Defining Immune Correlates of HIV Susceptibility in the Foreskin

by

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Abstract

HIV is a predominantly sexually transmitted infection that has infected over 60 million people and been responsible for 60 million deaths. To date, non-antiretroviral microbicides have failed to prevent HIV acquisition, or even increased it. This is likely because HIV preferentially infects activated immune cells (CD4+ T cells), taking advantage of the body's attempts to defend itself. Therefore, relative immunoquiescence, as opposed to immune activation, may be protective. I hypothesized that men who are biologically more susceptible to HIV would have increased foreskin CD4 T cell activation, while the opposite would be true of men who are relatively resistant. The foreskin has recently been identified as a major site of HIV acquisition, but little previous research has been performed on this tissue. I therefore developed novel techniques to isolate viable, immunologically functional T cells from foreskin tissue. I then worked with the Rakai Health Sciences Program in Uganda to identify men undergoing elective circumcision who are HIV-Exposed but have remained SeroNegative (HESN, relatively resistant to HIV), and men with Herpes Simple Virus-2 infection (HSV-2+, relatively susceptible to HIV). I collected sub-preputial swabs and foreskin tissue from these men, and characterized numerous immune parameters in their samples. I found that HSV-2+ men had an increased relative abundance of CD4 T cells co-expressing the HIV receptor CCR5. In contrast, I found that HESN men had a decreased relative abundance of activated T cells (CD4/8 T cells producing TNFα) and Th17

cells (a pro-inflammatory T cell subset known to be particularly susceptible to HIV). Additionally, foreskin secretions from HESN men were more likely to have antibodies (IgA) able to neutralize HIV, and had more innate anti-viral peptides. I therefore propose HIV resistance may be driven by decreased T cell activation in genital tissue, in combination with increased secretion of anti-HIV immune proteins.

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Table of Contents

List of Tables

List of Figures

List of Publications Arising from this Thesis

Prodger JL, Gray R, Kigosi G, Nalugoda F, Galiwango R, Hirbod T, Wawer M, Hofer SOP, Sewankambo N, Serwadda D, Kaul R. Foreskin T cell subsets differ substantially from blood with respect to HIV co-receptor expression, inflammatory profile and memory status. *Mucosal Immunology*, 5(2): 121-128 (2012).

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List of Abbreviations

AI	Anal Intercourse
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
APC	Allophycocyanin (a fluorophore)
ART	Antiretroviral Therapy
BV	Bacterial Vaginosis
C-type	Calcium dependent
cAMP	cyclic Adenosine MonoPhosphate
CD	Cluster of Differentiation
cDNA	copy DNA
CS	Cellulose Sulfate
CSW	Commercial Sex Worker
Ct	Comparative threshold
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific ICAM-3 Grabbing Molecule
DMSO	Dimethyl Sulfoxide
DNA	deoxyribonucleic acid
dpi	days post inoculation
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FGT	Female Genital Tract
FITC	Fluorescein isothiocyanate (a fluorophore)
FMO	Fluorescence Minus One
FOV	Field Of View
FoxP3	Forkhead box P3
GALT	Gut Associated Lymphoid Tissue
GFP	Green Fluorescence Protein
gp	glycoprotein

GUD	Genital Ulcer Disease
hBD	human β-defensin
HESN	HIV-Exposed SeroNegative
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HNP	Human Neutrophil Protein
HPV	Human Papillomavirus
HR-HPV	High Risk- HPV
HSV	Herpes Simplex Virus
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
iono	ionomycin
IP-10	Interferon-γ-induced protein
IRF-1	Interferon Regulatory Factor-1
LFA	Leukocyte Firm Adhesion
LLOQ	Lower Limit of Quantification
MC	Male Circumcision
MCP	Monocyte Chemotactic Protein
MHC	Major Histocompatibility Marker
MIG	Monocyte-Induced Gamma Interferon
MIP	Monocyte Inflammatory Protein
MR	Mannose Receptor
MSM	Men who have Sex with Men
N9	Nonoxyl-9
NFκB	Nuclear Factor-κB
NIAID	National Institute of Allergies and Infectious Diseases
NIH	National Institute of Health
NK	Natural Killer Cell
NKT	Natural Killer T cell

ns	not significant
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin (a fluorophore)
PerCP	Peridinin Chlorophyll Protein Complex (a fluorophore)
PHA-P	phytohemagglutinin
PMA	Phorbol 12-myristate 13-acetate
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RNA	ribonucleic acid
ROR	Retinoic acid-related Orphan Receptor
RPMI	Roswell Park Memorial Institute Medium
RQ	Relative Quantity
SC	Secretory Component
SDF	Stromal Derived Factor
sIgA	soluble IgA
SIV	Simian Immunodeficiency Virus
SLPI	Secretory Leukocyte Protease Inhibitor
STI	Sexually Transmitted Infection
T-bet	T-box Expressed in T cells
TCID50	Tissue Culture 50% Infectious Dose
TCM	Central Memory T Cell
TCR	T Cell Receptor
TEM	Effector Memory T Cell
TFH	Follicular Helper T Cell
TGFβ	Transforming Growth Factor-β
Th	helper T cell
TNF	Tumor Necrosis Factor
Treg	Regulatory T Cell
UBC	Ubiquitin C
UNAIDS	Joint United Nations program on HIV/AIDS
VCAM	Vascular Cell Adhesion Molecule

Chapter 1
Literature Review

1 Introduction

Human Immunodeficiency Virus-1 (HIV) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS), which has lead to the deaths of an estimated 30 million people, including 1.7 million last year [1]. HIV disproportionately affects poor and developing countries: 69% of HIV infections are in sub-Saharan Africa, where the prevalence of HIV is an astounding 4.9%, in contrast to North America where the prevalence is 0.6% [2]. HIV predominantly infects CD4 T cells so that infected individuals suffer from a gradual decline in CD4 T cell numbers, leading to immune dysfunction and increased susceptibility to cancers and opportunistic infections, collectively referred to as AIDS [3]. There is currently no cure for HIV, and without life-long treatment the median time to AIDS-related illness and death is 10 years [4]. While effective antiretroviral therapy (ART) can prevent the onset of AIDS, drug regimes are expensive ($23,000/person/year United States, [5]) and their provision is a significant barrier to economic development of resource-poor countries, where the burden of HIV is highest [2]. Even in resource-rich countries where ART is universally available, treatment regimes are complex and difficult to adhere to, and, despite effective treatment, HIV-infected individuals have significantly poorer health outcomes [6, 7]. As a result new, effective methods of preventing HIV transmission are desperately needed.

1.1 Study Rationale

There are three main types of HIV transmission: sexual, mother-to-child, and contaminated blood contact. Sexual HIV transmission accounts for 85% of global HIV cases, the majority of which occur through heterosexual exposure (~79% of sexually transmitted cases [8]). Contaminated blood contact accounts for 7.8% of infections, and mother-to-child transmission for 7.2% of infections [8].

Despite the staggering number of HIV infections acquired through sexual exposure, transmission by this route is surprisingly inefficient, and also highly variable (reviewed in [8]). Estimates of the probability of HIV transmission during a single act of vaginal intercourse range from 1/200-1/2000 for male-to-female transmission, and 1-700-1/3000 for female to male transmission. The large range in estimates of transmission probabilities is due both to variable infectiousness of the HIV-infected partner and also variable susceptibility of the HIV-uninfected partner. Infectiousness is highly dependent on the concentration of HIV (viral load) in genital secretions [9-11], demonstrated by the near absence of transmission when viral replication is fully suppressed by ART [12, 13]. Susceptibility of the uninfected partner has been epidemiologically attributed to numerous risk factors, including age[14, 15], race[16], sexually transmitted infections (STIs)[17], and circumcision status[18, 19].

While HIV infectivity is dictated by characteristics of the genital fluids of the infected partner, HIV susceptibility must be dictated by characteristics of the exposed tissue. However, it is unclear how epidemiologically observed states of increased susceptibility alter the genital mucosa to render an individual more vulnerable to HIV. This knowledge gap presents a significant barrier to the design of new modalities to prevent HIV infection: if we can understand the biological mechanisms that make someone more or less susceptible to HIV, we can design targeted interventions to dampen, or promote, these factors. *This body of work was undertaken to further elucidate what these factors might be at a single site of HIV infection: the foreskin*. To accomplish this, I used our current knowledge of mucosal HIV infection to identify plausible cellular/molecular factors that could contribute to HIV-susceptibility/protection. I then developed techniques to measure these factors in foreskin tissue. Finally, I examined if these factors were altered in states that have been epidemiologically linked to HIV susceptibility.

In this introduction I will first establish our rationale for investigating HIV susceptibility in the foreskin (Section 1.2). I will then review our current understanding of how HIV establishes infection, providing a rationale for the biological factors that we chose to investigate (Section 1.3). Finally, I will describe populations with increased or decreased HIV susceptibility, and review previous research providing biological explanations their altered HIV susceptibility (Section 1.4).

1.2 The Foreskin as a Site of HIV Infection

Evidence for the importance of the foreskin in HIV acquisition comes from studies showing male circumcision (MC) greatly reduces HIV susceptibility in heterosexual men. While 30-34% of men globally are circumcised, regional circumcision rates are highly variable and range from >5% to <80%[20]. Ecological studies comparing regions based on circumcision status have observed that areas with higher rates of circumcision also have lower HIV prevalence[21]. Epidemiological studies further supported a strong protective effect of MC, with a meta-analysis of 27 studies showing a 68% reduction in HIV risk for circumcised men[18]. These observations prompted three large, randomized trials of male circumcision for prevention of heterosexual HIV acquisition in South Africa, Uganda and Kenya [22-24]. All three trials demonstrated a significant reduction in HIV incidence among those men who received circumcision (51-60% reduction), despite significant differences in participant age, HIV incidence rates, the type of surgical procedure used, and trial setting (urban vs. rural). A meta-analysis of these three trials found little statistical heterogeneity between their results, and that the overall protective effect of MC was 54% at 24 months post circumcision in an intent-to-treat analysis[19].

In addition to a direct protective effect of circumcision on HIV susceptibility, circumcision also reduces other STIs that are associated with HIV susceptibility. The South African and Uganda randomized trials showed reductions in Herpes Simplex Virus type 2 (HSV-2) of 45% and 28%, and of high-risk strains of Human Papillomavirus (those associated with neoplasia, HR-HPV) by 35% and 34% [25-27], respectively. Additionally, the Uganda trial demonstrated a 47% reduction in genital ulcer disease (GUD)[25], the predominant cause of which is HSV-2, but syphilis and chancroid can also cause ulcers[28]. Circumcision has also been associated with reductions in bacterial STIs, such as gonorrhea, syphilis, and Chlamydia in some studies[29-31], but not others[32-34].

The effect of MC on HIV susceptibility of men who have sex with men (MSM) and transmission to female partners is less clear. Two previous prospective studies demonstrated that men who had been circumcised during childhood were less likely to transmit HIV to their female partners[35, 36]. As a result of these observations, a parallel study was run in Uganda examining the effect of MC of HIV-infected men on HIV transmission to their uninfected female

partner[37]. This study was stopped early due to futility, but at closure there were no difference in HIV acquisition between women whose partner was circumcised and those whose partner was not. A *post hoc* analysis revealed that HIV transmission in the first 6 months after surgery was significantly higher (over 3-fold) among couples who reported resumption of sexual activity prior to documented wound healing, compared to couples where the man remained uncircumcised. It is possible that any protective effects of MC on transmission to a female partner were masked due to the increased risk of transmission during wound healing. Randomized trials of male circumcision have shown to reduce bacterial vaginosis (BV), trichomonas, GUD[38], and HPV[39] in female partners, all of which are associated with increased female HIV susceptibility. The clear reduction in female-partner STIs, and potential long-term reduction in HIV transmission, provides a case for circumcision of HIV-infected men *if* resumption of sex before wound healing can be avoided.

A large longitudinal study of MSM found no protective effect of MC[40]. It is likely that the high risk of HIV acquisition during receptive anal intercourse (AI, 10x greater per-contact risk 1/20-1/300 [8]) outweighs the protective effect of circumcision during insertive AI. This is consistent with two cross-sectional studies that found, in the limited number of participants who practice exclusively insertive AI[41, 42], circumcision was associated with a lower prevalence of HIV. However, as the vast majority of MSM do not practice exclusively insertive AI[42], MC is unlikely to have an effect on HIV transmission in MSM couples.

The reproducibility of the protective effect of MC against heterosexual male HIV infection among the three clinical trials, and the consistency of the magnitude of this effect with previous epidemiological estimations, suggests that MC would be an effective intervention in areas where the epidemic is predominantly driven by heterosexual transmission. Mathematical modeling suggests that with 50% coverage a 25-41% reduction in HIV prevalence- in both men and women- would be achieved[21]. Circumcision also has the benefit of being a one-time intervention that will provide protection for life, and being relatively inexpensive (55-69USD per procedure)[43-49], and significant cost-saving measure when the expense of averted ART is accounted for[48]. Despite these benefits, both individual and national uptake of circumcision has remained low. Reasons for this low uptake at the individual level are driven by a fear of pain, potential lost income during healing, the necessary healing time before resumption of sex (usually about 6 weeks), and cultural concerns[50]. Circumcision globally is often deeply rooted

in cultural tradition, and as a result many men associated their circumcision status with their cultural identity[51]. These cultural distinctions are also likely a barrier to the implementation of circumcision for HIV-prevention at a national level. Additionally, while circumcision is a cost-savings measure over a long period, it requires financial and human resources input upfront from healthcare systems that are already overburdened in high HIV-prevalence areas[44]. As a result, roll-out of circumcision programs in UNAIDS priority countries has remained low[2].

Clinical trials of male circumcision and subsequent attempts to roll out circumcision programs at a population-level have demonstrated two things: (1) that the foreskin is an important site of HIV acquisition, and (2) that many at-risk men will choose not to be circumcised. As a result, new modalities to prevent HIV acquisition across the foreskin are necessary.

1.3 Cellular and Molecular Events in HIV Infection

1.3.1 HIV Virology

1.3.1.1 Structure

HIV-1 is a member of the family *Retroviridae* and the genus *lentivirus*. A unifying feature of retroviruses is that their genome is encoded in RNA that is reverse transcribed in the host cell into cDNA, which is then inserted into the host genome[3]. The genus lentivirus includes viral strains that can infect several different animal species, two of which infect humans: HIV-1 and HIV-2. While HIV-2 can also cause AIDS, it is far less pathogenic than HIV-1[52]. HIV will be used to refer to HIV-1 throughout this thesis.

An infectious HIV viral particle is composed of an envelope of host phospholipid membrane, acquired when the viral particle buds from the surface of an infected cell, encapsulating an inner viral core[3]. Embedded within the phospholipid membrane are viral envelope proteins (Env), comprised of non-covalently linked trimers of glycoprotein 120 (gp120)/ gp41 heterodimers. The gp41 trimer comprises the membrane-spanning portion of the envelope protein, and the gp120 trimer forms the extracellular domain[53]. It is the external gp120 trimer that is responsible for binding receptors on susceptible host target cells. Env is heavily glycosylated, with *N*-linked glycans contributing to almost half its molecular mass[54]. Just below the envelope is a lining of the viral protein matrix (p17)[55]. The viral core is a conical structure made of repeating units of the viral protein capsid (p24) containing two identical strands of viral RNA, and the enzymes reverse transcriptase, integrase, and protease[55]. Also within the viral particle are six regulatory proteins (Tat, Rev, Nef, Vif, Vpr, and Vpu) involved in replication and host immune evasion[3].

1.3.1.2 Replication

Viral particles bind to host target cells via interaction of the gp120 trimer with CD4 molecules on the cell surface. This attachment leads to conformational changes in both gp120 and CD4 that

expose a binding site on gp120 for one of two cellular co-receptors: CCR5 or CXCR4[56] (covered in detail in Section 1.3.2.1). Co-receptor binding causes further conformational changes in gp120, bringing the viral particle into closer proximity with the host cell membrane. Subsequently, gp41facilitates the fusion of the viral envelope with the host cell membrane allowing the viral core to enter the cell[57]. Upon cell entry, HIV RNA is released into the cell cytosol (uncoating) and a double stranded cDNA copy is made by the HIV enzyme reverse transcriptase[58]. This cDNA in incorporated into a prointegration complex with other viral proteins, including integrase and is transported to the nucleus where viral cDNA is inserted into one of the cells' chromosomes by the viral enzyme integrase[59]; the integrated viral DNA is referred to as a provirus, and in non-activated cells this integrated DNA can remain dormant, referred to as latent infection[60].

During viral replication, transcription of HIV DNA is controlled by the regulatory proteins Tat, Rev, and Nef[61]. The entire HIV genome consists of three major genes (*gag*, *pol*, and *env*) transcribed as a single mRNA transcript, which will serve both as mRNA for synthesis of viral proteins, and also later as genomic material to be packaged into new viral particles[62]. The single mRNA transcript is then differentially spliced to form the various transcripts required: the *gag* gene encodes structural proteins of the viral particle, the *pol* gene encodes regulatory proteins, and *env* encodes viral envelope glycoproteins[63]. The relative amounts of unspliced, singly spliced and multiply spliced mRNA are controlled by Rev to accommodate the relative abundance of viral proteins required to form infectious particles[61]. These differentially spliced mRNA transcripts are then translated into polypeptide chains, which are further cleaved by the viral enzyme protease to form functional viral proteins. Viral assembly takes place at the cell membrane, where viral RNA is packaged into a core using newly synthesized viral proteins[64]. This core buds from the surface of the cell, selectively incorporating host cell membrane proteins[65, 66] and lipids[67] in the process, as well as viral envelope trimers.

1.3.2 HIV Infection at the Level of the Cell

1.3.2.1 Cellular Entry

1.3.2.1.1 Primary HIV Receptor: CD4

The primary receptor for gp120 on host cells is the molecule CD4[68]. CD4 is predominantly expressed by T helper cells (Th cells), but is also expressed on dendritic cells[69], and macrophages[70]. On Th cells, CD4 is found in complex with the T cell receptor (TCR, which is in complex with CD3). The TCR consists of a highly variable domain that is specific for short amino acid sequences of foreign protein (antigen), and a constant region, including an intracellular domain and cytoplasmic tail. The variable region of the TCR recognizes antigen presented to the T cell by so-called antigen presenting cells (APCs; macrophages, B cells, and dendritic cells), in complex with an MHC (major histocompatibility complex) class II molecule on the APC surface. Antigen recognition by Th cells leads to cellular activation and effector functions. CD4 molecules are found in close proximity to the TCR, and bind a separate site in the constant region of the MHC molecule, increasing TCR sensitivity to presented antigen by 100-fold[62]. It is this co-stimulatory molecule, and thus Th cells, that are the primary receptor targeted by HIV.

1.3.2.1.2 HIV Co-Receptors: CCR5 or CXCR4

In addition to CD4, HIV requires one additional co-receptor to enter host cells; strains that use CXCR4 as a co-receptor are referred to as X4 tropic viruses, those that use CCR5 are referred to as R5 tropic viruses, and those that can use both are R5X4 tropic[56]. Both CCR5 and CXCR4 belong to a closely related family 7 transmembrane domain G-protein coupled receptors that bind a specific type of small cytokine referred to as chemokines. Chemokine binding to its receptor initiates a kinase-signaling cascade in the cell that results in cellular migration along the chemokine concentration gradient[71]. CCR5 has several natural ligands, including macrophage inflammatory protein-1α (MIP-1α/CCL3), MIP1β (CCL4) and regulated upon activation

normal T cell expressed and secreted (RANTES/CCL5)[72]. Monocyte chemoattractant proteins (MCP) 1-4 also bind CCR5, but with a lower affinity than the three main ligands[71]. Unlike CCR5, CXCR4 only has one known ligand: stromal-derived factor 1 (SDF-1/CXCL12)[71]. While both X4 and R5 tropic strains of HIV are prevalent among infectious individuals[73], and both CXCR4+ and CCR5+ CD4+ cells are present in the genital mucosa[74, 75], sexual transmission of HIV occurs almost exclusively through R5 tropic strains of HIV. Evidence for this comes from individuals who are homozygous for a truncated version of CCR5, called Δ32. In this variant there is a 32 base-pair deletion from the coding region of the CCR5 gene that leads to a frameshift mutation and a truncated protein[76-79]. The frequency of this mutation is quite high in Caucasian populations, 1% of individuals are homozygous for the Δ32 allele[77, 80], yet infection of these individuals is extremely rare[77] and only occurs through dual or X4 tropic viral strains[81, 82]. Additional evidence is provided by novel sequencing techniques that now allow for better identification and characterization of transmitted strains of HIV: transmitted strains of HIV are almost exclusively R5 tropic, with the only exceptions being rare cases of dual tropic strains[83, 84].

While transmission of HIV occurs through R5 tropic strains of HIV, X4 tropic strains develop in approximately 50% of individuals after 5 years of infection[73, 85], and the emergence of X4 tropic strains is associated with rapid progression to AIDS[73]. An R5 tropic virus can be converted to an X4 tropic virus with the change of only 2 or three amino acids[86-88]; given the high replicative ability of HIV and the high error rate of reverse transcriptase[89], the switch from R5 to X4 would be expected to occur frequently during the course of any infection[56]. It is not fully understood why sexual transmission of HIV is limited to R5 tropic strains of HIV, or why the emergence of X4 strains occurs so late in infection. Multiple hypothesis have been suggested, including the decreased replicative ability of X4 strains[82, 90], the high expression of CCR5 and reduced expression of CXCR4 in lymph nodes and gut-associated lymphoid tissue[91, 92] (GALT; the main sites of early HIV replication), or the preferential targeting of X4 strains by CTLs[93]. It may be that during HIV transmission and early in the course of infection R5 strains out-compete X4 strains, and later in disease progression gradual immune exhaustion and depletion of CCR5+ cells drives the emergence of X4 strains[56]. However, as CXCR4+ cells do not contribute to the sexual transmission of HIV, the remainder of this work will focus on the discussion of CD4/CCR5+ cell types.

1.3.2.1.3 Possible Additional HIV Receptors: Attachment Factors

While CD4 and CCR5 are essential receptors in virion-cell interactions during sexual transmission, other cell receptors are also capable of binding HIV, and therefore may facilitate HIV transmission. Many additional chemokine receptors have been shown to bind HIV *in vitro*[94], and these may contribute to virion capture, allowing for improved interactions with classical receptors (CD4/CCR5). The integrin $\alpha_4\beta_7$, involved in homing of Th cells to the GALT, has also been shown to bind gp120[95]. $\alpha_4\beta_7$ is found in close proximity to CD4[96] on activated Th cells that express high levels of CCR5[75], and $\alpha_4\beta_7$ extends approximately 3-times as far from the cell surface[95], and it has been hypothesized this may provide a way for R5 strains of HIV to specifically target highly vulnerable cells[95]. Finally, innate immune receptors such as Calcium-dependent (C-type) lectins, whose natural role is to recognizing sugar motifs on invading pathogens, can also act as receptors for HIV by binding to glycosylated regions of gp120. Several C-type lectins, such as DC-specific ICAM3-grabbing nonintegrin (DC-SIGN/CD209)[97], mannose receptor (MR/CD206)[98], and langerin (CD207)[99], have been shown to directly bind gp120. C-type lectins are not expressed on Th cells, but are expressed on macrophages and dendritic cells (DCs)[100], which may also be productively infected with HIV *in vitro*[101-103], albeit not as efficiently as Th cells[104]. DCs may also important in transferring HIV to Th cells in a process termed *trans* infection[97, 105] (discussed in detail in Section 1.3.2.2.2).

In addition to HIV-binding through gp120, cellular proteins that are selectively[106] incorporated into the budding virus can interact with cognate receptors on target cells and increase viral infectivity. One of the most abundantly incorporated host molecules is the MHC Class II molecule Human Leukocyte Antigen-DR (HLA-DR)[107], which binds to CD4; virions that incorporate HLA-DR are approximately 2 fold more infectious than those without HLA-DR[108]. Intracellular Adhesion Molecule-1 (ICAM) is also incorporated into budding virions[109]. ICAM-1 is an adhesion molecule that binds to Lymphocyte Function Associated molecule-1 (LFA-1) and is typically involved in leukocyte recruitment by the circulation by endothelial cells. CD4 T cells also express LFA-1, and therefore the incorporation of ICAM-1 into the virus envelope can facilitate adhesion to target cells via LFA-1 and increase infectivity of viral particles by up to 10-fold[110].

1.3.2.2 Cell-to-Cell Transfer of HIV

HIV infection of a susceptible CD4+ cell can occur in one of three ways. The first occurs when new viral particles bud from an infected target cell in a non-specific way; they are released into the extracellular space and reach new susceptible cells through diffusion, followed by attachment using CD4 or the other the cellular attachment factors discussed above. This is referred to as infection with "cell-free" virus. The second two mechanisms both involve the cell-to-cell transfer of HIV virions. This can occur either by directional budding of viral particles from an infected cell into a "virological synapse" formed with an uninfected cell, or through the transfer of intact viral particles from one uninfected cell to another- referred to as *trans* infection. Infection of CD4 T cells through cell-associated HIV (cell-to cell transfer through a virological synapse) is 100 to 1000-fold more efficient than infection with cell-free virus[111], and will be discussed further in this section.

1.3.2.2.1 Virological Synapse

Infected CD4 T cells express gp120 on their surface, and upon contact with another CD4 T cell, this gp120 can bind CD4 on the uninfected cell [112, 113] in a co-receptor independent fashion[114]. However, instead of leading to membrane fusion of the two cells[115], it triggers the formation of a stable intracellular junction between the two cells, referred to as a virological synapse [112, 116]. After gp120-CD4 engagement, there is co-polarization of HIV Env and CD4/co-receptors in the infected and target cell, respectively [112]. HIV structural proteins are then recruited to the site of cell-cell contact in the infected cell, and there is polarized virus assembly and budding of virions towards the target cell[116]. These viral particles do not fuse with the target cell membrane as in cell-free infection, but instead many immature viral particles are taken up at once by the target cell through endocytosis[114, 116, 117]. Once inside the target cell, viral particles mature and fuse with the endocytic compartment[118] and are released into the cell and viral replication can occur[119].

1.3.2.2.2 *trans* Infection

Cell-to-cell transfer of HIV to CD4 T cells can also occur through the transfer of intact viral particles from an uninfected cell, referred to as *trans* infection. It was originally observed that co-culture of DCs and T cells lead to enhanced infection of CD4 T cells, even without productive infection of the DCs themselves[105]. The exact details of how DCs transfer intact virions to CD4 T cells without becoming infected is currently a topic of significant debate; however, there is consensus that it occurs through the formation of an "infectious synapse" between the two cells. In this model, HIV is captured by cognate pathogen receptors on DCs, sequestered for long periods (from hours[120, 121] to several days[97, 122], depending on the report), and then presented as an intact virion to susceptible CD4 T cells. Normal DC processing of invading pathogens involves phagocytosis and subsequent targeting of ingested pathogen to the lysosolic compartment for degradation and presentation on MHC class II molecules. However, a portion of bound HIV virions escape degradation[122-124]; it is not clear if virions are fully internalized or remain bound to surface receptors in deep invaginations [125, 126](reviewed in [127]), but once the HIV-exposed DC comes into contact with a CD4 T cell, a stable infectious synapse forms between the DC and CD4 T cell[124, 128, 129], with recruitment of intact viral particles in the DC and CD4/CCR5 in the T cell allowing for efficient viral transfer and CD4 T cell infection[105]. Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) was the earliest DC receptor to be implicated in the *trans* infection of T cells[97, 122]), but since then it has been shown that *trans* infection is not always dependent on DC-SIGN[130-132], and that other lectin receptors (discussed in Section 1.3.2.1.3) may also be involved[133] and likely varies with the maturation state or sub-type of DC involved[132].

1.3.3 HIV Infection at the Level of the Tissue

Vaginal SIV inoculation of rhesus macaque monkeys has provided a model of the earliest events in mucosal HIV infection (see Figure 1.1, reproduced from [134]). In combination, these studies [135-139] show that while HIV virions penetrate the cervicovaginal epithelium in 30-60 minutes, several days pass before any viral replication can be detected. At 3-5 days post inoculation (dpi), small, localized, and extremely rare clusters of infected cells can be observed (one cluster of 30-40 infected cells in ~40 cervical sections per animal). Viral sequencing studies in humans show that a single viral strain is transmitted in 80% of heterosexual transmissions [84]. Together these data suggest that new HIV infections occur from a single cellular infection event, and that the population of infected cells expands through new viral production from that infected cell. Over the following days, this so-called founder population of infected cells expands and spreads within the cervix, and after several days of local expansion (~7dpi), HIV RNA can be detected in the lymph nodes draining the cervix, and rapidly after that (~10 dpi) if can be detected in the peripheral circulation.

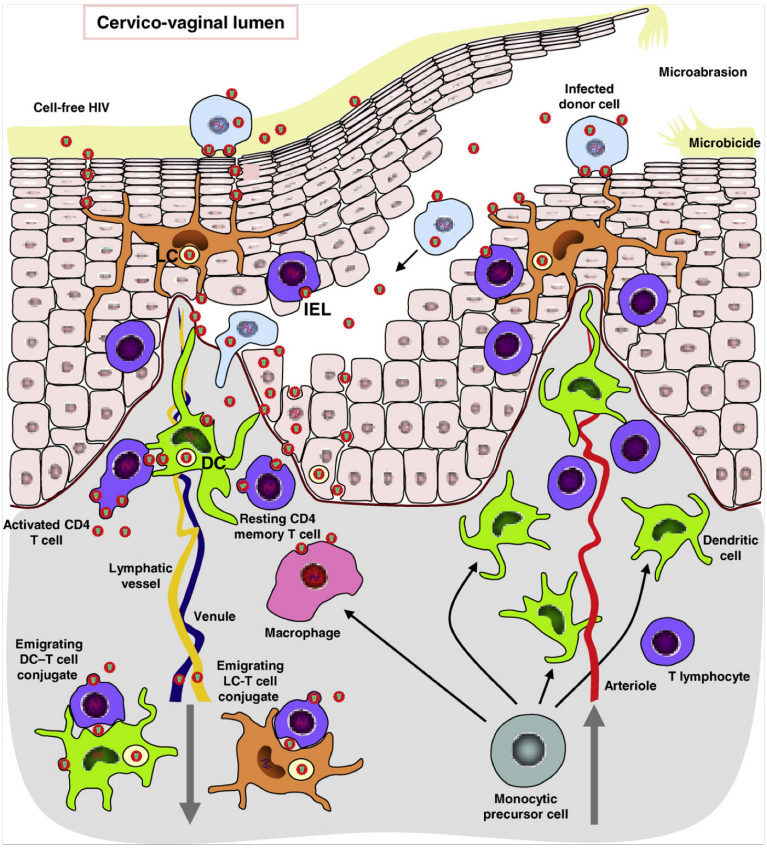

Figure 1.1 Sexual transmission of HIV in the female genital tract. Cell-free and cell-associated HIV penetrates the cervicovaginal epithelium through microabrasions, or by passive diffusion through intact tissue, reaching Langerhans cells (LC) and intraepithelial CD4 T lymphocytes (IEL) in the epithelium or dendritic cells (DC) and CD4 T cells in the lamina propria. Infection with one founder virus leads to local inflammation and T cell activation, fueling viral replication and causing chemokine-mediated recruitment of additional T cells. Dissemination of infected T cells, DC and LC from the initial cervicovaginal infection foci to draining lymph nodes leads to establishment of systemic infection. *From Hladik and Doncel, Antiviral Research, 2010.*

The point at which HIV spreads from the cervix to the draining lymph nodes is referred to as viral dissemination: the lymph nodes not only provide a rich, concentrated source of CD4 T cells for HIV to replicate in, but they also provides access to the lymphatic and circulatory system allowing for rapid viral spread throughout the body. Establishment of systemic infection poses two major issues: (1) it causes massive immune destruction, which is not fully functionally regained, even after halting CD4 T cell destruction with ART[140], and (2) it allows for widespread latent cellular infection to occur (Section 1.3.1.2), which will reactivate to produce virus upon ART interruption[141] (discussed further in Section 1.4). It would be ideal to halt HIV infection before viral dissemination occurs, and the rarity of successful infectious events suggests that this window period- when HIV infection exists only as a small, slowly growing cluster of infected cells- is a vulnerable period for the virus as it fights to establish infection[142]. It is possible that a balance between the availability of susceptible target cells, and host anti-viral immune defenses, determines whether or not an infection even is successful.

This knowledge of mucosal HIV transmission has been acquired through studies of cervicovaginal tissue in primates; only recently has adult male circumcision both identified the foreskin as a major site of HIV transmission and also provided a source of accessible penile tissue for research. As a result, Section 1.3.3 will draw heavily on studies using cervicovaginal tissue, with reference to the foreskin when data is available.

1.3.3.1 Crossing the Epithelial Barrier

Both the foreskin and the cervicovaginal mucosa are lined with epithelial cells that are not susceptible to infection[143, 144]. Below the epithelial layer of the foreskin lies the dermis, which contains the microvasculature along with different populations of dendritic cells and a much higher density of CD4 T cells[145-147]. To gain access to susceptible target cells in the underlying dermis, HIV must first cross this epithelial barrier. In women of reproductive age, the ectocervical and vaginal epithelium is comprised of 25-30 of flattened squamous epithelial cells, interspersed with immune cells such as dendritic cells and CD8 T cells, forming an epithelium approximately 200μm in depth[148-151]. The outer surface of foreskin tissue is lined with a layer of squamous epithelium that is similar to the vaginal epithelium (~100-200μm, and

interspersed with immune cells, see Figure 1.2[152])[145, 146, 152].

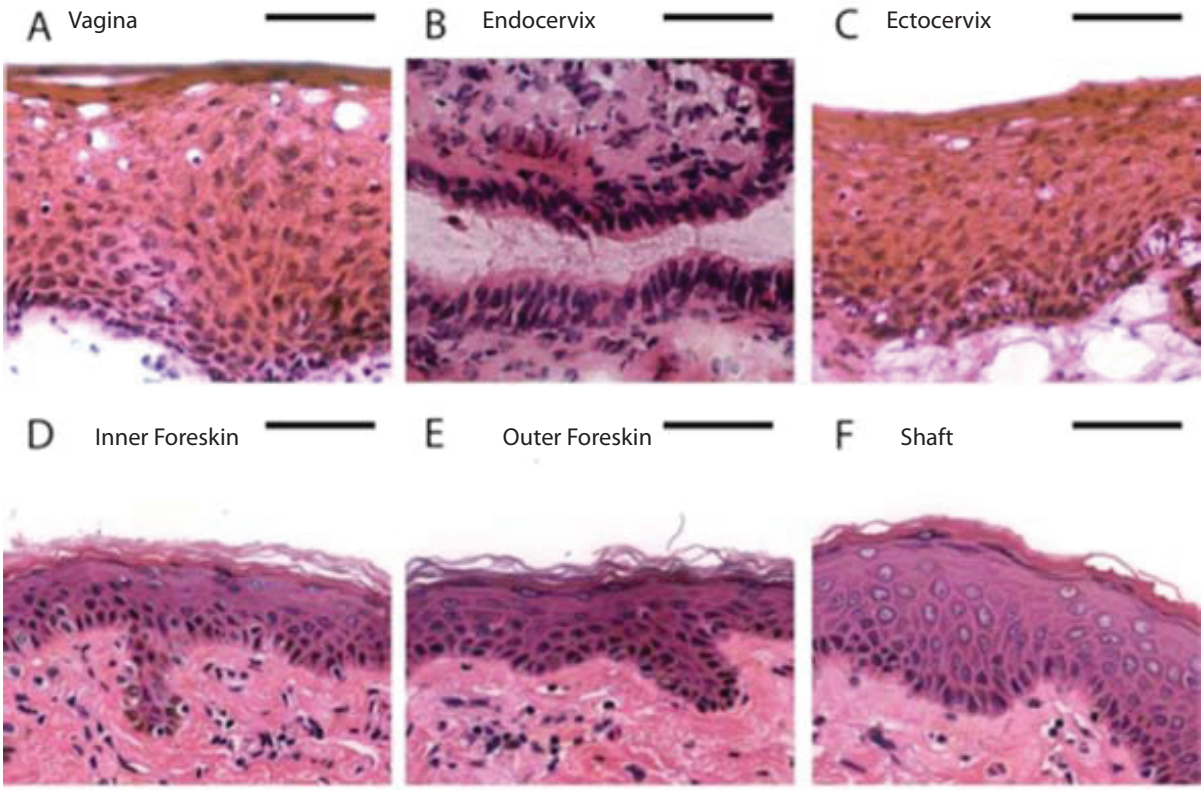

Figure 1.2 Various epithelia of the male and female genital tracts. The female genital tract (A-C) is comprised of the vagina (A, stratified squamous epithelium) and the cervix. The portion of the cervix that lies within the vagina is referred to as the ectocervix (C, stratified squamous epithelium), and the portion that lies beyond the cervical os is referred to as the endocervix (B, columnar epithelium). The skin of the foreskin (D-E) (and shaft, F) is covered by a stratified squamous epithelium, histologically similar to that of the vagina and endocervix. *From Dinh et al, Biol Rep 2012.*

The base of the epithelium is comprised of continually dividing basal cells (*stratum basale*); as these epithelial cells mature they are gradually pushed outwards and flatten, comprising the *stratum spinosum*. Epithelial cells in the *stratum spinosum* express cell surface proteins that interact with similar proteins on adjacent cells to form intracellular junctions. These intracellular junctions are important both in intercellular communication and also in preventing toxins or pathogens from entering the body[152]. In skin (including the foreskin), the *stratum spinosum* contains specialized epithelial cells called keratinocytes that produce large amounts of the fibrilar protein cytokeratin. As theses cells mature and are pushed outwards, they form the *stratum granulosum*, and secret lamellar bodies containing lipids and proteins that help to create the water-impermeable barrier function of the skin. Fully differentiated keratinocytes form a final

layer of cornified cells filled with keratin filaments and lacking nuclei and cytoplasmic organelles[153], referred to as the *stratum corneum* (~20μm)[154] (Figure 1.3, from [152]),, which is absent in non-cornified epithelia such as the ectocervix. Together with secretions from the *stratum granulosum*, they form an outer layer is highly water-insoluble, and thus has been hypothesized to provide an additional mechanical barrier to HIV compared to the ectocervix.

