacteroides fragilis* has been shown to trigger induction of T_{regs} and their production of the anti-inflammatory cytokine IL-10 (260).

γδT cells

Intestinal intraepithelial γδT cells play an extensive role in tissue surveillance, having a high migratory capacity and moving through the intestinal epithelium using occludin-mediated cell-cell contact (261). The majority of γδT cells in the human intestine express Vδ TCR (262) and have been associated with intestinal homeostasis *via* the production of keratinocyte growth factor 1 (KGF1) (263). Their significant contribution to gut homeostasis has been shown in γδT cell deficient mice, showing that mice lacking these cells have reduced intestinal epithelial cell turnover (264), increased susceptibility to dextran sulfate sodium (DSS)-induced colitis (263), and increased gut permeability (265). In humans, intestinal γδT expressing NKG2A have been shown to express TGF-β1, thereby dampening IFN-γ and granzyme B production by co-cultured αβT cells from patients with coeliac disease (266). Together, studies so far indicate that intestinal γδT cells have an important role in regulating tissue homeostasis and contribute to controlling inflammatory responses in the gut. However, a lot of open questions about their effector functions and interplay with other cells, such as APCs, in humans still remain.

DC-T cell composition in inflammation and infection

Chronic inflammatory diseases

Inflammatory bowel disease (IBD) is a well-known and well-studied chronic inflammatory condition in the intestine and covers ulcerative colitis and Crohn's disease. IBDs have been linked with multiple exogenous factors such as environmental factors, microbiota dysbiosis, and genetic background (267, 268), which culminate in an overall inappropriate immune cell activation in the gut. In IBD, DCs are known to contribute to disease pathology *via* TLR2/4-induced production of IL-12, IL-6, and IL-23 (269, 270), which further impacts T cell polarization and drives T_h17-mediated disease phenotypes. $CD103^+CD141^+CD1c^+$ cDCs are reduced in inflamed intestinal lesions, showing functional impairments such as decreased RALDH2 activity (271). Further, some findings have indicated that intestinal inflammation, such as seen in Crohn's disease, impairs normal DC trafficking which consequently leads to dysregulated T cell responses in the gut. For example, CCR7 expression on $CD83^+$DC-$SIGN^+$ intestinal cDCs is lower in patients with Crohn's disease (272). Further, it has been observed that leptin production in mesenteric fat is increased in early Crohn's disease patients (273), which has been associated with upregulation of CCR7, maturation and migration of cDCs (274). Whether CCR7 expression is timepoint dependent and what effect this has on T cell priming in Crohn's disease remains to be elucidated.

In recent years, the role of T_{rm} in IBD has become apparent. For example, $CD69^+CD103^+$ T_{rm}-like cells in the LP have been described to be increased in patients with ulcerative colitis and Crohn's disease. Further, the authors could show that increased levels of $CD4^+$ T_{rm} are associated with early IBD relapse (275). Along the same line, Bishu et al. described these $CD4^+$ T_{rm} as functionally competent TNF-α producers in inflamed tissue of patients with Crohn's disease (276). $CD8^+$ T_{rm} have also been implicated in IBD pathogenesis. Bottois et al. described two distinct subsets of $CD8^+$ T_{rm} expressing KLRG1 and CD103, showing that $CD103^+$ $CD8^+$ T_{rm} in Crohn's disease patients exhibit a T_h17-like phenotype, while highly proliferative $KLRG1^+$ $CD8^+$ T_{rm} present with increased cytotoxic effector function and are overrepresented during acute inflammation (277). Single-cell RNA-sequencing studies of ulcerative colitis also showed transcriptional changes in the $CD8^+$ T_{rm} compartment, with an increased inflammatory signature (278, 279). In a recent publication using mass spectrometry, HLA-DR^+CD38^+ $CD4^+$ T_{rm} were found to be enriched in lesions of Crohn's disease patients. The authors could further use imaging mass cytometry of tissue sections to show co-localization of memory $CD4^+$ T cells together with HLA-DR^+CD11c^+ DCs located below the epithelial layer in the inflamed regions of the intestine (280). T_{rm} with a regulatory signature have also been described to be reduced in IBD, characterized by $CD103^+Runx3^+$ and expression of the regulatory-associated molecules CD39 and CD73 together with IL-10 production (281). Furthermore, studies revealed a decrease in both the $CD103^+$ $CD8^+$ and $CD4^+$ T_{rm} compartment during active IBD, which recovered during remission phases, whereas the opposite observation was made for $CD103^-$ T_{rm} (282). These studies further demonstrate the heterogeneity of intestinal T_{rm} and are likely a reflection of T_{rm} plasticity during different phases of the inflammatory response.

Infection

While the physical barriers like the intestinal mucus protect against food-borne pathogens and harmful commensals (known as pathobionts), many microbial organisms have evolved to evade host defense and cause infections. Infections with such enteric pathogens are most commonly associated with diarrhea, which is a major cause of death worldwide (283). The most frequent enteric infections are with *Salmonella* spp. and *Campylobacter* spp (284)., with other examples being *Vibrio cholerae*, *Shigella* spp. and certain strains of *Escherichia coli* (285). The most common pathobiont infections are caused by *Enterococcus* spp (286). and *Clostridium difficile* (287).

While TLR2 is important in inducing T_{regs} (see above), TLR5, the receptor for bacterial flagellin (288), has been implicated in the host response to invasive pathogens such as *Salmonella* spp. $CD11c^+$ LP-resident DCs express TLR5, which is important in modulating DC movement, as TLR5-deficient mice have increased survival and lower dissemination when infected orally with *Salmo*

observed when mice were [illegible]. This observation indicates that trafficking to the mesenteric LN by DCs is impaired thereby preventing dissemination of the infection. Another important consequence of TLR-mediated activation of DCs is cytokine production. A crucial cytokine in the gut produced by DCs in response to infection is IL-23, which has been linked to infection with pathogens like *Salmonella* spp (290)., *C. rodentium* (291), and *C. jejuni* (292). The receptor for IL-23 in the gut is expressed on multiple immune cells such as T_h17, NKT, γδT cells and ILCs (293, 294). IL-23 receptor signaling in turn triggers production of IL-17 and IL-22. IL-17 appears to have time-dependent effects during intestinal infection. During early *Salmonella* spp. infection, IL-17 produced in the caecum is primarily mediated by T_h17 cells and to a lesser extent γδT and NKT cells (295, 296). Another example of the importance of T_h17-mediated immunity during infection has been shown in rhesus macaques where SIV-induced depletion of T_h17 cells leads to erosion of the mucosal barrier and increased dissemination of *S. enterica* Typhimurium to the mesenteric LNs (296). IL-17A or IL-17F deficiency in mice lead to increased pathology in response to *C. rodentium* infection (297).

