Characterization of Dendritic Cells in the Human Female Genital Tract and their Role in HIV-1 Pathogenesis

A thesis submitted by

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Abstract

The female genital tract (FGT) represents a complex and dynamic environment with specialized immune mechanisms uniquely designed to maintain a delicate balance between protection against invading pathogens and accommodating the unique physiological changes associated with reproductive function. It serves as a key entry point for sexually transmitted diseases like human immunodeficiency virus (HIV), prevention of which requires detailed understanding of cells involved in HIV pathogenesis. Furthermore, aging and menopause induces a systemic decline in functional properties of innate and adaptive immune cells. However, the immunological changes that happen in the FGT as women age are poorly understood. Dendritic cells (DCs) in the FRT constitute a diverse population of innate cells with critical roles in reproduction, mounting adaptive responses against pathogens, including HIV. Previous work from our group has demonstrated subset-specific, and age-dependent increase in ability of endometrial DCs to induce key tissue residency marker expression (CD103) on CD8+ T cells. These subset-specific functional differences extend to HIV response, where we established differential ability of FGT DC subsets in mediating HIV capture and observed rapid secretion of anti-HIV proteins upon HIV exposure. However, key mechanistic insight into factors driving DC subset-specific functional and phenotypic differences remains undefined. In this dissertation, I hypothesized that FGT DC's phenotypic and functional heterogeneity is driven through differential levels of activation status, ability to detect pathogens, and secrete cytokines and chemokines. To test this hypothesis, we processed hysterectomy samples from women aged between 27-77, to generate single-cell suspensions via enzymatic digestion. FGT resident CD14+ and CD1a+ DCs were isolated using magnetic beads and co-cultured with labeled allogeneic naïve T cells to measure T cell proliferation after 7 days using flow cytometry and levels of secreted proteins in supernatants with multiplex ELISA. Furthermore, we

characterized functional and phenotypic properties of FGT DCs using multi-omic spectral flow cytometry and CITEseq, Lastly, we investigated the transcriptional changes in FGT DCs upon exposure to HIV, mimicking exposure within the FGT. We established age-related induction of unconventional double negative T (DNT) cell proliferation by genital DCs. Specifically, CD1a+ DCs promote DN T cell proliferation in a TFG-β-dependent manner, which was more pronounced in older women. DNT cells in FGT exhibit unique profiles and functions compared to CD4+ and CD8+ T cells, with enhanced expression of inhibitory molecules and genes related to immune regulation and antiviral responses. Flow cytometry revealed expression of tissue residency markers, and cytotoxic molecules by DN T cells. Notably, age and location within the genital tract affected DN T cell distribution and function, where endometrial DN T cells become more cytotoxic with age, while ectocervical DN T cells were associated with reduced cytotoxicity. We found that the genital $CD11c^{+}HLA\text{-}DR^{+}$ myeloid population contains three DC subsets (CD1c+ DC2s, CD14+ monocyte-derived DCs and $CD14^{+}CD1c^{+}$ DC3s) and two monocyte/macrophage populations with distinct functional and phenotypic properties during homeostasis. Following HIV exposure, the antiviral response was dominated by DCs' rapid secretory response, activation of non-classical inflammatory pathways and host restriction factors. Further, we uncovered subset-specific differences in anti-HIV responses. CD14+ DCs were the main population activated by HIV and mediated the secretory antimicrobial response, while CD1c+ DC2s activated inflammasome pathways and IFN responses. These novel findings establish the role of FGT DC subsets in modulating tissue environment through mediating tissue homeostasis, detection of pathogens, secretion of cytokines, modulating T cell phenotype, and differential HIV response. Findings from this thesis could aid targeted therapeutic strategies to improve health outcomes at the mucosal tissue in FGT.

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13 and can probably identify the name of the font by looking at it. Thank you very much for your friendship and your mentorship Fran!

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*Equal contribution

List of Abbreviations

AMP – Antimicrobial peptides
APC – Antigen presenting cells
BCR – B cell receptor
cDC – conventional dendritic cells
CDP – Common dendritic cell progenitor
CITEseq – Cellular indexing of transcriptomes and epitopes by sequencing
CLR – C-type lectin receptors
CMP – Common monocyte progenitor
CTL – Cytotoxic T cell
DAMP – Damage-associated molecular pattern
DC – Dendritic cells
dNK – Decidual natural killer cells
DNT – Double negative T
dsDNA – Double-stranded DNA
dsRNA – Double-stranded RNA
ECX – Ectocervix
EM – Endometrium
END – Endocervix
FGT – Female genital tract
FSH – Follicle-stimulating hormone
GO – Gene Ontology
GPI – Glycosylphosphatidylinositol
GZA – Granzyme A
GZB – Granzyme B
HIV – Human immunodeficiency virus
ILC – Innate lymphoid cells
infMons – Inflammatory monocytes
IRAK – IL-1R-associated kinase
LH – Luteinizing hormone
LPS – Lipopolysaccharides
MAIT – Mucosal-associated invariant T
MHC-I – Major histocompatibility complex I

MHC-II – Major histocompatibility complex II

MoDC – Monocyte-derived dendritic cells

MoMac – Monocyte-derived macrophages

MyD88 – Myeloid differentiation primary-response gene 88

NCM – Non-classical monocytes

NET – Neutrophil extracellular traps

NK – Natural killer cells

NLR – Nucleotide-binding oligomerization domain-like receptors

PAMP – Pathogen associated molecular pattern

PCA – Principal component analysis

PCOS – Polycystic ovary syndrome

pDC – Plasmacytoid dendritic cells

PrEP – Pre-exposure prophylaxis

PRR – Pattern-recognition receptor

RLR – Retinoic acid-inducible gene-I-like receptors

ssRNA – Single-stranded RN

STI – Sexually transmitted infections

TCR – T cell receptor

Tfh – T follicular helper cell

Th – Helper T cell

TLR – Toll-like receptor

TNF – Tumor necrosis factor

TRM – Tissue-resident memory

UMAP – Uniform manifold approximation and projection

Chapter 1: Introduction

1.1 Features of the Immune System

The immune system represents an intricate defense mechanism in multiple organisms, crucial in safeguarding against various threats ranging from pathogens to cancer. It constitutes a remarkable network of cells, tissue, and organs that work together to maintain a healthy body and fight against pathogens. Multiple decades of research have further unraveled the specialized functions of the immune system, from recognizing, responding, and neutralizing foreign pathogens to an intricate system that allows long-term memory and rapid response upon subsequent encounters against the same pathogen [1-8]. The immune system is broadly categorized into systemic and mucosal immunity, each branch being associated with key characteristic features, including differences in immune cell function and phenotype.

Systemic immunity refers to the immune response occurring primarily in the bloodstream and the lymphatic system, providing defense against pathogens that enter the body through open wounds, insect bites or the bloodstream. Systemic immunity primarily involves the adaptive immune system, characterized by their ability to recognize specific pathogens and mount a targeted response. T cells and B cells play a crucial role in shaping systemic immune response in addition to antigen presenting cells (APCs) that present antigens and activate T cells.

The mucosal immune system serves as a diverse and vital cog in maintaining a balance between immune response against pathogens and preventing local tissue destruction [9]. Intricate physical barriers and cellular mechanisms in place mediate responses at mucosal barriers, which are continuously exposed to external factors such as air and food-borne allergens, bacteria, viruses, and other pathogens depending on the tissue location (**Figure 1.1**). Mucosal immune response is primarily mediated by the innate immune system, characterized by their ability to indiscriminately recognize pathogens through pre-existing mechanisms in our genes. Understanding the local tissue

environment at mucosal surfaces, could prove key to uncovering novel therapeutic interventions against invading pathogens. The work done in the thesis focuses on understanding the local tissue environment at mucosal surface of the female genital tract (FGT), which provides key insights into uncovering potential interventions against HIV infection.

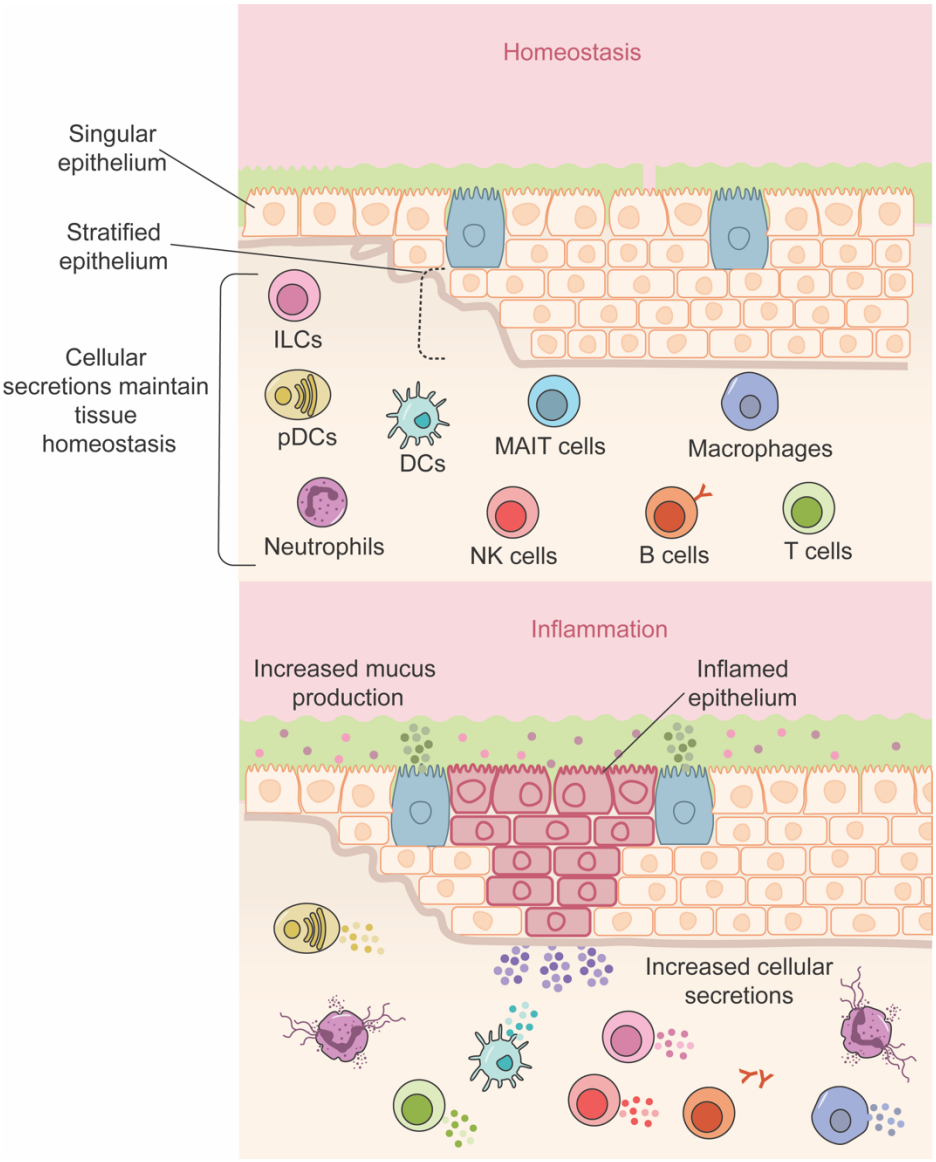

Figure 1.1 Physical, cellular and chemical barriers at mucosal sites.

Mucus and diverse epithelial cell layers mediate physical barriers at mucosal surfaces. Immune cells in the tissue mediate tissue homeostasis through secretions that promote healthy tissue environment. Upon initiation of local inflammation through exposure to

pathogens or wounds, several cellular and secreted mechanisms come into play to prevent infections and promote wound healing.

1.2 Mucosal Immunity

The main components of the mucosal immune system include the upper and lower respiratory tract, which mainly comes into contact with airborne pathogens; the gastrointestinal tract, which comes into contact with foodborne pathogens; and the urogenital tract, which comes into contact with surface and sexually transmitted pathogens [10]. The systemic and mucosal immune systems share salient features such as protection against invading pathogens, involvement of similar cell types such as B cells, T cells, macrophages, and dendritic cells influencing innate and adaptive immune responses, mechanisms mediating recognition of pathogens, and downstream effector functions [10]. Unique to the mucosal immune system, cell and cell-intrinsic soluble factors play a crucial role in promoting tolerance to benign antigens, such as food and commensal bacteria, while protecting against pathogens through an interplay between physical barriers, chemical barriers, and cellular barriers [9].

1.2.1 Physical barriers at mucosal sites.

Physical barriers, such as the mucus layer and epithelial cells, serve as the first line of defense against foreign pathogens (**Figure 1.1**). Encountering pathogens induces increased production of secreted factors such as enzymes, mucins, and antimicrobial peptides by epithelial cells and specialized mucus-secreting cells such as goblet cells. Increased mucus secretion traps pathogens in the mucus layer, preventing entry into the body [11]. The epithelium serves as a major barrier to prevent the entry of pathogens, signified by the presence of different types of epithelial layers with specialized functions. The singular epithelium is a single layer of epithelial cells providing a thin barrier and is responsible for absorption, filtration, and diffusion, mainly found in the lungs, kidneys,

intestines, and genital tract. The stratified represents multiple layers of epithelial cells, providing protection in areas subject to frequent abrasions and wear, such as the mouth, esophagus, and vagina. Pseudostratified epithelium layers are often ciliated and form a layer of epithelial cells of various heights and are involved in the secretion and movement of mucus like in the upper respiratory tract. The specialized epithelial layer known as transitional epithelium is found in the bladder and ureters, where they play an important role in the expansion and contraction of the organ without losing structural integrity [12].

1.2.2 Chemical barriers at mucosal sites.

Pathogen-induced activation or damage to epithelial cells initiates a cascade of signaling events that produce cytokines and chemokines that promote activation of mucosal-resident immune cells and infiltration of peripheral immune cells (**Figure 1.1**). Key enzymes and antimicrobial proteins, including myeloperoxidase, lysozymes, the family of alpha- and beta-defensins, and cathelicidins (LL-37), directly target and destroy pathogens [13]. Cytokines such as IL-4, IL-13, and IL-22 promote the production of mucins, antimicrobial peptides, and tight junction proteins and reinforce the barrier integrity function of epithelial cells in addition to activation of other immune cells [14-16]. Production of CCL2 attracts monocytes, CCL5, CCL20, and CXCL12 recruit lymphocytes [17-19], whereas CXCL8 plays a crucial role in the recruitment of neutrophils [20]. Growth factors such as EGF, TGF-β and VEGF production promote wound healing and modulate inflammation [21-23]. These intricate mechanisms and interactions play a crucial role in maintaining a balance between immune response and tissue damage. Additionally, differences in composition, structure, phenotype, and function of physical, cellular, and chemical barriers cater to the unique demands of the different mucosal surfaces in the human body.

1.2.3 Cellular barriers at mucosal sites.

Multiple innate and adaptive immune cells populate mucosal sites (**Figure 1.1**) [24]. Tissue intrinsic factors drive mucosal immune cell phenotype and function to maintain a critical balance between immune response and wound repair [25, 26]. These factors give rise to distinct subsets of macrophages, dendritic cells, monocytes, natural killer (NK) cells, and Th cells with unique phenotypes and functions. Additionally, immune cells such as innate lymphoid cells (ILCs), decidual NK cells (dNKs) – specific to the female reproductive tract, and mucosal-associated invariant T (MAIT) cells are uniquely found enriched at mucosal sites, with varying phenotypes and functions dependent on tissue location [27]. For example, in the lungs, phagocytosis of pathogens and wound healing is mediated by different macrophage subsets [28, 29], enrichment of Tregs in the gastrointestinal tract exert tolerance and modulate the immune response against commensal bacteria and food antigens [30, 31], dNK cells in the female reproductive tract play a crucial role in maintaining pregnancy and maternal-fetal interactions [32], further emphasizing compartmentalization of immune cells in a tissue-dependent manner. The contribution of different immune cells in mediating local tissue homeostasis and response to invading pathogens will be discussed in-depth below.

1.2.4 Innate Immune System

The innate immune system is the first line of defense against pathogenic insults. When pathogens breach the physical skin barrier, innate immune mechanisms emerge, relying on pre-existing mechanisms encoded in our genes. These factors serve as barriers against pathogens, including physical barriers (mucus, epithelial cells), chemical barriers (antimicrobials, cytokines, chemokines, enzymes), and cellular barriers (effector immune cells) [2, 33, 34]. Effector immune cells such as neutrophils, monocytes, macrophages, and dendritic cells play a crucial role in detecting pathogens, processing and

presentation of antigenic protein and cellular secretions to prevent infection and mediate wound repair to avoid the destruction of local tissue [2, 35] (**Figure 1.2**).

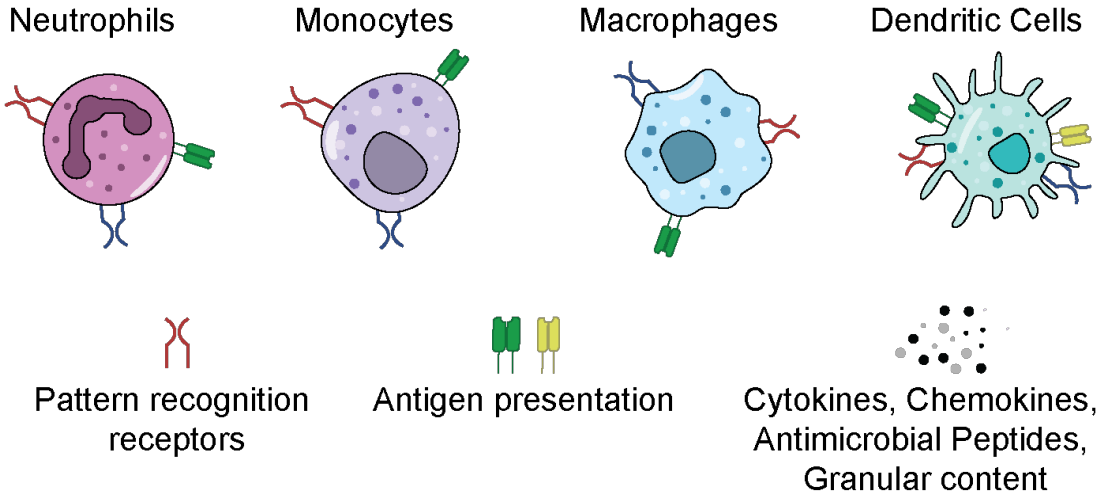

Figure 1.2 Characteristics of innate immune cells.

Immune cells such as neutrophils, monocytes, macrophages and DCs mediate innate immune responses through recognition of PAMPs and DAMPS through PRRs, present foreign antigens, and secrete proteins such as cytokines, chemokines, antimicrobial peptides and cytotoxic granules.

1.2.4.1 Detection of pathogens

Innate immune cells offer rapid protection against invading pathogens by recognizing damage-associated or pathogen-associated molecular patterns (DAMPs and PAMPs) via pattern-recognition receptors (PRRs). PRRs encompass a family of proteins, such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), C-type lectin receptors (CLRs), and retinoic acid-inducible gene-I-like receptors (RIG-I-like receptors, RLRs) that differentiate from healthy human cells (self) and microbes (non-self) [36, 37]. These receptors bind to various pathogenic moieties such as bacterial lipopolysaccharides (LPS), nucleic acids associated with viruses, components of fungi, and endogenous components associated with damaged and dying cells. The detection of pathogens at different stages of exposure, infection, and cellular damage is mediated through strategic distribution of PRRs on the cell surface,

endosomal and lysosomal compartments, and within intracellular compartments, which upon binding to PAMPs/DAMPs trigger signaling cascades leading to activation of immune cells, secretion of cytokines, chemokines, antimicrobial proteins, migration, and recruitment of other immune cells [7, 38]. In addition to the expression of PRRs, innate immune cells exert different functional responses depending on cell type, which varies by phenotype, location, and secreted factors.

1.2.4.2 Innate Immune Cells

Cells exerting immune function are broadly termed white blood cells, or leukocytes, which are continuously generated by the body through a process called hematopoiesis. Pluripotent hematopoietic stem cells give rise to erythrocytes (blood cells), megakaryocytes (platelets), and myeloid and lymphoid precursors that further differentiate into various cell types [39, 40]. In addition to cell type, immune cells perform specific functions in detecting, engulfing, and destroying pathogens, initiating inflammation, wound healing, and activating adaptive immune responses [2, 33, 37]. Neutrophils constitute the most abundant cell type circulating in the blood and often serve as the first and rapid response to various pathogens. Upon encountering a pathogen, neutrophils respond by releasing granules, chemokines/cytokines, and secreted proteins with antimicrobial properties [41-43]. A more recent discovery unraveled the ability of neutrophils to release neutrophil extracellular traps (NETs) composed of decondensed chromatin and DNA fragments coated with antimicrobial proteins that trap and inactivate viruses and bacteria [44-46]. In addition to protective properties, neutrophils can cause tissue damage at the site of infection, as seen in the recent COVID-19 pandemic, where increased infiltration and activation of neutrophils were detrimental to the host's health [47, 48].

Monocytes play a crucial role in mediating innate immunity and represent a critical line of defense against pathogens [49]. They circulate in the blood and are a versatile cell type

known for infiltrating tissues, where they can differentiate into macrophages or dendritic cells depending on the tissue environment [50{Wolf, 2019 #189]}. Monocytes' plasticity contributes to their ability to mediate wound repair and regulate inflammation, making them key players in maintaining a healthy body.

Macrophages populate all tissues and organs throughout the body to engulf and digest pathogens, clear cell debris, initiate inflammation, and mediate wound healing [51-53]. Their multifaceted role is due to the presence of different subsets with unique and specific functions that enable them to adapt to different tissues in response to local environmental cues. Additionally, macrophages can serve as antigen-presenting cells (APCs) by presenting peptide antigens to T cells, thereby activating them. These versatile functions make them key players in defense against pathogens and maintaining homeostasis.

Dendritic cells (DCs) epitomize the immune system’s complex and sophisticated surveillance and response capabilities, and they serve as orchestrators of innate and adaptive immune responses [54-56]. These specialized cells constantly survey their environment and, upon encountering pathogens or foreign substances, initiate a cascade of events leading to the development of a targeted immune response against the antigen. Notably, DCs are professional APCs due to their unique ability to educate naïve T cells, a type of adaptive immune cell that has not been primed by exposure to foreign antigens [54, 57-60]. DCs are pivotal contributors to immune regulation and tolerance, playing a delicate role in balancing robust responses against pathogens and, at the same time, preventing harmful responses against self-tissue [61, 62]. These specialized functions of DCs make them attractive targets for vaccination strategies in infectious diseases, cancer, and possible intervention targets in cases of tissue transplantation. Thus, these cells serve as the primary focus of my thesis and will be discussed in detail in the following sections, including their role in the adaptive immune

system, the constantly changing landscape of DC subsets, and efforts to identify key characteristics of DCs.

1.2.5 Adaptive Immune System

The adaptive immune system serves as a remarkable defense system against various threats. Unlike the innate immune system, which offers a rapid and nonspecific defense response against pathogens, the adaptive immune system exerts a response with high specificity and memory [3, 6]. The main cellular components of the adaptive immune system include T cells and B cells.

T cells represent a crucial component of the adaptive immune system through precise recognition of antigens, subsequently exerting specific immune response against them [63]. These cells originate in the bone marrow and develop in the thymus, a step crucial to their development in differentiating between self-tissue and foreign antigens [64-66]. Central to their function are T cell receptors (TCRs), which are specialized proteins that enable recognition of antigens presented by APCs. T cells are broadly classified into CD4+ helper T cells (Th) and CD8+ T cells, or cytotoxic T cells (CTLs) (**Figure 1.3**). Th cells play a pivotal role in coordinating the immune response by releasing cytokines and chemokines to eliminate pathogens, induce production of antibodies, and activate other immune cells [67]. CD8+ T cells, in contrast, directly target and destroy infected or abnormal cells which is crucial for eliminating intracellular pathogens and cancerous cells [68]. Moreover, T cells possess the remarkable ability to form immunological memory. Following an initial encounter with an antigen, a subset of T cells transitions into memory T cells that persist in the body, ready to mount a faster and more robust response upon subsequent encounters with the same antigen, thereby providing long-lasting immunity [69].

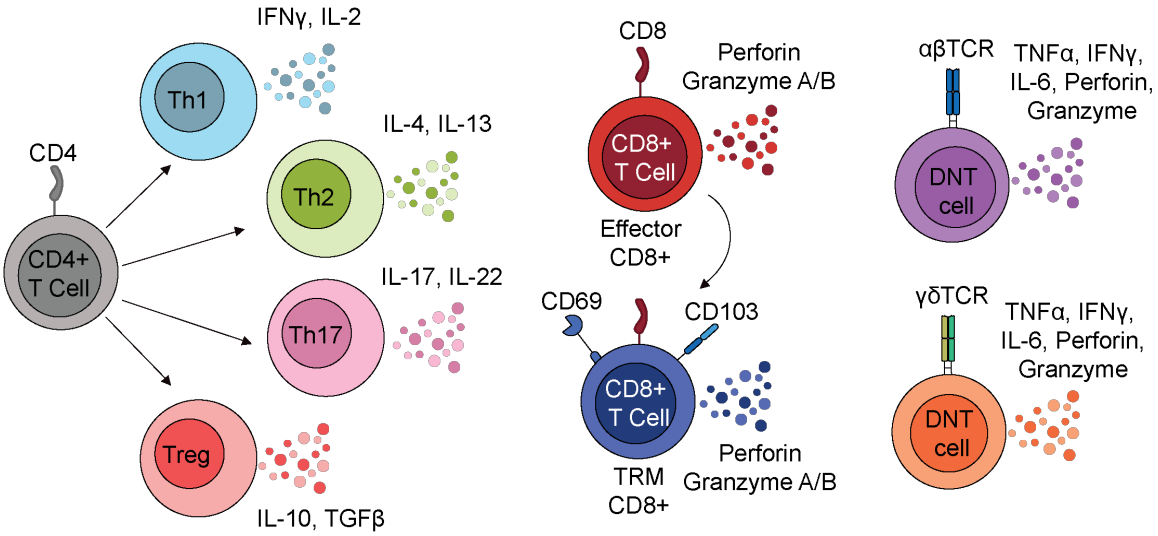

Figure 1.3 Human T cell phenotypes and function

CD4+ T cells in humans constitute 4 distinct subsets which are broadly characterized depending on key cytokine signatures, attributing to unique functional properties. Primarily, Th1 cells produce large amounts of IFN-γ and IL-2, Th2 cells IL-4 and IL-13, Th17 IL-17 and IL-22, and Tregs IL-10 and TGF-β. CD8+ T cells acquire an effector phenotype upon exposure to cognate antigen, and produce effector molecules such as perforin, and granzyme A/B. Upon resolution of pathogenesis, some CD8+ T cells transition to tissue resident-memory (TRM) cells that express key tissue residency markers such as CD69 and CD103. Unconventional T cells lacking expression of CD4 or CD8 on the surface, termed as double negative T (DNT) cells, represent a rare T cell subset with heterogenic functional properties.

1.2.5.1 CD4+ T Cells

CD4+ T cells, or Th cells, derive their name from the expression of CD4 receptor protein on their surface, which interacts with major histocompatibility complex class II (MHC-II) on APCs. These cells demonstrate tremendous diversity due to their ability to differentiate into distinct functional subsets such as T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), and regulatory T (Treg) cells, each with unique functions [70, 71]. Th1 cells largely mediate immunity against intracellular pathogens, whereas Th2 cells play a unique role in mediating the secretion of antibodies by plasma cells or directly secrete proteins against extracellular pathogens [72, 73]. Th17 cells are associated with response against bacteria, fungi and autoimmune inflammation, in addition to their

unique role in mucosal surfaces due to their ability to maintain barrier integrity [74, 75]. In contrast, Tregs are known for their role in maintaining tissue homeostasis and preventing autoimmune responses due to their primary function of suppressing excessive activation and inflammation [76, 77]. The multifaceted ability of CD4+ T cells to mediate protection against different factors makes them a crucial player in regulating the adaptive immune response.

1.2.5.2 CD8+ T Cells

CD8+ T cells are also known as CTLs due to their ability to directly target and eliminate cells infected with intracellular pathogens or tumor cells [63, 68]. Upon activation, CTLs release cytotoxic molecules such as perforins and granzymes, leading to the death of target cells. Additionally, CTLs produce cytokines to activate other cells and further enhance the immune response [68]. Moreover, upon resolution of the initial infection, the production of memory CTLs provide long-lasting immunity upon re-exposure to the same pathogen [78, 79]. Understanding the role of CTLs and memory CTLs is essential to generate immunotherapies to enhance immune responses and sustain long-term protection upon subsequent insults.

1.2.5.3 Double Negative T Cells

Double negative T (DNT) cells constitute a subset of T cells that lack expression of CD4 and CD8 on their surface [80]. However, a small population of DNT cells are present in blood and peripheral tissues, with known roles in autoimmune disease and cancer [81, 82]. Since these advances in the characterization of DNT cells have been recent [83-86], further research is required to elucidate the role of these cells in the immune system since they have shown the ability to mediate innate and adaptive immune functions.

1.3 Female Genital Tract

The female reproductive tract (FRT) is a unique mucosal surface with a challenge to prevent sexually transmitted infections (STIs) and enable successful pregnancy outcomes [87, 88]. The anatomy of the FRT plays a crucial role in the compartmentalization of barrier structure, integrity, composition, homeostasis, and immune response in the organ. Intricate mechanisms are in place to orchestrate this delicate balance, encompassing sex hormones, cellular secretions, and cell-cell interactions [87]. An in-depth understanding of the immune system in the FRT provides insight into several factors, such as adverse pregnancy outcomes, STIs, and even tumors.

1.3.1 Anatomy of the Female Genital Tract.

The FGT constitutes a series of connective tissues and organs, sectioned into the upper tract consisting of endometrium, fallopian tubes, and ovaries, and the lower tract consisting of the vagina and the cervix (**Figure 1.4**) [89]. The lower tract is constantly exposed to external pathogens, including STI-causing agents, whereas the upper tract is responsible for reproduction [87, 89]. Despite this distinction in location and function, the lower tract and upper tract communicate through secreted reproductive hormones, chemokines, cytokines, and cell-cell contact to condition the tissue for successful pregnancy outcomes or responses to invading pathogens.

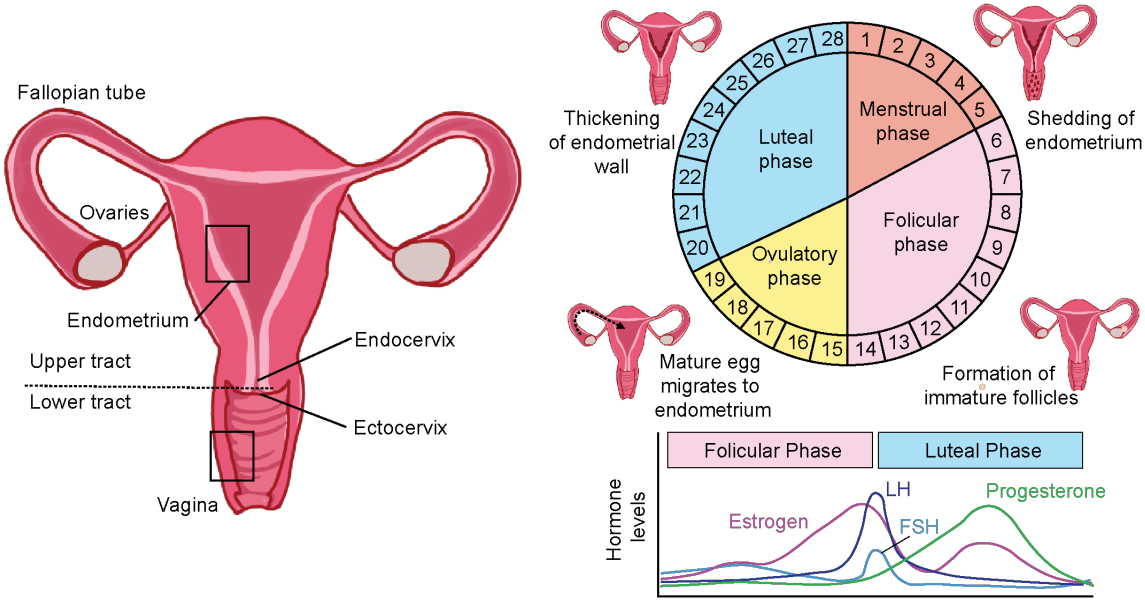

Figure 1.4 Anatomy of the FGT, the menstrual cycle and hormonal control.

The FGT is separated into the upper tract - consisting of ovaries, fallopian tube, endometrium and endocervix - and the lower tract consisting of the ectocervix and vagina. The menstrual cycle is characterized by physiological and hormonal changes under tight control.

1.3.1.1 Lower tract

The lower tract consists of the vagina and the ectocervix and has a distinctive stratified epithelial layer characterized by multiple layers of epithelial cells, which offers enhanced barrier protection against bacteria, viruses, and fungi. The vagina is located at the entrance to the FGT and is populated with commensal bacteria, which is crucial to maintaining tissue homeostasis. The major population of commensal microbiota is *Lactobacillus,* which contributes to a healthy microenvironment. Increased bacterial diversity and non-dominance of *Lactobacillus* contributes to vaginal dysbiosis [90]. Mucus secretions in the vagina not only offer physical protection against pathogens, enzymes and antimicrobial peptides (AMPs) in the mucus provide protection against pathogens [87]. The ectocervix is a part of the cervix that protrudes into the vagina and plays a crucial role in maintaining barrier protection in preventing pathogens from entering the uterine cavity, mucus secretions that change depending on the stage of the

menstrual cycle to allow or prevent the entry of sperm into the upper tract and offer structural support to the cervix and maintain the position of the uterus. Overall, the lower tract plays a significant role as a barrier against pathogens and maintains the demands of supporting pregnancy in the upper tract.

1.3.1.2 Upper tract

The upper tract consists entirely of connective tissue and organs responsible for tissue health and fertility. It includes the uterus—comprised of the endocervix, a canal leading to the opening of the uterus; the endometrium, which is the inner lining; and the myometrium, which is the outer lining of the uterus; fallopian tubes, the pathway through which eggs travel to the uterus; and ovaries that produce the eggs (ova) and hormones that regulate menstrual cycle and support reproductive function [91, 92]. The upper tract mediates the intricate mechanisms and signaling cascades required for a successful pregnancy outcome. Since the fetus is genetically distinct from the mother, a delicate equilibrium must be achieved in order to prevent rejection of the fetus but at the same time protect the mother from invading pathogens. Initial theories by Dr. Medawar regarding pregnancy and the tissue environment [93] suggested a highly immunosuppressive environment throughout the pregnancy term. Although immunosuppressive cytokines such as IL-10 and TGF-β are present in the second and third trimesters, recent studies demonstrate during a large part of the first trimester and the end of pregnancy where the tissue environment is very much pro-inflammatory [94]. The intricate and precise role of immune cells, cytokines, and hormones in maintaining equilibrium plays an important role in shaping the health of the fetus and the mother.