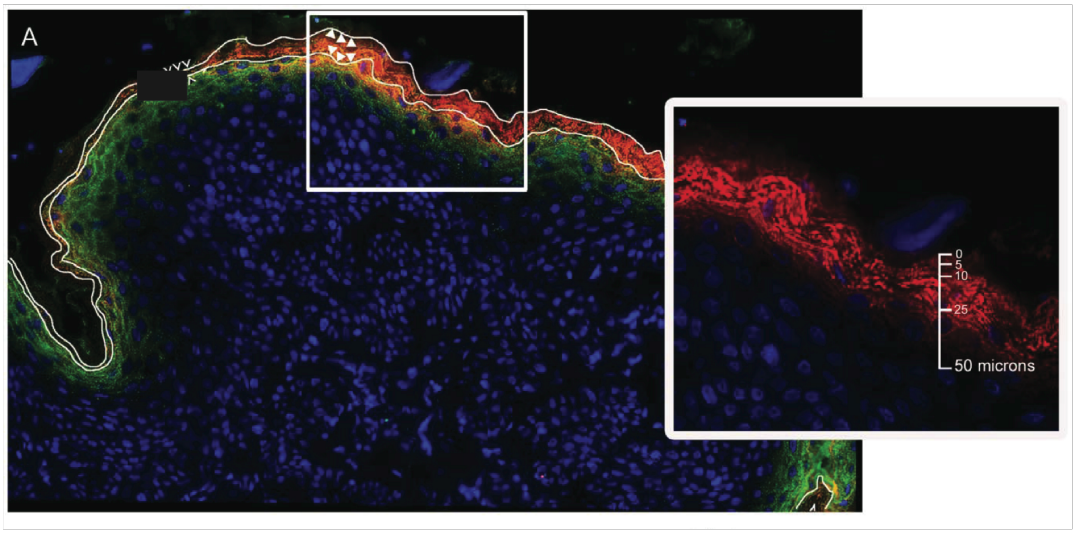

Figure 1.3 Immunofluorescent staining of keratin layer on foreskin epithelium. Involucrin (green) and filaggrin (red) used to stain maturing and terminally differentiated keratinocytes, respectively. Densely packed cell nuclei (blue) below keratin layer are epithelial cells. While this layer is highly variable in thickness (white 'VVV' symbols show areas of thinner keratin and white solid triangles show areas of thicker keratin), there is no difference in the median thickness of keratin from different anatomical regions of the foreskin (i.e. inner vs. outer aspects of the foreskin). Inset: magnification of boxed area with only filaggrin staining (red) shown. *From Dinh M, Prodger JL et al., PLoS One, 2012.*

In vitro studies in macaques or with explanted cervical tissue suggest that HIV crosses the epithelial barrier either directly by diffusion between epithelial cells[155] or through physical breaks in the epithelium[138], including microtears incurred during intercourse and ulcers due to STIs. Studies with explanted foreskin tissue and *in vitro* re-constructed multilayer epithelia from primary foreskin cells[156], show infected peripheral blood mononuclear cells (PBMCs) forming contacts with apical foreskin epithelial cells and subsequent directed budding of HIV virions. Within 1 hour, infectious viral particles can then found on the basal side of polarized explants, demonstrating newly formed virions are able to rapidly traverse the endothelial layer. Similarly, HIV virions can be observed penetrating intact ectocervical epithelium by diffusing between cells where cell-cell junctions are absent. Interestingly, studies viral penetration suggest that

keratin thickness may be a significant determinant in the ability of HIV to penetrate the epithelium, and our group has observed large inter- and intra-individual variation in keratin thickness on different areas of the foreskin[154]. The connection between keratin thickness and HIV susceptibility will be discussed further in Section 1.4.1.2.

1.3.3.2 Availability of Target Cells

Regardless of the mechanism, studies of SIV infected macaques suggest that HIV penetrates the epithelium rapidly, within 30-60 minutes[136]. Once HIV penetrates the epithelium, it has access to the numerous susceptible target cells that lie within and below the epidermal layer, such as dendritic cells, T cells and macrophages.

1.3.3.2.1 Mucosal Dendritic Cells

Studies of SIV infection in the female macaque show that initial founder populations of infected cells are comprised almost exclusively of CD3/4+ T cells[137, 139], with only limited reports of small numbers of infected dendritic cells (DCs)[157, 158]. Nonetheless there is substantial evidence that DCs play an important role in the establishment of the founder population of infected T cells.

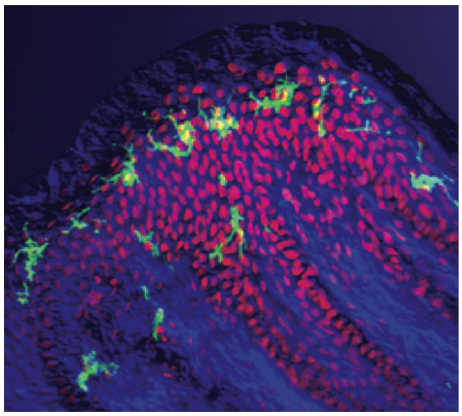

Figure 1.4 Langerhans cells (CD1a, green) within the foreskin epithelium. Densely packed cell nuclei, stained red (propidium iodide), show the epidermal layer. *From McCoombe et al, AIDS 2006.*

The physiological function of DCs is to take up foreign pathogens and proteins for presentation to naive T cells. As such, DCs have long cellular processes extending out through their cellular environment (Figure 1.3, [147]), and can be found in close proximity to the apical surface of mucosal tissues; the cell body of DCs can be found 61µm from the apical surface of foreskin, with processes reaching to within 24µm, by contrast, CD4 T cells are found at a median depth of 238µm[145, 147, 159]. Due to the proximity of DCs to the epithelial surface, and their natural proficiency at capturing foreign antigens, it is likely that DCs are the first cells to encounter HIV in the mucosa. Several of the innate antigen

receptors on DCs directly bind gp120, including the C-type lectin-receptors Langerin (CD207), DC-SIGN (CD209), and mannose receptor (MR, CD206) (see Section 1.3.2.1.3). Large, superficial DCs efficiently capture invading HIV virions, which, under normal circumstances, would be internalized by DCs, degraded in the lysosomal compartment, and the activated DCs would migrate out of the tissue in search of T cells for antigen presentation. However, as discussed in Section 1.3.2.2.2, some of the HIV viral particles captured by DCs evade degradation; it has therefore been suggested that DCs may be able to act as a "Trojan Horse", concentrating viral particles on susceptible T cell populations in the sub-epithelial space, which are then infected in *trans*[97]. Studies of explanted foreskin exposed to HIV in a polarized fashion show epidermal DCs first migrate towards the apical edge of the foreskin (1hr after HIV exposure)[156], and then to migrate back towards the dermis (4hrs), where they formed conjugates with foreskin resident CD4 T cells[160]. Viral particles could be visualized both within the DCs and also in the contact area formed between DC and CD4 T cell, suggesting that CD4 T cell infection *in trans* was occurring.

Distinct populations of dendritic cells are found dispersed throughout the stratified epithelium of the foreskin and vagina and in the sub-epithelial space (dermis or lamina propria, respectively). The epithelium of both the vagina[161] and the foreskin[159] contains a subset of dendritic cells referred to as Langerhans cells[162]. These cells can be distinguished from other DC cells by their unique expression of the C-type lectin langerin (CD207) and the MHC-like molecule CD1a, involved in the presentation of microbial lipids to T cells[163]. Langerhans cells are predominantly found in the epithelium of both the foreskin[145, 147, 164, 165] and the vagina. Dendritic cells of the dermis/lamina propria have a more varied phenotype[163], and can express DC-SIGN and/or Mannose Receptor[161, 166-168]. Several studies have shown that DC subtypes (both Langerhans and subepithelial DCs) that have been matured through exposure to bacterial antigens are less susceptible to direct HIV infection, but are more proficient at *trans* infection of CD4 T cells[132, 133, 169], and therefore altered genital flora or bacterial infections may increase *trans* infection of CD4 T cells.

1.3.3.2.2 T Cells

While dendritic cells may be important in initial cellular interactions with HIV, CD4 T cells are the main target of HIV in the mucosa[137]. However, CD4 T cells are heterogeneous, and the various CD4 T cells subsets differ in their permissiveness to HIV infection. The slow growth of the initially infected cluster of CD4 T cells suggests that the local abundance of CD4 T cell subsets that are particularly susceptible to HIV may be a significant determinant in establishment of the founder population.

Tissue damage or infection leads to leads to the local production of cytokines (such as tumor necrosis factor α (TNFα), interleukin-1α (IL-1α) and IL-1β) and chemokines that promote immune cell chemotaxis (such as IL-8 and RANTES) by epithelial cells and tissue resident dendritic cells and macrophages. This local inflammatory response both recruits tissue-resident immune cell populations and also increases expression of tissue-specific adhesion molecules on local endothelial cells (such as ICAM-1 and VCAM-1 (vascular cell adhesion molecule)), which recruits additional immune cell populations from the circulation, such as neutrophils, natural killer (NK) cells, memory T cells and monocytes/macrophages[170, 171]. When these infiltrating immune cell populations encounter pathogen they become activated, and produce large quantities of cytokines and chemokines as they perform their effector functions, further contributing to local immune activation and inflammation[172]. After the pathogen is cleared, most effector cells die off through apoptosis, including effector T cell populations. However, a subset of long-lived CD4 T cells revert to a resting state and remain in the tissue. These cells can rapidly be re-activated to proliferate and perform effector functions if they re-encounter antigen, and are hence termed "effector memory" T cells (T_{EM}) to distinguish them from the "central memory" T cells (T_{CM}) that remain in the peripheral circulation[172, 173].

1.3.3.2.2.1 Activated Th Cells

The number of resting T_{EM} in normal human skin is large- estimated at $1x10^6$ cells/cm^2 [174]– and in the absence of infection they vastly outnumber activated effector CD4 T cells

(those cells actively producing cytokines, proliferating, or expressing activation markers, such as CD69)[175]. While HIV can infect and replicate in both T_{EM} and activated effector CD4 T cells[176], its entry and replication are both more efficient in activated cells. Studies of vaginal SIV infection of macaques show that while activated cells make up only 1.4% of the cervical CD4 T cell population, they account for 10% of initially infected cells, and this proportion increases as the founder population expands[139, 177]. These initial studies of SIV infection in the macaque have been confirmed in other animal models[178] and human cervical explant tissue[179] and PBMC-based infectivity assays[180, 181], where the increased susceptibility to infection of activated CD4 T cells has been correlated with increased CCR5 expression[180]. In addition to being preferentially infected, CD4 T cells must be activated for viral replication to occur[182], and *in vivo* viral production by activated cells is several-fold higher than resting CD4 T cells[139, 177]. In keeping with this, blockage of immunomodulatory signaling in CD4 T cells (CTLA-4)[183] or immune activation with pro-inflammatory cytokines (IL-8)[184] leads to increased viral production. Increased viral replication in activated T cells is likely linked to the transcription factor nuclear factor-κB (NF-κB)[185], a central regulator of cellular immune activation. During immune activation, NF-κB translocates to the nucleus and binds to κB regulatory elements, up-regulating the expression of numerous pro-inflammatory genes[186]. HIV transcriptional regulatory elements also contain multiple NF-κB binding regions, so that HIV gene transcription is also driven during cellular activation[187]. Therefore, increased numbers of activated CD4 T cells in the foreskin or cervix may increase an individual's susceptibly to HIV, not only by providing increased numbers of a cell type that is preferentially infected by HIV, but also then by supporting high levels of viral reproduction, fueling the growth of the founder population.

In addition to overall differences in susceptibility between activated and resting cells, there are differences in HIV susceptibility between subsets of CD4 T cells. During naïve CD4 T cell antigen priming, signals from the priming dendritic cell, as well as local signals in the lymphoid tissue, lead to activation of lineage-specific transcription factors that will determine the eventual effector functions of the clonally expanded T cells, and therefore their role in pathogen defense[188]. Several subsets of Th cells have been identified based on their functional capacity and lineage-specific transcription factors: Th1, Th2, Th17, Th9,

T_{FH}, Th22 and regulatory T cells (Tregs). While there are counter-regulatory mechanisms involving the lineage-specific transcription factors that ensure that naïve T cells polarize towards one effector phenotype or another, there is also some plasticity and functional overlap between subtypes[188]. Th1 cells primarily provide co-stimulation to macrophages; Th2 cells promote IgE production from B cells and stimulate eosinophils; Th17 cells recruit neutrophils; Th22 cells are involved in the maintenance of epithelial integrity; T_{FH} (follicular helper) provide help to B cells; Th9 cells are involved in mucous production; and Tregs dampen the immune activation of other CD4 T cells (Figure 1.4). Of these subsets, Th17 cells, Th1 cells, and Tregs have been implicated in determining HIV susceptibility.

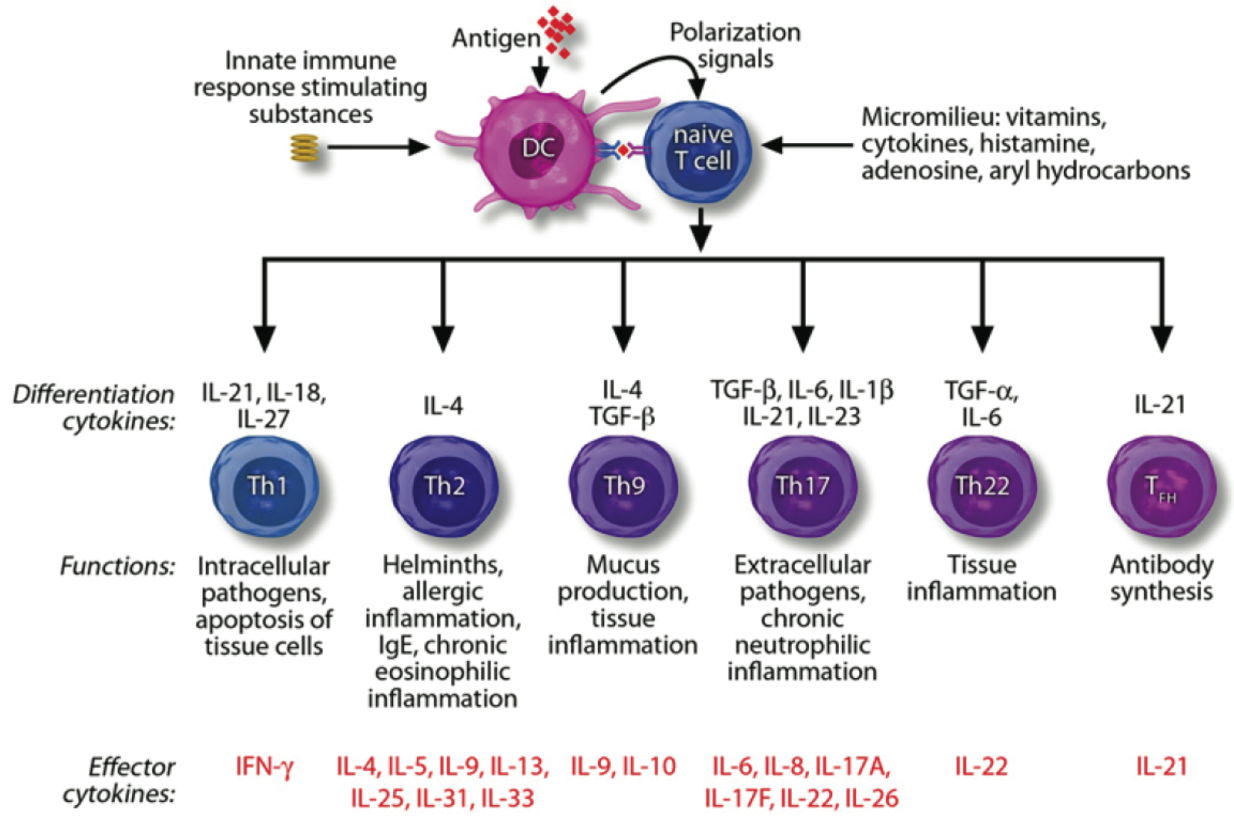

Figure 1.5 **Various Th subsets**. Polarization signals from the antigen presenting cell at the time of naive T cell stimulation, as well as signals from the local lymph tissue, promote the differentiation of effector (and subsequent memory) T cells with distinct effector functions. Subsets that have direct implications for HIV susceptibility are Th1 cells, Th17 cells, and Tregs. *From Akdis et al, Allergy Clin Immunol, 2012.*

1.3.3.2.2.2 Th17 Cells

Naïve CD4 T cell antigenic stimulation in the presence of IL-6, IL-1β, a low concentration of TGF-β, and the absence of IL-12, induces expression of the transcriptional factor Retinoic acid Orphan Receptor (ROR)-C2 (the human equivalent of mouse RORγt) and subsequent polarization towards a Th17 effector phenotype (Figure 1.4) [189-191]. IL-21 produced by initially differentiated Th17 cells acts as a positive feedback loop to drive further Th17 polarization[192, 193], and IL-23 is necessary for their expansion and terminal differentiation[194]. Th17 cells are characterized by the production of the cytokines IL-17A and IL-17F, but also produce IL-22, TNFα, IFNγ (interferon-γ), and IL-10 as important effector molecules[191]. All Th17 cells express CCR6[195, 196], but this is not an exclusive marker of this cell type, as Th22 cells[197] and other lymphocyte populations[198, 199] also express this receptor. IL17 (A and F) act on a wide variety of cell types, including epithelial, endothelial, and stromal cells as well as other leukocytes[200]. Through these cells, IL-17 promotes the local elaboration of the pro-inflammatory cytokines IL-1β[201] and IL-6[202], and the chemokines IL-8 (CXCL8)[201] and CXCL1[201, 203, 204], which are highly chemotactic for neutrophils[205].

Another important effector molecule of Th17 cells is IL-22. IL-22 is also expressed by Th22 cells[206] (which some believe to be a functional subset of Th17 cells[189]) and NK cells[207]. Unlike most other cytokines, IL-22 does not have direct effects on immune cells, but instead primarily signals to epithelial cells, including keratinocytes of the skin[191, 208]. In combination with IL-17, IL-22 promotes the production of antimicrobial peptides by keratinocytes[209, 210] and is protective against *Candida albicans*[211]. In addition to promoting antimicrobial proteins, IL-22 contributes to the maintenance of epithelial integrity by promoting keratinocyte proliferation[209] and is important in wound healing[212]. A major role of IL-22 may be to limit tissue damage during an inflammatory response, as can be seen in its protective role if administered during acute inflammation[213, 214]. However, the promotion of keratinocyte proliferation and migration that is important in wound healing also appears to contribute to the formation of plaques in psoriasis[208].

In keeping with this, Th17 cells are essential in the defense against extracellular pathogens; individuals with defects in the Th17 pathway suffer from severe infection by fungi and extracellular bacteria, such as *Candida albicans* and *Staphylococcus aureus*,[215, 216] underscoring the importance of Th17 cells in the immune defense of both the skin and genital tract. However, due to the highly inflammatory nature of Th17 cells, their dysregulation is also associated with autoimmune and inflammatory disorders such rheumatoid arthritis[217], multiple sclerosis[240], ulcerative colitis[241], and psoriasis[242-245].

1.3.3.2.2.3 Th1 Cells

Naïve T cells stimulated with antigen in the presence of IL-12 and IFNγ[218] upregulate the transcription factor T-bet (T-box expressed in T cells) and subsequently polarize towards a Th1 phenotype (Figure 1.4)[219]. Th1 cells are characterized by the production of IFNγ and expression of the chemokine receptor CXCR3[218]. The effects of IFNγ are extensive (reviewed in [220]), but can be grouped broadly into its effects on (1) macrophages, (2) endothelial cells, (3) non-immune cells, (4) B cells. Th1 cells upregulate macrophage environmental sampling, promote their microbial killing capabilities, and also lower their response threshold to TLR signaling. IFNγ also promotes chemokine production (IP-10, MCP-1, MIP-1α/β, RANTES, etc.) and adhesion molecule expression (ICAM-1, VCAM-1, etc.) by endothelial cells, contributing to additional leukocyte recruitment to the site of infection. IFNγ promotes MHC I upregulation and antigen presentation by non-immune cells, and upregulates intracellular antiviral enzymes, thereby helping to both uncover and clear intracellular infections. MHC I presentation of viral peptides stimulates cytotoxic T lymphocyte (CTLs, CD8 T cells) mediated killing, and IFNγ is also a main effector cytokine of CTLs, promoting antiviral and apoptotic pathways in infected target cells. Finally, IFNγ is involved in B cell class switching to IgG2a, and thereby promotes phagocytosis [218].

Another important effector molecule of Th1 cells is TNFα[221], although this more ubiquitous pro-inflammatory cytokine is also produced in large quantities by innate immune cells (epithelial cells, keratinocytes, DCs, NK cells and macrophages), as well as

CTLs and Th17 cells. TNFα has many overlapping functions with IFNγ, and in fact many IFNγ-inducible genes are also TNFα-inducible, and often the combined effects of IFNγ and TNFα are synergistic[220]. TNFα is important in rapid immune responses to invading pathogens, exemplified by its production by many innate or non-immune cells[62]. It up-regulates adhesion molecules on endothelial cells[222, 223] and promotes vasodilatation and vascular permeability[224], slowing blood flow and promoting leukocyte recruitment[225], adhesion, and extravasion into tissues. TNFα also upregulates compliment and opsonization of bacteria by macrophages, and subsequent microbial killing[226]. Finally, TNFα is important in the adaptive immune response, promoting dendritic cell maturation and local CD4 and CD8 T cell expansion upon antigen recognition[227]. The strongly pro-inflammatory role of TNFα is demonstrated by the success of pharmacological TNFα inhibition in treating a variety of inflammatory and autoimmune disorders, such as rheumatoid arthritis, inflammatory bowl disease, and psoriasis[228-230].

In combination, IFNγ and TNFα production makes Th1 cells particularly effective in the clearance of intracellular infections, which is demonstrated by their important role in the control of *Mycobacterium tuberculosis*[231, 232] and Leishmania major[233]. However, similar to Th17 cells, the highly pro-inflammatory nature of Th1 cells also means that their dysregulation leads to pathological inflammatory conditions, such as psoriasis[228].

Both Th1 and Th17 cells have been shown *in vitro* to be highly susceptible to entry by HIV[234], to support increased levels of replication[221, 234-236], and to be selectively depleted during early HIV infection[234, 237, 238]. Studies targeting Th17 cells have often used CCR6 expression to identify Th17 cells; while this receptor may not be exclusive to Th17 cells, it does select for a population enriched for Th17 cells, and these studies have found CCR6+ CD4 T cells to be a preferential target of HIV[221, 239]. Although *in vitro* studies consistently demonstrate that Th1 and Th17 cell are both highly susceptible to HIV, it is they are equivocal as to which of the two cell types is the preferred target of HIV: some studies showing increased infection of Th17 over Th1 cells[221, 236], and others showing increased infection of Th1 over Th17 cells[234, 235]. These discrepancies may be due to

the existence of cells with a mixed Th1/Th17 phenotype, which has been suggested to be a form of pathogenic Th17 cell[191], but may also be representative of plasticity among Th subsets[196]. These Th17/Th1 cells express both ROR-C2 and T-bet, have CXCR3 and CCR6 on their surface, and produce both IL-17 and IFNγ in response to stimulation[196, 221]. Those studies that have examined cells co-expressing IL-17 (or CCR6) and IFNγ have all found that this cellular phenotype is highly permissive to HIV infection[221, 234, 235, 238]. It is likely that an overrepresentation of Th1, Th17, or the mixed phenotype cell population at the site of HIV exposure would lead to an increased susceptibility to infection and more rapid founder population growth and dissemination.

The biological reasons for increased HIV susceptibility in these subtypes is not clear. Studies have shown that Th17 cells express higher levels of CCR5 and $\alpha_4\beta_7$,[75, 221, 239, 240] but there is evidence that the heightened HIV susceptibility of CCR6+ CD4 T cells is independent of $\alpha_4\beta_7$ expression[239]. The definition of Th1 and Th17 cells based on cytokine production also selects for activated cells, which, as discussed above, are highly susceptible to HIV. However, studies using chemokine receptor expression, as opposed to cytokine production, have also shown increased HIV susceptibility, and these studies would include resting T_{EM} cells. Understanding the biological mechanisms that render these cells vulnerable to HIV may provide new molecular targets for HIV prevention modalities.

1.3.3.2.2.4 Tregs

Regulatory T cells (Tregs) are a heterogeneous group of CD4 T cells involved in immune suppression. Tregs are essential in preventing both autoimmunity to self-antigens and in regulating the normal immune response to pathogens. Tregs can develop directly in the thymus after encountering high concentrations of self-antigen (often referred to as "natural Tregs"), or can develop in the peripheral lymphoid tissue from naïve CD4 T cells, similarly to effector T cells ("inducible" Tregs)[241]. Inducible Tregs develop when naïve CD4 T cells encounter antigen in the presence of IL-2[242] and high levels of TGF-β (transforming growth factor-β)[243, 244], and the absence of IL-6, IL-12 and IL-4[245]. Both types of Tregs are characterized by the expression of the transcription factor FoxP3 (forkhead box

P3)[246] and CD25[247], and both are involved in immunomodulation. Similar to effector T cells, Tregs express an array of cytokine receptors and homing addressins depending on the environmental cues they receive during development, including CCR4, CCR5, CCR6, CXCR3 and $\alpha_4\beta_7$, and thus can home to peripheral tissues along with effector T cells during an infection[245]. In the tissue, Tregs produce the immunomodulatory cytokines IL-10[248, 249], IL-35[250] and TGF-β that suppress T cell proliferation and production of effector cytokines[241]. Tregs may also produce Granzyme A/B and kill effector T cells by a contact dependent mechanism[245]. Although Tregs are antigen specific, once activated by their antigen they will suppress effector Tregs irrespective of whether these T cells share their antigen-specificity[251]. This immunomodulatory control ensures that effector T cell functions are only initiated when necessary, are resolved once pathogen is cleared, and to prevent excessive tissue damage during the inflammatory process[245]. In keeping with this, all of the aforementioned inflammatory disorders resulting from excessive Th1 and Th17 responses are also associated with Treg defects[252].

Due to their role in limiting immune activation in the skin and mucosa, and their ability to counter highly activated Th17 and Th1 cells, it is possible that increased numbers of Tregs at the site of HIV exposure might reduce the likelihood of infection, or slow the growth of a founder population. A recent study by Card *et al* [181, 253]found that peripheral blood from donors with high levels of Tregs had low levels of activated CD4 T cells and were relatively resistant to HIV infection *in vitro*. This observation is especially interesting in light of the finding that HIV can productively infect Tregs, to the same extent as activated CD4 T cells, a finding that has been supported by other studies[253-255], and the observation that Tregs not only express the necessary HIV-receptors CD4 and CCR5, but can also express $\alpha_4\beta_7$ and CCR6[245]. This implies that the reduced infectivity observed in samples with high frequencies of Tregs was not due to a lack of available target cells, but suggests that it might be due to the immunomodulatory effects of the Tregs themselves. A previous study by Moreno-Fernandez *et al* [256] found that co-culture of Tregs with effector T cells resulted in decreased HIV infection of effector cells in a mechanism dependent on cyclic adenosine 3', 5'-monophosphate (cAMP), a molecule involved in regulating T cell activation, and which has been shown to limit HIV replication and cell

entry[257]. In combination, these studies suggest that the *ratio* of Tregs to activated effector cell types may be important in determining HIV susceptibility.

1.3.3.3 Anti-Viral Defenses

Section 1.3.3.2 described how activated CD4 T cell subsets, normally involved in anti-viral defenses, are highly vulnerable to HIV entry and replication, and therefore their presence may contribute to increased HIV susceptibility. The following section will describe anti-viral immune mechanisms in the skin that may be protective against HIV infection. These include secreted peptides with anti-viral activity, innate cytotoxic cells, and HIV-specific CD8 T cells.

1.3.3.3.1 Secreted Innate Anti-Viral Peptides

The surface of the skin and mucosa contain many innate antimicrobial peptides that have direct anti-bacterial, anti-fungal and, importantly, anti-viral activity [258, 259]. There are several classes of antimicrobial peptides, of which the cationic peptides and antiproteases have been directly implicated as having anti-HIV activity.

Cationic peptides include α-defensins, β-defensins and the cathelicidin LL-37; α-defensins are primarily produced by neutrophils, while β–defensins are produced by epithelial cells and macrophages, and LL-37 is produced by both[259]. They carry a positive charge, allowing them to bind to anionic microbial membranes, and are amphipathic, allowing them either kill microbes by disrupting their cell membrane, or to pass through their membrane and target intracellular pathways involved in their replication[260, 261]. In addition to their direct antimicrobial activity, cationic peptides also serve as immune signaling molecules, and elicit numerous immunological responses including: epithelial cytokine production, migration and proliferation; macrophage recruitment and cytokine production; and DC maturation (reviewed in [262]). Purified α-defensins HNP1-4 (human neutrophil peptides 1 through 4)[263-265], β-defensins hBD1-3 (human β-defensins 1 through 3)[266-268], and LL-37[269-271], have all been shown to inhibit HIV replication *in vitro*. While the exact mechanisms of inhibition are not yet elucidated, studies have suggested roles in both preventing viral fusion/entry and in preventing

post-entry replication[263, 267, 270-273]. In additional to the ability of the purified peptides to inhibit HIV replication, their naturally occurring presence in vaginal secretions correlates with anti-HIV activity in *ex vivo* models[274-276]. However, the role of cationic peptides in HIV protection *in vivo* is less clear: increased production of hBD2 has been linked to increased viral load in HIV-infected individuals[277], and increased levels of LL-37 and α-defensins in the FGT were prospectively associated with genital infections and a higher risk of HIV seroconversion[278]. It is possible that this is due to their chemotactic and immune activating effects; for example, hBD2 is able to bind CCR6 and is chemotactic to highly HIV-susceptible CCR6+ cells[279, 280], and the other cationic peptides have also been shown to promote an adaptive immune response[262]. It may be that the indirect increase in HIV susceptibility due to the local recruitment and activation of CD4 T cell outweighs the direct antiviral effects of these molecules.

Anti-proteases are another class of secreted antimicrobial peptides that have been shown to have anti-HIV activity *in vitro*[281-286]. Secretory leukocyte protease inhibitor (SLPI) and elafin (including elafin's precursor, trappin-2) are anti-proteases that help to protect against neutrophil-mediated tissue damage during an immune response[287]. They also have anti-inflammatory properties, decreasing NFκB activation in T cells and IL-8, IL-6, and TNFα production by epithelial cells[281, 288, 289]. Their anti-HIV activity has been attributed both to their downregulation of NFκB[281], and to direct inhibition of viral fusion[290, 291] and replication[330]. It has been suggested that the anti-inflammatory characteristics of antiproteases will allow them to inhibit HIV replication in vivo without the concomitant recruitment and activation of CD4 T cells; in keeping with this, increased levels of trappin-2/elafin were prospectively associated with protection from HIV in a cohort of female sex workers[337]. However, there is recent evidence that SLPI and trappin-2/elafin may also have pro-inflammatory effects[292]. The ultimate *in vivo* ability of anti-proteases to protect against HIV remains to be determined.

1.3.3.3.2 Secreted HIV-Neutralizing Antibodies

Another component of secretions that is important in immune defenses is sIgA. sIgA is the secreted form of immunoglobulin A, and acts as a first line of defense by preventing pathogen adherence to the epithelium. Plasma cells just below the epithelium produce dimeric IgA, with two IgA molecules joined by a joining (J) chain. The dimeric IgA binds to polyclonal immunoglobulin receptors (pIgR) on the basolateral side of surrounding epithelial cells, and is transported through the cell in vesicles to the apical side, where it is released into the lumen. When the dimeric IgA is released the pIgR is cleaved to leave a portion bound to the dimeric IgA, which is referred to as the secretory component (SC). The released dimeric IgA, with SC, is referred to as sIgA (reviewed in [293]). The role of sIgA has primarily been studied in the columnar epithelial layers of the gut and the respiratory airways, but sIgA can also be detected in the vagina and on the skin. Once released into the lumen, sIgA prevents potentially harmful pathogens from transversing the epithelium (reviewed in [294]), which would theoretically be beneficial in preventing the establishment of a founder population, as it would prevent HIV from ever reaching susceptible cell populations. Indeed, mucosal IgA collected from the saliva or cervicovaginal secretions of some HIV-exposed, but seronegative (HESN) individuals has the capacity to both directly prevent HIV infection of CD4 T cells in suspension[295-301], and to inhibit the transcytosis of HIV across an epithelial layer[295, 301-303].

While HIV-neutralizing IgA in genital secretions of HESN women can be consistently detected, the exact target of this HIV-neutralizing IgA remains unknown. It is unclear if this IgA is specific for HIV and the result of adaptive immunity from HIV exposure, or if IgA is neutralizing HIV through a non-specific, "innate" mechanism. Confusing this issue in the literature is the distinction between HIV-*neutralizing* IgA and HIV-*specific* IgA. To demonstrate that IgA has the capacity to neutralize HIV, investigators have purified IgA from mucosal secretions and incubated it with primary HIV isolates, and then tested the subsequent ability of this HIV to infect PBMCs from a healthy donor. In this system, the target of the IgA remains unknown. In contrast, HIV-specific IgA is detected through the ability of IgA to bind HIV peptides. It is possible that HIV-neutralizing IgA may not be specific for HIV, and that HIV-specific IgA may not neutralize HIV. Note also that the protective effects of IgA *in vivo* may be multifactoral, as the ability of IgA to prevent HIV transcytosis across an epithelial layer may, theoretically, be distinct from its ability to prevent infection of PBMCs.

Many groups have either failed to detect HIV-specific IgA[304-307], or found that anti-HIV activity was not linked to HIV-specificity[308, 309]. A recent study examining the presence of both HIV-specific and HIV-neutralizing IgA by Horton *et al* found that the presence of HIV-specific IgA was very rare and did not correlate with HIV-neutralization[308]. However, several other groups *have* observed HIV-specific IgA in HESN populations[295, 301, 303, 310-314], and work by the laboratory of Mario Clerici has identified at least one epitope linked to HIV-neutralization and shown that this epitope is the result of somatic hypermutation[301]. These data suggest that while it may be possible to acquire anti-HIV IgA antibodies as a result of exposure to HIV, in many cases the actual protective function of IgA may not be HIV-specific. The target of HIV-neutralizing IgA may be a host receptor, allowing IgA to prevent HIV infection without binding HIV-itself. In keeping with this, there are several reports of anti-CCR5[315-317] and anti-CD4[318-320] antibodies in HESN populations. Additionally, both the IgA heavy chains and the SC are heavily glycoslyated by oligosaccharides that closely resemble the glycosylation on epithelial cells, allowing IgA to act as a non-specific decoy for pathogens attempting to bind the epithelium[294]. It may be that the anti-HIV activity of sIgA is not derived from the IgA molecules themselves, but instead from HIV interactions with the SC molecule.

