

Characterisation of Mucosal Tissue in the Foreskin after Voluntary Medical Male Circumcision

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A dissertation submitted in fulfilment of the requirements for the degree of

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"It is pointless to test your skills against something that does not challenge you"

Rushil Harjiparsad

List of Abbreviations

°C	Degree Celcius
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cells
ART	Antiretroviral Therapy
ARV	Antiretroviral Treatment
BP	Bandpass
BSA	Bovine Serum Albumin
BV	Bacterial Vaginosis
CCL	Chemokine Ligand
CCR5	Chemokine receptor 5
CD	Cluster Differentiation
CHB	Chris Hani Baragwanath Hospital
cm	Centimeter
cm²	Centimeter squared
cm³	Centimeter cubed
CMV	Cytomegalovirus
CT	Chlamydia Trachomatis
CTL	Cytotoxic T lymphocyte
CXCR4	C-X-C chemokine receptor 4
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3-Grabbing non-integrin
DCs	Dendritic Cells

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDH	Edendale Hospital
FBS	Fetal Bovine Serum
FGT	Female Genital Tract
g	Gram
GALT	Gut Associated Lymphoid Tissue
GIT	Gastrointestinal Tract
GM-CSF	Granulocyte macrophage colony stimulating factor
gp120	Glycoprotein 120
GUD	Genital ulcerative disease
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HPV	Human Papillomavirus
HREC	Health Sciences Research Ethics Committee
HSV	Herpes Simplex Virus
ICAM	Intercellular adhesion molecule
ICP27	Infected cell protein 27
IDL	Integrative Data Language
IDU	Intravenous Drug Users
IFN-γ	Interferon gamma
IFS	Immunofluorescent staining
IL	Interleukin
IP	Inducible Protein

IR	Infrared
Just ImageJ	Fiji
KZN	Kwa-Zulu Natal
LCs	Langerhans Cells
LN	Lymph Node
LPS	Lipopolysaccharide
MDM	Monocyte-Derived-Macrophage
MG	Mycoplasma Genitalium
MGT	Male Genital Tract
min	Minutes
ml	Milliliter
mm	millimeter
mm³	Millimetre cubed
MMC	Medical Male Circumcision
MSM	Men who have sex with men
MTC	Mother-to-child
NG	Neisseria Gonorrhoeae
NHLS	National Health Laboratory Services
NICD	National Institute for Communicable Diseases
NK	Natural Killer
NLO	Non-Linear Optical
nm	Nanometer
NRF	National Research Foundation
OCT	Optimal cutting temperature compound

PBMC	Peripheral Blood Mononuclear cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
PHRU	Perinatal HIV Research Unit
PrEP	Pre-exposure prophylaxis
PrEP	Pre-exposure prophylaxis
PRF	Poliomyelitis Research Foundation
R5	CCR5 Tropic Virus
RANTES	Regulated on activation, normal T expressed and secreted
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
RT	Room Temperature
SA	South Africa
SADH	South African Department of Health
SIV	Simian Immunodeficiency Virus
SSA	Sub-Saharan Africa
STIs	Sexually Transmitted Infetions
TCR	T cell receptor
Th	T Lymphocyte
TNF-α	Tumor necrosis factor alpha
TV	Trichomonas Vaginalis
U/ml	Units per milliliter

UCT	University of Cape Town
UNAIDS	Joint United Nations Programme on HIV/AIDS
VIVID	Violet Fluorescent reactive dye
vs	Versus
WHO	World Health Organisation
WКУHA	WhizzKids United Africa Aid
κ	Kappa
μg	microgram
μl	Microliter
μm	Micrometer

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Abstract

Background: Medical Male Circumcision (MMC) reduces the risk of HIV-1 acquisition by up to 60% as shown in a number of randomized controlled trials in Uganda, Kenya and South Africa. MMC has also been shown to reduce the prevalence of other sexually transmitted infections (STIs) like *Herpes Simplex Virus (HSV) -2* and *Human Papillomavirus (HPV)* by 25% and 35% respectively. Asymptomatic STIs may elevate the risk of HIV-1 acquisition by recruiting HIV-1 target cells to the foreskin. The higher permeability of the inner foreskin may play a role in HIV-1 acquisition as well as the number of target cells present in the foreskin. The more inflamed inner foreskin may be increasing the risk of a productive HIV-1 infection. The aims of this dissertation was to a) examine the levels of keratinisation in the inner and outer foreskins after MMC; b) investigate the number of Langerhans, Ki67+ and CD4+ T cells in the inner and outer foreskin and c) identify the impact of asymptomatic STIs on the numbers and proliferative capacity of foreskin-resident Langerhans and CD4+ T cells.

Methodology: A total of 150 young males (14-24 years) were recruited from Edendale Hospital in Kwa-Zulu Natal and the Perinatal HIV-1 Research Unit in Soweto, Johannesburg, South Africa. Foreskins, blood and urine were collected. Using immunofluorescent staining (IFS) of foreskin tissue sections, keratin thickness was measured and immune cells present in the inner and outer foreskin were characterised. We optimised the types and concentrations of collagenase, type of maceration and fresh versus frozen tissue for flow cytometry. Once techniques were optimized for IFS, we stained for CD4+, langerin+ (CD207) and Ki67+ cells as well as filaggrin for keratin thickness in a subset of 28 age-matched HIV-1 negative individuals (14 – 24 years) undergoing elective MMC. An average of 5 images per tissue was taken, keratin thickness and cell depth was measured and cell densities quantified using integrative data language (IDL). We compared all parameters between the inner and outer foreskin and assessed the impact of STIs (*C. trachomatis*, *N. gonorrhoea*, *M. genitalium*, *T. vaginalis*, HSV-1 & 2 and HPV) on HIV-1 target cell density and depth within foreskin tissues.

Results: Antibody dilutions for CD4, CCR5 and CD209 worked best at 1:100, 1:10 and 1:200 respectively and the use of Alexa647 was best for the detection of Ki67. Immunofluorescent staining for CD4, Ki67 and CD207 to identify proliferating immune cells and filaggrin for keratin layers showed significantly higher numbers of CD207+ Langerhans cells present in men with STIs, in both the inner (3.3 vs 0.3 cells/mm² in STI+ vs STI- men, p<0.0017) and the outer (4.4 vs 0.6 cells/mm², p<0.0005) foreskin. There were no significant differences in CD4+ T cell (4.4 vs 2.7 cells/mm² in the inner and outer foreskin) and Ki67 cell (3.8 vs 3.2 cells/mm² in the inner and outer foreskin) counts regardless of STI status and the keratin layer was significantly thicker (8.3873 vs 6.6748µm in outer vs inner foreskin, p=0.0058) with a median difference of 1.712µm. LCs were significantly closer to the keratin layer (75.25 vs 99.19 µm in outer vs inner foreskin, p<0.0001) with a median difference of 23.922µm. LCs in the outer foreskin were significantly closer (68.19 vs 83.608µm of STI+ and STI- men, p=0.0385) to the keratin layer with a median difference of 15.418µm.

Conclusion: This study successfully quantified HIV-1 target cells susceptible to infection present in the foreskin by using a formerly quantitative only method such as immunofluorescence. Lastly, the thicker keratin layer of the outer foreskin suggests that the level of keratinization provides a more robust barrier than the thinner keratin layer of the inner foreskin. Asymptomatic STI-induced inflammation and recruitment of immune cells may be elevating the risk of HIV-1 acquisition in uncircumcised men. Therefore, MMC removes the potential HIV-1 target cells present in foreskins of highly susceptible sexually active group of young adolescent uncircumcised men in South Africa and this in turn may lower the risk of acquiring an STI infection as well as reduce the risk of HIV-1 infection.

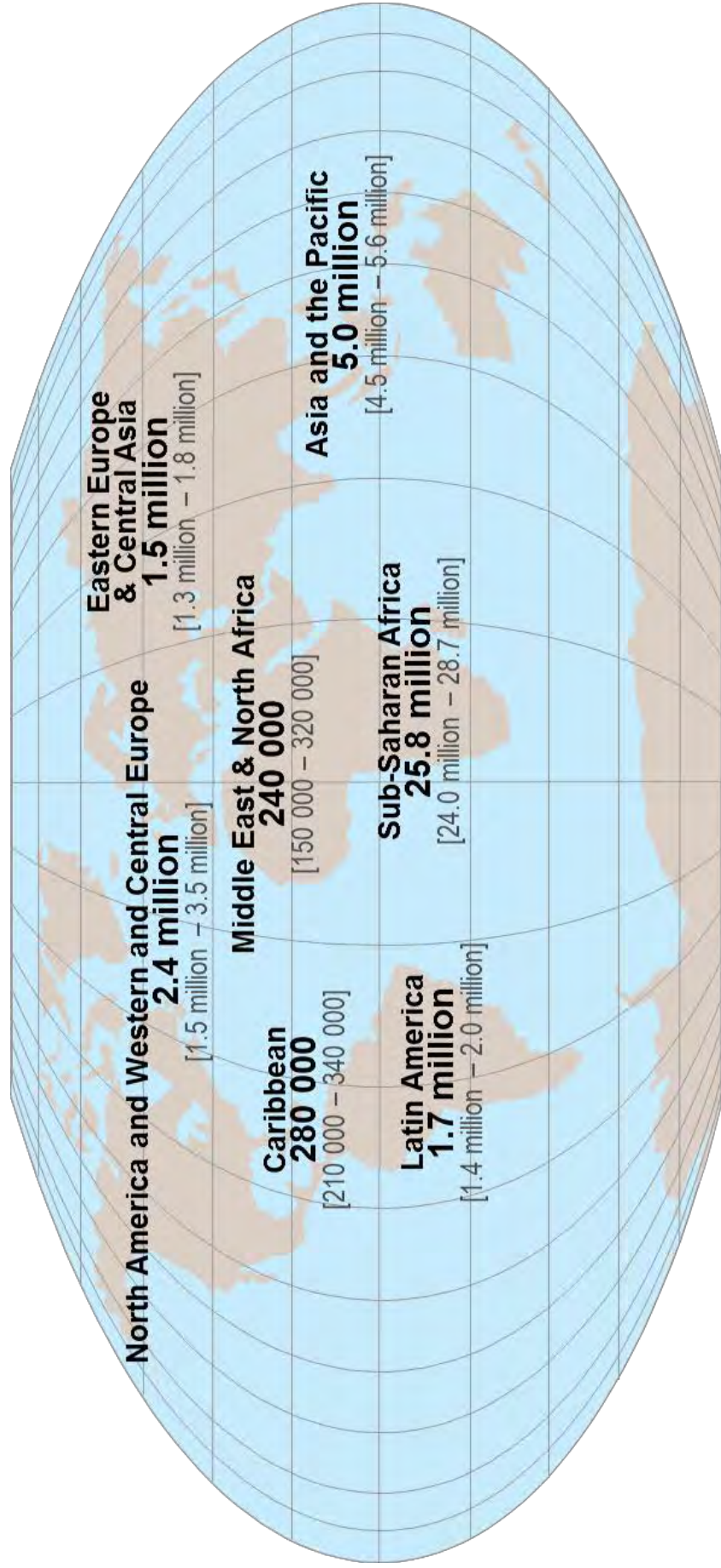
CHAPTER 1: LITERATURE REVIEW

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1.1 Introduction:

Since the discovery of Acquired immunodeficiency syndrome (AIDS) more than 30 years ago, there has been more than 25 million deaths globally and more than 60 million people infected. There are currently 36.9 million people living with human immunodeficiency virus (HIV-1) globally with an estimated 2 million new infections and 1.2 million deaths in 2014 as a result of AIDS (Unaids 2015). Sub-Saharan Africa (SSA) has been burdened with the highest rate of people living with HIV-1 for as long as the past 25 years. In 2014, there were an estimated 25.8 million people living with HIV-1 in SSA alone, accounting for as much as 70% of the global population living with HIV-1 [(Unaids 2015); Figure 1.1]. There were an estimated 1.4 million new HIV-1 infections and 790 000 AIDS-related deaths in SSA. There are 10.7 million people in SSA who have access to antiretroviral therapy (ART) with men comprising 36% and women 47% (Unaids 2015). Men in SSA accounts for an estimated 7.3 million people living with HIV-1 above the age of 15 and an estimated 1.9 million of these people are young adults between the ages of 15-24 (Unaids 2015). In South Africa, there are 6.4 million people that are living with HIV-1. Young male adolescents between the ages of 15-24 account for 28.4% (720 000) of individuals living with HIV-1 and only 14.3% (103 000) have access to ART (Shisana *et al.*, 2014).



Total: 36.9 million [34.3 million – 41.4 million]

Figure 1.1: Representation of the proportion of people that are living with HIV-1. Sub-Saharan Africa accounts for as much as 69.9% of the world's population that are living with HIV-1 (Taken from UNAIDS, 2015)

1.2 HIV-1 staging, acquisition and transmission rates

HIV-1 infection can be defined by three stages, the acute phase, the asymptomatic chronic phase and the progression to AIDS (reviewed by Coffin & Swanstrom 2013). Upon entry of the virus and infection of the first cell up until the virus is detectable in blood is termed the eclipse phase and can last between 7-21 days (Lindback *et al.* 2000). The eclipse phase is clinically silent and virus can be disseminated in CD4+ T cells present in mucosa, submucosa, draining lymphatics and to a lesser degree, the gut associated lymphoid tissue (GALT) and systemic lymphatic tissues (Haase 2010). In 2003, Fiebig and colleagues devised a laboratory staging system whereby they could identify acute and early infection (Fiebig *et al.* 2003). Fiebig staging can be categorised into stages I-IV extending from 10-30 days with each the specific stages lasting an average of 3-5 days, stage V lasting approximately 69.5 days and stage VI can be divided into recent versus early chronic infection (Fiebig *et al.* 2003). Figure 1.2 summarises the Fiebig stages and assays used in the ordered appearance in plasma of HIV-1 viral RNA (Fiebig stage I), viral p24 antigen (Fiebig stage II) and virus-specific antibodies (Fiebig stage III) which are detectable by recombinant protein-based enzyme-linked immunosorbant assay and then detected by using Western immunoblotting (Fiebig stage IV), diagnostic banding pattern but omitted p31 reactivity (Fiebig stage V) and lastly diagnostic banding pattern inclusive of p31 reactivity (Fiebig stage VI) (Fiebig *et al.* 2003; reviewed by Shaw & Hunter 2012). During the chronic phase of infection also defined as “clinical latency,” there is a constant increase in the viremia level by 1-100 000 copies/ml and gradual decrease in CD4+ T cell levels. Patients are not always aware that they are infected with the virus at this stage. The level of HIV-1-RNA decreases to less than 1% of the original viral load and remains relatively constant, this is known as the viral set point (reviewed by Fanales-Belasio *et al.* 2010). The last phase, AIDS, is characteristic of a decrease in the number of peripheral blood CD4+ T cells to ± 200 cells/ μ l. This is due to the deterioration of the immune system. The body unable to maintain immune control and as a result, opportunistic infections begin to appear (reviewed by Coffin & Swanstrom 2013). There is an increase in viral load and if left untreated, the combined effects of the opportunistic infection and a compromised immune system will ultimately lead to the death of the infected individual.

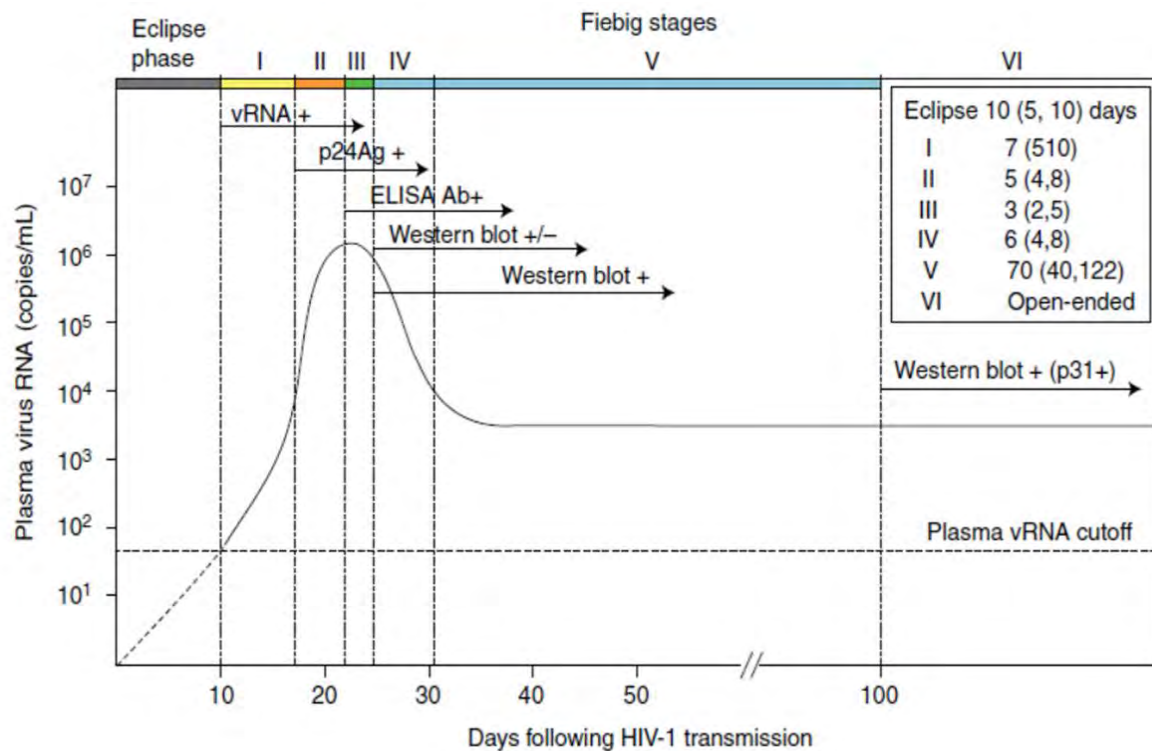


Figure 1.2: Fiebig stages (Taken from Shaw and Hunter., 2012).

Heterosexual intercourse may apply to both penile-vaginal and penile-anal intercourse. A number of trials, such as HPTN052, PARTNER and VOICE, have reported women engaging in penile-anal sex. Furthermore, women may be at an increased risk of contracting HIV-1 due to both penile-vaginal and penile-anal intercourse (Ramjee & Daniels 2013). There are as many as 90% of men in Africa that have acquired the virus through heterosexual intercourse (Donoval *et al.* 2006). HIV-1 transmission mainly occurs due to the exposure to virus at mucosal surfaces. It has been estimated that the transmission of HIV-1 during heterosexual encounters is more efficient from male-to-female than it is from female-to-male (Nicolosi *et al.* 1994; Carpenter *et al.* 1999). Powers *et al.*, had suggested that this is not the case and that during a single sex act, there is no difference in the transmission of HIV-1 from male-to-female or from female-to-male. Viral load plays a major role in determining the transmission risk of HIV-1 from one individual to another (Shaw & Hunter 2012). Patel *et al.*, has estimated that the risk of HIV-1 transmission through heterosexual intercourse in developed counties to be ~0.0008% for receptive and ~0.0004% for insertive penile-vaginal intercourse. The estimated risk of HIV-1 transmission for

males who had sex with males (MSM) was 5 times more than receptive penile-vaginal intercourse, with it estimated at ~0.005% through unprotected receptive anal intercourse. Patel *et al.*, has also estimated that the risk of HIV-1 transmission is ~0.001% per coital act. In contrast to the developed countries, a meta analyses in Eastern and Southern Africa involving a total of 14 sites, it was estimated that men and women are twice as likely to acquire HIV-1 through heterosexual intercourse, with 0.0010 and 0.0009 per coital act for men and women respectively (Hughes *et al.* 2012). These estimates of HIV-1 transmission do not explain the magnitude of the HIV-1 burden experienced by Sub-Saharan Africa. There may be many factors that influence the acquisition of HIV-1 in males. A systematic review by Patel *et al.*, and a meta-analysis by Powers *et al.*, suggested that factors such as a high viral load, genital ulcer disease, sexually transmitted infection (STIs), additional sexual partners, age, condom usage and whether or not the person is circumcised as well as the stage of disease play a role in HIV-1 acquisition.