The IL-23-T_h17 axis is also important in human intestinal infection. Patients suffering from *C. jejuni* infection show increased percentages of T_h1 and T_h17 cells, as well as increased levels of the respective effector cytokines. The authors could show that when intestinal epithelial cells were treated with IL-17A or IL-17F, intracellular survival of *C. jejuni* was significantly decreased, emphasizing the importance of these cytokines in human infection (292). Further, IL-17 expression was also detected in the duodenum of patients recovering from *V. cholerae* infection, the causative agent of cholera. Kuchta et al. observed that in patients suffering from acute cholera, IL-17 expression was increased compared to later disease stages or healthy subjects, suggesting that *V. cholerae* infection also induces an immediate mucosal T_h17 response (298).

The other IL-23-induced cytokine important in intestinal infection is IL-22. In general, IL-22 is associated with tissue repair and is known to be a major inducer of antimicrobial peptide production by mucosal epithelial cells (299, 300). In the context of infection, IL-22 has been found to increase colonization resistance to the pathobiont vancomycin-resistant enterococci (236). Similar to IL-17, IL-22 has also been shown to have time-dependent effects. During early infections, IL-22 is primarily produced by ILCs and only later on by T cells. This was demonstrated by Ahlfors et al, showing that during infection with *C. rodentium* IL-22 is initially produced by ILC3s and then by $CD4^+$ T cells (301).

Overall, it has become clear that the DC-induced IL-23-T_h17 axis is particularly important in response to intestinal infection by modulating epithelial microbial peptide expression and preventing dissemination of intestinal infection.

Female reproductive tract

The immune system in the FRT has a dual role as it protects the barrier tissue against pathogens transmitted during sexual intercourse, and promotes tolerance to foreign antigens necessary to allow fertilization and embryo development. As these two diametrical roles are important at specific times during the menstrual cycle, the composition of immune cells undergoes major fluctuations. During menstruation, a much higher density of $CD1a^+$ DCs was observed in the human uterus compared to proliferative and secretory phase (302). Uterine macrophages increase constantly in numbers during secretory phase and peak at menstruation, while the total number of T cells remains constant (303–305). The sex hormone progesterone does not only inhibit activation of DCs (306), but also causes polarization of T cells into T_h2 and T_{reg} direction (307, 308). Moreover, subsets of immune cells do not only change during the menstrual cycle, but also differ when comparing tissues from pre- and postmenopausal females (309). There are substantial differences between the structure and physiology of the female genital tract between the most frequently used animal model of mice compared to humans, as the murine uterus contains two uterine horns and also the estrous cycle has a length of around 5 days compared to 28 days in humans. However, due to the previously low interest in female reproductive health, scientists started only recently to investigate immune cell populations in large scale in the FRT of humans. Therefore, most knowledge on the female genital immune system was obtained in mice (310). With this section, we aim to shed light on specific features of antigen uptake and presentation as well as T cell responses in the female genital tract and raise awareness for inflammatory conditions and chronic infections.

The female genital tract is structured in several parts: the lower reproductive tract lined with multilayered stratified epithelia forming vagina and ectocervix, the endocervix as an interphase and the upper genital tract with single columnar epithelium forming the uterus, adjacent to the fallopian tubes stretch connecting the ovaries with the uterus which are composed of secretory and ciliated columnar epithelial cells (Figure 5). The main APC subsets in the human vaginal tissue are, similar to skin, in the epithelial layer LCs characterized by CD207 expression and in the lamina propria DCs characterized by expression of CD1c as well as CD14 on a specific subset (311, 312). In addition to DCs, another frequent APC subsets in the vagina are $CD1c^-CD14^+$ macrophages additionally having

CD163 on their surface (311, 312). In the cervix, the most frequent immune cell population are macrophages which make up more than 25% of all $CD45^+$ immune cells (55). $CD11c^+CD14^+$ DCs accounting for another approx. 20% of immune cells are the most common DC subset and a large proportion also express DC-SIGN. Other DC subsets such as $CD11c^+CD14^-$ myeloid DCs and $CD123^+$ plasmacytoid DCs were described in low numbers (55). The percentage of APC subsets within $CD45^+$ immune cells is quite similar in cervix and uterus, however, the APC compartment in the uterine endometrium shows some substantial differences. There are less DC-SIGN$^+$ DCs and DCs expressing $CD103^+$ involved in antigen sampling and migration were almost exclusively found in the endometrium (313). In the murine endometrium, both $CD103^+$ and $CD103^-$ DCs migrate to the local lymph nodes upon antigen challenge. The $CD103^+$ DCs preferentially present antigens to T_{regs}, whereas their $CD103^-$ counterparts were shown to stimulate an effective CD4 T cells response (314). In the murine uterus, DC in the decidua of pregnant females were shown to be trapped in the tissue, despite keeping responsiveness to pro-inflammatory stimuli and migration capacity towards CCL21 (315). This indicates that by preventing DC trafficking to the draining LN, T cell tolerance to fetal antigens is promoted. Single-cell sequencing of human uterine samples during secretory and proliferative phase revealed presence of myeloid cells during both phases, being composed of DCs as well as M1- and M2-polarized macrophages (316). $CD11c^+$ DCs can be further divided into $CD11b^{++}$ and $CD11b^{lo}$ DCs, with the $CD11b^+$ expressing DCs being the most abundant subset in all tissues of the FRT and correlating with CD14 expression (313). In the vagina, the ratio of CD4 to CD8 cells is almost equal, with an increasing ratio towards endocervix and ectocervix (55, 317). In the uterus, however, CD8 T cells represent the predominant subset (55). B and NK cells make up less than 5% of immune cells in the human ectocervix and are not in focus of this review (55).