Irrespective of the mechanism, IgA is ideally situated to prevent HIV infection, and naturally produced IgA has been consistently demonstrated to have anti HIV-activity. The presence of HIV-neutralizing IgA on the foreskin, and further work elucidating its mechanism(s) of protection is warranted.

1.3.3.3.3 Innate Cytotoxic Lymphocytes

There are several innate, and innate-like, lymphocyte populations in the epithelium with anti-viral activity. These include Natural Killer (NK) cells, γδ T cells (bearing a T cell receptor made of the alternative γ and δ chains, as opposed to the classical α and β chains), and NKT cells (which express both an αβ TCR and NK markers)[62]. None of these cell types recognize a specific antigen presented in MHC I molecules; instead they recognize a breadth of non-specific cues, such as cellular receptors upregulated during metabolic stress, alterations in levels of MHC I molecules characteristic of intracellular infection, viral proteins on the cell surface, or, in the

case of NKT cells, they recognize lipid antigen presenting in CD1 molecules[321-325]. In all cases their activation and expansion occurs locally in the tissue, and even in the case of a new infection, they do not require rounds of clonal expansion in lymphoid tissue to mount a response[323]. All three cell types are also able to kill pathogen-infected cells via perforin/granzyme mediated killing or Fas-induced apoptosis[321, 324]. As a result, these cytotoxic cells are able to mount an effective anti-viral immune response immediately upon tissue infection, without the time-delay necessary for naïve CD8 T cell priming in the setting of a new infection. This could potentially be of particular importance in the setting of HIV infection: viral dissemination from the time of inoculation to the time of HIV appearance in draining lymph nodes takes only a few days, a much shorter time than is necessary to prime and mount a CD8 T cell response. Additionally, NK, γδ, and non-lymphoid NKT cells, do not express CD4[321, 323, 324], and therefore are not direct targets of HIV themselves. Therefore, increased numbers of these cells in the foreskin could potentially be protective against HIV infection. However, these cell types also produce large amounts of pro-inflammatory cytokines when stimulated, including IFNγ, TNFα, IL-6, IL-17, RANTES, MIP-1α, and MIP-1β[321, 322, 324]. These cytokines promote the recruitment and activation of CD4 T cells, and in particular CCR5+ CD4 T cells (RANTES, MIP-1α, MIP-1β). As a result, the anti-viral benefits of these cells may be countered by the recruitment of new HIV target cells, and their protective effect *in vivo* nullified.

1.3.3.3.4 HIV-Specific Cytotoxic CD8 T Cells

In addition to innate cytotoxic lymphocytes, antigen-specific CD8+ cytotoxic T lymphocytes (CTL) are essential in control and clearance of viral infections. Immature CD8 T cells recognize viral antigens presented in MHC I molecules on the surface of antigen presenting cells (APC), but are not activated unless additional help is provided by a CD4 T cell: while the CD8 T cell binds to viral antigen presented in an MHC I molecule, the CD4 T cell binds to antigen presented in an MHC II molecule on the same APC. This stimulates the APC to upregulate co-stimulatory molecules (CD40) and the CD4 T cell to produce IL-2. These additional co-stimulatory signals license the CD8 T cell for effector functions[62]. Subsequently, when the CTL encounters antigen presented in an MHC I molecule on an infected cell, the CTL will kill the infected cell, either through Fas/Fas-L mediated apoptosis or through the release of cytotoxic granules

containing perforin/granzyme. CTLs also produce numerous cytokines in response to antigenic stimulation, including IFNγ, TNFα, IL-2, and MIP-1β[62, 326]. IFNγ can directly interfere with viral replication, while the release of other cytokines and chemokines promotes the recruitment and activation of other leukocytes to assist in viral clearance. The antiviral activity of CTLs is vitally important in the control of viral replication in SIV-infected macaques[327, 328], and the breadth of the cytokines they are able to produce has been linked to slower disease progression and a lower viral set-point in HIV-infected humans[329, 330]. This demonstrated ability to control (although not to clear) SIV/HIV infection suggests that if HIV-specific CTLs were present in the tissue at the time of HIV exposure they might contribute to protection by killing off infected cells and thereby limiting founder population growth. In support of this possibility, vaccinated macaques that develop strong vaginal CTL responses are protected from subsequent intravaginal challenge with SIV[331], and if CD8 T cells are depleted from animals prior to SIV challenge this protection is lost[332]. However, the strength of the CTL response elicited in protected macaques is much weaker than that elicited by vaccine candidates that failed to protect humans from HIV[331, 333], and also the strength of vaccine-elicited CTL response in both humans[333, 334] and macaques[331, 332] does not correlate with protection from infection. It may be that the pro-inflammatory effects of CTLs at the site of infection counteract their antiviral activities: vaccines creating strong CTL responses in macaques also create HIV-specific CD4 T cells and recruit additional activated CD4 T cells to the vaginal mucosa[331, 332], which results in enhanced viral replication over unvaccinated macaques when HIV-specific CTLs are depleted[335]. However, if HIV-specific CTLs could be generated on an background of immunoquiescent CD4 T cells, they may be effective in preventing infection, as is the case with live attenuated vaccination of macaques with SHIV: continuous low level antigenic stimulation leads to decreased frequencies of inflammatory plasmacytoid dendritic cells and increased frequencies of Tregs in combination with SIV-specific CTLs, and this immunomodulation is associated with subsequent protection from infection with SIV[336].

1.3.4 Summary of Susceptibility to HIV Infection

Successful HIV infection is an extremely rare event, with only 1-700-1/3000 exposures leading to infection. Additionally, this rare event generally begins (>80%) with the infection of a single tissue-resident cell, and the number of infected cells initially increases very slowly. This suggests that substrate availability is a limiting step in HIV infection, and a point of vulnerability for the virus. Animal and *in vitro* studies have shown that the primary substrate of HIV replication is CD4 T cells, and that activated CD4 T cells, particularly Th1 and Th17 cells, are most permissive to infection and produce higher levels of virus. Therefore, a decrease in the density of activated CD4 T cells at the site of HIV infection might be associated with a decreased risk of HIV, and anything that acts to recruit, or activate, CD4 T cells will be associated with increased HIV susceptibility.

Based on this background, I hypothesize that a relatively HIV-resistant genital tissue would be one with a low density of activated CD4 T cells, including fewer Th1 and Th17 cells, and a higher density of immunomodulatory Tregs. It would also contain lower levels of proinflammatory cell populations, such as TNFα-producing CD8 T cells, and lower overall levels of pro-inflammatory cytokines/chemokines, such as IL-8, MIP-1α, MIP-1α, MIP-1α, RANTES, MCP-1, CCL20, IL-6, TNFα, and IFNγ. Reduced levels of proinflammatory cytokines/chemokines would not only lead to reduced numbers of activated CD4 T cells, but might also reduce numbers and maturation of dendritic cells and hence reduce *trans* infection. This HIV-resistant tissue would be high in antiviral proteins that do not have pro-inflammatory properties, such as anti-proteases and sIgA. The role of peptides or cells that have both anti-viral and pro-inflammatory properties, such as HIV-specific CD8 T cells, innate cytotoxic cells, and cationic peptides, is less clear in this model and remains to be determined.

Evidence to support this hypothesis can be derived from microbicide trials that failed to protect against HIV. Nonoxynol-9 (N9) and cellulose sulfate (CS) were two early microbicides that had potent anti-HIV activity *in vitro*. However, instead of preventing HIV, both compounds tended to increase HIV acquisition in women[337, 338]. Subsequent *in vitro* and animal studies have shown that these compounds cause a strong inflammatory response in the vagina, inducing NFκB expression; release of IL-6, IL-1, and IL-8; expression of adhesion molecules VCAM-1, and P-selectin; and concomitant lymphocyte recruitment[339-344]. Conversely, compounds that

decrease mucosal inflammation, such as glycerol monolaurate and minocycline are both associated with reduced HIV infectivity. Macaques vaginally exposed to glycerol monolaurate had reduced levels of MIP-1α and IL-8 and were protected from SIV infection[137]. Vaginal exposure to minocycline had similar outcomes, inhibiting NFκB expression, decreasing CD4 T cell activation, and reducing cellular HIV susceptibility and subsequent viral production[345]. As a proof of both concepts, the addition of minocycline to vaginally applied cellulose sulfate abrogates its pro-inflammatory effects and also reduces HIV infection[384].

1.4 Establishment of Systemic Infection and Progression to AIDS

If HIV is able to overcome the paucity of susceptible target cells and natural antiviral defenses of the mucosa, a founder population of productively infected cells is established in the genital tissue. After approximately a week of local expansion, HIV virions, or infected cells, make their way to local lymph nodes draining the infected tissue (7 dpi)[346]. Here in the lymph nodes HIV has access to a concentrated supply of CD4 T cells, but also access to the circulation and lymphatics, and soon after reaching the lymph nodes HIV can be found systemically (10 dpi)[346]. HIV-specific immune responses have not yet developed, and therefore not only can infection not be detected serologically, but viral replication occurs largely unchecked, and HIV plasma viral loads peak at an average of 10^6 RNA copies/mL approximately 3 weeks after infection[141, 346] (see Figure 1.5). This window of rapid viral replication is referred to as acute infection.

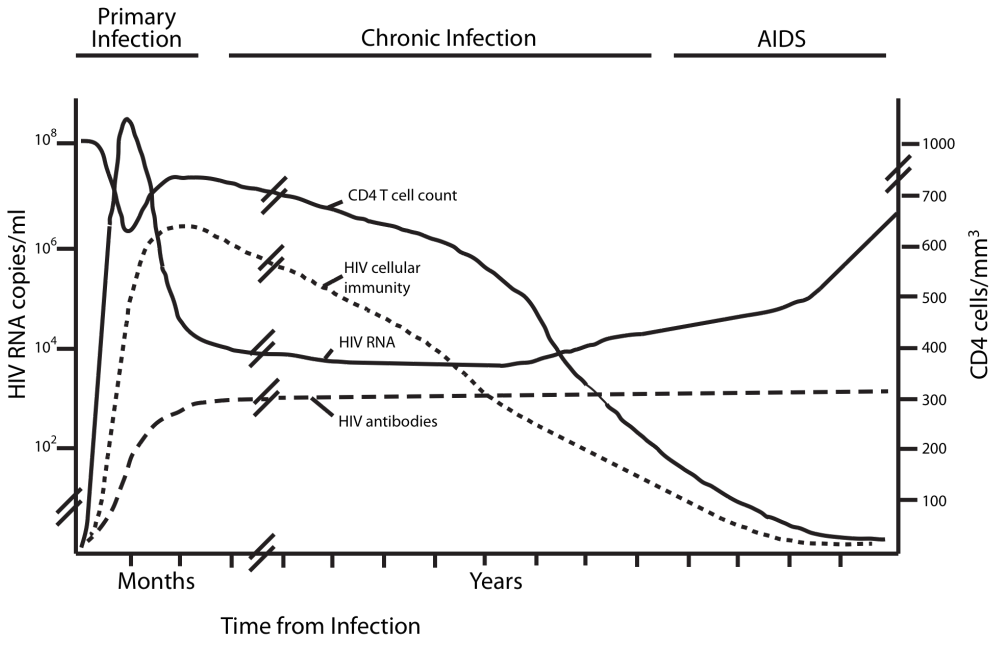

Figure 1.6 Changes in HIV RNA, CD4 T cell count, and HIV-specific immune responses over the course of HIV infection. *From Fields Virology, 2007.*

One major site of viral replication is the GALT, where the majority of the body's CD4 T cells reside[140]. During acute infection 30-60% of CD4 T cells in the GALT are infected and destroyed[141], including nearly all CCR5+ CD4 T cells[140]. HIV kills CD4 T cells through

both direct cytopathic effects in infected cells, indirectly through immune mediated killing, and also through bystander killing of uninfected cells via activation-induced apoptosis[3]. The latter reflects the dual nature of CD4 T cells in HIV pathogenesis: they are both necessary for the control of viral replication (by providing help to HIV-specific CD8 T cells and B cells), and also the substrate for its replication, with HIV-specific CD4 T cells being preferentially targeted[141]. Within weeks of infection HIV-specific CD8 T cells and antibodies (some of which are neutralizing) develop, and viral replication is brought under control so that plasma viral load is reduced to an average of 30,000 copies/mL through weeks 3-5 [141, 346](Figure 1.5). While HIV-specific CD8 T cells are able to kill CD4 T cells in which HIV is actively replicating, they are ineffective against latently infected cells, where HIV proteins are not being produced[141]. Periodically, viral replication from integrated HIV DNA in latent cells is re-activated, and therefore low-level viral replication continues to occur[347, 348]. Additionally, HIV replication occurs with an extremely high error rate (10^{-3}-10^{-4} errors per nucleotide base[349]), and therefore mutations that escape HIV-specific CTLs and neutralizing antibodies are constantly occurring, and new T cell and B cell clones must develop to combat the rapidly mutating virus[347]. The balance of this low level viral replication and the constantly adapting immune response maintains plasma viral load at a quasi steady state, and infected individuals remain largely asymptomatic[141]. This period of infection is referred to as chronic infection, and can vary in length from as little as 6 months to potentially indefinitely, with some individuals remaining symptom-free after over 25 years of documented infection[141]. However, this low level replication, along with leaking of microbial products into the bloodstream across the intestinal wall (after initial destruction of the GALT), causes continual systemic immune activation[141]. Successive rounds of naïve T and B cell stimulation and clonal expansion to combat continually mutating HIV strains eventually leads to immune exhaustion, and effector cell anergy[350]. T cell exhaustion is characterized by the expression of exhaustion markers, such as PD-1, LAG-3, Tim-3, and CTLA-4[350], and the gradual loss of effector functions: early in HIV infection HIV-specific CD8 T cells are able to produce numerous effector molecules in response to HIV; however, these effector functions are successively lost, with only monofunctional cells, producing largely IFNγ, remaining late in infection[351]. As the capacity for CD4 T cell replication is lost there is gradual decline in CD4 T cell numbers, and eventually viral replication can no longer be controlled and plasma viral loads rebound[350] (Figure 1.5). When CD4 T cell numbers fall to 500 copies/mL and lower, individuals begin to suffer from signs of immune

dysfunction, such as oral candidiasis, pneumococcal infections and tuberculosis[141]. This marks the beginning of advanced disease, and continued CD4 T cell loss is associated with progressively more severe immune deficiency. Individuals with advanced disease suffer life-threatening opportunistic infections, such as pneumonia, tuberculosis and systemic fungal infections, and cancers, especially those related to chronic viral infections (such as Kaposi sarcoma from reactivation of human herpes virus 8)[141]. Susceptibility to these infections/malignancies increases as CD4 T cell numbers decline, and AIDS is defined by these clinical manifestations of immunodeficiency. AIDS will eventually result in death if HIV replication is not brought under control by antiretroviral therapy (ART). ART is a combination of antiviral drugs that act by: (1) blocking viral attachment and fusion, (2) inhibiting reverse transcription, (3) inhibiting HIV protease and thereby polyprotein processing, (4) and preventing proviral integration[141]. Although drug resistance mutations do occur, combinations of available therapies can be used to control viral replication and allow for restoration of normal CD4 T cell numbers[141]. However, as ART targets actively replicating virus, it is ineffective against latently infected cells and therefore does not cure HIV: if ART is interrupted HIV replication will resume and progression towards AIDS continues. HIV-infected individuals therefore require lifelong treatment, which is prohibitively expensive in the countries where HIV prevalence is highest[1]. Even if ART was universally available, individuals on ART suffer from higher rates of cardiovascular disease, neurocognitive changes, and loss of bone density that may relate to persistent immune activation despite viral suppression below detectable levels[141]. While there are currently vigorous research efforts to find ways to clear the latent reservoir, a cure for HIV will not be available for several years, and an additional 7,000 people are infected with HIV every day[1]. Therefore, new ways to prevention HIV infection before systemic infection and viral latency are established, and that will be effective in the underdeveloped regions where HIV burden is highest, are desperately needed.

1.5 Epidemiologically Defined Groups at Increased/ Decreased Risk of HIV

As introduced in Section 1.1, epidemiological studies have identified groups of individuals who are at increased or decreased risk of HIV infection. One of the aims of the present work was to identify potential biological mechanisms in the foreskin to account for these divergences in risk. To accomplish this, I examined two groups of individuals with altered HIV susceptibility: men who have asymptomatic herpes simplex-2 (HSV-2) infection, and men who have been repeatedly exposed to HIV but have not become infected. This section will describe the epidemiological evidence that these individuals have altered HIV-risk and review proposed biological mechanisms for these alterations. Potential biological mechanisms for the protective effect of circumcision are also discussed.

1.5.1 Individuals at Increased Risk of HIV

1.5.1.1 HSV-2

Numerous STIs have been linked to increased HIV susceptibility, including both viral and bacterial infections. Possibly the most well documented, however, is the clear epidemiological link between HSV-2 infection and increased HIV risk. Numerous observational studies have examined this association, and a meta-analysis of 18 longitudinal cohort studies found that seroprevalent HSV-2 infection (which is generally asymptomatic) was associated with 2.7-fold increased risk of HIV acquisition in heterosexual men[352]. HSV-2 is an incurable sexually transmitted virus that causes lifelong infection. Virus in an infected partner's genital fluids initially infects epithelial cells of the genital skin/mucosa[353]. Infection is cytolytic, and in some cases ulcers form at sites of productive viral replication (genital ulcer disease, GUD). The virus rapidly spreads to sensory neurons innervating the infected tissue, and subsequent latent infection is established in the dorsal root ganglia. Periodically, viral replication is reactivated and HSV-2 virions are transported back by antegrade transport to the original site of infection, or new sites innervated by the same neuron[353]. Although initial infection (68% [354]) and

subsequent viral reactivation (80% [388]) are most often asymptomatic, in a minority of cases classical herpatic ulcers are apparent.

The cytolytic effect of HSV-2 infection on epithelial cells leads to a loss of epithelial integrity and hence facilitates HIV entry at the site of tissue damage[390]. Not surprisingly, when examining individuals with clinically apparent GUD (as opposed to any HSV-2 infection, which includes asymptomatic individuals), the per-contact risk of HIV transmission is much higher (5-fold increase in risk of HIV acquisition[355]). The strong epidemiological link between HSV-2/GUD and susceptibility to HIV initially raised hopes that HIV transmission could be reduced through HSV-2 treatment. While HSV-2 is not curable, antiviral treatment reduces the frequency of HSV-2 reactivation and prevents GUD[391-394]. However, randomized trials of HSV-2 treatment (acyclovir) failed to reduce HIV incidence, despite reducing HSV-2 associated ulcers by 63%[395-397]. This suggests that the increased risk of HIV acquisition observed with HSV-2 infection is not solely driven by the presence of breaks in the mucosal epithelium caused by genital ulcers.

Asymptomatic HSV-2 reactivation (absence of GUD) leads to local immune activation that could increase susceptibility to HIV. Control of HSV-2 replication is highly dependent on the recruitment and activation of CD8 T cells and NK cells in localized foci at the site of viral replication[398-401]. However, an increased number of CD4 T cells can also be found within these inflammatory foci[356], which may increase HIV susceptibility by providing additional substrate for HIV replication[390, 403]. Control of HSV-2 replication is usually rapid, and periods of viral replication are short lived (1-8 days[357]). However, the inflammatory cells recruited to the mucosa are retained for long periods of time: an increased density of CCR5+ CD4 T cells in close proximity to DC-SIGN+ dendritic cells can be found at the sites of healed herpetic ulcers after 20 weeks of acyclovir treatment, with no recurrence in HSV-2 reactivation[403]. While the maintenance of these effector T cells at the site of HSV-2 replication helps to control HSV-2 reactivation *in vivo*, they are readily infected by HIV *in vitro*, supporting 2-5-fold higher HIV replication that cervical tissue from unaffected areas[390, 403]. Additionally, HSV-2 infected women with no clinical history of GUD have a 10-fold increase in cervical dendritic cells and 3-fold increase in CCR5 expression on cervical CD4 T cells[405] and a recent report by our collaborators also found that asymptomatic HSV-2 infection was associated with an 2.5-fold increase in the number of CD4+ cells in foreskin tissue, although

activation status and HIV co-receptor expression were not investigated[358]. Because HIV selectively targets activated CCR5+ CD4 T cells for infection, it is likely that the recruitment of these cells to the genital skin/mucosa during asymptomatic HSV-2 reactivation contributes to increased HIV susceptibility, and explains the lack of efficacy of current HSV-2 treatment in preventing HIV acquisition: even high doses of acyclovir (3g daily) only reduces asymptomatic viral replication to ~16.5 times per year, on average this would equate to a burst of replication once every 3 weeks[388]. Since HIV-susceptible T cells are retained in the female genital tract for more that 20 weeks after reactivation[403], even on high does of suppressive therapy ongoing subclinical reactivation may be sufficient and frequent enough to maintain a high density of HIV target cells in the genital mucosa. This observation may explain the lack of efficacy of HSV-2 therapy in reducing HIV infections.

1.5.1.2 Uncircumcised Men

While randomized trials of male circumcision (MC) found a clear protective effect against heterosexual male HIV infection, the mechanism of this protection remains unknown. Four mechanisms have been proposed in the literature to date: (1) the foreskin has a thinner layer of keratin than other skin; (2) there is a higher density of HIV-susceptible cells in the foreskin; (3) immune cells in the foreskin are functionally different from those in other skin in ways that make them more susceptible; and, (4) the microbial flora of the uncircumcised penis causes immunological changes in the foreskin that make it more susceptible. The first three of these hypotheses are based on the idea that the foreskin cell populations/tissue structure is fundamentally different from other skin, and thus an ideal experimental design would be to compare foreskin tissue to matched biopsies of non-foreskin skin. However, this is practically very difficult, and so far instead studies have compared the “inner” and “outer” aspects of the foreskin tissue removed during circumcision (Figure 1.6). On the non-erect penis, the foreskin is folded over the glans, so that half of it is unexposed to the air: the so-called inner aspect. The outer aspect of the foreskin is continuous with the remaining skin on the shaft, and is exposed to the air even on the non-erect penis. During intercourse, the foreskin is pulled back off of the glans on the erect penis, and therefore both aspects are exposed. The two aspects of the foreskin are very similar in appearance, albeit with some pigmentation differences in most, but not all

men. By comparing the inner and outer aspects of the foreskin in experiments designed to explore why the foreskin is susceptible to HIV infection, the underlying assumption is that foreskin HIV infection occurs across the inner aspect of the foreskin, and that the outer aspect of the foreskin is representative of non-HIV-susceptible skin. While this assumption may not be entirely true, and therefore studies comparing the foreskin to skin from other anatomical sites are warranted, interesting differences have been observed between the inner and outer aspects (discussed below).

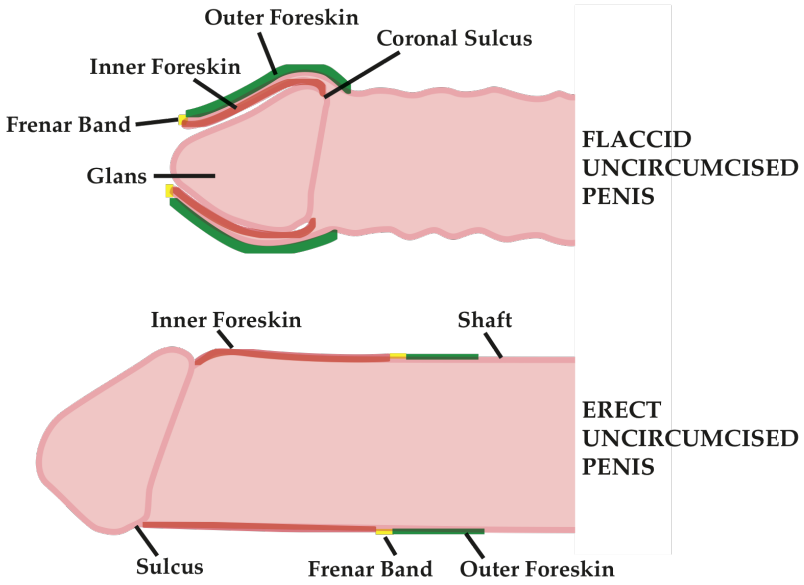

Figure 1.7 Inner and outer aspects of the foreskin. The inner aspect of the foreskin (red) is not exposed on the flaccid penis (top). However, during intercourse it is exposed on the erect penis. This is in contrast to the outer aspect of the foreskin (green), which is exposed on both the flaccid and erect penis. Both the inner and outer aspects of the foreskin are removed during circumcision.

The most widely hypothesized explanation for the susceptibility of the foreskin to HIV is that the inner aspect of the foreskin has a relatively thin layer of keratin, providing less of a barrier between HIV in genital secretions and underlying susceptible foreskin cell populations. This widely circulated early theory may have been based on observations of pigment differences between the inner and outer foreskin aspects, with empirical reports from only one study of cadaveric tissue[147] and two studies where thinner keratin on the inner aspect was observed, but not quantified[156, 359]. Subsequent studies have shown either no difference[189, 246] or a greater keratin thickness on the inner aspect[165], including a study by our own group, where samples were analyzed by two independent laboratories blinded to biopsy origin[154].

The density of possible HIV target cells in the foreskin has also been a topic of considerable investigation and debate. Under this hypothesis the foreskin would contain a greater number of HIV-susceptible cells (Langerhans cells or CD3/CD4+ cells) per mm^2 of tissue, making the foreskin stochastically more susceptible to HIV than other skin. Again studies to date exploring

this possibility have compared the inner and outer aspects of the foreskin, and have been inconclusive, with some studies showing an increased density of Langerhans cells or T cells[145, 156, 160, 359], another showing no difference[164], and two showing a decreased target cell density in the inner foreskin[147, 165]. The discrepancies between both keratin and cell density measurements underscore a need to perform immune studies in a rigorously blinded and quantifiable fashion, and also to consider variables such as participant STI history, age, and sample processing prior to analysis (cadaveric vs. cultured vs. fresh).

More recently (and after the initiation of the research presented in this thesis) it has been suggested that the foreskin may be susceptible to HIV due to functional differences in immune cell populations. As of yet this hypothesis is relatively new, and only two groups have investigated the possibility, both again focusing on differences between the inner and outer aspects of the foreskin. Work by Dr. Hope's laboratory[164] found that while the inner and outer aspects of the foreskin have similar densities of Langerhans and T cells, the Langerhans cells in the inner foreskin show increased environmental sampling and susceptibility to stimulation (express CD86 after TNFα exposure). Additionally, they observed increased CD4+ cell recruitment into the epidermis after exposure of the inner, but not outer, aspect to TNFα or MIP-1α. Subsequently, a collaboration between the laboratories of Dr. Ganor and Dr. Bomsel exposed explanted foreskin tissue and artificial foreskin constructs to HIV[156, 160]. They found that more HIV virions were able to enter the inner aspect of the foreskin than the outer aspect, and that more RANTES is released from epithelial cells in the inner foreskin, resulting in greater CD4 T cell recruitment into the epidermis. They also observed Langerhans cell migration, HIV uptake, and conjugate formation with CD4 T cells in the inner, but not outer aspects. In combination with the observations from Dr. Hope's laboratory[164], this suggests that it is increased immunological activity (increased Langerhans environmental sampling and propensity to mature, migrate and form conjugates with T cells, increased chemokine production by epithelial cells and increased T cell chemotaxis) that renders the foreskin susceptible to HIV.

The final theory thus far proposed to explain foreskin HIV susceptibility is that, instead of fundamental differences in foreskin compared to other skin, MC works by altering the inflammatory environment of the penile skin to generally reduce immune activation. Studies of MC in Rakai, Uganda found that both the total penile bacterial load and the representation of anaerobic bacteria in the penile microbiome, were substantially reduced after circumcision[360].

Altered bacterial flora, including increased anaerobic bacteria in the setting of BV, has been associated with increased vaginal inflammation and increased susceptibility to HIV in women[361, 362]. Some researchers have also suggested that alterations in the penile microbiome due to circumcision would not only decrease HIV target cells in the foreskin, but also in the urethra, thereby decreasing HIV acquisition at this penile site as well[363]. It is possible that increased bacterial load and relative quantity of anaerobic bacteria on the uncircumcised penis leads to both and increased density of foreskin HIV target cells and increased immune activation, and therefore increased HIV susceptibility.

It is clear that these four proposed mechanisms are not mutually exclusive, and HIV susceptibility in the foreskin is likely due to a combination of additive, or even synergistic, factors. Although outside the scope of the present work, fully elucidating the protective mechanisms, and understanding their relative importance and interactions, will not only provide new avenues for HIV prevention, but also allow us to focus the development of prevention modalities targeting the most practical, and impactful pathways.

1.5.2 Individuals at Decreased Risk of HIV

1.5.2.1 HESN

The variation in reports of per-contact risk of HIV infection[8] points to the fact that there is natural variability in HIV susceptibility among individuals. As described above, some of this variability is due to co-infections that increase HIV susceptibility[364], and this will inform the field as to what should be *avoided* in the design of new prevention modalities. Conversely, some individuals are naturally more resistant to HIV infection[408], and understanding the biological mechanisms for this resistance will be extremely important in identifying immunological targets for alteration with new prevention modalities.

One clearly defined group of individuals resistant to sexual acquisition of HIV is those with a 32 base pair deletion in the gene encoding CCR5 (Δ32). This base pair deletion leads to an early stop codon, and a truncated defective protein. Due to the redundancy in cytokine receptor-ligand specificity, individuals with the Δ32 mutation not susceptible to sexually acquired HIV[76, 78-80], but otherwise normal. However, a prospective observational cohort study of female commercial sex workers (CSW) in Nairobi, Kenya, identified a group of women who remained uninfected despite 10 years of exposure to HIV in a community whose annual incidence was 42%, representing a more than 100-fold decrease in susceptibility compared to the general population of CSW[408]. Importantly, these women did not have any CCR5 mutations (which are very rare in African populations), and their PBMCs were susceptible to HIV infection *in vitro*[409].

Since this report several other groups have described populations of individuals who are regularly HIV-exposed but remain seronegative (HESN), including other high-risk sexual exposure groups, injection drug users, individuals exposed to contaminated blood, and infants of HIV-infected mothers[365]. However, identifying correlates of protection from infection has proved difficult[366]. Numerous unique immunological characteristics have been identified, but these observations are not always consistent from study to study[412-415], suggesting that resistance to infection (outside of the Δ32 mutation) may be due to the combined protective effect of several factors, each of which is not completely protective on its own[367]. It follows that resistance does not necessarily denote complete immunity, and the *amount* of resistance may

vary from person to person, as exemplified by the late seroconversion of some CSW in Nairobi, despite their remaining seronegative much longer than would be expected based on local incidence rates within the community[368]. Additionally, the inclusion criteria for HESN studies often only enriches for resistant individuals: because the heterosexual per-act probability of HIV transmission is low[8], demonstrating true resistance requires years of follow-up and very high-level exposure[369], which is impractical in observational studies. Therefore, the magnitude of HIV exposure in most HESN studies is not sufficient to fully prove that all individuals are resistant, but instead it selects for individuals with some amount of known HIV exposure, providing a population enriched for resistant individuals compared to the general population[370]. The likely multifactoral nature of natural resistance to HIV, in combination with the difficulty in obtaining a "pure" population of resistant individuals, complicates the interpretation of HESN studies

There are also several important methodological aspects of HESN studies to consider with interpreting their results. One is the route of HIV exposure: immunological characteristics protecting an individual from one route of exposure may differ from other routes, so that the correlates of protection identified in individuals exposed to contaminated blood products may be different from those of individuals who are exposed to HIV sexually[365]. Additionally, among HESNs exposed to HIV sexually, there is evidence that systemic correlates of protection (blood) differ from those in the tissue at the site of exposure[418, 419]. Of note, all the HESN studies to date performed in the tissue have examined either the female genital tract (FGT) or the oral mucosa of MSM, and prior to the research presented in this thesis, no studies had been performed in the genital tract or foreskins of HESN men. Finally, the type of HIV exposure defining the HESN group, and importantly the control group they are compared to, differ from study to study and are very likely important. Generally there are two types of sexually exposed HESN populations: individuals with a high frequency of sex acts with many different partners (i.e. CSW, where HIV exposure is assumed stochastically), and individuals with a single HIV-infected partner (individuals in "discordant" relationships). Things such as frequency of sex (micro-abrasions and exposure to semen or HLA-mismatched cells during sex), exposure to other STIs, condom use, etc. differ between HESN and control groups, and are potential confounders as they can alter genital immunology[364]. Early studies of HESN populations of CSW used a comparison group of non-CSW women from the general population[371-374], and therefore

these studies may be heavily confounded by immune alterations associated with sex work, and not protection from HIV infection. Studies of individuals in discordant relationships remove many of these confounders, but there is still the necessary confounder of HIV-exposure itself; even low-level exposure to HIV induces immunological changes[375], and while it is possible that some of these may be protective (such as, theoretically, an effective adaptive immune response), many others may be deleterious (such as increased numbers of CCR5+ CD4 T cells). A longitudinal study by Suy *et al*. followed individuals in discordant relationships over time, and found increases in immune activation over time that correlated with their partner's viral load (similar changes were not observed in control individuals)[376]. Therefore, specifying a longer duration of discordant relationship, to enrich for an even greater number of resistant individuals, would also be expected to amplify confounding HIV-induced immune alterations. This confounder also affects more recent studies in CSW where resistant CSW, defined by a minimum duration of time in sex work, are compared to "non-resistant" CSW, who are, by definition, new CSW. While a CSW control group is much more appropriate for HESN studies in this population, the resistant CSW, by definition, will have been exposed to HIV for a longer period of time than new CSW.

Due to these possible confounders, immune characteristics that correlate with HESN status must be examined critically to ensure that they are indeed correlates of protection from infection, and not simply the result of high-risk behaviors or HIV-exposure itself. This is especially important since many of the potential confounders are associated with *increased* HIV-acquisition[364]; natural correlates of protection must be correctly distinguished from confounding immune activation that may actually increase HIV acquisition. In light of these important considerations, this section will describe correlates of protection identified in sexually exposed HESN populations, and will focus, when possible, on those defined in the tissue at the site of HIV exposure, as these are likely more relevant for preventing sexual transmission of than systemic correlates, and consideration will be given to studies where a causal link between the correlate and protection have been established.

1.5.2.1.1 Role of Secreted Anti-viral Proteins

Several research groups have demonstrated that cervicovaginal secretions are able to inhibit HIV infection of PBMCs and explanted cervical tissue *in vitro*[274-276, 278, 281, 282]. The mechanism for this HIV-neutralizing capacity has not been fully elucidated, and while many proteins with HIV-neutralizing capacity have been identified, no single factor has been identified that is wholly responsible for HIV-neutralization; instead neutralizing capacity appears to be due to a combinatorial effect of the multiple proteins present[276]. The depletion of cationic peptides from cervicovaginal fluids significantly decreases HIV-neutralization[274, 276, 282], and levels of LL-37, α- and β-defensins, and trappin-2/elafin in cervicovaginal fluids have all been shown to correlate with HIV-neutralizing capacity[275, 278, 282]. It is of note that while the protective role of SLPI has been well documented in the prevention of vertical HIV-transmission[419, 420], levels of SLPI in cervicovaginal secretions *inversely* correlate with HIV-neutralization, and the addition of recombinant SLPI to cervicovaginal secretions does not augment HIV-neutralization, while the addition of recombinant LL-37 or α-defensins does[274]. The association of these cationic peptides with HIV-neutralization has lead several groups to investigate if they are increased in HESN populations, and although comprehensive proteomic studies have found that cationic peptides (in particular anti-proteases) are uniquely upregulated in the genital secretions of resistant women[421, 422], studies directly examining levels of β-defensins, LL-37, and SLPI in cervicovaginal secretions found no differences between HESN and unexposed control women[311, 423, 424], and while one study observed increased α-defensins [425], another has not[274]. Only trappin-2/elafin has been consistently observed to be increased in the cervicovaginal secretions of HESN women[319, 426], and definitive evidence for its role of in protection from infection was provided by a prospective study showing CSW with increased levels of trappin-2/elafin were protected from HIV over time[426]. In contrast, despite correlating with HIV-neutralization *in vitro*, increased levels of LL-37 and α-defensins are associated with genital co-infections, and were predictive of subsequent seroconversion[278], suggesting that they may be correlates of high-risk behavior, and not of protection from infection. Interestingly, both LL-37 and α-defensins have pro-inflammatory properties[262], and it is possible that the anti-HIV activity of these compounds *in vitro* is counteracted by their chemotactic effects on HIV target cells *in vivo*, so that their net effect is to increase, instead of decreasing, HIV susceptibility. Conversely, trappin-2/elafin has been shown to decrease IL-8

secretion and NFκB signaling in cervical mononuclear cells, and these anti-inflammatory properties correlate with its anti-HIV activity[281].