1.2.1 HIV-1 Transmission and Acquisition

HIV-1 results from exposure to the virus at mucosal and percutaneous inoculation and can be transferred from one individual to another during sexual intercourse (Shaw & Hunter 2012). HIV-1 may be acquired by various means such as mother-to-child (MTC) transmission, heterosexual intercourse, MSM, sharing of needles by intravenous drug users (IDU) (WHO *et al.* 2014) and via abrasions in the skin.

1.3 The skin as a barrier to HIV-1

The skin consists of a highly specialized epithelium that is involved in prevention of water loss, toxin exclusion, resistance to mechanical stress and plays a role in immune responses. The skin is the first physical barrier of protection from invading organisms (reviewed by Simpson *et al.* 2011). Although people may not be infected through the skin, open cuts or wounds in the skin may make them susceptible to HIV-1 infection upon exposure to the virus. In an experiment by Kawamura *et al.* 2008, where they used abraded skin in explant models by using a wire brush, they showed that DCs were involved in virus dissemination (Kawamura *et al.* 2008). Keratinocytes are able to establish a barrier between the host and the environment by forming an adhesive network that has been organised into multiple layers. The skin has four main layers, namely the stratum basale, stratum spinosum, stratum granulosum and the stratum corneum (reviewed by Simpson *et al.* 2011; Figure 1.3B). Briefly, the keratinocytes are

able to replace cells that are shed from the body surface and the basale cells in the stratum basale are fixed to the matrix and proliferate. A small portion of their daughter cells are then able to enter the stratum spinosum where they exit the cell cycle and are able to grow and establish robust intercellular connections. The cells in the stratum granulosum flatten and are able to assemble an impermeable water cornified envelope that underlies the plasma membrane. Lastly, the keratinocytes in the stratum corneum are completely squamous and tightly crosslinked together to form the completed cutaneous barrier (reviewed by Simpson *et al.* 2011). There are many types of cells that are present in the skin and these can be divided to cells that are found in the epidermis and dermis of the skin. The epidermis is the outer most skin layer made up of stratified squamous epithelium and is separated from the dermis by a thin basement membrane zone (Strid *et al.* 2009). The dermis is the inner skin layer and consists of a collagen meshwork, dermal fibroblasts and a high concentration of immune cells (Strid *et al.* 2009). The cells present in the epidermis include keratinocytes which are involved in the replacement of cells shed from the body surface, langerhans cells (LCs), involved in antigen presentation and T cells involved in creation of immune response and clearance of pathogens while the dermis contains dendritic cells involved in antigen presentation and induction of T cell responses, macrophages involved in phagocytosis of foreign substances and T cells as mentioned above involved in the creation of an immune response and clearance of pathogens (reviewed by Summerfield *et al.* 2015). The skin structure is similar to foreskin structure and have similar types of cells that are also present in the foreskin.

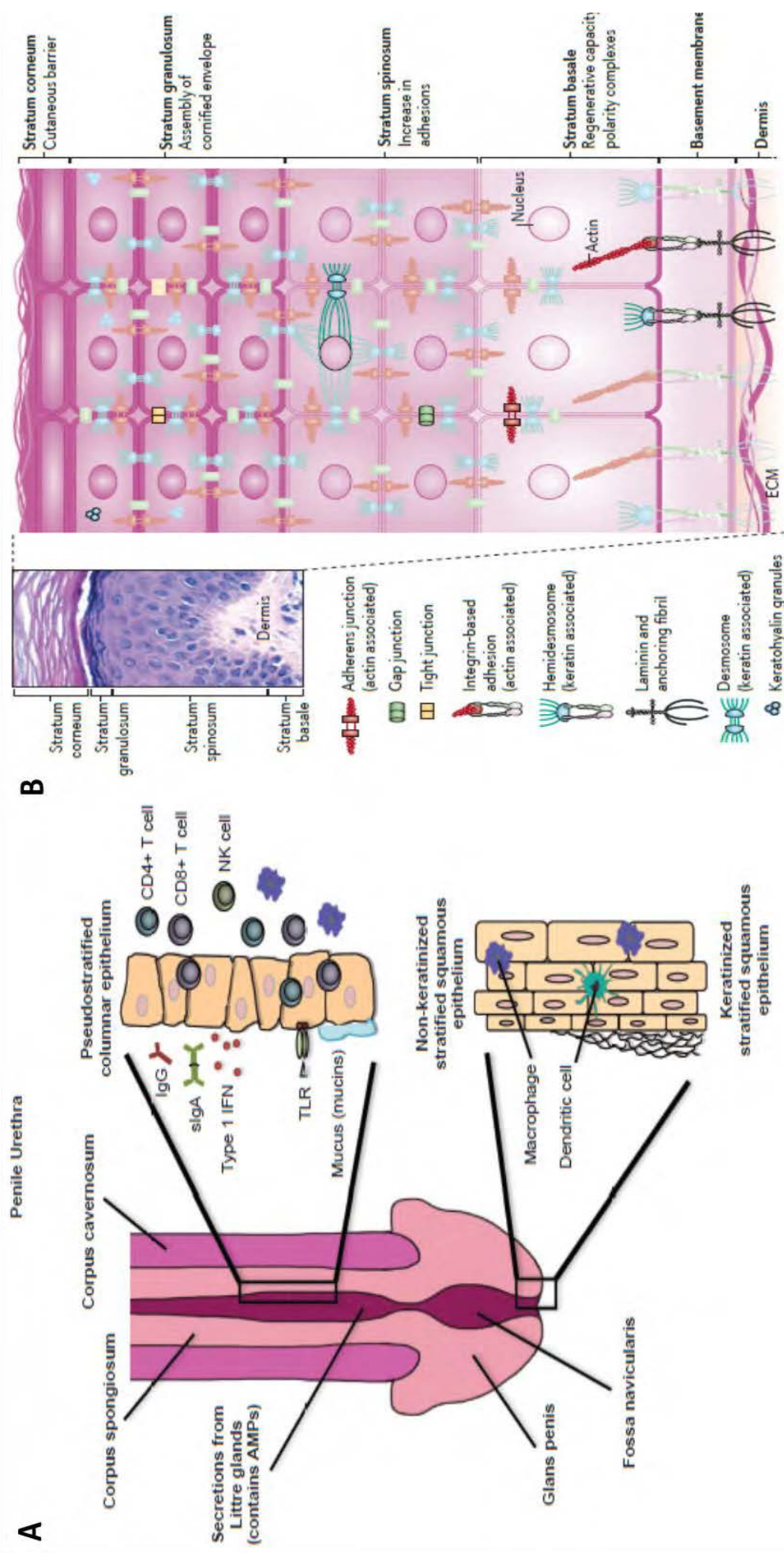


Figure 1.3: Penile anatomy and its immunological components. A The urethral pseudostratified columnar epithelium is an active immune microenvironment that contains key targets for HIV-1 infection of the urethra (Taken from Nguyen *et al.* 2014). **B** Skin structure and its components (Taken from Simpson *et al.* 2011).

1.4 HIV-1 at mucosal surfaces

The mucosal layer is less robust and consists of the epithelium and lamina propria (eds Bellanti *et al.*, 2012) compared to the 4 layers found in skin. The epithelial cells lining the mucosa play a role in innate immunity and provide a physical barrier (McGhee & Fujihashi 2012). The mucosal surfaces cover a total area of 400m² and are therefore the primary site of antigen entry (reviewed by Borges *et al.* 2010; Nagler-Anderson 2001). It is therefore not surprising that 90% of HIV-1 infections worldwide occur via mucosal surfaces (Kresina & Mathieson 1999). HIV-1 transmission occurs at the female and male genital tract, the intestinal tract, gut and draining lymph node (LN) (reviewed by Cohen *et al.* 2011; reviewed by Shaw & Hunter 2012; Deeks *et al.* 2013). The GALT has many of the lymphoid cells and CD4+ T cells are rapidly depleted during primary infection with HIV-1 (reviewed by Swanstrom & Coffin 2012). Majority of the CD4+ T cells reside within the gastrointestinal tract (GIT), LNs, and other lymphatic tissues compared to peripheral blood (Mowat & Viney 1997), furthermore the GIT can be extremely permissive to HIV-1 infection and replication. The gut mucosa contains a high concentration of CCR5 expressing CD4+ T cells compared to the LN (Brenchley *et al.* 2004). During acute HIV-1 infection, there is indirect epithelial injury as well as a rapid loss of CD4+ T cells in the GIT (Brenchley *et al.* 2004; Sankaran *et al.* 2008). The loss of mucosal integrity observed results in the sustained exposure within the gut mucosa to proinflammatory microbial products leading to microbial translocation (Mehandru *et al.* 2004) in turn leading to HIV-1-mediated CD4+ T cell depletion (Brenchley *et al.* 2004). Furthermore, the inflammatory response and tissue reconstruction during innate and adaptive immune responses prompted by HIV-1 replication leads to the destruction of the LN contributing to the depletion of CD4+ T cells (Schacker *et al.* 2002). Therefore, during HIV-1 infection, the LN, which is an organ of antigen presentation and homeostasis, becomes an organ of inflammation and fibrosis (Brenchley *et al.* 2004).

HIV-1 is able to gain entry into the body during sexual intercourse by crossing the epithelial barriers that covers the mucosal surfaces of both male and female genital tracts as well as anal/rectal epithelia (Figure 1.4B), once HIV-1 crosses the epithelial barrier, it is able to establish a founder population of infected cells within hours of infection (Tebit *et al.* 2012). A review by Haase using a simian immunodeficiency virus (SIV) rhesus macaque explains the early events in HIV-1 infection. After challenging

the rhesus macaques with high doses of SIV at the mucosal surface, the virus is able to cross the mucosal epithelial barrier within hours of infection and is able to establish a founder population of infected cells within lymphoid tissue as seen in humans (Haase *et al.* 1996). During the first week of infection, the infected cells undergo local expansion and are able to generate adequate virus and infected cells in order to disseminate and establish systemic infection in secondary lymphoid organs (Haase 2010). This results in the lytic destruction of susceptible CD4+CCR5+ host cells (reviewed by Veazey *et al.* 2001). In the second week of infection, the replication erupts in the lymphatic tissue where the virus is able to access susceptible target cells and the viral levels in blood and tissue peak (Figure 1.4A; Haase 2010). The first CD8 T cell response appears before the peak of viremia and as the T cell response evolves, the plasma viral load falls to stable levels by four weeks after exposure, also seen in humans (Haase 2010; M. S. Cohen *et al.* 2011). The most characteristic event in the acute phase is the decrease in the level of peripheral blood CD4+ T cells (Fauci *et al.* 1996; Clark *et al.* 1991). As the year's progress, the number of CD8+ T cells are depleted and opportunistic infection settles in and this induces additional mucosal damage. The opportunistic infection may be able to prime naïve CD4+ T cells which results in the increase of CD4+ T cell activation and expression of CCR5 and this forms viral target cells (Veazey *et al.* 2001). The sustained increase in viral target cells may explain the lymphoid depletion and increase in viral load over the years leading to the onset of AIDS.

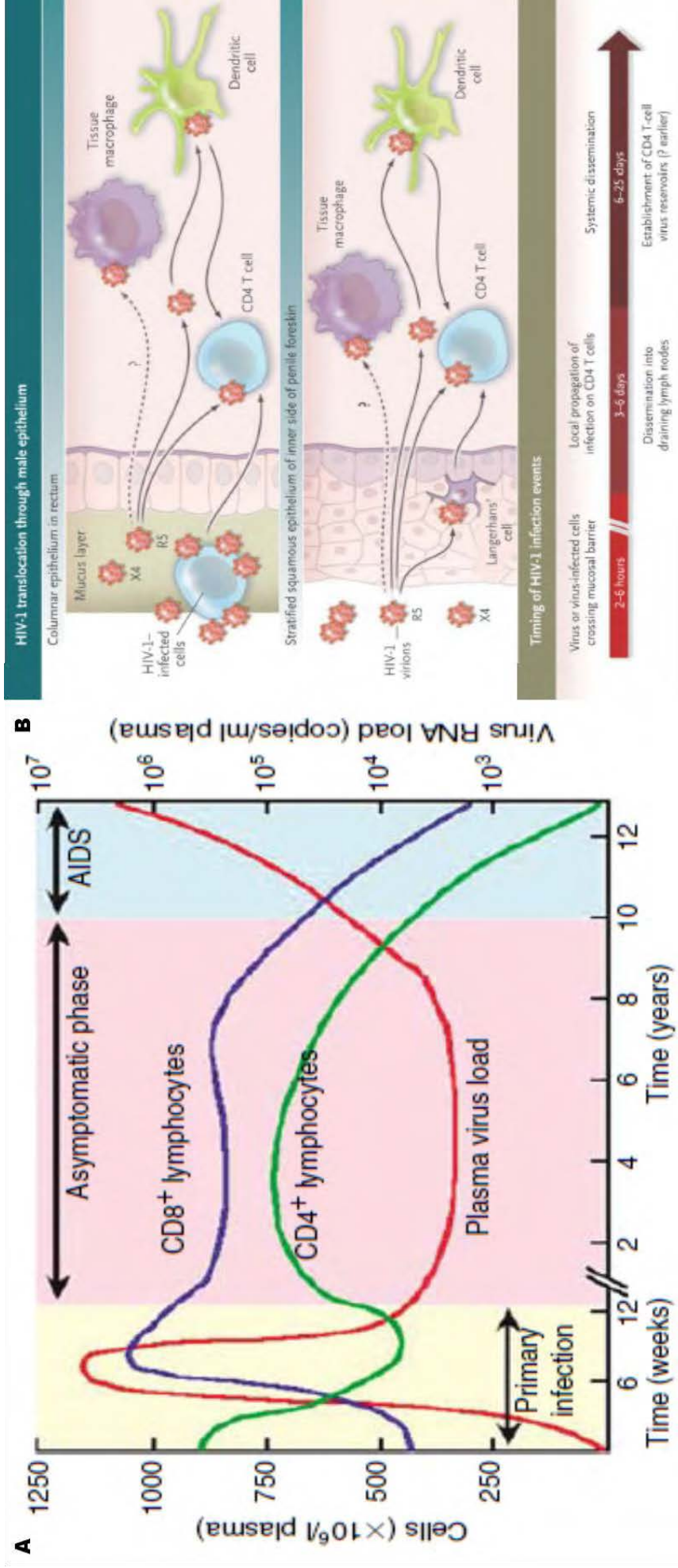


Figure 1.4: Time-course of HIV-1 infection. **A**) Stages and events of HIV-1 infection, the red line represents the plasma viral load, the green line represents the CD4⁺ T cells and the blue line represents the CD8⁺ T cells over time. **B**) Representative event of HIV-1 entering the mucosa of the rectum and inner penile foreskin during the first 25 days of infection (Taken from Munier & Kelleher 2007 and Cohen *et al.* 2011).

1.4.1 Anatomy of the male genital tract

1.4.1.1 The penis, testes and other organs

The male penis can be measured in a flaccid and an erect state. The flaccid length of an adult human penis is usually 5-6cm shorter than the erect adult penis which is an average of 14cm in length and 12.3cm in circumference (Wylie & Eardley 2007) which infers that the total surface area of an erect penis averages $\sim 200\text{cm}^2$ (Anderson *et al.* 2011). The male genital tract (MGT) consists of the testes, the accessory glands, the excurrent duct system and the penis (Cao & Hendrix 2008). The excurrent duct system is composed of the rete testes, efferent ducts, epididymis and vas deferens while the accessory glands are composed of the seminal vesicles, prostate and bulbourethral glands (Cao & Hendrix 2008). There are two types of epithelia in the MGT, namely the simple and stratified epithelia. The penile urethra that consists of keratinized stratified squamous epithelium transitions into non-keratinized stratified squamous epithelium in the fossa navicularis (Figure 1.3A; reviewed by Nguyen *et al.* 2014). The testes is an immune privileged site and has two distinct regions, the interstitial spaces between the tubules and the seminiferous tubules. The testis is responsible for the generation of sperm and production of testosterone. Leydig cells comprise majority of the cell population that is present within the interstitial space and is responsible for the synthesis of testosterone (reviewed by Nguyen *et al.* 2014).

1.4.1.2 The foreskin

The male has a sheath or covering over the penis known as the foreskin or prepuce. This foreskin consists of a double-sided layer of stratified squamous epithelium. The foreskin is a continuation of skin from the pubic mound, up the shaft of the penis and covers the glans penis and urethral meatus (WHO & UNAIDS 2007). The frenulum, which is a highly vascularised tissue, attaches the glans to the foreskin. The frenulum also forms the interface between the inner and outer foreskin (WHO & UNAIDS 2007). The outer foreskin is similar to the skin on the shaft of the penis while the inner foreskin has a similar composition to a mucous membrane. The foreskin epidermis is comprised of the stratum corneum, stratum granulosum, stratum spinosum and stratum basale. Upon erection, the inner surface of the foreskin is exposed and is thought to be more susceptible to viral entry (Hussain & Lehner 1995). The foreskin has been studied quite extensively as this was believed to provide protection for the penis in the past from abrasions and is believed to protect the sensitive (Simmons &

Jones 2007) glans of the penis but is now believed to be a key area in the acquisition of HIV-1 in males. Kigozi *et al.*, had observed that the size of foreskin had a significant correlation with the HIV-1 incidence rates. It was thought that the portal of entry for HIV-1 could be the glans, sub-prepuce and/or the urethra (Figure 1.5; Morris & Wamai 2012).

1.4.1.3 HIV-1 crossing the barrier

The foreskin is lined by stratified squamous epithelium and the external surface has been suggested to be more keratinised than the internal surface (McCoombe & Short 2006; Patterson *et al.* 2002). The inner foreskin surface and glans are wet mucosal epithelia and may be more susceptible to infection (Anderson, J. a. Politch, *et al.* 2011). The moist mucosa of the penis has been associated with HIV-1 acquisition in the MGT (O'Farrell *et al.* 2006). The foreskin of the penis folds back over the glans upon erection thereby exposing the inner foreskin mucosal epithelium to the external environment and an opportunity for HIV-1 entry (Figure 1.5). This exposes it to physical trauma and genital secretions of the female during heterosexual intercourse. The HIV-1 virions and infected cells of the infected partner may become trapped under the foreskin therefore increasing the risk of HIV-1 acquisition (Anderson, J. a. Politch, *et al.* 2011).

The outer foreskin has been suggested to provide a greater protective barrier to HIV-1 transmission due to the skin keratinisation. The urethra is more likely to become infected as it is lined with stratified, non-keratinised columnar epithelial cells (Hladik & McElrath 2008). The foreskin mucosa is believed to be rich in potential HIV-1 target cells such as LCs and CD4+ T cells in the squamous lining as well as T cells, macrophages and dendritic cells (DCs) in the underlying stroma (Hussain & Lehner 1995; McCoombe & Short 2006; Patterson *et al.* 2002; Donoval *et al.* 2006). These cells provide immune protection by killing infected cells. However, upon entry, it is possible that viral escape from HIV-1 specific T immunity in the foreskin can occur (reviewed by Ganor *et al.* 2010).