DC-T cell composition in homeostasis

Memory T cells

In general, most T cells in the female reproductive tract are T_{rm} being $CCR7^-CD45RA^-$. More than 80% of cervical T cells express CD69 within both stroma and epithelium (317, 318). The marker CD103 being associated with a T_{rm} phenotype in other tissues is in the cervix almost exclusively present on epithelial CD8 T_{rm} (318), but also enriched on vaginal CD4 T_{rm} (317). These vaginal $CD103^+CD69^+$ CD4 T_{rm} show a T_h17 signature including high expression of RORC, IL-17A, IL-17F and IL-22 (317). A recent publication used T_{rm} derived from human cervix to assess antigen-specific CD4 and CD8 response against HSV-2 (319). An elegant mouse study using parabiosis models revealed that CD8+ T_{rm} in the mucosa undergo proliferation *in situ* after mucosal rechallenge independently of CD11c+ DCs (114). On the other hand, bystander memory CD8 T cells consisting of T_{cm} and T_{em} are recruited during local challenge without antigen recognition and develop a T_{rm}-like phenotype by upregulating CD69, but not CD103 (114). To investigate how the recruitment of bystander memory cells to sites of infection as well as tissue autonomous amplification of local T_{rm} contributes to immunity in the human FRT, it is important to apply functional models with human cells and validate other experimental approaches in the future. To date, the T_{rm} subset is the best studied immune cell subset in the FRT and will be discussed further in the sections about the respective infectious diseases.

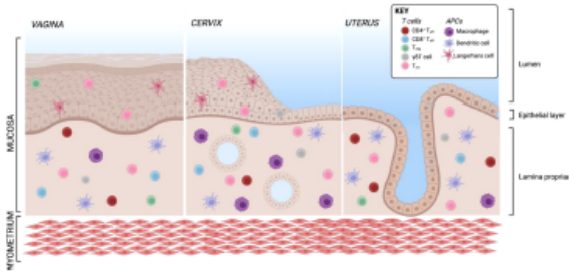

FIGURE 5
Resident T cells and APCs in the human FRT. Created with BioRender.com.

Regulatory T cells

Recently, T_{regs} were shown to make up around 15% of the CD4 population with comparable percentage within all tissues from the lower FRT, including vagina, endocervix and ectocervix (317, 320). It is reported that T_{regs} are induced in the decidua of mice and humans to protect the developing embryo from the immune system of the mother, nicely summarized in the following reviews (321–323). However, T_{regs} can also have an unfavorable role if they dampen the immune response against sexually transmitted infections such as human immunodeficiency virus (HIV), human papilloma virus (HPV) or chlamydia. In a mouse model of intravaginal *N. gonorrhea* infection TGF-β^+ T_{regs} were induced in cervix-draining lymph nodes, thus evading the immune response and enabling pathogen survival (324). The occurrence of T_{regs} in the mucosal tissue is described for several pathogens and conditions, while the mechanisms of their induction still need to be elucidated.

γδT cells

Human studies revealed a γδT cell percentage ranging from 5% to 10% of $CD3^+$ T cells depending on tissue sampling during the proliferative phase or secretory phase. The majority of them expressing Vδ1 (325, 326), but CCR5 can be found on the surface of both Vδ1 and Vδ2 (327). HIV infection significantly reduces the number of γδT cells in the cervix (327). Abnormal vaginal flora due to bacterial vaginosis was shown to change the composition of vaginal γδT cells to higher levels of Vδ2 (328). Beside their role during infection, γδT cells seem to be involved in tolerance induction during pregnancy. The decidua of women with spontaneous abortions showed increased numbers of γδT cells with an additional upregulation of $Vδ2^+$ cells (325). In the murine female genital tract, γδT cells represent a much higher proportion of immune cells and express preferentially IL-17A under steady state (329). As IL-17A was described to be essential for resistance against fungal infection, a murine study revealed that TCRγδ deficient mice are more susceptible to *C. albicans* growth in the FRT (330). To date, our knowledge about γδT cells in the FRT is still limited and remains to be addressed in different disease settings.

DC-T cell composition in infection

Viral infections

CD4 and the chemokine receptors CCR5 and CXCR4 are hijacked by HIV. Beside T cells, this repertoire of receptors is found on all four APC subsets in the vagina in different quantities, indicating a role of these cells during HIV acquisition and transmission to other cell types (311). It was shown that exclusively $CD14^+$ DCs take up HIV virus-like particles and express CCR5 ligands (313). The type-I interferon inducible lectin Siglec-1 expressed on $CD14^+$ DCs was identified to play an indispensable role in HIV uptake and transmission to CD4 T cells which can be blocked by anti-Siglec-1 antibodies (331). As $CD14^+$ DCs are most frequently occurring in the ectocervix, this tissue is highly relevant to study HIV transmission (313, 332). CD4 T_{rm} from the ectocervical region expressing CD69 are characterized by high CCR5, thereby function as a primary target for HIV infection and persistence (333, 334). Numbers of CD4 T_{rm} are significantly decreased in cervix tissue of infected individuals, but increased activation can be observed (333). In the same lines, $CXCR3^+$ T_{rm} in the skin and anal mucosa of HIV infected individuals starting antiretroviral therapy late remain constantly depleted, thereby creating an optimal environment for HPV related cancer development (335). HIV-infected individuals show increased T_{regs} and reduced T_h17 cells, the ratio between these two cell types can be restored by anti-retroviral therapy (ART) (336). The percentage of T_{regs} remained increased even under ART and was associated with a skewed ratio of CCL17/CCL20 in the ectocervix samples of these women (336), indicating that APCs as major source of those cytokines, are causing the disbalance of T cells in these conditions.

Infections with HPV are widespread and almost every human encounters HPV during their life time. There are several different types, with only some of them being transmitted sexually and causing infections that can lead to cancer development in the cervix. Patients with HIV infection possess an increased risk to develop HPV associated cancer with T cells as important players in the course of HPV-related malignancies (337, 338). Upon HPV infection, T cells in the cervix obtain a more activated profile by upregulation of HLA-DR, independent of HIV status of the patients (339). However, in patients with a co-infection of HIV and HPV, lower numbers of CD4 T cells were observed compared to HPV-negative HIV-infected patients (339). In individuals with HPV-associated genital warts, an accumulation of T_{regs} was reported (340). It was shown that T_{regs} are attracted by CCL17 and CCL22, which are mainly produced by $CD1a^+$ LCs and macrophages within the warts, respectively (340). Trafficking of APCs such as LCs is impaired in HPV lesions, as the chemoattractant for (CCL20) and activation pattern of LCs (CCR7, CD80 and CD86) seem to be decreased (341–343). Also, T_h17 cells seem to play a role in progression of HPV-related intraepithelial cervical neoplasia (CIN), as patients with high CIN or cervical cancer exhibit high numbers of T_h17 cell in the blood, which is correlated with high IL-17 levels in the cervix tissue (344). In a study assessing the T cell infiltration in cervical cancer patients, $CD103^+$ CD8 T cells infiltrate the tumors and are associated with good prognosis (345). These findings indicate that a T_h17 and T_{reg} response is correlated with progressive HPV infection, whereas CD8 T cells are beneficial. However, most studies focus on late stages in CIN progression/tumor development and little is known about early processes of HPV infection.