Natural chemokine ligands of CCR5 (CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES) have also been shown to have HIV-neutralizing capacity *in vitro*[427-431], and their levels correlate with the *in vitro* HIV-neutralizing capacity of saliva from HESN men[432]. Presumably these chemokines compete with HIV for CCR5, and chemokine binding to CCR5 induces CCR5 internalization, both decreasing its availability to HIV[433, 434]. Cervicovaginal secretions from HESN women have been shown to have increased levels of RANTES in some [423, 435], but not all studies[377]; however, increased RANTES was also associated with an increased density of CCR5+ cells in the cervical tissue[378], which may increase HIV susceptibility. While there is evidence that systemic levels of chemokines do prospectively correlate with protection against sexually acquired HIV in MSM[437], their strong chemotactic properties for HIV target cells in the genital mucosa warrant additional prospective observational studies to conclusively demonstrate their *in vivo* mucosal presence is protective.

Finally, IgA isolated from the cervicovaginal secretions of some individuals has been shown to neutralize HIV *in vitro*, and the HIV-neutralizing IgA is detected more frequently in the cervicovaginal secretions of HESN women than unexposed controls (covered in detail in Section 1.3.3.3.2). As previously discussed, a single, clear, protective mechanism of IgA has not been identified, and in some HESN individuals HIV-neutralizing IgA appears to be HIV-specific[295, 301, 303, 310-314], and the result of acquired immunity[301], while in other populations HIV-neutralizing capacity is not linked to HIV-specificity[308, 309], and therefore may represent natural "innate" immunity. While the exact mechanism by which IgA neutralizes HIV remains to be elucidated, it is clear that the cervicovaginal secretions of HESN women are more likely to contain HIV-neutralizing IgA than control populations. Additionally, the presence of HIV-neutralizing IgA in vaginal secretions is prospectively associated with protection from infection[300].

1.5.2.1.2 Role of HIV-Specific CD8 T Cells

Numerous groups have observed HIV-specific CD8 T cells in the blood of HESN populations, including infants born of HIV-infected mothers, individuals exposed to contaminated blood products, and individuals exposed to HIV sexually[379-390]. HIV-specific CD8 T cells have also been identified in the cervical tissue of HESN CSWs[391]. The proportion of women with HIV-specific CTLs increased with duration of sex-work, and continual sex-work was necessary for maintenance of responses, suggesting that these responses were acquired as a result of exposure[381, 392]. In keeping with this, seroconversion of women who had pre-existing HIV-specific cervical CTLs was associated with a recent break in sex-work and loss of HIV-specific CTL responses[368]. However, prospective studies have shown that neither MSM with systemic HIV-specific responses, nor CSWs with cervical HIV-specific CTLs, are any less likely than their peers to acquire HIV[300, 393, 394], suggesting that HIV-specific CTLs are a correlate of exposure to HIV, but are not sufficient to protect against infection. It is possible that the HIV-specific CD4 T cells necessary for their activation, or the release of chemotactic/proinflammatory cytokines by activated HIV-specific CTL at the site of exposure may counteract any beneficial effects of clearing infected cells. While it may be that future vaccines eliciting strong mucosal CTL responses with high avidity and a large array of effector functions will prove to be protective, their role in natural protection from HIV infection is far from established.

1.5.2.1.3 Role of Inflammation

Based on mechanistic studies of HIV infection, we hypothesized that the HESN phenotype would be associated with generally reduced immune activation. However, cross-sectional studies are dichotomous, either finding that HESNs are characterized generally by immune quiescence[393, 395-401] or by immune activation[371-374, 376, 402-404]. The previously discussed confounders of HESN studies likely contribute to these discrepancies, with many early studies comparing CSW to non-CSW controls[371-374]. However, some study designs may overcome these confounders, such as prospective studies, which delineate correlates of protection from exposure, or genetic associations, which are not confounded by behavior.

Importantly, the only prospective study to measure immune activation in HESN individuals found that highly exposed men who remained seronegative had decreased levels of activation markers on CD4 T cells (HLA-DR/CD38), and showed reduced proliferation of both CD4 and CD8 T cells[393]. Additionally, genetic studies examining HESN women who lack the Δ32-CCR5 mutation have identified polymorphisms associated the *irf-1* gene, that lead to decreased expression of Interferon Regulatory Factor-1 (IRF-1)[405], which is involved in the regulation of a number of genes with diverse pro-inflammatory effects[406]. While these studies suggest a protective role of generalized reduced inflammation, there is also evidence that, as opposed to ubiquitous immunoquiescence, protection from infection is due to down-modulation of specific pro-inflammatory immune populations/pathways, with upregulation of others. This may also help to explain discordant results when measuring inflammation in bulk PBMCs, or levels of proteins secreted in sera/cervicovaginal fluids of HESN populations. Gene expression analysis of purified CD4 T cells shows largely decreased gene expression, including several pathways linked to HIV replication, including TCR signaling[396]. However, specific populations that have anti-HIV properties, such as NK cells[373, 407], are upregulated. Further examination of specific cell populations shows that anti-inflammatory Tregs[395] are upregulated in HESN individuals, while populations that are particularly susceptible to HIV, such as Th17 cells[398] and Th1 cells[399, 405], are downregulated. Further HESN studies, focusing on the activity of well-defined cell populations, are warranted to explore this possibility.

1.6 Research Aims and Hypotheses

Based on our understanding of the earliest events in HIV transmission, I hypothesized that states of increased or apparent decreased HIV susceptibility would correlate with foreskin immune activation.

Specifically, I hypothesized that the foreskins of HIV-susceptible men would have increased numbers of activated CD4 T cells and highly susceptible subsets, such as Th1 and Th17 cells, and increased local levels of pro-inflammatory chemokines and cytokines, while in the foreskins of relatively HIV-resistant populations these would be decreased. Additionally, I expected HIV-resistant individuals to have increased levels of innate anti-microbial peptides and HIV-neutralizing IgA in foreskin secretions and to have HIV-specific CD8 T cells within their foreskin tissues.

1.6.1 Specific Aims

SA-1: To develop techniques to isolate live, immunologically functional T cells from foreskin tissue, and characterize foreskin T cell populations in comparison to those in the systemic circulation.

SA-2: To characterize foreskin T cell populations in men with increased susceptibility to HIV; specifically, men with asymptomatic HSV-2 infection

SA-3: To characterize foreskin T cell populations in men with decreased susceptibility to HIV; specifically, men in discordant relationships (HESN).

Chapter 2
Participants and Common Methodology

2 Participants and Common Methodology

The research presented in this thesis represents analyses of data collected from a single, large cross-sectional study, with recruitment deliberately structured to permit several hypotheses to be tested. Chapters 3-7 represent the analyses of sub-groups of men meeting the inclusion criteria necessary to answer each research question. The overlap in participants between analyses was an intentional aspect of the study design for efficiency in participant recruitment. To ensure blinding to participant status, the laboratory assays performed on site in Rakai (diagnostics and flow cytometry) were performed for every participant. Additionally, assays performed at University of Toronto (CD3 immunohistochemistry (IHC) and sub-preputial cytokine detection) were performed on all participants. The methodologies for these assays, which are common to all analyses, are presented in this chapter.

2.1 Participants

Participants were monogamous heterosexual couples recruited from an established community cohort in Rakai, Uganda[36], of which the male partner had elected to undergo adult circumcision at the Rakai Health Sciences Program in Kalisizo, Uganda. Each partner within the couple completed a behavioral questionnaire before sample collection/surgery (summary demographics provided in Table 2.1). All participants provided written informed consent, and formal ethical approval was obtained at the University of Toronto, the Uganda Virus Research Institute's Scientific and Ethical Committee, Karolinska Institute and Western IRB (Olympia, WA).

Table 2.1 Summary of participant demographics.

		Men	Women
Age		34.5 (22-53)	30.0 (19-49)
HIV+	Seropositive	20/110 (18.2%)	36/110 (32.7%)
	CD4 count /mm^3	409.0 (6.2 - 934.6)	553.3 (72.2 - 1,591.7)
	Viral Load /mL	30,693 (≤39 - 335,903)	2,562 (≤39 - 389,942)
HSV-2+		57/107 (51.8%)	71/107 (66.4%)
Condom use	never	78/110 (70.9%)	90/110 (81.8%)
	sometimes	24/110 (21.8%)	12/110 (10.9%)
	always	8/110 (7.3%)	8/110 (7.3%)
sex partners in last year	single	82/110 (74.5%)	106/110 (96.4%)
	multiple	28/110 (25.5%)	4/110 (3.6%)
Self reported STI symptoms (last 30 days)	Ulcer	2/110 (1.8%)	11/110 (10.0%)
	Warts	0/110 (0.0%)	5/110 (4.5%)
	Balanitis/itching	5/110 (4.5%)	n/a
extramarital relationship	yes	15/110 (13.6%)	4/109 (3.6%)
	no	95/110 (86.4%)	105/109 (95.5%)

2.2 Sample Collection

Women provided 8 mL of venous blood. Men underwent a physical examination prior to surgery, and circumcision was deferred until after treatment if urethral discharge or clinically apparent genital ulceration was present; 16 mL of whole blood and a swab from the sub-preputial space were then collected prior to surgery. A single sub-preputial swab was collected from each participant immediately prior to surgery using a FLOQswab (COPAN Diagnostics, Murrieta, CA USA) pre-moistened in sterile PBS and rolled once around the coronal sulcus and once down the frenulum. Swabs were collected by the same medical officers throughout the study, and care was taken to collect each swab in a consistent manner. Swabs were then resuspended in 1mL of AMPLICOR® STD Specimen Transport Kit medium (Roche Diagnostic Systems, Branchburg, NJ, USA) and stored at 4^{o}C until division into two aliquots (one for cytokine detection and one

for IgA/innate factor analysis), which were stored at -80^{o}C. Foreskin tissue was processed immediately upon surgical excision in a room adjoining the surgical suite: two sections were snap-frozen into cryomolds in Optimal Cutting Temperature (OCT) compound (Fisher Scientific, Toronto, Canada) for IHC; two sections were placed in RNAlater (Applied Biosystems, Carlsbad, CA) at 4^{o}C for 8 hours, drained, and then cryopreserved at -80^{o}C for PCR; and one large section for T cell isolation collected into RPMI 1640 media supplemented with: 10% heat-inactivated FBS, 10 U/mL penicillin, 10 µg/mL streptomycin, 250 ng/mL amphotericin B, and 2mM L-Glutamine (all from Gibco, Invitrogen; Carlsbad, CA, USA; henceforth referred to as R10 medium). All foreskin tissues were labeled with a unique identifier and provided to research personnel blinded to participant study group for subsequent processing and immune analysis.

2.3 Diagnostics

Diagnostics were performed by the Clinical Laboratory staff at the Rakai Health Sciences Program. The HIV infection status of both partners was determined using two HIV ELISAs (Murex HIV-1.2.O, Abbott, Abbott Park, Illinois, USA; and Vironostika HIV Uni-Form II plus O Mircoelisa System, bioMerieux; Marcy l'Etoile, France). Discordant results were confirmed by Western blot (GS HIV-1 Western Blot, BioRad; Hercules, CA, USA). All participants were also screened for acute HIV infection using real time PCR. RNA was extracted from plasma samples using the Abbott Sample Preparation System, and amplification was performed using the Real Time HIV-1 Amplification Reagent Kit (Abbott) and run on the M2000rt (Abbott) with a lower limit of detection of 40 copies/mL. CD4 counts were performed using Tritest Reagent in combination with Trucount Tubes and were analyzed on a FACSCalibur platform (BD Biosciences, San Jose, CA, USA). HSV-2 infection status was determined by ELISA (Herpes Simplex Type 2 IgG ELISA, Kalon Biological Ltd., Guildford, UK), with cutoffs previously validated in Rakai[408].

2.4 T Cell Characterization

2.4.1 T Cell Isolation

Foreskin samples for T cell isolation were always processed within 15 minutes of surgery, since additional time caused the dermal morphology to change substantially, with gross macroscopic tissue edema. Tissue was first sectioned into longitudinal strips including both inner and outer foreskin and containing both epidermal and dermal tissue. These strips were then further sectioned to create pieces of approximately 0.25cm^2. Each piece was placed in a 1.5 mL conical tube containing 1.0 mL of 500 U/mL Collagenase Type I (Gibco) and 42.5 U/mL of DNAse (Invitrogen) in RPMI 1640 media supplemented with 10 U/mL penicillin, 10 µg/mL streptomycin, 250 ng/mL amphotericin B, and 2mM L-Glutamine (henceforth referred to as RPMI, all from Gibco). Initial immune studies have used dispase for foreskin tissue digestion, but we found that treatment with as little as 1.0 U/mL of dispase (Gibco) for 30 minutes at 37^{o}C lead led to the loss of CD4 expression and decreased CD8 expression in both peripheral blood and foreskin-derived T cells (Figure 2.1).

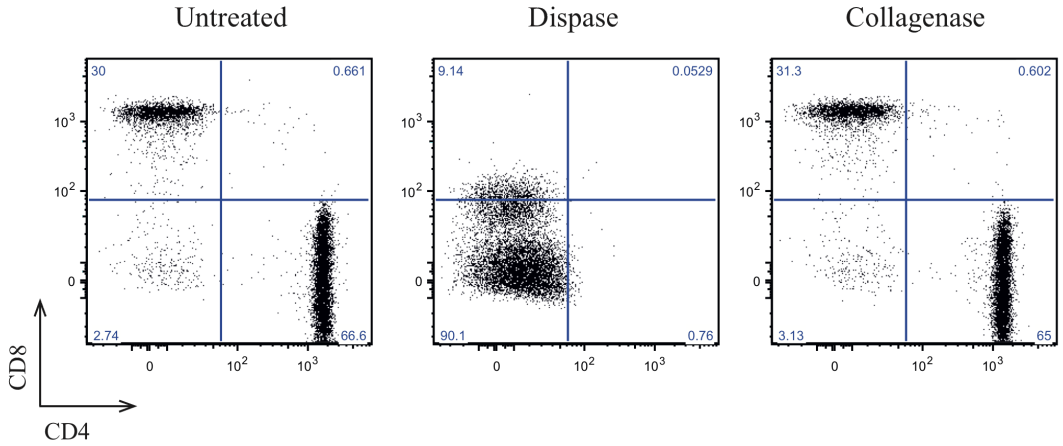

Figure 2.1 Effect of dispase treatment on CD4 expression in blood lymphocytes. PBMCs were isolated from 3 individuals by density centrifugation and either left untreated, treated with 1.0U/mL dispase, or treated with collagenase I for 30 minutes. PBMCs were then stained with CD3-FITC, CD4-PE and CD8-PerCP. Plots show CD3+ events from one representative individual.

Scissors were used to mechanically disrupt each piece of tissue, and tubes were then placed on a shaker (Eppendorf Thermomixer; Hamburg, Germany) for 30 minutes of enzymatic digestion at 37^{o}C with shaking at 900 rpm. The cellular suspension obtained from each tube was pooled, FBS (to 10%) was added to stabilize cells, and was then filtered through a 100µm cell strainer

(BD Biosciences; Franklin Lakes, NJ USA) to remove any remaining undigested tissue. Filtered cells were washed once to remove collagenase, resuspended in R10, and allowed to rest under normal growth conditions (37°C, 5% CO_2, humidified atmosphere) for 3-7 hours. This combination of collagenase 1 and gentle mechanical digestion allowed for the retention of CD4 expression and gave a single cell suspension containing CD3+ T cells that showed a similar CD4 and CD8 expression profile to PBMCs from the same individual (Figure 2.2).

PBMCs were isolated by density gradient centrifugation (Ficoll-Paque Plus; Amersham Biosciences; Uppsala, Sweden).

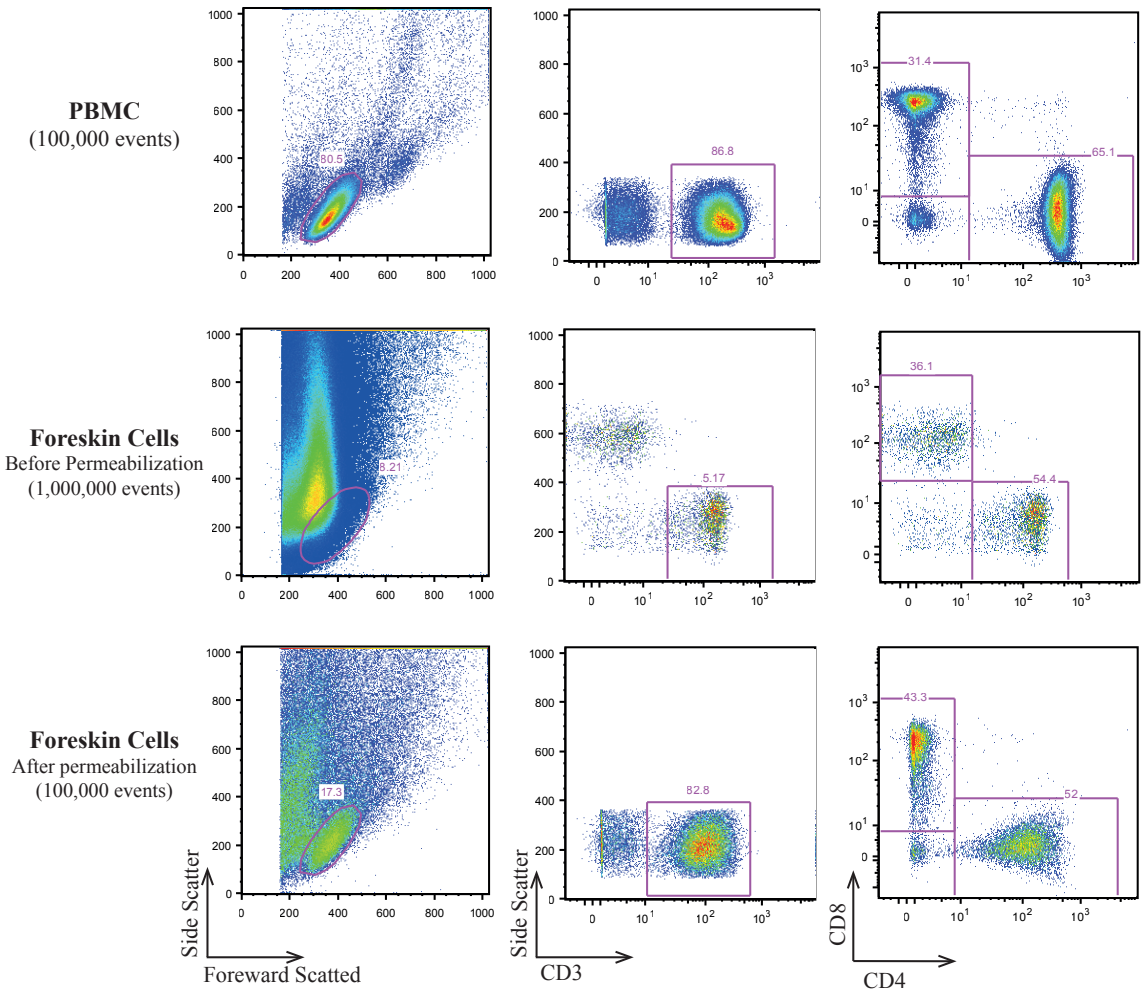

Figure 2.2 Gating protocol for foreskin T cell subset analysis. PBMC and foreskin cells were gated based on forward and side scatter, and stained with CD3-APC, CD3-PE and CD8-PerCP (representative plots).

2.4.2 Flow Cytometry

Both PBMC and foreskin cell numbers were determined by trypan blue exclusion. $1x10^6$ PBMCs and $10\text{-}20x10^6$ foreskin cells (depending on yield) were plated in 500µl culture medium and stimulated for 9 hours at 37°C in the presence of 5 µg/mL Brefeldin A (GolgiPlug, BD Biosciences), with either: 1 ng/mL phorbol-12-myristate-13-acetate (PMA) and 1 µg/mL ionomycin (both from Sigma; St. Louis, MO, USA); or 102 µg/mL of a pool of 51 HIV peptide epitopes (9-11 amino acids long, JPT Peptide Technologies, Berlin, Germany) previously found by our group to be highly antigenic in an East African population[392]; or vehicle (0.1% DMSO). Samples were then washed with cold 2% FBS in PBS and stained with fluorochrome-labeled monoclonal antibodies specific for CD3 (UCHT1), CD4 (RPA-T4), CD8 (SK1), CCR5 (2D7/CCR5), and CD25 (M-A251; all BD Biosciences). Excess surface antibody was removed by washing with 2% FBS in PBS. Samples for intracellular staining were permeabilized using either the eBioscience fixation/permeabilization solution for Treg identification (eBiosciences; San Diego, CA, USA) or the BD Cytofix/Cytoperm solution (BD Biosciences) for all others. Cells were washed in permeabilization wash buffer and stained with fluorochrome-labeled monoclonal antibodies specific for combinations of the following intracellular cytokines/transcription factors: TNFα (MAb11; BD Biosciences), IFNγ (B27; BD Biosciences), IL17a (eBio64DEC17; eBioscience), IL22 (22URTI; eBioscience), and FoxP3 (PCH101; eBioscience). Samples were acquired using a FACSCalibur flow cytometer (BD Systems) and data analysis performed using FlowJo analytical software version v.9.3 (Treestar; Ashland, OR, USA).

Unpermeabilized foreskin cells were gated based on forward and sideward scatter (Figure 2.2). This gate was created based on the location of CD3+ T cells in the PBMC sample from the same patient. Back gating was used to confirm that this gate corresponded with the location of CD3+ cells in the foreskin sample (CD3+ cells representing ~0.1-0.6% of total events in the foreskin sample). A clearly visible population of CD3+ cells in the foreskin samples could then be identified. For unpermeabilized foreskin cells, 10^6 events were recorded, while only 10^5 events were recorded for PBMC and permeabilized foreskin cells, due to the large amount of other cell types present in unpermeabilized foreskin samples. After permeabilization the T cell population was enriched (3-5% of total events) and could be directly identified on the forward by side scatter plot (Figure 2.2).

2.4.3 CD3 Immunohistochemistry

CD3 IHC was performed as a clinical service by the Toronto General Hospital Department of Pathology; Image scanning and Definiens software use was performed with the Spatio-Temporal Targeting and Amplification of Radiation Response Program (STTARR). In order to translate flow-derived cell proportions into an absolute tissue density of foreskin T cells, two sections of foreskin where collected from each participant and IHC was performed. OCT cryopreserved tissues were sectioned to 8µm, fixed in 2% formaldehyde, and frozen for batch staining. For CD3 staining, frozen sections were thawed and air-dried at room temperature. Endogenous peroxidase and biotin activities were blocked using 0.3% hydrogen peroxide and avidin/biotin blocking kit (Vector Labs), respectively, followed by 10% normal goat serum. Sections were then incubated with rabbit anti-human CD3 antibody (DAKO, Vector Labs), followed by biotin-labeled goat anti-rabbit secondary and Alkaline Phosphatase Streptavidin Labeling Reagent (all Vector Labs). Color development was performed with freshly prepared Alkaline Phosphatase Substrate Kit Vector Red (Vector Labs). Finally, sections were counterstained lightly with

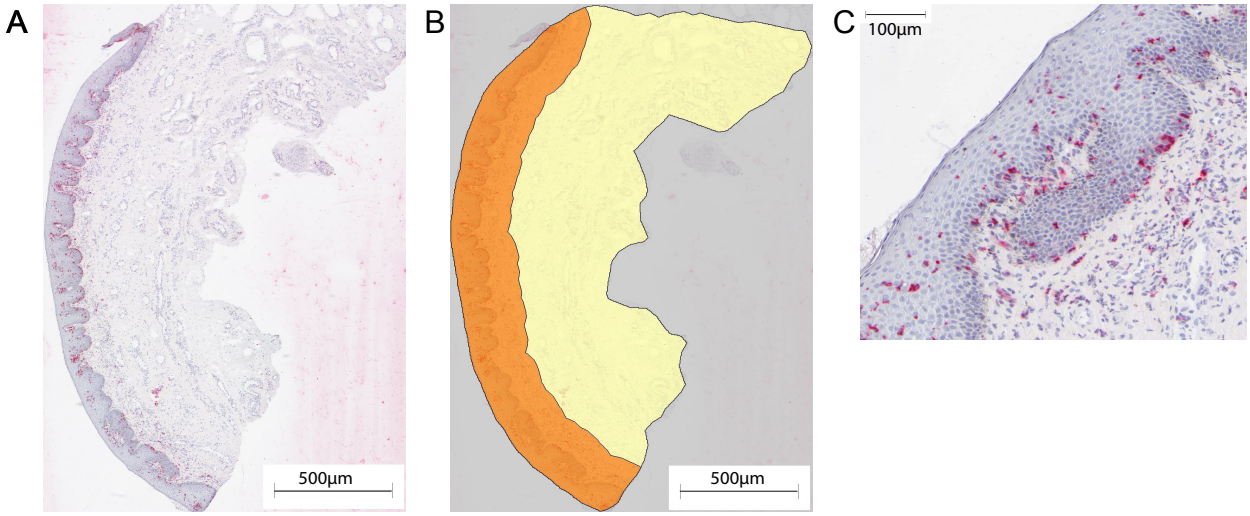

Figure 2.3 **Immunohistochemistry to determine average density of CD3 T cells in foreskin tissue.** Snap frozen tissue was sectioned and stained for CD3. Whole sections were scanned (A) and Defensins software was used to delineate the outer epidermal edge and trace a region 300µm in from this edge (orange area, B). Higher magnification images were created for the entire traced region (C), and CD3 positive events were manually counted in each image to obtain an average number of CD3 T cells per mm^2 for each section. Two sections from distal sites on the foreskin were analyzed to obtain an average number of CD3 cells per mm^2 for each patient.

Meyer's Hematoxylin, dehydrated in alcohols, cleared in xylene, and mounted in Permount (Fisher Scientific). The number of CD3+ T cells per mm^2 of tissue for each patient was derived from the average of two tissue sections taken from distal locations on the foreskin. A median of 6.10mm^2 of foreskin tissue was analyzed by IHC per patient. Whole sections were scanned at 0.5μm/pixel using the TissueScope 4000 (Huron Technologies, Waterloo, Canada). Image analysis software (Definiens, München, Germany) was used to delineate the apical edge of the epidermis and create fields of view (FOV) of the entire length of each section to a depth of 300μm (excluding artifacts or folds, see Figure 2.3). CD3 cells in each FOV were manually counted by an investigator blinded to group status. A CD3 positive cell was defined as nuclear hematoxylin staining overlapping with, or directly adjacent to, Vector Red staining.

2.5 Cytokine and Chemokine Analysis of Sub-preputial Swabs

Cytokine analysis was performed by Kamnoosh Shahabi, a technician in Dr. Kaul's laboratory. One 500μL aliquot of sub-preputial swab was assayed for cytokine levels using an electrochemiluminescent detection system. Two custom Human Ultra-Sensitive 7-spot kit from Meso Scale Discovery (Rockville, MD, USA) was utilized to assay the following 14 cytokine/chemokines in undiluted sub-preputial swabs: IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-17, TNFα, MCP-1, MDC (macrophage-derived chemokine), MIG (monokine induced by γ-interferon), MIP-1β, MIP-3α, RANTES, and IP-10 (Interferon gamma-induced protein-10). Plates were imaged using the Sector Imager 2400A platform (Meso Scale Discovery). The manufacturer suggests determining the lower limit of quantification (LLOQ) for each analyte on a per-plate basis. However, due to the large number of samples analyzed in this study multiples were required and as a result of inter-run variability slightly different LLOQ values were obtained. For initial analysis, sample measurements below the study LLOQ were imputed as the value of the LLOQ: IL-1α= 10.3 pg/mL; IL-8= 1.5 pg/mL; MCP-1= 0.6 pg/mL; MDC= 1250.0 pg/mL; MIG= 0.3 pg/mL; MIP-3α= 46.2 pg/mL; RANTES= 3.0 pg/mL; IL-10= 10.5 pg/mL; IL-1β= 9.9 pg/mL; IL-17=10.0 pg/mL; IL-6= 2.6 pg/mL; IP-10= 20.6 pg/mL; MIP-1β= 20.6 pg/mL; TNFα= 2.6 pg/mL. Subsequently, due to the low analyte concentrations in swabs, only levels of IL-8 were quantified, and all other analytes were treated as a binary outcome of either "detectable" or "undetectable".

Chapter 3
Foreskin T Cell Subsets Differ Substantially from Blood with Respect to HIV co-Receptor Expression, Inflammatory Profile, and Memory Status

3 Foreskin T Cells Subsets Differ Substantially from Blood with Respect to HIV co-Receptor Expression, Inflammatory Profile, and Memory Status

3.1 Introduction

As of 2009 there were 33.3 million people infected with HIV-1 (HIV), and only a third of those requiring treatment were receiving it[2]. In addition there were an estimated 2.6 million new infections in that year, the majority transmitted through heterosexual sex, emphasizing the urgent need for better HIV prevention strategies. Clinical trials have demonstrated that circumcision reduces HIV acquisition by 50-60% in heterosexual men, proving that the foreskin is the site of most acquisition in uncircumcised men exposed to HIV during insertive vaginal sex[22-24]. Although other penile sites such as the urethra may also play a role[363], the central role of the foreskin in HIV acquisition was further supported by the observation that an increased foreskin surface area correlated with increased risk of HIV acquisition[410]. However, the immune events that surround acquisition and establishment of productive infection in the foreskin are poorly defined[411]. Understanding the immunopathogenesis of HIV acquisition in the foreskin remains an important priority for the development of new prevention modalities, despite the efficacy of male circumcision, as evidenced by the fact that only a third of eligible men opted to avail themselves for free male circumcision during a recent HIV vaccine trial in South Africa[412].

In the cervix, HIV and SIV infection is initiated by a small founder population of infected $CD4^+$ T cells that expands through the local production of chemoattractant cytokines, followed by subsequent recruitment of activated memory $CD4^+$ T cells[137, 139]. It is likely that the efficiency with which this founder virus population expands depends on the immune milieu in

the genital mucosa at the time of exposure to HIV[413]. While resting $CD4^+$ T cells can be infected, viral replication within such cells is less efficient, and HIV propagation and dissemination from the site of initial infection is driven by the rapid recruitment of activated $CD4^+$ T cells in which the virus can more readily replicate[137, 177]. Recruitment of these activated $CD4^+$ T cells to the initial site of exposure may be assisted by HIV-induced changes in the local immune milieu, including the expression of chemokines such as MIP-3α and MIP-1β by epithelial and plasmacytoid dendritic cells[137, 142].

The presence or absence of certain T cell subsets at the mucosal site of HIV exposure may be an important determinant of HIV susceptibility. Genital herpes is associated with an increase in activated $CD4^+$ T cells within the foreskin and female genital tract[358, 414, 415], perhaps contributing to the three-fold increase in HIV susceptibility associated with this infection[352]. The pro-inflammatory Th17 cells that normally protect skin and mucosal sites against bacterial and fungal infection are present at high frequency in the female genital mucosa and display enhanced HIV susceptibility[75, 234]. IL-22 is an important effector molecule of Th17 cells, playing a role in epithelial integrity and repair. IL-22 is also produced by pro-inflammatory Th22 cells, which may be preferentially infected by HIV[416]. Conversely, $CD25^+$/$FoxP3^+$ (Forkhead box P3) T regulatory cells (Tregs) play an important role in controlling inflammation, and higher Treg frequencies in the blood have been linked to reduced HIV susceptibility[395]. Furthermore, individuals who are HIV exposed but seronegative (HESN) show a quiescent immune phenotype with reduced basal T cell cytokine production and lower proportions of activated T cells[371, 395, 396, 400].

While immunohistochemistry is able to demonstrate the tissue position of specific cells in three dimensions, the ability of this technique to define cellular immune function is very limited. Therefore we have developed techniques to isolate a single cell suspension from fresh foreskin tissues, and to characterize the frequency and function of foreskin T cell subsets using multiparameter flow cytometry. Our results demonstrate that the foreskin constitutes a pro-inflammatory immune environment that is enriched for HIV-susceptible T cell subsets.

3.2 Methods

3.2.1 Participants

Participants were recruited from men in an established community cohort in Rakai, Uganda[417], who had elected to undergo adult circumcision at the Rakai Health Sciences Program in Kalisizo, Uganda.

3.2.2 Samples and Assays Included in this Study

Foreskin tissue and blood was analyzed for T cell populations (flow cytometry).

3.2.3 Statistical Analysis

T cell populations were compared between blood and foreskin by paired Wilcoxon rank sum test. Statistical tests were run on SPSS v.17.0 for Mac (IBM; New York, NY, USA). Flow cytometry data was analyzed in FlowJo v.9.3 and Excel (Microsoft; Redmond, WA, USA) prior to statistical testing.

3.3 Results

3.3.1 Study Population

Participants were 46 HIV-negative men enrolled in a broader study of foreskin immunology[409].

3.3.2 T Cell Proportions in the Blood and Foreskin

Foreskin T cells were identified based on the expression of CD3, and comprised between 0.1-0.6% of total recorded events from digested, filtered foreskin tissue (Figure 2.2). Upon permeabilization, many contaminating events (non-$CD3^+$) were removed from the cell solution, so that $CD3^+$ events constituted 10-15% of total recorded events, allowing for easier identification of lymphocytes based on forward and side scatter alone. Due to differences in the size of foreskin samples and to variation in tissue physical properties leading to differential cell loss during the digestion procedure, the absolute number of CD3 cells per foreskin was not defined. Rather, we report proportions of cells, standardized to $CD3^+$, $CD3/4^+$ or $CD3/8^+$. The

majority of foreskin $CD3^+$ cells were found to express either CD4 (mean, 51.4% of $CD3^+$ cells) or CD8 (mean, 35.1%). Peripheral blood cells isolated from the same participants in parallel contained a higher proportion of $CD4^+$ cells (63.4%, p=0.0001) and slightly lower proportion of $CD8^+$ cells (31.8%, p=0.005, Figure 3.1A), resulting in a substantially reduced CD4/CD8 ratio in the foreskin compared to blood (1.53 vs. 2.27; p<0.0001). A small proportion of $CD3^+$ cells in both the foreskin and peripheral blood were found to express both CD8 and CD4 (0.41% and 0.84%, respectively, not significantly different). Of note, the foreskin contained more than twice as many $CD4^-/CD8^-$ (double negative) $CD3^+$ cells as the blood (12.4% vs. 5.1%, p<0.0001, Figure 3.1B).

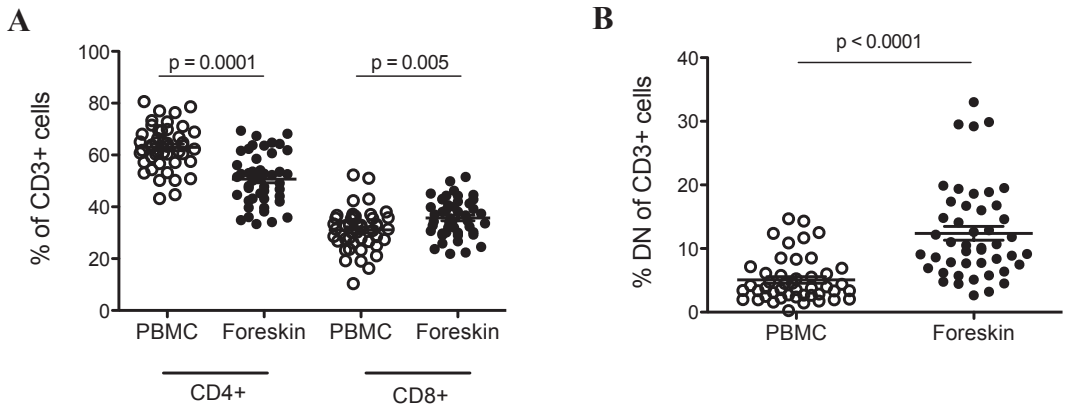

Figure 3.1 CD4+ and CD8+ T cell subsets within the foreskin and peripheral blood. PBMC and foreskin cells from 46 men were stained with CD3-FITC, CD4-PE, and CD8-PerCP. Graphs show percentages of $CD3^+$ cells within PBMC or foreskin cells that co-express (A) either CD4 or CD8, or (B) expressed neither CD4 nor CD8 (double negative, DN, T cells). Statistical comparisons were performed using the Mann-Whitney *U* Test.