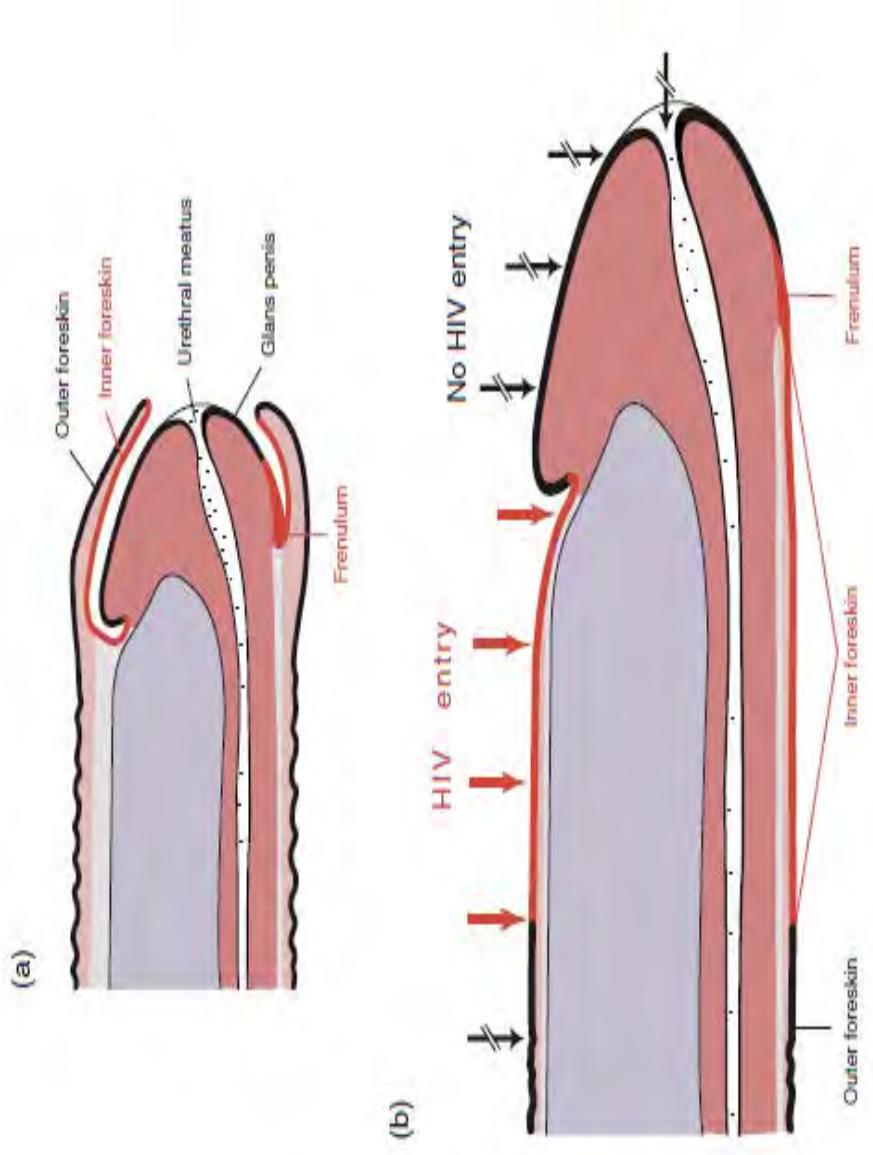


Figure 1.5: HIV-1 entry in the penis. A) Flaccid penis with the foreskin covering the glans of the penis. The inner foreskin is not exposed to the external environment. B) An erect penis with the foreskin retracted back over the glans of the penis exposing the inner foreskin to HIV-1 entry (Taken from McCoombe & Short 2006).

1.5 Target cells for HIV-1 within foreskin tissue

1.5.1 Langerhans Cells

Similar to the skin, the foreskin has an epidermis and a dermis with various types of cells present. The mucosal surface of the inner foreskin is rich in LCs (Szabo & Short 2000). LCs are antigen presenting cells that sense foreign antigens in the environment with the aid of their dendrites (Merad *et al.* 2008), are present in the epidermis of the foreskin (Merad *et al.* 2013) and are thought to be the first immune cells to encounter HIV-1 (Patterson *et al.* 2002; Kawamura *et al.* 2005; de Witte *et al.* 2007). LCs are also part of the dendritic cell lineage and express the C-type lectin langerin (Patterson *et al.* 2002). Langerin is responsible for the generation of birbeck granules believed to be involved in the internalisation and degradation of viral particles (Romani *et al.* 2010; de Witte, Nabatov, Pion, *et al.* 2007). Upon capture of foreign antigens, the LCs migrate to the lymph node and signal to T cells in order to elicit an appropriate immune response, therefore LCs are important in the first immunological barrier against invading pathogens (Merad *et al.* 2008). LCs have the ability to bind to the HIV-1 envelope glycoprotein subunit gp120 using their unique C-type lectin, langerin (de Witte, Nabatov, Pion, *et al.* 2007). In the past, LCs have been shown to transmit the virus to T cells upon infection (Blauvelt *et al.* 2000; Kawamura *et al.* 2001), but recent data shows that LCs have been able to provide protection against HIV-1 as well as the prevention of HIV-1 dissemination in the host (de Witte, Nabatov, Pion, *et al.* 2007). However, this is only possible at low viral concentrations ($\leq 10\ 000$ copies/ml), while at high viral concentrations ($\geq 100\ 000$ copies/ml) (de Witte, Nabatov, Pion, *et al.* 2007), the protective effect of langerin is inhibited allowing for the transfer of the internalized virus to T cells (Hladik & McElrath 2008). The role of langerin may be important in the fight against HIV-1. Several studies have found that LCs were detected at higher densities in the epithelium of the foreskin (Donoval *et al.* 2006) and many have observed that there was a greater density of LCs in the inner compared to the outer foreskin (Liu *et al.* 2014). LCs express the primary HIV-1 receptor as well as the co-receptor CCR5, this makes LCs an ideal target for HIV-1 infection and transfer to CD4+ T cells (Kawamura *et al.* 2001). A study by Ganor *et al.*, using an *ex vivo* foreskin explant model to determine the early events of HIV-1 exposure in the foreskin revealed the abundance of LCs in the inner foreskin. This showed that HIV-1 entry through the inner foreskin was more efficient than in the outer foreskin (Ganor *et al.* 2010;

Kawamura *et al.* 2008). An *in vitro* model by Ganor *et al.*, showed that infection was more efficient in HIV-1 infected cells compared to the cell-free virus. Upon high viral loads (200 μ l of HIV-1 infected PBMCs or cell free virus) in the foreskin, the LCs were infected and the formation of LC-T cell conjugates were observed in the inner foreskin compared to the outer foreskin (Figure 1.6; Ganor *et al.* 2010). In the inner foreskin, HIV-1 infected cells form viral synapses and results in HIV-1 particle budding [1]. At low viral concentrations (100 μ l of HIV-1 infected PBMCs or cell free virus), LCs are induced within the epidermis and LCs internalise HIV-1 and degrade it [2]. In a high viral concentration, the cells infected with HIV-1 [3] attract LCs to the mucosal surface where they capture HIV-1 [4] and migrate back to the epidermal-dermal interface. LCs are able to form LC-T cell conjugates allowing for HIV-1 transfer to T cells [5]. The conjugates are then able to disseminate the infection [6] (Figure 1.6; Left). In the outer foreskin, HIV-1 infected cells are able to form viral synapses with apical foreskin surface [7]. HIV-1 virions become trapped within thick keratin layer at mucosal surface and thereby preventing HIV-1 dissemination [8,9] (Figure 1.6; Right). This could be representative of events that take place *in vivo*, with LCs playing a role in HIV-1 dissemination to T cells.

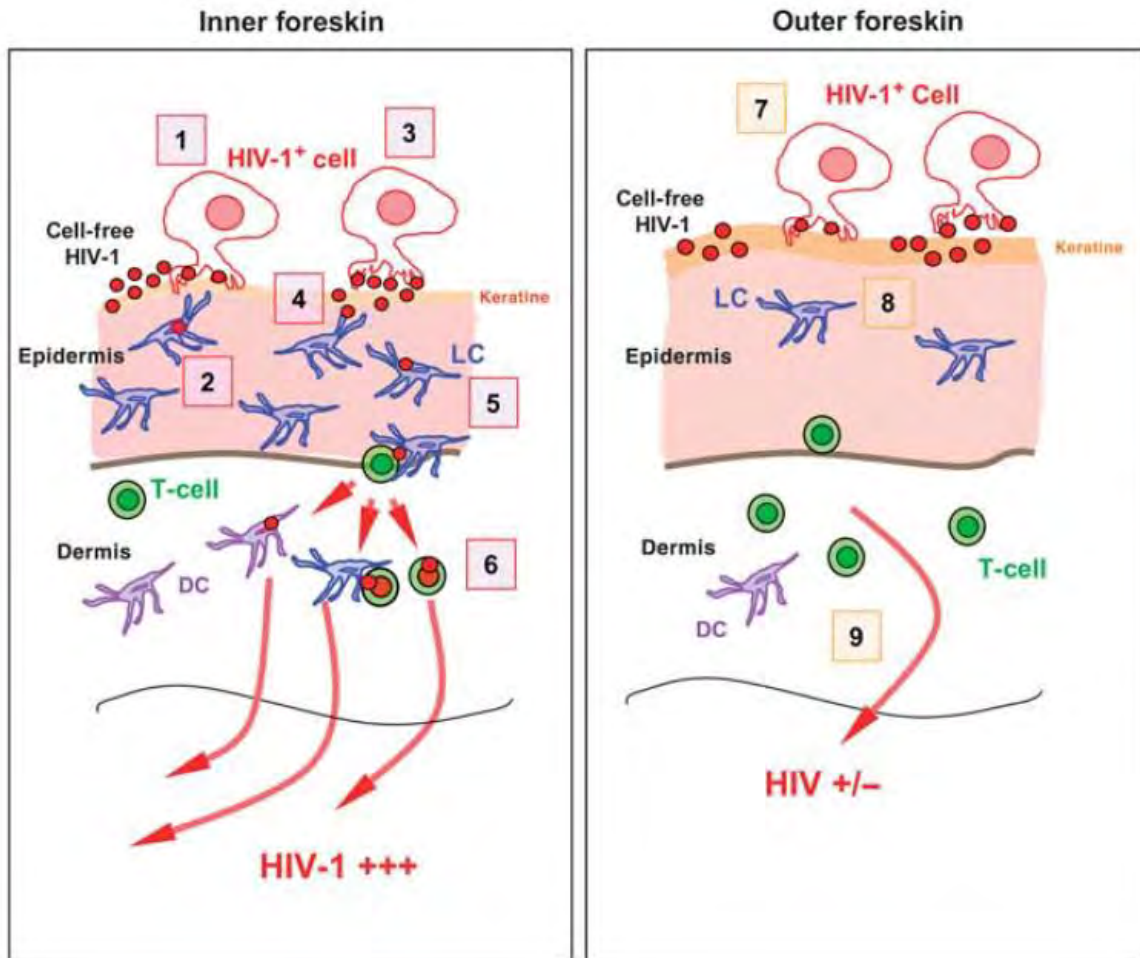


Figure 1.6: Initial HIV-1 transmission events in the inner and outer foreskin
(Taken from Ganor *et al.*, 2010).

1.5.2 CD4 T cells and co-receptors

Lynch *et al.*, showed that a small population of CD1a positive LCs were positive for CD4 expression. CD4 is a cell surface molecule which is expressed by a subtype of T lymphocytes and is a primary receptor for HIV-1 (Patterson *et al.* 2002). CD4+ T cells are present in the mucosal layer of the foreskin and are targets for HIV-1 infection (Patterson *et al.* 2002). Donoval *et al.*, showed that the CD4+ T cells are found deep in the tissue and are therefore not likely the first cells to come into contact with HIV-1. DCs, LCs and CD4+ T cells are present in the foreskin (Donoval *et al.* 2006; Patterson *et al.* 2002; Liu *et al.* 2014). CD4+ cells are the main targets of HIV-1 infection, which is transported across epithelial barriers covering the mucosal surface of the penis (Tebit *et al.* 2012) most likely by LCs and DCs. *In vitro*, LCs have been shown to form

LC-T cell conjugates at high viral concentrations and LCs transferred the virus to T cells (Ganor *et al.* 2010). However, this process has not been identified *in vivo*. There has recently been a penile SIV infection model that has shown encouraging results (Ma *et al.* 2011). Studies of vaginal mucosa has shown that the stratified squamous epithelium of the vagina contains LCs (Hladik & McElrath 2008; Nguyen *et al.* 2014). An *ex vivo* experiment showed that LCs can be infected by HIV-1 and move to the submucosa (Hladik *et al.* 2007). The submucosa contains DCs which may also be infected and transmit HIV-1 to CD4+ T cells (Hladik *et al.* 2007). Both DCs and CD4+ lymphocytes are able to be infected via receptor dependant mechanisms that allow the virus to spread to lymph nodes and subsequently the bloodstream and viral replication occurs extensively in the lymphatic tissue of infected mucosae and lymph node (Spira *et al.* 1996; Hu *et al.* 2000). Infected CD4+ can undergo lysis or allow for the establishment of latent infection, which may act as permanent reservoirs (Aleaki *et al.* 2008). It can be hypothesized that similar events that are seen with vaginal models may occur in the penis, but further research needs to be conducted to identify how HIV-1 is acquired in the male genital tract. CD4+ T cells are heterogeneous in their expression of the CCR5 co-receptor and the expression of CXCR4 is predominantly expressed on memory and naïve T cells (reviewed by Swanstrom & Coffin 2012). Upon HIV-1 infection, the gradual decrease in CD4+ T cell numbers eventually lead to AIDS (Mehandru *et al.* 2004). There are a greater proportion of the CD4+ T-lymphocyte population in the gastrointestinal mucosa and other secondary lymphoid organs compared to those found in peripheral blood (Brenchley *et al.* 2004). Prodger *et al.*, have shown that there are four times the amount of CD4+ T cells in the foreskin mucosa compared to that of blood. There have been 3 groups that have found different results with the number of CD4+ T cells found in the foreskin, Hussain and Lehner found no differences in the number of CD4+ T cells between the inner compared to the outer foreskin, McCoombe and Short found more CD4+ T cells in the outer compared to the inner foreskin and Patterson *et al.*, found more CD4+ T cells in the inner compared to the outer foreskin. The differences in results from these three groups may be due to confounder effects in the individuals such as the presence of an infection and the different methodologies used by each group. These may include the staining technique, method of analysis and source of foreskin tissue.

1.5.3 Dendritic cells and Macrophages

CD4 expression has also been shown on DCs and is involved in the inhibition of DC-mediated HIV-1 transmission to CD4⁺ T cells (Wang *et al.* 2007). DCs are able to express the C-type lectin Dendritic cell specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), and this functions as an adhesion molecule (van Kooyk & Geijtenbeek 2003). DC-SIGN binds Intercellular adhesion molecule (ICAM)-3 on T cells with high affinity thereby allowing for a stable DC-T cell immunological synapse (Van Gisbergen *et al.* 2005). DC-SIGN is also able to bind virion-associated HIV-1 Env (reviewed by Coleman *et al.* 2013) but overexpression of DC-SIGN may promote HIV-1 entry and infection (Lee *et al.* 2001). It is postulated by several studies that DC-SIGN on DCs bind HIV-1 virions and may allow for transfer of HIV-1 from DCs to CD4⁺ T cells due to the binding of HIV-1 envelope glycoprotein, gp120 to DC-SIGN (Geijtenbeek *et al.* 2000). DC-SIGN similarly to langerin plays a role in the internalisation of HIV-1 perhaps protecting the virus from degradation prior to viral synapse formation and CD4⁺ T-cell engagement (reviewed by Coleman *et al.* 2013). Macrophages are found in the lamina propria which is adjacent to the mucosal epithelium, they may come into contact with HIV-1 due to genital ulcerations and abrasions in the mucosal epithelium (Zaitseva *et al.* 1997). The exchange of information between the macrophages and CD4⁺ T cells plays an important role in the transmission of HIV-1 from macrophages to CD4⁺ T cells (Crowe *et al.* 1992; Groot *et al.* 2008). Macrophages infected with HIV-1 are able to survive for long periods as a viral reservoir (Gorry *et al.* 2014) and comprise up to 10% of infected cells (Zhang *et al.* 1999). HIV-1 tropism for macrophages is determined by the CD4 receptor as well as both CCR5 and CXCR4 co-receptor expression (Duncan & Sattentau 2011). Baxter *et al.*, conducted a series of experiments and found that primary monocyte-derived macrophages (MDMs) are able to selectively capture HIV-1 infected CD4⁺ T cells leading to the infection of macrophages and efficient transfer of HIV-1 from cell-to-cell. They co-cultured MDMs with CD4⁺ T cells expressing either CCR5 or CXCR4 or uninfected CD4⁺ T cells and showed that cell death of HIV-1 infection of CD4⁺ T cells increased their uptake by macrophages showing the efficient capture of the HIV-1 infected CD4⁺ T cells independent of co-receptor specificity of HIV-1. This uptake of HIV-1 infected CD4⁺ T cells could potentially lead to the infection of macrophages leading to the diffusion of HIV-1 *in vivo* (Baxter *et al.* 2014). Although other receptors such as DC-SIGN and langerin are present throughout the epithelia of the inner lining,

HIV-1 is only able to cause infection of cells that it is able to enter (Patterson *et al.* 2002).

1.6 Factors enhancing HIV-1 infection

HIV-1 disease progression is characterised by the damage and loss of CD4+ T cells as well as an impaired function of a range of immune cells. The hyper-activation of the immune system and activation induced cell death has been shown as a major mechanism driving HIV-1 disease progression (Hunt 2007; Douek *et al.* 2009). There may be other factors that contribute to the elevation in HIV-1 infectivity rates such as STIs and other co-infections (Stillwaggon & Sawers 2015).

1.6.1 Immune activation and inflammation during HIV-1 infection

Inflammation can be defined as the complex biological response of vascular tissues to injury or harmful stimuli such as those inflicted by pathogens, damaged cells and irritants (eds Bellanti *et al.*, 2012) and immune activation is the activation of cellular components of the immune system (Hsu & Sereti 2014).

Mucosal inflammation enhances HIV-1 transmission rates from an HIV-1-infected to an uninfected individual (Kaul *et al.* 2015). Inflammation leads to plasma and immune cell extravasation to the site of injury resulting in the clearance of the pathogen and promotion of tissue healing. The early mediators of inflammation are produced following innate immune activation and involves pro-inflammatory cytokines and chemokines released by several cell types such as epithelial cells, tissue-resident DCs and macrophages as well as innate lymphoid cells amongst others (Bamais *et al.* 2014). The prototypic pro-inflammatory cytokines include interleukin (IL) -1a and tumor necrosis factor alpha (TNF- α), to a lesser extent, IL-8, IL-6 and interferon gamma (IFN- γ) (Dinarello 2000). The increased expression of these cytokines at the mucosal surface enhances HIV-1 transmission (Kaul *et al.* 2015). TNF- α is involved in the direct reduction of epithelial barrier integrity by the disruption of tight junction proteins, namely ZO-1 and occludin thereby enhancing HIV-1 viral entry to the submucosal targets (Nazli *et al.* 2010). The chemoattractant properties of the cytokines and chemokines leads to the recruitment of activated CD4+ T cells to the mucosa resulting in increased target cells for HIV-1 (Kaul *et al.* 2015). The higher levels of pro-inflammatory cytokines seen in genital secretions of an infected person is directly

correlated with the increased HIV-1 RNA viral load seen in semen (Olivier *et al.* 2014) and vaginal secretions (Gumbi *et al.* 2008).