Bacterial infections

Infections with chlamydia are the most common bacterial sexually transmitted infection in humans. However, most of our knowledge of immune reactions during chlamydia infections was obtained in mice, as studying immunity against *chlamydia trachomatis* (Ct) is connected with many difficulties, such as the high number of asymptomatic cases and the development of tolerance instead of immunity when using inactivated bacteria. The later problem was addressed in a mouse model by Stary et al. showing that live and UV-inactivated Ct are taken up by either $CD103^-$ and $CD103^+$ DC subsets, causing priming of immunogenic effector T cells or T_{regs}, respectively (314). In mice, induction of T_h1 cells plays a huge role in conveying protective immunity, whereas stimulation of $CD8^+$ T cells was suggested to play a role in chronical inflammation and cause tissue destruction rather than advancing protective immunity in mice (314, 346, 347). T_h1 polarization initially relies on IL-12 production by DCs, as IL-12 deficient mice had prolonged times of chlamydia shedding (348). In fact, the most important immune mechanism for chlamydia clearance is IFN-γ, as T-bet deficient mice could not control *chlamydia* growth, but T cells shifted to a more T_h17 response, whereas IFN-γ or IFN-γ-receptor deficient mice die from systemic infection (349, 350). T_{rm} of the FRT seemed to be essential to protect against subsequent chlamydia infection (314). However, a recent publication suggests that also circulating memory T cells can protect against infection without being primed in the tissue (351). Apart from conveying protective immunity, T cells can also be involved in undesirable responses causing FRT pathology and chronic inflammation. Especially activation of non-antigen-specific CD4 as well as CD8 bystander cells can exacerbate the pathology in a mouse model of chlamydia infection (352). The presence of T_{regs} was on the one hand shown to exacerbate Ct infection (314), on the other hand, they are described to skew T cell differentiation into a T_h17 direction, which was correlated with increased pathology in a *chlamydia muridarum* mouse model of infection (353). Together, these findings suggest that the T cell response during Ct infection is highly plastic and the induction of a certain cytokine milieu is essential.

Discussion

All the same: Commonalities and differences in tissue APC-T cell crosstalk

When comparing the three different tissues summarized in this review, some overarching themes are apparent: The majority of T cells in tissues are T_{rm} cells (7), closely followed by T_{regs} (19, 20), both cell types reflecting the constant exposure to environmental compounds and antigens in barrier tissues and the need for a balance between immune tolerance and reaction. Further, DC subsets are responsible for controlling this balance, but they are often described by different markers in different tissues and their subsets appear more tissue-specific than those of T cells, whose identity is often easier to define across tissues. However, some clear differences exist also in T cells. Expression of CD69 and CD103, canonical T_{rm} markers in the skin (13, 111) and FRT (317, 318), seem dispensable for T_{rm} establishment in the intestine (7). T_{rm} are relatively stationary within the respective tissue, however, there are quite substantial differences in motility between T_{rm} in different tissues, as T_{rm} in the FRT move up to 5-times faster compared to T_{rm} in skin epidermis, probably depending on the architecture of the tissue and density of the structural cells (114). While $CD4^+$ and $CD8^+$ T_{rm} exist in all discussed barrier tissues, the skin harbors more $CD8^+$ T_{rm} than the intestine and the FRT, where the distribution of $CD4^+$:$CD8^+$ T_{rm} is approximately equal (218, 225). Further, T_{reg} induction in the intestine is highly dependent on RA produced by local DCs (254–256) and in the FRT, progesterone (307), independently of DCs, appears to take a similar role, while no hormones or metabolites are yet identified to induce T_{regs} in the skin. In general, it appears, that while all barrier tissues are continuously exposed to microbial antigens, only the intestine has dedicated DC subsets to specifically induce T_{regs} to promote tolerance against the microbiome (191). This observation fits with the fact that, in the skin, most T_{regs} respond and get activated by non-antigenic stimuli while most T_{regs} (127) in the gut are antigen specific (240–242). In general, aside from their function in maintaining immune tolerance, the function of T_{regs} in different tissues is often diverse, ranging from direct suppression of activated immune cells to aiding in tissue repair (19, 20), thereby emphasizing the need to characterize these cells and their non-canonical functions in a tissue context better. Similar to this, γδT cells exhibit both regulatory and cytotoxic functions across tissues even though their distribution is tissue-specific (Vδ1 in the skin, Vδ7 in the intestine, Vδ1 and Vδ2 in the FRT) (149).

During an immune challenge in barrier organs, such as during infection, T_{rm} are poised locally in all three tissues, reacting to previously encountered antigenic stimuli directly. Further immune responses are induced by APCs which traffic to the respective draining lymph nodes and recruit T_{eff} cells to the tissue. T_h17 responses are crucial in controlling infections, both bacterial and viral (313, 332). Interestingly, the same responses and effector cytokines are also often the ones that are pathogenic in chronic inflammatory diseases (153, 154). How and why exactly these exacerbated immune responses cannot be controlled by tissue-resident T_{regs}, which are present in barrier tissues in great abundance under homeostatic conditions, has yet to be elucidated. However, all chronic inflammatory diseases discussed in this review are characterized by a decrease in tissue T_{regs}, but whether this is cause or effect of chronic tissue inflammation and what role APCs play in this shift of T cell subsets during chronic inflammation remains a big question that should be the topic of further research.