3.3.3 CCR5 Expression and $CD4^+$ Th17 and T Regulatory Subsets in the Foreskin

The great majority of sexually transmitted viruses use CCR5 as a co-receptor[418]. Therefore, we compared the expression of CCR5 on $CD3/CD4^+$ between T cells isolated from the foreskin and the blood of study participants (Figure 3.2A). The proportion of foreskin $CD4^+$ T cells expressing CCR5 was over four-fold higher than that in blood (41.7% in the foreskin vs. 9.9% in PBMCs, p<0.0001, Figure 3.2B).

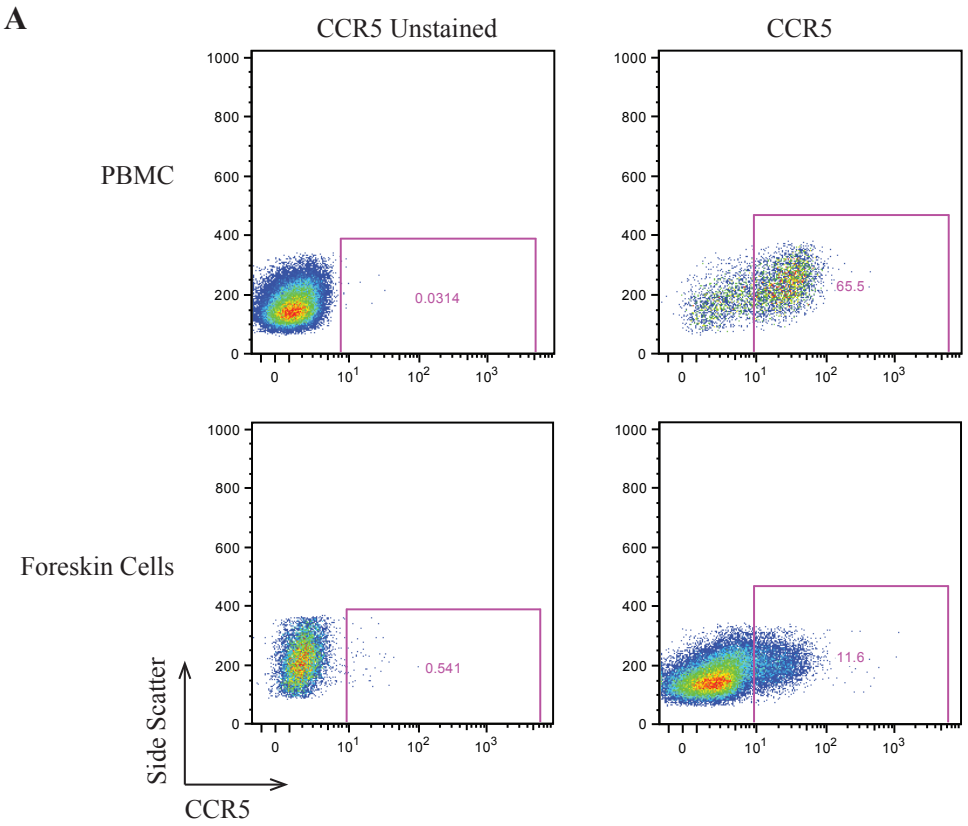

Figure 3.2 CCR5 expression on CD4+ T cells from the foreskin and peripheral blood. PBMC and foreskin cells from 46 men were stained with CD3-APC, CD4-PE, and CCR5-FITC. Plots in (A) were created by gating on $CD3^+/CD4^+$ events. The gate defining $CCR5^+$ events was created based on PBMC staining for this marker and applied to foreskin plots. (B) Proportions of $CD3^+/CD4^+$ cells in PBMC and foreskin cells co-expressing CCR5. Statistical comparisons were performed using the Mann-Whitney *U* Test.

Th17 cells may be preferentially infected by HIV[234] and the ratio of mucosal Th17/Treg cells is important in HIV immunopathogenesis.[419] Regulatory T cells (Tregs) were defined as

$CD3^+/CD4^+$ cells that co-expressed CD25 and the transcription factor FoxP3, and Th17 cells were defined as $CD4^+$ T cells producing IL17a, either at rest or upon stimulation with PMA-ionomycin. Substantial differences in Th17 subsets were seen between foreskin and blood. Specifically, a much higher proportion of isolated foreskin $CD4^+$ T cells produced IL17a, both unstimulated (3.3% of $CD3/4^+$ cells vs. 0.30%, $p<0.0001$) and after stimulation (7.4% vs. 3.8%, $p<0.0001$; Figures 3.3A and C). In addition, a higher proportion of $CD4^+$ T cells from the foreskin produced the Th17-associated cytokine IL22 than in the peripheral blood, both at rest (0.166% vs. 0.579%; $p<0.0001$), and after stimulation (1.09% vs. 2.88%; $p<0.0001$, Figures 3.3A and D). However, no difference was observed in the frequency of Tregs between the foreskin and blood (3.9% of foreskin $CD3/4^+$ cells, vs. 3.7% in blood; Figures 3.3B and E). As a consequence, the Th17/Treg ratio was considerably higher in the foreskin that in the blood (4.1 vs. 1.3, respectively; $p<0.0001$)

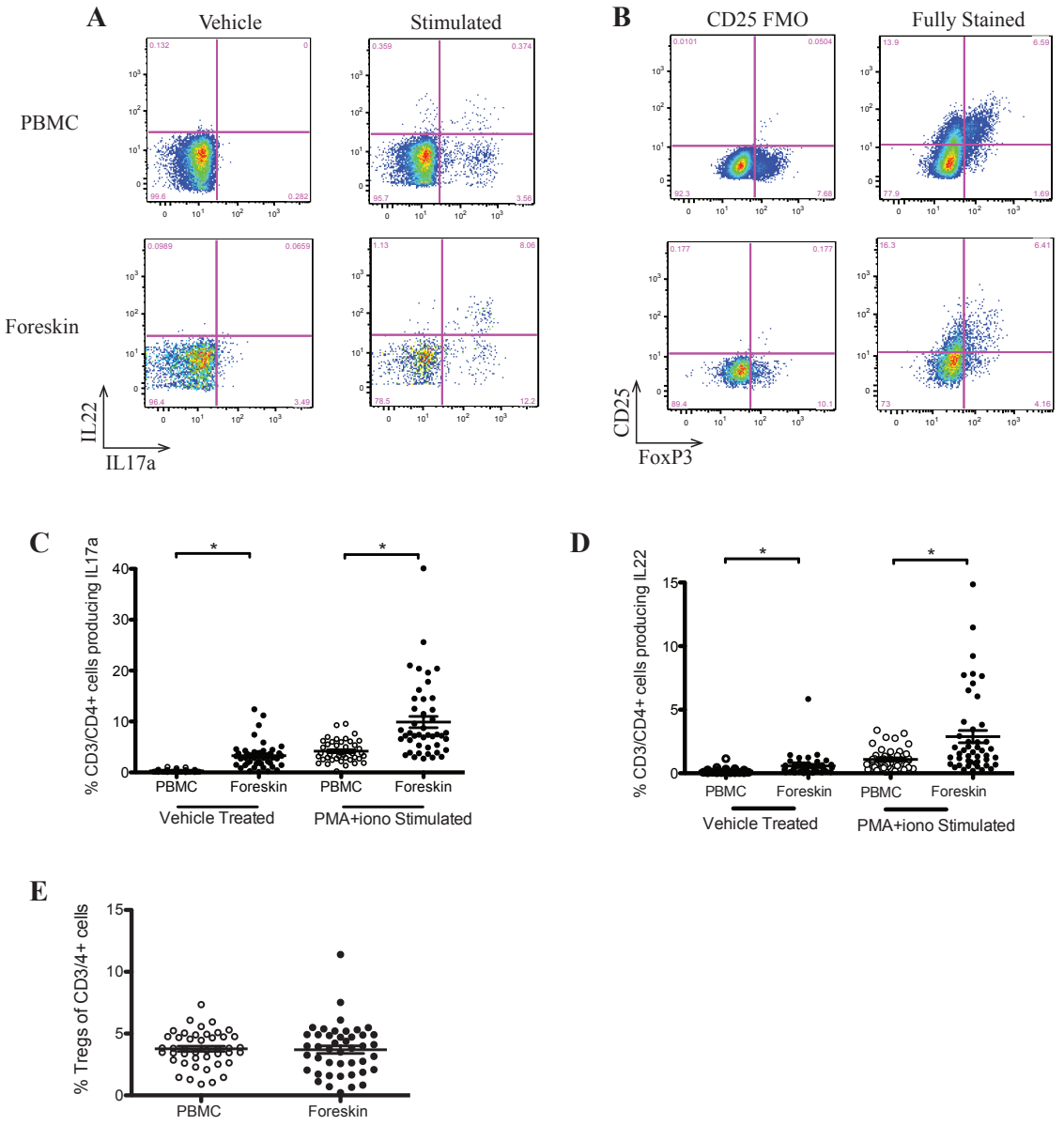

Figure 3.3 Increased production of IL-17/IL-22 by foreskin CD4 T cells, with no change in Treg frequency. PBMC and foreskin cells were either left unstimulated (vehicle/Treg) or treated with PMA-ionomycin (stimulated). Representative plots are shown (A, B). The gates in (A) defining IL17a+ and IL22+ events were created based on unstimulated PBMC staining for each patient, and then applied to stimulated PBMC and foreskin plots. The gate defining CD25+ events in (B) was created based on CD25-FMOs (fluorescence minus one = CD3, CD4 and FoxP3). (C) Proportions of Th17 cells in PBMC and foreskin samples ($CD3^+/CD4^+/IL17a^+$). (D) IL22 production in stimulated PBMC and foreskin CD4 T cells. (E) Proportions of PBMC and foreskin $CD3^+/CD4^+$ cells that are Tregs. Statistical comparisons were performed using the Mann-Whitney *U* Test, *p<0.0001

3.3.4 Foreskin $CD4^+$ T Cells Display a Predominantly Effector Memory Phenotype

The memory phenotype of foreskin and blood $CD4^+$ T cells was assessed in a subset of 3 individuals (representative plots; Figure 3.4) by staining with CD45RA to distinguish naïve ($CD45RA^+$) from memory T cells ($CD45RA^-$), and CCR7 to further delineate central (T_{CM}; $CD45RA^-/CCR7^+$) and effector (T_{EM}; $CD45RA^-/CCR7^-$) memory cells[173]. While blood contained approximately equal proportions of naïve and memory T cells, the foreskin contained few naïve T cells (ranging from 1.2 to 5.8%). Of the memory $CD4^+$ T cells in the foreskin, the majority was of the T_{EM} phenotype (72.6-89.5%).

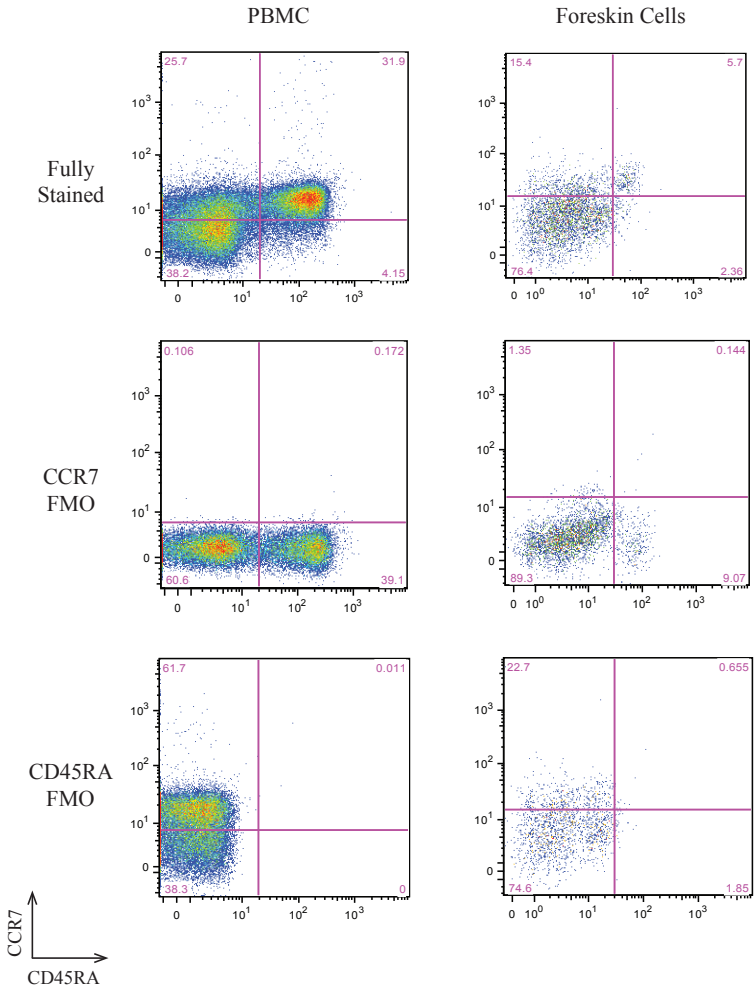

Figure 3.4 The foreskin contains primarily effector memory CD4 T cells (T_{EM}). Memory phenotype of PBMC and foreskin mononuclear cells was assessed on a sub-group of three men by staining with CD3-FITC, CD4-PerCP, CCR7-PE and CD45RA-APC. Representative plots were created by gating on $CD3^+/CD4^+$ events. The gates defining CD45RA+ and CCR7+ events were created based on FMO (Fluorescence minus one) staining for each cell type.

3.3.5 Capacity of Foreskin T Cells to Produce Pro-inflammatory Cytokines

Since an inflammatory mucosal immune milieu may enhance HIV acquisition[413], we next assessed the production of the cytokines IFNγ and TNFα by $CD8^+$ and $CD8^-$ T cell subsets, both at rest and after stimulation (Figure 3.5A). A relatively low frequency of foreskin T cells produced pro-inflammatory cytokines prior to stimulation, but this frequency was higher than blood T cells for both TNFα (2.8% vs. 0.47%; $p<0.0001$; Figure 3.5B) and IFNγ (0.33% vs. 0.19%; $p=0.048$; Figure 3.5C). Likewise, a higher frequency of foreskin $CD8^+$ T cells produced pro-inflammatory cytokines after mitogen stimulation: this was the case for both TNFα (45.3% vs. 39.4%, $p=0.0029$; Figure 3.5B), IFNγ (48.2% vs. 41.3%, $p=0.025$; Figure 3.5C), and for bi-functional cells co-producing both cytokines (35.2% vs. 28.6%, $p=0.035$; Figure 3.5D).

While both peripheral blood and foreskin contained a small proportion of "double negative" ($CD4^-/CD8^-$) T cells, the great majority of $CD3^+/CD8^-$ cells were $CD4^+$ T cells (Figure 3.1). Therefore, we also quantified TNFα and IFNγ production in these $CD8^-$ T cells as a proxy for $CD4^+$ T cells. A greater frequency of foreskin $CD8^-$ T cells than blood produced pro-inflammatory cytokines prior to stimulation (0.98% of foreskin cells produced TNFα vs. 0.29% of those from blood, $p<0.0001$; 0.48% of foreskin cells produced IFNγ vs. 0.18% of blood, $p<0.01$). After mitogen stimulation the foreskin contained more $CD8^-$ T cells producing IFNγ (45.1% of foreskin T cells vs. 40.9% of blood T cells, $p=0.0006$) and more bi-functional cells (20.6% vs. 15.9%, $p<0.0027$), although no differences in the frequency of cells producing TNFα were apparent between compartments.

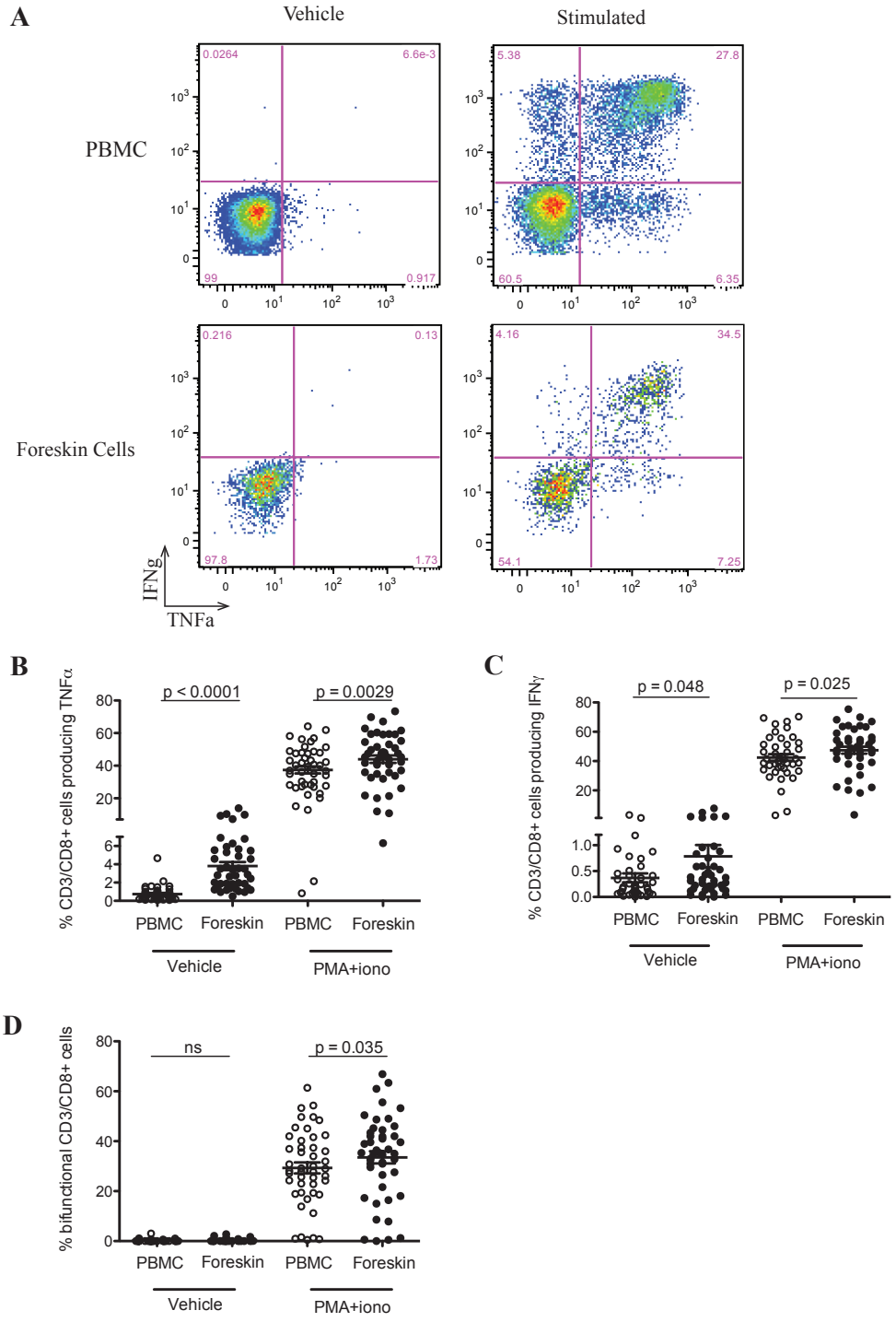

Figure 3.5 Enhanced production of pro-inflammatory cytokines by foreskin CD8+ T cells. PBMC and foreskin cells from 46 men were either left unstimulated (vehicle) or treated with PMA-ionomycin. Cells were then stained with CD3-FITC, CD8-PerCP, TNFα-PE and IFNγ-APC. Plots in (A) were created by gating on $CD3^{+}/CD8^{+}$ events. The gates defining TNFα+ and IFNγ+ events were created based on unstimulated PBMC staining for each patient, and then applied to stimulated PBMC and foreskin plots. (B) Proportions of CD8 T cells producing TNFα, (C) IFNγ, and (D) of bi-functional CD8 T cells (producing both TNFα and IFNγ). Statistical comparisons were performed using the Mann-Whitney *U* Test.

3.4 Discussion

While circumcision reduces the incidence of HIV by up to 60% in heterosexual African men[22-24], providing strong evidence that the foreskin is the main site of male HIV acquisition during vaginal sex[420], the immunobiology of HIV acquisition in the foreskin is poorly understood. Previous studies of genital immunology as it relates to the sexual acquisition of HIV have focused on the female genital tract and gut since samples are more easily obtained from these sites[421, 422]. While results of the recent circumcision trials have focused interest on the foreskin, immunology studies have often used cadaveric or fixed/cryopreserved tissues, precluding functional immune studies[147, 159, 165, 358, 423-425]. In collaboration with a clinical site providing safe and free male circumcision as an HIV prevention tool[23], we have developed field techniques utilizing expedited tissue processing and use of collagenase I for tissue digestion to isolate viable T cells from foreskin tissue with retention of the expression of T cell markers and the functional ability to produce multiple cytokines. This has allowed for the characterization of functional foreskin T cell subsets.

Several clear differences were evident between foreskin and blood T cell subsets, both in terms of proportions, expression of HIV co-receptor CCR5, memory phenotypes, and the production of pro-inflammatory cytokines. There was a relative enrichment of $CD8^+$ T cells in the foreskin compared to the blood, contributing to a significantly reduced CD4/CD8 ratio in the foreskin. However, although the proportion of $CD4^+$ T cells was reduced, the proportion of CD4 T cells in the foreskin that co-expressed CCR5 was over four times higher than in blood, potentially enhancing susceptibility to HIV infection. Increased expression of CCR5 relative to the blood has also been observed in the cervix, where CD4 T cells are 10x more likely to express CCR5[75]. HIV strains that use CCR5 as an entry co-receptor (R5 strains) are almost always responsible for sexual HIV transmission *in vivo*[418], and an *ex vivo* model has demonstrated that the foreskin is susceptible to infection with R5-tropic but not X4-tropic viruses[145, 359]. These results suggest that the substantial enhancement of CCR5 expression on foreskin-derived T cells may have direct implications for HIV acquisition.

Interestingly, the proportion of double negative T cells (i.e. $CD3^+$ but $CD4^-/CD8^-$) was twice as high in the foreskin than in blood. Various $CD3^+$ T cell populations may be contained within this subset, including NKT cells[426] and T cells bearing the variant T cell receptors (TCR) γδ[427]

or the regulatory TCR $\alpha\beta^+$[428]. Double negative $CD3^+$ T cells have been associated with protection against SIV immunopathogenesis in some primate species[429]. Elucidating the identity of these double negative cells using multiparameter flow cytometry and investigating their possible relevance for HIV transmission will be important areas for future study.

While phenotypic characterization of foreskin T cells has been possible using fixed or cryopreserved tissues, we were particularly interested to define their function directly *ex vivo*. Two $CD4^+$ T cell subsets that may be particularly relevant to HIV acquisition and pathogenesis are Th17 and regulatory T cells (Tregs)[419]. Th17 cells are $CD4^+$ T cells producing the cytokine IL17a, and play a prominent pro-inflammatory role in mucosal immune defense against invading bacterial and fungal pathogens through the IL17 mediated recruitment of neutrophils, induction of antimicrobial peptides, and maintenance of epithelial integrity[430]. Th17 cells display enhanced susceptibility to HIV *in vitro*[221, 234] and are preferentially depleted from the blood and particularly the mucosa of HIV-infected individuals[234, 421], suggesting their enrichment at mucosal surfaces might enhance HIV acquisition. Tregs have immunomodulatory effects that are thought to play an important role in counterbalancing Th17-induced inflammation, despite sharing a common precursor, chemokine receptors, and mucosal homing properties with Th17 cells[419]. We found that Th17 proportions were substantially increased in the foreskin compared to blood in the absence of any corresponding enrichment in Treg cells. Although Tregs were not examined, previous studies in the cervix have also reported an increased proportion of Th17 cells compared to the blood[75]. This increased Th17/Treg ratio in the foreskin suggests that this tissue is biased towards a predominantly pro-inflammatory immune environment, which could enhance HIV acquisition[413].

Our data showing enhanced production of the cytokines TNFα and IFNγ by foreskin T cells, both at rest and after non-specific stimulation, supports the concept of the foreskin as a pro-inflammatory tissue. This enhanced cytokine production is likely to be related to the high proportion of effector memory T cells (T_{EM}) found in the foreskin tissues, since this cell subset is primed to migrate to tissue sites and to carry out immediate effector functions[173].

It is likely that both the function and proportions of T cell subsets in the foreskin would be impacted by common bacterial and viral genital co-infections[358, 414], and any such differences might well have implications for HIV susceptibility. While men with symptomatic

genital infections were excluded from male circumcision due to the potential increased risk of post-surgical infection and other complications, asymptomatic genital infections such as HSV-2 and HPV are common in these men[431, 432]. While the purpose of our initial analysis was to compare T cell subsets in the foreskin and blood, recruitment of a larger participant sample size is ongoing with the goal of characterizing the immune impact of these infections.

While our study examined pooled T lymphocytes derived from both the inner and outer foreskin, there is *in vitro* evidence to suggest that HIV acquisition may be more efficient across the inner surface of the foreskin[156], defined as the portion of the foreskin that sits against the glans on the non-erect penis but is exposed on the erect penis during intercourse. It was initially assumed that this increased susceptibility was due to a thinner keratin layer on the inner foreskin[147, 156], but studies using freshly processed foreskin samples have shown no difference in this layer between the inner and outer foreskin[165, 424]. While reports of differences in the density of HIV target cells between these two sites have been contradictory[145, 147, 164, 359, 425], it does seem that cells of the inner foreskin may be functionally different to those of the outer foreskin, both in their responsiveness to cytokines such as TNFα and MIP1α[164] and in their production of chemokines after HIV exposure[156, 160, 164]. Better elucidation of the functional differences between T cells derived from the inner and outer foreskin will constitute an important area for future research.

In summary, we have developed novel techniques to purify a single-cell suspension from fresh foreskin tissues, and to characterize the functional characteristics of foreskin T cell populations. Compared to blood, the foreskin manifested a pro-inflammatory immune environment that was enriched for highly HIV-susceptible $CD4^+$ T cell subsets such as Th17 cells and those expressing the HIV co-receptor CCR5. These observations have important implications for HIV susceptibility in the foreskin, and will permit larger immuno-epidemiology field studies aiming to define the immune correlates of HIV susceptibility in the foreskin.

Chapter 4
Chemokine Levels in sub-Preputial Swabs Correlate with Foreskin Tissue Density of HIV-susceptible CD4 T Cell Populations

4 Chemokine Levels in sub-Preputial Swabs Correlate with Foreskin Tissue Density of HIV-susceptible CD4 T Cell Populations

4.1 Introduction

Male circumcision reduces HIV acquisition in heterosexual men by ~60%[433], suggesting that most HIV is acquired across the foreskin. However, despite the provision of free, safe circumcision services, many men decline services in Africa[434], making it important to understand risk factors for HIV transmission across the foreskin.

The sexual transmission of HIV is inefficient, and individual susceptibility to HIV is very heterogeneous. Studies of early SIV infection in macaques show that infection begins with a small population of infected CD4 T cells that grows through the recruitment of additional susceptible cells (for review see[142]). Therefore, the likelihood of HIV infection after exposure may be influenced by the availability of vulnerable CD4 T cell populations at the site of viral exposure (for review see [435]). Certain CD4 T cell subsets appear to be particularly susceptible to HIV, including activated CD4 T cells, Th1 cells, and Th17 cells, and the sexual transmission of HIV almost exclusively involves viral strains that use CCR5 as a co-receptor. This may be why conditions that increase local T cell density or activation are also associated with increased HIV acquisition[435], and suggests that it may be important to monitor the effect of candidate microbicides and other clinical interventions on mucosal HIV-susceptible cell populations. However, while longitudinal sampling of cervical and gut cells[436] is possible, this cannot easily be done in the foreskin. As a result, a surrogate for foreskin HIV-susceptible cell populations, which can be followed in a non-invasive fashion, is needed.

We sought to determine whether cytokines previously associated with T cell recruitment and activation[378, 437] could be detected in swabs taken from the sub-preputial space of uncircumcised men, and whether the level of these cytokines correlated with T cell density in foreskin tissue obtained after elective circumcision.

4.2 Methods

4.2.1 Study Participants

Participants were 89 HIV-negative men enrolled in a broader study of foreskin immunology[409].

4.2.2 Samples and Assays Included in this Study

Foreskin tissue and blood was analyzed for T cell populations (flow cytometry and CD3 IHC).

4.2.3 Statistical Analysis

Cytokine concentrations were correlated with T cell densities by simple linear regression; values below the LLOQ were imputed as the value of the LLOQ. Multiple comparisons were corrected for using a Bonforonni correction (adjusted $\alpha = 0.005$ to account for correlation with 10 different cell populations). Statistical tests used SPSS v.20.0 for Mac (IBM; New York, NY, USA) and graphs were created using Prism v5 for Mac (GraphPad Software; La Jolla, CA, USA). Flow cytometry data was analyzed in FlowJo v.9.5.2 (Treestar; Ashland, OR, USA).

4.3 Results

Participants included 89 HIV-uninfected men free of symptomatic genital infections undergoing elective adult circumcision (Table 4.1; no behavioral characteristics correlated with cytokine levels). Of the 14 cytokines/chemokines assayed, only IL-8, MCP-1, MIG, and RANTES were detectable in the sub-preputial swabs. IL-8 was detectable in the majority of men (89.9%) at a median concentration of 23.0 pg/mL (IQR 4.07-125.31 pg/mL, max 5140.48 pg/mL). MIG was detectable in 52.8% of men at a median concentration of 0.34 pg/mL (IQR <0.30-1.32 pg/mL, max 18.40 pg/mL). MCP-1 and RANTES were undetectable in the majority of swabs (MCP-1 detected in 47.2% of men, max 13.63 pg/mL; RANTES in 12.4%, max 8.98 pg/mL, Table 4.2).

The robust detectability of IL-8 (median concentration in swabs was more than 15-fold higher than the LLOQ, and it was detected over a $\log_{10}$ 3 range of concentration between men) allowed it to be correlated with the density of T cell populations in foreskin tissue.

Table 4.1 Participant demographics.

		Frequency	Percent
Age	18-29	22/89	24.7
	30-34	26/89	28.1
	35-39	19/89	21.3
	40+	23/89	25.8
HSV-2	negative	48/87	55.2
	positive	39/87	44.8
Self Reported STI Symptoms (past 30 days)	urethral discharge	0/89	0.0
	balanitis/itching	3/89	3.4
	ulcers	0/89	0.0
	warts	0/89	0.0
Condom use	never	69/89	77.5
	sometimes	17/89	19.1
	always	3/89	3.4
Sex partners in the last year	single	66/89	74.2
	multiple	23/89	25.8
Current extramarital relationship		13/89	14.6

Table 4.2 Cytokine and chemokine levels in sub-preputial swabs.

	Frequency of detection (%)	Median (IQR) (pg/mL)	Maximum (pg/mL)
IL-8	80/89 (89.9)	23.00 (4.07, 125.31)	5140.48
MCP-1	42/89 (47.2)	--	13.63
MIG	47/89 (52.8)	0.34 (<0.30, 1.32)	18.40
RANTES	11/89 (12.4)	--	8.98

Foreskin T cell subsets were quantified using flow cytometry, and the tissue density of each subset was subsequently calculated using CD3 IHC. Levels of IL-8 in the prepuce correlated with an increased foreskin density of almost all CD4 (CCR5+, Th17, Th1, TNFα+) and CD8 (IFNγ+ and TNFα+) T cell subsets examined, albeit with a high degree of variability (all p≤0.02); T regulatory cells, whose presence may decrease HIV susceptibility, were the only exception (p=0.2). After correcting for multiple comparisons, correlations with the density of bulk CD4 T cells remained significant (p=0.005), including significant correlation with density of HIV-susceptible Th17 cells (p=0.005) and TNFα-producing CD4 T cells (p=0.002) (Figure 4.1). Of note, we have previously found the relative abundance of these specific CD4 T cell populations to be reduced in men who are HIV-exposed but remain seronegative (HESN, see Chapter 7).

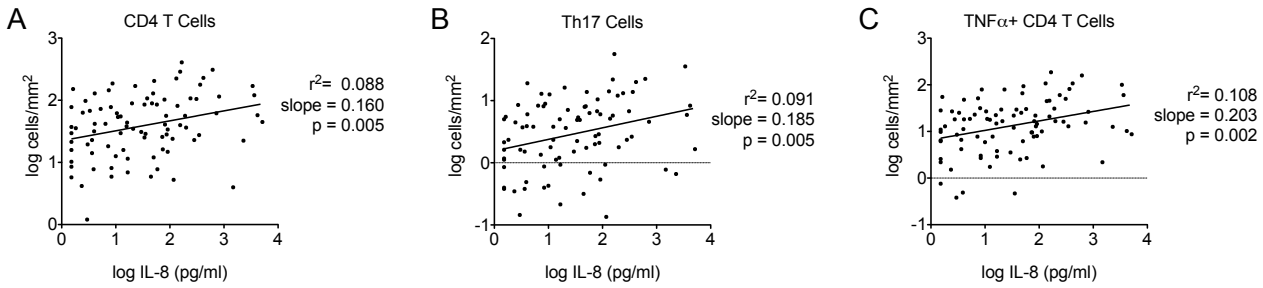

Figure 4.1 Correlation of concentration of IL-8 in the sub-preputial space with densities of T cell populations in foreskin tissue. IL-8 was measured in sub-preputial swabs taken immediately prior to circumcision, and densities of T cell subsets were measured in foreskin tissue using flow cytometry and immunohistochemistry. Correlations of $\log_{10}$IL-8 with T cell populations that remained significant after correcting for multiple comparisons ($p<0.005$) are displayed (all $\log_{10}$ transformed): (A) bulk CD4 T cells/mm^2, (B) Th17/mm^2, and (C) TNFα+ CD4 T cell/mm^2. Statistical significance of correlation made with Spearman's rho.

4.4 Discussion

We assayed sub-preputial swabs for levels of 14 chemokines and cytokines associated with T cell recruitment and activation. While MCP-1, MIG and RANTES could be detected in swabs, only concentrations of IL-8 were high enough to assess correlations with T cell density in the underlying foreskin tissue. We found that IL-8 concentration in swabs correlated with the overall density of CD4 T cells in the foreskin, as well as with the density of both Th17 cells and TNFα-producing CD4 T cells.

IL-8 was the most robustly detected cytokine in the sub-preputial swabs, due to a combination of the high concentration of IL-8 in swabs (median 23.0 pg/mL) and the relatively low LLOQ of our assay for this analyte (1.5 pg/mL). Although statistically very robust, the strength of the correlation between IL-8 and T cell populations was relatively weak. This may reflect complexity in the causal relationship between IL-8 levels and T cell density. IL-8 is a chemokine produced by both T cells and many other cell types, including epithelial cells and various immune cell subsets. It is highly chemotactic for T cells and other leukocyte subsets, especially neutrophils[438]. Therefore, it is impossible in this cross-sectional study to determine if higher concentrations of IL-8 lead to increased T cell recruitment, or if they are reflective of it, or both. Nonetheless, IL-8 concentrations correlate with the density of both Th17 and TNFα-producing CD4 T cells: T cell population previously shown to be reduced in men who have been exposed to HIV but remained uninfected. We therefore propose that IL-8 concentration could be used as an approximate indicator of HIV-susceptible T cell numbers, such that an intervention associated with increased IL8 levels on the foreskin would likely reflect an increase in tissue HIV-susceptible T cell populations.

While the presence of MCP-1, MIG and RANTES was also associated with increased foreskin T cell density (data not shown), these cytokines were less easily detected, and the associations were less robust. Interestingly, the only association of these cytokines that remained significant after correction for multiple comparisons was the presence of MIG with CD8 T cell density ($p<0.005$), which is consistent with MIG's known role as a CD8 T cell chemoattractant[439].

The low concentration of analytes in foreskin swabs was a limitation of our study. Many of the cytokines assayed were below the LLOQs for our assay (IL-1α, IL-1β, IL-6, IL-10, IL-17, TNFα,

MDC, IP-10, MIP-3α, and MIP-1β) or were detected only in a limited number of participants (MCP-1, MIG and RANTES). The inability of our assay to detect low levels of these cytokines does not preclude them as important markers of tissue T cell activation, but suggests that they may be less useful surrogate endpoints for swab-based field studies. Further refinement of collection techniques, including collection into a smaller volume of transport medium, may improve detection of additional cytokines/chemokines.