Chronic inflammation has been shown to play a central role in the pathogenesis of HIV-1 infection without treatment. Acute HIV-1 infection has been associated with the rapid and intense release of cytokines such as IFN- α , IFN- γ , inducible protein 10 (IP-10), TNF, IL-10, IL-6 and IL-15 (Stacey *et al.* 2009). During acute HIV-1 infection, the frequency of activated T cells is increased drastically in peripheral blood and almost 50% of which are CD8⁺ T subsets. The potential sources of inflammation may involve HIV-1 replication directly contributing to T cell activation (Papagno *et al.* 2004), contribution of other opportunistic infections such as herpes viruses enhancing high level T cell activation (Smith *et al.* 2013; Wittkop *et al.* 2013), HIV-1-mediated breakdown in the gut mucosa and chronic exposure to gut microbial products such as lipopolysaccharide (LPS) (Brenchley *et al.* 2006) and the impaired function of immunoregulatory factors that may also contribute to the persistence of inflammation in lymphoid tissues (Deeks *et al.* 2013). A large percentage of CD4⁺ T cells are also capable of producing cytokines in situ, indicating that they are terminally differentiated effector cells (Mowat & Viney 1997). Importantly, the cytokines and chemokines that are secreted by epithelial, stromal and immune cells are able to recruit neutrophil, monocytes, macrophages, DCs, T cells and natural killer (NK) cells (reviewed by Rancez *et al.* 2012). CD4⁺ T cells are a subset of helper T lymphocytes (Th) and are the main target for HIV-1. HIV-1 is also able to infect macrophages and DCs that express the CD4 molecule on its surface. CD4⁺ T cell levels are decreased in three ways, the direct viral killing of the cells, increase of apoptosis in infected cells and killing of the infected cells by cytotoxic T lymphocytes (CTL) (Huang *et al.* 2012). The Th cells do not play any role in the cytotoxic or phagocytic activity directed towards pathogens and do not kill any infected cells or pathogens. Their role involves the activation and proliferation of CTL and B cells as well as the determination of B cell antibody class switching (Huang *et al.* 2012).

The gut mucosa contains CD4⁺CCR5⁺ T cells at a high concentration (Deeks *et al.* 2013). During acute HIV-1 infection, the virus is disseminated rapidly throughout the GALT which leads to the direct loss of CD4⁺ T cells and indirectly to epithelial injury (Sankaran *et al.* 2008). Up to 60% of the T cells found within mucosal surfaces express CCR5 and can be infected by HIV-1. (reviewed by Lackner *et al.* 2012).

1.6.2 Inflammatory milieu

The increase in HIV-1 viral load corresponds with the surge of inflammatory cytokines led by IFN- α and IL-15 (Stacey *et al.* 2009). DCs are the earliest cells to produce cytokines and later during the infective process, cells such as monocytes, macrophages, NK cells and T cells are able to produce cytokines (Borrow & Bhardwaj 2008). NK cells play an important role in the innate immune system and in anti-viral and anti-tumour responses. Upon activation, NK cells function primarily by killing virus-infected or tumour cells or they produce large amounts of cytokines, IFN- γ in particular, to activate macrophages to kill intracellular bacteria (Wira *et al.* 2005). Both DCs and macrophages are sentinels of the mucosal immune system and are constantly surveying and processing antigens from the external environment in turn providing important information and signals to the host immune system (reviewed by Nguyen *et al.* 2014). T cells are able to proliferate in response to cytokines in T-cell receptor (TCR) independent manner (Geginat *et al.* 2003; reviewed by Seder *et al.* 2008). IL-2, TNF- α and IFN- γ are the main effector cytokines that are involved in T-cell differentiation and formation of T-cell responses (Mahnke *et al.* 2013). IL-2 production determines whether T cells become effector or memory cells due to their impact on proliferation, differentiation and survival of antigen-specific T cells (Kalia *et al.* 2010). Thus, cytokine production may enhance antiviral immune responses in acute HIV-1 infection but the prolonged cytokine storm may also contribute to the harmful immune activation and loss of CD4⁺ T cells (reviewed by Cohen *et al.* 2011). Other factors contributing to the inflammatory milieu are STIs. STIs are able to increase an already inflamed surface. Symptomatic STIs, such as herpes simplex virus (HSV)-2, have been shown to be associated with increased inflammation and immune activation (Johnson *et al.* 2009). Asymptomatic STIs, such as *Chlamydia Trachomatis* (CT), have also been associated with the increase in inflammation in the genital region (Kokab *et al.* 2010; Buckner *et al.* 2016).

1.6.3 Bystander activation

Inflammation due to the influx of cells to the site of the infection caused by an invading pathogen/virus may induce the activation of surrounding immune cells, this is known as bystander activation (Moir *et al.* 2011). During HIV-1 infection, CD4⁺ T cells are depleted from lymphoid systems, by the virus itself and as a consequence of bystander activation-induced cell death (Douek *et al.* 2009). HIV-1 infection has been shown to

cause changes to the inflammatory milieu, contributing to bystander activation, with the introduction of cytokines such as IL-2, IL-6, IL-7 and IL-12 capable of increasing the susceptibility of surrounding resting cells to HIV-1 infection (Unutmaz *et al.* 1999). Lemos *et al.*, found differences in the inflammatory milieu in the inner versus outer foreskin and the presence of this inflammatory milieu was supported by the increased secretion of granulocyte macrophage colony stimulating factor (GM-CSF), IP-10 and regulated on activation, normal T expressed and secreted (RANTES) which increased the density of CCR5+ and CD4+CCR5+ cells. Prodger *et al.*, found that HIV-1 exposed-resistant men had lower levels of Th17 cells and TNF- α producing CD4 and CD8 T cells compared to unexposed controls. The reduction of IL-17 and TNF- α may reduce bystander activation of other CD4+ T cell and dendritic cell populations (Jong *et al.* 2008) and indirectly reduce the number of HIV-1-susceptible cells (Prodger *et al.* 2014). One could therefore hypothesize based on findings by Lemos *et al* and Prodger *et al* that changes in inflammatory milieu in the foreskin can lead to bystander activation of T cells and increase in HIV-1-susceptibility.

1.6.4 The impact of STIs on bystander activation and HIV-1

Symptomatic STIs such as HSV-2 along with other co-infections facilitate HIV-1 transmission and acquisition (Gray *et al.* 2001; Korenromp *et al.* 2005; Reynolds *et al.* 2003; Stillwaggon & Sawers 2015). STIs and other genital infections have also been linked to the increase of HIV-1 genital shedding (Corbett *et al.* 2002; Cohen & Pilcher 2005; Anderson *et al.* 2010). These infections include cytomegalovirus (CMV), gonorrhoea, chancroid, syphilis, genital herpes, bacterial vaginosis (BV) as well as candidiasis (reviewed by Kaul *et al.* 2008). The biological findings support the mechanism that STIs increase HIV-1 acquisition and transmission by the direct mucosal disruption, recruitment of HIV-1 target cells to the genital tract and by the increase in HIV-1 plasma viral load and genital secretions (reviewed by (Ward & Rönn 2010). Studies that have been conducted on foreskins from men that had an asymptomatic STI (HSV-2) or previous history of a STI have shown that the infections were associated with inflammation and an increase in the number of HIV-1 target cells (Patterson *et al.* 2002; Johnson *et al.* 2009).

STI prevalence rates are high in South Africa and the prevalence of some STIs exceeds those estimated in various African households (Johnson *et al.* 2005). There are different types of STIs such as HPV and HSV which are viral STIs, *Mycoplasma*

Genitalium (MG), *Neisseria Gonorrhoeae* (NG) and CT which are bacterial STIs and *Trichomonas Vaginalis* (TV) which is a protozoan STI.

STIs enable HIV-1 transmission by increasing both the infectiousness (Korenromp *et al.* 2005; Gray *et al.* 2001) and susceptibility (Reynolds *et al.* 2003; Wawer *et al.* 2005; Freeman *et al.* 2006). A large influence is the presence of genital ulcer disease (Wawer *et al.* 2005; Gray *et al.* 2001) such as NG (Macdonald *et al.* 2008), chancroid, infectious syphilis (Buchacz *et al.* 2004), TV (McClelland *et al.* 2007) and HSV-2 which increases the susceptibility to HIV-1 (Cameron *et al.* 1989; Anderson *et al.* 2010). Biological findings have shown that STIs such as HSV-2, NG and HPV increases susceptibility (Cohen *et al.* 1997; Freeman *et al.* 2006; Auvert *et al.* 2009), and HSV-2 has been shown to increase HIV-1 target cell recruitment to the genital tract (reviewed by Johnson & Lewis 2008).

1.7 Medical Male Circumcision as an HIV-1 prevention method

There have been three randomised controlled trial in Africa that have shown male circumcision provides up to 60% protection against the acquisition of HIV-1 (Gray *et al.* 2007; Bailey *et al.* 2007; Auvert *et al.* 2005), furthermore an uncircumcised man has a 1.8 to 8.2-fold increased chance of acquiring HIV-1 compared to a circumcised man (Donoval *et al.* 2006). The foreskin also has a higher density of HIV-1 target cells and may be susceptible to epithelial disruptions during sexual intercourse, providing additional portals of entry for HIV-1 (Weiss & Quigley. 2000; Weiss *et al.* 2006). The removal of the foreskin would reduce the number of target cells for HIV-1 (McCoombe & Short 2006; Fischetti *et al.* 2009). Circumcision has also been shown to reduce the chances of STI acquisition (Auvert *et al.* 2001; Weiss *et al.* 2001; Bwayo *et al.* 1994; Cook *et al.* 1994; Baldwin *et al.* 2003) and will be discussed further below.

1.7.1 Male Circumcision prevalence

Medical male circumcision (MMC) is the complete surgical removal of the foreskin that covers the glans of the penis under local anaesthesia (Figure 1.7). There are currently on 30% of men worldwide that circumcise due to religious, cultural or medical reasons (WHO & UnaidS 2007). WHO and UNAIDS have set established recommendations to reach 80% of male circumcisions in men aged 15-49 in 14 countries in East and Southern Africa where there is a high prevalence of HIV-1 and low male circumcision

rates, by 2016 (reviewed by Tobian *et al.* 2014). The total number of MMCs aimed to achieve is 20.8 million and between 2011 and 2014 there have been a total of 9.1 million (44%) circumcisions in the 14 countries (WHO 2015; World Health Organization/Joint United Nations Programme on HIV-1/AIDS 2011). In SSA there has been a total of 5.8 million circumcisions achieved to date. South Africa has achieved 1.8 million of the total 4.3 million target of MMCs. The continued scale up of MMCs is needed to help achieve the target of 80% set out by WHO and UNAIDS. A review on the acceptability of circumcision in SSA suggested that 65% of uncircumcised men are willing to undergo circumcision, 69% of women prefer partners who are circumcised and that 71% of men and 81% of women would consider circumcising their sons (Westercamp & Bailey 2007).

1.7.2 Protection against other STIs

Circumcision has been shown to be protective against STIs. The randomised circumcision trials in Uganda and Kenya was associated with the decreased frequency of genital ulceration (Gray *et al.* 2007; Mehta *et al.* 2012). Prior to these circumcision trials, other observational studies showed that male circumcision may play a role in the decrease of infection with high-risk HPV (Baldwin *et al.* 2003; Lajous *et al.* 2005). In HSV-2, MMC decreased the chance of infection by 25% and HPV by 35% (Tobian *et al.* 2010). Male circumcision was also evaluated on the risk of bacterial STI acquisition among men. The South African trial found that male circumcision decreases TV and CT (Sobngwi-Tambekou *et al.* 2009) but had no impact on NG (Auvert *et al.* 2009) and the Kenyan trial found no protective effect for NG, CT or TV but only found a protective effect for MG (Mehta *et al.* 2009).

1.7.3 Immunological basis for protection

Although male circumcision has been shown to reduce the prevalence of STIs in heterosexual men, the effects among MSM is not clear, while some studies have shown that male circumcision is associated with decreased risk of infection (Buchbinder *et al.* 2005). Prodger *et al.*, found a higher proportion of CD4+ and CD8+ T cells in the foreskin compared to blood and Lemos *et al.*, suggested that the combination of increased inflammation and alterations to epithelial barriers in the inner foreskin may contribute to STIs and HIV-1 in uncircumcised men therefore emphasising the need for circumcision. This was further supported by Liu *et al.*, who found the inner foreskin was enriched with HIV-1-susceptible immune cells compared

to the outer and suggested that this provides a biological explanation for male circumcision in preventing HIV-1 infection. MMC may also confer protection for female partner. Two studies have shown that MMC reduces the risk of cervical cancer in females (Castellsagué *et al.* 2002; Drain *et al.* 2006) while only one study has shown no protective effect (Brinton *et al.* 1989).

1.7.4 Other HIV-1 prevention methods

There has been much research that has been put into the development of a HIV-1 vaccine over the years to prevent HIV-1 transmission. Although there have been many studies that have acquired a high efficacy of protection, none have been able to provide one hundred percent protection. Globally, in 2015, there were 15.8 million people that had access to ART with 41% of adults accessing ART in 2014 compared to 23% in 2010. In SSA alone, there were 10.7 million people who had access to antiretroviral (ARV) treatment up from fewer than 100 000 people in 2002 (Unaids 2015). HIV-1 prevention tools such as abstinence, condom usage, monogamy, microbicides, ART and oral pre-exposure prophylaxis (PrEP) is needed to aid in the prevention of the HIV-1 transmission. HIV-1 has a vast rate of mutation, thereby enabling it to evade host immune responses (Korber *et al.* 2009). Vaccine design has been focused on the induction of cell-mediated immune responses that lower viral loads as well as eliciting neutralising antibodies capable of providing sterilising immunity (Kim *et al.* 2010; reviewed by Walker & Burton 2008). Biomedical measure to prevent the transmission of HIV-1 have shown a great promise. The use of ARV drugs in PrEP has been shown to protect against the sexual transmission of HIV for both men and women in four clinical trials: the CAPRISA 004 (Karim *et al.* 2010), iPrEx (Grant *et al.* 2010), TDF2 (Thigpen *et al.* 2012) and Partners in Prevention (Baeten *et al.* 2012). Only partial protection was observed in the CAPRISA 004 trial. The HPTN 052 trial was a treatment for prevention strategy and showed that antiretroviral treatment was 96% efficacious in preventing transmission (Cohen *et al.* 2011). The RV144 vaccine trial however, showed a modest efficacy of 31% at 3 years (Rerks-Ngarm *et al.* 2009; Pitisuttithum *et al.* 2013). Furthermore, a study in 2015 found that PrEP reduced the risk of HIV-1 infection by 86% in MSM (McCormack *et al.* 2016). The initiation of highly active anti-retroviral drug combination therapy (HAART) decreases the viremia in plasma and blood CD4 counts rise (reviewed by Lackner *et al.* 2012). Although, highly active antiretroviral therapy (HAART) has been successful

in the reduction of HIV-1 replication, it is unable to completely eliminate infection. Therefore, the development of prevention strategies such HIV-1 vaccines, ARV-based treatments and circumcision are important combined strategies.

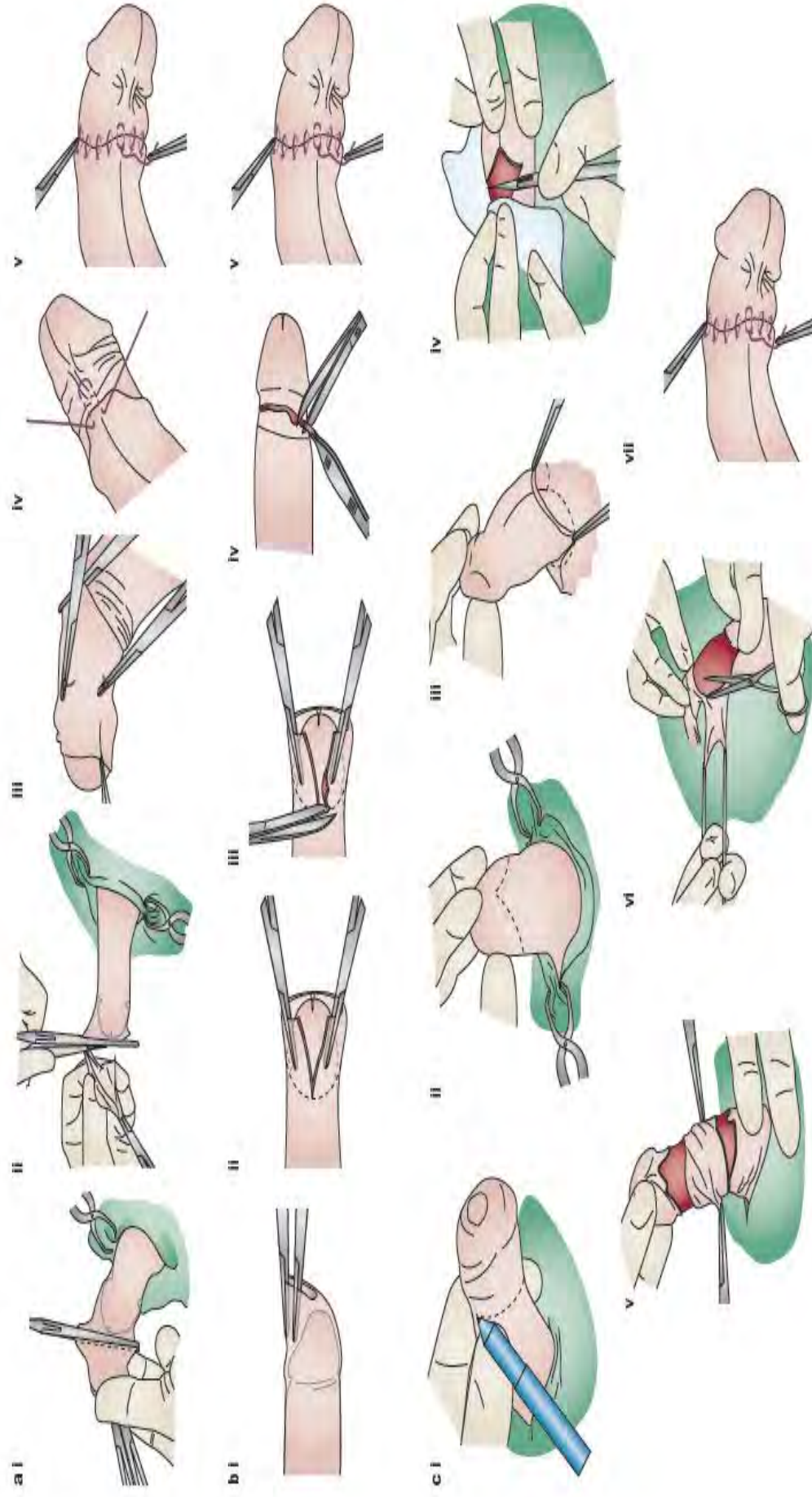


Figure 1.7: Various surgical methods used in MMCs. a) Forceps guided method b) Dorsal-slit method c) Sleeve resection technique (Taken from Tobian *et al.* 2015).

1.8 Aims and Objectives

Overall aim of thesis

The aim of this thesis was to optimise the techniques used to retrieve the maximum amount of viable cells from the inner and outer foreskin of fresh and frozen tissue and investigate the number of immune cells present in the inner and outer foreskin by immunofluorescence.

Specific objective 1:

To isolate the optimal amount of viable cells from foreskin tissue.

Hypothesis:

There will be a higher percentage of viable cells retrieved from fresh tissue.

Specific Objective 2:

To examine the levels of keratinisation in the inner and outer foreskins after medical male circumcision.

Hypothesis:

There are no differences in the keratinisation of the inner and outer foreskin.

Specific Objective 3:

To investigate the numbers of CD4⁺ T cells in the inner and outer foreskin.