Into the (un)known: On big data, future perspectives, and individualized therapies

Previous dogmas of dividing immune responses strictly into pro- and anti-inflammatory immune cell subsets are outdated. The more we learn about tissue-specific immune responses, the more we understand that there is not the one beneficial and harmful immune cell subset to every disease. It is more a fine-tuned balance act between APCs and T cells to enable immunity against pathogens but protect the host from autoimmunity. With current advances in single-cell RNA sequencing (scRNA-seq) and multichannel flow cytometry, we will be able to get a better insight, which players are involved in regulating immunity during homeostasis. scRNA-seq has specifically enabled much greater insight into molecular mechanisms of tissue immunity as well as led to the discovery of new immune cell subsets or new definitions of existing subsets. This is especially valuable since this approach allows for the acquisition of a large amount of data from, often limited, human material. Further, a lot of information that is derived from these big data experiments would be impossible to acquire using traditional experimental models as it is now possible to also model *in vivo* dynamics from these datasets, such as the interplay between different cell types (354, 355) and temporal dynamics across the development of organs (356–358), and tracking T cell clones across tissues (359, 360). Analyses like these have revealed novel regulatory T cell-APC interactions at the maternal-fetal interface important for embryo implantation (361), a renewed focus on pDCs in skin inflammation (362), novel Vδ1 T cell effector subsets (363), and detailed profiling of different immune niches and interactions across the human intestine (54). Further, a better understanding of tissue adaptation of different immune cells is becoming appreciated, highlighting basic principles of immune biology in barrier tissues but also appreciating that these cells have the potential to specifically adapt to the local tissue environment and how this changes in disease (126, 364–366). As highlighted in this review, communication between different immune cell types is absolutely essential in determining the outcome of an immune response and understanding this interplay at a deeper level in local tissues is an important step towards developing new therapeutic avenues that can act in a much more targeted manner than previously possible. Further, the plasticity of immune cell subtypes, especially APCs and T cells, is becoming more appreciated as having whole transcriptome data can separate cell types that were previously indistinguishable and is an important step towards understanding fundamental changes during disease development. As this knowledge progresses, it will be interesting to see if we will gain a better understanding of responses to immunotherapy and why some patients benefit while others do not. Moreover, this technical evolution will also allow to come away from animal models and help uncover tissue-specific differences as well as overarching themes in immune defense in barrier tissues. In addition, we want to emphasize the importance of investigating the interplay of different human immune cell subtypes in complex 3D model systems to further validate findings from big data-based models and how these can be translated to patient care. It will be crucial to define the function of rare DC subsets, T_{regs} or γδT cells as they seem to have a major role in immune balance despite their low frequencies. Especially the mechanisms balancing different γδT cell subset or T_{regs} and T_h17 cells will be an important focus for further studies. In the future, integrating different large datasets will be highly valuable in better understanding more complex disease systems, such as metabolic dysregulation as well as epigenetic modifications. Together, these data will yield a clearer picture of biological networks and how they are perturbed in different diseases. Currently, we are at the start of a new era of understanding biological mechanisms that lead to disease and disease progression. In the future, insights gained from these basic studies will in turn re-shape how therapeutics are developed and most likely emphasize the importance of more patient-specific approaches to health care.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Review: Models for sexually transmitted infections

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Models for sexually transmitted infections

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Extensive research on sexually transmitted infections (STI) in the last century has led to a better understanding of disease prevention and treatment options. Nowadays, bacterial infections with chlamydia, gonorrhea or syphilis are curable with antibiotics. Also, infection with HIV has lost its terror as there exists a treatment allowing patients a normal life. Pre- and post-exposure prophylaxis of HIV with anti-retroviral drugs prevents infection of sexual partners. However, health organizations report that STI are on the rise again, being problematic as many infections remain undetected, thereby causing cancers, infertility or congenital infection. To circumvent this, development of vaccines against different STI are urgently needed. As it is difficult to study interaction between host and pathogen in humans, model systems including animal models and *in vitro* approaches are necessary. Within this review, we give an overview on animal models of STI with a focus on chlamydia infection, discussing advantages and disadvantages of modeling infection with species-specific or human pathogens. Moreover, we present available *in vitro* models of STI, pointing out current advances in the development of three-dimensional (3D) culture systems closely resembling human tissue architecture. This allows to explore infection under physiological conditions in human cells without ethical concerns. A toolbox full of tightly coordinated *in vivo* and *in vitro* infection models will be required to advance STI research and vaccine design in the future.

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Introduction

Sexually transmitted infections (STIs) are spread within populations worldwide for centuries affecting the course of history. Groundbreaking advances in medicine during the previous century including diagnostic methods, monitoring of bacterial susceptibility to antibiotics by culture-based and molecular surveillance, the development of specific therapies against viral and bacterial infections as well as a better understanding of disease prevention with exposure prophylaxis and sex education dramatically decreased the prevalence of once fearsome diseases like syphilis, gonorrhea and human immunodeficiency virus infections (HIV) [1,2]. However, the world health organization and national health services report that STI are on the rise again [3]. For example, the US Centers of Disease Control and Prevention (CDC) report steadily increasing chlamydia infection rates from 250 to more than 500 cases per 100,000 population in the last 20 years [4]. One general

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challenge with STI despite their relative straight-forward treatment is that they remain undetected and asymptomatic in many cases. Still, they might be the cause of genital cancers, pelvic inflammatory disease resulting in ectopic pregnancy and infertility or congenital infection in newborns of infected mothers. Another reason why an increase of STI is alarming is that clinicians experience that pathogens of STI become resistant against broadly used therapy options. Health reports warn that *Neisseria gonorrhoeae* is becoming less sensitive to standard antibiotics treatment and multi resistant strains are becoming more frequent [5]. To prevent the spread of STI, one possibility is to extend national screening programs to treat asymptomatic patients before they can infect others or develop effective vaccines [6]. While the effectiveness of population-wide screening programs is still under debate and resource intense, vaccination against human papilloma virus infection (HPV) as the main cause of genital cancers and genital warts is a good proof-of-principle of a vaccine preventing STI [7]. As the development of novel treatments and vaccines requires model systems closely resembling natural infection, we will review current animal and *in vitro* models being used to study STI. In focus will be possibilities to model *Chlamydia trachomatis* infections, as this intracellular pathogen is the most common cause of bacterial STI with extensive studies trying to unravel pathogenetic factors of disease for decades to ultimately develop a protective and save prophylactic vaccine [8,9].