In conclusion, the concentration of IL-8 in sub-preputial swabs correlated with tissue density of CD4 T cells, including highly HIV-susceptible T cell populations such as TNFα-producing and Th17 cells. The longitudinal assessment of IL-8 in preputial swabs may therefore provide information regarding foreskin T cell populations in the context of male-focused HIV prevention trials, or in other studies of HIV risk among men who choose to remain uncircumcised.

Chapter 5
Impact of Asymptomatic Herpes Simplex Virus-2 Infection on T Cell Phenotype and Function in the Foreskin

5 Impact of Asymptomatic Herpes Simplex Virus-2 Infection on T Cell Phenotype and Function in the Foreskin

5.1 Introduction

Susceptibility to HIV is heterogeneous[441], and chronic infection by Herpes simplex virus type 2 (HSV-2) has been associated with an approximately three-fold increase in HIV acquisition by both men and women[352]. However, most HSV-2 is asymptomatic and suppressive acyclovir in HSV-2 seropositive individuals did not reduce HIV acquisition despite reductions in clinical ulceration[442], suggesting that increased HIV susceptibility is not solely due to compromised epithelial integrity. A recent study has shown the density of CD4 T cells expressing the HIV co-receptor CCR5 is increased at the site of herpetic ulcers, even after 20 weeks of acyclovir treatment with no recurrence of ulceration and no detectable HSV DNA[415]. Additionally, a higher proportion of CD4 T cells in the cervix of HSV-2 seropositive women co-express CCR5[414], even in the absence of prior symptomatic herpes or asymptomatic local viral reactivation[443], suggesting that changes in the cervical immune microenvironment may increase a woman's HIV susceptibility during asymptomatic HSV-2 infection.

Less is known about the immune correlates of HIV susceptibility in HSV-2^{+} men. The rollout of male circumcision (MC) in sub-Saharan Africa as an HIV prevention strategy has increased opportunities to address this gap. Studies from Rakai, Uganda demonstrated an increased density of foreskin CD4 T cells in HSV-2-infected men[358], but the functional characteristics of mucosal CD4 T cells may also be an important determinant of HIV susceptibility[435]. We recently established techniques to isolate viable T cells from preputial tissues[409], and demonstrated that, compared to the blood, the foreskin manifests a pro-inflammatory immune

microenvironment, with high production of TNFα and IFNγ by CD8 T cells and high expression of CCR5 by CD4 T cells. We also found the foreskin to contain a more Th17 cells, which are important in mucosal defense against bacterial and fungal infections but are highly susceptible to HIV infection [75, 221, 234] and promote a pro-inflammatory immune environment. However, there was no corresponding increase in immunomodulatory T-regulatory cells (Tregs), which decrease local immune activation and may reduce HIV susceptibility *in vivo* [395].

Based on these findings, we hypothesized that asymptomatic HSV-2 infection would be associated with increased levels of CCR5 expression, enhanced inflammatory cytokine production, and more Th17 cells within the foreskin.

5.2 Methods

5.2.1 Participants

Participants were 87 HIV-negative men enrolled in a broader study of foreskin immunology[409].

5.2.2 Samples and Assays Included in this Study

Foreskin tissue and blood was analyzed for T cell populations (flow cytometry).

5.3 Results

5.3.1 Study Population

Foreskins and blood were obtained from 87 HIV-negative men, 39 of whom were found to be positive for HSV-2 by serology. All participants were free of symptoms or signs of genital infections (ulceration, dysuria or urethral discharge) at the time of surgery; one HSV-2 seropositive participant reported mild balanitis that was not apparent on physical exam. HSV-2 seropositive participants were older (median 36 vs. 32 years; Pearson p=0.013) and were more

likely to have more than one sex partner over the past year (38.5% vs. 16.7%; p=0.022), although condom use did not vary by group (data not shown).

5.3.2 T Cell Proportions in the Blood and Foreskin

The proportion of $CD3^+$ cells expressing CD4 was non-significantly higher in both the blood (65.2 vs. 61.3%, p = 0.172) and the foreskin (53.1 vs. 49.3%, p = 0.096) of $HSV\text{-}2^+$ individuals, and foreskin T cell density was not assessed. Since sexual HIV-infection occurs almost exclusively with viral strains using CCR5 as a co-receptor, expression of CCR5 on these CD4 T cells was also measured. A significantly higher proportion of foreskin CD4 T cells from $HSV\text{-}2^+$ men co-expressed CCR5 (45.6 vs. 37.7%, p = 0.024; Figure 5.1A), and this association remained after controlling for age (p=0.017) and number of sex partners (p=0.009). CCR5+ expression did not vary by HSV-2 status in the blood compartment (p = 0.582).

However, there were no associations between HSV-2 infection and the frequency of $CD4^+$ Th17 and T regulatory cells (Tregs) in the foreskin (foreskin: Th17: 6.1 vs. 6.2%; Treg: 3.4 vs. 3.3%; Figure 5.1B). Likewise, HSV-2 infection was not associated with differences in the capacity of foreskin or blood $CD8^+$ T cells to produce the pro-inflammatory cytokines TNFα or IFNγ (foreskin: TNFα: 39.6 vs. 37.5%; IFNγ: 44.9 vs. 41.1%; Figure 5.1C).

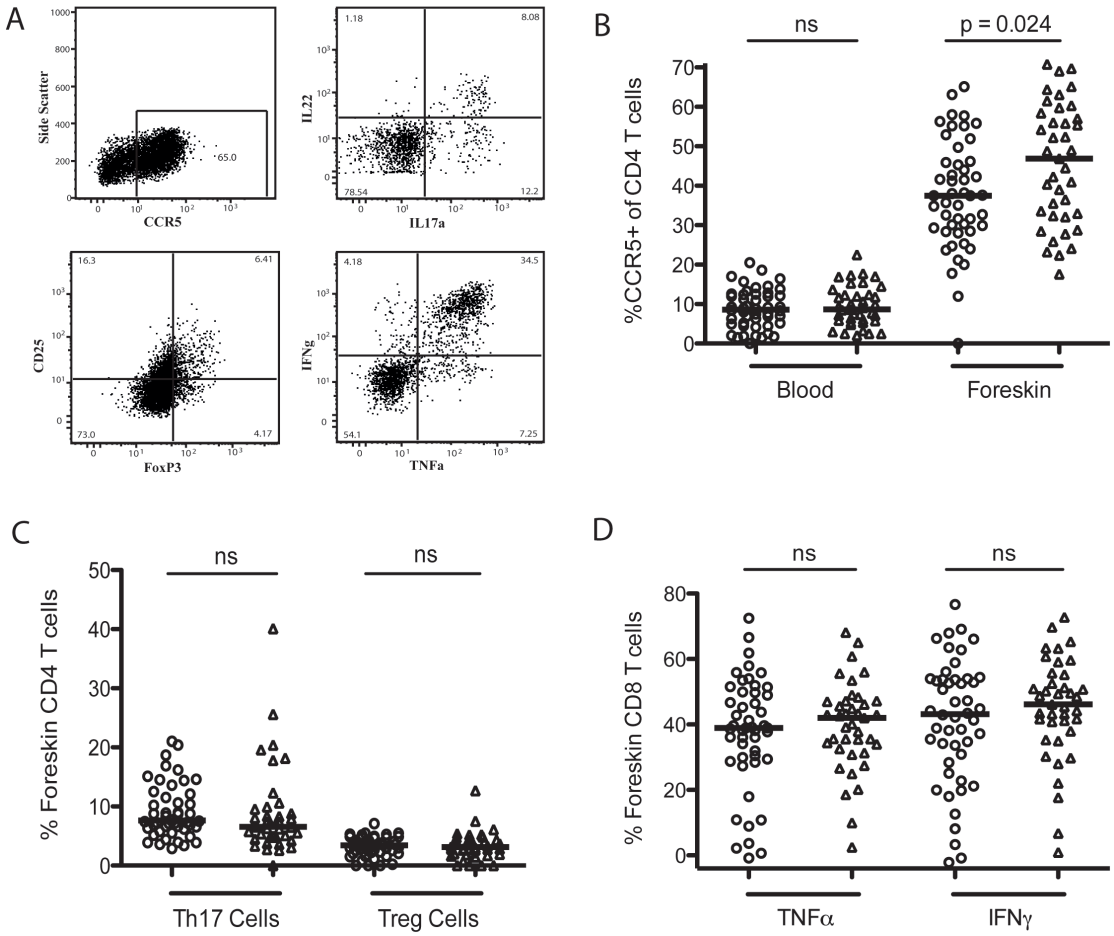

Figure 5.1 Immune associations of HSV-2 infection in the foreskin.

Impact of HSV-2 infection status on: the proportion of CD4 T cells from the blood and foreskin expressing the HIV co-receptor CCR5 (B); the proportion of CD4 T cells from the blood and foreskin that are Th17 cells (producing IL-17a upon stimulation) or Tregs (co-expressing CD25 and FoxP3; (C); and on the capacity of CD8 T cells to produce the pro-inflammatory cytokines IFNγ and TNFα upon stimulation (D). Representative flow plots presented in (A); plots presented in more detail in Chapter 3. Statistical comparisons were performed using the Mann-Whitney *U* Test.

5.4 Discussion

The foreskin is the site of most HIV acquisition in uncircumcised men, in part because (compared to blood) it constitutes a pro-inflammatory mucosal immune environment. Specifically, the foreskin demonstrates higher CD4 T cell expression of the HIV co-receptor CCR5, Th17 cell frequency and CD8 T cell production of pro-inflammatory cytokines[409]. While we hypothesized that increased HIV susceptibility in HSV-2-infected men might relate to

accentuation of each of these factors, we found that the major immune association of asymptomatic HSV-2 infection in the foreskin was an increase in the expression of CCR5 by CD4 T cells. Therefore, HSV-2 is not only associated with an increased density of CD4 T cells in the foreskin, as we have shown previously[358], but these CD4 T cells also express higher levels of CCR5. This compartmentalized increase of CCR5 expression on foreskin CD4 T cells may contribute to HSV-2-associated increases in HIV susceptibility.

Chapter 6
HIV Infection in Uncircumcised Men is Associated with Altered CD8+ T cell Function but Normal CD4+ T cell Numbers in the Foreskin

6 HIV Infection in Uncircumcised Men is Associated with Altered CD8+ T cell Function but Normal CD4+ T cell Numbers in the Foreskin

6.1 Introduction

Infection with Human Immunodeficiency Virus type 1 (HIV) is associated with CD4 T cell loss in the peripheral blood, systemic immune activation and exhaustion, and a progressive increase in the risk of opportunistic infections and certain cancers. There is also substantial CD4 T cell loss within the gut mucosa, which is thought to impair the gut mucosal barrier, leading to microbial translocation and systemic immune activation [140, 444]. CD4 depletion is also apparent within the female genital tract mucosa [445, 446], which may lead to increased host susceptibility to genital infections such as herpes, candidiasis and new/persistent human papillomavirus (HPV).

Male circumcision reduces HIV incidence by 50-60%[22-24], proving that the foreskin is the site of most HIV acquisition in heterosexual, uncircumcised men. In addition, circumcision reduces male susceptibility to HPV and herpes simplex virus 2 (HSV-2), suggesting that these pathogens are also acquired via the foreskin. While the immune defenses of the foreskin are poorly understood, normal adult foreskin contains numerous T cells and dendritic cells, with enrichment of T cell subsets such as Th17 cells that have important antibacterial and antifungal functions. The effect of HIV on these functional T cell subsets is not known.

HIV-infected men are more susceptible to herpes simplex virus-2 (HSV-2) infection and reactivation than their uninfected counterparts, and experience more frequent genital ulcers (GUD)[447-449]. In addition, HIV-infected women have an increased incidence of HPV,

gonorrhea, chancroid, candidiasis and trichomoniasis [447, 450-453]. As well as being more susceptible to new infections, HIV-infected individuals are less likely to clear an HPV infection[454]. Additionally, HIV-infected men are susceptible to subsequent HIV infection with distinct strains of HIV, referred to as superinfection[455]. This increased rate of genital co-infections has important implications for the sexual health of HIV-infected individuals, and the resulting local inflammation may also increase HIV shedding and the likelihood of HIV transmission to sexual partner(s)[456-464].

We hypothesized that the increased susceptibility to foreskin-acquired infections in HIV-infected men might be caused by HIV-induced immune changes in the foreskin, particularly the loss of CD4 T cell subsets such as Th17 cells. In order to investigate this hypothesis, we collected blood and foreskin tissue from adult men undergoing elective, male circumcision at the Rakai Health Science Program (Uganda), and used flow cytometry and immunohistochemistry (IHC) to determine if HIV infection was associated with alterations in foreskin T cell populations.

6.2 Methods

6.2.1 Study Participants

Participants consisted of 90 men enrolled in a broader study of foreskin immunology[409]. HIV-exposed seronegative (HESN) men who reported regular unprotected sex with an HIV-infected woman were excluded from this analysis to avoid any potential bias due to mucosal immune changes in this population (see Chapter 7). Participants were offered voluntary HIV counseling and testing, and HIV-infected men were referred to the Rakai Health Sciences HIV care and treatment program.

6.2.2 Samples and Assays Included in this Study

Sub-preputial swabs were analyzed for cytokine levels; foreskin tissue was analyzed for T cell populations (flow cytometry, PCR and CD3 IHC).

6.2.3 Detection and Quantification of mRNA

Detection and quantification of mRNA was performed by our collaborator, Dr. Taha Hirbod, at the Karolinska Institute, as they have previously described[159]. In brief, foreskin samples treated with RNAlater solution were thawed and disrupted in lysis buffer using a mechanical rotor. RNA was then extracted using RNeasy kit according to the manufacturer's protocol (QIAgen; Hilden, Germany) and converted in equal dilutions to cDNA in a single reverse transcriptase reaction using superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexanucleotide primers (Roche, Basel, Switzerland). Amplification of Ubiquitin C (UBC), CD3, CD4, and CD8 cDNA was performed using the ABI PRISM 7700 sequence detection system and commercial FAM dye-labeled TaqMan MGB probes and primers (Applied Biosystems, Foster City, CA, USA). Each sample and control was run in triplicate. Relative quantity (RQ) of target cDNA was computed by using the comparative threshold (Ct) method[465]. Ct values for target cDNA were normalized to UBC by using the normalized expression ratio 2^{-dCT}, so that amounts are described as a relative quantity to UBC.

6.2.4 Statistical Analysis

T cell populations and levels of mRNA and IL-8 were compared between HIV-infected and negative men by Mann-Whitney U test. Variables found to be associated ($p<0.1$) with HSV-2 status or condom use were controlled for by multivariate general linear regression and adjusted p-values are reported. Proportions of men with HIV-specific CD8 T cell responses and detectable MCP-1, MIG, and RANTES were compared by Fisher's exact test; differences in the magnitude of HIV-specific response between blood and foreskin CD8 T cells were determined using paired Wilcoxon signed rank test. Statistical tests were run using SPSS v.20.0 for Mac (IBM; New York, NY, USA). Flow cytometry data was analyzed in FlowJo v.9.5.2 (Treestar; Ashland, OR, USA) and Excel v.12.3.5 (Microsoft; Redmond, WA, USA) prior to statistical testing.

6.3 Results

6.3.1 Study Population

Participants consisted of men with prevalent HIV infection (n=20) and HIV-uninfected men (n=70) from Rakai, Uganda who were undergoing elective male circumcision. HIV-infected men had a mean viral load of 30,690 copies/mL (range ≤39 - 335,900 copies/mL) and a mean peripheral blood CD4 T cell count of 409 cells/cm^3 (range 6-935 cells/cm^3). HIV-infected men were more likely to be co-infected with HSV-2 than HIV-uninfected men (90% vs. 35%, p<0.001; Table 1), and all subsequent immune analyses were controlled for HSV-2 status.

Table 6.1: Patient demographics

		HIV+ (n=20)	HIV- (n=70)	sig.
Age (yrs)		38 (28-50)	35 (22-51)	ns
HSV-2 serology (%)		90.0	35.7	<0.001
Viral load (copies/mL)		30,693 (≤39 - 335,903)	≤39	n/a
CD4 count (cells/mm^3)		409 (6 - 935)	878 (144-2,172)	<0.001
ART (%)	ART-naive	85.0	n/a	n/a
	Recent initiation*	15.0	n/a	n/a
On TMP/SMX** (%)		25.0	n/a	n/a
Condom use (%)	Always	10.0	17.1	<0.001
	Sometimes	40.0	2.9	
	Not using	50.0	80.0	
Sexual partners in past year (%)	Single partner	75.0	86.0	ns
	Multiple partners	25.0	14.0	
Extramarital relationship (%)		10.0	14.3	ns

* Defined as initiation within 6 months, with detectable viral load at the time of circumcision; exclusion of these 3 individuals did not alter analysis outcome.
** TMP/SMX: trimethoprim and sulfamethoxazole.

6.3.2 Foreskin CD4 and CD8 T Cell Proportions

In HIV-uninfected participants the blood T cell CD4/CD8 ratio was 2.2 (64.9% CD4 vs. 30.1% CD8; Figure 6.1B), while the foreskin had a higher proportion of CD8 T cells, resulting in a ratio of 1.5 (51.7% CD4 vs. 34.0% CD8, $p<0.001$ vs. blood; Figure 6.1A). In HIV-infected individuals, the CD4:CD8 T cell ratio was significantly lower in both the blood (0.6 in HIV-infected men, vs. 2.2 in HIV-uninfected men, $p<0.001$) and the foreskin (0.3 vs. 1.5, $p<0.001$). While the decreased CD4:CD8 ratio in the blood of HIV-infected individuals was due to both an increase in CD8 T cell numbers and a loss of CD4 T cells (absolute count 409.0 vs. 877.8 CD4 T cells/mm^3 blood, in HIV+ vs. HIV-, $p<0.001$, Figure 6.1D), in the foreskin the reduced ratio was driven solely by an increase in the absolute number of CD8 T cells. HIV-infected men had over 4-fold more CD8 T cells/mm^2 of foreskin tissue (108.8 vs. 23.1/mm^2, $p<0.001$, Figure 6.1C), with no reduction in the absolute number of CD4 T cells (43.0 vs. 33.7/mm^2, $p=0.67$). This was observed in conjunction with increased levels of chemokines in the sub-preputial space; HIV – infected men had increased level of IL-8 (97.1 vs. 18.8 pg/ml, $p=0.04$, Figure 6.1F) and were more likely to have detectable MIG (85.0 vs. 54.3% of men, $p=0.002$, Figure 6.1G) than HIV-uninfected men. Frequency of detection of MCP-1 and RANTES did not differ between HIV-infected and uninfected men. IL-1α, MDC and MIP-3α were not detected in sub-preputial swabs.

Tissue levels of CD3, CD4 and CD8 mRNA were then quantified by PCR in corresponding foreskin biopsies (Figure 6.1E). Confirming our flow cytometry and immunohistochemistry results, we found no change in expression of CD4 mRNA in the foreskins of HIV-infected men, but instead an increase in the expression of both CD3 ($8.0x10^{-3}$ vs. $5.6x10^{-3}$ RQ, $p=0.047$) and CD8 ($17.8x10^{-3}$ vs. $7.3x10^{-3}$ RQ, $p<0.001$) mRNA.

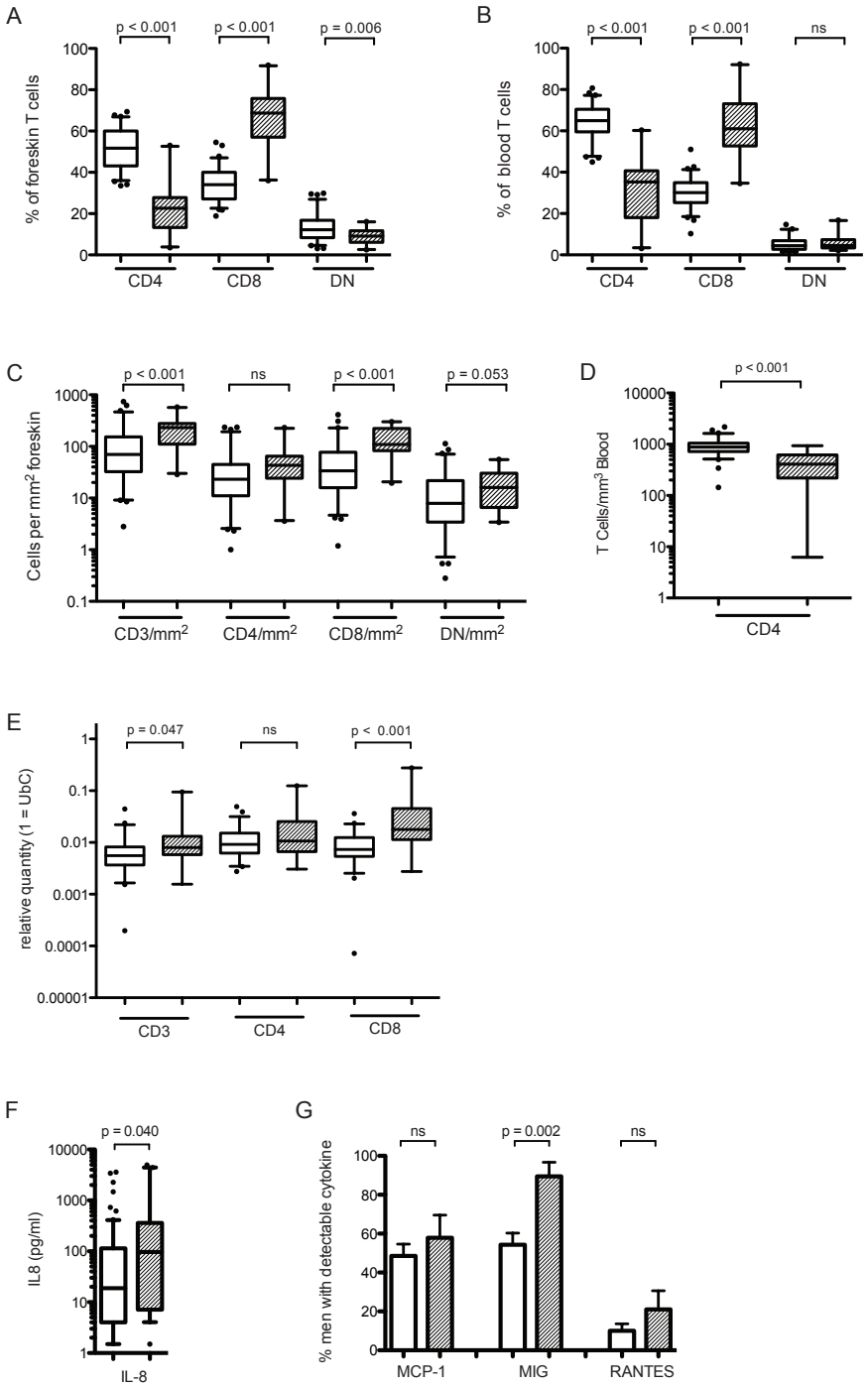

Figure 6.1 T cell subsets in the blood and foreskin of HIV-infected (hatched bars) and uninfected (open bars) men. Relative proportions of CD4, CD8 and double negative (DN) CD3+ T cells were measured in the foreskin (A) and blood (B) using flow cytometry. CD3 T cells per mm^2 (C) was obtained through IHC and used to calculate the absolute numbers of cells from flow cytometry proportions; absolute numbers of blood CD4 T cells were obtained from clinical CD4 counts (D). Foreskin tissue quantities of T cell markers were confirmed with PCR (E). Statistical comparisons made by Mann-Whitney U test; CD3, CD4 and CD8 T cell densities controlled for HSV-2 status by multivariate general linear regression and adjusted p-values are reported.

6.3.3 CD4 T Cell Subsets in the Foreskin

We next assessed the impact of HIV infection on the frequency of CCR5 expression, Th17 cells and T regulatory cells (Tregs) in the blood and foreskin. The relative proportion of each CD4 T cell type was determined using flow cytometry, and these proportions were converted to absolute numbers using either the blood CD4 T cell count or the foreskin CD3+ cell density measured by IHC.

After controlling for HSV-2 status, there were no differences in the proportion (Figure 6.2A) or absolute number (Figure 6.2B) of foreskin CD4 T cells expressing CCR5. There were also no changes in the proportion of blood CD4 T cells expressing CCR5 in HIV-infected men (Figure 6.2A). However, due to the overall depletion of CD4 T cells from the blood, the absolute number of CCR5+ CD4 T cells in the blood of HIV-infected men was decreased (25.7 vs. 72.6 cells/mm^3, p=0.001, Figure 6.2B).

Th17 cells were defined as CD4 T cells producing the cytokine IL17a in response to mitogen stimulation. HIV infection was not associated with any alteration in the proportion of Th17 cells in either the foreskin or the blood (Figure 6.1C); the absolute number of Th17 cells was significantly decreased in the blood (15.2 vs. 30.9 cells/mm^3, p=0.019) but not the foreskin (2.61 vs. 2.71 cells/mm^2, p=0.694) of HIV-infected men (Figure 6.1D).

Tregs were defined as CD4 T cells co-expressing CD25 and the transcription factor FoxP3. While the proportion of Tregs was increased in the blood of HIV-infected men (4.7 vs. 3.5%, p=0.041, Figure 6.2E), the absolute number of Tregs/mm^3 was decreased in HIV-infected men (16.4 vs. 30.3 cells/mm^3, p=0.003, Figure 6.2F). There were no HIV-associated alterations in either the proportion or absolute number of Tregs in the foreskin (Figure 6.2E and 6.F).

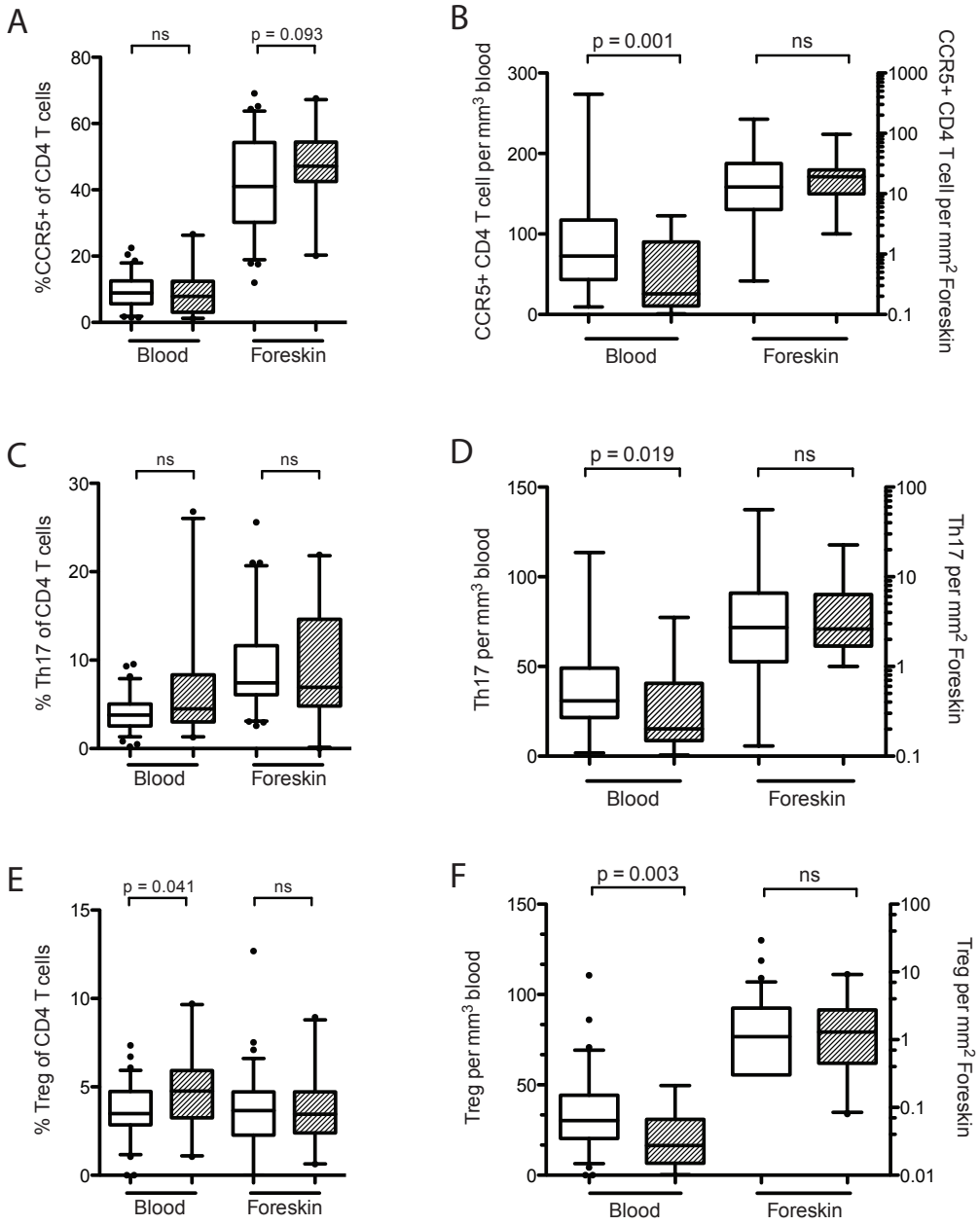

Figure 6.2 CD4 T cell subsets in the blood and foreskin of HIV-infected (hatched bars) and uninfected (open bars) men. Tregs were defined as CD4 T cells co-expressing CD25 and FoxP3; Th17 cells were defined as CD4 T cells producing IL17a in response to PMA and ionomycin stimulation; CCR5+ cells were CD4 T cells expressing the HIV-coreceptor CCR5. The proportion of CD4 T cells that were Tregs (A), Th17 cells (B), or expressed CCR5 (C) were measured using flow cytometry. Proportions were converted into absolute numbers of each cell type (B, D, E) using either the CD3 T cell density obtained through IHC, for foreskin tissue, or clinical CD4 counts, for blood. Statistical comparisons made by Mann-Whitney U test; CCR5+ T cell proportions and densities controlled for HSV-2 using multivariate general linear regression and adjusted p-values are reported.

6.3.4 HIV Status and CD8 T Cell Cytokine Production in the Foreskin

We next measured the proportion and absolute number of CD8 T cells producing the cytokines TNFα and IFNγ in the blood and foreskin after mitogen (PMA-ionomycin) stimulation (Figure 6.1C). In the foreskin of HIV-infected men, a greater proportion of CD8 T cells produced only IFNγ (16.6 vs. 9.4%, $p<0.001$), while the proportions of CD8 T cells producing both TNFα and IFNγ (bi-functional cells, 26.5 vs. 35.0%, $p=0.014$), or only TNFα (3.3 vs. 7.3%, $p<0.001$; Figure 6.3A), were reduced. Therefore this increase in foreskin CD8 T cell IFNγ mono-production was driven by a decrease in the proportion of cells able to produce TNFα, as opposed to a gain in IFNγ production: when bi-functional cells were included there was no overall increase in the proportion of cells producing IFNγ, but instead a decrease in the proportion of cells producing TNFα (31.2 vs. 47.6%, $p<0.001$, Figure 6.3C). However, despite this proportionate reduction in TNFα production, the increased CD8 T cell infiltration in the foreskin of HIV-infected men meant that there was actually an increase in the absolute number of foreskin CD8 T cells producing TNFα/mm^3 (4.8 vs. 1.5 cells/mm^3, $p=0.014$, Figure 6.3D).

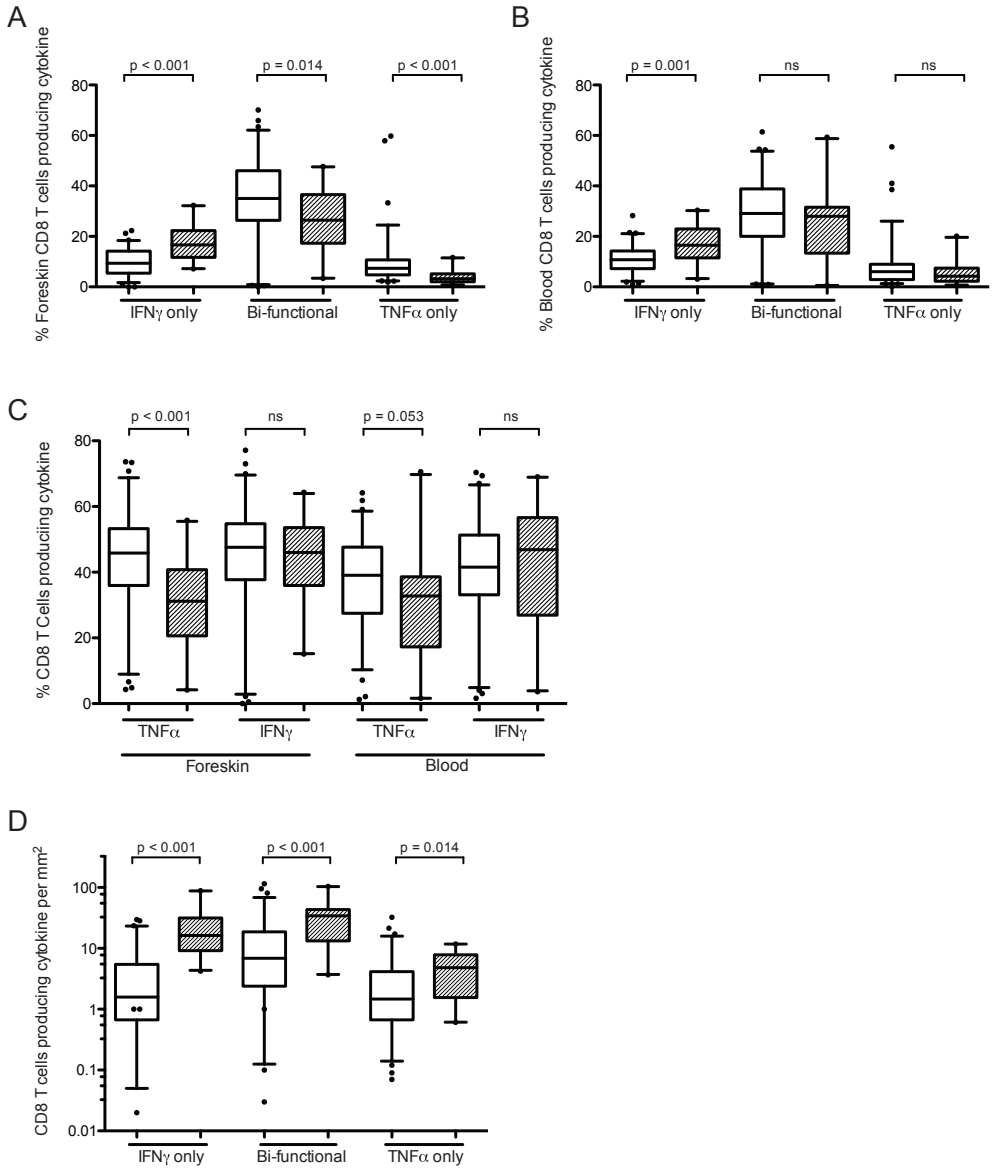

Figure 6.3 Production of inflammatory cytokines by CD8 T cells in the foreskin and blood of HIV-infected (hatched bars) and uninfected (open bars) men. T cells isolated from foreskin tissue (A) and blood (B) were stimulated with PMA and ionomycin and subsequent TNFα and IFNγ production by CD8 T cells was measured by flow cytometry (A), allowing for identification of cells producing only IFNγ, both IFNγ and TNFα (bi-functional cells), or cells that produce TNFα only. Panel (C) show the overall proportion of cells producing each cytokine (values include bi-functional cells). Proportions of foreskin cell populations were converted into absolute numbers (D) using IHC (Figure 1). Statistical comparisons made by Mann-Whitney U test.

6.3.5 HIV-specific CD8+ T Cell Responses in the Foreskin

Blood and foreskin cell suspensions were stimulated with a pool of optimized HIV class I epitope peptides previously shown to be highly antigenic in a cohort of East African women[392], and TNFα and IFNγ production were measured by flow cytometry. A positive HIV-specific response was defined as: (1) ≥0.3% of CD8 T cells producing either TNFα or IFNγ in response to HIV peptides; and (2) a percentage of CD8 T cells producing cytokine after peptide stimulation that exceeded the vehicle-only control by at least three-fold.

HIV-specific CD8 T cell responses were present in both the blood and foreskin of a large proportion of HIV-infected men. However, while HIV-specific CD8 T cells in the blood produced both IFNγ (60% HIV+ men vs. 7.1% HIV- men, $p<0.001$) and TNFα (34% HIV+ men vs. 8.6% HIV- men, $p=0.007$, Figure 6.4A), HIV-specific CD8 T cells in the foreskin produced IFNγ almost exclusively (40.0% HIV+ men vs. 10.0% HIV- men, $p=0.004$, Figure 6.4B). HIV-specific TNFα responses in the foreskin were very infrequent, and their frequency did not differ from HIV-infected men (5.0% vs. 2.9%).