1.3.2 Menstrual Cycle.

The menstrual cycle, central to female reproductive health, is a series of physical transformations, hormone fluctuations, and changes in immune cell composition to

facilitate the maturation and release of an egg from the ovaries, preparation of uterine lining for implantation, and if pregnancy does not occur, shedding of the uterine wall through menstruation [92]. The cycle spans approximately 28 days and can be divided into 4 distinct phases, characterized by physical and hormone fluctuations (**Figure 1.4**). The menstrual phase marks the beginning of the cycle, characterized by the shedding of endometrial tissue, which thickens in preparation for pregnancy. The proliferative (follicular) phase follows menstruation and generally lasts from days 1-14 and is characterized by increased levels of follicle-stimulating hormone (FSH), responsible for forming immature egg-containing follicles. During the mid-cycle ovulatory phase on day 14, the mature egg-containing follicles release estrogen, which triggers a surge in luteinizing hormone (LH), thereby causing the follicle to rupture and release the egg into the fallopian tube. Subsequently, in the secretory (luteal) phase from days 15-18, the ruptured follicle transforms into the corpus luteum, which mediates the secretion of progesterone and some estrogen. Progesterone serves as a key pregnancy hormone important to the maintenance of a thick endometrial lining, ensuring an environment conducive to embryo implantation. If fertilization and implantation do not occur, the corpus luteum degrades, leading to a drop in progesterone and estrogen levels. Declining levels of these hormones trigger the shedding of the thick endometrial lining, marking the onset of menstruation and the start of a new cycle. The menstrual cycle is an important indicator of reproductive health and physical well-being [92]. Regular menstrual cycles suggest hormonal balance and tissue equilibrium, whereas irregularities could indicate underlying issues such as polycystic ovary syndrome (PCOS), thyroid disorders, or any other hormonal irregularities. Ongoing research and advancements in reproductive health play a significant role in the understanding of the menstrual cycle, revealing new opportunities for improving health and pregnancy outcomes.

1.3.3 Aging and Menopause.

Aging is a natural process with multifaceted effects on the human body, which extend to every organ, including the FGT. Changes in the FGT due to age significantly affect women's overall health, fertility, and quality of life. Aging affects integral features of the FGT, including changes to ovarian, uterine, and vaginal morphology, function, and hormone levels [87]. The decline in ovarian reproductive function leads to a decrease in the number of ovarian follicles, causing lower levels of estrogen and progesterone production [95]. Uterine changes include thinning of the endometrium and reduced responsiveness to hormonal signals, whereas in the lower tract, decreasing estrogen levels lead to thinning of vaginal walls, decreased elasticity, and reduced lubrication [96]. These changes begin slowly in the early to late thirties and accelerate once approaching forties and fifties.

The onset of menopause signals the end of reproductive capability and is defined as the cessation of menstruation for 12 consecutive months [96]. Humans are unique due to their ability to live several decades post-menopause [97], making it crucial to understand menopause-induced changes for the health and well-being of the individual. Significantly lower levels of estrogen and progesterone compared to pre-menopause women translate to changes in vaginal microbiome, secreted proteins such as cytokines, chemokines, antimicrobial proteins, and immune cell function and phenotype [98]. Changes in these factors contribute towards increased susceptibility to sexually transmitted infections (STIs), cardiovascular, and bone disease, further highlighting the integral role of the FRT in maintaining the health of women [98]. Given the delicate balance between reproduction, response to invading pathogens, and how significant the effects of aging and menopause are on integral mechanisms that mediate key tissue function and homeostasis, it is of utmost importance to better understand these changes. Projections estimate an increase in the number of people above the age of 65

could represent about 30% of the worldwide population by 2050, therefore adding further pressure to fully understand the effects of aging and menopause to develop interventions and improve quality of life.

1.3.4 Role of Immune Cells in the FGT

Immune cells in the FRT play an important role in maintaining tissue reproductive function, homeostasis, and response to pathogens. The tissue environment in the FRT is constantly evolving to support the needs during pregnancy and the menstrual cycle, which reflects on immune cell composition, phenotype, and function. In addition to fluctuating hormone levels, changes in levels of cytokines and chemokines throughout the menstrual cycle and pregnancy exert their effects on immune cells [87]. Epithelial cells, fibroblasts, and leukocytes in the FGT initiate innate and mediate adaptive immune responses in the tissue. Given the anatomy of the FGT, there exist changes in the composition of immune cells depending on location withing the FGT (**Figure 1.5**). Leukocytes in the FRT account for 6-20% of total cells, with CD3+ T cells being the most dominant subset, followed by NK cells, neutrophils, macrophages, dendritic cells, and B cells, with a higher frequency of leukocytes in the upper tract compared to the lower tract [99]. Changes in the menstrual cycle, aging, and menopause exert their effects on phenotype and function.

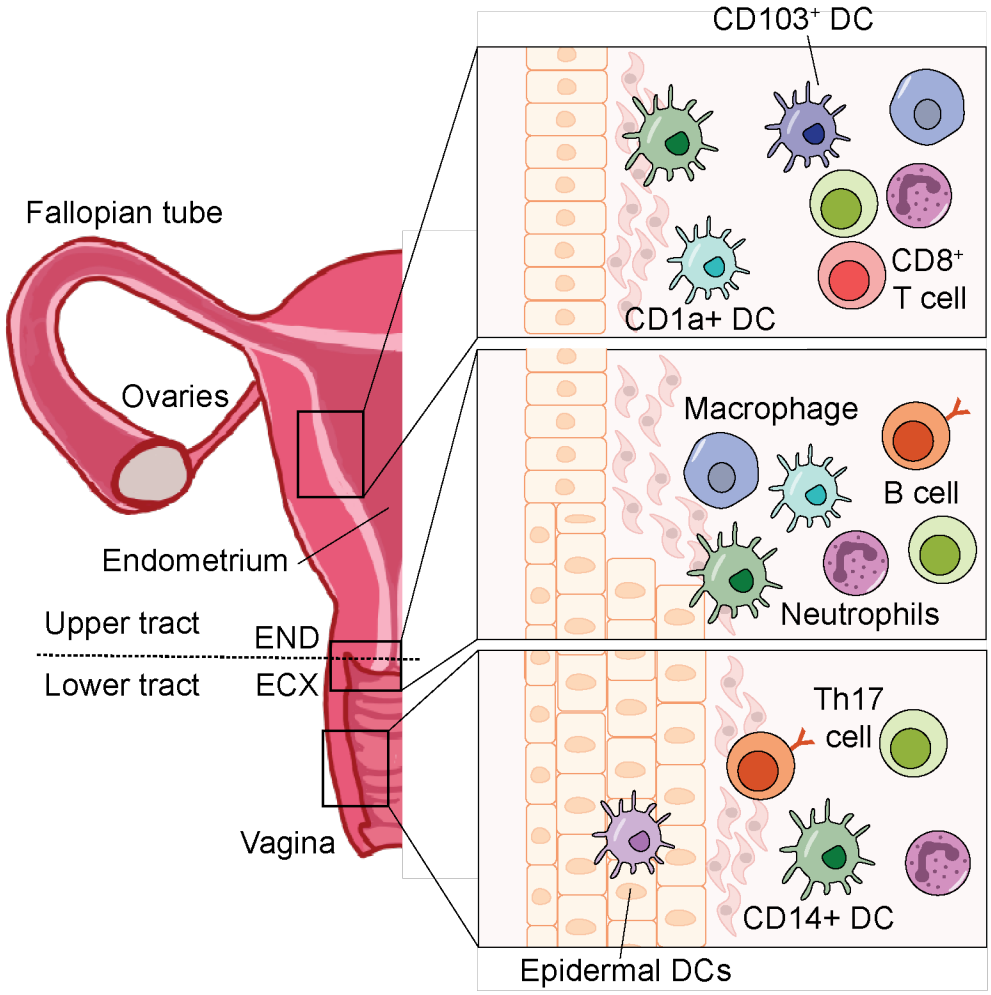

Figure 1.5 Composition and compartmentalization of immune cells in the FRT

The FGT is characterized by compartmentalization of immune cells, with unique phenotype and function depending on tissue location. It is comprised of macrophage, T cell and DC subsets with unique functional properties, including CD8 T cells, B cells, and neutrophils. Characterization of DC populations, reveal unique subsets in different locations. CD14 DCs and conventional CD1a+ DC populations are present throughout the FGT, whereas CD103+ DCs are uniquely enriched in the endometrium.

1.3.4.1 T Cells

The majority of T cells in the FGT have a memory phenotype and are distributed throughout the FGT. Studies have shown that the direct cytotoxic activity of tissue-resident memory (TRM) cells, identified based on CD69 and CD103 expression, exhibit tissue-specific differences based on menopause, with increased levels of TRMs in

endometrium compared to the cervix in post-menopause women [100-102]. CTLs within the FGT mediate the rejection of the fetus, which leads to recurrent pregnancy loss and infertility [103, 104]. Although mechanisms leading to the suppression of CTLs are unknown, studies have shown that the cytotoxic ability of CTLs increases, and TGF- mediated suppression is decreased post-menopause [101]. Additional functional changes to CTLs include increased degranulation ability and higher levels of proinflammatory Granzyme A phenotype post-menopause [101].

Th17 cells in the FRT play a crucial role in the maintenance of epithelial barrier function and are the most abundant Th cell population in the cervix of pre-menopause women but present at lower numbers in the endometrium. However, there is an increase in the number of Th17 cells in the endometrium with no changes to the cervix post-menopause, suggesting a level of relevance for the successful outcome of reproductive function [105]. Menopause-dependent fluctuations in the composition, phenotype, and function of T cells within the FGT warrant further studies to understand and improve the health outcomes of post-menopausal women.

Little is known about DN T cells in humans, where they have largely been studied under pathological conditions such as tumors, transplantation and autoimmune diseases, but their distribution, function and phenotype in tissues is largely unknown. Although the presence and function of FGT resident DN T cells and their contribution to pregnancy have been described in mice ([106]), however their role in the FGT remains poorly understood. Given their important role in multiple disease settings, characterization of DN T cells under homeostatic conditions and their response under pathogenic conditions represents an important gap in knowledge requiring further studies.

1.3.4.2 Myeloid cells

Mononuclear phagocytes (MNPs) such as monocytes, macrophages and DCs, and multi-nucleated neutrophils constitute a majority of myeloid cell populations in the FGT.

These cell types serve as sentinels that play crucial roles in recognizing and responding to pathogens, in addition to maintaining tissue homeostasis. Monocytes and macrophages in the FGT play a crucial role in menstruation and pregnancy by maintaining tissue homeostasis, wound repair, clearance of debris and implantation of the fetus [107, 108].

The role of neutrophils in the FGT remains a topic of controversy due to a significant lack of knowledge. Our group has previously demonstrated the role of neutrophils in HIV pathogenesis with their ability to trap and inactivate HIV, however their role under homeostatic conditions remains unknown.

DCs in the FGT play an important role in surveillance of tissue environment, determining response to pathogenic insults in addition to tuning the microenvironment conducive to pregnancy. The composition, distribution, phenotype and function of DCs in the FGT is the main focus of this thesis and is discussed in further detail in subsequent chapters.

1.4 HIV

Human immunodeficiency virus (HIV) remains a global problem, with approximately 38 million people currently living with HIV infection - 19.2 million of them being women (UNAIDS 2019) [109]. In endemic regions like Sub-Saharan Africa, the rate of HIV acquisition in women is significantly higher compared to men and represents an urgent unmet need for intervention [110]. Several factors, such as educational awareness, socio-economic disparities, and sexual violence, contribute to the difference in HIV acquisition [110]. The development of current strategies, derived from in-depth knowledge of HIV viral structure and viral life cycle, has proven successful in suppressing and delaying the progression of HIV infection. Although pre-exposure prophylaxis (PrEP) prevents the virus from establishing permanent infection upon exposure [111], the lack of an effective vaccine against HIV remains elusive.

The main route of HIV acquisition in women is through sexual transmission, and CD4+ cells in the FGT serve as target cells for HIV infection and replication at the portal of entry [112]. HIV gains access to target cells within mucosal sites via tears in the epithelial walls or interactions with immune cells beneath the epithelial surface [113, 114]. However, the rate of HIV transmission per sexual act is relatively low, indicating the presence of innate immune responses against HIV. Understanding the tissue environment at the primary portal of entry remains crucial to developing intervention strategies and reducing the risks of HIV infection.

1.4.1 HIV structure

HIV is an enveloped virus with a complex structure that facilitates its entry into host cells and its ability to evade the immune system [115]. The virus is relatively spherical in shape, with a diameter of approximately 100-120 nanometers. Its structure can be divided into three main components: the envelope, the matrix, and the core (**Figure 1.6**).

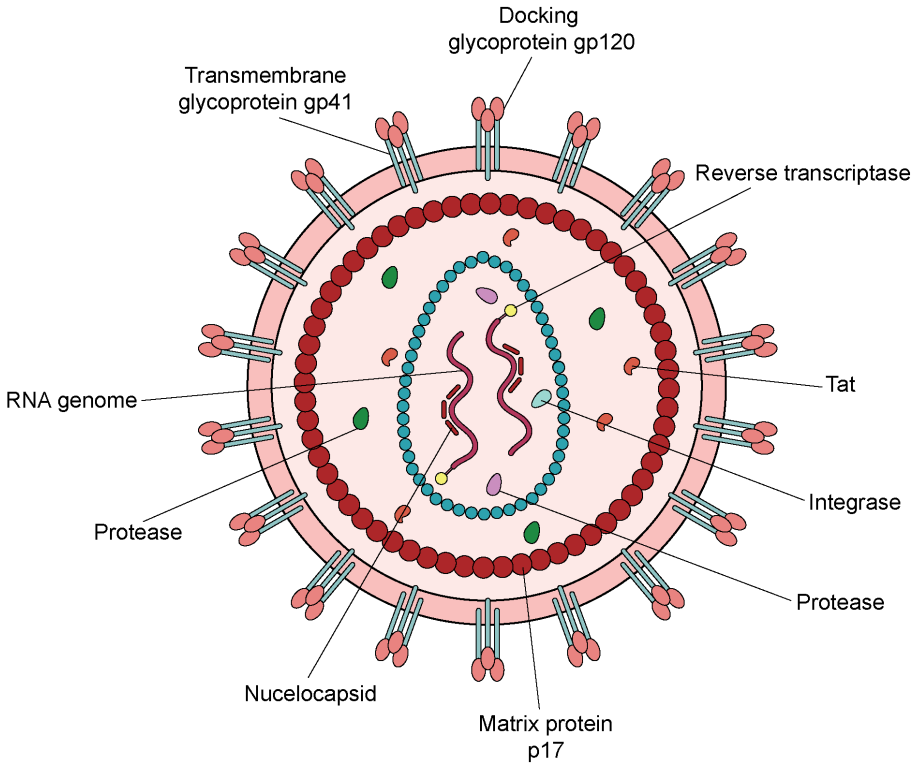

Figure 1.6 Structural composition of HIV.

The structure of HIV is composed of the envelope, consisting of glycoproteins gp120 and gp41, the matrix which contains matrix protein p17, the viral core or the nucleocapsid composed of the viral RNA genome and associated proteins.

1.4.1.1 Envelope

The envelope of HIV is a lipid bilayer derived from the host cell membrane. It surrounds the virus particle and is an integral part of the virus's ability to infect host cells. Embedded in this lipid bilayer are viral glycoproteins, specifically gp120 and gp41 [115]. These glycoproteins play a key role in the virus's entry into host cells. gp120 is a glycoprotein that protrudes from the viral envelope and is essential for the initial interaction with host cells. This is mainly due to its high binding affinity towards CD4 receptors on the surface of cells [116, 117]. Additionally, gp120 also interacts with co-receptors such as CCR5 or CXCR4, which are required for the virus to enter the cell [118]. The glycoprotein gp41 spans the viral envelope and is involved in the fusion of the viral envelope with the host cell membrane. The fusion process allows the viral core to enter the host cell, initiating the infection.

1.4.1.2 Matrix

Underneath the envelope lies the matrix, which constitutes a layer of proteins that provide structural support and help to stabilize the viral particle. The matrix is composed primarily of the protein p17, which forms a shell-like structure [119]. This matrix protein is essential for maintaining the integrity of the virus and for the proper assembly and budding of new viral particles [120].

1.4.1.3 Core

The core of HIV, also known as the nucleocapsid, is a complex structure composed of the viral RNA genome and associated proteins. The core is roughly conical or cylindrical in shape and is surrounded by a protein shell called the capsid [121]. The capsid is

made up of the protein p24, which forms a dense, conical shell that encloses the viral RNA and associated proteins. The capsid plays a critical role in protecting the viral genetic material during its passage through the host cell and its delivery to the cell's nucleus. HIV carries two identical strands of RNA as its genetic material. This RNA serves as a template for reverse transcription, a process by which the viral RNA is converted into DNA by the enzyme reverse transcriptase. This DNA is then integrated into the host cell's genome. The core also contains several essential accessory proteins, including reverse transcriptase, integrase, and protease. Reverse transcriptase is responsible for synthesizing DNA from the viral RNA, integrase facilitates the integration of the viral DNA into the host cell's genome, and protease is involved in the maturation of new viral particles by cleaving viral proteins into their functional forms [122].

1.4.2 Viral Replication Cycle

The structure of HIV is intricately designed to maximize its ability to infect host cells and replicate. To understand how HIV undermines the immune system and propagates, it is important to fully understand the viral replication cycle. This cycle comprises several distinct phases, each crucial for the virus's replication and persistence within the host. From entry into the host cell to the release of new virions, the HIV replication cycle exemplifies the intricate interplay between viral mechanisms and host cellular processes (**Figure 1.7**) [123].

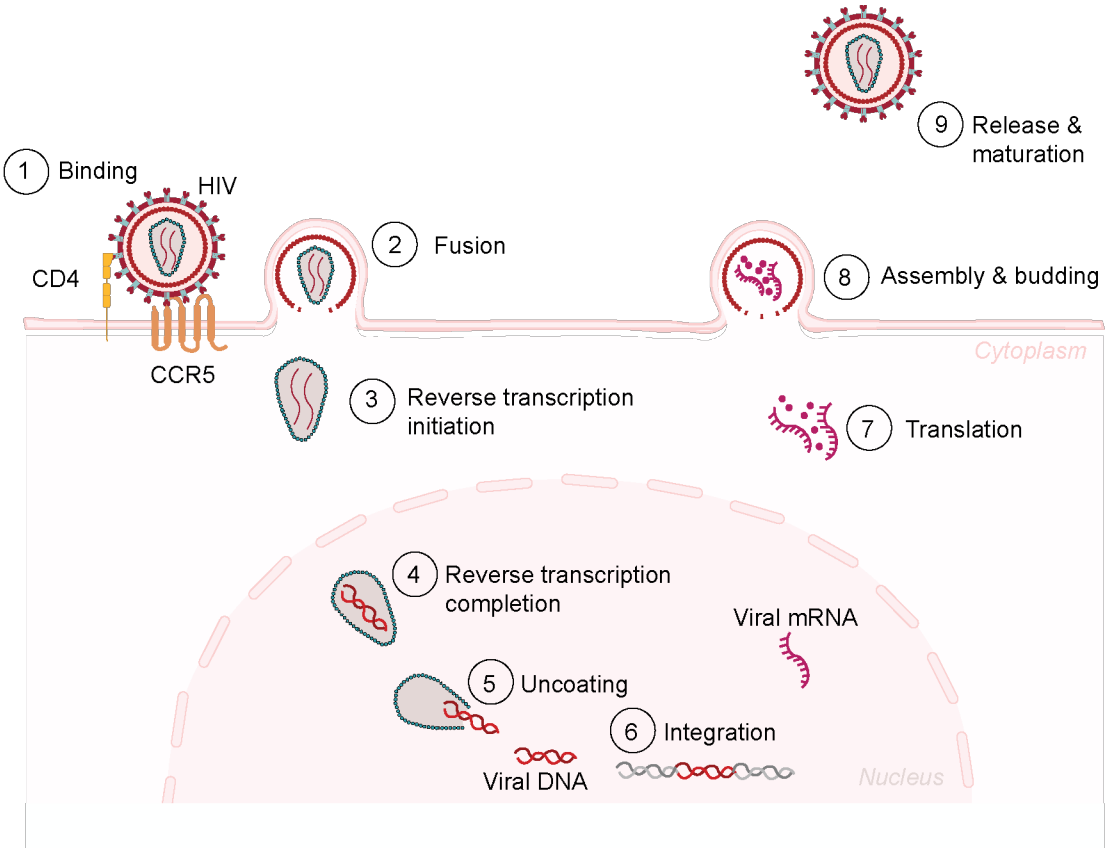

Figure 1.7 Processes involved in viral replication cycle of HIV.

The viral replication cycle of HIV is comprised of several distinct phases, crucial for productive infection to occur. These steps include binding of the virus to tropic surface proteins, fusion to the cell membrane, initiation of reverse transcription, followed by entry into the nucleus where reverse transcription is completed, the viral DNA is uncoated and integrates into the host genome producing viral mRNA. The viral mRNA is then translated into viral proteins, followed by assembly and budding of the virus, and finally release of mature virions.

1.4.2.1 Viral entry into host cell

The replication cycle of HIV begins with the virus's entry into the host cell. The primary target cells are CD4+ T cells, which are central to the immune response [116]. HIV's envelope glycoprotein gp120 binds to the CD4 receptor on the surface of the T cell. This interaction is highly specific and crucial for the virus's entry. The binding of gp120 induces conformational changes that expose a secondary binding site on gp120, which interacts with a co-receptor, classically CXCR4 or CCR5 [118]. Studies have shown different receptors and co-receptors for viral entry into host cells, including DC-SIGN,

Siglec1,[124, 125] and CD49d, among others [126]. Following co-receptor binding, the envelope glycoprotein gp41 facilitates the fusion of the viral envelope with the host cell membrane. This fusion process allows the viral core to enter the host cell's cytoplasm [127].

1.4.2.2 Reverse transcription

Once inside the host cell, HIV's RNA genome must be converted into DNA to integrate into the host cell's genome. This conversion is carried out by the viral enzyme reverse transcriptase [128]. This enzyme catalyzes the synthesis of complementary DNA (cDNA) from the viral RNA. This process involves several intermediate steps, including synthesizing a DNA strand complementary to the viral RNA and degrading the RNA strand to form double-stranded DNA.

1.4.2.3 Integration into the host genome

The newly synthesized viral DNA is then transported into the host cell's nucleus, where it undergoes integration into the host genome. The enzyme integrase facilitates the insertion of the viral DNA into the host cell's chromosomal DNA [129]. This integration is a critical step, as it allows the viral DNA to be replicated along with the host cell's DNA, ensuring that each new cell division will contain a copy of the viral genome.

1.4.2.4 Transcription and Translation

Once integrated into the host genome, the viral DNA is transcribed and translated to produce viral proteins and RNA genomes [130]. The integrated viral DNA serves as a template for the transcription of viral mRNA by the host cell's RNA polymerase. This mRNA includes instructions for synthesizing viral proteins and for producing new viral RNA genomes. Subsequently, the viral mRNA is translated into viral proteins in the host cell's cytoplasm. These proteins include structural proteins (such as gp120, gp41, and p24) and enzymes (such as reverse transcriptase, integrase, and protease).

1.4.2.5 Assembly and Maturation

The newly formed viral components are assembled following protein synthesis into new virions [123]. The viral proteins and RNA genomes are assembled into a conical or cylindrical core structure, which includes the viral RNA and essential enzymes. The assembled virion buds from the host cell's membrane, acquiring its lipid envelope during the process. This envelope contains viral glycoproteins (gp120 and gp41) essential for the virus's ability to infect other cells. The viral protease enzyme cleaves the polyprotein precursors into their functional forms. This cleavage is crucial for the maturation of the virus, as it ensures that the structural proteins are properly arranged and functional for a new round of infection.

1.4.3 HIV Infection in the FGT

The FGT is the primary portal of entry in cases of sexual transmission of HIV [112]. Since the FGT is populated by multiple immune cells, there are potentially multiple layers of protection and, conversely, different targets for HIV infection (**Figure 1.8**) [89, 131]. To develop successful interventions and therapeutic strategies, it is imperative to potentiate protective mechanisms and suppress HIV infection immediately upon exposure to the virus. To better understand protective mechanisms, it is important to fully understand the immune cell milieu in the FGT.

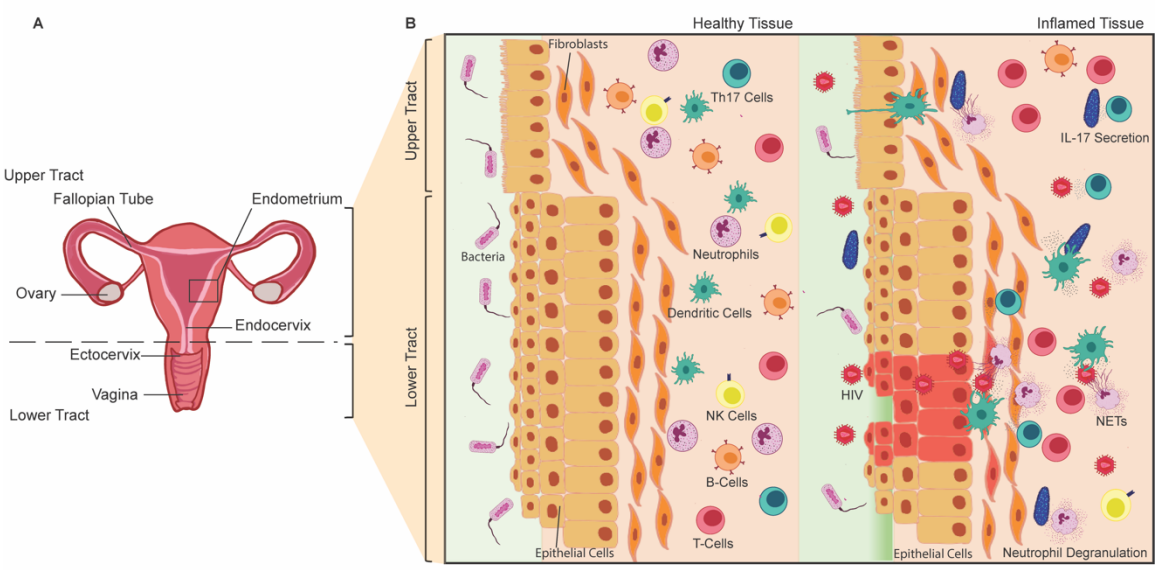

Figure 1.8 Immune cell distribution and responses in the female genital tract under healthy and inflammatory conditions[1].

A) The female genital tract (FGT) is divided in different anatomical compartments. The upper tract, lined by a single layer of epithelial cells, includes the endocervix, endometrium, Fallopian tubes and ovaries. The lower tract, lined with multiple layers of squamous epithelium, includes the ectocervix and vagina. B) Under physiological conditions (left panel), healthy genital tissues contain immune cells with specialized functions and unique phenotypes modified by the tissue environment. Important for HIV, immune cells including neutrophils, dendritic cells and T cells (CD8+ and CD4+ Th subsets) are present throughout the FGT in healthy women. The epithelium serves as a physical and immunological barrier, is coated with mucus, supports a Lactobacillus-dominated microbiome with anti-inflammatory and anti-HIV properties. Under inflammatory conditions (right panel), altered microbiome profiles, STIs or other exogenous factors induce the production of cytokines, chemokines and other inflammatory molecules by epithelial cells and immune cells. These cytokines and bacterial ligands recruit and activate immune cells and disrupt the epithelial barrier. These factors facilitate access of HIV to a mucosal environment rich in susceptible target cells such as Th17 cells and other CD4+ T cells expressing CCR5, increasing the likelihood for HIV acquisition.

1.4.3.1 CD4$^+$ T Cells

A majority of leukocytes within the upper and lower tract of the FRT are T cells, where

CD4+ T cells represent preferential targets for HIV infection due to the high density of

CD4 receptors on the surface and CCR5 expression [126, 132, 133]. Additionally, CD4+

[1] Reprinted with permission from: LM de Lara, RS Parthasarathy, M Rodriguez-Garcia. Mucosal Immunity and HIV Acquisition in Women. COPHYS 2021.

T cells supporting HIV replication have been shown to express CD69 and markers consistent with a Th17 phenotype. Preferential infection and depletion of this subset are observed in women living with acute HIV infection, suggesting that Th17 cells play an important role in HIV infection within the FGT.

1.4.3.2 Neutrophils

Neutrophils are present throughout the FRT and represent 10-20% of total leukocytes in the tissue. They are involved in maintaining tissue homeostasis and defense against pathogens and have been shown to be functionally and phenotypically distinct from blood neutrophils. Similar to blood neutrophils, within minutes of HIV exposure, neutrophils release NETs that trap and inactivate HIV, thereby preventing infection of target cells [46].

1.4.3.3 Mononuclear phagocytes

Macrophages and DCs make up a majority of mononuclear phagocytes (MNPs) within FGT leukocytes. HIV replication in both macrophages and DCs is restricted due to elevated levels of SAMHD1 expression, a host-restriction factor that prevents reverse transcription of the virus [134]. Although in vitro studies suggest robust replication of HIV in macrophages [135], the impact of tissue-resident macrophages in HIV pathogenesis remains unclear.

DCs within the FGT represent a heterogeneous population, as previously mentioned. In addition to subset-specific differences in phenotype and function, studies by us and others reveal that these differences extend to HIV pathogenesis, including viral capture and protein secretion [102, 136-141]. The role of DC subsets in HIV pathogenesis will be discussed in depth below.

1.5 Dendritic Cells

The specialized distinction between first encountering a pathogen and response to subsequent exposure is broadly classified into the innate and adaptive immune systems, respectively, as described in the sections below. Rapid advances in understanding cellular mechanisms and the composition of cell and cell-derived components of the innate and adaptive immune systems have served as cornerstones for understanding health, disease pathologies, and groundbreaking medical interventions such as vaccines and cell- and gene-based therapies, among others.

1.5.1 DCs – Bridging the innate and adaptive immune system

The discovery of DCs by Dr. Steinman and Dr. Cohn serves as a seminal cornerstone in understanding immunology [60, 142, 143]. Initial studies revealed the presence of a cell type consisting of dendrites and shown to play a significant role in shaping T cell response. Further studies unraveled the potential of DCs in bridging the gap between innate and adaptive immunity. DCs serve as professional APCs, capable of capturing, processing foreign antigens, and presenting cognate peptides to T cells. Their unique role lies in their ability to shape T cell response through induction of naïve T cell proliferation and shaping T cell phenotype through secretion of key cytokines, which to this day is harnessed for therapeutic purposes [144]. Advances in technology have further unraveled distinct DC subsets with unique phenotypes and specialized functions [145-148]. DCs consist of a diverse group of APCs that are classified into distinct subsets depending on their phenotype, location, and specialized function in the immune system, where they are broadly categorized into conventional DCs (cDCs), plasmacytoid DCs (pDCs), and monocyte-derived DCs (MoDCs). The heterogeneity of DC populations has initially been described on the basis of surface protein expression and transcriptome. However, recent advances in high-dimensional data have enabled the identification of previously overlooked DC populations and their functional properties,

including response to pathogens and tumors, cytokine secretion, and induction of T cell phenotype [80, 145, 147, 149]. Therefore, to fully utilize the immense therapeutic potential of DCs, it is important to fully understand their phenotypic and functional characteristics in order to develop potent therapeutic interventions.

DCs, being professional APCs, play a pivotal role in initiating and shaping T cell response, including T cell antigen-specificity, Th cell phenotype, and T cell memory. This is primarily due to their unique ability to prime naive T cells through sophisticated signaling cascades, broadly classified into three steps [57-60] (**Figure 1.9**).

The first step involves processing and presenting antigen fragment peptides on major histocompatibility complex I (MHC-I) or II (MHC-II) to naïve T cells that recognize cognate peptide antigen through their TCRs (CD3) and further strengthen the complex by binding CD8 or CD4 to MHC-I or MHC-II, respectively. This establishes an immune synapse between DCs and T cells. DCs upregulate the expression of key co-stimulatory markers, such as CD80, CD86, 4-1BBL, and OX40L, to further strengthen the immune synapse between DCs and T cells and promote efficient priming of T cells. Lastly, depending on the antigenic stimuli, DCs secrete different cytokines that influence T cell phenotype (**Figure 1.9**). Recent studies have shown DC-subset specific differences in induction of Th cell phenotype to further highlight the importance of understanding and characterizing DC subsets [150] and resolve the complexity of the role of DCs in bridging the gap between innate and adaptive immunity.

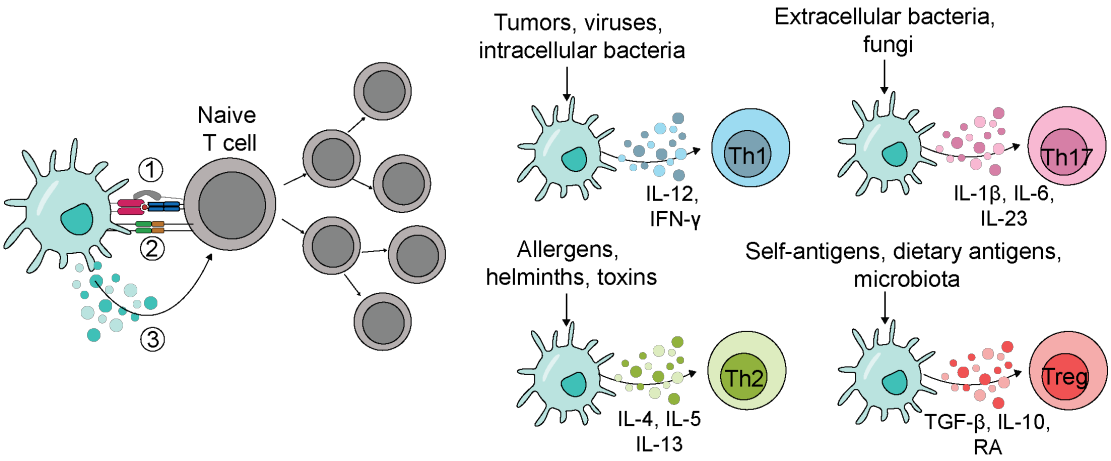

Figure 1.9 Activation of naive CD3 T cells and induction of Th cell phenotype by DCs.

DCs are professional antigen cells due to their unique property to induce proliferation of naïve T cells. This is done through three signals with the first being antigen presentation, second signal through engagement of co-stimulatory molecules, and third signal involving secretion of cytokines that modulate T cell function. DCs also play a critical role in modulating Th cell phenotype depending on antigenic stimuli, where DC exposure to tumors, viruses, and intracellular bacteria leads to production of IFN-γ and IL-12 influencing Th1 phenotype, extracellular bacteria and fungi exposure leads to production of IL-1β, IL-6, and IL-23 influencing Th17 phenotype, exposure to allergens, helminths, and toxins induces production of IL-4, IL-5, and IL-13 by DCs influencing Th2 phenotype, whereas exposure of DCs to self and dietary antigens, and microbiota leads to production of TGF-β, IL-10 and retinoic acid (RA) thereby influencing Treg phenotype.

1.5.2 Conventional DC populations

The development of DCs from their progenitors has offered insight into the differentiation of different DC subsets and the genetic mechanisms imprinted to drive these differences. DCs develop from monocyte/DC progenitors [140, 145] (**Figure 1.10**), which in turn give rise to common monocyte progenitors (CMPs), common DC progenitors (CDPs), and the recently described preDC3 progenitors depending on IRF8 expression levels. CDPs serve as progenitors for cDCs, which are further classified into cDC1 and cDC2 in humans and serve as homologs for murine CD8α^+ and CD11b$^+$ DCs, respectively. CDPs also serve as progenitors for pDCs in humans. preDC3s give rise to the novel DC3 population that shares cDC and MoDC characteristics, whereas CMPs give rise to a bonafide monocyte population that can be differentiated into MoDCs *ex-vivo* or through

tissue intrinsic factors driving differentiation *in-vivo*. Although murine DC development has been characterized in depth through the identification of key transcription factors driving DC subset development, the lack of similar tools in human systems remains a significant challenge in tracing human DC lineages.