Animal models for STI research

STI do not only occur in human, but also in different animal species. Animals can be infected experimentally *via* different routes by species-specific or human pathogens or naturally *via* transmission during sexual intercourse. Some human STI, such as *Chlamydia trachomatis* or HIV have common ancestors to animal pathogens [8,10]. For *Chlamydia trachomatis* for example, there exist several equivalent bacteria in the animal world with similar pathogenesis as observed in humans, such as *Chlamydia muridarum*, *Chlamydia caviae* or *Chlamydia suis* infecting mice, guinea pigs or pigs, respectively [8]. Also *Treponema pallidum*, the agent causing syphilis, has a closely related pathogen namely Treponema paraluiscuniculi, which infects rabbits [11]. Therefore, infection models using pathogens adapted to the host to study pathogenesis, immune responses, treatment and prevention measures is an obvious and essential tool to gain a better understanding of infectious diseases or vaccination trials (Fig. 1). However, studying human strains in animals has the advantage that relevant antigens for human disease can be targeted and analyzed. Consequently, mouse models for *Chlamydia trachomatis* or rabbit models for *Treponema pallidum* were established that resemble the clinical course of human disease [11,12] (Fig. 1). Due to their size, practicability, available reagents and easy handling, mice are the most commonly used animal model for various infections, including STI like chlamydia. Apart from mouse models, guinea pigs, pigs and non-human primates are used for vaccination trials against chlamydia [8]. Thereof, the model best resembling human physiology, menstrual cycle and infections are non-human primates. However, due to ethical reasons, costs and difficult handling, this animal model of chlamydia infection is not widely spread [13]. Additionally, alternatives to research with non-human primates are also needed for other STIs, such as HIV or gonorrhea. As infection with *Neisseria gonorrhea* is highly specific to humans, the only other animal in which an infection was successfully established are chimpanzees. However, it is possible to obtain a robust infection in mice, if the estrous cycle is previously modulated by treatment with estradiol [14]. In case of HIV, where a lot of knowledge was gained using non-human primates being a natural host to the related simian immunodeficiency virus (SIV), infection can also be studied with humanized mice, having human CD4+ T cells infected with HIV [15] (Fig. 1).

Immune response in animal models for chlamydia infection

The final goal of STI research is the development of protective vaccines and curative treatments. Therefore, *in vivo* models are essential as surrogates for immune responses in humans. STI can lead to a broad spectrum of clinical symptoms, ranging from spontaneous clearance of infectious agents and asymptomatic courses to the development of persistent infections with long-term sequelae. This can be explained by a unique immunological background and history of contacts with different pathogens by every individual, thereby developing a different quality of innate and adaptive immune response. To study immune responses under standardized conditions, mice are the model of choice as there is no genetic variability in immune responses as most mouse strains are inbred [9]. Many studies in the field of chlamydia research were performed using *Chlamydia muridarum*, as this pathogen is more pathogenic in mice than human chlamydia strains and mice are the natural host, therefore it can be applied intravaginally and subsequently ascends in some animals into the upper genital tract. However, as a strong adaptive immune response is induced, leading to clearance of the pathogen within 4 weeks and immunity against re-challenge with the same strain, long-term persistence as hallmark for chlamydia infection in humans cannot be studied with this model [16,17]. The main players in immunity against *Chlamydia muridarum* are CD4+ type1 T helper (Th1) cells, which are crucial for initial elimination of the bacteria by interferon-γ (IFN-γ) release and B cells, which promote antibody-mediated immunity protecting against re-infection [18,19]. Another mouse model of chlamydia infection uses the human strain *Chlamydia trachomatis*. Because this pathogen causes less inflammation in mice, the intravaginal infection only leads to mild infection and barely ascends into upper genital tract. Therefore, the infectious agent is inoculated

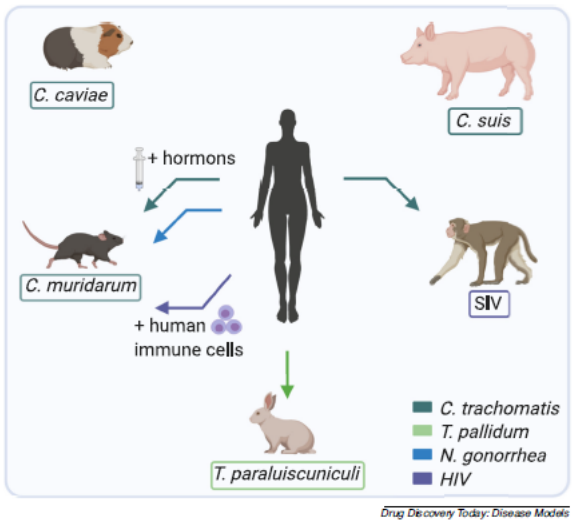

Fig. 1. Overview of host and model organisms of STI. Human pathogens of STI are depicted in different colors. Colors of arrows and bordered boxes indicate which animals and animal pathogens are used to model human STI.

directly into the uterus of mice with normalized estrous cycle by treatment with medroxyprogesterone to cause pathology in the uterus, uterine horns and oviducts and ovaries [12]. CD4+ T cells secreting IFNγ being specific to *Chlamydia trachomatis* were shown to also play a major role in clearance of *Chlamydia trachomatis* and promote partial protection against subsequent chlamydia challenge [12]. In detail, CD4+ T cells are activated by CD11b+CD103- dendritic cells (DC) upon mucosal infection with *Chlamydia trachomatis*, giving rise to tissue resident memory and circulating memory T cells [20]. On the other hand, CD11b-CD103+ DC were shown to take up inactivated chlamydial particles, thereby stimulating regulatory T cells, which reduce the clearance of an subsequent infection [20]. CD8+ T cells are not essential for pathogen elimination or protection, even though they can produce IFNγ upon infection with *Chlamydia trachomatis* [18]. Moreover, CXCR3-driven influx of unspecific CD4+ and CD8+ T cells into upper genital tract contributes to immunopathology instead of protective immune response [21]. Also activation and recruitment of neutrophils into the upper genital tract does not have an effect in clearance of chlamydia, but rather has an immunopathological effect [21]. Both *Chlamydia muridarum* and *Chlamydia trachomatis* induce a serum IgG response, however, a main difference between these two infection models is that only *Chlamydia muridarum* causes a long lasting IgA response [22].

In-vitro infection models

Apart from studying STI in animals, *in vitro* models are a widely used tool to investigate the interaction between pathogens and human cells under standardized conditions. This is now even possible with *Treponema pallidum*, as a method to culture and expand this pathogen *in vitro* was published in 2018 [23]. For virally induced infections like HPV, HIV and herpes simplex virus, several *in vitro* models including 2D culture of polarized epithelial cells as well as complex 3D culture models consisting of several cell types or organotypic cultures are established and nicely summarized in two reviews [24,25]. To study the course of infection with *Chlamydia trachomatis* and *Neisseria gonorrhea*, monolayers of cervical, endometrial or colorectal cancer cell lines, such as HeLa, HEC-1B or Caco-2 cells, are extensively used [26–33]. The advantages of 2D cell culture models comprise being easy to handle due to fast proliferation of established cell lines, already proven susceptibility to infection and availability. However, they do not necessarily resemble the physiological situation as structural aspects of infections are missing (Fig. 2). This led to development of approaches with primary cells and 3D models, which will be discussed in more detail.