Foreskin HIV-specific CD8 T cell responses were both less frequent and of a smaller magnitude than blood responses. The proportion of HIV-infected men with a response detected in the foreskin was lower than that with a response in the blood (40.0% vs. 60.0%, $p=0.005$, Figure 6.4C). Furthermore, the frequency of HIV-specific foreskin CD8 T cells was generally lower than that in the blood (median difference between foreskin and blood -0.7%, $p=0.006$, Figure 6.4D).

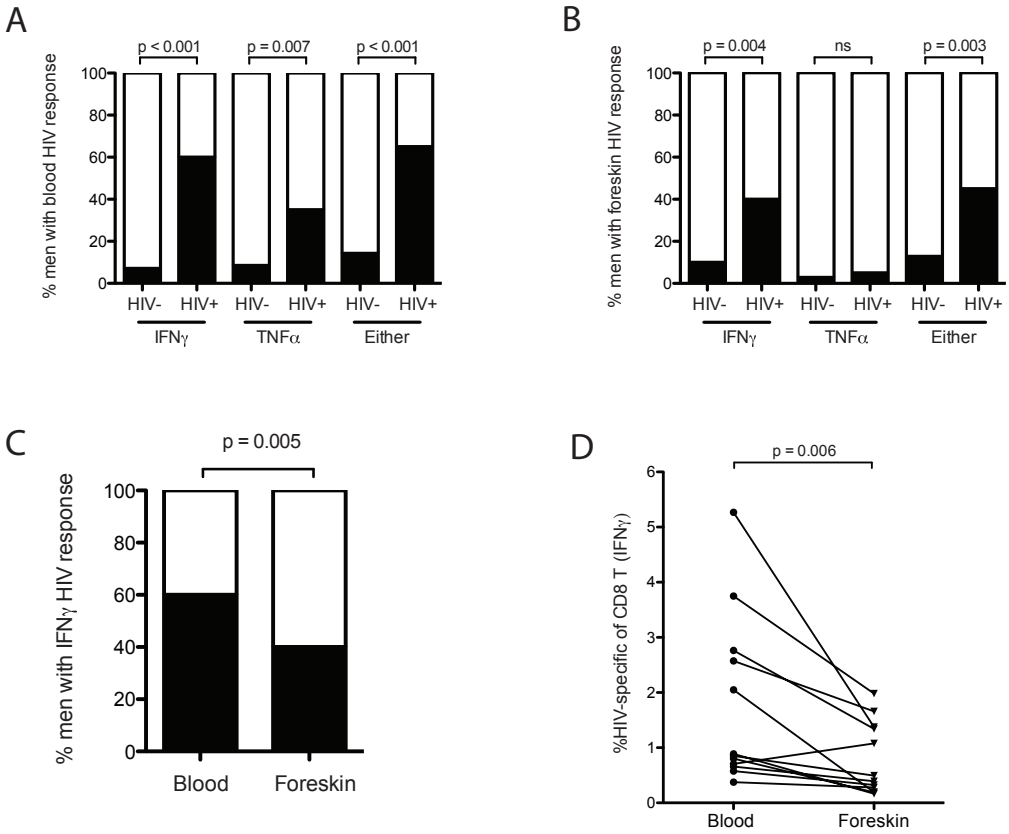

Figure 6.4 HIV-specific CD8 T cells responses in the blood and foreskin.

CD8 T cells isolated from foreskin tissue or blood were challenged with a pool of cross-clade HIV peptides previously shown to be highly antigenic in an East African population. TNFα and IFNγ production by CD8 T cells was measured by flow cytometry; an HIV-specific response was defined as cytokine production 3 times greater than that observed in unstimulated cells, and a minimum of 0.3% of cells responding (shown in black). The proportion of men with HIV-specific cytokine production in the blood (A) and foreskin (B) is shown (Fisher's exact test). Panel (C) compares the proportion of men with a blood vs. foreskin IFNγ response (Fisher's exact test). Among HIV-infected men who had an HIV-specific response, panel (D) compares the percentage of CD8 T cells that were HIV-specific in the blood and foreskin (Wilcoxon signed rank test).

6.4 Discussion

We found that HIV infection was associated with a four-fold increase in the tissue density of CD8 T cells in the foreskin with no reduction in the density of foreskin CD4 T cells. The proportion of CD8 T cells in the foreskin that were able to produce TNFα was decreased with HIV infection, leading to a functional skewing from bi-functional cells able to produce both

TNFα and IFNγ, towards IFNγ mono-production. While HIV-specific CD8 T cells were readily detected in the foreskin tissue of HIV-infected men, the frequency and diversity of cytokines, and the magnitude of foreskin HIV-specific responses were lower than those seen in the blood.

Maintenance of CD4 T cell numbers in foreskin tissue during HIV infection despite a substantial reduction in absolute blood CD4 counts was in contrast to mucosal pathogenesis in the cervix of HIV-infected women, where significant CD4 depletion was observed in some [445, 466] but not all [467] studies. Previous studies in the foreskin showed that mono-infection by HIV (i.e. without HSV-2) was associated with a loss of CD4+ cells from the foreskin, but that no CD4+ loss was observed in men co-infected with both HIV and HSV-2 [358]. This may be because the host immune response against HSV-2 is characterized by long-lasting mucosal infiltration with antigen-specific CD4+ T cells [468]. Since the great majority of HIV-infected men in our study were co-infected by HSV-2, this limited our ability to assess the impact of HIV mono-infection on foreskin CD4 T cell numbers. However, we found no HSV-2 associated increase in CD4 T cell density among HIV-uninfected controls, and point estimates of outcomes were similar after stratification for HSV-2 infection status (significance unaltered by stratification, data not shown).

In contrast to HIV mucosal pathogenesis in the gastrointestinal tract[421], we did not observe any HIV-associated loss of CCR5+ CD4 T cells or Th17 cells from the foreskin. Sexual transmission of HIV occurs almost exclusively through R5-tropic viral strains[418], and Th17 cells are highly susceptible to HIV infection *in vitro*[237, 239]. This raises the intriguing possibility that the maintenance of highly HIV-susceptible CCR5+ CD4 T cells and Th17 cells in the foreskin of HIV-infected men may permit HIV superinfection, something that has been observed with a relatively high frequency in this population-based cohort[455]. Superinfection might also be aided by the fact that virus-specific CD8 T cell responses were reduced in frequency in the foreskin of HIV-infected men, as has been observed in the female genital mucosa[422, 466], and had a functional profile that was skewed towards IFNγ mono-production.

HIV infection was both associated with an increased tissue density of CD8 T cells in the foreskin, and with changes in the quality of their cytokine production. Specifically, the proportion of CD8 T cells producing TNFα was significantly decreased, as was the proportion of CD8 T cells producing only TNFα, and of bi-functional cells producing both TNFα and IFNγ. Since TNFα production is a key component of the host immune response to several genital

infections, including HSV-2[469], syphilis[470], and gonorrhea[471, 472], this may impair immune responses to these pathogens and contribute to their increased incidence (syphilis, gonorrhea, HPV), duration (HPV) and/or recurrence (HSV-2) in HIV-infected men.

Whether the T cell alterations that we observed in the foreskin of HIV-infected men are causally related to a subsequent increase in the risk of genital infections or HIV superinfection cannot be determined in observational studies. Animal models and/or ex vivo foreskin explant models may be useful ways to explore the direction of causality in the future. In addition, our on-site FACSCaliber flow cytometer only permits the measurement of four immune parameters, which limited our capacity to assess T-cell polyfunction (such as production of additional cytokines, including IL-2, MIP-1β or perforin). Based on the importance of T cell polyfunctionality in HIV disease progression[473], future studies are warranted to explore these T cell functions in the foreskin.

In conclusion, we found that foreskin CD4 T cell numbers, including Th17 cells and CCR5+ CD4 T cell subsets, were maintained during HIV infection, while overall foreskin CD8 T cell numbers were increased. However, a reduced functional capacity in both bulk and HIV-specific CD8+ T cells in the foreskin may impair local immune defenses against genital co-infections and HIV superinfection.

Chapter 7
Immune Correlates of HIV Exposure Without Infection in Foreskins of Men from Rakai, Uganda.

7 Immune Correlates of HIV Exposure Without Infection in Foreskins of Men from Rakai, Uganda.

7.1 Introduction

HIV-1 (HIV) is primarily transmitted through unprotected sex. Despite the high global prevalence of HIV, transmission of the virus during insertive vaginal sex is both relatively inefficient and heterogeneous, with the estimated per-contact risk of female-to-male transmission ranging from 1/200 to 1/2000[8]. To rationally design new tools to prevent HIV transmission we need to understand the mucosal determinants of transmission. Individuals who are regularly HIV exposed but seronegative (HESN) may provide important insights into the mucosal immune correlates of resistance to HIV infection.

A number of previous studies have examined these immune parameters in HESN individuals exposed to HIV through sero-discordant sexual relationships or commercial sex work (CSW). Mucosal secretions (cervical and salivary) from HESN individuals contain higher levels of several antimicrobial peptides and C-C chemokines that have been shown to have antiviral or HIV-neutralizing capacity *in vitro*. Upregulated antimicrobial proteins include the cathelicidin LL-37[274, 278], α-defensins (human neutrophil peptides 1-3, HNP1-3)[274, 278], β-defensins (hBD2) [266, 474], protease inhibitors[401, 475, 476] (SLPI and trappin-2/elafin), and IFNα[374]. Upregulated C-C chemokines include MIP-1α[477], MCP-1[477], and RANTES[374, 404]. Furthermore, cervico-vaginal secretions and saliva from HESN individuals may contain HIV-neutralizing IgA[296, 299], which was associated with a reduced risk of HIV acquisition in a prospective study[300].

These data suggest that higher mucosal levels of certain innate/adaptive immune molecules may provide protection against HIV acquisition. However, some mucosal immune factors that

neutralize HIV *in vitro* also activate or recruit HIV target cells, thereby negating any protective neutralizing effect of the peptide, or even increasing HIV susceptibility *in vivo*, as appears to be the case for LL-37 and α-defensins[160, 278, 378]. Indeed, there is evidence to suggest that reduced immune activation at the site of HIV exposure may correlate with resistance to infection. Cervical secretions from HESN women in Nairobi contain lower levels of C-X-C chemokines and the pro-inflammatory cytokine IL-1a as compared to new (non-HESN) CSWs[401]. Furthermore, T cells from the blood and genital tract of HESN women have increased regulatory T cells (Tregs)[395, 478], lower CD4 T cell expression of the activation markers HLA-DR[400, 479] and CD38[479, 480], and reduced production of the inflammatory cytokines IL-17, IL-22, IL-1β, IL-6 and TNFα [396, 398]. Taken together, these studies suggest that relative resistance to HIV infection may require a delicate balance between the levels of antimicrobial peptides in genital secretions and of activated/highly susceptible target cells the mucosa[435].

Randomized trials of male circumcision have demonstrated that the foreskin is the main site of HIV acquisition in heterosexual men[22-24], underlining the need to better characterize the immune correlates of HIV susceptibility in this anatomic site. Compared to blood, T cells in the foreskin produce more cytokines, express more CCR5, and are enriched for highly HIV-susceptible Th17 cells[409]. Additionally, foreskin tissue has been shown to produce several soluble peptides that have *in vitro* antiviral activity[160]. Therefore, we performed an investigator- blinded study to define the immune correlates of reduced HIV susceptibility in the foreskins of HESN men from Rakai, Uganda.

7.2 Methods

7.2.1 Study Participants

Participants consisted of 77 heterosexual couples enrolled in a broader study of foreskin immunology[409]. HESN men were HIV-seronegative and in a stable relationship with an ART-naïve, HIV-seropositive female who had a detectable HIV plasma viral load, and reported inconsistent or no condom use with this female partner despite risk-reduction counseling and the provision of free condoms. Unexposed control men were HIV-seronegative and in a monogamous relationship with an HIV-seronegative woman.

7.2.2 Samples and Assays Included in this Study

Sub-preputial swabs were analyzed for cytokines, HIV-neutralizing IgA, and innate factors. Foreskin tissue and blood were analyzed for T cell populations (flow cytometry and CD3 IHC).

7.2.3 IgA Purification and PBMC Neutralization Assays

HIV-neutralization assays were performed by our collaborator, Dr. Taha Hirbod, at the Karolinska Institute. Neutralizing IgA activity was assessed in a subset of samples (18 HESN men and 37 unexposed controls) where samples were available. One aliquot of sub-preputial swab (500µL) was thawed and centrifuged at 1500 rpm (5 min, 4°C) to remove cellular debris. IgA was purified as previously described[298]. Briefly, 400µL undiluted swab solution was added to 200µL jacalin/agarose beads (Vector Labs, Burlingame, CA, USA), mixed for 2 hours at 4°C, and centrifuged to separate the IgA-depleted fraction (stored at -80°C for innate factor analysis). Jacalin/agarose beads were thoroughly washed with PBS pH 7.4, and bound IgA was eluted overnight at room temperature by adding 500µL 0.8 M D-galactose pH 7.5. The supernatant (purified IgA) was collected, diluted 1:2 with RMPI 1640 medium (Invitrogen AB, Lidingö, Sweden) and stored at -80°C. HIV neutralization assays were performed according to a predefined protocol and neutralization cut-off[300]. R5 tropic primary isolates (clade C: ZA009, obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) were collected from PBMC stimulated with phytohemagglutinin (PHA-P) and interleukin-2 (IL-2) (both Sigma-Aldrich Sweden AB, Stockholm, Sweden). The $TCID_{50}$ was determined and supernatants were aliquoted and stored at -80ºC. Since the $TCID_{50}$ may differ between PBMC donors, three viral dilutions were used in each assay. IgA fractions were diluted 1:2 with RMPI 1640 medium (Invitrogen AB, Lidingö, Sweden) and 75µL of this diluted sample was incubated with 75µL of each virus dilution (in duplicate) for 1 hour at 37ºC to allow for viral neutralization. Virus was mixed with $1x10^5$ PHA-P-stimulated mixed PBMCs from 2-3 donors; after 24h incubation at 37ºC, PBMCs were washed and cultured for 6 days in 200µL of fresh RMPI 1640 medium supplemented with bovine serum albumin and IL-2, with half the medium replaced on day 3. Supernatants were collected on day 6 for analysis of virus production with a p24 antigen ELISA (Vironostika HIV-1 Antigen; Electra-Box Diagnostica AB, Stockholm, Sweden). Percent neutralization was defined as the reduction in p24 production compared to a reference sample (created by pooling IgA from 5 unexposed control men). However, there is significant inter-assay variability in the quantity of p24 antigen produced as a result of PBMC

donor variability. We therefore treated IgA neutralization as a binary outcome, where successful neutralization was defined as a ≥67% percent neutralization. A cut-off of ≥67% provides robust reproducibility of successful neutralization [296], has been shown to correlate prospectively with protection from infection[300]. Positive control samples (HIV IgG positive serum) were included in each assay. Samples that did not have consistent absence/presence of neutralization in all three viral dilutions were re-tested until a consensus was achieved.

7.2.4 Innate Factor Analysis

Innate factor analysis was performed by our collaborator, Dr. Taha Hirbod, at the Karolinska Institute. Trappin/Elafin, Human Neutrophil Peptides 1-3 (HNP-1-3), human β-defensin 2 (HBD-2) and Secretory leukocyte protease inhibitor (SLPI) were quantified in IgA depleted fractions of sub-preputial swabs. Commercial ELISA kits were used according to the manufacturers protocols as follows (additional dilutions in parentheses): Trappin/Elafin (1:10), α-defensins HNP-1-3 (1:10) (all from HyCult Biotechnology, Uden, The Netherlands), β-defensins HBD-2 (1:10-1:100) (Phoenix Pharmaceuticals, Burlingame, CA, USA) and SLPI (1:5-1:100) (RD Systems Europe, Abingdon Oxon, UK).

7.2.5 Statistical Analysis

All immune assays were performed by research personnel blinded to participant study group; immune data files were cleaned and finalized prior to study group linkage. Innate factors, chemokines, cytokines, and T cell populations were compared between HESN and unexposed controls by Mann-Whitney U test. Associations between demographic factors and immunological parameters were tested by Spearman's correlation coefficient. Demographic variables found to be associated with HESN status (HSV-2 status and concurrent sexual partners) were controlled for by multivariate general linear regression and adjusted p-values are reported. Proportions of men with HIV-specific IgA and CD8 T cell responses were compared by Fisher's exact test. Statistical tests were run using SPSS v.19.0 for Mac (IBM; New York, NY, USA). Flow cytometry data was analyzed in FlowJo v.9.5.2 (Treestar; Ashland, OR, USA) and Excel (Microsoft; Redmond, WA, USA) prior to statistical testing.

7.3 Results

7.3.1 Participant Characteristics

HESN men (n=20; see Methods for definition) had been in a primary sexual relationship with an HIV-infected woman for a median of 5 years (range, 1-10 years; see Table 7.1). HESN men reported using condoms either "sometimes" (30%) or "never" (70%), and the median plasma viral load of the HIV-seropositive female partner was 3.74 $\log_{10}$ RNA copies/mL (range 1.62 – 5.22; Table 7.1). All HESN participants were HIV PCR negative at the time of the study. HESN men and HIV-unexposed control men did not differ in terms of age, condom use or the number of sexual partners in the last year. However, HESN men had a higher HSV-2 seroprevalence than unexposed controls (70 vs. 31.5%, p=0.004) and were more likely to report concurrent sexual relationships (3/20 vs. 0/57, p=0.016); therefore multivariate linear regression was used to control for HSV-2 status and concurrent sexual relationships in all subsequent analyses, and adjusted p-values are reported. Of note, while concurrent sexual relationships were not associated (p<0.1) with any of the immunological parameters investigated in this study, in a larger analysis of the effect of HSV-2 infection on foreskin T cell populations[440] we previously found that HSV-2 was associated with increased CCR5 expression on foreskin CD4 T cells, but not with any other immune parameters investigated.

Table 7.1: Participants demographics.

		HESN (n=20)	Unexposed (n=57)	sig.
Age (yrs)	man	33 (23-53)	34 (22-51)	0.419
	woman	29 (20-41)	29 (19-49)	0.819
HSV-2 serology		14/20 (70.0%)	17/54 (31.5%)	0.004
Partner viral load (copies/mL)		5,589 (42-165,288)	n/a	
Partner CD4 count (cells/mm^3)		553 (295-1,592)	1,152 (492-2,019)	<0.001
Length of HIV exposure (yrs)		5 (0.5-15)	n/a	
Condom use (%)	always	1/20 (5.0%)	0/57 (0.0%)	0.114
	sometimes	5/20 (25.0%)	9/57 (15.8%)	
	not using	14/20 (70.0%)	48/57 (84.2%)	
Sexual partners in past year	single	15/20 (75.0%)	49/57 (86.0%)	0.304
	multiple	5/20 (25.0%)	8/57 (14.0%)	
Concurrent sexual partners (%)		3/20 (15.0%)	0/57 (0.0%)	0.016

7.3.2 HIV Neutralization by Foreskin-Derived IgA

The ability of purified sub-preputial IgA to neutralize infection of activated PBMCs by a primary clade C HIV isolate was then assessed by research personnel blinded to participant study group. Using a predefined cut-off of ≥67% neutralization compared to the reference sample, IgA purified from sub-preputial swabs of 9/18 (50%) HESN men was able to inhibit HIV infection of activated PBMC, while IgA from only 4/37 (10.8%) unexposed controls had this neutralizing capacity (Figure 7.1; prevalence risk ratio: 4.63; 95% CI: 3.00-6.26%; Fisher exact p=0.002). The absolute quantity of IgA in HESN samples was similar to that in controls (2146 pg/mL in controls vs. 1649 pg/mL in HESN, p=0.9), and a sensitivity analysis using an increased cut-off of ≥90% neutralization yielded similar results (neutralization seen in 9/18 HESN men vs. 2/37 control men, p<0.001). Within the HESN group, IgA neutralization capacity did not correlate with the quantity of IgA, female partner viral load, length of time of HIV exposure, or the presence of HIV-specific CD8 T cells in his blood or foreskin. Presence of HIV-neutralizing IgA also did not correlate with HSV-2 status or concurrent extramarital relationships (data not shown).

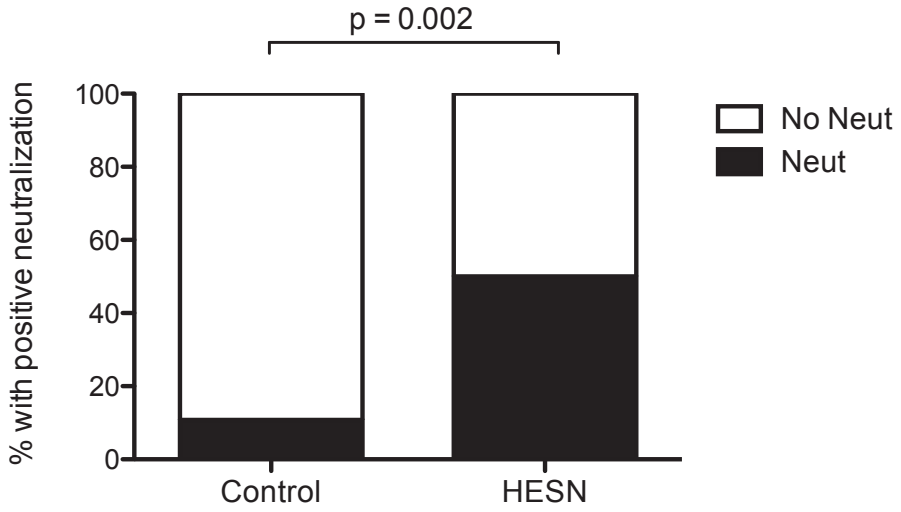

Figure 7.1 The ability of IgA isolated from the foreskin to neutralize HIV.
IgA was purified from foreskin secretions and incubated with a primary clade C viral isolate, which was subsequently challenged with PBMCs. The ability of IgA-treated virus to infect PBMCs was measured through p24 production. Capacity to neutralization HIV was defined as 67% less p24 production compared to reference sample. The proportion of men in each group with HIV-neutralizing IgA compared by Fisher's exact test (HESN n=18; Controls n=37).

7.3.3 Preputial Levels of Soluble Innate Immune Factors and Cytokines

Levels of innate anti-microbial peptides were quantified in the IgA-depleted fraction of sub-preputial swab eluate. Levels of the innate antimicrobial peptides Trappin2/Elafin, HNP-1-3, HBD-2 and SLPI are shown in Figure 7.2. The α-defensins HNP 1-3 was present at a significantly higher level in swabs taken from the sub-preputial space of HESN men compared to controls (3027 pg/mL vs. 1795 pg/mL; adjusted p=0.011); which among HESN men correlated with the presence of neutralizing IgA (correlation coefficient 0.503, p=0.033) but not partner viral load or length of HIV exposure. To ensure that IgA neutralization was not a result of HNP-1-3, IgA fractions were also analyzed for presence of HNP-1-3: no HNP-1-3 was detected in any IgA fractions (data not shown). Levels of Trappin2/Elafin, HBD-2 and SLPI were similar between HESN men and unexposed controls.

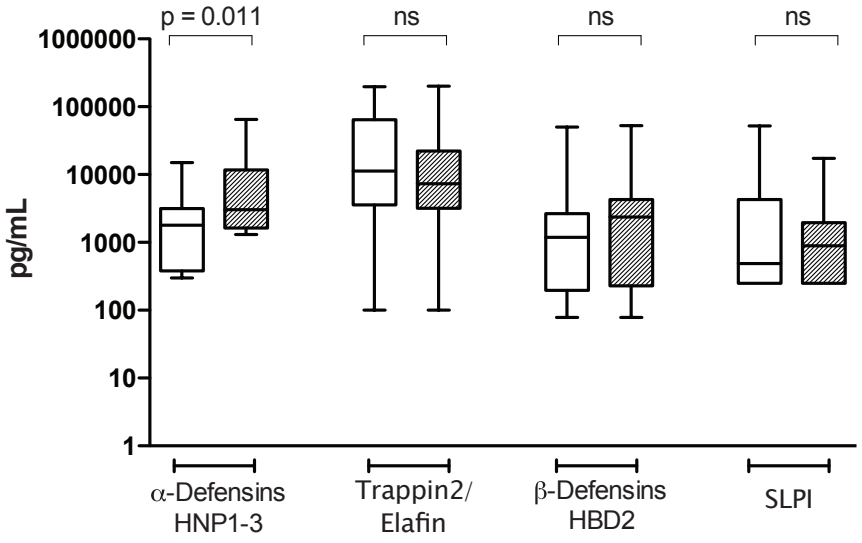

Figure 7.2 Levels of soluble innate immune proteins in foreskin secretions.
HESN (hatched bars) and unexposed control men (open bars). Sub-preputial swabs collected before surgery were assayed for levels of soluble innate immune proteins by ELISA. Median concentration (with range) of innate peptide in 1 mL collection volume is displayed (HESN n=18; Controls n=37). Statistical comparisons made by Mann-Whitney U test.

Additionally, levels of cytokines and chemokines were assayed in a separate aliquot of undiluted sub-preputial swab samples (Table 7.2). IL-8 was present at a level that could be quantified in the majority of men; however, the median IL-8 levels did not differ significantly between HESN and control men (33.7 vs. 29.8 pg/mL in HESN vs. control men, p=0.40). MCP-1, MIG, and

RANTES were present in <50% of HESN men, and therefore the frequency of detection of these cytokines, as opposed to the concentration of analyte, was compared between groups. There were no HESN-associated differences in the frequency of detection of MCP-1, (42.1 vs. 50.9% of HESN vs. control men, p=0.6), MIG (47.4 vs. 54.5%, p=0.6), or RANTES (21.1 vs. 10.5%, p=0.3). IL-1α, MDC and MIP-3α were not detected in sub-preputial swabs. No HSV-2 associated differences in levels of cytokines, chemokines, and soluble innate factors were observed.

Table 2. Chemokine and cytokine levels in sub-preputial swabs.

	LLOQ (pg/mL)	HESN (n=19)		Unexposed controls (n=57)		sig.*
		Frequency of detection (%)	Median (pg/mL)	Frequency of detection (%)	Median (pg/mL)	
IL-1α	10.3	0 (0.0%)	-	0 (0.0%)	-	-
IL-8	1.5	18 (94.7%)	33.73	52 (91.2%)	29.84	0.40
MCP-1	0.6	8 (42.1%)	-	29 (50.9%)	0.69	0.60
MDC	1250.0	0 (0.0%)	-	0 (0.0%)	-	-
MIG	0.3	9 (47.4%)	-	31 (54.4%)	0.34	0.61
MIP-3α	46.2	0 (0.0%)	-	0 (0.0%)	-	-
RANTES	3.0	4 (21.1%)	-	6 (10.5%)	-	0.26

* IL-8 medians compared between groups using Mann-Whitney *U*, else frequency of detection was compared using Fisher's exact test.

7.3.4 T cell Subset Density and Relative Proportions in the Foreskin

The proportions of T cell subsets in the blood and foreskin were assessed by flow cytometry, and the average tissue density of foreskin T cell subsets was calculated by combining these proportions with immunohistochemical determination of CD3 T cell density/mm^2 of tissue. The proportion of T cells expressing CD4 or CD8 did not differ between HESN men and unexposed controls (54.1 vs. 51.3% and 32.7 vs. 34.8% of T cells, respectively; ns). However, HESN men had a significantly higher density of CD3 T cells/mm^2 of tissue after controlling for HSV-2 serostatus (151.9 vs. 69.9 cells/mm^2, adjusted p=0.018, Figure 7.3C), and therefore had higher absolute numbers of both CD4 and CD8 T cells/mm^2 of foreskin tissue (84.6 vs. 31.3 cells/mm^2,

p=0.022; 45.6 vs. 22.7 cells/mm^2, p=0.013; Figure 7.3C). Density of foreskin T cells did not correlate with partner viral load.

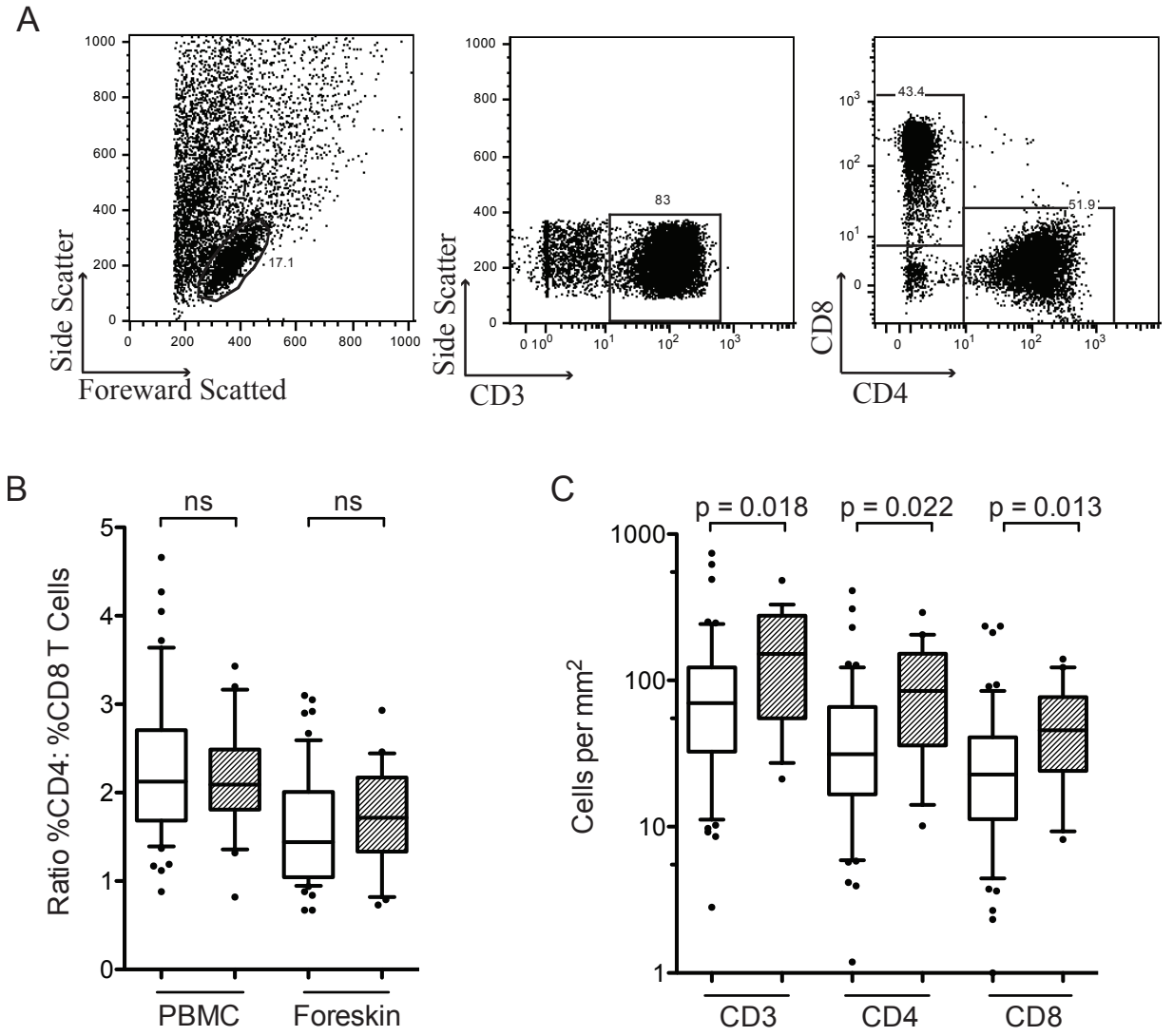

Figure 7.3 T cell subsets in the blood and foreskin.
HESN (hatched bars) and unexposed control men (open bars). The proportion of CD3+ T cells expressing either CD4 or CD8 was measured on PBMCs and foreskin cells using flow cytometry (B); representative flow cytometry plots showing the gating strategy for foreskin cells are shown in A. Proportions of T cells subsets were normalized to the number of CD3 T cells per mm^2 of foreskin tissue (obtained using IHC) to obtain absolute numbers of each cell type (C) (HESN n=20, Controls n=57). Statistical comparisons made by Mann-Whitney U test.

7.3.5 Phenotype and Function of Foreskin T Cell Subsets

Flow cytometry was used to further characterize the proportion of CD4 T cells classified as Th17 cells (production of IL-17a upon stimulation), T-regulatory cells (Tregs; co-expression of CD25 and FoxP3), and expressing the HIV co-receptor CCR5 (representative plots of foreskin staining shown in Figure 7.4A and D). Compared to unexposed controls, HESN men had a decreased proportion of Th17 cells in both the blood and the foreskin (2.6 vs. 3.8% of blood CD4 T cells, p=0.042; 6.1 vs. 8.0% of foreskin CD4 T cells, p=0.007; Figure 7.4E, but a similar proportion of Tregs (4.1 vs. 3.7% of blood CD4 T cells, ns; 2.9 vs. 3.7% of foreskin CD4 T cells, ns) and of $CCR5^{+}$ CD4 T cells (7.8 vs. 8.7% of blood CD4 T cells, ns; 44.3 vs. 40.0% of foreskin CD4 T cells, ns; Figure 7.4B). This decreased relative abundance of Th17 cells did not correlate with partner viral load.

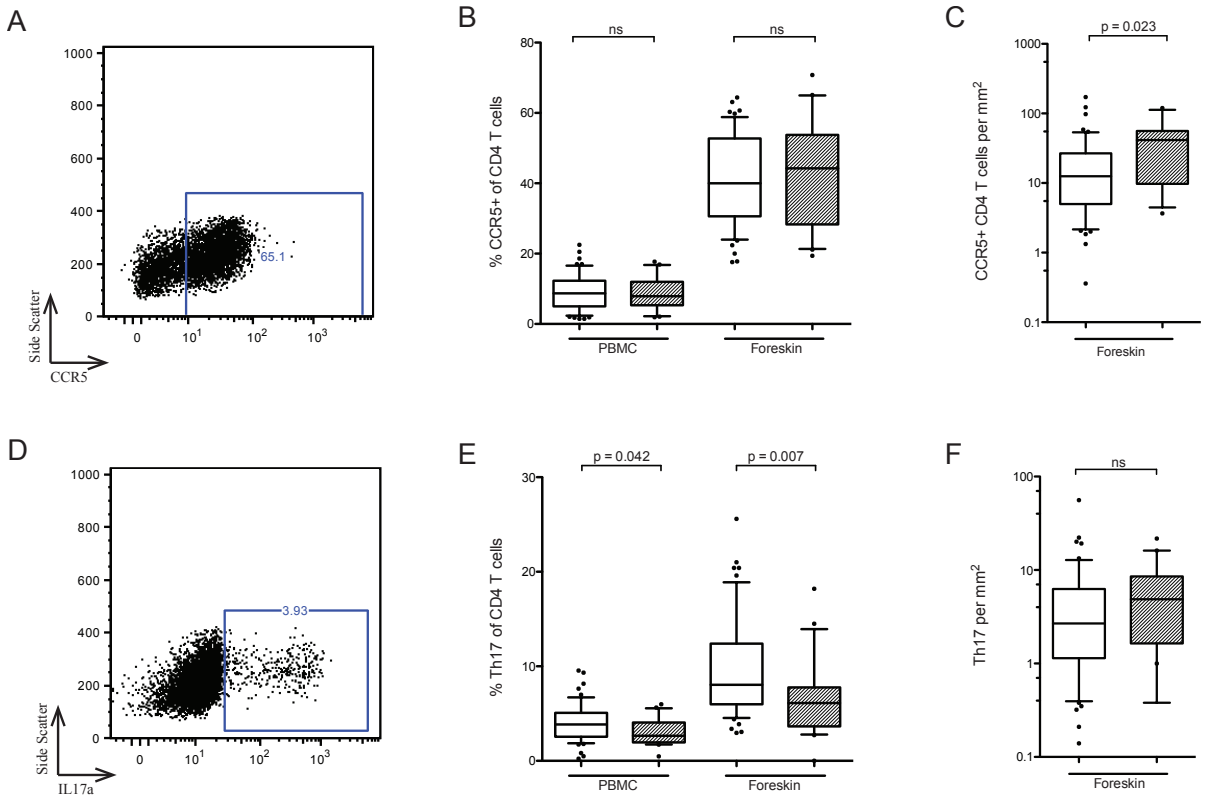

Figure 7.4 CD4 T cell subsets in the blood and foreskin. HESN (hatched bars) and unexposed control men (open bars). CCR5 expression was measured on PBMCs and foreskin CD4 T cells using flow cytometry (representative foreskin plot in A; plot shown in more detail in Figure 3.2). Th17 cells were identified by production of IL17a by CD4 T cells in response to stimulation with PMA and ionomycin (representative foreskin plot in D; plot shown in more detail in Figure 3.3). Summary data of the proportion of CCR5+ CD4 T cells and Th17 cells in the blood and foreskin are shown in B and E. Absolute numbers of cells, obtained by IHC of

CD3, are shown in C and F (HESN n=20, Controls n=57). Statistical comparisons made by Mann-Whitney U test; CCR5+ T cell proportions and densities controlled for HSV-2 using multivariate general linear regression and adjusted p-values are reported

Given the higher overall tissue density of T cells in the HESN foreskin, this translated into a similar absolute number of Th17 cells/mm^2 in HESN men (4.9 vs. 2.7 cells/mm^2, ns; Figure 7.4F, and an increased absolute number of CCR5$^+$ CD4 T cells/mm^2 (41.5 vs. 12.5, p=0.023; Figure 7.4C).