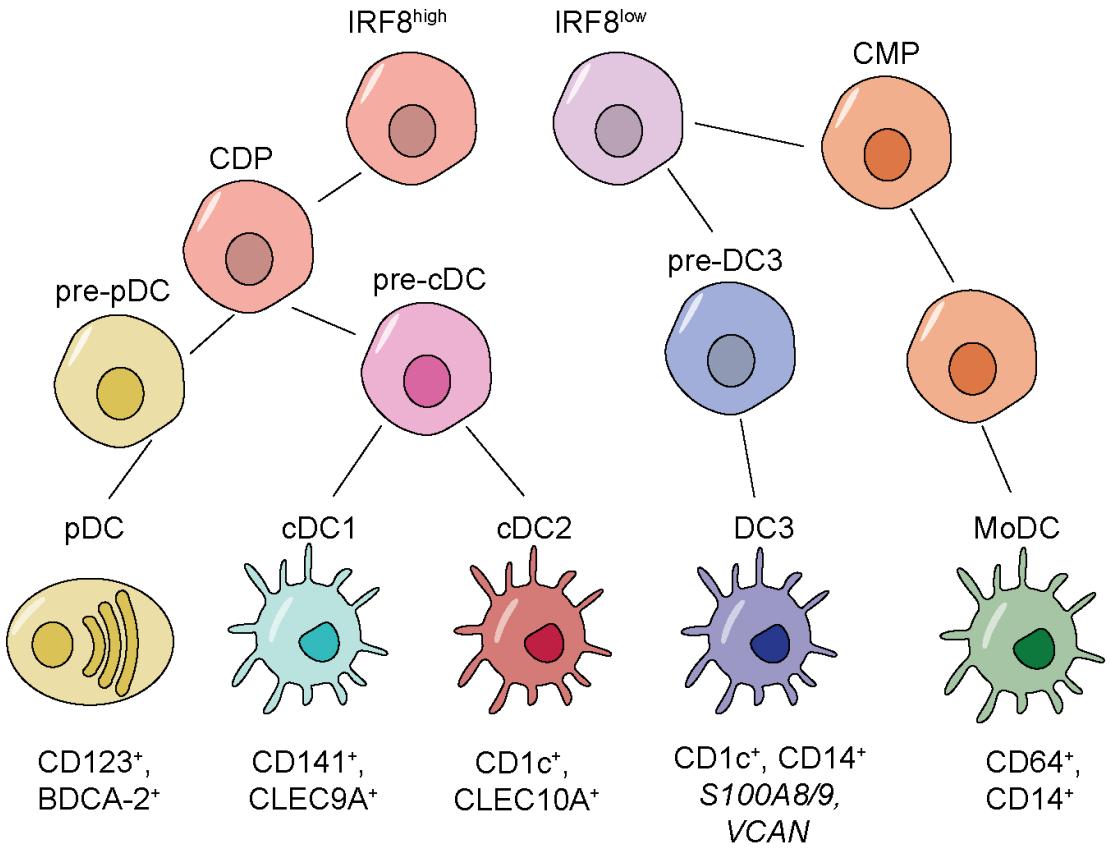

Figure 1.10 Ontogeny of human DC subsets.

DC precursors rise from granulocyte-monocyte-DC progenitors in an IRF8 dependent manner. pDCs, cDCs, and DC3s are derived from distinct progenitors compared to MoDCs, which are differentiated from monocytes. Identification of these subsets is heavily reliant on a combination of surface marker expression for conventional DC subsets, whereas novel DC3 subsets require a combination of surface protein and RNA expression (in italics) to reliably delineate from other DC populations.

1.5.2.1 pDCs

pDCs are characterized by their ability to produce large amounts of type I IFNs upon TLR stimulation, making them an integral part of the response against intracellular

pathogens. pDCs are capable of recognizing virus-infected cells through cell-cell mediated contact, subsequently triggering long-lasting IFN response [151, 152]. pDCs are less adept at triggering naïve T cell proliferation and differentiation than other DC subsets [153]. However, recent studies suggest that upon encountering pathogens, a subset of pDCs can differentiate into APCs with enhanced T cell priming potential [154]. pDCs are characterized through the expression of CD123 and BDCA-2 on the cell surface [140, 145]. In addition to producing large amounts of type-I-IFNs, pDCs produce multiple soluble factors that mediate the recruitment and activation of other DC subsets, NK cells, monocytes, and macrophages, making them key orchestrators of antiviral response [151, 152].

1.5.2.2 cDC1s

cDC1s are found in peripheral blood, tissue, and lymphoid organs and are known for their enhanced cross-presentation ability compared to other DC subsets, working efficiently to prime $CD8^+$ T cells [155]. Expression of TLR3 and TLR9 enables the detection of dsRNA and DNA, thereby leading to the production of type III IFNs and IL-12 [156]. cDC1s represent a homogenous population and human cDC1s are identified through the surface expression of CD141, CADM1, and the CLR CLEC9A and have been shown to prime Th1, Th2, and CTL responses [145].

1.5.2.3 cDC2s

cDC2s represent a distinct and major population in peripheral blood and tissue and are enriched in lymphoid organs where they play a crucial role in T follicular helper (Tfh) cell function [157]. cDC2s efficiently polarize naïve Th cells into Th1, Th2, and Th17 cells due to their ability to secrete a wide variety of soluble factors upon TLR stimulation [158]. Although cDC2s are classified based on high levels of CD1c expression, recent advances in the field of DC biology have shown that, unlike cDC1s, cDC2s represent a

heterogeneous population [147]. In the spleen, cDC2 populations are distinguished based on CLEC10A^{+}/CLEC10A^{-} [147], whereas in blood, initial studies reported CD14^{+}CD1c^{+} DCs as a subset of CD1c+ cDC2s. Subsequent studies, however, showed that CD14+CD1c+ DCs (DC3s) represent a distinct population with shared features of MoDCs and cDC2s [147]. These findings highlight the constantly evolving field of DC biology and warrant further questions into the role of cDC2s in different tissues.

1.5.3 CD14 Expressing DC subsets

CD14 is traditionally used to distinguish monocytes from other cell types, and although *in vitro* generated MoDCs express CD14 on the surface, until recently, DCs have been considered to lack CD14 expression in blood. However, we and others have previously described the presence of CD14 DCs in human tissue, considered to be DCs of monocyte origin. Recent studies [147, 149] have established the presence of unique CD14-expressing DC populations in the blood with distinct ontogeny compared to cDCs [147]. These studies have broadened the understanding of DC subsets, suggesting that CD14 DCs represent a mature or inflammatory subset due to their phenotype. However, key phenotypic and functional differences between CD14-expressing DCs and cDCs remain poorly understood.

1.5.3.1 Role of CD14 signaling pathway in inflammation

CD14 is a surface receptor anchored to the cell membrane by glycosylphosphatidylinositol (GPI) and expressed by monocytes, macrophages, and DCs. Due to its ability to directly bind to LPS, CD14 plays a crucial role as a co-receptor in LPS signaling despite lacking a transmembrane signaling domain [159]. Initiation of LPS signaling begins at the plasma membrane by the interactions between LPS-CD14 and TLR4-MD2 complex, which begins a series of signaling cascades thereby activating MAPK pathways and NF-kB, which leads to an inflammatory immune response through

the production of chemokines and cytokines. In addition to its role in LPS-TLR4 signaling, CD14 also serves as a coreceptor for other TLRs, such as TLR1 and TLR2, which bind to lipoproteins, TLR3, TLR7, and TLR9, thereby participating in nucleic-acid-mediated activation. Recent studies have demonstrated the crucial role of CD14 in capturing inflammatory lipids from dying cells leading to hyperactivation of DCs [160], further highlighting the promiscuous nature of CD14 interactions with various ligands. This multifaceted role of CD14 interaction with different ligands makes it important to fully understand the function of CD14-expressing DCs and their role in innate immune response.

1.5.3.2 MoDC

As the name suggests, MoDCs are derived from monocytes that differentiate under specific cytokine and growth conditions to give rise to a distinct DC subset. MoDCs are found in tissues, where they are considered to be differentiated from monocytes, recruited to the particular tissue upon injury/inflammation, and further undergo differentiation into MoDCs [50, 161]. MoDCs are characterized by the expression of CD14; however, they lack CD1c expression compared to DC3s. A considerable challenge remains in distinguishing these cells from monocyte-derived macrophages (MoMacs) since they share several markers. However, MoDCs can be distinguished by their ability to induce the proliferation and differentiation of naïve T cells, a hallmark of DCs, which currently serves as a reliable, functional method to distinguish between MoDCs and MoMacs. Similar to cDCs and DC3s, MoDCs prime Th1, Th17, and CTL responses. Due to their ability to be differentiated *ex-vivo,* these cells represent a workhorse for DC-based cell therapies.

1.5.3.3 DC3s

As mentioned in the section above, DC3s were initially reported to be a subset of cDC2s due to similarities in phenotype and transcriptional characteristics between the two, in addition to sharing monocyte-like features. First described in peripheral blood by Vilani et al. [149] as a subset of cDC2s, subsequent studies established DC3s as a distinct population with a unique precursor and GM-CSF-dependent differentiation as opposed to FLT3-L-dependent differentiation of cDCs. Additionally, DC3s have been shown to expand in the human body under inflammatory conditions/diseases such as SLE and infiltrate solid tumors [147]. DC3s are characterized based on the expression of CD14 and CD1c on the cell surface, in addition to key transcripts such as *VCAN*, *S100A8*, and *S100A9* [147, 149]. Similar to cDCs, DC3s are potent inducers of naïve T cell proliferation and differentiation, priming Th1, Th17, and CTL responses [147]. Additionally, DC3s have been shown to induce expression of tissue-homing and memory T cell marker CD103 on $CD8^+$ T cells [147], further highlighting their importance in the induction of T cell responses. Despite studies establishing the presence of DC3s in several tissues under pathological conditions, the role of DC3s under homeostatic conditions remains poorly characterized.

1.5.4 DCs in the FGT

DCs in the FGT have been shown to decrease in number with age. However, studies assessing their ability to induce the proliferation of naïve T cell proliferation, a hallmark of DC function, are lacking. Additionally, The presence of DCs in low numbers within the FRT makes these studies challenging and requires innovative techniques to evaluate the effects of aging on DCs.

1.5.5 Characteristics of DCs in the FGT

Efficient function of DCs in the FGT is crucial to maintenance of tissue environment, prevention of infections and successful pregnancy outcomes. However, DC dysfunction has been related to pregnancy-associated diseases such as preterm birth and spontaneous abortion. To that extent, DCs from post-menopausal women have been shown to possess an enhanced ability to induce expression of CD103 on proliferation CD8 T cells compared to DCs from pre-menopausal women in a TGF-dependent manner [102]. Although these studies highlight modifications to DCs and their ability to influence T cell proliferation and phenotype, further studies are required to elucidate any differences in innate immune function, including the detection of pathogens, secreted protein profile, and ability to prime antigen-specific responses between pre- and post-menopausal women. Additionally, our group and others have demonstrated the presence of multiple DC subsets in the FGT with unique phenotypic properties [102, 136-138, 140, 141]. However, with recent advancements in the field of DC biology which has unveiled novel DC populations, it is important to put into context these recent findings and expand current understanding of FGT DC subsets. To better understand the role of DCs in mediating tissue homeostasis and innate and initiating adaptive immune response, it is important to characterize FGT DCs and understand subset-specific properties to improve health outcomes.

1.5.5.1 Genital CD14 DCs

Identification of DCs through expression of CD11c and CD11b has established the presence of distinct subsets, with CD11b+CD14+ DCs (CD14 DCs) being the most abundant throughout the FRT [136, 137]. CD14 DCs have been shown to express CD1c on the surface and lack expression of macrophage marker CD163. In addition to lacking macrophage markers, CD14 DCs induce the proliferation of naïve CD4 and CD8 T cells,

a hallmark of DC function [101]. Additionally, CD14 DCs have been shown to induce CD103 expression on CD8+ T cells, highlighting their ability to potentiate tissue-resident phenotypes in CD8+ T cells [101]. Given the role of CD14 in initiating inflammatory response, it is important to fully characterize this subset to understand their role in homeostasis and response to pathogens. Furthermore, the identification of CD14+CD1c+ DCs in the periphery as a distinct subset, warrants further investigation into the presence of this subset within the mucosa [136, 138, 141, 162].

1.5.5.2 Genital CD1a+ DCs

CD1a-expressing DCs have been identified by us and others in the upper and lower tract of the FRT [102, 136, 137, 139, 141]. CD1a DCs in the endometrium, endocervix, and ectocervix of the FRT express high levels of DC maturation markers HLA-DR, CD86, and CD83, suggesting that this subset represents a distinct mature population within the FRT [136, 137]. Magnetic isolation of CD1a DCs from the FRT reveals the presence of a homogenous DC population, with T cell proliferation induction levels similar to that of MoDCs [136, 137]. CD1a+ DCs have also been described in the vaginal epithelium, characterized by the expression of CD207 (Langerin) with a distinct phenotype compared to Langerhans cells [139]. Interestingly, CD1a DCs in the endometrium, endocervix, and ectocervix lack expression of Langerin, suggesting differences in phenotype dependent on tissue location [102]. However, studies in blood and tissue CD1c+ DCs have shown that TGFβ treatment induces upregulation of CD1a expression and acquisition of Langerhans cell-like phenotype by CD1c+ DCs, with increased expression of CD40, CD80, and CD86 correlating with enhanced Th cell expansion and differentiation into Th2 cells [163]. Given the elevated levels of TGF-β in the endometrium, this is of important physiological relevance.

1.5.5.3 Genital CD103+ DCs

CD103 is a surface protein that is associated with epithelial cell interactions through interactions with E-Cadherin expressed on the surface of epithelial cells. CD103 expression has been observed on DCs in the lungs, gut, and, more recently, FGT DCs [102]. In the gut, CD103+ DCs are considered to promote tolerance towards commensal bacteria and food antigens. In the FGT, CD14 DCs lack CD103 expression, whereas lack of CD14 expression on DCs is associated with high levels of CD103 expression found exclusively in the endometrium [102]. Given the role of CD103+ DCs in the lung and gut to promote tolerance, their presence exclusively in the endometrium could suggest an important role in mediating pregnancy. However, low CD103+ DC numbers in the FRT make it challenging to fully understand the role of this DC subset. Although CD103+ DCs in mice have been shown to sample antigens and demonstrate enhanced lymph node migration capabilities [164], FGT DCs lack expression of CCR7, indicating a lack of ability to migrate to lymph nodes and highlighting disparities in mice and human DCs.

1.5.6 Role of DCs in HIV pathogenesis

The ability of DCs to process, present antigens, and induce the proliferation of T cells is harnessed for several therapeutic applications, including HIV. However, DCs could prove to be a double-edged sword in response to HIV infection (**Figure 1.11**). Inefficient processing of HIV by DCs can lead to dissemination of the virus to CD4+ cells upon migration to secondary lymphoid organs [124, 141, 162, 165, 166]. Conversely, studies have shown that efficient capture followed by processing and presentation of HIV leads to the expansion of antigen-specific T cells [167]. Traditionally, DC models for HIV pathogenesis have relied on in vitro differentiated MoDCs. However, advances in the DC field have led to the reclassification of DC subsets and the identification of novel ones in

blood and tissue, making it important to understand subset-specific differences in HIV response, if any.

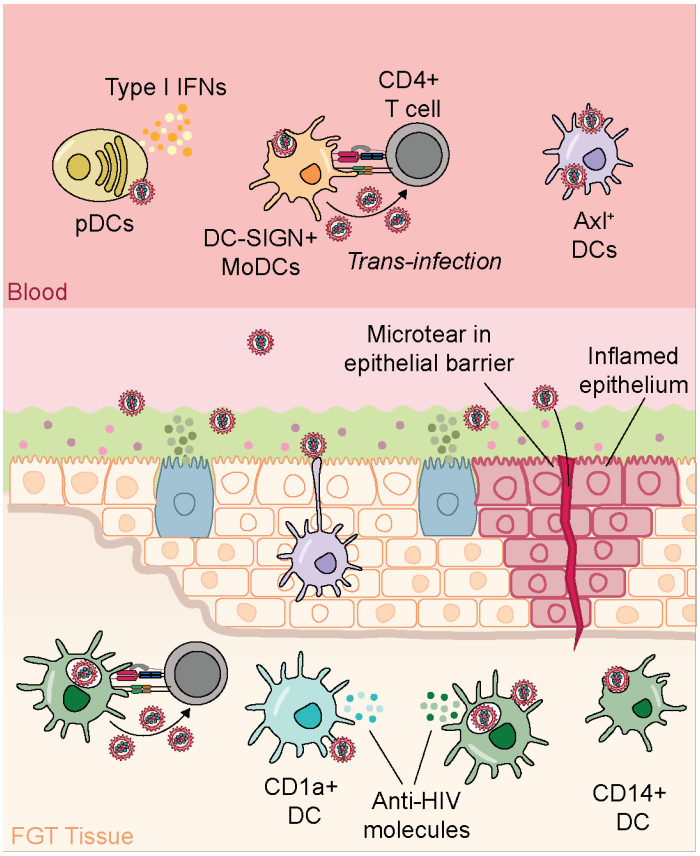

Figure 1.11 Role of human DCs in HIV pathogenesis.

Human DCs play a crucial role in the pathogenesis of HIV. In the blood, pDCs respond to HIV through release of type I IFNs, MoDCs capture HIV through DC-SIGN resulting in improper processing of the virus. The virus can then be transferred to CD4+ T cells through a process known as trans-infection, resulting in productive infection. Axl+ DCs in the blood have also been shown to interact with HIV, in a differential manner depending on how they virus is sensed. In the FGT, HIV gains access to target cells through microtears in the epithelial barriers. Additionally, it is proposed that DCs interact with the virus and sense them through extensions of their dendrites between epithelial cell layers. CD14+ DCs in the FGT capture the virus in a preferential manner, however, both CD1a+ and CD14+ DCs rapidly secrete anti-HIV molecules upon exposure to HIV. Additionally, studies have shown that CD14+ DCs from anogenital regions inefficiently process HIV and can trans-infect CD4+ T cells.

1.5.6.1 MoDCs

In-vitro assays using MoDCs have demonstrated their ability to capture HIV and migrate towards a CCL19 or CCL21 gradient (mimicking migration to lymph nodes), consequently transmitting the virus to CD4+ T cells [168]. However, complete inactivation of HIV could lead to efficient capture, processing, and presentation of HIV antigens to trigger an adaptive immune response against HIV [167]. Despite low levels of CD4 expression, HIV has been shown to bind to MoDCs independent of CD4, with DC-SIGN and Siglec-1 playing key roles in HIV binding to DCs. MoDCs are also less susceptible to HIV infection and replication due to their innate ability to prevent viral replication through elevated levels of SAMHD1 expression [134]. Additionally, HIV has been shown to significantly modulate MoDC functional responses by impairing DC autophagy, maturation, T cell activation, IL-12 production, and inducing IL-10 production.

1.5.6.2 Peripheral DCs

Few studies have been performed with fresh primary human DCs from blood. Initial studies have shown that endocytosis of HIV by pDCs leads to their activation through TLR-viral RNA interactions and activation of bystander DCs [169]. HIV has also been shown to harness DC-SIGN^{+} MoDCs from blood to undergo transcription and replication, leading to productive infection [125]. Increased resistance against HIV by CD141^{+} cDC1s, attributed to increased expression of RAB15, further highlights differences in HIV response by DC subsets [58]. A recent study by F. Brouiller et al. shows differential sensing of HIV by Axl+ peripheral blood DCs leading to the activation of different transcriptional events [167]. NF-kB-mediated response leading to maturation of Axl^{+} DCs translated to efficient CD4+ T cell activation, whereas STAT1/2 activation leads to type I IFN and ISG responses. However, these responses do not translate to cDC2s except when viral replication is allowed using an HIV-1 strain overexpressing the SAMHD1 degrading protein Vpx. Additionally, the study identified a subset of Axl+ DCs actively

replicating HIV and demonstrated a mixed NF-kB and type I IFN/ISG response. These studies contribute to the growing knowledge of DC subset-specific responses to HIV, highlighting key differences between homogenous MoDC and heterogenous, differential primary peripheral blood DC response to HIV.

1.5.6.3 FGT DCs

The FGT represents a mucosal site where immune cells first encounter HIV in cases of sexual transmission of the virus, which includes heterogeneous DC populations within the mucosa. Our study shows that in stark contrast to peripheral blood DCs and MoDCs, FGT DCs have been shown to capture HIV in a DC-SIGN independent manner, with CD14 DCs mediating preferential capture of the virus [136]. Interestingly, although only CD14-expressing DCs capture HIV, both CD14-expressing DCs and CD1a+ DCs in the FGT secrete anti-HIV proteins in a rapid manner upon exposure to the virus, suggesting differential sensing and activation pathways in different FGT DC subsets [136]. Further studies using anogenital DCs confirmed our findings and showed that anogenital DCs were capable of transferring HIV to CD4 T cells [141]. Additionally, cervical and vaginal epidermal DCs have been shown to capture HIV and transfer them to T cells [139, 162]. However, the mechanisms enabling differential response to HIV and in-depth characterization of FGT DC subset response to HIV remain lacking. To develop preventative therapies against HIV, it is important to identify innovative strategies to model sexual transmission of HIV *in vivo* and, more importantly, to characterize the events that occur promptly upon exposure to the virus and precede productive infection.

Chapter 2: Aging Modifies Endometrial Dendritic Cell Function and Unconventional Double Negative T Cells in the Human Genital Mucosa[2]

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2.1 Introduction

The immune system in the female reproductive tract (FRT) has the double task of protecting against infections while facilitating implantation and pregnancy [87]. To accomplish this, immune cell populations in the FRT are tightly controlled by sex hormones with their distribution and functions compartmentalized in that each anatomical region displays unique immunological characteristics [87]. While most research efforts are focused on understanding the immune system in the FRT of premenopausal women as it relates to fertility and pregnancy, little is known about the immunological changes that take place in the non-pregnant endometrium in the years following menopause, when reproductive function is no longer present [88, 98].

The global population is aging rapidly, with women representing about 60% of individuals 60 and older [170]. Genitourinary infections and gynecological cancers increase with age, significantly affecting women's morbidity and mortality [171]. Sexually transmitted infections (STI) are increasing in older women, and while the overall prevalence is lower than in younger women, the increase rate is higher, representing a public health problem [172, 173]. In women, the aging process is intimately related to menopause, when ovarian hormone production ceases marking the end of the reproductive function. Thus, it is critical to understand how the immune system changes in the FRT before and after menopause as women age. We have previously described how menopause and aging induce multiple changes in immune cell distribution and function of different immune cells in the FRT, including Th17 cells, CD8+ T cells and dendritic cells (DCs) [100-102, 105, 174, 175].

DCs that reside in the mucosa provide immune surveillance against pathogens and trigger specific adaptive immune responses [143]. Unlike macrophages, DCs are specialized in priming naïve T cells and sustaining the cytokine environment during

antigen presentation that shapes the immune response [176]. The tissue environment in which DCs reside strongly influences DC phenotype and function [176]. In the FRT, DCs also have the unique task of being tolerant to allogeneic antigens in order to facilitate reproductive function [88, 177]. We have previously demonstrated that different subsets of DCs exist throughout the FRT, including CD1a+ DCs, that display characteristics of classical DCs, and CD14+ DCs which co-express CD11b and resemble monocyte-derived DCs [136]. Both DC subsets can induce proliferation of naïve T cells and upregulation of tissue residency markers (CD103) on naïve CD8+ T cells, suggesting their ability to control tissue resident memory T cells in the FRT [102]. Interestingly, the ability of endometrial DC to induce CD103 expression on CD8+ T cells is age-dependent and enhanced after menopause [102].

In addition to DCs, FRT tissues are populated with different T cell populations, which have distinct roles in defense during the menstrual cycle, throughout pregnancy and following menopause [87]. We have previously reported that endometrial CD8+ T cell function is strongly regulated throughout the menstrual cycle and increases after menopause [100, 174, 175]. We also demonstrated that Th17 presence increases in the EM after menopause [105]. However, little is known about other unconventional T cell subsets, such as double negative TCRαβ+ (DN) T cells, which are thought to play an important role in reproduction in mice [85]. The origin and function of DN T cells in humans are poorly defined and may include multiple cells with different origins [86]. Further, in humans, DN T cells have been mostly studied in pathological conditions, such as solid tumors (melanoma), transplantation, and autoimmune diseases [81, 82, 86], but little is known about tissue distribution and function in the steady state. Two recent studies described the presence of Mucosal-associated invariant T cells (MAIT) in the endometrium and cervicovaginal tissues, which represents a subset of unconventional DN T cell population with antimicrobial properties [178, 179]. However, a gap remains in

our knowledge about the heterogeneity, distribution, and function of TCRαβ+ DN T cells throughout the human FRT and if these cells change with aging.

Here we investigated how endometrial DCs' ability to induce T cell proliferation is modified by aging and unexpectedly found a DN (CD4-CD8-) naïve T cell population that was being stimulated to proliferate in a DC subset-dependent and age-dependent manner. Characterization of unconventional DN T cells through single-cell sequencing and flow cytometry in human genital tissues revealed a heterogeneous tissue-resident population with unique adaptive and innate-like functions and uncovered a selective decline of this population in the cervix with aging. Our findings provide essential information to understand immune responses related to reproductive success and defense against gynecological cancers and infections over the course of women's lives.

2.2 Materials and Methods

2.2.1 Study Design

Studies were approved by Dartmouth College Institutional Review Board and the Committee for the Protection of Human Subjects (CPHS) and by the Health Sciences Institutional Review Board at Tufts University. Written informed consent was obtained before surgery from HIV-negative women undergoing hysterectomies at Dartmouth-Hitchcock Medical Center (Lebanon, NH) or at Tufts Medical Center (TMC, Boston, MA). Patients with gynecological cancer were excluded from the study. Surgery was performed to treat benign conditions including fibroids, prolapse, dysmenorrhea and abnormal uterine bleeding. For a subset of patients, information about specific surgical indication was not available. Trained pathologists selected tissue samples from endometrium (EM), endocervix (CX) and ectocervix (ECX) free of pathological lesions and distant from the sites of pathology. Women were HIV- and HPV- but no additional

information regarding other genital infections was available. A total of 94 women were included in the study, ranging from 27 to 77 years of age (median=55).

2.2.2 Tissue Processing

Tissues from the EM, CX and ECX were obtained after hysterectomy. In some cases, only endometrial tissue was provided by pathology. Vaginal tissues were not available. Tissues were processed to obtain a stromal cell suspension as described previously [102, 136, 137], using 0.05% collagenase type IV (Sigma-Aldrich, St. Louis, MO) and 0.01% DNAse (Worthington Biochemical, Lakewood, NJ). After filtering through a 20 μm mesh screen (Small Parts) to separate epithelial cells from stromal cells, stromal cells underwent dead cell removal (Dead Cell Removal Kit, Miltenyi biotech, Auburn, CA) as previously described [105]. This protocol results in more than 90% cell viability by trypan blue staining. After dead cell removal, mixed cell suspensions were used for phenotypical analyses by flow cytometry, or further processed for cell isolation.

2.2.3 CD14+ and CD1a+ cell isolation

Mixed cell suspensions were centrifuged by standard Ficoll gradient as described previously [102, 136, 137], prior to DC isolation using positive magnetic bead selection with either the CD14+ or CD1a+ isolation kits (Miltenyi Biotec) according to the manufacturer's instructions. After two rounds of positive selection, purity of the CD14+ and CD1a+ population was about 90% [102, 136, 137]. Isolated DCs were plated in round bottom ultra-low attachment 96-well plates (Corning, Corning, NY) in Xvivo15 media (Invitrogen Waltham, MA) supplemented with 10% human AB serum (Valley Biomedical Winchester, VA) for *in vitro* allogeneic stimulation.

2.2.4 Allogeneic Naïve T Cell Stimulation Assay

Naïve T cells were purified from cryopreserved peripheral blood mononuclear cells (PBMCs) using the naïve Pan T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA). After

purification, isolated naïve T cells were >99% CCR7+ CD45A+ as previously described [102]. Naïve T cells were stained with Cell Proliferation Dye eFluor-670 (eBioscience, San Diego, CA) as recommended by the manufacturer. Purified mucosal CD1a+ or CD14+ cells ($5x10^3$ cells) were plated with naïve T cells ($7.5x10^4$ cells) (1:15 ratio) in round-bottom 96-well plates, in Xvivo 15 media (Invitrogen, Waltham, MA) supplemented with 10% human AB serum (Innovative Research, Novi, MI). DC to T cell ratio was selected based on our prior studies [102, 136, 137] and *in vivo* animal models [180]. Naïve T cells were isolated from four different blood donors and each donor was used in co-culture with DCs from at least two patients. After 6 days in culture, proliferation of T cells was assessed by flow cytometry after staining with zombie yellow dye (Biolegend, San Diego, CA) and CD3-APC-Cy7, CD8-FITC (Tonbo, San Diego, CA), CD4-PE, CD103-PE-Cy7 (eBioscience, San Diego, CA) and CD11c-PerCp-Cy5.5 (Biolegend, San Diego, CA). Naïve T cells alone were used as a negative control. For a subset of experiments, TGFβ Receptor 1 blocker, SB431542 (10 μM, Tocris Cookson Inc) was added to the media at the beginning of each co-culture as described before [102].

2.2.5 Cytokine Secretion Determination

After 6 days in culture, supernatants from the DC-T cell cocultures were collected and a panel of 13 cytokines was detected using Luminex Assay (GM-CSF, IFNγ, GROα, IL-10, MDC, IL-13, IL-15, IL-17A, IL-1Rα, IL-5, IL-8, IP-10 and MCP-1). Undetectable values were assigned a value of 0.1 to allow for presentation in log scale.

2.2.6 Flow Cytometry

Mixed cell suspensions were stained for surface markers with combinations of the following antibodies: CD45-vioblue450, CD8-FITC, CD19-APC (Tonbo, San Diego, CA), HLA-DR-FITC, CD3-viogreen, CD103-PE (Miltenyi Biotec, Auburn, CA), CD11c-PerCp-

Cy5.5, CD103-PE-Cy7, CD4-PE (eBioscience, San Diego, CA), CD56-APC (BD Pharmingen, San Diego, CA), CD69-BrilliantViolet510 (BioLegend, San Diego, CA). Dead cells were excluded with 7AAD (Southern Biotech, Birmingham, AL) or zombie dye yellow staining (BioLegend, San Diego, CA). For spectral flow cytometry, the following antibodies were used: CX3CR1-PE eFluor610 (Thermo Fisher, Waltham, MA), CD3-VioGreen (Miltenyi Biotec), CD4-BrilliantUV805, CD10-BrilliantViolet650, CCR5-PE-Cy5, CD45-BrilliantUV395 (BD Biosciences, Franklin Lakes, NJ), HLA-DR-AlexaFluor700, CCR7-BrilliantViolet750, CD8-SparkBlue550, CD62L-BrilliantViolet605 (BioLegend). Analysis was performed on Gallios (Beckman Coulter, Brea, CA) or Cytek Aurora 5 lasers configuration (Cytek, Fremont, CA) flow cytometers and data analyzed with FlowJo (Tree Star, Inc., Ashland, OR) or OMIQ software (www.omiq.ai). Expression of surface markers is shown as percentage of positive cells. Fluorescence minus one (FMO) strategy was used to establish appropriate gates.

2.2.7 Intracellular staining of Cytotoxic Molecules

Detection of perforin, GZA and GZB was performed on mixed cell populations after dead cell removal as described [100, 174]. Cells were surface stained first and then fixed and permeabilized with Cytofix/cytoperm kit (BD Bioscience, Franklin Lakes, NJ) according to instructions. Intracellular staining of perforin, Granzyme A and B were done using combinations of the following antibodies: anti-human Perforin-PE/Dazzle, Granzyme A-AF647, Granzyme A-PerCp-Cy5.5, Granzyme B-AF647 (BioLegend, San Diego, CA) and Granzyme B-BV421 (BD Bioscience, Franklin Lakes, NJ) as described [100, 174]. Analysis was performed on BioRad ZE5 flow cytometers (BioRad) using Everest software and data analyzed with FlowJo software (Tree Star, Inc. Ashland, OR). Expression of surface markers was measured by the percentage of positive cells. Fluorescence minus one (FMO) strategy was used to establish appropriate gates.

2.2.8 Sample Preparation for Single-Cell Antibody and RNA Sequencing

Single cell antibody and RNA sequencing was performed using the BD Rhapsody platform (BD Biosciences). Hysterectomy tissues (endometrium and ectocervix) from one patient were minced and enzymatically digested with Tumor Dissociation Kit (Mitenyi Biotec) and dissociated in a gentleMACS Octo Dissociator (Miltenyi Biotec) followed by sequential filtering to obtain mixed single-cell suspensions. Mixed cell suspensions from EM and ECX were incubated with oligo-conjugated antibodies and barcoded sample tags to differentiate between the two samples. 20 minutes post incubation, cells were washed to remove unbound protein and barcoded sample tags. Cells were counted and 10,000 cells from each tissue combined and loaded onto a BD Rhapsody™ cartridge. RNA-capture beads containing unique molecular identifier (UMI) barcodes were subsequently loaded into the cartridge followed by a lysis step to release cellular RNA. Sequencing libraries are then generated as instructed by the manufacturer.

2.2.9 Sequencing and Data Processing

A total of 20,000 cells were sequenced in an S4 cell of NovaSeq6000 to generate raw FASTQ files with 100 base pair read length. Sequencing parameters were calculated to generate 60,000 reads per cell for whole transcriptome analysis, 600 reads per cell for sample tag barcoding and 850 reads per oligo-conjugated antibody per cell. FASTQ files were subsequently trimmed to 75 base pair read length, aligned, and annotated with the human genome to obtain gene counts by using manufacturer provided analysis pipeline. Gene counts were then uploaded to Partek® Flow® software, v10.0 for further analysis. Data was cleaned up to filter out cells expressing higher than 30% mitochondrial genes, less than 200 and greater than 4000 features per cell. Data matrix was split to separate out RNA and protein data and subsequently normalized. To enrich for immune cells, we selected cells expressing *PTPRC* (CD45) gene followed by generating principal components. Thereafter we selected cells expressing CD3 protein to enrich for T Cells

(**Fig. 5B**). To visualize our cells of interest, we performed principal component analysis followed by generating UMAP plots. To integrate protein expression and RNA expression data, we performed weighted-nearest neighbor analysis tool on the software. To evaluate transcriptional differences between the T cell subsets, we performed differential expression analysis using ANOVA and identified uniquely upregulated and downregulated genes ($p<0.05$; $-1.2<Log_2(FoldChange)>1.2$). Subsequently we generated hierarchical-clustering heat maps by calculating the mean expression of genes across the different subsets. We curated lists of genes for specific categories to generate hierarchical clustering heatmaps. To generate a list of gene ontology (GO) processes, we used gene lists of upregulated and downregulated genes mentioned above and considered significant processes with $p\leq0.05$. To detect uniquely upregulated pathways, we used the upregulated gene list for comparing with the KEGG database and considered significant pathways with $p\leq0.05$. Bubble plots were generated using GraphPad Prism v9.0.

2.2.10 Statistics

Data analysis was performed using the GraphPad Prism 8.0 software. A two-sided P-value <0.05 was considered statistically significant. Comparison of two groups was performed with the non-parametric Mann Whitney U test or Wilcoxon paired test. Comparison of three or more groups was performed applying the non-parametric Kruskal-Wallis or paired Friedman test followed by Dunn's post-test. Correlation analyses were performed applying non-parametric Spearman test. Power and sample size calculations were performed using anticipated means based on our prior publications [102, 174]. Data are represented as the median ± interquartile range.

2.3 Results

2.3.1 Endometrial antigen-presenting cell subsets maintain differential cytokine secretion profiles during the induction of T cell proliferation.