Uterus and endocervix are composed of columnar epithelial cells, whereas the ectocervix is lined with stratified squamous epithelial cells. It was proposed that the structure of the epithelium has an impact on the susceptibly to chlamydia

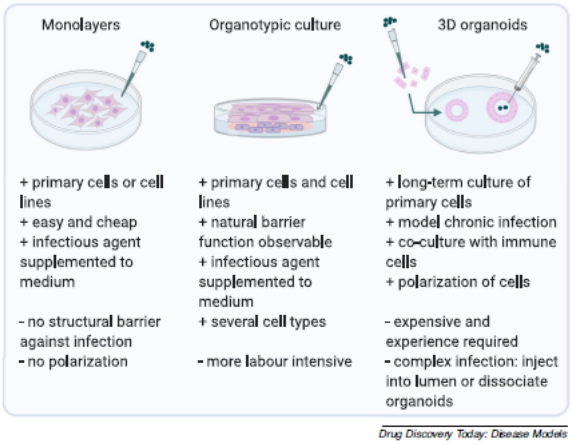

Fig. 2. *In vitro* culture systems to model STI of female genital tract. Schemes illustrate the different possibilities to culture genital epithelial cells: Monolayers of epithelial cells, organotypic culture of epithelial cells on top of collagen rafts seeded with stromal cells or 3D organoids of epithelial cells embedded in extracellular matrix. Pipettes and syringes demonstrate the mode of infection with pathogens of STI as chlamydia. Advantages and disadvantages of all described *in vitro* models are listed below.

infection as endocervical cells show higher levels of infection than ectocervical explants [34,35]. This finding was taken up by Nogueira et al., who created organotypic stratified squamous epithelial models of human immortalized keratinocytes HaCaT cultured on collagen rafts and NIH 3T3 fibroblasts. It was proposed that squamous epithelial acts as a natural barrier against infection with *Chlamydia trachomatis* with only the uppermost layer presenting with chlamydia inclusions [36]. Deng et al. describe a method to efficiently isolate and culture patient derived primary endo- and ectocervical cells derived from human cervix biopsies. They tested several commercially available keratinocyte culture media and found that cells from different cervical zones grow better under distinct culture conditions. Moreover, when culturing endo-, ecto- and transcervical epithelial cells on top of stromal cells in organotypic rafts, it was observed that epithelia cell morphology is similar to the *in vivo* situation, thereby providing a tool to study differences in infection susceptibility between cervical epithelia [37] (Fig. 2). As STI can also infect the anal and colorectal mucosa, culture models for these tissues are required. The anal mucosa consists of a stratified keratinized epithelium, while the colorectal mucosa is lined by a columnar epithelium [25]. Primary colon epithelial cells as well as colorectal explants are very short-lived in culture. However, advances were made in culturing colorectal epithelium in self-renewing 3D organoids and monolayers when provided with a tissue environment [25,38].

3D organoid cultures of female genital tract epithelia

Non-immortalized primary cells are not applicable for long term culture, as they become senescent or differentiated after certain days in culture. For cervical epithelial cells, the median life span of culture was reported to be around 5–6 weeks [37,39]. Another issue is that patient material is limited and size and quality of biopsies varies, thereby making it impossible to establish cell lines from every patient [39]. Recent efforts to grow organoids from cervical and endometrial epithelium were successful and present a novel tool to perform experiments with self-renewing epithelial cells in a more physiological environment. Cervical organoids were derived from stem cells isolated of human cervical biopsies. They are embedded in extracellular matrix and cultured in specifically supplemented organoid growth medium. Depending on the presence or absence of Wnt3A and R-spondin1, the cells developed into columnar endocervical or squamous stratified ectocervical organoids, respectively [40].

To create human endometrial organoids, endometrial biopsies were dissociated, dispersed in extracellular matrix and subsequently cultured in growth-factor enriched medium [41]. Endometrial organoids can be maintained for more than 4 months and it was shown that they even respond to hormone treatment: Estradiol causes Ki67 upregulation and growth of the organoids, resembling the proliferative cycle phase, whereas progesterone treatment reduced the number of proliferating cells, mimicking the secretory-phase of the cycle with increased mucin production [41]. Another study describes the establish-

ment of organoids from patients suffering from endometriosis or hyperplastic endometrium, thereby providing an instrument to study very heterogenous endometrial diseases and allow to think about using it for personalized drug screens [42]. Boretto et al. describe the use of murine endometrial organoids to explore chlamydia infection. They adopted protocols to generate murine endometrial organoids of uterine horns [41]. The organoids are mechanically dissociated, before infecting the fragmented organoids with chlamydia. The infected cells were embedded again in extracellular matrix to allow reformation of organoids [43] (Fig. 2). In a manuscript which was recently uploaded to bioRxiv, a different approach was used to infect the organoids: they injected chlamydial elementary bodies with microneedles into the lumen of organoids, as it was already performed in stomach organoids infected with *Helicobacter pylori* [44] (Fig. 2). Additionally, they co-culture their infected organoids with neutrophils, giving a glimpse on what powerful applications this approach might have for future studies of immune responses after infection [45]. These first studies were performed in mouse endometrial organoids, but in principle it should be possible to adapt these infection models also to human tissue-derived endometrial organoids. Another 3D model uses a rotating wall vessel bioreactor to study the interaction between the endometrial adenocarcinoma cell line HEC-1A and *Neisseria gonorrhea* and other vaginal bacteria [46]. For this technique, the cells are maintained in free fall, thereby forcing them to aggregate around beads used as growth scaffolds [46,47]. Within this approach, endometrial epithelial cells exhibited microvilli on the apical site, produced mucus and where able to be colonized with microorganisms [46], therefore providing all prerequisites to be challenged also with chlamydia allowing a more natural environment for infection.

Human fallopian tube organoids are obtained by digesting fallopian tube biopsies, before embedding epithelial cells into extracellular matrix and culturing in organoid growth medium. It was shown that the organoids can be maintained for more than a year in culture [48]. Fallopian tube organoids were successfully infected with several *Chlamydiatrachomatis* strains by mechanical disruption before the actual infection. Strikingly, in contrast to 2D infection models, the organoids survived for several months after infection, with detectable but decreasing numbers of inclusions. This allows for the first time to study chronic chlamydia infections in an *in vitro* model, which hopefully increases our understanding of how chronic low-grade infection induces pathologies like fibrosis, infertility and ectopic pregnancy [49].