Production of the cytokines IFNγ and TNFα by foreskin and blood T cells after PMA-ionomycin stimulation was assessed by intracellular cytokine production (representative plots of foreskin staining shown in Figure 7.5A and F), with CD8$^-$ T cells used as a proxy for CD4 T cells. A decreased proportion of HESN CD8 and CD4 T cells produced TNFα in both the blood (CD8 T cells, 29.4% vs. 38.8%, p=0.032; CD4 T cells, 31.4% vs. 43.2%, p=0.007) and foreskin (CD8 T cells, 36.9% vs. 45.7%, p=0.004; CD4 T cells, 34.3% vs. 41.8%, p=0.037; Figures 7.5D and I). This decrease did not correlate with partner viral load. The absolute number of TNFα-producing CD8 or CD4 T cells was not statistically different in HESN and unexposed control foreskin tissue (15.9 vs. 9.1 CD8 T cells/mm^2, Figure 7.5E, ns; 18.3 vs. 12.7, Figure 7.5J, ns). The proportion of CD8 and CD4 T cells producing IFNγ was similar between groups (38.1 vs. 41.5% PBMC CD8, ns; 43.2vs. 47.6% foreskin CD8, ns, Figure 7.5B; 12.8 vs. 16.5% PBMC CD4, ns; 17.5 vs. 23.7% foreskin CD4, ns, Figure 7.5G, ns), and so the increased overall T cell density in the HESN foreskin translated into a higher absolute number of IFNγ-producing CD8 T cells (19.7 vs. 8.3 cells/mm^2, p=0.011; Figure 7.5C), but not CD4 T cells (11.7 vs. 6.94cells/mm^2, ns; Figure 7.5H).

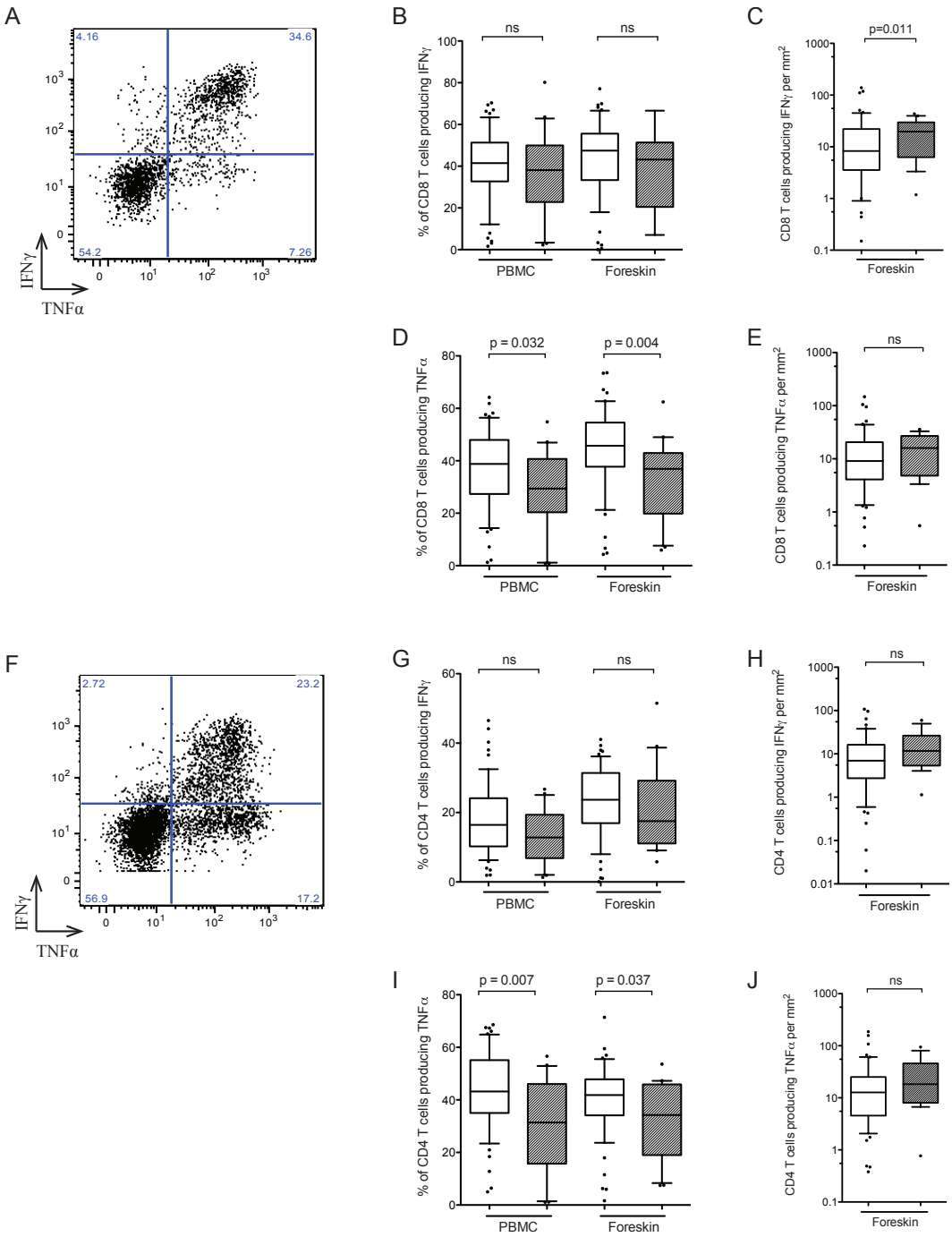

Figure 7.5 Inflammatory cytokine production by CD8 (A-E) and CD4 (F-J) T cells. HESN (hatched bars) and unexposed control men (open bars). Foreskin T cells were challenged with PMA and ionomycin and subsequent TNFα and IFNγ production by either CD8+ T cells or CD8- T cells (a proxy for CD4 T cells) was measured by flow cytometry (representative plots shown for CD8 (A) and CD4 T cells (F); plots shown in more detail in Figure 3.5). Summary data for CD8+ T cells shown in B and D, respectively. Absolute numbers of each cell type, obtained through CD3 IHC, shown in C and E. Similarly, proportions of CD4 T cells shown in G and I, and absolute numbers of each cell type are shown in H and J (HESN n=20, Controls n=57). Statistical comparisons made by Mann-Whitney U test.

7.3.6 HIV-Specific T Cell Responses in the Foreskin

Blood and foreskin cell suspensions were stimulated with a pool of pre-defined, optimized CD8 T cell peptide epitopes (see Methods, above). To be considered a positive HIV-specific response: (1) ≥0.3% of CD8 T cells had to produce either TNFα or IFNγ in response to peptide stimulation (background subtracted), and (2) cytokine production in response to peptides had to exceed background cytokine production by at least threefold. HIV-specific CD8 T cell responses were observed in both the blood and the foreskin of a minority of participants (representative plot, Figure 7.6A), but there was no significant difference in the frequency of an HIV-specific response between HESN men and unexposed controls (blood, 3/20 (25.0%) vs. 7/57 (12.3%); foreskin, 2/20 (20.0%) vs. 8/57 (14.0%); both $p>0.2$, Figure 7.6B). Among participants with an HIV-specific response, the magnitude of that response was also similar in HESN men and controls for both the blood (mean % CD8 T cells producing TNFα amongst responding HESN men = 1.51% vs. 1.47% in unexposed controls, ns; mean % producing IFNγ = 0.71% vs. 0.37%, ns) and the foreskin, where only IFNγ responses were observed (0.37% vs. 1.02%, ns). The presence of HIV-specific responses in HESN men did not correlate with partner viral load or length of time of exposure to HIV, and did not correlate with other immune parameters found to be associated with HESN status (data not shown).

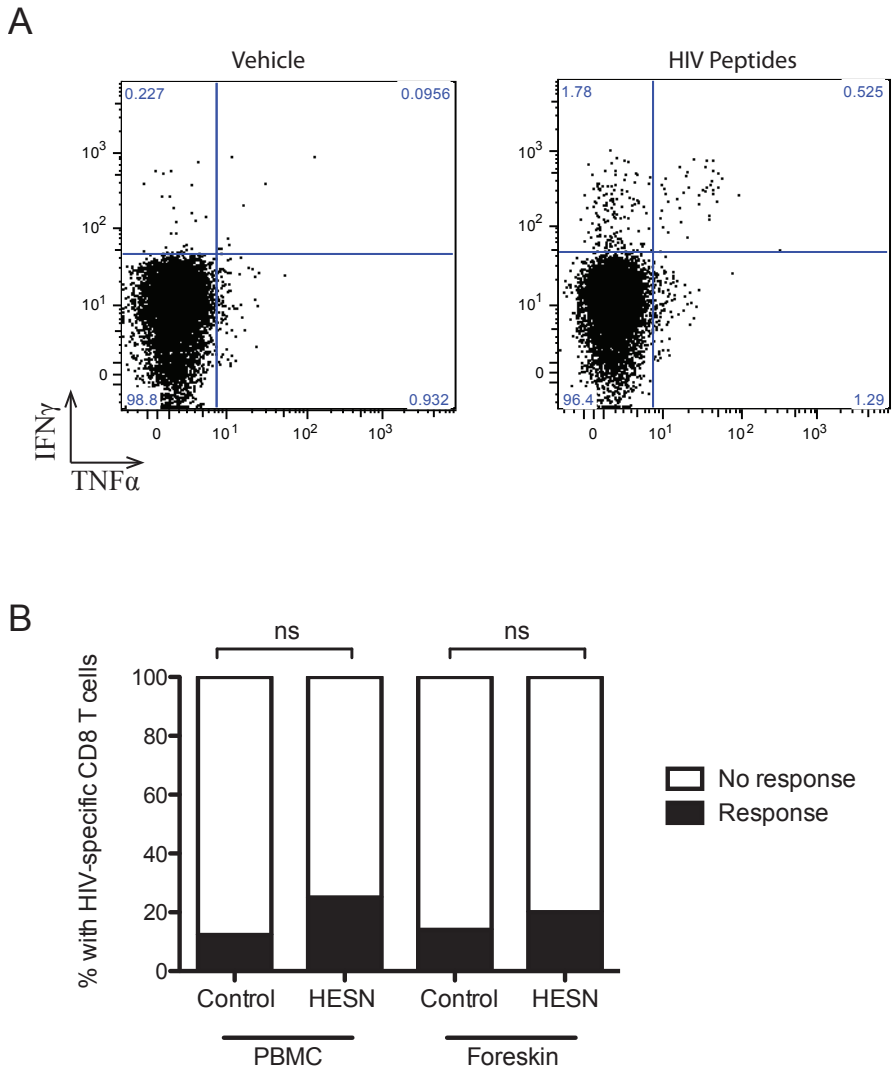

Figure 7.6 HIV-specific CD8 T cells responses in the foreskin.
CD8 T cells isolated from foreskin tissue were challenged with a pool of cross-clade HIV peptides previously shown to be highly antigenic in an East African population. (A) TNFα and IFNγ production by CD8 T cells was measured by flow cytometry. (B) Frequency of response for HESN and unexposed control men. An HIV-specific response was defined as cytokine production 3 times greater than observed in unstimulated cells and a minimum of 0.3% (HESN n=20, Controls n=57). Statistical comparisons made by Fisher's exact test.

7.4 Discussion

Natural susceptibility to HIV is heterogeneous, and elucidation of the mucosal immune correlates of reduced HIV susceptibility at sites of sexual exposure may provide important lessons for the HIV vaccine and microbicide fields. We combined epidemiological data collected through the Rakai Community Cohort Study with blinded immune studies of sub-preputial swabs and foreskin tissues to explore the immune correlates of HIV exposure without infection in the foreskin. We found secretions from the HESN foreskin to be enriched for α-defensins and HIV-neutralizing IgA. HESN foreskin tissue had an increased overall density of CD3+ T cells, but these T cells contained disproportionately fewer Th17 cells and produced less of the pro-inflammatory cytokine TNFα. We did not find that HIV-specific T cell responses were associated with the HESN phenotype.

Our observation that HESN men were more likely to have IgA with the capacity to neutralize HIV is consistent with numerous previous reports, including studies in diverse HESN populations examining several mucosal sites[295-300, 310, 311, 385]. While the presence of IgA with the ability to neutralize HIV in HESN populations is well established, the *specificity* of this IgA remains unknown[481]. Detection of HIV-specific IgA by a binding assay such as an ELISA or Western blot is rare[308, 482], and it is of note that the presence of HIV-neutralizing IgA did not correlate with foreskin HIV-specific CD8 T cells in this study. It may be that the target of this IgA is not HIV itself, but a host factor involved in HIV binding, such as CCR5, a lectin receptor[483, 484], or the integrin $\alpha_4\beta_7$[485]. Determining the target, mechanism of action, and source of this frequently observed neutralizing IgA should be a research priority.

While the sub-preputial space contained significant levels of several innate immune factors and cytokines, only the α-defensins HNP-1-3 were enriched in HESN sub-preputial swabs. Our group previously found that both HNP-1-3 and IgA in female genital secretions of Kenyan sex workers were correlated with *in vitro* HIV neutralization[278]. However, while HIV-neutralizing IgA was prospectively associated with HIV protection, increased α-defensins were associated with the presence of other genital co-infections as well as with an unexpected increase in the risk of HIV acquisition[278]. The latter finding may reflect the T cell chemoattractant properties of some antiviral innate factors and cytokines, as demonstrated by the association of

vaginal RANTES levels with increased mucosal $CD4^+$ target cells *in vivo*[404], and emphasizes that *ex vivo* antiviral activity does not always predict *in vivo* protection against HIV[364].

We found that HESN men had an increased density of $CD3^+$ T cells/mm^2 in their foreskin tissue, independently of their HSV-2 serostatus. While this was unexpected, increased numbers of cervical T cells have been observed previously in HESN female CSWs[404]. Despite having an increased overall number of $CD3^+$ T cells/mm^2 of foreskin tissue, a smaller proportion of these T cells were Th17 cells or had the capacity to produce the pro-inflammatory cytokine TNFα. Studies in an SIV-rhesus macaque model have shown that initial infection in the FGT begins with a small founder population of infected T cells[138] which grows through the recruitment of new, activated target cells driven by local inflammation[137]. Levels of susceptible target cells and local inflammation may therefore be a determinant in individual susceptibility to HIV. Th17 cells have been shown to be highly HIV-susceptible *in vitro*[221, 239] and to be selectively depleted during early HIV infection[221], while TNFα increases HIV replication in a paracrine fashion and is produced by activated T cells that are more permissive to HIV infection[177] and replication[445]. TNFα has also been shown to recruit CD4 T cells to foreskin tissue *ex vivo*[164], and to activate dendritic cells, which may pass HIV to susceptible CD4 T cells through viral synapses[156, 483]. Therefore, decreased production of TNFα and decreased proportion of Th17 cells in the HESN foreskin may inhibit the establishment of a founder population of infected cells after HIV exposure.

A decreased relative abundance of highly susceptible cell populations in conjunction with an increased overall number of T cells implies that there is an increased abundance of other T cell subsets. Further elucidation of these T cell populations in the HESN foreskin using multiparameter flow cytometry, as opposed to the 4-colour flow system available at our field site, should be a priority in future studies. This would also allow for characterization of other cell types that have been newly identified as important in HIV susceptibility, such as Th22 cells[486] or cells expressing the gp120-binding integrin $\alpha_4\beta_7$[485]. Furthermore, the position of susceptible target cells within the foreskin may also be very relevant to HIV susceptibility, particularly their depth below the epithelial surface and/or proximity to other immune cell subsets. Ideally, future studies should identify exclusive surface markers for relevant T cell subsets, which would permit immunohistochemical analysis of these parameters.

It is important to note that the sexual behavior of HESN individuals is, by definition, different to that of low risk men. Therefore cross-sectional studies of HESN genital immunology cannot distinguish which unique immune characteristics (if any) are protective against HIV, and which are related to increased sexual risk and may actually enhance susceptibility. Furthermore, while prospective studies of HIV acquisition would seem to be a way to address this issue, a drawback of using tissues obtained during male circumcision is that prospective studies of HIV acquisition in the foreskin then become impossible (as the foreskin is absent in future sexual exposures). Therefore animal models and/or improved *ex vivo* explant models of foreskin HIV infection may be useful ways to further characterize the direction of causation of the HESN immune associations that we have described.

In summary, we have combined epidemiology and mucosal immunology to define the unique immunological features of foreskins from men from Rakai, Uganda who are regularly exposed to HIV but have remained uninfected. We find that the foreskin of HESN men is characterized by increased overall T cell density in the context of reduced Th17 frequencies and reduced pro-inflammatory cytokine production, and by the presence of HIV-neutralizing IgA and elevated α-defensin levels in foreskin secretions. The ability of these immune parameters, either separately or in combination, to protect against HIV acquisition in the foreskin merits investigation in future research studies.

Chapter 8
Conclusions and Future Directions

8 Conclusions and Future Directions

8.1 Conclusions

HIV is predominantly sexually transmitted[1], and the main site of HIV acquisition in heterosexual men is the foreskin[22-24]. However, little is known about HIV infection in the foreskin due to the previous difficulty in obtaining biopsies from this site. The vast majority of sexual exposures to HIV do not result in infection[8], and sequencing analysis has revealed that when infection does occur, it is usually through a single successful virion using CCR5 as a co-receptor[84]. Studies of SIV infection in the macaque female genital tract [135-139] show that infection spreads locally in the cervicovaginal mucosa through new viral production from this initially infected cell, and that growth of this "founder population" of infected cells occurs through the recruitment and activation of additional CD4 T cells. Activated CD4 T cells are more susceptible to HIV infection and produce more virus once productively infected[139, 177, 180-182], and certain subtypes of effector CD4 T cells are particularly susceptible to infection, such as Th17 cells[221, 234-238]. Based on the rarity of the original infection event, and the slow initial growth of the founder population, I propose that access to susceptible CD4 T cells in the genital exposure site determines whether or not exposure to HIV will result in productive infection. Specifically, I hypothesized that the foreskins of HIV-susceptible men (infected by HSV-2[352]) would have increased numbers of activated and highly susceptible T cell subsets, while the opposite would be true in the foreskins of relatively HIV-resistant men (HESN[487]). Additionally, I expected HIV-resistant individuals to have increased levels of innate anti-microbial peptides and HIV-neutralizing IgA in foreskin secretions, and to have HIV-specific CD8 T cells within their foreskin tissues.

To test this hypothesis, I collected foreskin tissue and sub-preputial swabs from 110 men undergoing elective adult circumcision at the Rakai Health Sciences Program in Uganda, who were previously enrolled in the Rakai Community Cohort Study (RCCS): a well-established cohort that collects epidemiological and STI data from both male and female members of the

community[36]. I first developed novel techniques to digest foreskin tissue to a single-cell suspension containing T cells that were viable, able to produce cytokines in response to both antigenic and non-specific mitogen stimulation, and retained normal expression of phenotypic markers (CD4/8). In this initial study, I characterized foreskin T cell populations and compared them to T cell populations from the systemic compartment of the same man. I found the foreskin is relatively pro-inflammatory; containing a higher proportion of CCR5+ CD4 T cells, Th17 cells, and activated CD4 and CD8 T cells ($TNF\alpha^+/IFN\gamma^+$) than the blood (Chapter 3). I then examined cytokine levels in the sub-preputial space, and found that the density of pro-inflammatory T cell populations in foreskin tissue correlated with levels of IL-8 and other chemokines on the surface of the foreskin (Chapter 4).

After characterizing foreskin T cell populations, I sought to test my hypothesis that these populations would be over-represented in men known to be at increased risk of HIV, and under-represented in relatively resistant men. I found that men with asymptomatic HSV-2 infection had increased numbers of CCR5+ CD4 T cells in their foreskin, suggesting that increased rates of HIV acquisition in HSV-2 infected men may be in part due to increased HIV co-receptor expression in the foreskin (Chapter 5). I then examined HIV-uninfected men who have had regular sexual exposure to HIV, a population expected to be enriched for men who are relatively resistant to HIV. In collaboration with investigators at the Karolinska Institute, we found that the foreskin secretions of HESN men were more likely to have HIV-neutralizing IgA and had increased levels of α-defensins, both of which could contribute to HIV protection by preventing HIV from ever crossing the epithelial layer of the foreskin. In the foreskin tissue itself, I found that HESN men had a reduced proportion of Th17 cells and TNFα-producing CD4 and CD8 T cells compared to unexposed controls. HESN men also had a reduced proportion of HIV-susceptible T cells in their blood, but the reductions were more pronounced in the foreskin (Chapter 6). This reduction in Th17 cells and TNFα-producing T cells in the foreskin would be expected to reduce HIV susceptibility in two ways. First, because Th17 cells and activated CD4 T cells are particularly vulnerable to HIV[180-182, 221, 234-238], it represents a direct stochastic reduction of HIV-susceptible cells in the foreskin. Second, a reduction in local levels of IL-17 and TNFα would reduce bystander activation of other CD4 T cell and dendritic cell populations[472], indirectly reducing the abundance of HIV-susceptible cells. And because IL-

17 and TNFα act synergistically[200, 488], the combinatorial effect of a reduction in both cytokines may be larger than the individual reductions in either cytokine alone.

Finally, I examined HIV-susceptible T cell populations in the foreskins of HIV-infected men, because these men are at risk of becoming infected a second time with a distinct stain of HIV (superinfection). The ability of HIV to superinfect an individual seems counterintuitive because: (1) HIV-infection depletes blood CD4 T cells, thereby reducing the number of available target cells; and (2) HIV-infection induces adaptive immune responses that are capable of controlling viral replication[141], and might be expected to protect against subsequent infection. However, a recent epidemiological study confirmed that rates of superinfection in HIV-infected men in Rakai were surprisingly high, and similar to rates of incident HIV in the general population[455]. I examined foreskin tissues from HIV-infected men and found they had a similar density of CD4 T cells in their foreskin tissue to uninfected men, including both CCR5+ CD4 T cells and Th17 cells, and that this maintenance of foreskin CD4 T cell density occurred despite declining CD4 T cell counts in the blood. While HIV-specific CTL responses were found in the foreskins of some HIV-infected men, these responses were less common than blood responses, involved a smaller proportion of the total CD8 T cells, and involved only IFNγ production (as opposed to both IFNγ and TNFα production in the blood) (Chapter 7). The maintenance of HIV-susceptible CD4 T cells in foreskin tissue, in combination with weak and poorly functional HIV-specific CD8 T cell responses, may explain the high rates of superinfection observed in HIV-infected men.

8.2 Future Directions

Based on this body of work, I propose the following model of an HIV-resistant foreskin (Figure 8.2): the tissue of the foreskin contains a reduced relative abundance of CCR5+ CD4 T cells, Th17 cells and TNFα-producing T cells, while foreskin secretions contain increased levels of α-defensins and HIV-neutralizing IgA.

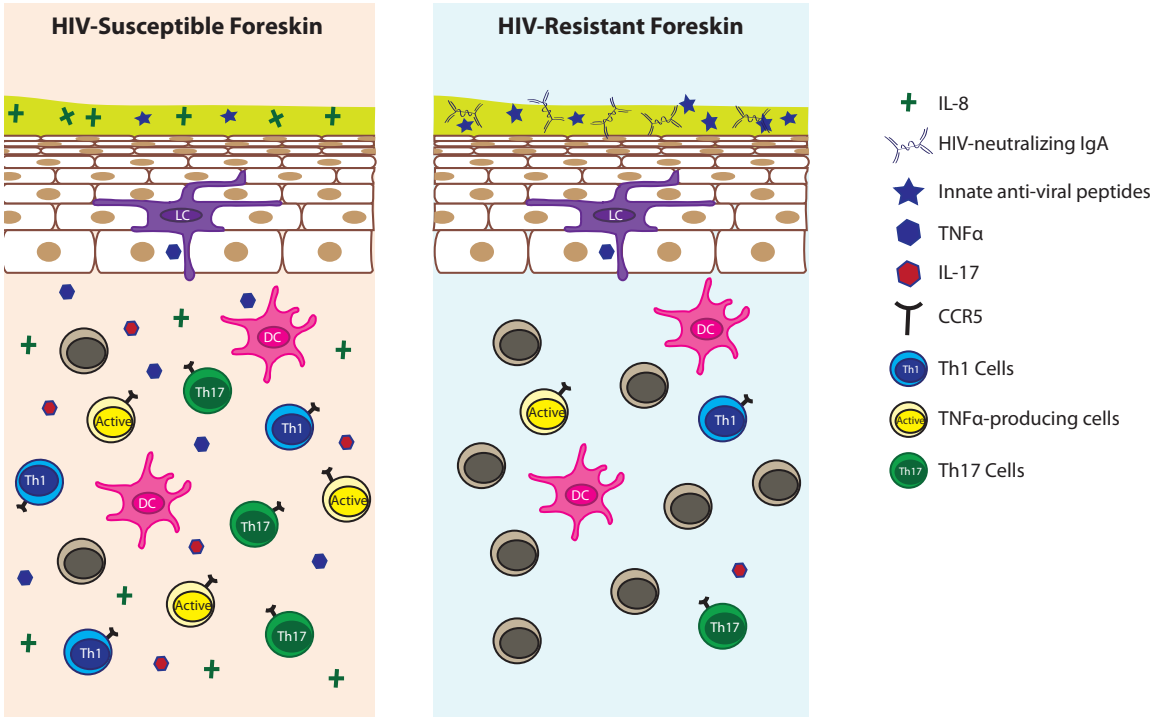

Figure 8.1 Proposed Model of an HIV-Resistant Foreskin. The HIV-resistant foreskin has a reduced relative abundance of HIV-susceptible cell types, such as Th17 cell, Th1 cells, and activated CD4 T cells, with increased secretion of HIV-neutralizing IgA and anti-viral peptides such as α-defensins.

However, as discussed in Section 1.5.2, cross-sectional observational studies such as the present work are effective in identifying potential correlates of protection from infection, but are naturally confounded by the immunological effects of exposure to HIV. Therefore, each element of the proposed model must be confirmed/refuted in mechanistic studies. The necessity of these confirmatory studies are demonstrated by the case of α-defensins: despite their anti-HIV activity *in vitro*[272], increased levels of α-defensins *in vivo* were prospectively associated with an increased risk of seroconversion[278]. This may be due to the fact that in addition to directly neutralizing HIV they also have diverse pro-inflammatory effects[262], and therefore any protective benefit is outweighed by the recruitment and activation of additional HIV target cells. In contrast, HIV-neutralizing IgA in cervicovaginal secretions is associated with decreased risk of seroconversion[300]. Therefore, prospective studies are necessary to determine if each potential correlate of protection truly contributes to HIV-resistance *in vivo*.

The ideal study design to test this would be a longitudinal prospective study with seroconversion as an outcome. For soluble immune factors, such as α-defensins and HIV-neutralizing IgA, it

would be possible to follow men who choose to remain uncircumcised for several years, collecting sub-preputial swabs and performing HIV testing at regular intervals. Unfortunately, cellular parameters could not be measured directly in such a prospective study because removal of the foreskin to measure these cell populations means any subsequent seroconversion will be independent of those cells. However, I found that IL-8 in sub-preputial swabs is associated with increased density of HIV-susceptible CD4 T cell populations and CD8 T cell cytokine production. Therefore, measuring IL-8 prospectively could provide some indications if the reduced Th17 and TNFα-producing T cells observed in HESN men are actually protective. Such a study would also provide a general proof-of-concept that foreskin inflammation predicts HIV susceptibility. However, because IL-8 levels correlated with densities of all pro-inflammatory T cell populations examined (all populations except immunomodulatory Tregs), such a study would not provide information on the roles of *specific* T cell populations, such as Th17 cells. Additionally, even in highly affected areas such as Uganda, annual HIV incidence ranges from less than 1% to 5%, and therefore any prospective studies with seroconversion as an endpoint would require large numbers of participants and several years of follow-up to obtain sufficient power to detect statistically significant differences.

An alternative approach would be to recapitulate potential correlates of protection in *ex vivo* models of foreskin HIV infection. One *ex vivo* infectivity model that has been used successfully with foreskin tissue is a long-term culture assay, where small pieces ($4mm^2$) of foreskin tissue are inoculated with an R5-tropic strain of HIV for several days (4-14) and levels of p24 production in the supernatant are measured[145]. This model would be able to demonstrate whether a correlate of protection was recapitulated, the ability of the foreskin tissue to support productive viral infection and replication is diminished. However, during the extended duration of culture time necessary for p24 production, the integrity of the foreskin tissue is lost, and therefore this assay may not be able to account for the effects of the barrier function of the epidermis or spatial positioning of cell populations, for example the depth of Langerhans cells from apical epidermal surface. Additionally, the bulk p24 read-out does not provide information on the exact cell types participating in establishment of HIV infection.

A second model that would provide some of this missing information would be a short-term culture assay (4-24 hours) using a virus expressing PA GFP-tagged Vpr. Tissue exposed to fluorescently tagged HIV can then be examined with IHC to determine how many virions were

able to penetrate the epithelial barrier, and also to explore what cell types the virus first makes contact with. This model was recently been successfully used in explanted cervical tissue to study how HIV crosses the intact cervical epithelium[155]. The short-term culture does not allow sufficient time for productive infection, but it would maintain tissue integrity. Additionally, IHC can be used to determine the initial cell populations that the fluorescently tagged virus interacts with upon entering the foreskin, although it would be unclear if HIV actually enters these cells.

A third complementary *ex vivo* infectivity model that could be used would be to infect foreskin cells obtained through the digestion techniques presented in this thesis with a pseudovirus incorporating a reporter system, such as a β-lactamase-Vpr (BlaM-Vpr) chimeric protein. This pseudovirus was originally used to examine HIV entry of PBMCs[489], and has recently been adapted in our lab for use in cervical mononuclear cells. If HIV successfully fuses with the cell membrane (entering it) viral BlaM-Vpr is delivered into the cytosol. The cells are then loaded with the BlaM substrate CCF2-AM. CCF-2AM is a membrane-permeant form of the fluorescent molecule CCF, which contains two fluorophores, 7-hydroxycoumarin and fluorescein, linked by a β-lactam bond. In the absence of virally-delivered β-lactamase, CCF2-AM emits green light at 520nm. In infected cells, β-lactamase (delivered by BlaM-Vpr) cleaves the β-lactam bond of CCF2-AM, altering the emission spectrum so that blue light is emitted at 447nm[489]. In combination with the flow cytometric techniques described in this thesis, this blue emission could be used to identify the exact foreskin cell populations that are entered by HIV. However, similarly to the long-term culture model, this model does not take into account tissue structure and spatial positing of cells, and similarly to the short-term culture model it does not take into account replicative capacity of HIV.

While each of these three models has its limitations, in combination they might effectively be used to test if the potential correlates of protection from infection identified in this thesis truly are protective. For example, if α-defensins were added to each model, their anti-HIV activity may protect against viral penetration/cellular entry in the short-term culture model but their pro-inflammatory effects would be expected to increased viral replication in the long-term culture assay. The ability of HIV-neutralizing IgA to protect against HIV infection could be tested in a similar fashion. The protective effect of reduced T cell activation could be tested with the addition of topical tacrolimus, which is a potent calcineurin inhibitor and has the advantage of

being FDA-approved if it were found to be efficacious. Reduced TNFα and IL-17 production and could be specifically recapitulated with monoclonal blocking antibodies, such as the anti-TNFα monoclonal antibody infliximab, which is also approved to treat inflammatory skin conditions[490], and the anti-IL-17 monoclonal antibody secukinumab, which has been shown to reduce dermal IL-17 production[236, 491]. In addition to blocking the local inflammatory effects of IL-17, numbers of Th17 cells can be reduced by blocking proliferation of skin resident Th17 cells with the monoclonal anti-IL-12/23p40 antibody ustekinumab, which has been shown to prevent T cell-mediated immunopathology in patients with psoriasis [492, 493]. Finally, CCR5-blockers such as maraviroc[494] could be tested to demonstrate the relevance of CCR5 availability in HIV susceptibility.

Once it has been determined which of the correlates of protection identified in this thesis are protective in *ex vivo* systems, new microbicides or prevention modalities targeting these immune characteristics should be developed. However, while many of the modalities suggested above are either being developed, or are already approved for topical use, they may require frequent application to be efficacious. Previous microbicide trials using a vaginally-applied gel formulation of tenofovir (an antiretroviral drug ART) have shown that efficacy is highly dependent on compliance[495], and compliance rates at a population level were extremely low[496]. This shows that frequent application of a microbicide is not practical for many at-risk individuals and therefore will be less effective in a real-world setting. Instead, determining the root cause for altered foreskin immune parameters and targeting these may allow for more long-term activity and necessitate less frequent application. One potential root-cause that warrants further exploration is the effect of the foreskin microbiome on immune parameters. Circumcision both eliminates the anaerobic conditions of the sub-preputial space and also prevents the accumulations of smegma: an oily substance comprised of sloughed epithelial cells and lipid secretions that can provide a substrate for bacterial colonization[497] and is associated with inflammation in the foreskin[146]. Recent characterization of the penile microbiome before and after circumcision has found that circumcision reduces both total penile bacterial load and reduces the relative abundance of specific anaerobic genera[360]. Th17 cells are associated with defense against fungal and bacterial infections[215, 216], and therefore are very likely affected by the microbial communities colonizing the foreskin. Additionally, alterations in vaginal bacterial communities (bacterial vaginosis) are associated with increased risk of HIV

acquisition[361, 362]. It may be that reduced HIV susceptibility, caused by decreased Th17/activated CD4 T cell populations, is the result of a less inflammatory penile microbiome. To explore this hypothesis, bacterial strains/community types could be correlated with foreskin T cell populations *in vivo* in a cross-sectional study with a similar design to the present work, and their effect on HIV susceptibility could be tested in the above described *ex vivo* infectivity models and prospective/longitudinal studies with seroconversion as an endpoint. If the microbiome is found to be responsible for the immune parameters that affect HIV susceptibility, strain-specific targeted antibacterial agents might be tested to shift the microbiome towards a less harmful one, without causing large disturbances in the natural microbiome[498].

There are two final, but important tasks that arise from this work: (1) determining the specificity of HIV-neutralizing IgA, and (2) characterizing the infiltrating T cells in the HESN foreskin. Although the specificity of HIV-neutralizing IgA was not explored in the present work, pervious HESN studies have suggested that it is not HIV-specific[308, 309]. To be able induce production of this IgA, or design prevention modalities with a similar mode of action, we need to determine the source and specificity of this HIV-neutralizing IgA so often observed in HESN populations[295-300, 310, 311, 385]. Determining the phenotype of the additional T cells in the HESN foreskin may also provide new targets for HIV-prevention. While the relative abundance of Th17 cells and TNFα-producing T cells was decreased in the HESN foreskin, the overall number of T cells was increased. Therefore, there was in increase in T cells that were not Tregs, and did not produce IL-22, IL-17, TNFα or IFNγ when stimulated. These uncharacterized T cells may also play an important role in HIV-protection, and should be characterized in future HESN studies.

In conclusion, the present work describes novel methods to isolate live, functional T cells from foreskin tissue and measure several secreted factors in the sub-preputial space, and represents the first description of correlates of protection from HIV in the foreskin. The findings of this study support the growing body of evidence that reductions in the relative abundance of highly HIV-susceptible T cell populations at the site of HIV-exposure is protective against infection. I suggest that reduced CCR5+ CD4 T cells, Th17 cells and TNFα-producing T cells in the foreskin, in combination with HIV-neutralizing IgA in preputial secretions, may protect against HIV infection.

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