We have previously identified different subsets of antigen-presenting cells in the human endometrium and demonstrated their ability to induce proliferation of naïve T cells [102, 136], a hallmark of DC function [4, 142, 181]. Here, we wanted to define the cytokine secretion profile induced during the naïve T cell proliferation process to identify DC-specific functional differences between endometrial antigen-presenting cell subsets.
We purified CD1a+ and CD14+ cells from the same human endometrial samples and co-cultured them with allogeneic naïve T cells from blood as described before [102, 136, 137]. After magnetic bead selection, the CD1a+ and the CD14+ purified populations expressed high levels of CD11c and HLA-DR (**Appendix Fig. 5.1A**). The CD14+ selected population contained about 20% of cells co-expressing CD1a (**Appendix Fig. 5.1B**). Supernatants from the allogeneic co-culture were collected after 6 days to evaluate cytokine secretion in a multiplex assay as described in the methods. Compared to naïve T cells alone, co-culture with CD1a+ DCs resulted in a significant upregulation of most cytokines in our panel, including pro-inflammatory (GM-CSF, IFNγ), anti-inflammatory (IL-10, IL-5, IL-13) and chemokines (IL-8, GROα, MDC, IP-10) **(Fig. 2.1A)**. Co-culture of CD14+ cells with naïve T cells resulted in significant upregulation of the same cytokines as CD1a+ DCs and also IL1Rα and MCP-1 **(Fig. 2.1B)**. IL-17a, IL-15, IL-6 and IFNα2 were undetectable (not shown). To further determine the main cell source for cytokine production, we were able to include a control with CD14+ cells alone for a subset of patients (**Fig. 2.1C**). As seen in figure 2.1B, CD14+ cells alone produced high levels of GROα, MDC, IL1Rα, IL-8, IP-10 and MCP-1, which were further upregulated in the co-culture (**Fig. 2.1B**). CD14+ cells alone did not contribute to the

production of IFNγ, IL-13 or IL-5. We then compared the cytokine profiles induced during the T cell proliferation process by CD1a+ and CD14+ cells. As seen in **Fig. 2.1D**, CD1a+ DCs induced higher levels of GM-CSF, IFNγ, IL-13 and IL-5 compared to CD14+ cells, while CD14+ cells induced higher secretion of MCP-1, with no differences for the rest of cytokines and chemokines analyzed.

Next, we investigated if menopausal status could influence cytokine secretion profiles. For these analyses, results from CD1a+ and CD14+ cells were combined to increase power, since not enough co-cultures with CD1a+ DCs from premenopausal women were available for DC subset comparisons. Endometrial CD1a+ and CD14+ cells from postmenopausal women induced significantly increased secretion of multiple cytokines (GM-CSF, IFNγ, GROα, IL-10, MDC, IL-13, IL1Rα, IL-5, and IL-8) during T cell proliferation compared to premenopausal women (**Fig. 2.1E**). Both CD1a+ and CD14+ subsets contributed to the observed increased secretion in postmenopausal women, with the exception of IP-10, which was enhanced by CD14+ cells but not CD1a+ DCs (**Fig. 2.1E**).

These findings demonstrate that cytokine secretion profiles induced by endometrial CD1a+ and CD14+ cell subsets are distinct and change as women age, suggesting differential induction of T cell activation profiles.

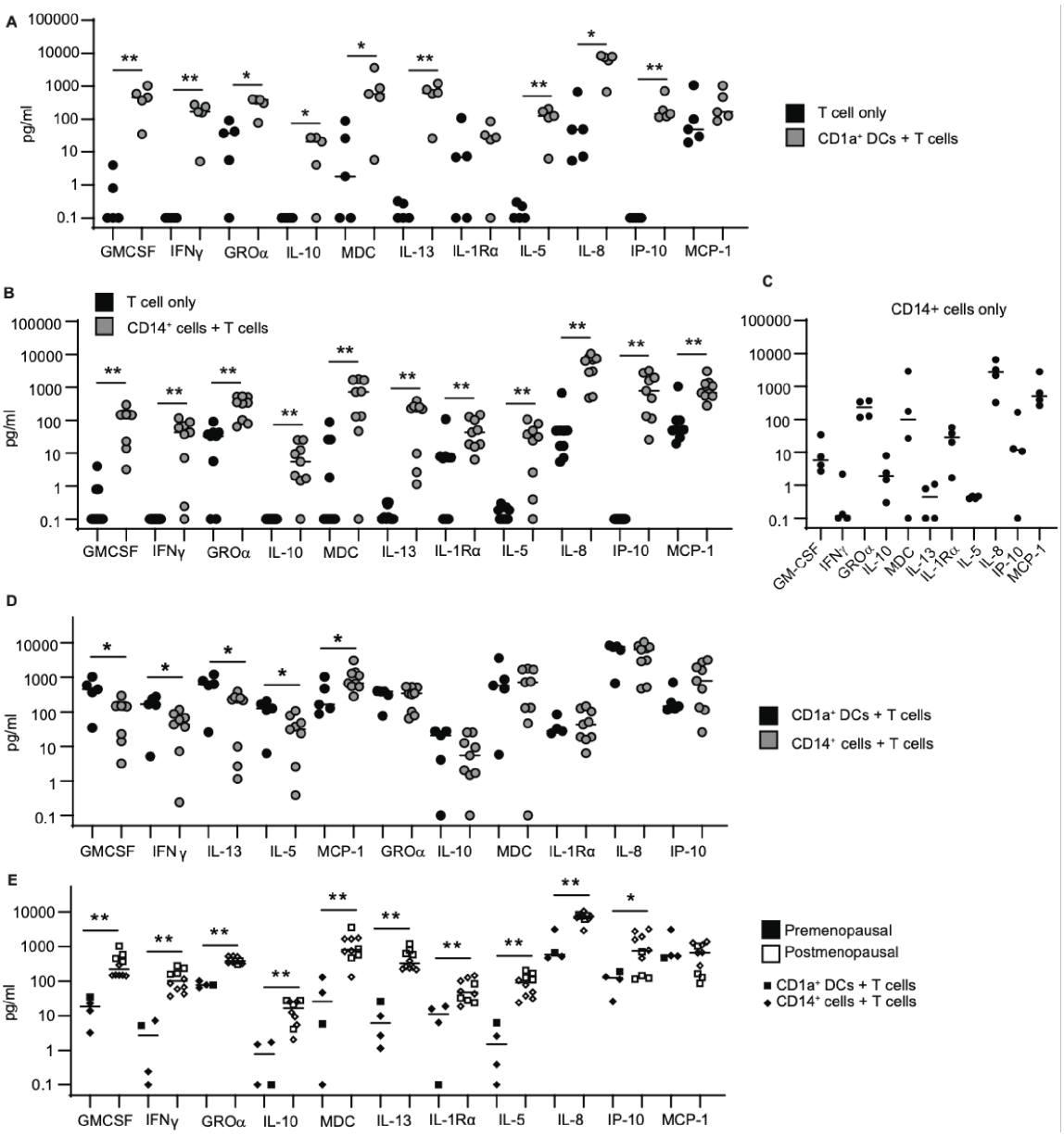

Figure 2.1: Menopause enhances cytokine profile induction by endometrial antigen presenting cells.

(A) Comparison of cytokine induction in co-culture of blood naïve T cells with allogeneic endometrial CD1a+ DCs and **(B)** CD14+ cells isolated from premenopausal (n=4) and postmenopausal patients (n=10). Black dots represent blood naïve T cells alone. **(C)** Production of cytokines by CD14+ cells alone (N=4). **(D)** Comparison of proliferating T cell cytokine induction by CD1a+ (n=5) and CD14+ (n=9) endometrial cells in co-culture. **(D)** Comparison of cytokine production in co-culture of blood naïve T cells with allogeneic endometrial antigen presenting cells (CD1a+ (squares) and CD14+ (diamonds)) from pre (black) and postmenopausal women (white). Mann-Whitney U test was used to compare independent groups. *p<0.05, **p<0.01, ***p<0.001.

2.3.2 Endometrial DCs induce naïve T cell proliferation in a subset-specific manner.

Since we observed differential secretion profiles during the T cell proliferation process between CD1a+ and CD14+ cells, we next investigated the extent to which these cell populations differ in their ability to induce naïve T cell proliferation, a DC-specific function [4, 142, 181]. Endometrial CD1a+ and CD14+ cells from the same human endometrial samples were co-cultured with allogeneic naïve T cells from blood and proliferation evaluated by flow cytometry as described [102, 137]. Consistent with our previous findings, after 6 days in culture we detected naïve T cell proliferation in the presence of both CD1a+ and CD14+ cells (**Fig. 2.2A**). However, CD1a+ DCs induced significantly more T cell proliferation compared to CD14+ cells isolated from the same patients (**Fig. 2.2B**).

Next, we analyzed proliferation of CD4+ and CD8+ T cell populations to identify potential differences in the type of T cell subset induced. As seen in **Fig. 2.2C**, CD1a+ and CD14+ cells induced proliferation of CD4+ and CD8+ T cells, but CD1a+ DCs showed enhanced ability to induced proliferation of both naïve T cell populations compared to CD14+ cells (**Fig. 2.2D**). These results indicate that endometrial CD1a+ DCs have enhanced ability to induce naïve T cell proliferation compared to CD14+ cells.

2.3.3 Endometrial DCs induce proliferation of double negative (CD4-CD8-) T cells.

During our analysis we noticed the presence of a double negative (DN) (CD4-CD8-) naïve T cell population that was also being stimulated to proliferate in addition to CD4+ and CD8+ T cells (**Fig. 2.2E**). Therefore, we examined the ability of each antigen presenting cell subset to induce DN T cell proliferation. Both CD1a+ and CD14+ cells induced DN T cell proliferation **(Fig. 2.2F)**, however, CD1a+ DCs were superior at

inducing DN T cell proliferation compared to CD14+ cells from the same patients (**Fig. 2G**), similar to our results with the other T cell subsets (**Fig. 2.2D**).

We have previously demonstrated that the TGFβ signaling pathway regulates genital DC ability to induce CD103+ CD8+ T cells without affecting overall proliferation induction capacity [102]. To investigate if this same pathway could be involved in induction of DN T cell proliferation by genital DCs, we blocked TGFβ signaling using the TGFβ receptor 1 blocker SB431542 during the proliferation process as described before [102]. As seen in **Figure 2.2H**, TGFβ blockade resulted in increased proliferation of DN T cells by 2-fold, while no changes were detected in the proliferation of CD4+ and CD8+ T cells. To better understand the underlying mechanisms responsible for enhanced proliferation of DN T cells after TGFβ blockade, we evaluated cytokine production during the proliferation process in the presence of SB431542. TGFβ blockade significantly enhanced production of IFNγ by both CD1a+ and CD14+ cells, and suppressed production of GRO, IL-8 and MCP-1 (10%, 37% and 60% reduction respectively) (**Fig. 2.2I**). GM-CSF and IP-10 followed opposite patterns by CD1a+ and CD14+ cells (**Fig. 2.2I**). Next, we assessed whether proliferation of DN T cells was associated with upregulation of CD103 and if it would be modified by TGFβ. As shown in **Figure 2.2J**, in contrast to CD8+ T cells, we detected minimal upregulation of CD103 on DN T cells during the proliferation process, with expression similar to that detected on CD4+ T cells, and TGFβ blockade did not modify CD103 expression (**Fig. 2.2K**).

Taken together, these results establish the ability of endometrial DCs to induce DN T cell proliferation through a TGFβ-dependent mechanism and demonstrate that CD1a+ DCs have enhanced ability to induce naïve T cell proliferation, including CD8+, CD4+ and DN T cell proliferation.

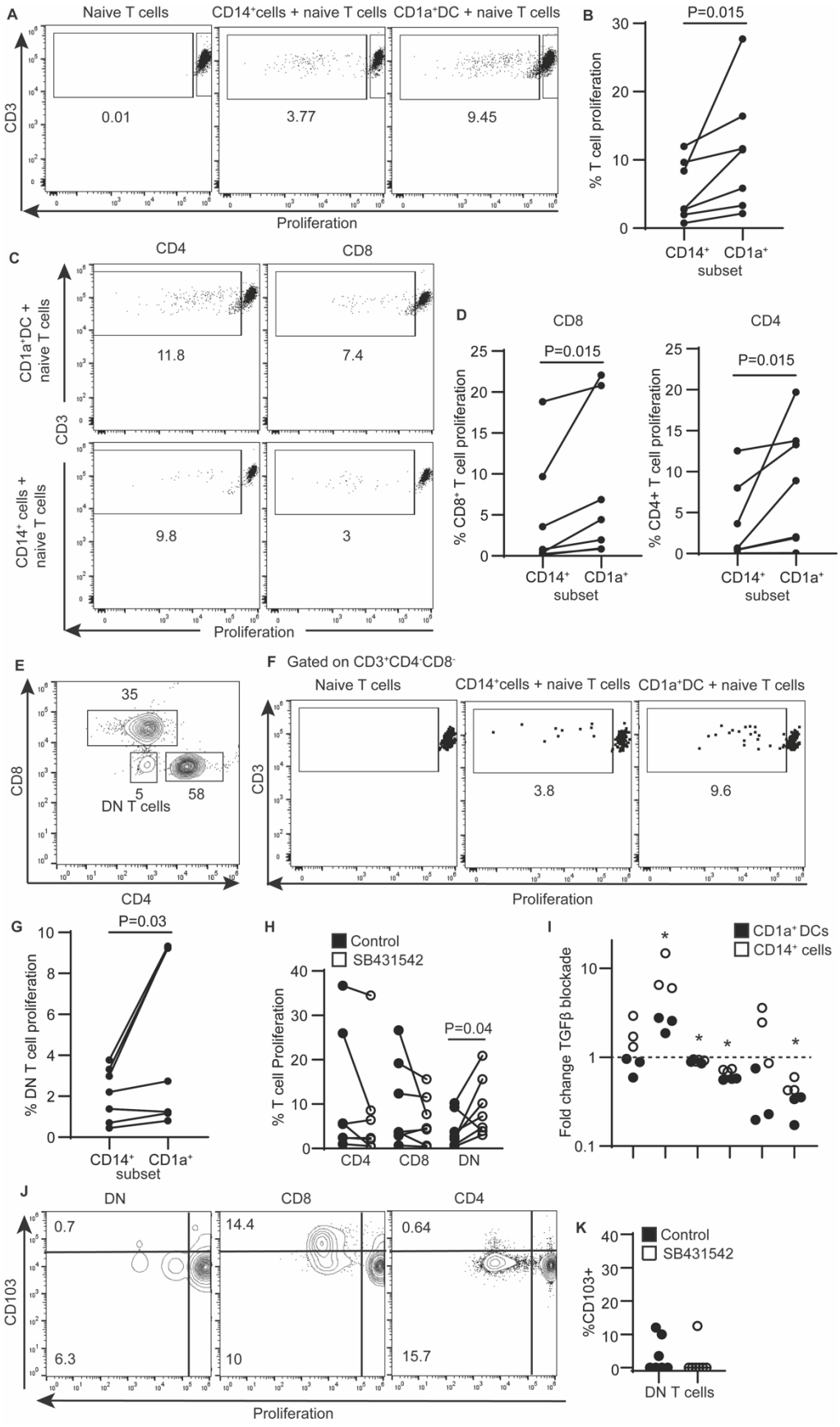
A
Naive T cells
CD14+cells + naive T cells
CD1a+DC + naive T cells
CD3
0.01
3.77
9.45
Proliferation
B
P=0.015
% T cell proliferation
CD14+
CD1a+
subset
C
CD4
CD8
CD1a+DC + naive T cells
CD14+ cells + naive T cells
CD3
11.8
7.4
9.8
3
Proliferation
D
CD8
CD4
P=0.015
% CD8+ T cell proliferation
% CD4+ T cell proliferation
CD14+
CD1a+
subset
E
35
5
58
DN T cells
CD8
CD4
F
Gated on CD3+CD4-CD8-
Naive T cells
CD14+cells + naive T cells
CD1a+DC + naive T cells
CD3
3.8
9.6
Proliferation
G
P=0.03
% DN T cell proliferation
CD14+
CD1a+
subset
H
Control
SB431542
% T cell Proliferation
P=0.04
CD4
CD8
DN
I
CD1a+ DCs
CD14+ cells
Fold change TGFβ blockade
J
DN
CD8
CD4
CD103
0.7
14.4
0.64
6.3
10
15.7
Proliferation
K
Control
SB431542
%CD103+
DN T cells

Figure 2.2: Endometrial DCs induce naïve T cell proliferation in a subset-specific manner.

Allogeneic naïve T cells from blood were co-cultured with CD14+ or CD1a+ cells isolated from the endometrium. **(A)** Representative plot demonstrating proliferation of naïve T cells in the absence and presence of CD14+ or CD1a+ endometrial cells. **(B)** Comparison of the percentage of naïve T cell proliferation induced by CD14+ and CD1a+ endometrial cells from the same women (n=7). **(C)** Representative example of CD4+ and CD8+ naïve T cell proliferation induced by CD14+ and CD1a+ endometrial antigen presenting cells. **(D)** Proliferation rate of CD8+ or CD4+ naïve T cells in the presence of CD14+ or CD1a+ cells. **(E)** Flow cytometry plot of different blood naïve T cell populations after proliferation assay. **(F)** Representative plots demonstrating induction of DN T cell proliferation by CD14+ or CD1a+ endometrial antigen presenting cells. **(G)** Proliferation percentage of DN T cells in response to the different endometrial antigen presenting cell subsets. Wilcoxon pair comparison was used. **(H)** Percentage of CD4+, CD8+ and DN T cell proliferation in the absence (control) or presence of the TGFβ signaling inhibitor SB431542 (n=7). Non-parametric Friedman test for multiple comparisons was used. **(I)** Fold change of cytokine production in co-culture of blood naïve T cells with allogeneic endometrial antigen presenting cells (CD1a+ (black; n=3) and CD14+ (white; n=3)) in the presence of the TGFβ signaling inhibitor SB431542 relative to control conditions. One sample Wilcoxon signed-rank test was used to compare the median of the TGFβ blockade group to the normalized value of the controls (1). *$p<0.05$. (**J**) Representative plots showing expression of CD103 on DN, CD8+ and CD4+ T cells during the proliferation process induced by CD14+ or CD1a+ endometrial antigen presenting cells. (**K**) Percentage of CD103 expression on proliferated DN T cells in the absence (control) or presence of the TGFβ signaling inhibitor SB431542 (n=7).

2.3.4 Aging selectively enhances endometrial CD1a+ DC ability to induce T cell proliferation.

Since we observed differences in secretion profiles induced by antigen presenting cells from premenopausal and postmenopausal women, we next explored if the ability of each DC population to induce naïve T cell proliferation could be differentially affected by aging.

To identify age-dependent effects, we purified CD1a+ and CD14+ cells from premenopausal and postmenopausal women, evaluated their ability to induce proliferation of naïve CD4+, CD8+ and DN T cells, and performed correlation analyses between the age of the women from which endometrial antigen presenting cells were purified and the level of proliferation of the different T cell subsets that each antigen presenting cell population induced. As seen in **Fig. 2.3A**, with increasing age, CD1a+

DCs induced increasing levels of CD8+, CD4+ and DN T cell proliferation. In contrast, no correlation was found between age and T cell proliferation levels induced by CD14+ cells (**Fig. 2.3B**).

We have previously reported that CD1a+ DCs express higher levels of CD83, a co-stimulatory molecule, than CD14+ cells [136] and therefore hypothesized that changes in CD83 expression with aging could be responsible for the age-dependent differences in induction of proliferation. However, we found no evidence of changes in the expression of CD83 in these subsets with increasing age (**Fig. 2.3C**).

These results indicate that aging selectively increases CD1a+ DC ability to induce proliferation of naïve T cells, including DN T cells, whereas CD14+ cell ability to induce proliferation remains largely unchanged as women age.

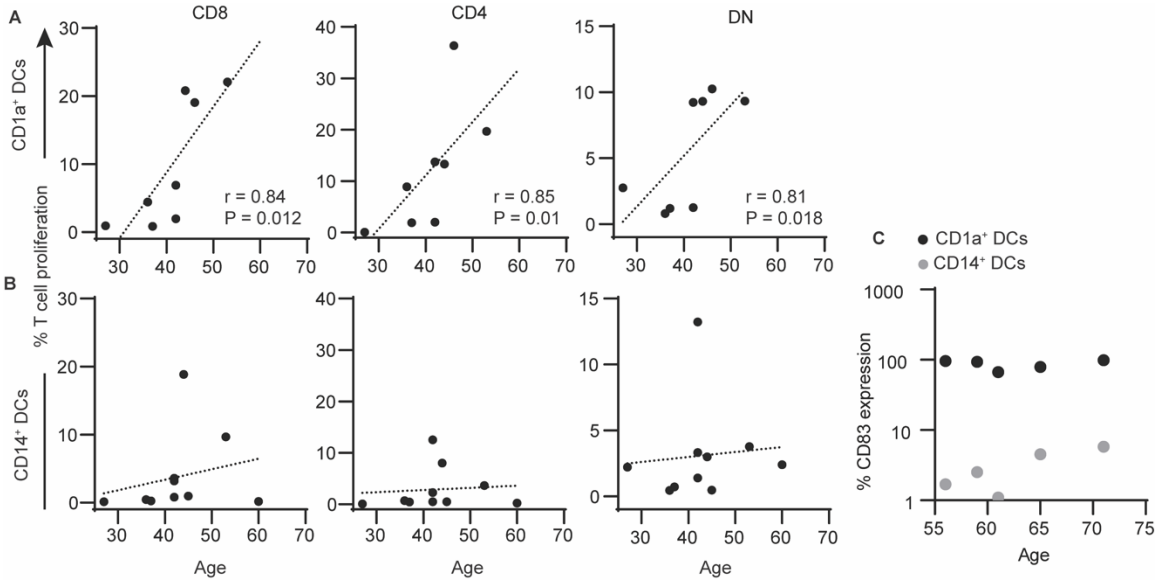

Figure 2.3: Aging selectively enhances endometrial CD1a+ DC function.

Correlation between age and proliferation of CD8+, CD4+ and DN T cells induced by **(A)** CD1a+ endometrial DCs (n=8) and (**B**) CD14+ endometrial cells (n=10). (**C**) Expression of CD83 by CD14+ and CD1a+ endometrial cells from the same patients with respect to age of patient (n=5). Spearman correlation test was used. P and r values are indicated when the correlation was significant.

2.3.5 DN T cells are more abundant in the endometrium than in the cervix and are redistributed with increasing age in a site-dependent manner.

Recognizing that DN T cells remain undefined in human genital tissues and considering our *in vitro* findings that endometrial DCs induce DN T cell proliferation in an age-dependent manner, we next investigated DN T cell distribution in FRT tissues, and any potential modifications with age.

Mixed cell suspensions were generated after digestion of endometrial, endocervical and ectocervical tissues from the same patients to identify DN T cells by flow cytometry. After gating on the T cell population (CD45+CD3+), a DN (CD4-CD8-) T cell subset was clearly identified that represented less than 10% of the total CD3+ T cell population (**Fig. 2.4A**). DN T cells were detected in endometrium (EM), endocervix (CX) and ectocervix (ECX), but were significantly more abundant in the EM compared to the other two sites **(Fig. 2.4B**).

Next, we evaluated if the distribution of DN T cells could be affected by menopause by comparing samples obtained from premenopausal and postmenopausal women. Because no significant differences were detected in DN T cell abundance between endo and ectocervical samples (**Fig. 2.4B**), data from these two locations were combined as “cervical” for menopause and aging comparisons. As seen in **Figure 2.4C**, when samples from premenopausal women were compared, no differences were found between DN T presence in endometrium and cervix, but, after menopause, DN T cell presence was significantly reduced in the cervix compared to the endometrium.

To evaluate if aging in the years following menopause had any additional effects on DN T cell tissue levels, we performed correlations with age in the postmenopausal group and found that DN T cells significantly declined in cervical samples but remained

constant in the endometrium (**Fig. 2.4D**).

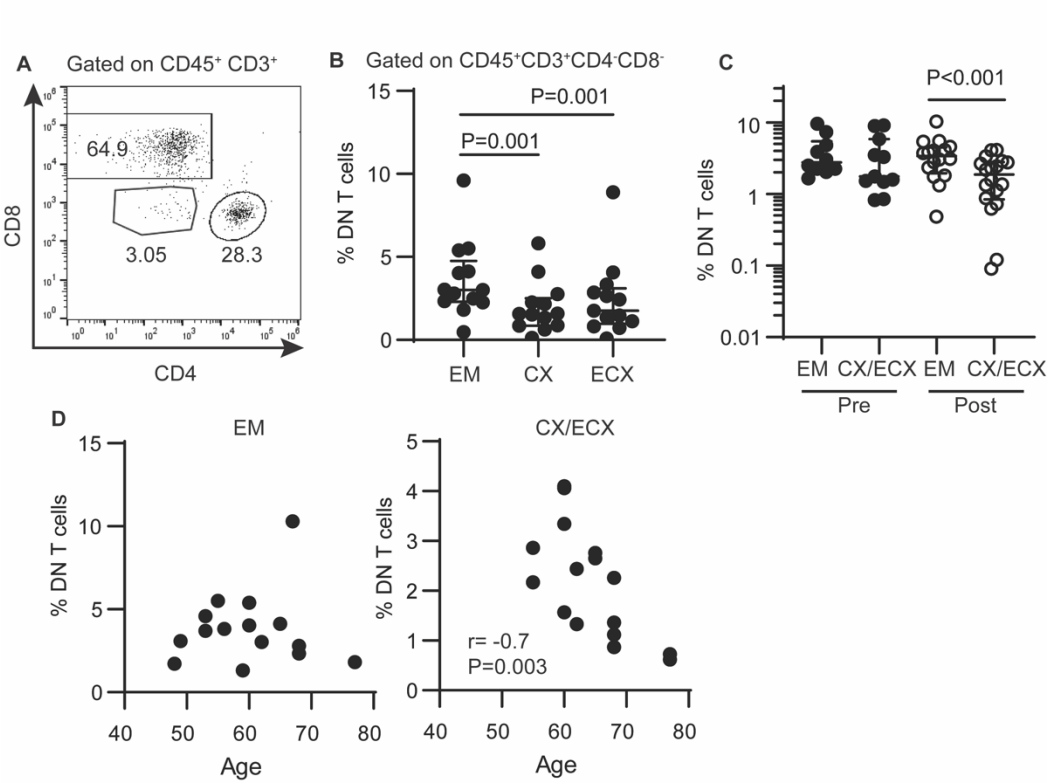

Figure 2.4: DN T cells are present in FRT tissues, are more abundant in the endometrium and show redistribution with aging in a site-dependent manner.

Flow cytometry analysis of T cell populations in different anatomical regions of the FRT. **(A)** Representative plot of T cell populations in the FRT (EM). (**B**) Percentage of DN T cells in endometrium (EM), endocervix (CX) and ectocervix (ECX) from the same patients (n=13; Friedman test). **(C)** Comparison of DN T cell percentages in EM and CX/ECX in premenopausal (pre; EM=10, CX/ECX=11) and postmenopausal women (post; EM=16, CX/ECX=19). (**D**) Correlation between age and percentage of DN T cells in EM (left) and CX/ECX (right) following menopause. Non-parametric paired Friedman **(B)** and unpaired Kruskal-Wallis **(C)** test followed by Dunn's post-test**.** Spearman test was used in D, and P and r values are indicated when the correlation was significant.

2.3.6 DN T cells represent a discrete T cell subset in the FRT with distinct transcriptional profiles.

Since DN T cells are poorly characterized in the human FRT, to better understand their phenotype and function, we optimized a protocol to simultaneously determine DN T cell surface expression markers and the whole transcriptome profile at the single-cell level through oligo-conjugated antibody tags and RNA sequencing.

Mixed cell suspensions from endometrium and cervix were enriched for immune cells, incubated with oligo- conjugated antibodies and whole transcriptome profile of single cells determined by sequencing as detailed in methods (**Fig. 2.5A**). Analysis of surface proteins via oligo-conjugated antibodies identified the presence of DN T cells (CD3+, CD4-, CD8-) (**Fig. 2.5B,** purple), reproducing our flow cytometry findings (**Fig. 2.4A**). When protein expression, gene expression profile or RNA+protein integrated data were represented using uniform manifold approximation and projection (UMAP), DN T cells were identified in multiple clusters, preferentially clusters containing CD8+ T cells (**Fig. 2.5C**), suggesting heterogeneity of the DN T cell population.

To further evaluate the transcriptional differences between DN T cells, CD4+ and CD8+ T cells, we compared their whole transcriptome expression profiles. As expected, the majority of transcripts detected in DN T cells were shared with CD4+ and CD8+ T cells, although uniquely expressed genes were also identified (**Fig. 2.5D**). Analysis of differentially expressed genes (DEG) between DN T cells and CD4+ T cells identified 869 genes significantly upregulated in DN T cells, and 237 downregulated in comparison to the CD4+ T population (**Fig. 2.5E, left panel**). Similarly, comparison of DN T cells and CD8+ T cells, identified significant upregulation of 767 genes and downregulation of 128 in DN T cells (**Fig. 2.5E**, right panel), demonstrating that DN T cells are transcriptionally different from both CD4+ and CD8+ T cells.

To further characterize the phenotype of DN T cells, we analyzed the expression of surface markers included in our oligo-conjugated antibody panel. Hierarchical clustering of cell surface markers separated DN T cells from CD4+ and CD8+ T cells (**Fig. 2.5F)** and demonstrated enhanced protein surface expression of inhibitory check point molecules (PD-1, PD-L1 and CTLA-4), chemokine receptors (CX3CR1 and CCR5), molecules involved in T cell priming/activation (HLA-DR) and apoptosis (CD117 and CD10) [182, 183]. Hierarchical clustering of RNA sequencing data for these same

markers also segregated DN T cells from CD4+ and CD8+ T cells (**Fig. 2.5G**) and demonstrated concomitant upregulation of protein and gene expression for some molecules, such as PD1, PDL1, CD117 (*PDCD1*, *CD274*, and *KIT*), while other markers showed opposite profiles, such as CD10 (*MME*) and CTLA4 (**Fig. 2.5G**). Intermediate expression of CD62L (*SELL*), CD69 and CD103 (*ITGAE*) in DN T cells was consistent between protein and RNA results (**Fig. 2.5F and 2.5G)**. These findings highlight the importance of using integrated protein + RNA data to characterize these cells. Because CD69 and CD103 are expressed on tissue-resident T cells [184], we investigated in more detail protein/gene profiles related to tissue residency to determine if DN T cells represent a tissue resident population. Tissue resident profile can be defined by high expression of CD69 and downregulation of S1PR1 and CCR7, with CD103 expression related to residency in epithelial tissues [184]. As seen in **Fig. 2.5H**, about 20% of DN T cells expressed CD103 protein, approximately half of DN T cells expressed CD69 transcripts, less than 20% of DN T cells expressed SRP1 and around 12% expressed CCR7 transcripts, similar to CD8+ T cells. This transcriptional profile suggests the presence of tissue resident DN T cells.

To further validate our single-cell antibody findings, we performed flow cytometry staining of some of the markers identified in DN T cells and confirmed surface expression of CD10, HLA-DR, CD62L, CCR7, CX3CR1 and high expression of CCR5 (the main HIV coreceptor for mucosal infection [132]) on the DN T cell population in the different anatomical regions of the FRT **(Appendix Fig. 5.2).** Of note, CCR7 surface expression was detectable but very low **(Appendix Fig. 5.2E)**, consistent with the transcriptional signature and with a tissue-resident phenotype [184]. Next, we investigated additional differentially expressed genes that encode surface markers not included in our antibody panel that could help identify and characterize DN T cells. As seen in **Figure 2.5I**, DN T cells expressed genes that encode NK cell markers (*KIR3DL1, KIR3DL2, NCR1*), the

chemokine receptor CCR3, markers involved in apoptosis (*TNFRSF9, CEACAM5*), cytokine receptors (upregulation of *IL13RA*, but downregulation of *IL4R* and *IL7R*), and markers involved in adhesion to extracellular matrix and human papillomavirus viral entry (*PVR*). We also detected expression of genes encoding TGFβ receptors (*TGFBR1* and *TGFBR2*), suggesting that DN T cells may respond to tissue derived TGFβ.

Because of the NK cell related signatures and heterogeneous profile detected, we further investigated potential contribution of known T cell subsets that lack CD4 and CD8 expression, such as NKT cells, γδ T cells and MAIT cells. As shown in supplementary fig. 3, DN T cells expressed low levels of CD56, but lacked CD16 expression, making it unlikely that NKT cells represent a major contributing subset to the DN T cell population [185] (**Appendix Fig. 5.3A**). Furthermore, DN T cells expressed less than 2% of γδTCR as determined by flow cytometry, but about 10% of DN T cells co-expressed *TRGC1/TRGC2* and *TRDC* as determined by RNA sequencing, which encode the gamma and delta chains of the γδTCR (**Appendix Fig. 5.3B**). Lastly, we detected less than 3% MR1 expression on DN T cells and by did not detect *MR1*, *SLC4A10* or *TRAV1-2* RNA expression (**Appendix Fig. 5.3C-D**), genes characteristic of MAIT cells [186]. Overall, these results suggest that these populations were not major contributing subsets to the observed signatures.

We also identified a number of transcription factors upregulated in DN T cells, relative to that seen on CD4+ and CD8+ T cells, many of which were involved in embryonic development (**Fig. 2.5l)**. Of particular interest to immune and reproductive function in the FRT, we identified upregulation of transcription factors critical for ovarian development and function (*FOXL2, CREB5*), differentiation and function of female reproductive organs (*ZBED6, HOXD2, HOXA11, LHX1*), regulation of the Wtn/β-catenin pathway (*SALL4, CDX4, SOX2, ZBED3*), PPARα pathway (*AHRR, GRHL1*) and IL-4 and IL-13 signaling pathway (*SOX2*), and cellular senescence and response to DNA damage (*KIN, SCMH1*).

We also detected upregulation and downregulation of genes encoding secreted factors (**Fig. 5I**). Remarkably, DN T cells specifically expressed high levels of genes related to secreted molecules involved in tissue homeostasis and remodeling including basement membrane formation and function and epithelial-mesenchymal regulation (*LAMA3*), wound healing (*FGF2*) and extracellular matrix binding (*OLFML2A*). DN T cell also had upregulated expression of *CCL5* and *CCL4*, CCR5-ligands important for innate anti-HIV protection, *FGFBP2* which is secreted selectively by cytotoxic lymphocytes, *ZBED3* and *DKK3* which are related to the Wtn/β-catenin pathway and IL-10, with immunoregulatory function.

To better understand the immunological functions of DN T cells in comparison to CD4+ and CD8+ T cells we next performed gene ontology (GO) analysis of significantly upregulated genes in DN T cells. When compared to CD4+ T cells, DN T cells had significant enrichment of terms related to cytotoxicity (**Fig. 2.5J and Table 2.1, Additional file 1**), including T cell mediated (T cell mediated cytotoxicity, regulation of cell killing, positive regulation of IFNγ production) and NK cell mediated cytotoxicity, terms related to MHC-I and MHC-II peptide binding and T cell signaling/activation, and terms involved in innate-like defense responses (TLR signaling, CCR1 chemokine receptor binding, response to bacterium and external stimulus), including anti-viral responses (regulation of viral life cycle, TLR7 signaling pathway) (**Fig. 2.5J**). When compared to CD8+ T cells, a smaller number of terms was significantly enriched, mostly related to regulation of immune response (regulation of IL-2 production and leukocyte/T cell activation), regulation of T cell differentiation (proliferation, Th1, Th2 and cytotoxic differentiation) and MHC-I peptide binding (**Fig. 2.5J**).

Consistently, KEGG pathway analysis, revealed enrichment of pathways related to antigen processing and presentation, graft vs host disease, NK cell mediated cytotoxicity, allograft rejection, the PI3K-akt signaling pathway (involved in cell survival

and apoptosis) and several viral infection pathways, including human papillomavirus infection (HPV) (**Fig 2.5K**). These pathways were significantly enriched in DN T cells when compared to CD4, and were present when compared to CD8 T cells, but did not reach significance (**Fig 2.5K**).