Conclusion

For many years, monolayers of female reproductive tract cells like HeLa cells were standard to study molecular mechanisms of infections with sexually transmitted pathogens, such as *Chlamydiatrachomatis*. In addition, animal models allowed to get insight into the interplay between the host organism and the infectious agent. Animal models also helped us to understand immune mechanisms involved in combating STI as valuables tools for vaccination studies. However, exploring specific features of a pathogen interacting with the human organisms remained difficult, as most human cell lines originate from cancers. Only recent advances in culturing primary epithelial cells as well as being able to generate organoids from different human genital tract tissues will enable us to study the pathology of infectious diseases in a physiological environment without ethical concerns. Organoids will also allow testing different conditions and immune cell pathways in a high-throughput manner. This will help us to study interaction of different host cell types within "mini-organs" in steady-state and diseased tissue. However, organoids are more labor-intensive and expensive than traditional cell culture and will not entirely replace *in vivo* studies in animals and clinical studies. Therefore, this review provided an overview of different experimental models and strategies for STI research, which need to be tightly coordinated for future study designs.

Conflict of interest

The Authors declare that they don't have a conflict of interest.

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Curriculum Vitae

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Nationality: Austrian

Education

2019 – current	PhD in Immunology, CeMM PhD program, Medical University of Vienna
2016 – 2019	MSc in Molecular Medicine, Medical University of Innsbruck
2013 – 2016	BSc in Molecular Medicine, Medical University of Innsbruck
2013	Matura with distinction, PORG Volders

Study projects

PhD thesis	10/2019-current	"Preventive strategies and early immune responses in Chlamydia trachomatis infection" Department of Dermatology, Medical University of Vienna. Advisor: Dr. Georg Stary
Master's thesis	02/2018-09/2019	"Understanding the role of the PIDDosome in hepatocyte development and transformation" Division of Developmental Immunology, Medical University of Innsbruck. Advisor: Dr. Andreas Villunger
Bachelor's thesis	02/2016-03/2016	"Characterization of natural killer cells in a spontaneous melanoma mouse model" Laboratory for Langerhans Cell Research, Medical University of Innsbruck. Advisor: Patrizia Stoitzner, PhD
Rotation	11/2019-12/2019	"Illuminating the role of RhoG in cytotoxic granule release and actin remodeling at the immunological synapse" St. Anna Children's Cancer Research Institute. Advisor: Dr. Kaan Boztug

Internship	08/2017	Division of Cell and Developmental Biology, Medical University of Vienna. Advisor: Dr. Dietmar Abraham

Skills

Languages	German – native speaker English – excellent Italian – basic
Laboratory skills	mammalian cell culture, microbiology, DNA and RNA isolation, immunoblotting, qRT-PCR, flow cytometry, working with mice, immunofluorescence microscopy, migration assays, basic programming in R
Extracurricular	President of the Next Generation Immunologists 2022 – 2024 (Young immunologists activities group of the ÖGAI), Organization of the 1st NGI summer school in Vienna 2024, Co-organization of the young immunologists sessions at the DGFI/ÖGAI joint meeting 2022, Participation in xbio entrepreneurship course 2025, Scouting, Badminton

List of publications

Neuwirth N, Malzl D, **Knapp K**, Tsokkou P, Kleissl L, Gabriel A, … Stary G. The polyamine-regulating enzyme SSAT1 impairs tissue regulatory T cell function in chronic cutaneous inflammation. Immunity. 2025; 58(3):632-647

Knapp K, Klasinc R, Koren A, Siller M, Dingelmaier-Hovorka R, Drach M, … Stary G. Combination of compound screening with an animal model identifies pentamidine to prevent Chlamydia trachomatis infection. Cell Reports Medicine. 2024; 5(7):101643.

Ökengin D, Noori T, Alemany A, Bienkowski C, Liegon G, İnkaya AC, Carillo J, Stary G, **Knapp K**, Mitja O, Molina JM. Prevention Strategies for Sexually Transmitted Infections, HIV, and viral hepatitis in Europe Series. The Lancet Regional Health – Europe. 2023; 34:100760.

Kretschmer L, Pecht T, **Knapp K**, Bennstein S. Community perspective: the importance of in-person meetings for young immunologists. Eur J Immunol. 2022; 52(12):1876-1879.

Neuwirth T, **Knapp K**, Stary G. (Not) Home alone: Antigen presenting cell - T Cell communication in barrier tissues. Frontiers in Immunology. 2022; 13:984356.

Knapp K, Stary G. Models for sexually transmitted infections. Drug Discov Today Dis Model. 2020; 32:1–6.

Sladky V, **Knapp K**, Szabo TG, Braun VZ, Bongiovanni L, van den Bos H, … Villunger A. PIDDosome-induced p53-dependent ploidy restriction facilitates hepatocarcinogenesis. EMBO Rep. 2020; 21(12):e50893. (master thesis)

Sladky V, **Knapp K**, Soratroi C, Heppke J, Eichin F, Rocamora-Reverte L, … Villunger A. E2F-Family Members Engage the PIDDosome to Limit Hepatocyte Ploidy in Liver Development and Regeneration. Developmental Cell. 2020; 52(3):335-349. (master thesis)

Contribution to Conferences

Österreichischer Infektionskongress, Saalfelden, 2025, oral presentation

IUSTI Europe Congress, Zagreb, 2024, oral presentation

European Congress of Immunology, 2024, Dublin, poster presentation

ISDS International Skin Disease Summit, Vienna, 2023, Poster presentation

ÖGAI Annual meeting, Linz, 2023, Poster presentation and session chair

ADF Annual meeting, Innsbruck, 2023, Poster presentation

MESIA 5th Meeting of Middle Europe Societies of Immunology and Allergology 2022, Prague, oral presentation

11th Conference on Rare and Undiagnosed Diseases 2022, Vienna, oral presentation

Joint Meeting of DGfI and ÖGAI, Hannover, 2022, poster presentation and session chair

Julius Schachter 15th International Symposium on Human Chlamydial Infections 2022, San Antonio, Texas, oral presentation

ÖGDV Science Days, 2021, 2022, 2023, 2024, 2025, Austria, oral and poster presentations

Awards

Österreichischer Infektionspreis of OEGIT for the Cell Rep Med paper, Knapp *et al*; 2025

Wissenschaftspreis of ÖGDV for the Cell Rep Med paper, Knapp *et al*, 2024

IUSTI Presidents Award for best oral presentation, 2024

ÖGDV Science Days Mobility Award for best flash talk, 2022

FEMS Travel Grant, 2022

EFIS-EJI Travel Grant for MESIA conference, 2022