Hypothesis:

There are no differences in CD4⁺ T cell density in the inner and outer foreskin

Specific Objective 4:

To identify the impact of asymptomatic STIs on the numbers and activation status of foreskin-resident CD4⁺ T cells.

Hypothesis:

Males with an asymptomatic STI have higher levels of activated immune cells in the inner foreskin compared to the outer foreskin.

CHAPTER 2: MATERIALS, METHODS AND OPTIMISATION OF METHODS

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A. Materials and Methods

2.1 Study participants and setting

A total of one hundred and fifty participants between the ages of 14 and 24 was recruited.

Cohort 1: One hundred and nine participants were recruited from Edendale Hospital (EDH), Pietermaritzburg, Kwa-Zulu Natal. This hospital offered district and regional level services to a catchment area of approximately 1 million people, including Medical Male Circumcision (MMC) for boys and men from the Edendale Valley.

The participants were recruited from the Research Ready Cohort that attended the WhizzKids United, Africa Aid (WKUHA), an adolescent-friendly care clinic and recreation centre at the EDH. WKUHA offers HIV-1 testing, MMC, antiretroviral treatment, sexual risk assessment, screening for sexually transmitted infections as well as primary healthcare services such as support for orphans and vulnerable children, and career guidance. This facility and services represented a multi-stake partnership between WKUHA, the Kwa-Zulu Natal Department of Health and Edendale Hospital.

Cohort 2: Forty one participants were recruited from the Khula Ndoda circumcision clinic at the Chris Hani Baragwanath (CHB) Hospital, Diepkloof, Gauteng. The Khula Ndoda circumcision clinic was opened by the Perinatal HIV-1 Research Unit (PHRU) in 2010 and engages in research, training, policy-formulation and advocacy that concerns HIV-1-positive people and their children. Khula Ndoda offers free MMC to residents of Soweto and surrounding communities. They provide HIV-1 testing, antiretroviral treatment, sexual risk assessment, screening for sexually transmitted infections and other primary healthcare services.

The following inclusion criteria were applied:

1. Scheduled for elective MMC
2. Age 14– 24 years
3. HIV-1 seronegative tested within the past 30 days
4. Informed consent or parental/guardian consent and participant assent if <18 years
5. Reside within approximately 5km of the clinic

The following exclusion criteria was applied:

1. Inclusion criteria not met
2. Unknown HIV-1 status or HIV-1 seropositive
3. Informed consent/assent not obtained

Ethics approval for the study was obtained from the University of Cape Town (UCT) Faculty of Health Sciences Research Ethics Committee (HREC Ref: 566/2012).

2.2 Sample Collection

Three follow-up visits were scheduled for the participants at the Edendale Hospital and two follow-up visits at Chris Hani Baragwanath Hospital. Participants were evaluated in accordance with the study schema shown in Table 1.

Table 1: Participant evaluation

Procedure	At Booking	At MMC	2 weeks post MMC	12 weeks post MMC	24 weeks post MMC
Assent/ Consent	X				
Confirm consent/assent		X			
Risk reduction counseling, routine post-operative check		X	X	X	X
MMC		X			
Foreskin collection		X			
Penile swab		X	X	X	X
HIV-1 and STI testing		X		X	X
Hormone testing		X			

Samples:

To test for a panel of STIs, first pass urine was self-collected and deposited in a 40ml blue-capped specimen jar (Lasec, Cat. No.: PLASEC15839R) at the EDH site while urethral swabs were taken at the CHB site. All urine samples and swabs were sent to the National Health Laboratory Service (NHLS) for testing. To assess changes in the bacterial microbiome, a swab was taken from the coronal sulcus (head ridge) and glans of the penis underneath the foreskin. This was placed in a labelled (S1) Primestore tube. (Primestore MTM, Longhorn Vaccines & Diagnostics, San Antonio, Lot no. 2684C145). A second swab was taken from below the head of the penis, around the shaft of the penis. This was placed in a labelled (S2) Primestore tube. Blood samples (6ml) were collected to test for testosterone levels, HIV-1, and peripheral blood mononuclear cell (PBMC) extraction. Blood collection was done in a purple top EDTA vacutainer [Greiner Bio-One (Lasec, Cat. No.: VGRV456043)] and a further 6ml of blood

was taken in a green top NaHep vacutainer [Greiner Bio-One (Lasec, Cat. No.: VGRV456028)]. The foreskin was excised by a surgical team subsequent to a pre-surgical examination of the foreskin and penis for any visible complications or any symptomatic STIs. If there were no complications, a local anaesthesia was applied to the sites of excision and either a Tara Klamp (EDH, KZN) or forceps (CHB, Gauteng) was used to facilitate the circumcision. The excised tissue was then placed into a 50ml (BD Falcon (Scientific Group, Cat. No.: 352070) blue-capped tube containing 15ml of R15 (Table S2) media, Penicillin/Streptomycin (Gibco, Cat. No.: 15070-063) and Fungin (InvivoGen, Cat. No.: ant-fn-2)]. Samples were then shipped by an overnight courier on ice from the KZN site to the lab at UCT for processing within 24 hours and samples from the JHB site were transported by car from the hospital to the NHLS for processing within 4 hours.

2.3 Sample processing and storage

Upon arrival of the foreskin samples in the lab, the R15 media in the 50ml container was poured out. The foreskin was rinsed twice in 40ml phosphate buffer saline (PBS) (Table S2) to ensure that all blood was rinsed off the tissue. The foreskin was then placed in a petri dish (epidermis facing up) and spread out as flat as possible using disposable forceps (B&M Scientific, Cat. No. XFPB0015) to facilitate the dissection of the inner from the outer foreskin. The inner foreskin was separated from the outer foreskin (Figure 2.1) using a sharp disposable scalpel (Lasec, Cat. No. HFLS13242), cutting in a 'see-saw' motion. The inner and outer foreskin was cut into small blocks of approximately 0.8-1cm³ for maceration and the remainder was cut into 5-7mm³ blocks for tissue sectioning and subsequent immunofluorescent staining. To be able to normalise using weight, the weight of each block was recorded before being placed in labelled cryovials containing 1 ml of 10% freezing media. The freezing media was added dropwise to the cryovials. The cryovials were then placed in ice-cold Mr Frosty's (Nalgene®; C1562, Sigma-Aldrich) and stored at -80°C overnight. The samples were then transferred to liquid nitrogen the next day for long-term storage. To ensure adequate space for tissue blocks, Cryo-molds were created by folding strips of double-sided aluminium foil (10x10cm) around the top end of a tube (usually a 15ml tube) to make a round foil cup with

flattened bottoms. The molds were then filled with Optimal Cutting Temperature (OCT) liquid (Leica, Cat. No. 14020108926) to approximately 1cm height. A piece of foreskin tissue was then placed in each mold ensuring that the tissue was placed in the correct orientation (Figure 2.2A) and immediately placed at -80°C overnight. The open ends of the cryo-molds were then closed the next day and stored away in labelled plastic bags that were placed in 96-well cryoboxes.

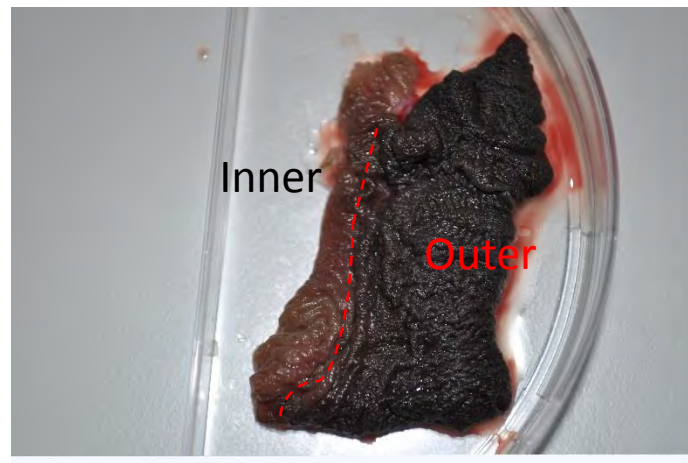


Figure 2.1: Foreskin tissue sample. The red dotted line separates the Inner (light in colour) from the Outer (dark in colour) foreskin.

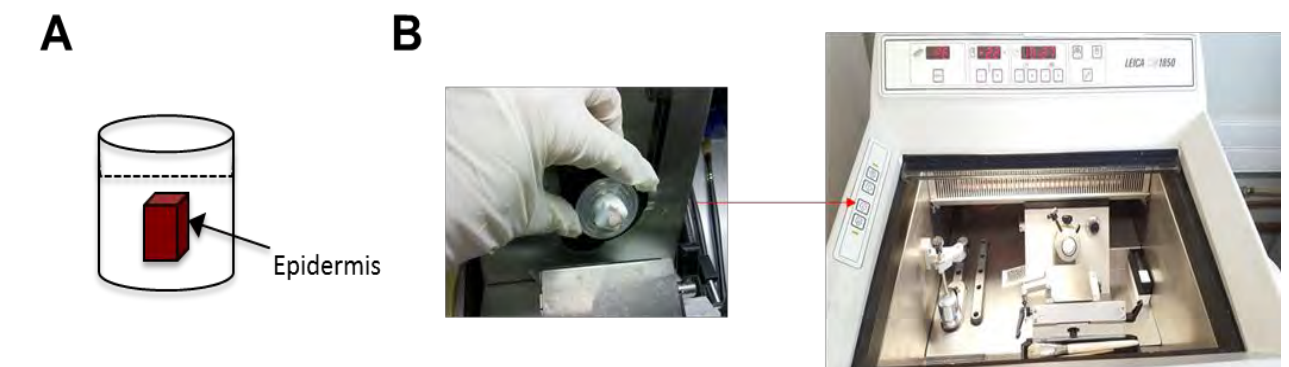


Figure 2.2: Tissue orientation and sectioning **A)** Correct orientation of tissue in OCT. **B)** OCT block placed on chuck (left) in preparation for sectioning using the cryostat machine (right).

2.4 PBMC isolation and storage

PBMC isolation: Upon arrival of blood samples in respective laboratories, 3ml of ficoll was pipetted in 12ml leucosep tubes and centrifuged at 1500 revolutions per minute (rpm) for 2 minutes (min). The blood tubes were inverted a few times before being carefully poured in 12ml leucosep tubes and centrifuged at 1500rpm for 5 min. For testosterone and internal HIV-1 testing, approximately 500µl of plasma layer was removed using a 1ml pipette and placed in two labelled cryovials and this was stored at -80°C. To isolate PBMCs, gradient separation using 12ml Leucosep (Greiner Bio-one; Cat. No. 89048-932) tubes with 3ml Ficoll (Table S2) were used. To facilitate a homogenous suspension, blood tubes were inverted several times and layered on leucosep dividers above the Ficoll. The tubes were centrifuged at 2500rpm for 15 min. A fresh sterile pasture pipette was used to transfer the 'buffy' layer of PBMCs into 15ml tubes and 1% wash buffer (Table S2) was used to top up the volume to 15ml. The tube was centrifuged at 1200rpm for 10 min to wash away all contaminating ficoll. The supernatant was discarded and the pellet was gently re-suspended. Following a second wash step, the supernatant was discarded, the pellet re-suspended and transferred to a cryovial. Fetal Bovine Serum (FBS) (Table S2) was added to the pellet to make up 500µl in total. A further 500µl of freezing media (Table S2) was then added dropwise with intermittent gentle shaking. The cryovial was then placed in a Mr Frosty which was kept at 4°C and then transferred to -80°C overnight before being transferred to a vapour phase liquid nitrogen cryotank (Cryo 200, Forma Scientific) the next day.

2.5 Urine samples:

Processing and storage: To ensure that all possible bacteria were flushed out for STI testing, self-collected first pass urine received in the laboratories was aliquoted into 4 labelled cryovials of 1.5ml each and stored at -80°C. Within the same week of sampling, 1 tube was sent to the NHLS for STI screening.

STI screening: STI detection was performed using the urine samples from the Edendale site and urethral swabs from the Johannesburg site to screen for the following STIs: *N. gonorrhoea* (NG), *C. trachomatis* (CT), *T. vaginalis* (TV), *M.*

genitalium (MG), HSV-1 and HSV-2. Multiplex real time Polymerase Chain Reaction (PCR) was used for the detection of these STIs. Briefly, this method allows for the amplification of several DNA sequences simultaneously. This process amplifies the DNA in the samples by use of multiple primers as well as temperature mediated DNA polymerase in a thermal cycler. The bands produced are visualised using gel electrophoresis, this allows for the detection of a STI (Chamberlain *et al.* 1988). *Human Papilloma Virus* (HPV) was detected by DNA extraction from penile cells that were collected using a urethral swab. This was performed using a MagNa Pure Compact Nucleic Acid Isolation kit (Roche) and an automated MagNa Pure Compact machine (Roche). The HPV typing was performed by using a Roche Linear Array HPV Genotyping assay as per manufacturer's instructions.

2.6 Foreskin cell isolation

2.6.1 Liberation of cells

To liberate cells for flow cytometric analysis, pieces of inner and outer foreskin were cut into strips and then further sectioned to create 24 pieces of approximately 0.25 cm². To enzymatically digest collagen within skin, each piece was placed in a 1.5ml eppendorf tube containing 0.5ml of 500U/ml⁻¹ Collagenase Type 1 (Life Technologies, Cat. No. 17018-029) and 200U/ml⁻¹ DNase 1 (ThermoFisher Scientific, Cat. No. 89836) in RPMI 1640 media. Stainless steel scissors with curved edge blades were used to mechanically disrupt each piece of tissue in a 1.5ml eppendorf. A further 0.5ml of collagenase media (Table S2) was added to each eppendorf and was then placed on a shaker at 900rpm for 30 min at 37°C. In a separate 50ml tube, 1.5ml of FBS was added. After incubation, the cellular suspension was added to the tube containing the 1.5ml FBS. A further 0.5ml of R10 (Table S2) was added to each eppendorf to rinse all contents in the eppendorf tube and the contents were added to a 50ml tube. The resulting suspension was filtered using a 100µm filter to ensure that only liberated cells were collected (BD cell strainer, 100µm, Cat. No. BD/352060). In order to liberate the optimal amount of cells, the tube that contained the undigested tissue aggregate was rinsed with 5ml of R10 (Table S2) and added through the filter. The tube was centrifuged at 1860rpm

for 10 min, the supernatant discarded and the cells re-suspended. To determine the number of viable cells, a cell count was done using the TC20 cell counter (BIO RAD, Model no. TC20™ Automated Cell counter). A total of 10ml of R10 and 150U/ml⁻¹ DNase 1 was added to the cells and centrifuged at 1860rpm for 10 min. The supernatant was discarded and cells re-suspended in 400µl R10 and 200 µl was transferred to two wells in a 96-well V-bottom plate for subsequent flow cytometric staining (Protocol adapted from Prodger *et al.* 2012).

2.6.2 Flow Cytometry and foreskin cell staining

Continuing from above, the plate was centrifuged at 2100rpm for 3 min. The supernatant was then discarded. A volume of 100µl of 1% wash buffer (Table S2) was added to each well, the cell pellet was re-suspended and transferred to one well. The plate was then centrifuged at 2100rpm for 3 min, flicked onto a stack of paper and blotted dry. To measure viability, a Vivid (Life technologies, Cat. No. L34955,) dye solution was prepared (1:40 i.e. 39µl PBS + 1µl Vivid) and 1µl was added to a 100µl of staining volume in each well and cell pellets re-suspended. The plate was incubated at room temperature (RT) for 20 min in the dark. Vivid binds to the free amine groups in the cytoplasm of damaged cells but it is unable to bind to free amines on an intact cell, allowing for the differentiation of live and dead cells (Perfetto *et al.* 2010). To ensure that all free amine groups were removed, the cells were washed twice with 1% wash buffer (150µl and 200µl for the first and second washes respectively) and the plate centrifuged at 2100rpm for 3 min. A total of 100µl of cytofix/cytoperm (Table S2) was added to the pellets and re-suspended. The plate was incubated at RT for 20 min. Permwash [BD Biosciences, Cat. No. 554723] (Table S2) was added twice to wash the cells as before and the plate centrifuged at 2100rpm for 3 min. A four colour panel anti-CD3 V450, anti-CD4 PerCP-Cy5.5, anti-CD8 V500 and anti-CD45 APC (see table 2 below) was applied. An antibody mastermix was prepared using anti-CD3, anti-CD8 and anti-CD4 and anti-CD45 before adding 100µl of antibody mastermix to the cell pellets. The plate was then incubated at RT for 20 min. The cells were washed twice as before and the plate centrifuged at 2100rpm for 3 min. To ensure the cross-linking of membrane bound proteins in order to stabilize the bond between the antibody

and protein, 200µl of cell fix was added to cell pellets and re-suspended before being transferred to labelled 5ml Falcon tubes (BD Biosciences). A further 100-200µl of cell fix (Table S2) was added to the wells and contents added to falcon tubes. Samples were acquired within 24 hours of staining using the BD LSRII flow cytometer and data obtained was analysed using FlowJo analytical software version v.9.8.5 (Treestar, Ashland, OR).

Table 2: List of Antibodies used in Flow Cytometry

Antibody Specificity	Fluorochrome	Catalog number	Company	Clone	Titrated Volume
CD3	V450	560365	BD Biosciences	OKT3	1µl
CD4	PerCP-Cy5.5	560650	BD Biosciences	RPA-T4	1µl
CD8	V500	560775	BD Biosciences	RPA-T8	1µl
CD45	APC	555485	BD Biosciences	H130	10µl
Live/dead fixable Violet dead cell stain kit		L34955	Life Technologies		1µl

2.6.3 Preparation of compensation tubes

A new set of compensation tubes were prepared for every experiment in order to prevent spectral overlap by using artificial latex beads. To create a positively labelled population, CompBeads come in a pair and consist of two microparticle populations, CompBeads anti-mouse Ig,κ (positive) and the CompBeads negative control. The positive microparticles bound to mouse κ chain immunoglobulins create the positive population. The negative CompBeads (BD Biosciences, Cat. No. 51-90-9001291) control have no binding capability, therefore are unable to bind to any antibody and forms the unstained population. Before addition of a single drop of negative and positive (BD Biosciences, Cat. No. 51-90-9001229) compbeads to individual FACS tubes containing 100µl PBS, the compbeads were vortexed thoroughly for about a minute to avoid clumping of the beads. The pre-determined volumes of anti-CD3, anti-CD4, anti-CD8, anti-CD45 and anti-CD14 PacBlue (for compensation of Vivid) were added to their respective tubes and was incubated at 4°C for 20 min. The beads were then fixed by adding 100µl of cell fix. Tubes were wrapped in aluminium foil and kept in the dark at 4°C until acquisition on the LSRII flow cytometer.

2.7 Immunofluorescence

Tissue blocks stored at -80°C were transported on dry ice and 8-10µm thick sections were cut using a cryostat (Leica CM1850 Cryostat) (Fig 2.2B). The sections were placed onto microscope slides (Leica, Cat no. 38002e) with at least 3 sections per microscope slide. Slides were then stored for a short period of time at -80°C until staining.