Overall, these findings demonstrate that DN T cells are transcriptionally different than CD4+ and CD8+ T cells and suggest that DN T cells may display functions involved in reproduction, immune regulation and defense, including anti-viral functions, through cytotoxicity and innate-like mechanisms.

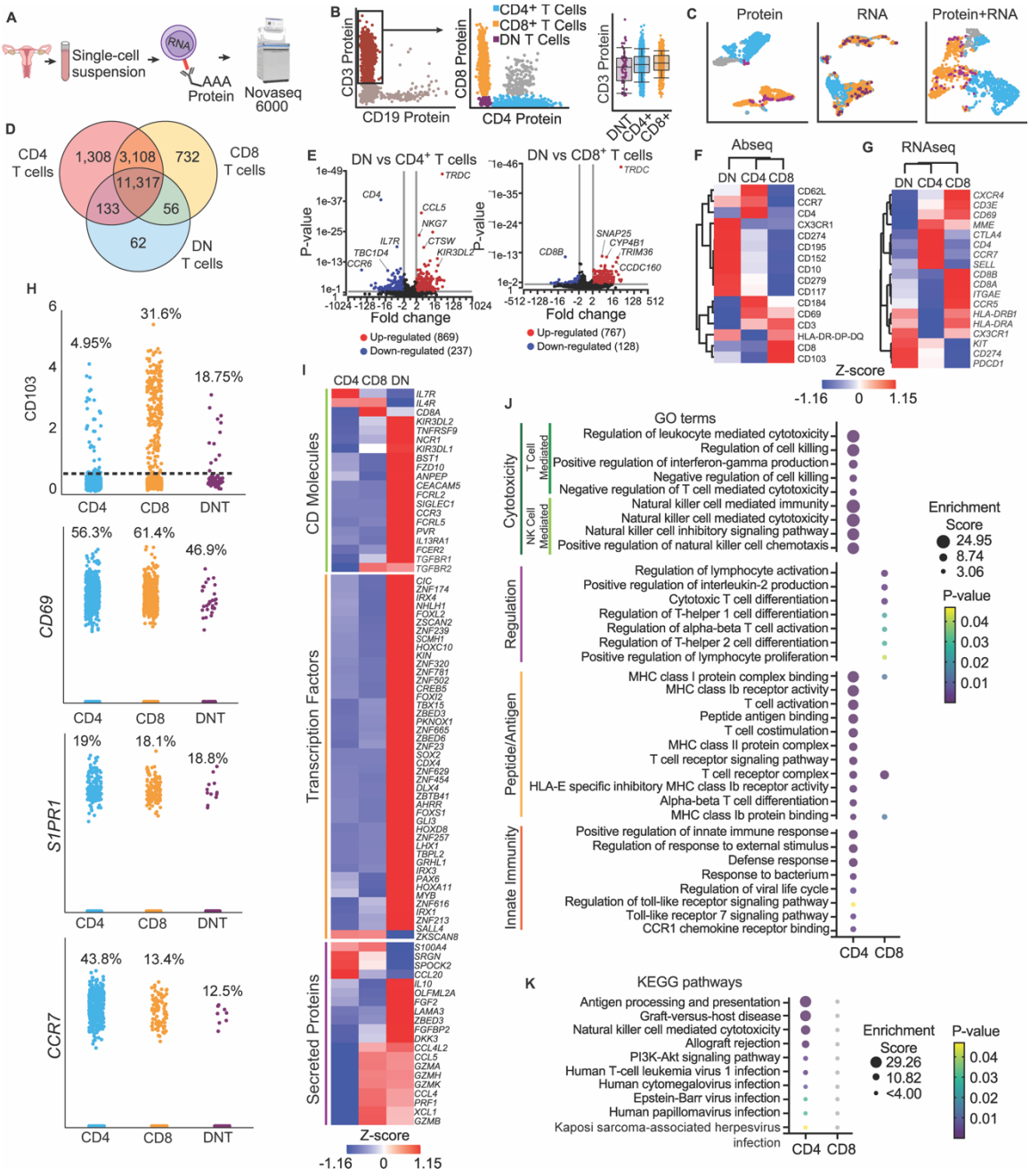

Figure 2.5: DN T cells constitute an unconventional subset of T cells in the FRT with a distinct gene expression profile.

(A) Diagram of experimental procedures. **(B)** Identification of CD3+ DN T cells via oligo-conjugated antibody expression**. (C)** Analysis of protein expression, RNA expression or protein + RNA integrated data plotted in Uniform manifold approximation and projection (UMAP) graphs from T cell subsets: CD4+ (blue), CD8+ (yellow), DN (purple) and double positive T cells (grey). **(D)** Venn diagram shows genes expressed in DN T cells, CD4+ and CD8+ T cells. **(E)** Volcano plot showing differentially expressed genes comparing DN vs CD4+ T cells (left panel) and DN vs CD8+ T cells (right panel) (significance p≤0.05; -2 < Fold Change >2). **(F)** Hierarchical clustering heatmaps

comparing relative expression levels of surface markers (AbSeq) and **(G)** analogous RNA expression. (**H**) Percentage of each T cell population expressing tissue-resident related profiles: CD103 oligo-conjugated antibody, and transcripts for CD69, S1PR1 and CCR7. **(I)** Heat map of selected CD molecules, transcription factors and secreted proteins differentially expressed in DN, CD4+ and CD8+ T cells. (Significance $p \leq 0.05$; -1.2 < Fold Change >1.2). **(J)** Bubble plot shows the enriched gene ontology (GO) terms significantly upregulated in DN T cells vs CD4+ or DN T cells vs CD8+, that are associated to functions related to cytotoxicity, regulation, antigen presentation and innate immunity. **(K)** Bubble plot shows enriched KEGG pathways in DN T cells vs CD4+ and DN T cells vs CD8+ T cells.

2.3.7 DN T cells express tissue residency markers and cytotoxic molecules.

Our single-cell data analysis identified transcripts related to tissue residency and cytotoxicity in the DN T cell population. To directly determine tissue residency and cytotoxic potential of DN T cells in FRT tissues, we measured surface expression of CD69 and CD103 (markers that identify tissue-resident memory T cells and intraepithelial residency) [184, 187], and intracellular content of cytotoxic molecules by flow cytometry.

DN T cells expressed high levels of CD69, with a subset of cells co-expressing CD103, indicating tissue-resident phenotype (**Fig. 2.6A**). Comparison of DN T cells from different anatomical compartments within the FRT (EM, CX and ECX) revealed CD69 expression on 20-95% of cells, with expression significantly higher in EM compared to CX and ECX (**Fig. 2.6B**). Next, we investigated whether aging affects CD69 expression, but no correlation was detected between the percentage of CD69+ DN T cells and age, regardless of the FRT site analyzed (**Fig. 2.6C**). DN T cells expressed CD103 at levels comparable to CD8+ T cells (**Fig. 2.6D**). DN T cells from the three FRT sites analyzed (EM, CX and ECX) expressed CD103 at equal levels, with a wide range of expression (**Fig. 2.6E**). We investigated potential effects of menopause and aging on CD103 expression, however no correlations were found (**Fig. 2.6F**).

Next, cytotoxic potential of DN T cells was evaluated by measuring intracellular content of perforin, granzyme (GZ) A and B. Intracellular staining of resting cells revealed

expression of perforin, GZB and GZA, at levels intermediate in between the CD8+ and the CD4+ T cell populations (**Fig. 2.6G**). While the percentage of cells expressing perforin was similar to the CD8+ T cell population, the expression of GZB and GZA resembled the CD4+ T cell population (**Fig. 2.6G**). We have previously described that CD8+CD103- T cells express higher levels of cytotoxic molecules compared to CD8+CD103+ T cells [100, 174]. To determine if this differential expression was also present in the DN T cell population, we measured cytotoxic molecules in CD103+ and CD103- DN T cells. The intracellular content of perforin, GZA and GZB was significantly higher in CD103- compared to CD103+ DN T cells, and this difference was observed in the three anatomical sites analyzed (**Fig. 2.6H**). Perforin was expressed in less than 10% of CD103+ DN T cells, while the mean percent of CD103+ DN T cells expressing GZB and GZA was around 25% (**Fig. 2.6H**). In comparison, CD103- DN T cells had significantly higher expression of cytotoxic molecules, with more than double the percentage of perforin+ and GZA+ cells compared to CD103+ DN T cells (**Fig. 2.6H**). Finally, we evaluated whether expression of intracellular molecules changes with aging. DN T cells expressing GZB and GZA did not change with aging (not shown), however, we observed age-dependent changes in CD103+perforin+ DN T cells in a tissue-specific manner. In the EM, perforin-expressing DN T cells increased after menopause as women aged, while a significant decline was observed in the ECX, but no effects of aging were detected in the CX (**Fig. 2.6I**). Interestingly, age-dependent changes were detected only in the tissue resident CD103+ DN T cell population (**Fig. 2.6I**), with no significant changes observed in CD103- DN T cells (**Fig. 2.6J**).

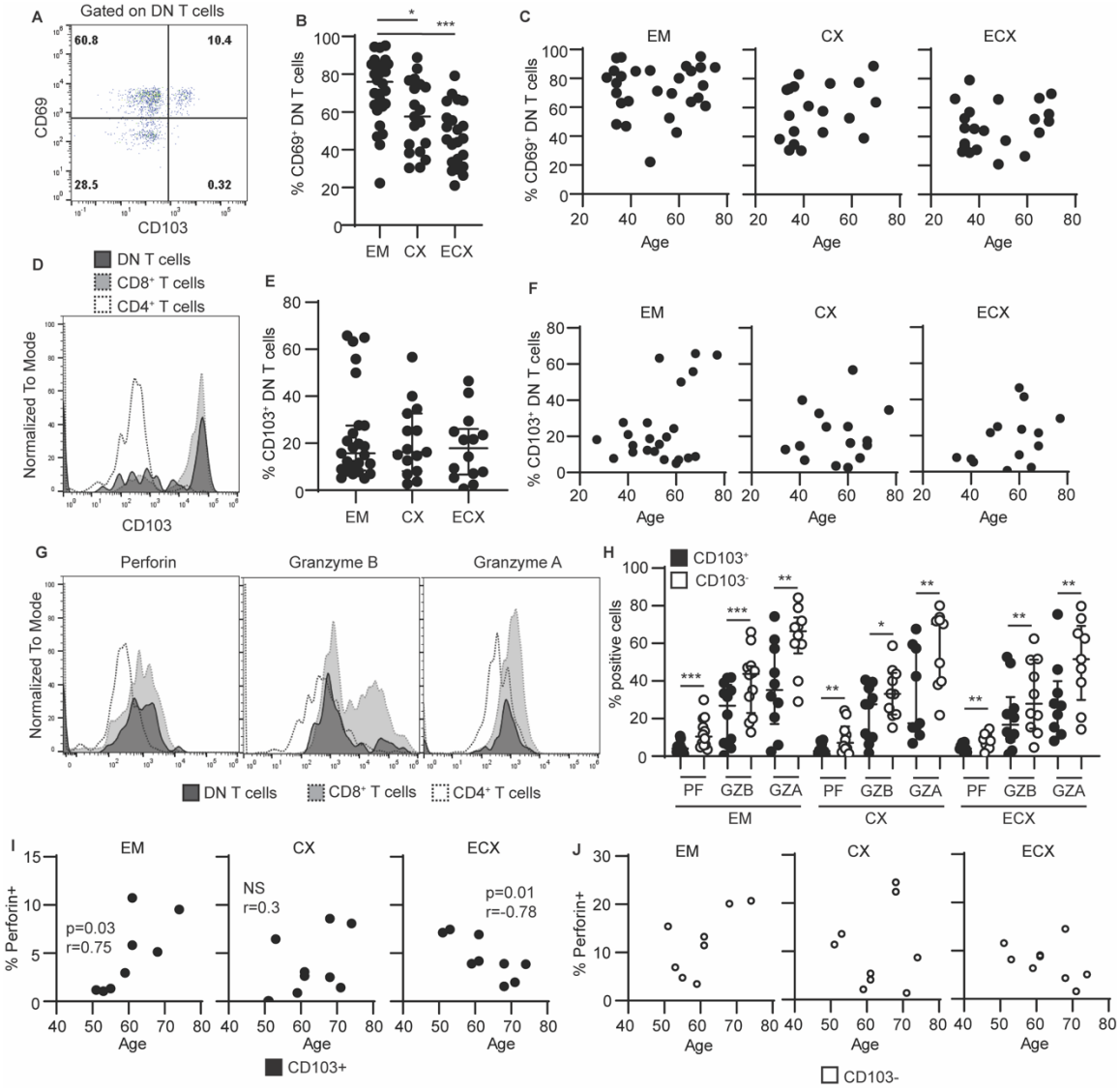

Figure 2.6: DN T cells express tissue residency markers and intracellular cytotoxic molecules.

(**A)** Representative contour plot of CD69 and CD103 expression on DN T cells. (**B**) Percentage of CD69+ DN T cells in different anatomical regions of the FRT (EM=28, CX=19, ECX=22). (**C**) Lack of correlation between CD69+ DN T cells and age in endometrium (EM=28), endocervix (CX=19) and ectocervix (ECX=22). (**D**) Representative overlay histogram of CD103 expression by DN T cells (black), CD8+ (gray) and CD4+ (clear) T cells. **(E)** Percentage of CD103+ DN T cells in different anatomical regions of FRT (EM=24, CX=15, ECX=14). (**F)** Lack of correlation between CD103+ DN T cells and age in EM (left), CX (center) and ECX (right). **(G)** Representative plots of Perforin (right), Granzyme B (center) and Granzyme A (left) expression by DN (black), CD8+ (gray) and CD4+ (clear) T cells in the FRT. (**H**) Comparison of percentage of CD103+ (black) and CD103- (white) DN T cells expressing perforin (PF), granzyme B (GZB) and granzyme A (GZA) in the endometrium (EM=12), endocervix (CX=10) and ectocervix (ECX=10). Wilcoxon paired test was used to compare CD103+ and CD103- DN T cells within each tissue. *p<0.05, **p<0.01,

***p<0.001. **(I)** Correlation between age in postmenopausal women and perforin+ CD103+ DN T cells and **(J)** perforin+ CD103- DN T cells in EM (N=8), CX (N=9) and ECX (N=9). Spearman correlation analysis was performed. Each dot represents a different patient.

2.4 Discussion

Using human genital samples from women ranging from 27 to 77 years of age, we demonstrate for the first time a compartmentalized regulation of DN T cell induction, distribution and function by aging in the female genital tract. We demonstrate that human genital DCs, particularly CD1a+ DCs, induce proliferation of DN T cells, and that this function is enhanced with aging following menopause (**Fig. 2.7**, left panel). Further we demonstrate that DN T cells in genital tissues represent a functionally heterogeneous tissue-resident population with potential homeostatic, regulatory, cytotoxic, and innate-like antiviral functions and that their presence and function are selectively regulated by aging in a site-specific manner (**Fig. 2.7**, right panel). Our deep characterization of DN T cells opens new avenues for research and therapeutic potential of targeting DN T cells to improve fertility and pregnancy outcomes, gynecological cancers and antiviral protection in the female genital tract as women age.

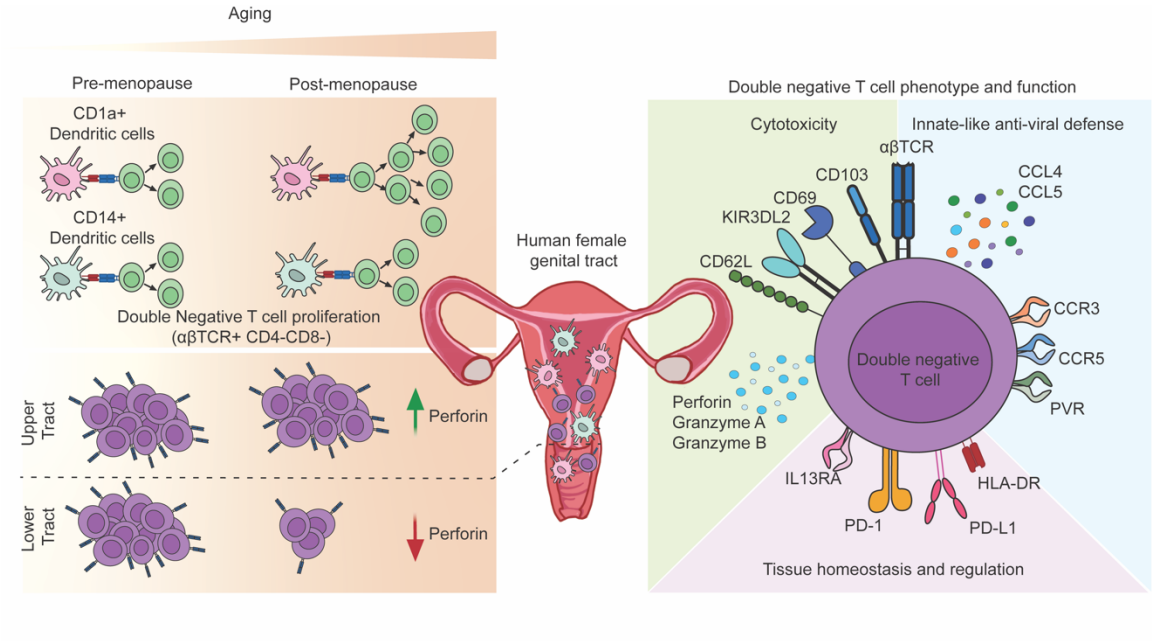

Figure 2.7: Aging regulates DN T cell induction by DCs and DN T cell distribution and function in the FRT.

Schematic summary of age-dependent and DC subset specific induction of DN T cell proliferation and changes with age found in this study (top left panel); anatomical region-specific differences in DN T cell numbers with respect to aging and differences in perforin expression (bottom left panel); and phenotypic and functional characteristics of FRT DN T cells illustrating molecules associated with cytotoxicity, innate-like anti-viral defense, tissue homeostasis and regulation (right panel).

Most studies investigating functional alterations in DCs induced by aging utilize models of *in vitro*-generated monocyte-derived DCs, known to be transcriptionally different than *bona fide* DCs [148, 188]. However, how aging affects different subsets of DCs in tissues is largely unknown, partly due to the technical difficulty in obtaining human tissues and purifying DC subsets. This is particularly important because tissue-resident DC subsets are conditioned and unique to the local tissue environment [176]. In this study, using primary antigen presenting cell subsets purified from the human endometrium, we demonstrate that aging and menopause regulate DC function selectively in a subset-specific manner, with CD1a+ DCs being particularly sensitive to functional changes after menopause. CD1a+ DCs induced DN T cell proliferation better than CD14+ cells, and this differential function between CD1a+ and CD14+ cells was most pronounced as

women aged due to selective enhanced proliferation induction capacity in CD1a+ DCs. Unexpectedly, we found an age-dependent enhanced ability of CD1a+ DCs to induce overall T proliferation (including CD4+, CD8+ and DN T cells) when compared to CD14+ cells in our *in vitro* allogeneic system. This contrasted with our previous observation that no changes in DC proliferation induction capacity occurred with age [102]. These apparently disparate findings were due to an analysis in which both antigen presenting cell subsets were combined in our previous report [102], highlighting the importance to study subsets individually. Whether DN T cell induction is a specific function of CD1a+ DCs or a consequence of an overall enhanced ability to induce naïve T cell proliferation remains to be determined. Enhanced ability of CD1a+ DCs to induce naïve T cell proliferation is in agreement with our prior studies indicating that genital CD1a+ DCs represent a homogeneous group of *bona fide* DCs, while the CD14+ population is more heterogeneous and comprises different monocyte-derived populations with phenotype and functions characteristic of DCs, including a subset of CD14+ CD1c+ DCs [136]. Interestingly, although CD14+ cell ability to induce T cell proliferation remained constant after menopause, the cytokine profile induced during the proliferation process by both CD1a+ and CD14+ cells changed, showing enhanced production of Th1 cytokines (IFNγ) and Th2 cytokines (IL-5, IL-10, IL-13) after menopause. Because the cytokine environment during antigen presentation is known to determine the function of induced T cells, this postmenopausal environment might support enhanced cytotoxic activity in CD8+ T cells after menopause. This would be congruent with our previous observations that cytotoxic activity of FRT CD8+ T cells is increased in postmenopausal women [100, 101, 131, 175]. Further, our studies suggest enhanced induction of Th2 differentiation following menopause. This is in agreement with previous reports of age-dependent decline in the Th1/Th2 ratio in blood, particularly in women [189]. Of note, increased IP-10 (CXCL10) production after menopause was only detected in co-cultures with CD14+

cells. Recognizing that IP-10 has been involved in impaired T cell function and proliferation in the context of chronic viral infections [190, 191], IP-10 may be an interesting candidate molecule to explore in future studies to explain the differences in proliferation induction capacity between CD1a and CD14 DCs and changes with age detected in our study.

From a mechanistic standpoint, changes in DC proliferation induction capacity and cytokine production could be driven by the lack of hormonal control after menopause, intrinsic defects in aging DCs and the influence of the senescent tissue environment. We and others have previously shown that estradiol treatment modifies innate immune function of blood DCs [192, 193] and genital DCs [136]. We have also demonstrated that overall DC numbers decline in the FRT as women age together with tissue-resident T cells [102]. Our findings here indicate that EM CD1a+ DC function is actively suppressed during the premenopausal years, most likely to prevent rejection during pregnancy, and/or that CD1a+ DCs are more susceptible to intrinsic or environment-induced aging effects. Future studies to investigate the involvement of sex hormones in regulation of CD1a+ DC function in the FRT is warranted.

Another novel contribution of our study is the deep phenotypic, molecular and functional characterization of DN T cells in the FRT. We demonstrate that DN T cells are present throughout the human FRT and that they are more abundant in the endometrium, which may be consistent with their previously reported involvement in reproduction in animal models [85]. The phenotype and function of DN T cells in humans remains unclear due to the low frequency of these cells and the lack of standardized definitions, particularly as it relates to human tissues. Here, using oligo-conjugated antibody tags and RNA single-cell sequencing in combination with flow cytometry, we describe for the first time that genital DN T cells represent a transcriptionally distinct and functionally heterogeneous population of unconventional T cells with a broad spectrum of potential

functions, including cytotoxicity, T cell regulation, antigen presentation and innate-like functions. Our transcriptional identification of functionally heterogeneous DN subsets is consistent with a recent characterization of DN T cells in mouse spleen using single-cell sequencing and the identification of T helper, cytotoxic and innate immune functions [83, 194]. These shared transcriptional signatures between our study and prior studies may represent common defining profiles for DN T cells. In addition, we identified potentially unique characteristics of genital DN T cells that may be relevant for homeostatic functions in the genital tract (such us maintenance of barrier function and reproductive function) and the potential involvement of DN T cells in genital viral infections, such as HPV and HIV infection. Involvement in mucosal HIV infection is further supported by a previous report describing DN T cell presence at the endocervical mucosal surface of female sex workers [84]. These unique signatures may be driven by the tissue environment as described previously for tissue-resident memory CD8+ T cells [184], a topic that deserves further investigation. We report that a large proportion of DN T cells display a tissue-resident phenotype (CD69, CD103 and low CCR7) [184, 187], and that the majority of DN T cells display an effector memory or effector cell phenotype (CD62L-CCR7-), consistent with what has been described for other T cell subsets in the FRT [89, 132, 195]. Further, we identify a number of markers with regulatory functions, including KIR3DL, inhibitory checkpoint molecules, HLA-DR, as well as cytokine and chemokine receptors and secreted factors that can be further researched for potential therapeutic purposes. Interestingly, in contrast to previous reports with mice DN T cells [83], we did not detect a Th17 signature in our samples, which could be unique to the FRT and consistent with the reported negative effect of Th17 cells in pregnancy outcomes [196]. Another unique finding in our human genital samples was that DN T cells expressed higher levels of *IL13Rα1* when compared to CD4+ and CD8+ T cells, instead of *IL7R* as described in splenic mouse DN T cells [83]. IL13 receptor α1 dimerizes with IL4 receptor

and mediates signaling by IL13 and IL4. Importantly, IL13 signaling has been implicated in implantation and embryo tolerance [197, 198], supporting specific roles of DN T cells in reproduction as described by others [85, 199].

Regarding cytotoxic potential of DN T cells, we demonstrate that genital DN T cells express low levels of perforin, similar to those found in FRT CD8+ T cells [100, 174], and lower levels of GZA and GZB than FRT CD8+ T cells [100, 174], which overall would suggest low cytotoxic potential under resting conditions. However, granzymes are also known to have other immunoregulatory functions, particularly GZA has inflammatory properties [200, 201] and therefore further studies are needed to define the role of perforin and granzyme positive DN T cells in the FRT.

Recent reports have described the presence of DN MAIT cells in the EM and cervix [178, 179], which play important roles in antimicrobial control. However, MAIT cells minimally contributed to the DN T cell signatures in our single-cell preparations, or in flow cytometry analysis of MR1. The reason for this discrepancy remains to be address and may be due to the rare nature of these cells in human genital tissues. Future studies are needed to determine tissue distribution and functional differences between αβTCR+ DN T cells and DN MAIT cells in the human FRT.

Finally, we uncovered site-specific effects of aging on DN T cell distribution and function. While we observed no changes in endometrial DN T cells, we detected a significant reduction in CX and ECX from postmenopausal women as they aged. We have previously described a reduction in the proportion of tissue-resident CD103+CD8+ T cells in the cervix with no changes in the EM [46]. In contrast, in the present study we did not observe any changes in the proportion of tissue-resident CD103+ DN T cell population with aging, suggesting a selective decline of CD103+CD8+ T cells with aging, but not of all CD103+ expressing T cells. This age-dependent differential control may be physiologically relevant since CD103 allows interactions with epithelial cells, and

CD103+CD8+ T cells represent the majority of human intraepithelial lymphocytes [202]. Our results suggest that as women age declining numbers of CD8+ intraepithelial lymphocytes may be replaced by DN CD103+ T cells. Further, we detected specific modifications in the proportion of perforin-expressing CD103+ DN T cells with aging in a tissue-specific manner. Reduction of DN T cell presence in the lower FRT, and reduced production of perforin by ectocervical DN T cells, could represent a novel factor to explain increased inflammation and reduced protection against genital infections in women as they age. Future studies including microscopy are needed to address changes in intraepithelial lymphocyte composition with aging and their potential functional consequences.

In contrast to the ectocervix, enhanced perforin production by endometrial CD103+ DN T cells may represent a mechanism of sustained endometrial barrier defense in older women, potentially triggered by lack of sex hormones, decreased CD8+ T cell presence, epithelial alterations and microbiome changes with aging [98]. While the mechanisms involved in the site-specific decrease of selected T cell populations between the EM and cervix with age as well as the functional consequences remain to be explored, it is tempting to speculate that enhanced function of endometrial CD1a+ DCs observed in our study may contribute to the maintenance of defined T cell subsets in the EM. Studies are needed to determine additional functional changes in DN T cells with aging as well as the interplay between CD8+ T cells and DN T cells as women age and their role in reproduction and immune protection.

Finally, limitations of our study need to be taken into consideration for data interpretation. Our sample size is limited and additional studies with larger populations are needed to validate and expand our results. Particularly, our single-cell sequencing data needs to be confirmed and expanded with multiple women throughout their lifespan. The reduced number of DN T cells obtained from our hysterectomy samples, given their rare nature,

limits the number of comparisons that can be made and the study of DN T cell subsets contributing to the functional heterogeneity of this population. Lastly, we only evaluated DC function with the allogeneic reaction assay due to lack of access to matching naïve T cells from blood or lymph nodes from the same women, and therefore autologous proliferation assays remain to be tested in future studies to fully characterize DC functional changes with age.

Chapter 3: Human Genital Dendritic Cell Heterogeneity Confers Differential Rapid Response to HIV Exposure[3]

[3] Reprinted with permission from: RS Parthasarathy, LM de Lara, FJ Carrilo-Salinas, A Werner, A Borchers, V Iyer, A Vogell, JM Fortier, CR Wira, M Rodriguez-Garcia. Human genital dendritic cell heterogeneity confers differential rapid response to HIV-1 exposure. *Frontiers in Immunology* *https://doi.org/10.3389/fimmu.2024.1472656*

3.1 Introduction

Human Immunodeficiency Virus (HIV) infection is an ongoing epidemic affecting about 38 million people worldwide. Women represent half of the people living with HIV, but in endemic regions HIV prevalence in women is higher than in men [110] [109]. The main mechanism of HIV transmission is attributed to sexual intercourse [112] therefore, understanding HIV pathogenesis at the primary portal of entry, the female genital tract (FGT), remains a high priority to develop effective prevention strategies.

HIV gains access to the FGT by crossing the mucosal epithelial barrier during heterosexual transmission via seminal fluid, microtears in epithelial barriers, or transepithelial migration [113, 114]. Different immune populations within the FGT supply target cells for HIV infection or act as protective innate effector cells that limit HIV acquisition [46, 89, 105, 131, 203]. Potent antiviral innate defense followed by generation of protective local adaptive immune responses would be necessary to prevent HIV infection through repeated exposures.

Dendritic cells (DCs) are critical in shaping mucosal immunity against pathogens and maintaining tissue homeostasis [204]. DCs express pattern recognition receptors (PRRs) that enable pathogen recognition and capture, specifically through C-type lectin receptors (CLRs) [162, 205-207]. Following antigen processing, DCs have the unique ability to prime naive T cell function, making DCs ideal targets for vaccination and therapeutic strategies against cancers and infections, including HIV [208]. However, in HIV pathogenesis, DCs are considered a double-edged sword due to their ability to secrete anti-viral proteins and resist viral replication, but capture and transfer active viral particles to target $CD4^+$ T cells [124, 141, 162, 165, 166]. Different models using in vitro monocyte-derived DCs, Langerhans cells and bona fide DCs have demonstrated that HIV-transfer from DCs to T cells can occur through infection-independent mechanisms

via CLRs in early phases, or in an infection-dependent manner through HIV receptors and viral replication at later time points [141, 209-212].

We previously demonstrated rapid secretion of antimicrobial peptides with anti-HIV activity by genital DCs upon HIV exposure, and subset-specific uptake of viral-like HIV particles by CD14+ DCs preferentially [136]. Other studies further demonstrated that genital $CD14^+$ DCs were capable of capturing and transferring HIV to $CD4^+$ T cells [141, 162, 165]. However, the role that different DC subsets may play in mucosal HIV acquisition remains unclear.

DCs and mononuclear phagocyte populations are highly specialized depending on the tissue of residence [213]. Specifically in mucosal regions like the FGT, resident DCs display unique subset-specific functions in balancing immune protection and reproduction [88, 177, 214-216]. Mucosal sites are populated with conventional DCs (cDCs) derived from DC precursors that seed the tissues [217]. In addition, *in-vivo* recruitment and differentiation of monocytes into DCs or macrophages is mediated by acute inflammation in different disease models and at various mucosal sites [218]. Characterization and delineation of DC subsets is classically dependent on surface protein expression patterns [219]. However, recent advances in single-cell RNA sequencing (scRNAseq) have enabled better discrimination of human DC populations, revealing novel subsets and the inherent heterogeneity of $CD14^+$ mononuclear populations [147, 149]. These recent advances highlight the need to combine surface protein and RNA expression to fully characterize DC subsets [80].

Recent studies have enhanced our understanding of the DC and mononuclear phagocyte subsets that populate different human female genital mucosal surfaces relevant for HIV acquisition [136, 139, 141, 162, 212, 215, 220]. Multiple of these studies identified a variety of CD14-expressing populations that remain poorly defined [221]. Understanding the heterogeneity of DC and mononuclear cell populations in the FGT,

along with their unique contribution to HIV pathogenesis is key for targeted interventions [222].

Here we use a combination of cellular indexing of transcriptomes and epitopes sequencing (CITE-seq) and spectral flow cytometry to define DC and mononuclear cell subsets in the FGT and determine their immediate responses to HIV exposure. Identification of phenotypic and functional properties of FGT-resident DCs and their role in HIV acquisition could inform future therapeutic and vaccination strategies against HIV.

3.2 Methods

3.2.1 Tissue Processing.

Tissues obtained from hysterectomies were separated by endometrium (EM), endocervix (END) and ectocervix (ECT) by pathologists and transferred to the laboratory post-surgery. Tissues were processed as described previously [137, 203, 215]. Briefly, tissues were minced into 1-2 mm fragments in Roswell Park Memorial Institute (RPMI) medium (Gibco) containing enzymes from Tumor Dissociation Kit, human (Miltenyi Biotec) and 0.01% DNAse (Worthington Biochemical) and transfered into sterile gentleMACS^{TM} C Tubes (Miltenyi Biotec). Enzymatic digestion was performed on genlteMACS^{TM} Dissociator (Miltenyi Biotec) using “37C_h_TDK_1” program. Digested tissue was filtered through 100μm, 70μm and 30μm MACS^{TM} SmartStrainers (Miltenyi Biotec) to generate stromal single cells suspensions.

3.2.2 Flow Cytometry.

Mixed single-cell suspensions from tissues were washed in Phosphate-Buffered Saline (PBS; Gibco). Subsequently, cells were incubated in the dark at room temperature with LIVE/DEAD Blue (ThermoFisher) dye for 10 minutes. Anti-CD16 (BD Biosciences) was then added and incubated for 5 minutes, followed by anti-CCR7 (BioLegend), anti-CXCR4 (BioLegend), anti-CCR5 (BioLegend) and anti-CX3CR1 (BioLegend) for another

5 minutes. Finally, cells were incubated with an antibody cocktail containing remaining antibodies (**Table 5.1**) at room temperature for 15 minutes, washed with MACS buffer, fixed in 2% paraformaldehyde (PFA;Thermo Fisher), and analyzed for surface marker expression on Cytek Aurora (5 laser, 64 detector configuration; Cytek Biosciences). Expression of surface markers were quantified using OMIQ (Dotmatics).

3.2.3 HIV-1 viral stock propagation.

HIV-BaL (R5) isolates obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH [223] were propagated through infection of peripheral blood mononuclear cells (PBMCs) activated with phytohemagglutinin (PHA) (2.5 μg/mL; Sigma, St. Louis, MO) and IL-2 (50 U/mL; AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Human rIL-2 from Dr. Maurice Gately, Hoffmann – La Roche Inc) for 6-8 days. Stocks were harvested when p24 concentrations reached 100 ng/mL. Titration of viral stocks were performed using PHA and IL-2 activated PBMCs [224].

3.2.4 Sample preparation for multi-omics single-cell RNA sequencing.

Mixed single-cell suspensions from hysterectomy samples obtained from 4 healthy female donor, consisting of 4 endometrium (EM), 2 endocervix (END) and 4 ectocervix (n =10 tissues), were enriched for immune cells by magnetic bead removal of $CD3^+$ (CD3 MicroBeads, human; Miltenyi Biotec), $CD19^+$ (CD19 MicroBeads, human; Miltenyi Biotec), $CD235a^+$ (CD235a (Glycophorin A) MicroBeads, human; Miltenyi Biotec) red blood cells and fibroblasts (Anti-fibroblast MicroBeads, human; Miltenyi Biotec). For homeostatic conditions, cells were washed thoroughly with PBS containing 5% HS to remove any excess magnetic beads and incubated with oligo-conjugated antibodies (AbSeq; **Table 5.2**) and barcoded sample tags (BD^{TM} Hu Single Cell Sample Multiplexing Kit; BD Biosciences), to differentiate between tissue sites, for 20 minutes at

room temperature in PBS containing 5% HS. For HIV stimulation experiments, cells were incubated with HIV-BaL (MOI = 0.5) in XVIVO-15 or media alone for 30 minutes. Cells were washed thoroughly with PBS+5% HS to remove unbound virus and subsequently incubated with oligo-conjugated antibodies and barcoded sample tags as mentioned above. Cells were subsequently washed twice in excess PBS+5% HS to remove unbound antibodies and sample tags. Cells were counted and 10,000 cells from each tissue were combined to obtain a total of 30,000 cells each for control and HIV conditions. Cells were then subsequently loaded onto separate BD Rhapsody™ Cartridge (BD Rhapsody™ Cartridge Kit; BD Biosciences) followed by RNA capture on polyA tail capture beads containing unique molecular identifiers (UMIs) (BD Rhapsody™ Enhanced Cartridge Reagent Kit V3; BD Biosciences) on BD Rhapsody™ Express Single-Cell Analysis System (BD Biosciences) followed by cDNA, whole-transcriptome amplification and single-cell indexing libraries according to manufacturer's protocol (Protocol-Rhapsody WTA+AbSeq+ST; BD Biosciences).

3.2.5 Multi-omics RNA sequencing analysis.

FASTQ files containing unaligned reads were generated on NovaSeq6000 sequencing system (Illumina). Gene counts were generated by aligning and annotating reads to the human genome (GRCh38.p12 v29). To assess capture of HIV, reads from respective experiments were also aligned to the HIV genome FASTA file. Count tables were subsequently uploaded to Partek Flow (Partek, an Illumina company) for downstream quantification and visualization of data. Cells with mitochondrial gene expression >25% number of detected features per cell less than 200 or greater than 4300 were excluded from analysis, according to recent studies published by us [215] and others [225]. RNA and protein data were split and normalized respectively. To identify DCs, we first selected immune cells expressing *PTPRC* genes, which encodes CD45 protein. Next, we used protein information to exclude remaining T and B cells by selecting $CD3^{-}CD19^{+}$

cells, followed by selection of CD11c+HLA-DR-DP-DQ+ cells. Since genital NK cells and neutrophils can express CD11c and HLA-DR [226, 227], we excluded neutrophil contamination by selecting CD15- cells within this population, and NK cell contamination by gating out cells expressing *NCAM1* RNA (**Fig. 3.1A**). PCA was performed on normalized data followed by UMAP for visualization. Additionally, we also performed weighted-nearest neighbor analysis to integrate protein and RNA data. Subsequently, we performed k-nearest neighbor analysis and graph-based analysis to identify cell clusters and used "compute biomarker" function in Partek Flow (Partek – an Illumina company) to generate biomarkers associated with the clusters (**Supp File 1**). To identify differentially expressed genes (DEGs) between DC subsets, we performed non-parametric ANOVA to identify significantly upregulated and downregulated genes with significance of $p \leq 0.05$ and $Log_2(FoldChange) \pm 1.2$. GO Biological processes [228, 229] and Reactome [230] were generated using gene lists of upregulated and downregulated genes. To identify unique expression of genes within each cluster, "compute biomarker" function in Partek Flow Analysis. Curated gene lists were used to assess expression of CD markers, cytokines, chemokines, PRRs, antimicrobial proteins and gene lists associated with GO processes for AUCell [231] (**Supp. File 2**) were used from GSEA analysis available in Partek Flow (Partek – an Illumina company).

3.2.6 $CD14^+$ DC isolation and HIV stimulation.

$CD14^+$ DCs were isolated from mixed-cell supsensions using magnetic bead separation (CD14 MicroBeads, human; BD Biosciences) as per previous studies [136, 137, 215, 216]. Isolated cells were incubated with HIV-1-BaL (R5) isolates (MOI = 0.5) for 3 hours. Supernatants were collected and stored at -80C until multiplex assay analysis to determine secreted protein levels.

3.2.7 Luminex assay.

Analytes from HIV stimulation experiments were quantified using Millipore human cytokine multiplex kits according to manufacturer's instructions. Signal was measured using Magpix software with five-parametric-curve fitting for data analysis. Molecules measured included IL-1β, IFNγ, IL-5, IL-13, IL1Rα, GM-CSF, G-CSF, CCL11, CCL22, CXCL1, CXCL10 and CX3CL1.

3.2.8 Statistical analysis.

To identify differentially expressed genes, significance threshold was set as ($p<0.05$; -1.2 <FC>1.2) on non-parametric ANOVA. Statistics for flow cytometry analysis were done using Friedman's multiple comparison, ($p\leq0.05$ *; $p\leq0.01$ **; $p\leq0.001$ ***; $p\leq0.0001$ ****). Hierarchical clustering heatmaps were performed on significant, differentially expressed genes, by generating pseudo-bulk expression of groups (eg. HIV vs control; cluster 1, cluster 2, cluster 3, cluster 4 comparison) and visualized using bubble plots, with size of the bubble referring to percentage of cells expressing the particular gene, shade and color referring to Z-score expression of the gene. For Reactome and GO analysis, terms with FDR≤0.05 were chosen as significant values and bubble plots were visualized using GraphPad Prism.

3.2.9 Study subjects.

Written informed consent was obtained before surgery from HIV-negative women undergoing hysterectomies at Tufts Medical Center (Boston, MA, USA). Studies were approved by Tufts University Institutional Review Board and the Committee for the Protection of Human Subjects. Surgery was performed to treat benign conditions including fibroids, prolapse, and menorraghia. Trained pathologists selected tissue samples from endometrium (EM), endocervix (END), and ectocervix (ECX), free of

pathological lesions and distant from the sites of pathology. Women were HIV− and HPV− but no additional information regarding other genital infections was available.

3.3 Results

3.3.1 Identification of phenotypically and transcriptionally unique DC subsets in the genital tract.

Prior studies have identified different genital DC and mononuclear phagocyte populations within the anogenital mucosa [136-138, 141, 162, 205, 212, 215, 220]. However, transcriptional, phenotypical, and functional characterization of the heterogenous CD14-expressing populations in the genital tract remains poorly defined. To address these gaps, we optimized a protocol to define DC subsets in the FGT phenotypically and transcriptionally at the single-cell level. We generated single cell suspensions from human hysterectomy samples and enriched the target immune cells as detailed in methods. We labeled the enriched cells with oligo-conjugated antibodies targeted towards surface proteins (**Table 5.2**), lysed the cells to release RNA, and sequenced the RNA to simultaneously determine the whole transcriptome profile and surface protein expression at the single-cell level. To identify the genital resident DCs, we took advantage of CITE-seq's combined surface protein information and gene expression and developed a gating strategy to select the $CD11c^{+}HLA\text{-}DR^{+}$ population (**Fig. 3.1A**), which contains DCs. This approach eliminates the need for lengthy fluorescence activated cell-sorting (FACS) prior to sequencing, thereby limiting processing steps that can alter primary tissue resident immune cells [232].
Unbiased clustering analysis discriminated four distinct clusters of $CD11c^{+}HLA\text{-}DR^{+}$ cells within the genital mucosa (**Fig. 3.1B**). We determined the top RNA and surface protein (Abseq) expression unique to each cluster by using "compute biomarkers" function (**Fig. 3.1B**). Cluster 1 was characterized by RNA expression of *VCAN*, *S100A8*, *S100A9* and

surface protein expression of CD14, CD11b and CD64 **(Fig 3.1C),** confirming the presence of a $CD14^+$ monocyte-derived DC population in the genital mucosa as previously described by us and others [136, 138, 141, 215, 216, 233]. Cluster 2 was characterized by surface protein expression of CD1c, the DC maturation marker CD83, and the pro-survival and tissue homing marker CXCR4 [234, 235], suggesting enrichment of cDC2s in this cluster **(Fig. 3.1C**). Clusters 3 and 4 displayed phenotypes similar to monocyte populations. Cluster 3 expressed elevated CD16 and CD127, whereas cluster 4 displayed elevated expression of CD64, HLA-DR and low levels of CLEC12A protein expression **(Fig. 3.1C**). Additionally, cluster 3 expressed RNA transcripts such as *FCGR3A*, *SMIM25* and *SPN (***Fig 3.1B***)*, consistent with non-classical monocyte (NCM) signature expression [236]. Cluster 4 expressed elevated levels of complement encoding RNA transcripts *C1QC* and *C1QB*, consistent with inflammatory monocytes/macrophages (infMons) [237]. These two clusters indicated the presence of two distinct monocyte populations within $CD11c^+HLA\text{-}DR^+$ cells.

Recent studies identified a novel DC population in peripheral blood named DC3 that co-expresses CD14 and CD1c and displays and intermediate phenotype and function between monocytes and classical myeloid DCs (cDC2s) [147, 238]. Interestingly, we and others have previously described the presence of $CD14^+CD1c^+$ cells in the FGT [136, 138, 141]. To determine whether the DC3 population was present in the FGT, we analyzed our CITE-seq data using AUCell [231] with the top 50 genes described by Villani et. al in their peripheral blood DC3 cluster (**Supp. File 2**)[149]. We observed an enrichment of the DC3 signature in cluster 1 (**Fig. 3.1D**), indicating the presence of DC3s within the $CD14^+$ DC cluster.

Overall, our multi-omics data indicates the presence of four distinct $CD11c^+HLA\text{-}DR^+$ populations in the FGT, consisting of two DC and two monocyte populations, with unique transcriptional and phenotypic signatures. Furthermore, we observed the presence of

novel DC3 gene signature in the CD14^{+} DC cluster, suggesting inherent heterogeneity of CD14^{+} DCs within the FGT.

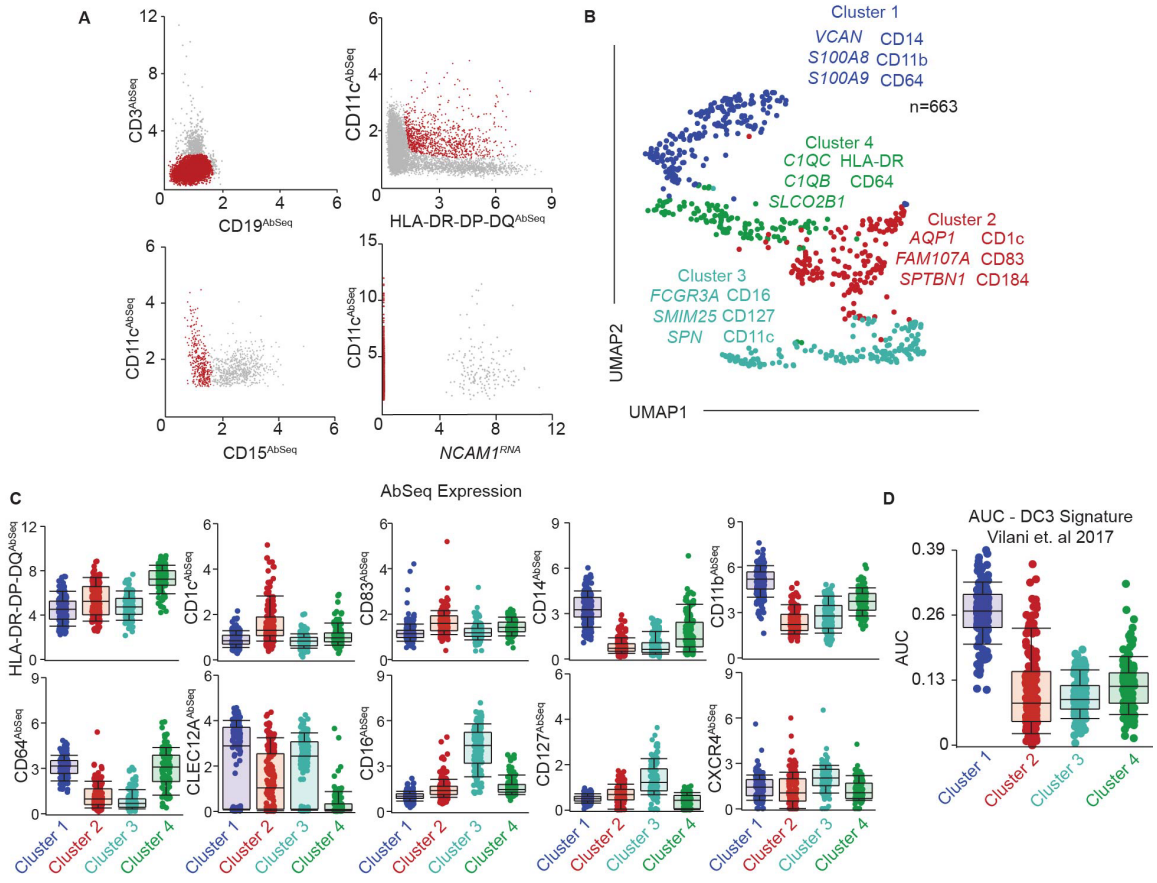

Figure 3.1 Identification of phennotypically and transcriptionally unique DC subsets in the genital tract.

(A) Representative gating strategy to identify FGT resident CD11c+HLA-DR+ cells using a combination of surface protein expression (AbSeq) and RNA expression in CITEseq dataset. **(B)** Representative UMAP visualization of FGT resident CD11c+HLA-DR+ cells through unbiased clustering, depicting expression of discriminating RNA expression (left) and surface protein expression (right) in distinct subsets, cluster 1 (blue), cluster 2 (red), cluster 3 (teal) and cluster 4 (green). **(C)** Scatter plot comparing AbSeq expression between different FGT CD11c+HLA-DR+ subsets. (D) AUCell analysis of top 50 genes in DC3 cluster observed by AC Villani et. al

3.3.2 Functional specialization of genital DCs under homeostatic conditions

Next, we investigated whether our phenotypical clustering was associated with distinct subset-specific functions for each subset under homeostatic conditions.

To determine if the distinct populations would be differently posed to detect and interact with invading pathogens, including HIV, we compared expression levels of PRRs (**Supp. File 2**) including toll-like receptors (TLRs), C-type lectin receptors (CLRs) and NOD-like receptors (NLRs). Using a list of known PRRs (**Supp. Table 2**) we compared expression levels between subsets through hierarchical clustering (**Fig. 3.2A**). We observed no TLR9 expression across all subsets, ruling out the possibility of plasmacytoid DC contribution to the gene signature of each subset. Compared to the other clusters, Cluster 1 ($CD14^+$ DCs) showed relatively higher expression of TLRs and CLRs associated with detecting and binding to HIV, including *CLEC4A*, *CLEC4E* and *TLR4* [239, 240]. Additionally, in Clusters 1 and 3, we observed shared expression of *CLEC12A,* which has been linked to improved antigen delivery and cross presentation by DCs [199], and *CLEC7A,* previously described in $CD14^+$ DCs from blood and lungs [241]. Additionally, Clusters 1, 3 and 4 also shared expression of *TLR2,* which binds HIV *[242]*. Cluster 2 (cDC2s) had no unique gene expression pattern. The highest expression in Cluster 2 was observed for the CLRs *CLEC10A* and CLEC1A, involved in antigen internalization and presentation [243, 244]. When compared to the other clusters, Cluster 3 (NCM) displayed high preferential expression of *IFIH1* (encoding MDA5), *DDX58* (encoding RIGI) which mediate detection of cytosolic viral RNA [245, 246], and *TLR8,* which recognizes endolysosomal ssRNA [247]. Cluster 4 (infMons) expressed *MRC1* (encoding mannose receptor (MR)) and *CD209*, consistent with a monocyte/macrophage phenotype [161, 194]. Cluster 4 also expressed high levels of *CLEC5A* which forms heterodimers with CD209 and MR upon antigen exposure to enhance viral internalization [248]. Further, compared to the other clusters, Cluster 4 had preferential expression of *TLR1* and *TLR6,* known to dimerize with TLR2 for detection of bacterial lipopeptides; *TLR7* and *TRL3* which recognize ssRNA and dsRNA, respectively [249, 250]. Finally, a small percentage of cells in Cluster 4 preferentially expressed

AIM2, involved in recognition of cytosolic dsDNA [251]. Altogether, our analysis reveals subset specific differences in genital DC recognition of different moieties of pathogens, Cluster 1 being enriched with PRRs associated with membrane HIV recognition and binding, Cluster 2 expressed PRRs for antigen uptake, Cluster 3 and Cluster 4 were enriched for PRRs that detects cytosolic and endolysosomal ssRNA, dsRNA and dsDNA.

Using the same analytical approach, we next focused on cytokine and chemokine expression under homeostatic conditions, to understand functional differences in innate secretory functions involved in maintenance of mucosal barrier integrity, immune cell recruitment and anti-microbial protection. We identified the top 5 cytokine and chemokine gene signatures unique to each cluster (**Fig. 3.2B**; **Supp. File 2**). Cluster 1 ($CD14^+$ DCs) was characterized by *CCL20, CXCL8* and *CXCL2* expression, genes involved in antimicrobial activity [252], chemotaxis for Th17 cells, neutrophils, monocytes and DCs [253-256]. Expression of *IL1B* and its inhibitory receptor *IL1RN* were also elevated in Cluster 1, suggesting production of IL1β but prevention of autocrine/paracrine actions of IL1β on $CD14^+$ DCs. Cluster 2 (containing cDC2s), specialized in expression of genes involved in T cell chemotaxis: *IL33,* an alarmin that controls tissue homeostasis and type 2 immunity [257]; *CCL17,* a chemoattractant for helper T cells expressing CCR4 (such as Th2 and regulatory T cells) [258] [259]*; CXCL12,* a CXCR4-ligand; *CX3CL1* (fractalkine) which play critical roles in menstruation [260]; and *IL7,* suggesting an important role for cDC2s in control of genital T cell populations and favoring Th2 and T regulatory profiles. Cluster 3 (NCMs) expressed high levels of *IL16,* a cytokine that exclusively binds and signals through CD4 [261]; and *CXCL16,* important for control of MAIT cells and NK cells and expressed by non-classical monocytes in blood [262-264]. In addition, a smaller percentage of cells in cluster 3 expressed CCR3 ligands *CCL24*, *CCL26 and CCL13,* with antimicrobial

properties and potent chemoattractants for eosinophils and basophils [265] [266]. Cluster 4 (infMons) was characterized by expression of CCR5 ligands *CCL3*, *CCL4, CCL5* and CXCR4 ligands *CXCL12* and *CCL4L2*, suggesting a potential role in mediating anti-HIV activity. Cluster 4 also expressed high levels of IL18, a proinflammatory cytokine described in tissue-resident macrophages that induces IFN-γ production by T cells in an inflammatory environment, or Th2 differentiation in the absence of inflammatory cytokines [267, 268].

To further understand functional contributions to mucosal homeostasis and defense, we performed AUCell analysis [231] of genes associated with immune protection (**Fig. 3.2C**; **Supp. File 2**). Cluster 1, containing $CD14^+$ DCs, was enriched for genes associated with inflammatory and defense response, suggesting enhanced potential to mediate innate immune protection. Furthermore, enrichment of genes associated with positive regulation of myeloid leukocyte migration within this cluster suggests the presence of a migratory DC population. Cluster 2 (cDC2s) and Cluster 4 (infMon) were enriched for genes related to activation of the complement classical pathway, correlating with increased expression of complement associated genes. Lastly, no differences were observed for enrichment of antigen processing and presentation or positive regulation of T cell proliferation genes in genital $CD11c^+$HLA-DR^+ subsets under homeostatic conditions.

Overall, our data demonstrates distinct functional specialization of genital $CD11c^+$HLA-DR^+ subsets under homeostatic conditions.

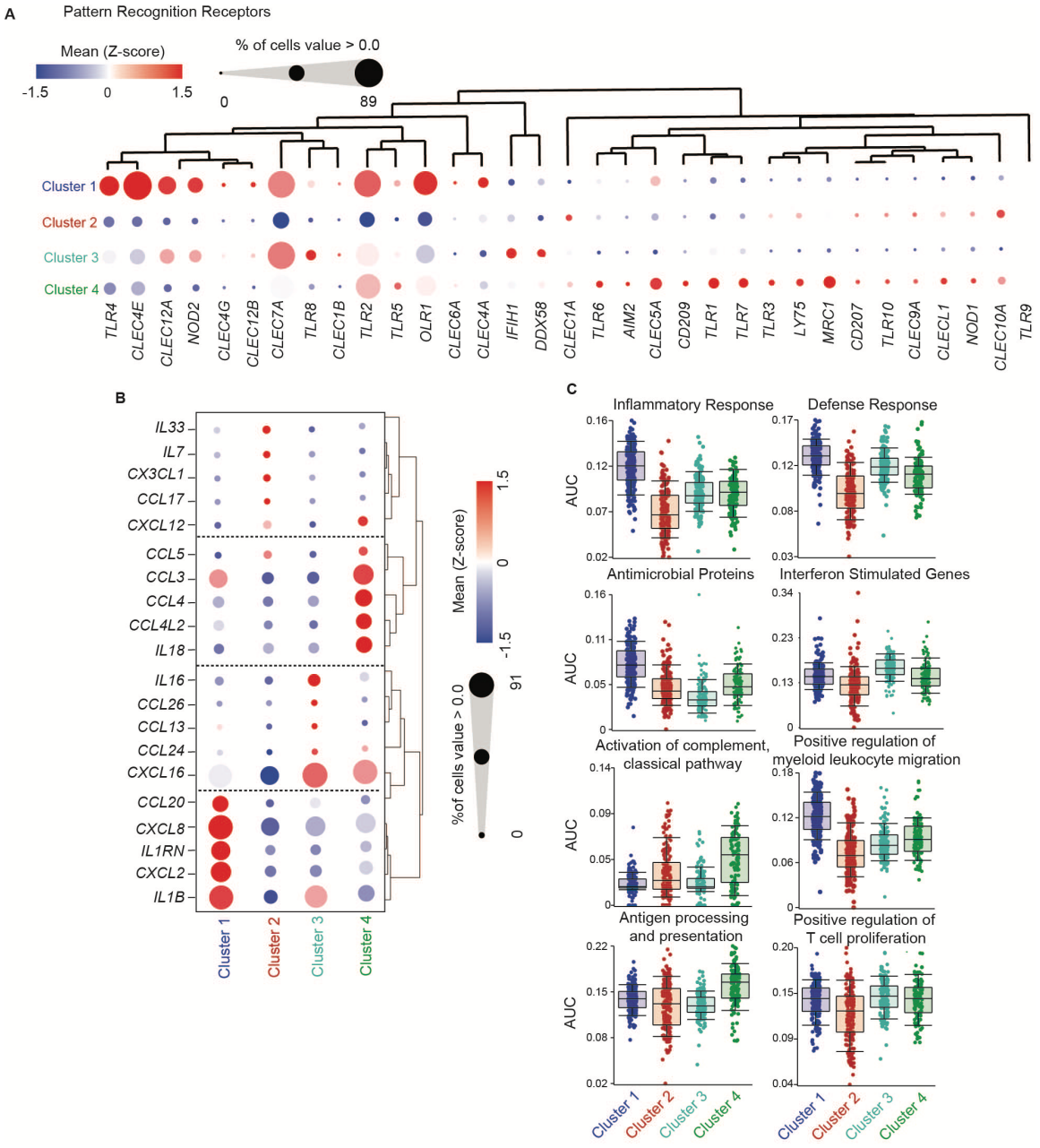

Figure 3.2 Functional specialization of genital DCs under homeostatic conditions.

(A) Hierarchical clustering heatmap comparing gene expression of significant pattern recognition receptors (PRRs) between FGT resident CD11c+HLA-DR+ subsets. **(B)** Hierarchical clustering heatmap comparing gene expression of top 5 interleukin and chemokines in each cluster. **(C)** AUCell comparison of key gene ontology (GO) terms associated with dendritic cell function between DC subsets. Significance - non-parametric ANOVA (p<=0.05; -1.2 <FC>1.2).

3.3.3 $CD14^+$ DCs represent a heterogenous, activated population within the genital mucosa.

Data in this study highlights the inherent heterogeneity of DC subsets within the mucosa, with unique transcriptional and phenotypic properties under homeostatic conditions. To validate our CITE-seq findings in a larger number of patients and further delineate genital DC populations, we developed a multi-parameter spectral flow cytometry panel (**Table 5.1**). We gated on $CD11c^+HLA\text{-}DR^+$ cells (**Fig. 3.3A**) and identified four different populations based on CD1c and CD14 surface expression (**Fig. 3.3B)**, main discriminatory markers identified in our CITEseq data (**Fig. 3.1C**). As seen in **Fig. 3.3C**, $CD14^{high}CD1c^-$ cells ($CD14^+$ DCs) were the most abundant subset (53%), followed by $CD14^{low}$CD1c- (29.1%), $CD14^+CD1c^+$ DCs (DC3s) (7.44%) and $CD14^-CD1c^+$ (cDC2s) cells (6.5%). To determine if $CD11c^+HLA\text{-}DR^+$ subset distribution varies throughout the genital tract, we compared the abundance of each subset in the endometrium (EM), endocervix (END), and ectocervix (ECX) (**Fig. 3.3D**). We observed an increased abundance of $CD14^+$ cells in the ECX compared to the EM, whereas the opposite was true for the $CD14^{low}$CD1c- subset, with no significant changes observed in $CD1c^+$ and $CD14^+CD1c^+$ cells.

In our CITE-seq data set we identified two distinct $CD14^-CD1c^-$ populations with differential expression of CD16 and CLEC12A (**Fig. 3.1C**). Therefore, we further characterized the $CD14^-CD1c^-$ population to determine if this group was composed of two different cell subsets. Consistent with our CITE-seq findings, the $CD14^{low}CD1c^-$ population contained two different subsets: $CD14^-CD16^+$ and $CD14^-CD16^-$ (**Fig. 3.3E**). Furthermore, these two populations could be distinguished by CLEC12A expression (**Fig. 3.3E**, histogram), suggesting $CD14^-CD16^+CLEC12A^+$ cells are similar to NCMs, whereas lack of CLEC12A expression in $CD14^-CD16^-$ cells corresponds to the inflammatory/activated monocyte population (infMon).

Next, we analyzed expression of surface markers associated with DC activation status and function (**Fig. 3.3F**). First, we assessed maturation status by gating on HLA-DR^{high} cells consistent with our previous publication (Rodriguez-Garcia et al., 2017). We observed that a large proportion of CD14^{+}CD1c^{+} and CD1c^{+} cells were HLA-DR^{high}, significantly higher than other CD11c^{+}HLA-DR^{+} subsets. Compared to the other subsets, CD14^{+}CD1c^{+} and CD14^{+} cells showed higher expression of CD11b and CD64, associated with cell adhesion and antigen uptake. CD14^{+}CD1c^{+} cells expressed the highest levels of CD54, a molecule involved in enhanced immune synapse formation between DCs and T cells [269]. CD14^{-}CD16^{+} cells displayed the highest levels of CLEC12A (a myeloid inhibitory receptor), and along with CD14^{+}CD1c^{+} cells, showed high expression of CX3CR1, the receptor for CX3CL1 (fractalkine).

Previous studies from our group and others have shown that CD14-expressing FGT DCs preferentially capture HIV viral-like particles [136, 141, 162, 165], indicating subset-specific interactions with HIV. Based on this, we evaluated expression of receptors and coreceptors for HIV in the different subsets (**Fig. 3.3G**). We observed that all three DC subsets expressed significantly higher levels of CD4 and CXCR4 compared to CD14^{-} CD16^{-} monocytes. Importantly, CD14^{+}CD1c^{+} DCs expressed the highest levels of CCR5, which is crucial for HIV acquisition in the mucosa [132]. In addition to the classical co-receptors, CD49d ($\alpha 4\beta 7$ integrin) expression was significantly higher in CD14^{+}CD1c^{+} DCs and CD14^{-}CD16^{+} monocytes compared to other subsets. CD49d acts as a tissue homing marker and non-classical HIV co-receptor important for mucosal infection [126]. Altogether, our flow cytometry data demonstrates the presence of phenotypically distinct genital DC populations and further supports the presence of CD14+ DCs and DC3s within the CD14+ population. We demonstrate that genital DC3s expressed levels of activation markers similar to conventional cDC2s (CD1c^{+} DCs) and share expression of classical monocyte-derived DC markers with CD14^{+} DCs. Additionally, we demonstrate

elevated expression of HIV coreceptors in DC3s compared to other DC populations, suggesting predisposed differential response to HIV within the genital mucosa.

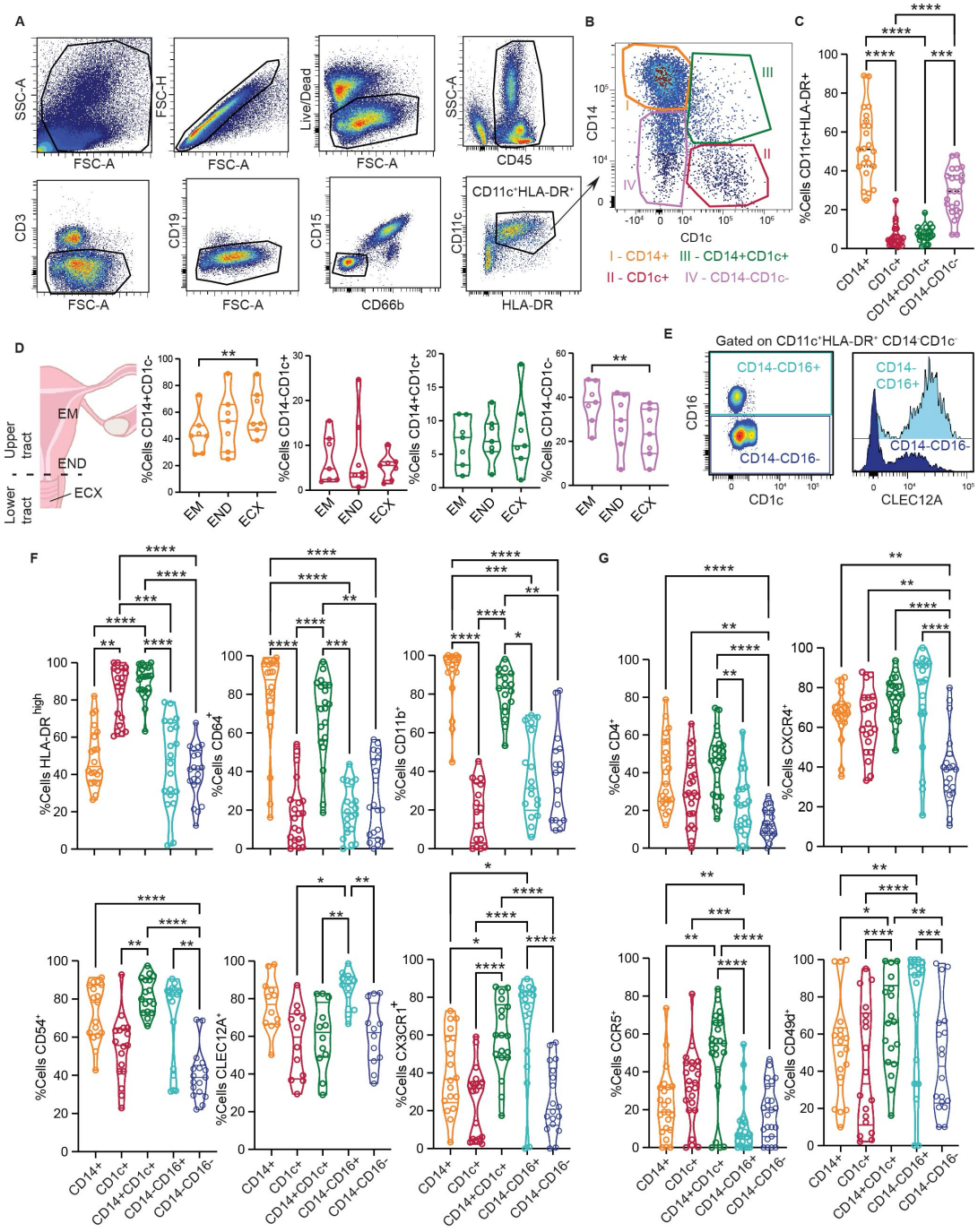

Figure 3.3 CD14 DCs represent a heterogenous, activated population within the genital mucosa.

(A) Representative flow cytometry gating strategy to identify FGT resident CD11c+HLA-DR+ cells. **(B)** Representative plot of HLA-DR+CD11c+ subsets based on CD14 and

CD1c expression; CD14+CD1c- (I, orange), CD14-CD1c+ (II, red), CD14+CD1c+ (III, green) and CD14-CD1c- (IV, pink). **(C)** Comparison of CD11c+HLA-DR+ subset frequencies in the FGT. **(D)** Comparison of CD11c+HLA-DR+ subset distribution across different anatomical regions of the FGT, endometrium (EM), endocervix (END) and ectocervix (ECX). **(E)** Representative gating to differentiate CD14-CD1c- cells based on CD16 expression (left); Histogram representation comparing CLEC12A expression between CD14-CD16+ and CD14-CD16- cells (right). **(F)** Comparison of surface protein expression between different CD11c+HLA-DR+ cells; HLA-DR^{high} (top right), CD64 (top middle), CD11b (top right), CD54 (bottom left), CLEC12A (bottom middle) and CX3CR1 (bottom left). **(G)** Comparison of HIV tropic surface proteins between different CD11c+HLA-DR+ cells; CD4 (top left), CXCR4 (top right), CCR5 (bottom left) and CD49d (bottom right). Statistics – Friedman's multiple comparison, paired non-parametric ANOVA (p<=0.05 *; p<=0.01 **; p<=0.001 ***; p<=0.0001 ****).

3.3.4 Genital DCs undergo rapid transcriptional changes in response to HIV stimulation.

Earlier studies investigated the role of DCs in HIV pathogenesis using monocyte-derived DCs over a time course from 6-48 hours to assess their responses to HIV during viral uptake and infection [210]. However, most studies evaluating the role of genital DCs in HIV pathogenesis have investigated events that occur 12 hours or more after viral exposure, focusing on DC susceptibility to HIV infection and *trans-infection* to CD4$^+$ T cells [139] [141, 162, 165]. We have previously shown that genital DCs release chemokines and antimicrobials in a rapid manner, within 3 hours after HIV stimulation, [136], indicating a role for DCs in triggering the initial mucosal innate response against the virus. However, the overall antiviral response induced by HIV immediately following challenge of genital DCs, prior to viral replication, integration, or productive infection, remains uncharacterized.

To address this gap, we adapted our CITEseq approach to identify early transcriptional responses of genital DCs to HIV. Single cell suspensions generated from human hysterectomy samples were incubated with HIV in vitro for 30 minutes, prior to proceeding with the CITE-seq protocol described in detail in methods, to determine whole transcriptome profile and surface protein expression simultaneously at the single-cell level (**Fig. 3.4A**). The time of 30 minutes was chosen to identify pathways involved

in viral recognition and immune response independent of viral replication, integration and productive infection. We used the surface protein expression information to select $CD11c^{+}HLA\text{-}DR^{+}$ cells as done in **Fig. 3.1A**, which contains the DC and monocytic populations, and performed differential gene expression analysis between HIV and unstimulated control conditions to define transcriptional changes induced by viral exposure. This analysis identified a significant transcriptional shift induced by HIV within 30 minutes, with 333 genes differentially upregulated and 4148 genes downregulated in response to HIV (**Fig. 3.4B; Supp. File 1)**.

To define this HIV response, we first focused on genes associated with DC-mediated immune protection, inflammation, and antiviral response within the top 50 differentially expressed genes based on fold change and p-value (**Fig. 3.4B; Supp. File 1**).

Within the upregulated genes, we detected increased expression of the interferon-stimulated genes (ISGs) *IRF1* and *IFITM2,* indicating initiation of innate immune activation and anti-viral response. We also observed a strong upregulation of genes associated with interleukin and chemokine signaling pathways (*IL1B*, *CXCL8*, *CXCL10*, *CISH, SOCS3),* indicating rapid induction of innate secreted responses. Consistent with increased secretory response, we observed upregulation of serglycin gene expression (*SRGN)* in response to HIV, involved in storage and secretion of innate molecules in intracellular vesicles in blood monocytes and other cell types [270]. Additionally, we detected upregulation of *ACOD1*, a gene involved in antimicrobial and antiviral responses of innate cells, and a negative regulator of TLR-mediated inflammatory responses [271].