2.7.1 Foreskin cell staining

Slides were taken from -80°C and allowed to warm up to RT for 10 min. To fix tissue sections, 4% paraformaldehyde solution (Santa Cruz, Cat no. SC-281692,) was used to cover all sections on the slide for 10 min. The solution was dumped off the slide and washed twice in Coplin jars (Sigma Aldrich Product no. S5766) containing ice cold 1x PBS. The remainder of the solution was removed using a vacuum pump (Millipore, Model no. WP6122050). To

block any non-specific binding, 2% Bovine Serum Albumin (BSA) (Santa Cruz, Cat no. sc-2323,) was added to slides and incubated for 30 min. The BSA was then poured off and the remainder of the liquid suctioned up. The tissue sections were then outlined using a hydrophobic pen (Leica, Cat. No. Z672548) to keep the antibody within the staining area. The primary antibody was then added, anti-langerin (Table 3) and incubated overnight at 4°C ensuring it was away from light. The wash step was repeated. A secondary antibody was added to detect the primary antibody, Rhodamine-Red (Table 3) for 30 min in the dark at RT. The wash step was repeated. A second primary stain was then applied using anti-Filaggrin (Table 3) with the use of Zenon kits (Table 3; Figure 2.3) as recommended by the manufacturer. Briefly, the Zenon kits allow the formation of a complex between an intact IgG antibody and a fluorophore directed against the Fc portion of the IgG. This eliminates non-specific binding and ensures the high affinity selectivity for the Fc portion of the corresponding antibody. A master mix was prepared by adding 5µl of anti-filaggrin antibody to 5µl of Zenon Alexa fluor 647 reagent A, mixed and incubated in the dark for 5 min. To ensure there was no non-specific binding by unbound Fc portions, 5µl of Zenon blocking reagent B was added and incubated in the dark for 5 min. To make a dilution of 1:50, 758µl of PBS was added to the solution before being added to the slides and incubated in the dark for 1 hour. The solution was dumped off and washed twice in PBS and the remaining liquid suctioned up. In order to detect the nucleus of the cell, a 1:5000 dilution of Hoechst (Table 3) was added as a counterstain to the slides and incubated for 15-20 min in the dark. The wash step was repeated. A small drop of fluorescent mounting medium (Sigma, Cat no. F4680) was added to each tissue and a coverslip (Leica, Cat. no. 14071135635) was then placed on the slides ensuring no air bubble formations. This was allowed to dry for 5 min before sealing the edges of the coverslip with fast drying clear nail polish. After ensuring that the nail polish was dry, slides were stored in slide boxes (Leica, Cat. No. 71459-B) and stored at 4°C until imaging the next day.

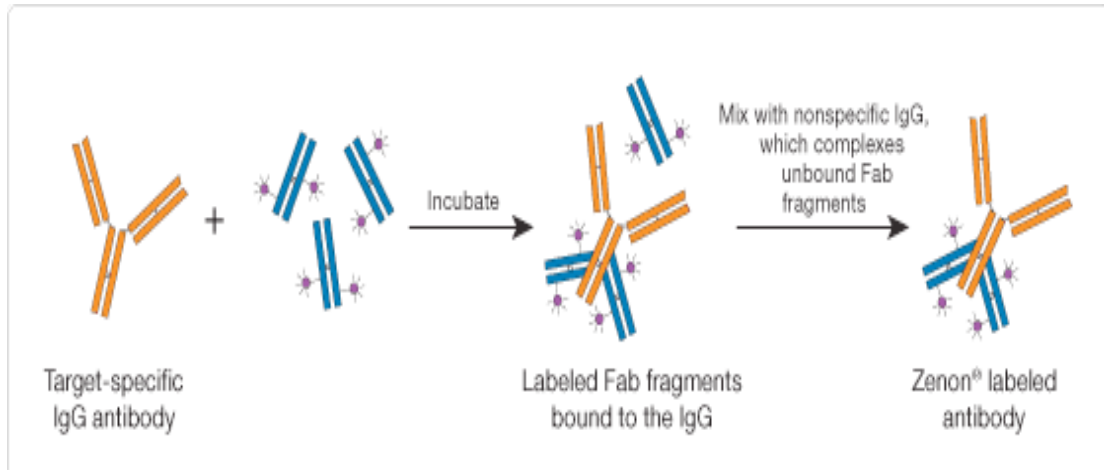


Figure 2.3: Zenon kit labelling: The Zenon fragments were specifically designed to target and bind to the Fc portion of the primary antibody only to allow for rapid, noncovalent labelling of small quantities of primary antibody (Taken from Life Technologies),

(www.thermofisher.com/za/en/home/references/molecular-probes-the-handbook/antibodies-avidins-lectins-and-related-products/zenon-technology-versatile-reagents-for-immunolabeling.html)

It should be noted that the CCR5 antibody used for IHC staining was kindly provided by Prof. Mathias Mack at the University of Regensburg, Germany (Table 3).

Table 3: List of antibodies used for immunohistochemistry staining

Antibody	Clone	Catalog no.	Company	Details	Dilution/Amount used
Ki67	MIB-1	M724001	Dako	Monoclonal Mouse anti-human	1:40
CCR5				Monoclonal Mouse anti-human	1:10
CD4	Q4120	C1805	Sigma	Monoclonal Mouse anti-human	1:100
Alexa Fluor 647	Poly4053	405322	Biolegend	Goat anti-mouse IgG	1:500
Filaggrin	AKH1	sc-80609	Santa Cruz Biotechnology	Mouse monoclonal IgG1	5µl
Langerin (CD207)	DCGM4	PN IM3449	Santa Cruz Biotechnology	Mouse monoclonal IgG1	1:350
CD206	5C11	Ab117644	Abcam	Anti-mannose receptor Ab	1:200
CD209 (DC-SIGN)	9E9A8	330112	Biolegend	A647 anti-human	1:200
Cy3			AEC amersham	Cy3-AffiniPure Donkey Anti-mouse	1:500
Alexa Fluor 647			AEC amersham	Cy5-AffiniPure Donkey Anti-mouse	1:500
Hoescht (Dapi)		H21491	Invitrogen	Hoechst 33258, pentahydrate (bis-benzimide) FluoroPure grade	1:5000
Zenon Kit (A)		Z25408	Life Technologies	A647 human IgG	5µl
Zenon Kit (B)		Z25408	Life Technologies	Blocking reagent	5µl
Negative control		X0931	DAKO	Mouse IgG1	1:500
Rhodamine Red		715-295-150	Jackson Immuno Research	Donkey Anti-mouse IgG	1:500

2.7.2 Confocal Imaging

Images of tissue sections were taken on the Zeiss LSM510 Meta with Non-Linear Optical (NLO) confocal using the following lasers: An Argon Green emission with excitation at 488 nano meter (nm), A Solid state laser Red Emission with excitation at 561nm excitation, A HeNe laser Far red emission with excitation at 633nm and it has a two photon laser for ultra violet excitation and detection of the nuclear stain at 750nm. All voltages were constant for all images taken. The red laser has a Bandpass (BP) 575-630 Infrared (IR) filter, the far red laser has a BP 650-710 IR filter and the ultra violet laser has a BP 390-465 IR filter. The purpose of the filter is to allow all wavelengths in between the specified filter range (eg. BP 575-630 would allow all wavelengths between 575nm and 630nm to pass through) and infrared light to pass through, while all other wavelengths are reflected. Images for CD4/Ki67 and CCR5/CD4 were collected using the oil objective at 63x magnification and CD207/Filaggrin was collected using the water objective at 40x magnification. The number of Z-sections varied from section to section, with the z-sections being 0.98 μ m apart and an average of five images collected per slide. To facilitate imaging of larger areas of tissue, tile scanning was performed by stitching of 3x3 separate images (or tiles) for CD4/Ki67 and CCR5/CD4 while 3x2 tiles were stitched together for CD207/Filaggrin.

2.8 Data analysis

2.8.1 Flow Jo

All samples were acquired on the LSRII flow cytometer ensuring that all photomultiplier tubes (PMT) voltages were adjusted according to quality control checks and compensation matrices. Flow cytometry analysis was then performed using FlowJo analytical software version v.9.8.5. (Treestar, Ashland, OR) The following gating strategy was applied (Figure 2.4).

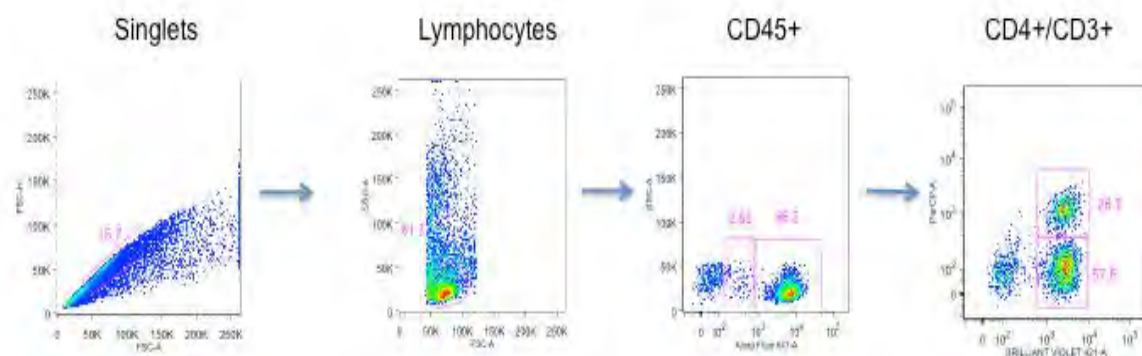


Figure 2.4: Gating strategy from a foreskin sample applied to FlowJo analysis

2.8.2 Fiji and Integrative Data language

To ensure the optimal and accurate analysis of the images captured, images were analysed using Just ImageJ (Fiji) software imaging version 2.0.0-rc-23/1.49m (GNU General Public License v3, open source) to utilize captured Z-stacks where necessary to ensure counting positive staining events and aid in the quantitative application of integrative data language (IDL) version 7.1.1 with X11 (XQuartz-v2.7.7). Z-stacks combines multiple images taken at different focal distances to provide a composite image with a greater depth (Ray., 2002), enabling clearer vision of cells that were imaged. IDL is a scientific programming language used in the analysis and visualisation of multidimensional data sets. Using an IDL generated algorithm (Table S1), LCs (CD207+) and keratin layers (Filaggrin+) were measured as follows (Figure 2.5): lines K1 (outer keratin layer) and K2 (inner keratin layer) were drawn carefully using a mouse and the LCs were marked using the mouse by clicking on the LCs that were visible. Once completed, IDL generated algorithms

automatically drew lines between K1 and K2 as well as lines from K2 to all LCs. The measured distances were automatically generated in summarised excel spreadsheets. A minimum of 5 images for both the inner and outer foreskin was taken and the averages from these images were used for data analysis. The same analysis was applied and another algorithm used to analyse the CD4/Ki67 images with the line K1 drawn and the CD4 and Ki67 cells marked using the mouse. Cell numbers were calculated using a cell density formula (Table S1).

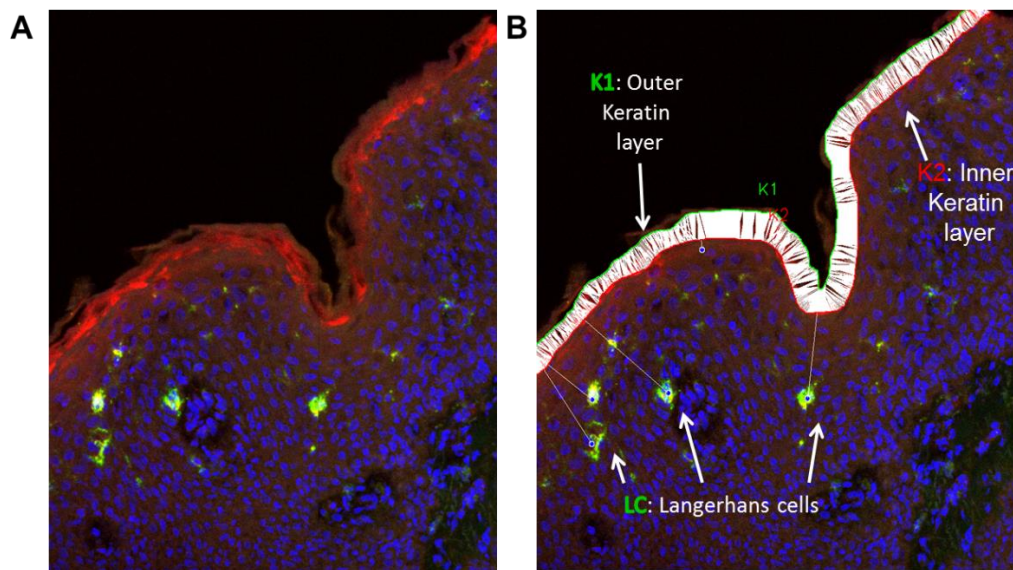


Figure 2.5: IDL image analysis. A) An image before analysis using IDL. The keratin layer, stained using anti-filaggrin (red), LCs stained using anti-langerin (green) and nuclei stain using dapi (blue). **B)** An image that has been analysed using IDL. Line K1 (green) represented the outer layer of keratin of the epidermis. Line K2 (red) demarcated the separation between the bottom keratin layer and the epidermal cells. LCs were counted by placing a computer generated dot over each visibly stained LC.

2.9 Statistical analysis

All the statistical analyses were performed using GraphPad Prism 5[®] (GraphPad Software, San Diego California USA). The non-parametric Mann-Whitney U test was used for unpaired data. Paired t-test was used for the comparison of matched data. The Spearman rank correlation test is a statistical measure of the strength between two continuous or ordinal variables. This is based on the ranked values for each variable rather than the raw data. The Pearson test is a statistical measure of a correlation between two continuous variables. A p-value of ≤ 0.05 was considered to be statistically significant.

B. Optimisation for Flow Cytometry and Immunohistochemistry

All protocols used in the optimisation of techniques were novel to this dissertation. Table 4 shows a summary of the different experiments carried out to optimise the number of viable cells isolated from foreskin tissue. Fresh samples were used for all experiments unless it was stated that frozen samples were used. All tissues used were of equal quantity. The same participant was used for all experiments except where fresh tissue was compared to frozen tissue. The time period for storage of frozen samples was not recorded (Figures 2.5; 2.7; 2.9; 2.11). The purpose of optimisation experiments was to ensure there was an optimal yield of viable cells from the foreskin. We first optimised the type of collagenase, collagenase A (Life Technologies) and collagenase B (Sigma Aldrich) used to determine which would facilitate the digestion of foreskin tissue effectively to get the maximum number of viable cells. Next we determined the optimal concentration of collagenase to use to digest the foreskin tissue as well as test if the use of scissors or an automated machine aided tissue digestion. The purpose of the next experiment was to determine if digesting fresh tissue compared to frozen tissue yielded a greater amount of viable cells. Table 5 shows a summary of the different antibody dilutions that were optimised in order to stain the foreskin for cells using immunohistochemistry. The purpose of the experiments was to determine the optimal concentration of antibody that gave the brightest signal upon cell staining. We first optimised the anti-CD4 antibody to visualise CD4⁺ resident T cells present in the foreskin. Next we stained using anti-Ki67 with the use of Zenon kits and a secondary detection antibody, Alexa647 to determine if the use of the Zenon kit or secondary antibody facilitated a better detection of Ki67. Thereafter, we optimised the staining dilution for anti-CCR5 as this is a known co-receptor for CD4. Lastly, we optimised the staining dilution for anti-CD209 (DC-SIGN) which is expressed on DCs and is involved in HIV-1 capture.

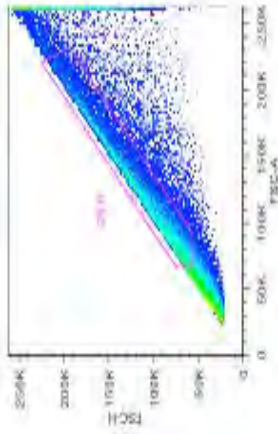
Table 4: Methodology used to optimise the foreskin cell isolation protocol

Experiment	Purpose	Method	Figure/s
Collagenase A vs Collagenase B	To determine the best type of collagenase to use	Protocol followed as per 2.5.1 above with use of collagenase A and B in the two experiments respectively	2.5
Collagenase A at different concentrations using scissors and BD MediMachine	To determine the optimal amount of collagenase to use in tissue digestion in conjunction with scissors or Medimachine	Protocol followed as per 2.5.1 with the following amendments: Collagenase was used at 62.5, 125, 250 and 500 Units/ml. Tissue was placed in an Eppendorf and macerated using scissors as per protocol 2.5.1. When using the medimachine, tissue was placed in a Medicon with the collagenase. It was then placed in the MediMachine where the blade in the medicon was turned and macerated the tissue automatically. The rest of the protocol was followed as in 2.5.1 above.	2.7
Use of BD Machine vs Scissors at optimal Collagenase A concentration	To determine if the use of scissors or BD Medimachine was better in aiding tissue digestion	Protocol followed as per 2.5.1 with the following amendments: Tissue was placed in an Eppendorf and macerated using scissors as per protocol 2.5.1. When using the medimachine, tissue was placed in a Medicon with the collagenase. It was then placed in the MediMachine where the blade in the medicon was turned and macerated the tissue automatically. The rest of the protocol was followed as in 2.5.1 above.	2.9
Fresh tissue vs Frozen tissue	To determine if tissue digestion using fresh compared to frozen tissue yielded a greater number of viable cells	Protocol followed as per 2.5.1 with the following amendments: Tissue was placed in an Eppendorf and macerated using scissors as per protocol 2.5.1. When using the medimachine, tissue was placed in a Medicon with the collagenase. It was then placed in the MediMachine where the blade in the medicon was turned and macerated the tissue automatically. The rest of the protocol was followed as in 2.5.1 above.	2.11