We detected downregulation of genes associated with pro-viral replication of HIV such as *GRIP1*, *EIF4A2, SRRM2, XRN1,* and *MALAT1* [272-275], suggesting suppression of host mechanisms to prevent HIV replication in genital DCs.

To uncover markers that enable the identification of phenotypic changes induced by HIV in genital DCs, we evaluated differences in expression of genes associated with surface protein expression (CD marker list, **Supp. File 2**), focusing on molecules associated with DC function such as activation, migration, and T cell co-stimulation (**Fig. 3.4C**). We observed that the majority of genes related to CD markers were downregulated, but detected strong upregulation of three genes: *CD69,* which mediates tissue retention*; CD99,* involved in diapedesis of monocytes and dendritic cells into inflamed tissue [276]; and *ANPEP* (encoding CD13), which mediates DC cross-presentation in human DC populations [277]. Analysis of the downregulated genes revealed suppression of markers associated with classical DC activation and T cell co-stimulatory ligands (*CD83*, *CD86, CD58*) and molecules important for migration of DCs (*PECAM1*, *CD37*) [278, 279], indicating potential suppression of T cell interaction molecules and retention of genital DCs within the mucosa at early timepoints. We also observed decreased expression of disintegrin and metalloproteinase family of genes (*ADAM10*, *ADAM17)* which mediate shedding of TNF-α [280], suggesting suppression of classical pro-inflammatory responses. Additionally, relative to the control, the HIV-stimulated DCs had decreased expression of *CLEC12A,* and genes encoding the inhibitory leukocyte immunoglobulin-like receptor subfamily B (*LILRB1*, *LILRB2*, *LILRB3*), suggesting a non-classical mechanism of DC activation [281].

Next, we performed Gene Ontology (GO) analysis of genes upregulated and downregulated by HIV stimulation (FDR≤0.05) (**Fig. 3.4D; Supp. File 1**). Analysis of the upregulated genes revealed an enrichment of terms associated with cytokine mediated signaling pathways, innate sensing, and metabolic processes (**Fig. 3.4D; Supp. File 1**). Downregulated genes (**Fig. 3.4D**) were associated to terms related to antigen presentation, T cell responses, regulation of classical inflammatory cytokines, type I IFN responses, and canonical proinflammatory pathways, but also suppression of viral cycle

and viral replication. These GO terms suggest suppression of classical inflammation but activation of innate host antiviral responses within 30 minutes of exposure to HIV. Based on these GO terms, we further explored the secretory response to HIV and compared expression of genes encoding cytokines, chemokines and antimicrobials between control and HIV conditions (**Fig. 3.4E**). In response to HIV we detected upregulation of an innate secretory response, including pro-inflammatory genes *IL1B* and *CXCL10, CXCL8* and *CCL2,* chemoattractant for neutrophils, DCs and monocytes; and *CCL8, a* CCR5 ligand with antiviral activity, [282]. Compared to the control group, we observed decreased expression of genes encoding inflammatory cytokines *IL6* and *IL18,* which act as Th1 polarizing cytokines; *IL16,* a $CD4^+$ cell specific chemoattractant; *CCL28,* a $CCR10^+$ T cell chemoattractant; and *CCL21,* which mediates homing to lymphoid tissues, suggesting potential inhibition of CCR7+ DC migration towards lymph nodes. Comparison of antimicrobial peptide gene expression ([283]; **Supp. File 2**) revealed increased expression of *RNASE1* and *S100A12* in the HIV group compared to control (**Fig. 3.4E**). Interestingly, we observed suppression of *SAA1* (encoding serum amyloid A), which contributes to induction of pathogenic Th17 cells [284], known to be preferential targets for HIV infection [105, 133, 285]. Additionally, we observed suppression of *ADM* (encoding the anti-bacterial peptide adrenomedullin), which also acts as suppressor of inflammatory cytokines [286].

Taken together, our data indicates that mononuclear phagocytic populations respond to HIV in a rapid manner within 30 minutes of exposure, with upregulation of endogenous host restriction factors, activation of non-classical inflammatory responses, and increased gene expression of cytokines, chemokines and antimicrobials, suggesting initiation of a localized antiviral response.

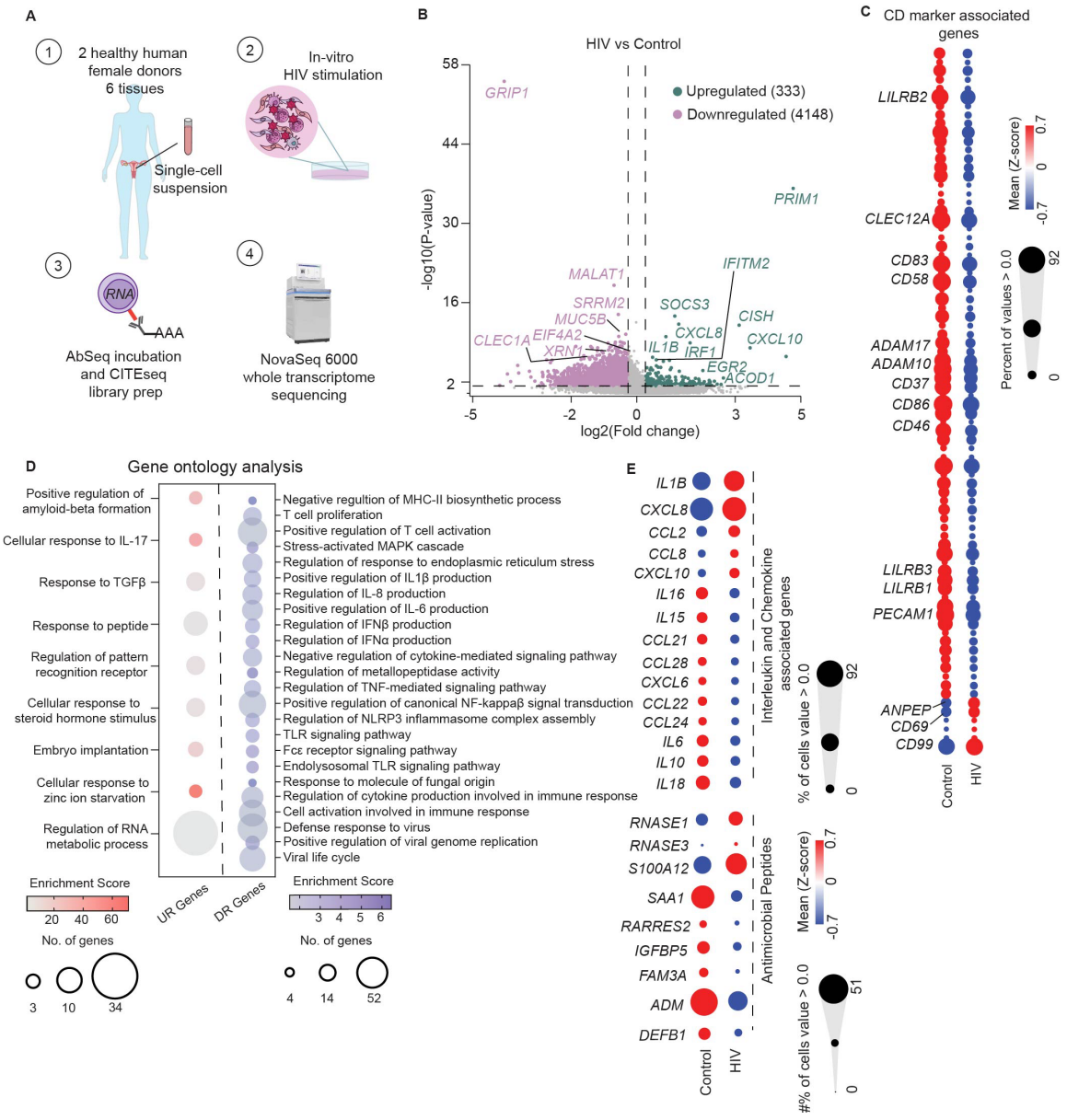

Figure 3.4 Genital DCs undergo rapid transcriptional changes in response to HIV stimulation.

(A) Graphical depiction of CITEseq protocol to identify transcriptional changes induced by HIV in FGT resident CD11c+-HLA-DR+ cells. **(B)** Volcano plot of significantly upregulated (n=333) and downregulated genes (n=4148) by HIV in CD11c+HLA-DR+ cells. **(C)** Hierarchical clustering heatmap comparing gene expression of cluster differentiation markers between control and HIV treated cells. **(D)** Bubble-plot visualization of significant GO terms enriched in genes upregulated (UR genes; red) and downregulated (DR genes; blue) in response to HIV by CD11c+HLA-DR+ cells. **(E)** Hierarchical clustering heatmap comparing genes expression of secreted factors such as interleukins, chemokines and antimicrobial proteins between control and HIV treated CD11c+HLA-DR+ cells. Statistics – Volcano plot, non-parametric ANOVA ($p<=0.05$; -1.2 <FC>1.2); GO terms significance of FDR<=0.05.

3.3.5 CD14^{+} DCs largely mediate the rapid innate secretory signature observed in genital DC response to HIV while cDC2s mediate antiviral inflammatory responses.

Transcriptional changes in genital mononuclear phagocytic populations exposed to HIV indicates rapid activation of endogenous host restriction factors and localized non-classical inflammatory response, but also an overall suppression of gene transcription. Since our results identified distinct DC subsets with differential homeostatic activation and functions, we next determined how each of these subsets contributes to the overall response after HIV challenge. To define cell clusters and determine their responses following HIV exposure, we used the CITEseq libraries generated from single-cell suspensions stimulated with HIV for 30 minutes shown in figure 4. We performed PCA, followed by unbiased clustering and visualization using UMAP. This analysis revealed four clusters (**Fig. 3.5A**): CD14 DCs, cDC2s, infMons and NCMs, consistent with our findings under homeostatic conditions (**Fig. 3.1B**). Overlay of unstimulated and HIV treated cells, revealed contribution from control and HIV stimulation conditions to each cluster, except for the NCM cluster, where we found a very limited number of cells from the HIV stimulated condition, and were therefore unable to analyze NCM response to HIV (**Fig. 3.5B**).

First, we utilized the overall transcriptional signature detected after HIV exposure shown in **Fig. 3.4B** to perform AUCell analysis and determine whether this signature was enriched in specific genital DC subsets (**Fig. 3.5C**). Interestingly, the overall HIV response signature (including upregulated and downregulated genes) was preferentially enriched in cDC2s and infMons. However, we observed enrichment of HIV upregulated genes in CD14 DCs, whereas HIV downregulated genes were enriched in cDC2s and infMons, suggesting that HIV challenge preferentially suppresses transcriptional changes in cDC2s and infMons compared to CD14 DCs. Furthermore, we detected an

enrichment of cytokine, chemokine and antimicrobial peptide signatures within the $CD14^+$ DC population, but no differences in enrichment of antiviral ISGs (**Fig. 3.5C**), suggesting that $CD14^+$ DCs largely mediate innate secretory response to HIV at early stages.

We next analyzed differential gene expression within each subset to identify transcriptional changes induced by HIV exposure (**Fig. 3.5D**; **Supp. File 1**). All three subsets shared upregulation of *AF033819.3* (the viral transcript from viral input), *SOCS3,* and *CXCL10* (**Fig. 3.5D)**. CD14 DCs and cDC2s shared expression of anti-viral genes *IRF1*, *IFITM2* and *ACOD1* and the cytokine signaling genes *CISH*, *IL1A* and *IL1B*. CD14 DCs and infMons shared expression of zinc finger proteins and non-protein coding genes, except for *CSF3* (encoding the granulocyte colony stimulating factor (G-CSF)) (**Supp. File 1**). cDC2s and infMons both upregulated *CXCL8* expression upon HIV stimulation.

Next, we analyzed genes uniquely upregulated by each subset in response to HIV. We observed that CD14 DCs upregulated *TIFA* and *FOSL1*, genes associated with initiation of inflammation; *EMP1*, previously described in type I interferon stimulated DCs [287]; and *CDKN1A*, a gene involved in p53 transcription previously shown to be protective at early stages of HIV infection [288]. cDC2s uniquely upregulated ISGs *IFIT2* and *IFIT3*, both possessing antiviral functions, *CCL3*, *CCL4* and *CCL4L2,* encoding chemokines capable of binding HIV receptors (Diamond and Farzan, 2013; *Lambert et al., 2007*) and *NLRP3,* encoding inflammasome protein and indicating rapid induction of inflammation. infMons upregulated *RNASE1 (*encoding RNA degrading secreted protein) [289], and *LIFR,* which encodes CD118 described to function as an alternative receptor for HIV [290].

Downregulated genes were more abundant, and we focused our analysis on genes associated with DC function and HIV pathogenesis (**Fig. 3.5D; Supp. File 1**). All 3

subsets shared lowered expression of genes associated with HIV transcription such as *GRIP1* and *SRRM2,* which promote HIV replication, and interestingly *SAMHD1*, which plays a role in preventing replication of HIV. Additionally, all three subsets showed decreased expression of inflammation associated genes *JUN, CSF3R* and *IL6R*. CD14 DCs and infMons shared downregulation of *HSPA1L* (encoding a heat shock protein) and genes associated with adaptive immunity such as *CD36*, *ITGAL*, *LILRA1* and *SIRPB1*. CD14 and cDC2s shared suppression of HIV-replication promoting gene *XRN1*, and interferon signaling associated genes *TRIM2, TRIM4, CIITA* and *MX1*. infMons and cDC2s shared downregulated expression of genes associated with unfolded protein response, such as *EDEM1, HYOU1, KLHDC3* and *KAT2A* [291]. CD14 DCs uniquely downregulated inhibitory genes *CLEC12A, LILRB1*, and classical DC activation marker *CD83*, suggesting non-canonical activation of this subset. We also observed downregulation of HIV binding and nuclear localization protein encoding gene *DDX21* [292], suggesting inhibition of nuclear import. cDC2s downregulated genes associated with promoting HIV replication such as *EIF4A1*, *EIF4A2* (HIV translation initiation) [293] and RNA polymerase II encoding genes *POLR2F, POLR2G* [294]. We also observed downregulation of the TNFα degrading gene *ADAM17* and *A2M* encoding alpha2-macroglobulin, that binds to inflammatory interleukins with high affinity, suggesting inhibition of HIV replication and promoting inflammatory environment. infMons downregulated expression of *NOD2*, *CD80,* and *IFNAR* indicating decreased cell activation.

We performed pathway analysis using Reactome to gain information about biological interactions [230] (**Fig. 3.5E; Supp. File 1**). Analysis of upregulated genes revealed shared involvement of CD14 DCs and cDC2s in cytokine signaling pathways (IL-10, IL-4/IL-13 pathways). cDC2s uniquely upregulated Type I IFN and IFN-γ signaling, while CD14 DCs uniquely upregulated transcriptional activation of p21, a known restriction

factor against HIV infection in monocyte-derived DCs [295]. Analysis of downregulated genes revealed shared signatures between $CD14^+$ DCs and infMons for heat shock response, and a unique signature for infMons related to downregulation of viral replication.

Finally, to validate our findings of a secreted response at the protein level, we purified genital CD14 DCs by magnetic bead selection and incubated them with HIV as described in methods. Supernatants harvested 3 hours post stimulation from untreated and HIV treated cells were assayed to quantify secreted proteins (**Fig. 3.5F**). In response to HIV, we detected increased secretion of cytokines and chemokines (IFN-γ, IL-5, IL-13, IL1Rα, GM-CSF, CXCL1, CXCL10 and CCL22). These results confirm rapid innate secretory response in response to HIV.

Overall, we demonstrate subset-specific responses to HIV, with a preferential suppression of genes in cDC2s and infMons compared to CD14 DCs, with $CD14^+$ DCs largely mediating the secretory innate response after HIV stimulation.

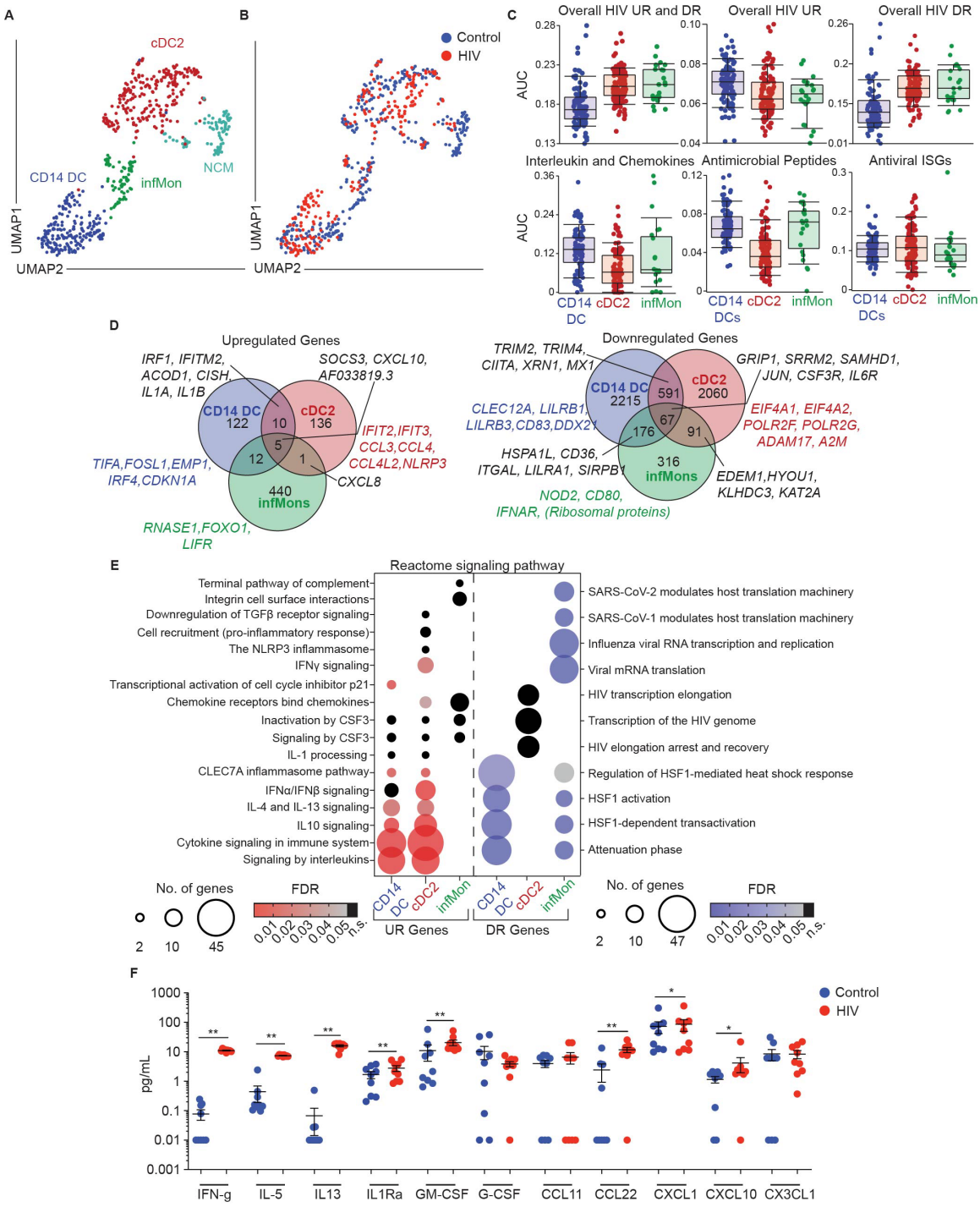

Figure 3.5 CD14+ DCs largely mediate rapid protective host response observed in genital DC response to HIV.

(A) Representative UMAP visualization of CD11c+HLA-DR+ clusters and **(B)** overlay of control and HIV treated cells. **(C)** AUCell comparing enrichment of overall upregulated and downregulated HIV signatures (top row) and key gene ontology (GO) terms associated with dendritic cell function (bottom row) between CD11c+HLA-DR+ subsets in HIV treated samples. **(D)** Venn-diagram representation comparing expression of

shared and unique genes significantly (pvalue<=0.05; -1.2 <FC>1.2) upregulated (top) and downregulated (bottom) between CD11c+HLA-DR+ subsets in response to HIV stimulation. **(E)** Bubble-plot representation of significant (FDR<=0.05; black dots indicate non-significant (n.s.) FDR values) reactome signaling pathways comparing pathways enriched in genes upregulated (red) and downregulated (blue) in response to HIV stimulation between different CD11c+HLA-DR+ subsets.(F) Comparison of secreted protein levels in supernatants of media (control; blue) and HIV treated (HIV; red), FGT resident CD14+ cells. Significance – Paired non-parametric Wilcoxon-test (p<=0.05 *; p<=0.01 **).

3.4 Discussion

In this study we evaluated dendritic cell heterogeneity in the human female genital mucosa and how DC subsets respond to HIV immediately after exposure. We found that the $CD11c^{+}HLA\text{-}DR^{+}$ myeloid population in the genital mucosa includes three DC subsets and two monocyte/macrophage populations with distinct functional and phenotypic properties under homeostatic conditions. However, following HIV exposure, the antiviral response is dominated by DCs' rapid secretory response, activation of non-classical inflammatory pathways and host restriction factors. Further, we report subset specific differences in genital DC response to HIV, where CD14 DCs are the major subset activated by HIV and responsible for the secretory antimicrobial response, while cDC2s activate inflammasome pathways and antiviral IFN responses. Recognizing that DCs play a crucial role in responding to invading pathogens and recruiting adaptive immunity, identifying DC subsets with anti-HIV properties could aid targeted HIV prevention and vaccination strategies.

We and others have reported the presence of different DC subsets in the genital mucosa [124, 136, 139, 141, 162, 215, 216], however in-depth characterization of these subsets, specifically the CD14-expressing DC compartment, throughout the female genital tract according to recent advances in the field remains lacking [80]. Mucosal DC characterization in humans is technically challenging due to their rare nature and the difficulty in isolating specific subsets for functional characterization. To overcome these barriers, we utilized multi-omics approaches to characterize protein surface expression

and transcriptome profile of DCs at the single-cell level in mixed cell suspensions from human hysterectomies. Our analysis revealed that the CD11c^{+}HLA-DR^{+} population is highly heterogeneous, including multiple DCs and monocyte/macrophage subsets. Within the DCs populations, we identified CD14^{+} DCs, CD1c^{+} cDC2s and CD14^{+}CD1c^{+} DC3s, with CD14^{+} DCs representing the dominant population, consistent with prior reports using flow cytometry [136]. Importantly, we identified two monocyte/macrophage subsets within CD11c^{+}HLA-DR^{+} cells, indicating that CD11c and HLA-DR expression are not sufficient to define DCs in the genital tract.

Under homeostatic conditions the different myeloid populations displayed differential expression of PRRs, chemokines/cytokines and antimicrobials, indicating subset-dependent roles in tissue homeostasis and differential predisposition to sense and respond to pathogens, including HIV. Specifically, PRRs previously associated with HIV membrane binding and detection (such as *CLEC4A*, *CLEC4E*, *TLR2* and *TLR4)* were preferentially expressed by CD14 DCs, suggesting that CD14 DCs may play a principal role in early detection of HIV upon exposure within the genital tract. Although prior studies using in vitro generated monocyte-derived DCs demonstrated that DCIR (encoded by CLEC4A) was responsible for viral capture and transinfection to CD4+ T cells [239, 296], future functional studies are needed to test whether DCIR is involved in HIV capture by genital CD14+ DCs. Interestingly, our data indicates a lack of gene expression by CD14+ DCs of other classical HIV-binding lectins (CD207, CD209 and MRC1) which were uniquely expressed in the infMons subset that shares characteristics of macrophages. Further research is needed to define the mechanisms responsible for viral capture by genital DCs. Cytoplasmic and endosomal sensors for viral RNA (*TLR7/8, DDX58, IFIH1*) were enriched in the monocyte/macrophage subsets, possible indicating delayed responses or roles in later HIV detection following viral replication. Cytokine/chemokine expression patterns further pointed for CD14^{+} DCs to play a role in

antibacterial defense and inflammatory responses, while cDC2s were involved in maintenance of tissue homeostasis, regulation of inflammation, and promotion of a Th2/T regulatory environment. Overall, this suggests that the CD14 DC population is pre-armed to generate rapid innate responses against incoming pathogens. Interestingly, between the subsets, no differences were observed in antigen processing and presentation, and T cell proliferation pathways, indicating that these populations share their antigen presenting cell properties under homeostatic conditions, consistent with our prior observations [215, 216].

Our validation of this myeloid subset classification using flow cytometry further allowed phenotypical comparisons between the $CD14^+$ DC, $CD14^+CD1c^+$ DCs and $CD1c^+$ cDC2 populations and the establishment of markers to discriminate the activated monocyte/macrophage populations (CD16, CLEC12A). Consistent with studies evaluating blood cells [147, 149, 238], we found that the $CD14^+CD1c^+$ subset displayed an intermediate phenotype between the monocyte-derived $CD14^+$ DCs and the classical myeloid DCs ($CD1c^+$ cDC2s), with high expression of HLA-DR but also monocyte origin-associated markers (CD64, CD11b, CX3CR1). This suggests that $CD14^+CD1c^+$ DCs in the genital mucosa are a homolog of DC3s described in peripheral blood and tumors [147, 149, 238], but in the genital tract they are present under steady-state conditions. In addition, the DC3 subset showed increased expression of classical and non-classical HIV coreceptors (CCR5, CD49d, CX3CR1) relevant for mucosal HIV pathogenesis [126, 132, 297], suggesting enhanced ability for viral uptake by $CD14^+CD1c^+$ DC population as described previously by us and others [136, 141]. These phenotypic differences in expression of activation, origin, antigen uptake and HIV tropic markers highlight the importance of understanding genital DC subsets to develop targeted strategies.

A novel aspect of our study is the identification of early transcriptional signatures in genital DCs immediately following HIV exposure. While DC infection and trans-infection

to T cells have been evaluated previously by others in blood [125, 167] and mucosal tissues [141, 162, 165], the early antiviral responses induced immediately following mucosal HIV exposure remained uncharacterized. In this study, we uncovered that, at early time points, before viral replication takes place, HIV exposure induces a rapid secretory response at the transcriptional and protein levels, activation of host restriction factors (*IRF1, IFITM2, ACOD1*), upregulation of genes involved tissue retention (CD69), and suppression of genes involved in T cell activation (*CD83, CD86, CD58*). Taken together, our data suggests that shortly after exposure, DCs remain in the mucosa and play a role in initiating local innate antiviral protection. However, several inflammatory markers were also upregulated and therefore the consequences for tissue protection and potential attraction and activation of HIV target cells remains to be determined. Another novel contribution of our study is the discrimination of responses following HIV stimulation in different subsets of genital DCs. We found that all subsets shared activation of genes related to secretion of cytokines and chemokines, although $CD14^+$ DCs and cDC2s were the predominant subsets involved in this response. Here, we observed shared upregulation of genes associated with inflammation and antiviral properties (*IRF1, IFITM2, ACOD1, CISH, IL1A, IL1B*) between cDC2s and $CD14^+$ DCs upon exposure to HIV. These findings are consistent with earlier studies using monocyte-derived DCs and macrophages which demonstrated the upregulation of IRF1 transcripts and protein, in addition to other ISGs [210, 298]. In addition, we uncovered unique pathways elicited by HIV stimulation in each subset. CD14 DCs were the main players in overall antimicrobial defense, responses to TLR activation and initiation of inflammation (*TIFA, FOSL1, EMP1*), while cDC2s displayed a more specific antiviral response with activation of type I interferon (*IFIT2, IFIT3, IRF1*) and inflammasome (*NLRP3*) pathways. Importantly, both subsets activated mechanisms to prevent HIV replication. CD14 DCs induced transcriptional activation of p21, a host restriction factor

in monocyte-derived DCs [295], while cDC2s downregulated genes necessary for HIV transcription (*EIF4A1, EIF4A2, POLR2F, POLR2G*). In contrast to the DC subsets, infMons were not involved in initial antiviral protection, but downregulated pathways related to viral cycle, suggesting inhibition of HIV replication. However, future time-course studies are needed to better understand the kinetics and mechanisms by which HIV modifies DC function in a subset-specific manner to promote infection, *trans-infection* and HIV dissemination. Additionally, our study only used HIV-BaL, a laboratory adapted strain, but our findings were not confirmed with HIV transmitted/founder (TF) strains. Although prior studies with DCs and macrophages found no differences between HIV-BaL and TF strains [141, 212, 299], future studies are needed to define DC subset-specific responses to TF strains immediately following exposure to HIV. Finally, we validated the transcriptional signatures by characterizing the anti-HIV secretory response at the protein level in supernatants from CD14 DCs purified from the genital tract. Despite our observation of upregulation of *IL1B* at the transcriptional levels, we did not detect IL1-β production in our cultures. However, we detected production of IFN-γ, GM-CSF and chemokines with inflammatory and antiviral properties (CXCL10, CCL22). These results complement our prior identification of early secretion of CCR5-ligands and antimicrobial proteins by $CD14^+$ DCs in response to HIV [136]. Production of GM-CSF, IFN-γ, and CXCL10 has been shown to be induced by TLR stimulation in DCs [300, 301], suggesting that TLR signaling in CD14 DCs may be responsible for induction of cytokine and chemokine secretion. Interestingly, while IFNγ and CXCL10 are involved in mediating Th1 and $CD8^+$ T cell adaptive immunity [302, 303], we also detected production of the Th2 cytokines IL5 and IL13, involved in allergic inflammation and activation of Th2 $CD4^+$ T cells (Johnson-Huang et al., 2009). While we have previously described that under homeostatic conditions $CD14^+$ DCs induce proliferation of $CD8^+$ T

cells with tissue-resident memory phenotype [216], and proliferation of $CD4^+$ T cells and double negative (DN) T cells [215], future studies are needed to determine how these cytokine profiles modify tissue environment, susceptibility to HIV infection, and modify T cell induction profile.

Our study has several limitations mainly due to the rare nature of mucosal DCs and the technical difficulties in isolating human DC subsets. First, due to the very low frequency of cDC2s within the genital mucosa, we were unable to isolate cDC2s from mixed cell suspensions to determine their secretory response to HIV at the protein level. Similarly, lack of distinct surface markers and low cell numbers in the genital tract prevented isolating DC3s ($CD14^+CD1c^+$) and resulted in clustering and isolation of this subset together with the $CD14^+$ DCs. Therefore, quantifying cDC2 and DC3s individual response to HIV at the protein level will require innovative strategies. Furthermore, our analysis of subset-specific responses was unable to evaluate non-classical monocytes due to low numbers of cells in the HIV stimulated condition, and therefore the contribution of this subset to HIV pathogenesis remains to be elucidated. Despite these limitations, our study provides valuable novel information using an experimental model to evaluate initial mucosal responses to HIV exposure that allows the study of DC subsets without lengthy processing time that could potentially modify primary DCs.

Overall, we demonstrate that the female genital mucosa is populated with different subsets of DCs that specialize under homeostatic conditions and that, immediately following HIV exposure, initiate a local secretory antiviral response and activate host mechanisms to prevent HIV replication in a subset-specific manner. Our findings contribute to the field of mucosal HIV acquisition and provide a map to identify therapeutic targets that trigger local protective innate immune responses against HIV without inducing detrimental tissue inflammation.

Chapter 4: Discussion

4.1 Summary and Significance

The work done in this thesis addresses the effects of aging on subset-specific changes in the induction of DN T cell proliferation by FGT resident DCs, characterization of DN T cell distribution, and phenotype, followed by in-depth characterization of FGT DC subsets under homeostatic conditions and their response to HIV exposure. First, we observed that both CD14+ and CD1a+ DCs induce the proliferation of DN T cells; however, CD1a+ DCs exhibit an enhanced ability to induce proliferation, which is further augmented with aging following menopause, compared to CD14+ DCs. Additionally, in the DC-T cell co-cultures, we observed differential cytokine secretion between CD1a+ and CD14+ DCs, with additional aging-associated effects further modifying the cytokine secretion profile of FGT DCs. Furthermore, we establish the presence of transcriptionally and phenotypically unique DN T cell subset with cytotoxic, innate-like anti-viral defense, tissue homeostasis, and regulation in the FGT. Interestingly, we observed compartmentalization of DN T cells, with age-associated decline being limited to the lower tract in the FGT. These findings highlight FGT DC subset-specific function in the induction of unconventional DN T cell proliferation, which was previously unknown. Following up on these findings, we subsequently identified and characterized CD11c+HLA-DR+ myeloid cells in the FGT and established the presence of 4 distinct populations, namely CD14-expressing DCs, CD1c+ cDC2s (conventional DCs), and two monocyte-like subsets – non-classical monocytes and inflammatory monocytes – in the FGT. To that extent, we establish differences in the expression of PRRs, chemokine, interleukin, and antimicrobial peptide genes, outlining key functional differences between the subsets under homeostatic conditions. Next, we developed a model to decipher transcriptional changes induced by HIV in FGT DCs, which mimic in vivo conditions where genital DCs are exposed to the virus through sexual contact. Our data reveals rapid activation of secretory response at transcriptional and protein levels, activation of

host restriction factors, upregulation of tissue retention factors, and suppression of genes involved in T cell activation. Another novel aspect of our study is the delineation of DC subset-specific responses to HIV, where we demonstrate that CD14+ DCs and cDC2s were the predominant subsets involved in the activation of genes related to the secretion of cytokines and chemokines, in addition to the shared expression of genes associated with inflammation, antiviral properties, and HIV prevention mechanisms. Although inflammatory monocytes were not involved in the initial antiviral protective signature that was observed, they downregulated pathways related to the viral cycle suggesting an inhibition of HIV replication at initial stages. Validation of anti-HIV transcriptional signatures observed in CD14+ DCs was performed through purification of CD14+ DCs from the FGT followed by HIV exposure, after which supernatants harvested consisted of elevated levels of inflammatory and anti-HIV proteins compared to untreated control. Taken together, the work done in this thesis advances the field of DC biology, and more specifically, DC biology in the FGT, which is crucial to understanding the dynamics of balance between tissue homeostasis and immune protection. Additionally, we identify early and key transcriptional changes in FGT DCs upon HIV exposure.

4.2 FGT DCs exhibit differential cytokine secretion and T cell induction in a subset-specific and age-dependent manner.

Most studies on the effect of aging on DCs have relied on *in vitro*-generated MoDCs, which are transcriptionally distinct from primary DCs in the human body [148, 188]. Due to challenges in obtaining sizeable human tissues and isolating primary DCs from them, how aging affects different DC subsets is poorly understood. However, tissue-resident DCs are conditioned to the unique tissue environment of the FGT, making it crucial to put this unique tissue environment into perspective to broaden existing knowledge of the

effects of aging on primary DCs [26, 176]. In chapter 2 of this thesis, we demonstrate that aging and menopause uniquely regulate DC function in a subset-specific manner, with CD1a+ DCs being particularly sensitive to age-associated changes after the onset of menopause compared to CD14+ DCs. This phenomenon was highlighted through enhanced induction of DN T cell proliferation by CD1a+ DCs compared to CD14+ DCs pre-menopause, which was further augmented post-menopause. Interestingly, this was in contrast with previous observations from our group, where we observed no differences in the ability of DCs to induce T cell proliferation in an age-dependent manner [102]. These contrasting findings were largely due to a difference in analysis where both DC subsets were combined in our previous study, highlighting the importance of studying each subset individually. The enhanced ability of CD1a+ DCs to induce naïve T cell proliferation corroborated the findings from our previous study [102], suggesting that these represent bona fide DCs in the FGT, consisting of a homogenous population. However, CD14+ DCs in the FGT could possibly represent a heterogenous population comprised of different monocyte-derived subsets, sharing the function and phenotype of DCs due to their ability to induce naïve T cell proliferation. In our DC-naïve T cell co-culture model, we observed a significant shift in the cytokine profile induced during the proliferation process by both CD1a+ and CD14+ DCs following menopause. This was characterized by enhanced production of Th1 (IFNγ) and Th2 cytokines (IL-5, IL-10, IL-13) in the co-culture system. Since the cytokine milieu is critical to shaping T cell function and phenotype [150], the post-menopausal environment could be more amenable to support the cytotoxic activity of CD8+ T cells as observed in previous studies [100, 101, 174]. Additionally, enhanced production of Th2 cytokines post-menopause is in agreement with previous studies that show an age-dependent decline in the Th1/Th2 ratio in the blood of women [189]. Interestingly, increased IP-10 (CXCL10) production was only observed in CD14+ DC co-cultures post-menopause. IP-

10 has been shown to be involved in impaired T cell function and proliferation in the context of viral infections [190, 191], making this an interesting candidate for explaining the differences in proliferation induction capability between CD1a+ and CD14+ DCs in the FGT. Since aberrant DC function has been associated with poor pregnancy outcomes such as recurrent spontaneous abortion (RSA) and preterm birth (PTB) [304], our findings here suggest that endometrial CD1a+ DC function is actively suppressed during premenopausal years, more likely to support a successful pregnancy outcome. This could be due to intrinsic environmental factors, which are altered due to aging. Factors such as sex hormones could contribute to changes in DC proliferation induction capabilities and cytokine production, further augmented by the lack of hormonal control post-menopause. Studies by our group and others have shown estradiol-mediated modifications of innate immune mechanisms in blood and genital DCs [192, 193]. However, the role of sex hormones in the regulation of CD1a+ DC function is not fully known and requires further investigation.