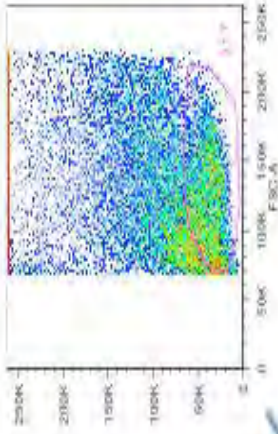
A

Life Collagenase 1

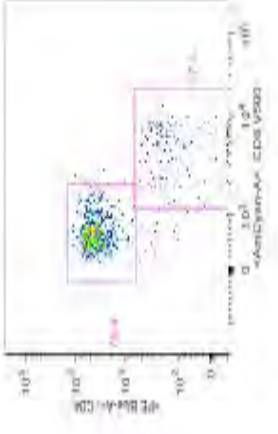
Singlets



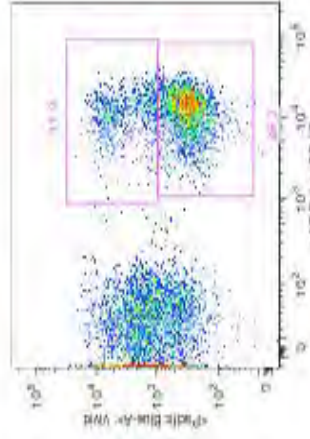
Lymphocytes



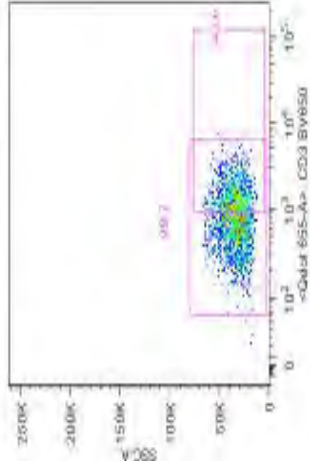
Gated CD3+



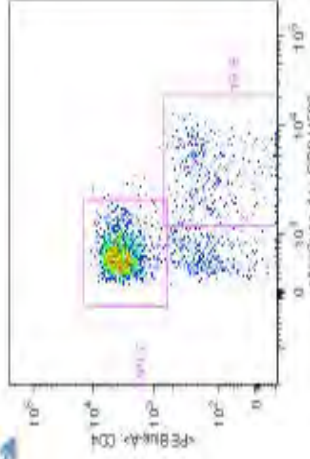
Live/Dead



CD3+ Lymphocytes



Whole CD3+



B

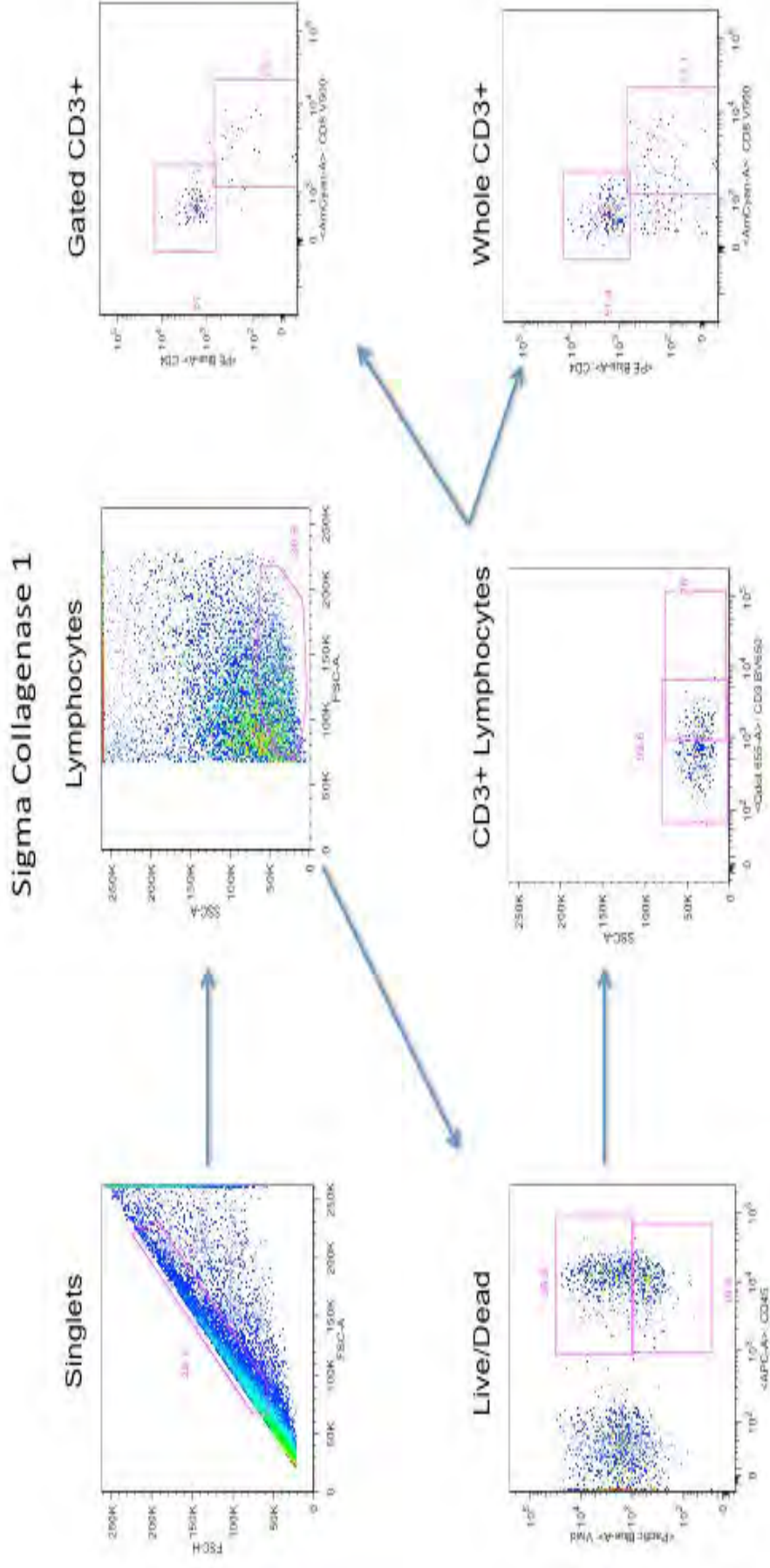


Figure 2.5: Comparison of the two types of Collagenase; A) Collagenase A B) Collagenase B

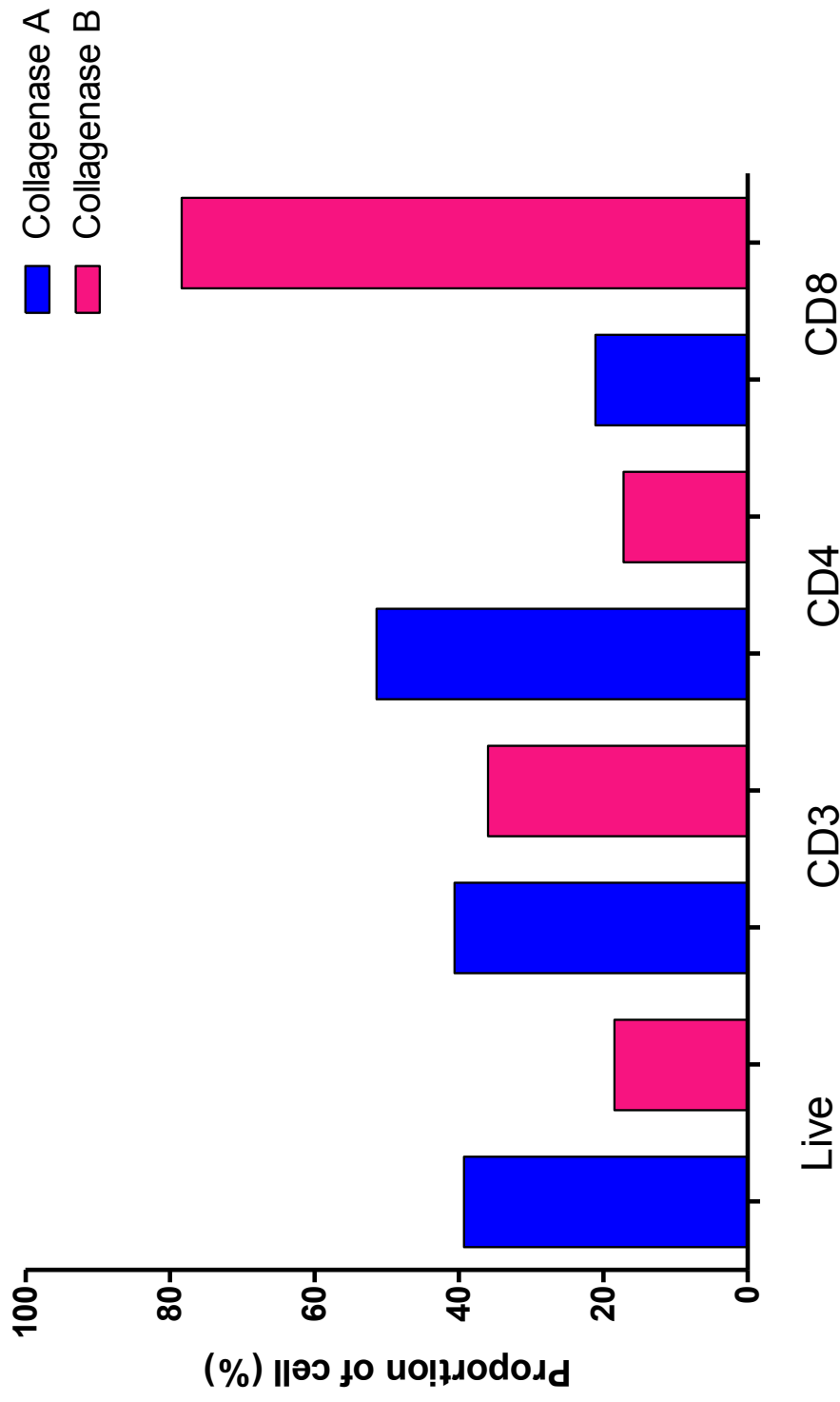
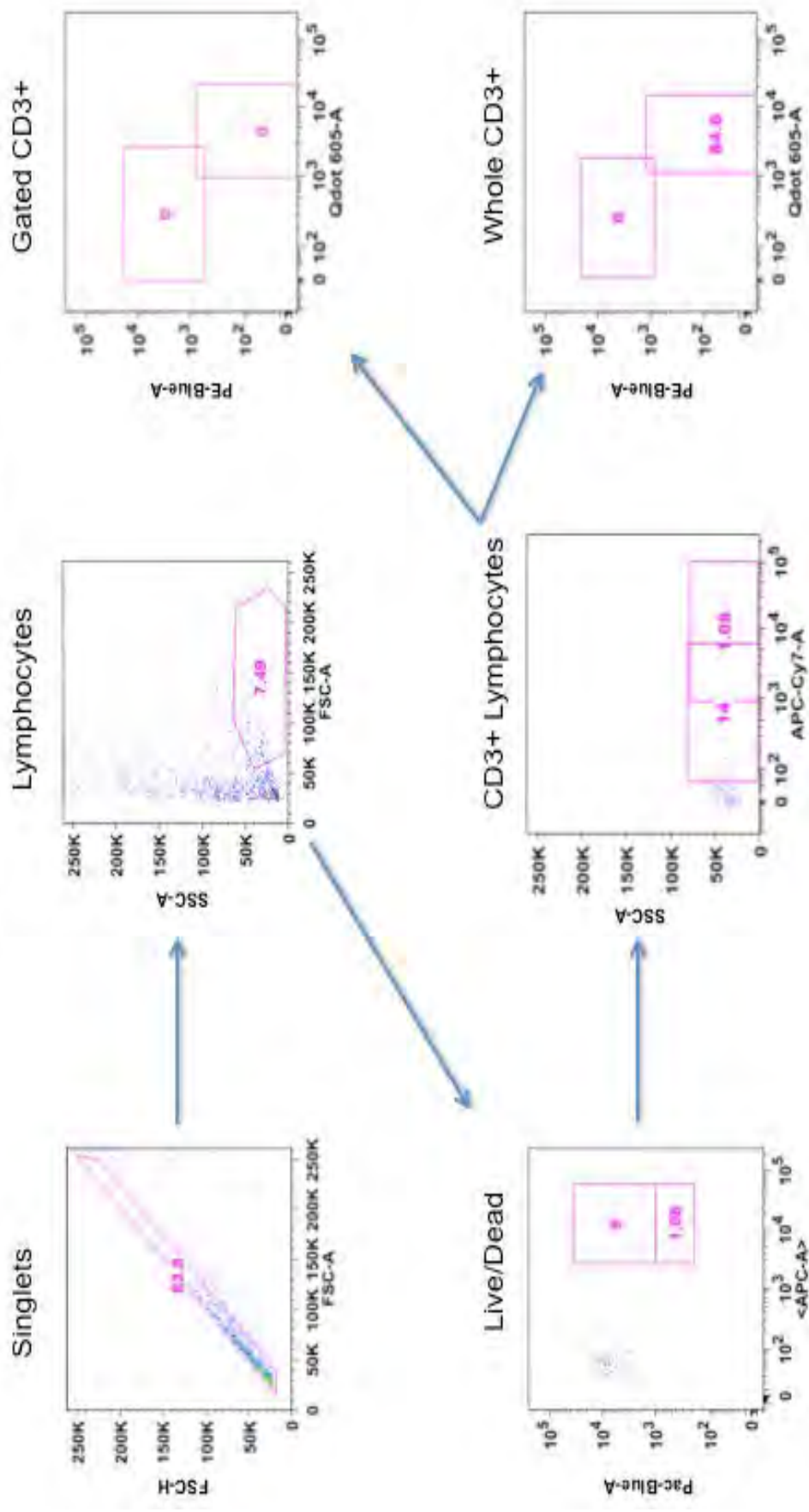


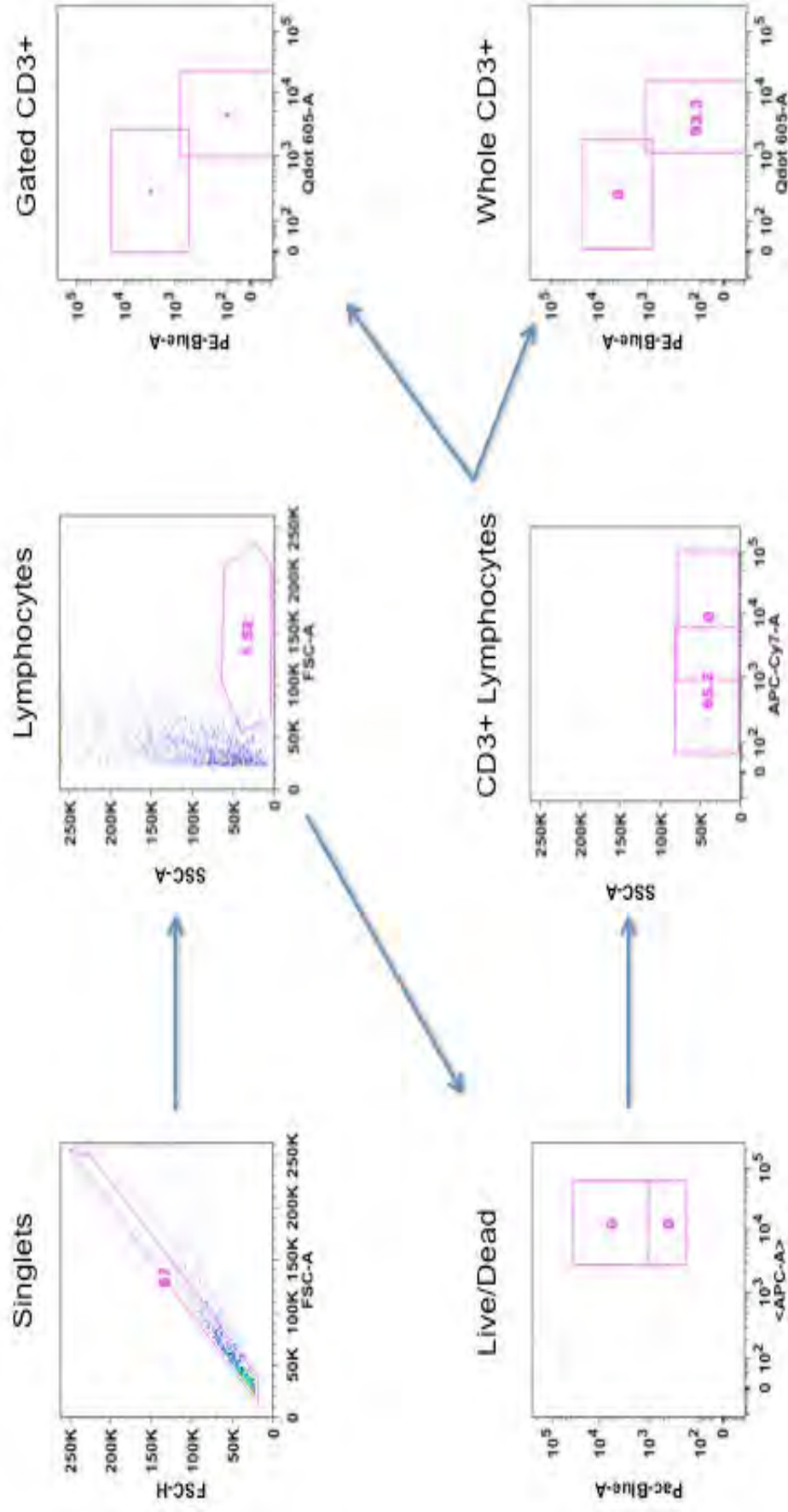
Figure 2.6: Proportion of cells (%) after tissue digestion using two collagenase type. Collagenase A is represented in blue while Collagenase B is represented in pink.