4.3 DN T cells represent a distinct T cell population with age-dependent changes in distribution through the FGT.

Another novel contribution of our study is the deep characterization of the unconventional DN T cell population in the FGT. We establish the presence of a distinct DN T cell population that is more abundant in the endometrium than the cervix, which could be consistent with animal models. Low frequencies of these cells in human tissues are a significant challenge in efforts to identify the functional role of these cells. To overcome these challenges, we used oligo-conjugated antibody tags and RNA single-cell sequencing in combination with flow cytometry to describe the presence of a transcriptionally distinct and functionally heterogeneous population of unconventional DN T cells for the first time. DN T cells exhibited a broad spectrum of potential functions, seen through the enrichment of genes associated with cytotoxic, T cell regulation,

antigen presentation, and innate-like functions, which are consistent with a recent characterization of mouse DN T cells in the spleen, which may represent a common defining profile of DN T cells [83]. Additionally, we observed potentially unique functions of genital DN T cells, which could be relevant for the maintenance of barrier function, reproductive function, and involvement in response to genital viral infections such as HPV and HIV. Based on previous studies on TRM CD8+ T cells suggesting that their phenotype and function may be driven by the tissue environment [184], the unique signatures observed in DN T cells could also be driven by similar mechanisms. In addition to broad spectrum of biological function, we also observed that DN T cells expressed high levels of CD69, CD103, and low CCR7 levels, consistent with key tissue-resident phenotype markers [184, 187]. Additionally, we observed that DN T cells demonstrated an effector memory phenotype through low levels of CD62L and CCR7 expression, consistent with other T cell subsets in the FGT [98, 132, 195]. Lack of Th17 signature in DN T cells could be unique to the FGT [83], consistent with reported negative pregnancy outcomes associated with elevated Th17 levels [196]. Additionally, increased expression of the gene encoding IL13 receptor α1 (*IL13RA1*) which dimerizes with IL4 receptor and mediates signaling by IL13 and IL4 [83], with the former being associated with implantation and embryo tolerance [197, 198], supporting the role of DN T cells in reproduction as described in other studies . To validate the cytotoxic signature observed in our single-cell dataset, we subsequently assessed levels of cytotoxic intracellular proteins and observed that DN T cells expressed low levels of perforin, similar to FGT CD8+ T cells [100, 174], and lower levels of GZA and GZB than CD8+ T cells [100, 174], suggesting low cytotoxic potential under resting conditions, which could be unique to the FGT. Finally, we observed age-dependent changes in the distribution and function of DN T cells in different anatomical sites of the FGT, with a significant reduction in the cervix and endocervix from postmenopausal women. Although previous

studies from our group have shown a decline in the proportion of tissue-resident CD103+CD8+ T cells in the cervix with no changes in the endometrium [101], we did not observe similar changes in CD103+ DN T cells with aging suggesting a selective decline of CD103+CD8+ T cells and not all CD103+ T cell populations. This could be of physiological relevance since CD103 allows interactions with epithelial cells and suggests that as women age, although CD103+CD8+ T cells decline in numbers, they may be replaced by DN CD103+ T cells. Additionally, we observed increased perforin production by endometrial CD103+ DN T cells compared to the ectocervix, which may represent a mechanism of endometrial barrier defense in older women.

4.4 FGT DCs comprise phenotypical, transcriptional, and functionally distinct subsets.

Studies by us and others reveal that FGT DCs are not a homogenous population but comprise distinct subsets that differ in phenotype, transcriptional profile, and function. This finding underscores the immune system's complexity in the FGT and the need for further research to understand the unique roles of these subsets. Previous work from our group [136] has shown the presence of a distinct CD1c+CD14+ DC subset, lacking further in-depth characterization of this subset. Given our earlier findings from Chapter 2 demonstrate distinct functional differences in DC induction of T cell proliferation in a subset-specific manner, we next sought to identify underlying mechanisms driving these differences. In line with recent advances in the field of DC biology [80], we used a multi-omics approach to phenotype, classify, and characterize FGT DC subsets based on surface expression and transcriptome profile of DCs at the single-cell level in mixed cell suspensions from human hysterectomies. Our data establishes the presence of highly heterogeneous CD11c+HLA-DR+ subsets, including distinct DC subsets, such as CD14+ DCs, CD1c+ cDC2s, and CD14+CD1c+ DC3s, where CD14+ DCs represent a dominant population consistent with previous publications. Additionally, we identify the

presence of heterogenous monocyte subsets lacking CD14 expression within CD11c+HLA-DR+ FGT cells, warranting the identification of additional markers to delineate monocyte subsets from DCs in the FGT efficiently. We observed functional differences in DC subsets through differential gene expression of PRRs, chemokines/cytokines, and antimicrobials under homeostatic conditions suggesting subset-specific roles in tissue homeostasis and predisposed sensing and response to pathogens. Interestingly, we observed differences in PRRs associated with HIV binding and detection (such as *CLEC4A*, *CLEC4E*, *TLR2,* and *TLR4*), which were preferentially expressed by CD14 DCs, suggesting that CD14 DCs may play a crucial role in early detection of HIV upon exposure to the virus. In contrast, monocyte subsets were enriched for cytoplasmic and endosomal sensors of viral RNA, such as *TLR7/8*, *DDX58*, and *IFIH1*, indicating a possible role in delayed responses or later in HIV detection following viral replication. CD14+ DC expression of cytokine/chemokine-associated genes points towards their potential role in mediating antibacterial defense and inflammatory response, whereas cDC2s were involved in tissue homeostasis and regulation of inflammation through the promotion of a Th2/ T regulatory environment. Taken together, the data suggests that CD14+ DC population is pre-armed to respond in a rapid manner against invading pathogens. Interestingly, we observed no differences in antigen presentation capabilities between the subsets under homeostatic conditions, consistent with our previous observations with premenopausal women, which could suggest tissue intrinsic factors driving suppression of DC activation under homeostatic conditions. Further validation of these subsets using spectral flow cytometry allowed in-depth phenotypical comparison between CD14+ DC, CD14+CD1c+ DC and CD1c+ cDC2 populations and establishment of key markers to discriminate them from monocyte populations lacking CD14 expression. CD14+CD1c+ DCs demonstrated an intermediate phenotype between CD14+ DCs and cDC2s, consistent with recent studies evaluating

blood cells [147, 149, 238]. CD14+CD1c+ DCs shared elevated expression of HLA-DR with cDC2s compared to CD14 DCs, and shared expression of monocyte origin-associated markers CD64, CD11b and CX3CR1 with CD14 DCs compared to cDC2s, suggesting that this population in the FGT are a homolog of DC3s described in peripheral blood and tumors. Additionally, the DC3 subset showed elevated expression of classical (CCR5) and non-classical (CD49d, CX3CR1) HIV co-receptors relevant for mucosal HIV acquisition [126, 132, 297], suggesting an enhanced ability for viral uptake by CD14+CD1c+ DCs described by us and others. Characterization of these subsets furthers the field of DC biology, specifically in the FGT, allowing in-depth understanding of FGT DC subsets' role under homeostatic conditions and identifying key markers to discriminate FGT DCs from non-classical/activated monocyte subsets. This is of high importance to efficiently target therapeutic interventions to either maintain tissue homeostasis, prevent tissue destruction or enhance response against pathogens by targeting different subsets in a clinical setting.

4.5 HIV induces rapid transcriptional changes in FGT DCs, with subset differences in secretory, antiviral, and inflammatory response.

Another novel aspect of the work done in this thesis is the identification of key transcriptional signatures in FGT DCs immediately following HIV exposure. Although the role of DC infection and *trans-infection* has been evaluated by us and others in blood [125, 167] and mucosal tissues [141, 162, 165], the events preceding viral infection and early antiviral responses induced immediately upon mucosal HIV exposure remained uncharacterized. Through the work done in Chapters 3.4-5, we uncovered innate immune mechanisms upon HIV exposure encompassed by induction of rapid secretory response at transcriptional and protein levels, activation of host restriction factors such as *IRF1*, *IFITM2*, *ACOD1*, upregulation of tissue retention (*CD69*) and suppression of T cell activation-associated genes such as *CD83*, *CD86,* and *CD58*. Our data suggests

that shortly after HIV exposure, FGT DCs remain in the mucosa and play a role in initiating a local innate antiviral response. Finally, to determine the contribution of DC subsets to early innate response to HIV exposure, we compared transcriptional changes induced by HIV between different FGT myeloid subsets, namely CD14 DCs, cDC2s, and infMons. We found that all three subsets shared activation of genes associated with the secretion of cytokines and chemokines, with CD14+ DCs and cDC2s being predominant subsets in this response. We observed shared upregulation of *IRF1, IFITM2, ACOD1, CISH, IL1A,* and *IL1B*, genes associated with inflammation and antiviral properties, between cDC2s and CD14+ DCs upon exposure to HIV. Unique pathways were upregulated due to HIV exposure in each subset with CD14+ DCs mediating antimicrobial defense, response to TLR activation, and initiation of inflammation, while cDC2s were responsible for a more specific antiviral response with activation of type I interferon pathway genes *IFIT2*, *IFIT3*, *IRF1*, and inflammasome pathway through NLRP3 activation. We observed that both CD14 DCs and cDC2s activated mechanisms to prevent HIV replication, with CD14 DCs inducing host restriction factor in MoDCs through transcriptional activation of p21, while cDC2s downregulated *EIF4A1, EIF4A2, POLR2F,* and *POLR2G,* genes necessary for HIV replication. In contrast to DCs, infMons did not play a role in early antiviral protection but downregulated viral cycle pathways, suggesting initial inhibition of HIV replication. To validate the transcriptional signatures, we next characterized anti-HIV secretory response at the protein level in supernatants of isolated CD14+ DCs from the FGT, comparing control and HIV-treated conditions. Despite significant upregulation of *IL1B* at the transcriptional level, we did not detect IL-1β production in our cultures. However, we detected the production of cytokines/chemokines with inflammatory (IFNγ, GM-CSF) and antiviral properties (CXCL10, CCL22), complementing previous findings of early secretion of CCR5-ligands

and anti-microbial proteins by CD14+ DCs in response to HIV [136]. Previous studies have shown that secretion of IFN-γ, CXCL10, and GM-CSF is induced upon TLR stimulation of DCs [300, 301], suggesting that CD14 DC response to HIV is possibly initiated by TLR signaling-mediated induction of chemokines/cytokines. Since DCs play a crucial role in defining T cell function and phenotype, the mixed signatures detected in the levels of secreted proteins suggest that upon exposure to HIV, FGT DCs potentially initiate varied T cell induction capabilities. This was characterized by the secretion of IFNγ and CXCL10, involved in mediating Th1 and CD8+ T cell adaptive immunity [302, 303], and Th2-associated cytokines IL-5 and IL-13 which are involved in allergic inflammation and activation of Th2 CD4+ T cells. Overall, our data identifies early transcriptional events induced by HIV in FGT DCs and subsequently establishes shared suppression of HIV replication by FGT DC subsets and the distinct role of CD14+ DCs in mediating early secretory response at transcriptional and protein levels, whereas cDC2s mediate early transcriptional activation of antiviral response. These findings advance the understanding of FGT DCs in HIV pathogenesis and identify key differences in mechanisms driving differential response to HIV in DC subsets.

4.6 Study limitations and future directions.

Our study has several limitations due to the rare nature of mucosal DCs and associated technical difficulties in isolating human DC subsets. Firstly, cDC2s in the FGT are present in low numbers and lack key discriminatory markers to isolate this subset since other cells, such as B cells, share CD1c expression with cDC2s. To that extent, we utilized CD1a as a discriminatory marker to isolate cDC2s. However, studies have shown that TGFβ induces CD1a expression on DCs [163]; therefore, whether these cells represent a distinct subset of cDC2s or are present in a transitionary state remains unknown. Additionally, whether differences in levels of sex hormones due to the

menstrual cycle and aging exert their effects on the phenotype and function of FGT DCs remains unknown. Studies linking fluctuating sex hormone levels through the menstrual cycle pre-menopause and further post-menopause and FGT DC phenotype and function, could shed light on whether tissue intrinsic factors drive FGT DC mechanisms. Similar to challenges in isolating cDC2s, the isolation of genital DC3s requires an innovative approach due to a lack of key discriminatory markers. Furthermore, due to the rare nature resulting in low numbers in the FGT, we did not observe a distinct clustering of DC3s from CD14 DCs. Therefore, quantifying individual responses of cDC2s and DC3s to HIV remains challenging and requires the development of isolation strategies. Additionally, given our findings on the effects of aging on FGT DC induction of T cell proliferation, the effects of aging on FGT DC response to HIV remain unknown. Longitudinal studies comparing the effects of aging on FGT DC's ability to respond to HIV are required to fully understand their role in HIV pathogenesis at the mucosal site. Another limitation of our study was the lack of characterization of monocyte response to HIV due to the low number of cells in the HIV-stimulated conditions. Therefore, the contribution of monocytes to HIV pathogenesis remains to be elucidated. Given that DN T cells displayed innate immune-like properties, their role in HIV pathogenesis remains to be understood, including any changes to their function depending on age of the patient. Additionally, studies with larger populations are needed to validate and expand our results due to the limited sample size in our single-cell sequencing data with DN T cell population. Furthermore, due to their rare nature, the reduced number of DN T cells in hysterectomy samples adds additional complexity in identifying mechanisms contributing to the functional heterogeneity of this subset. We relied on allogeneic T cell proliferation assay in our DC-T cell co-cultures due to lack of access to matching naïve T cells from blood or lymph nodes of the same women, therefore the ability of FGT DCs to mediate autologous naïve T cell proliferation remains to be tested in future studies.

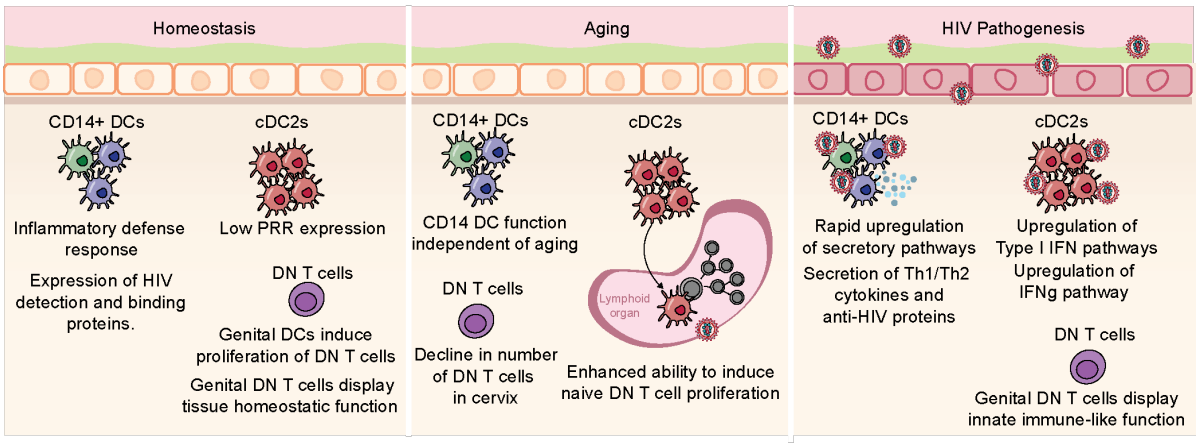

Figure 4.1 Graphical summary of genital DC phenotype and function.

In tissue homeostasis (left), CD14-expressing DCs constitute a heterogenous population that includes monocyte-derived CD14+ DCs and the novel DC3 subset, whereas cDC2s represent a homogenous subpopulation of classical DCs each with unique homeostatic function. The DC subsets shared ability to induce naïve T cell proliferation including the unconventional DN T cell population. With aging (middle) the ability to induce naïve T cell proliferation and induction of DN T cells is specifically enhanced in cDC2s, whereas tissue-intrinsic effects are not observed on CD14 DCs suggesting a replenishing population in the tissue. In HIV pathogenesis (right), CD14 DCs upregulate secretory pathways and in turn secrete Th1/Th2 cytokines and anti-HIV proteins upon exposure to HIV, whereas cDC2s upregulated type I IFN and IFN-γ pathways. Although genital DN T cells display innate immune-like function, their role in HIV response is unknown.

4.7 Concluding remarks and relevance

Taken together, the work done in this thesis highlights tissue intrinsic effects of aging on the function of FGT DC subsets and identifies key functional differences in the induction of innate immune response to HIV (**Fig 4.1**). Although genital DCs play an important role in maintaining a balance between reproductive function and response to pathogens, whether this is done by a single DC subset or multiple DC subsets with unique roles is unknown. Data from our study demonstrates the strong influence of the tissue environment on genital cDC2s, where we observe an increase in cDC2 activation and T cell proliferation ability with increased age, correlating with the gradual loss of reproductive function. Interestingly, tissue intrinsic effects do not extend to genital CD14 DCs, which are responsible for rapid innate immune functions as shown by our data,

suggesting that this population could represent a replenishing niche within the genital tract. Given the ability of CD14 DCs to produce molecules associated with anti-HIV properties, exploring topical applications targeting these populations prior to sexual intercourse could elevate levels of anti-HIV molecules in the mucosa.

Additionally, our findings highlight the subset-specific differences in induction of unconventional DN T cell proliferation by genital DCs. DN T cells have been shown to play a crucial role in the maintenance of pregnancy in mice, however their role in humans, specifically in the female genital tract remained poorly characterized. To our knowledge, we are the first study to demonstrate the ability of genital DCs to induce proliferation of DN T cells in a subset-specific manner. Our dataset significantly contributes to the field of unconventional DN T cells in the genital mucosa through identification and characterization of genital DN T cells using multi-omics datasets to establish their multi-faceted role in mediating tissue homeostasis, possessing cytotoxic and innate immune-like function. Given a complete lack of studies exploring the role of DN T cells in humans and their significant role in maintaining pregnancy in mice, our data highlights the importance of understanding this unconventional subset and explore their role in adverse pregnancy outcomes. Further, our data analysis demonstrating antiviral innate-like functions of DN T cells sets the stage for future studies investigating their potential contribution to HIV pathogenesis. Overall, our findings contribute to the field of mucosal immunity and provide a map to identify therapeutic targets to trigger local protective innate immune responses against HIV, and potentially modulate the adaptive arm.

Chapter 5: Appendix

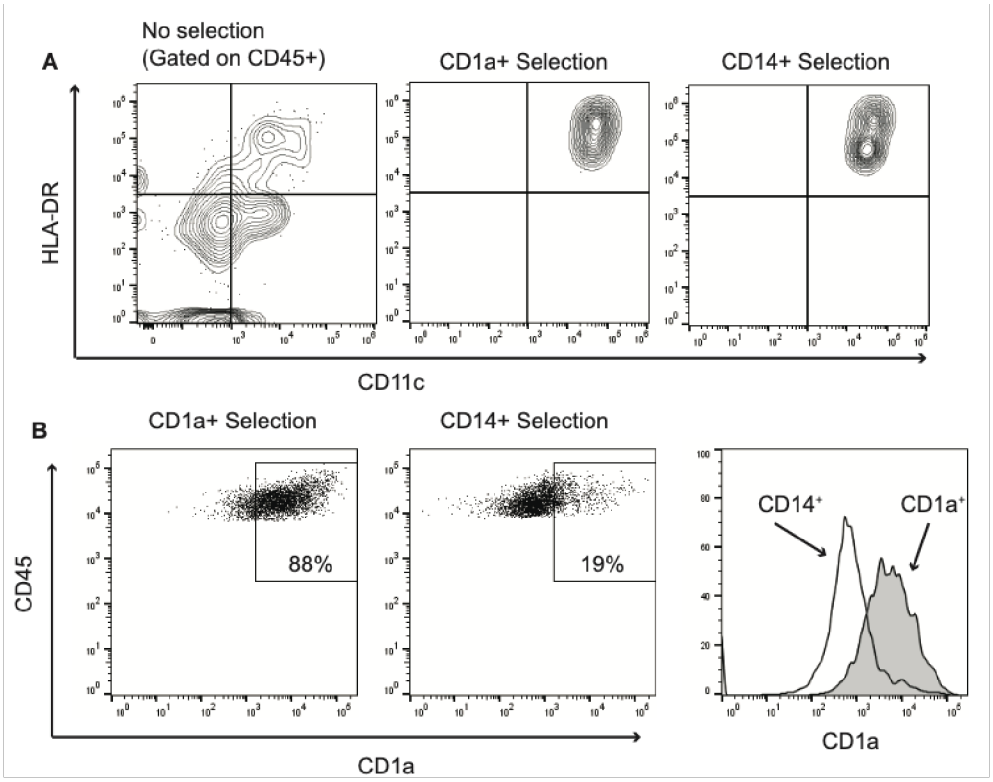

Figure 5.1: Cell phenotype after magnetic bead selection.

(A) Representative flow cytometry contour plots of CD11c and HLA-DR expression, before selection (no selection) and after CD1a+ and CD14+ DC selection. (B) Representative flow cytometry dot plots of CD1a expression and overlay histogram of CD1a expression following CD1a+ and CD14+ DC selection.

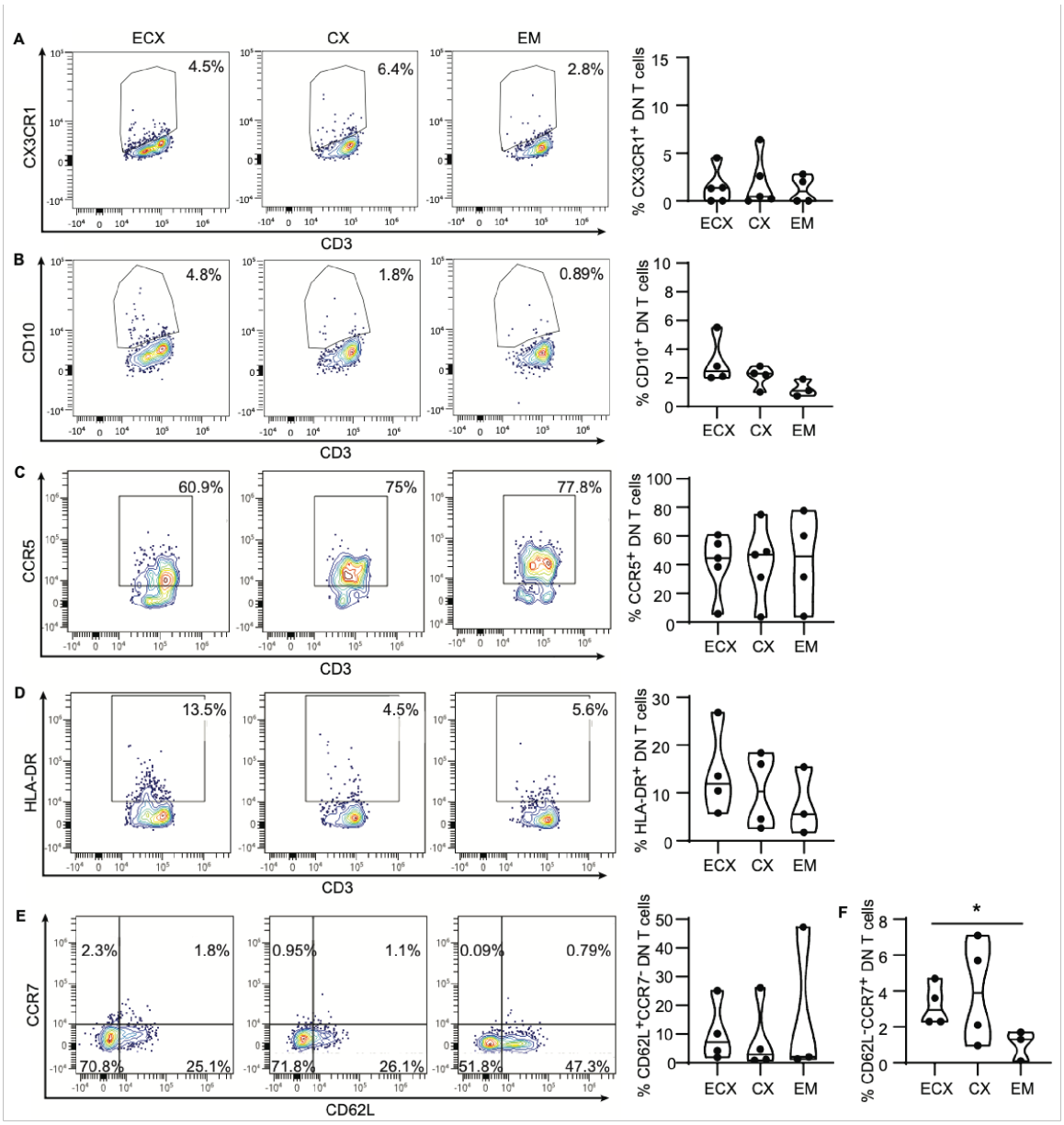

Figure 5.2: Phenotype of DN T cells in the FRT.

Ectocervical, endocervical and endometrial representative flow cytometry plots and percentage of DN T cells expressing CX3CR1+ (A), CD10+ (B), CCR5+ (C), HLA-DR+ (D), CD62L+ CCR7- (E) and CD62L- CCR7+ (F). Each dot represents a different patient. Wilcoxon test was used for statistical analysis. *p<0.05. ECX: ectocervix; CX: endocervix; EM: endometrium; DN: double negative.

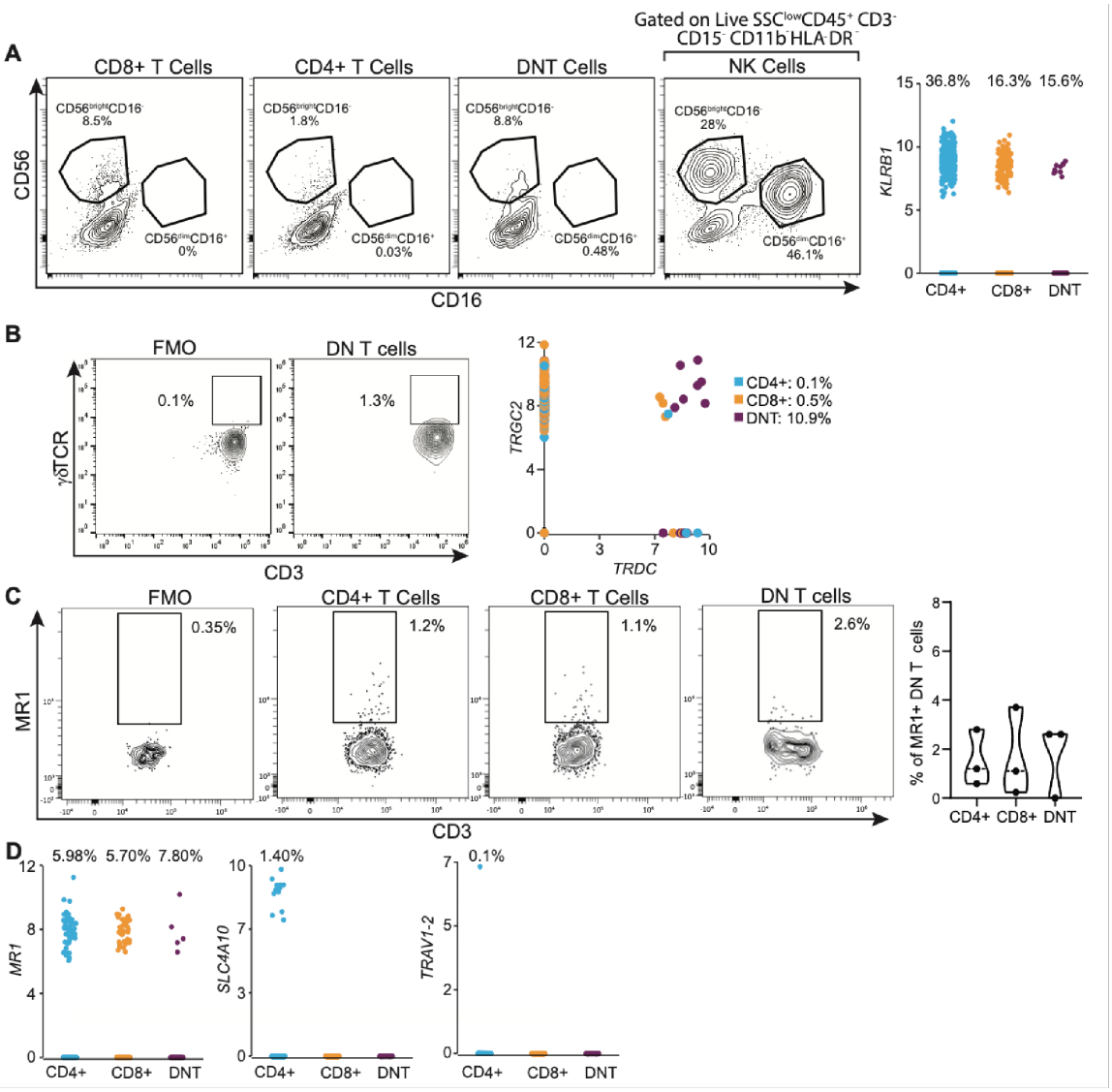

Figure 5.3: Characterization of CD3+CD4-CD8- T cell subpopulations.

(A) Determination of NKT cell presence. Representative flow cytometry plots of CD56 and CD16 expression in CD8+, CD4+, DNT and NK cells in the FRT, and KLRB1 gene expression across T cell subsets in the FRT. (B) Determination of γδ T cells. Representative flow cytometry plot assessing expression of γδTCR chain in DNT cells from the FRT (FMO=fluorescence minus one) and scatter plot comparing T cell subsets in the FRT for expression of TRGC2 and TRDC genes. Numbers indicate the percentage of T cells co-expressing TRGC2 and
TRDC genes within each population. (C) Representative flow cytometry plots and quantification of MR1 expression on T cell populations. (D) Percentage of T cell populations expressing canonical MAIT cell genes MR1 (left), SLC4A10 (center) and TRAV1-2 (right).

Table 5.1 Fluorescence-conjugated antibodies used for spectral flow cytometry experiments.

Target	Clone	Fluorophore
CD45	H130	BUV395
Viability	-	Live/Dead Blue
CD16	3GB	BUV496
CD19	SJ25C1	BUV563
CD64	10.1	BUV737
CD4	SK3	BUV805
CD54	HA58	BV421
CD66b	G10F5	Pacific Blue
CD14	MϕP9	BV480
CD3	REA613	VioGreen
CD11b	ICRF44	BV570
CD49d	SK11	BV711
CCR7	G043H7	BV750
CXCR4	12G5	BV785
CD8	SK11	SparkBlue
CD15	H198	PerCP-Cy5.5
CD1c	L161	PE
CX3CR1	2A9-1	PE-eF610
CCR5	2D7/CCR5	PE-Cy5
CLEC12A	50C1	APC

Target	Clone	Fluorophore
HLA-DR	LN3	AF700
CD11c	Bu15	APC-Cy7
Perforin	dG9	PE/Dazzle
Granzyme A	CB9	AF647
Granzyme B	GB11	BV421

Table 5.2: List of oligo-conjugated surface protein antibodies (AbSeq) used for CITEseq experiments.

Antibody Tag	Catalog #	Clone	Oligo-nucleotide sequence
CD103	940067	BER-ACT8	AAATAGTATCGAGCGTAGTTAAGTTGCGTAGCCGTT
CD274	940035	MIH1	ATCGTAAGGCTCGTGGTTCGTAAGTAAGTTCGTATC
CD69	940019	FN50	CAATAACGGGTCATAGTAAGTCGCGAGTAAGAGGGC
CD152	940034	BNI3	TAGTATCCGTAGTAGTTATCTGCCCGTTCGTTATGC
CD14	940005	MPHIP9	TGGCCCGTGGTAGCGCAATGTGAGATCGTAATAAGT
CD62L	940041	DREG	ATGGTAAATATGGGCGAATGCGGGTTGTGCTAAAGT

CD10	940045	HI10A	CCTGTTTGATGCGTACGGAGATTTAGCGGATTTATG
CD16	940006	3G8	TAAATCTAATCGCGGTAACATAACGGTGGGTAAGGT
CD184	940056	12G5	CAGTGTTTAGAGCGGGTTGCATATGTCGTTTAGAGG
CD163	940058	GHI/61	TATTATGTGCGAACTATGGTATCCGTATTGAGGGCT
CD1a	940063	HI149	TTGGTTGCAGTGCGGTCGAAGATACGTAGTGAGATT
CD195	940050	2D7/CCR5	ATGGTTTAGTCGTACGTGGGTTTAGATTGGCGGTGC
CD83	940054	HB15e	AAGCTTGGACGATGGTATATTAACGATTGAGAGTGC
CD1c	940083	F10/21A3	ATAGATTACATTCGTTTAGCGTTGGGTTCGGTCCGT
CX3CR1	940216	2A9-1	GGGTTCACGAGGTTTAAAGCGGTAGTATAGGATGCC
HLA-DR	940235	TU39	GAATCGAGTTTATAGGTGGCGTTAGTAGTTGTGGGC
CD19	940247	HIB19	AAGCGGTAAATCGGGAGTAAGTCGTGTTCTAGCAGT
CD117	940250	104D2	ATGGAGAGCGATTGCGTGAGGATATGCGAGATTGTT

CD11c	940265	S-HCL-3	GTCGGTTCGTGATTTAGTTAGTGCGTCTTAGTGTCC
CD11b	940266	ICRF44	ATGGATTCGGTTCGGTGTTTGGATAGATAGGCTGCG
CD279	940467	MIH4	ACGAGAAATGCGCGGAATGGGTGAGTTAGTAAGACG
BDCA-2	940282	V24-785	TAAGGTAGGCAGTAGATAACGGGACGAATGATGAGC
CD4	940304	RPA-T4	AATGTGCGGCGGTGTATATGATCGAGTCCAACGTCT
CD8	940305	SK1	AGGACATAGAGTAGGACGAGGTAGGCTTAAATTGCT
CD3	940307	UCHT1	AGCTAGGTGTTATCGGCAAGTTGTACGGTGAAGTCG
CCR7	940394	2-L1-A	AATGTGTGATCGGCAAAGGGTTCTCGGGTTAATATG
CD15	940274	W6D3	ATAGGCATGGACGACGTAGATAATAAGTGGCGGGTT
CD64	940262	MD22	ATGTAGTCTGTATAGCGGTGTAGCGGATTAAAGGCG
CD127	940012	HIL-&R-M21	AGTTATTAGGCTCGTAGGTATGTTTAGGTTATCGCG

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