EDH091 Machine Inner 62.5U/ml Collagenase 1

A

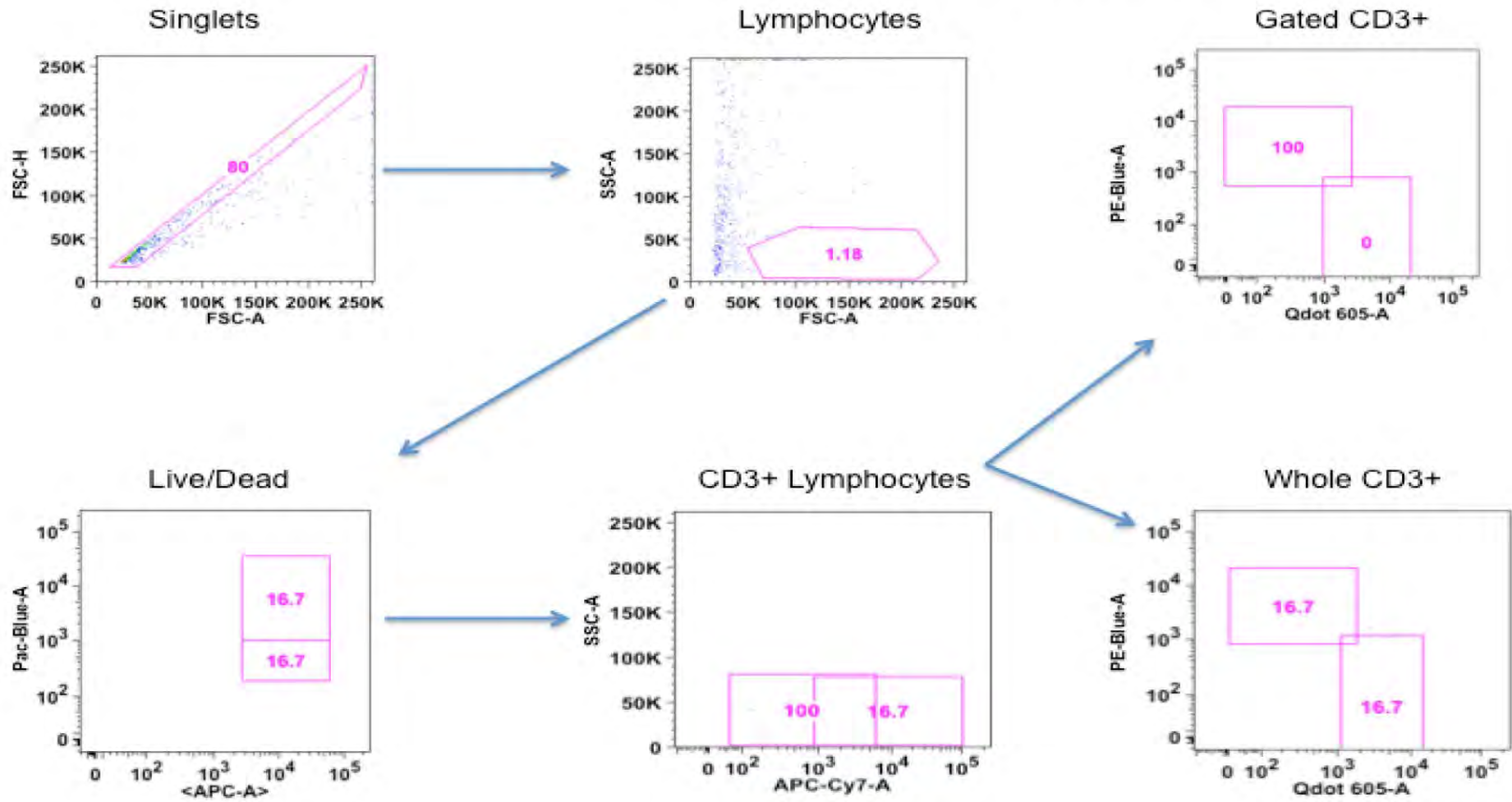


B EDH091 Scissors Inner 62.5U/ml Collagenase 1



C

EDH091 Machine Inner 500U/ml Collagenase 1



D

EDH091 Scissors Inner 500U/ml Collagenase 1

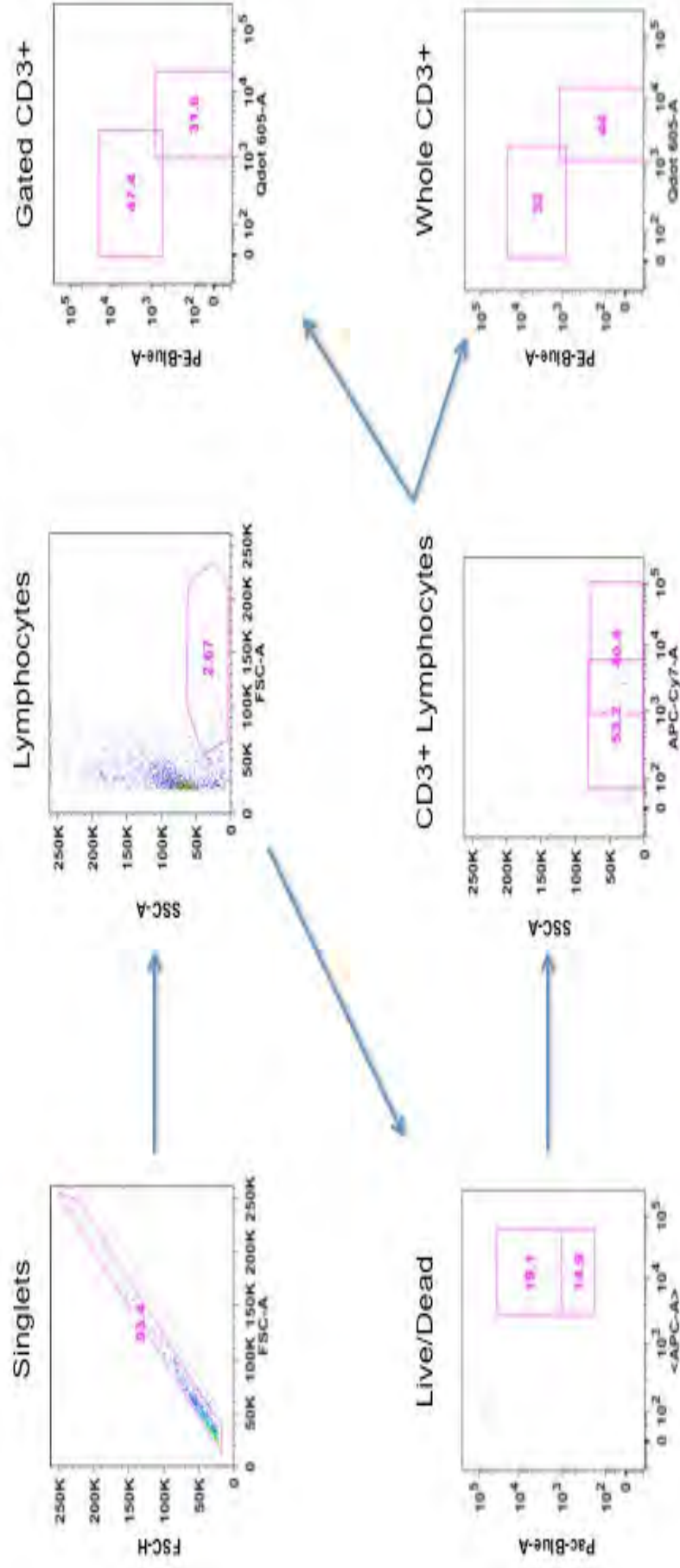
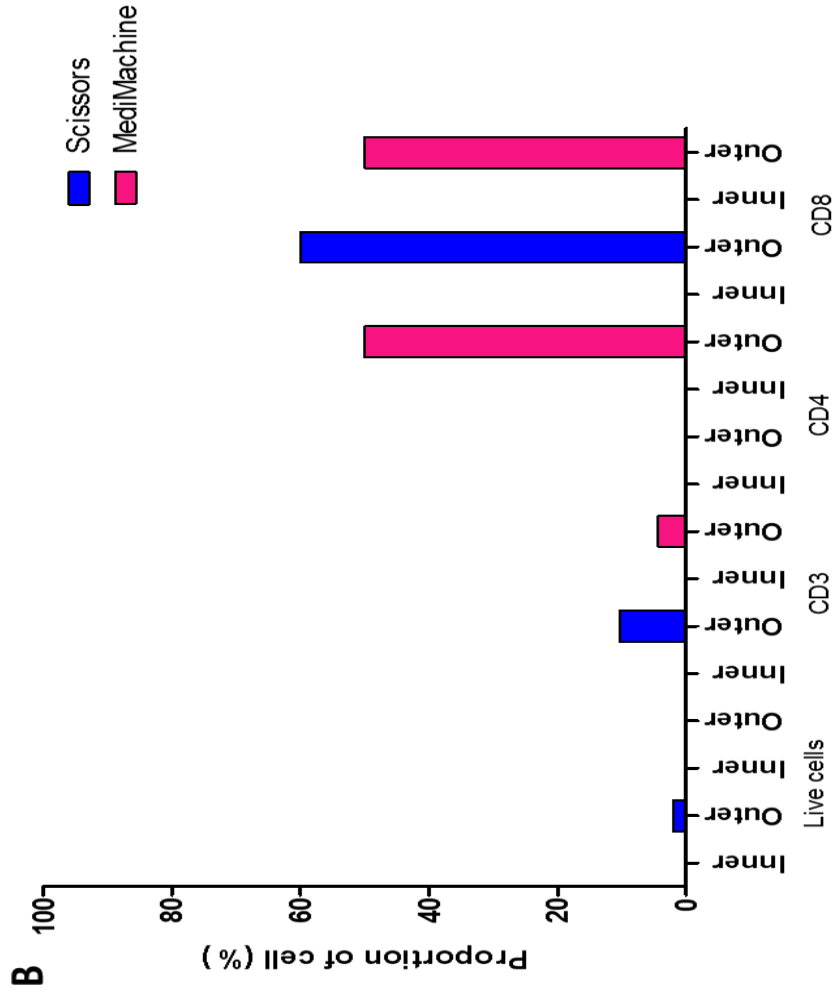
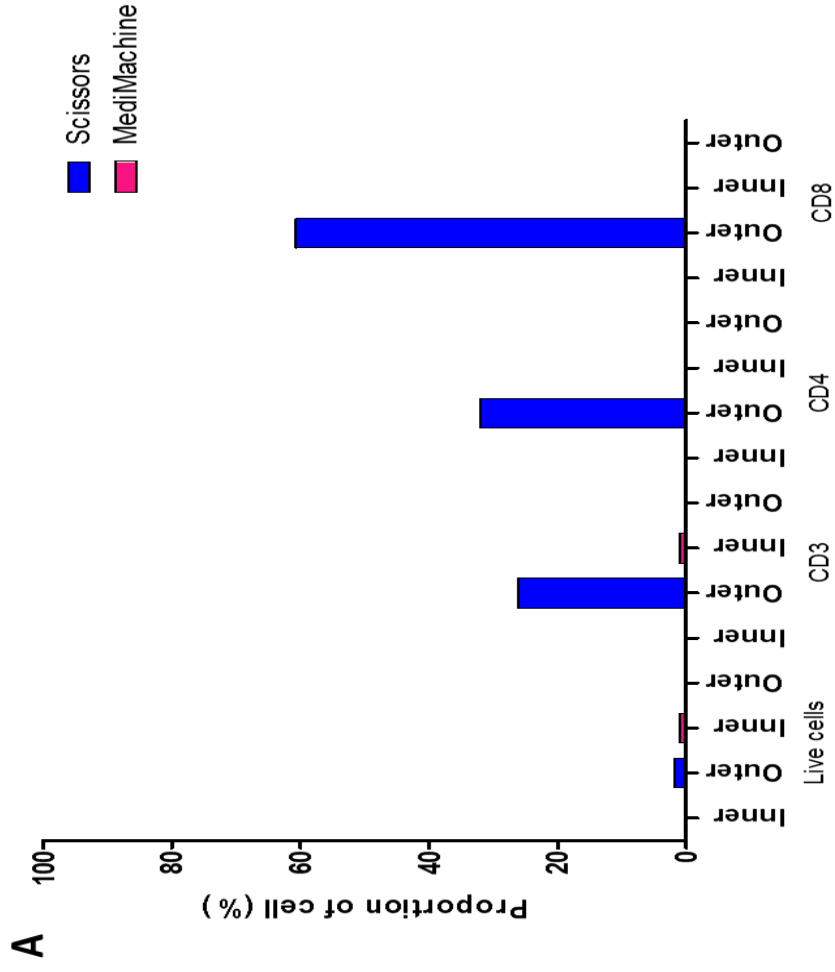


Figure 2.7: Comparison of different concentrations of collagenase A using BD Medimachine and Scissors.



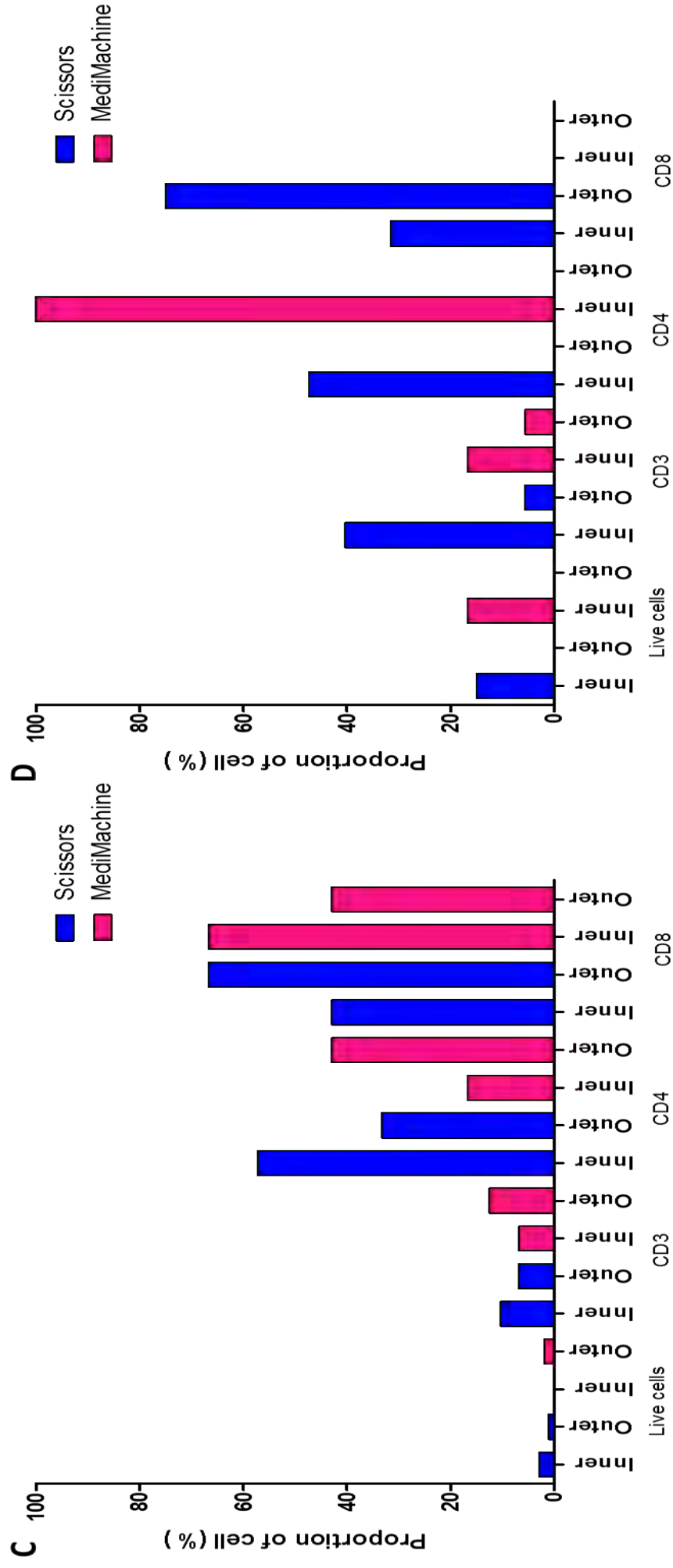
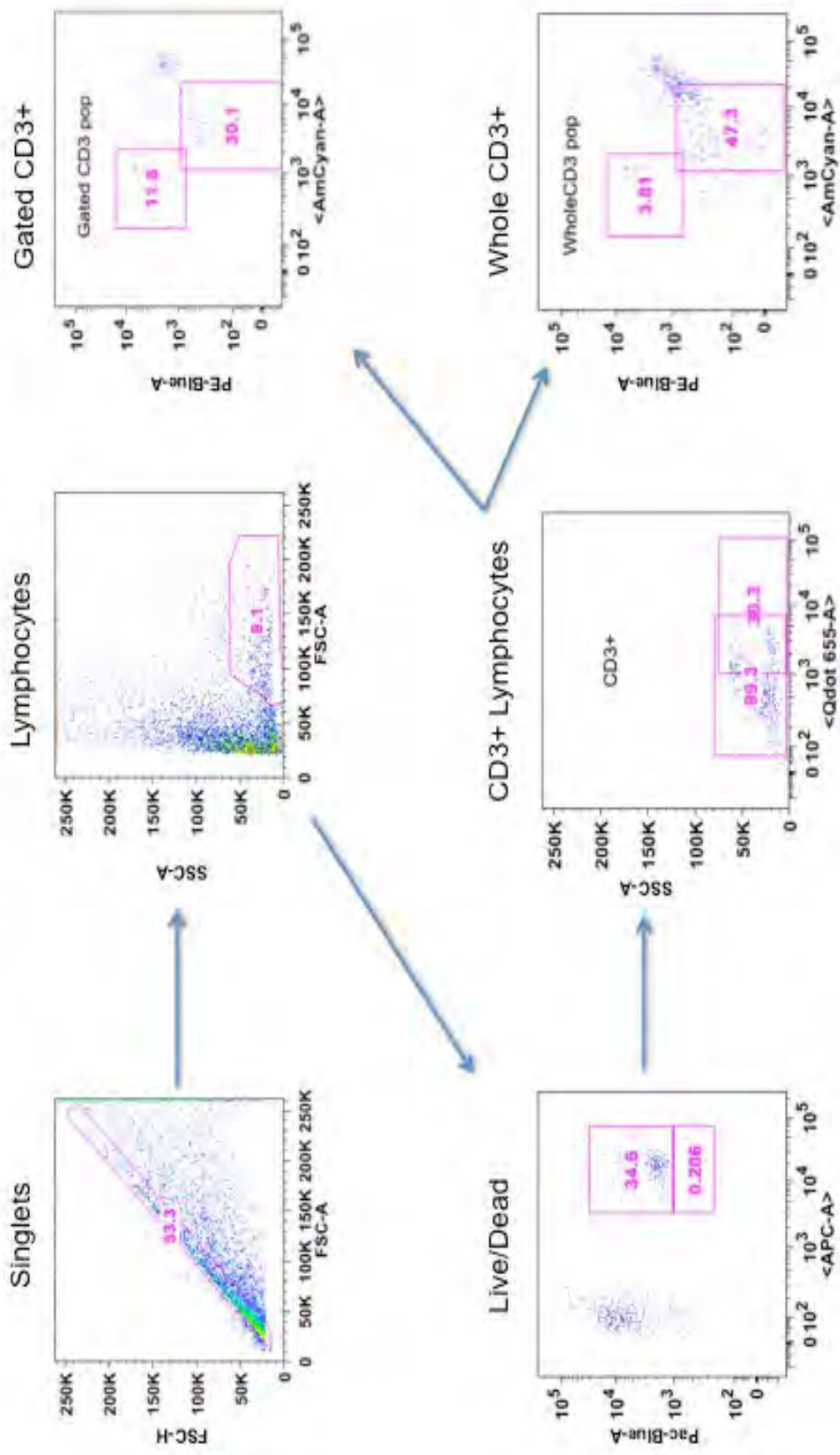


Figure 2.8: Proportion of cells (%) after tissue digestion with collagenase A at various concentrations using scissors and medimachine. A) 62.5U/ml B) 125U/ml C) 250U/ml D) 500U/ml

A

EDH087 Machine Inner 500U/ml Collagenase 1



B

EDH087 Scissors Inner 500U/ml Collagenase 1

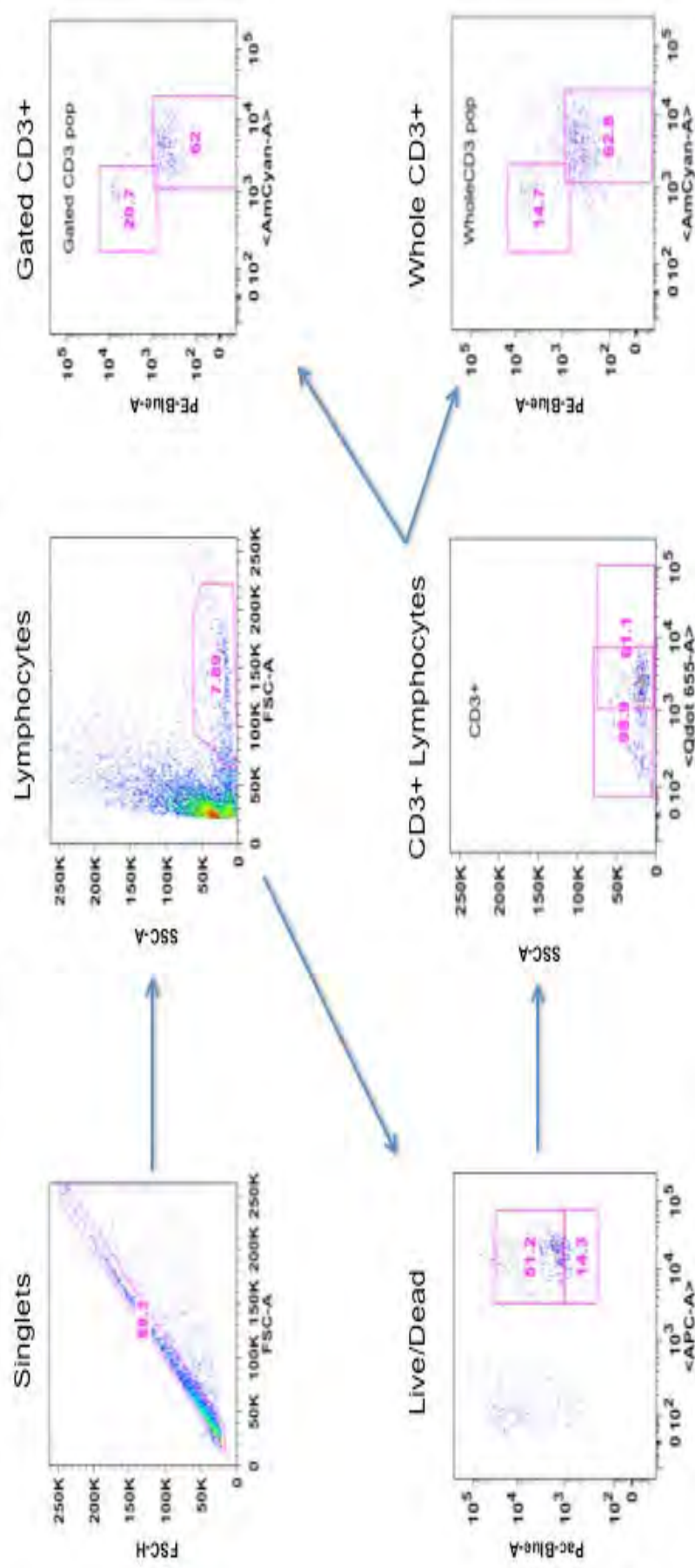


Figure 2.9: Comparison of scissors and BD Medimachine at optimal collagenase concentration. A) Medimachine B) Scissors

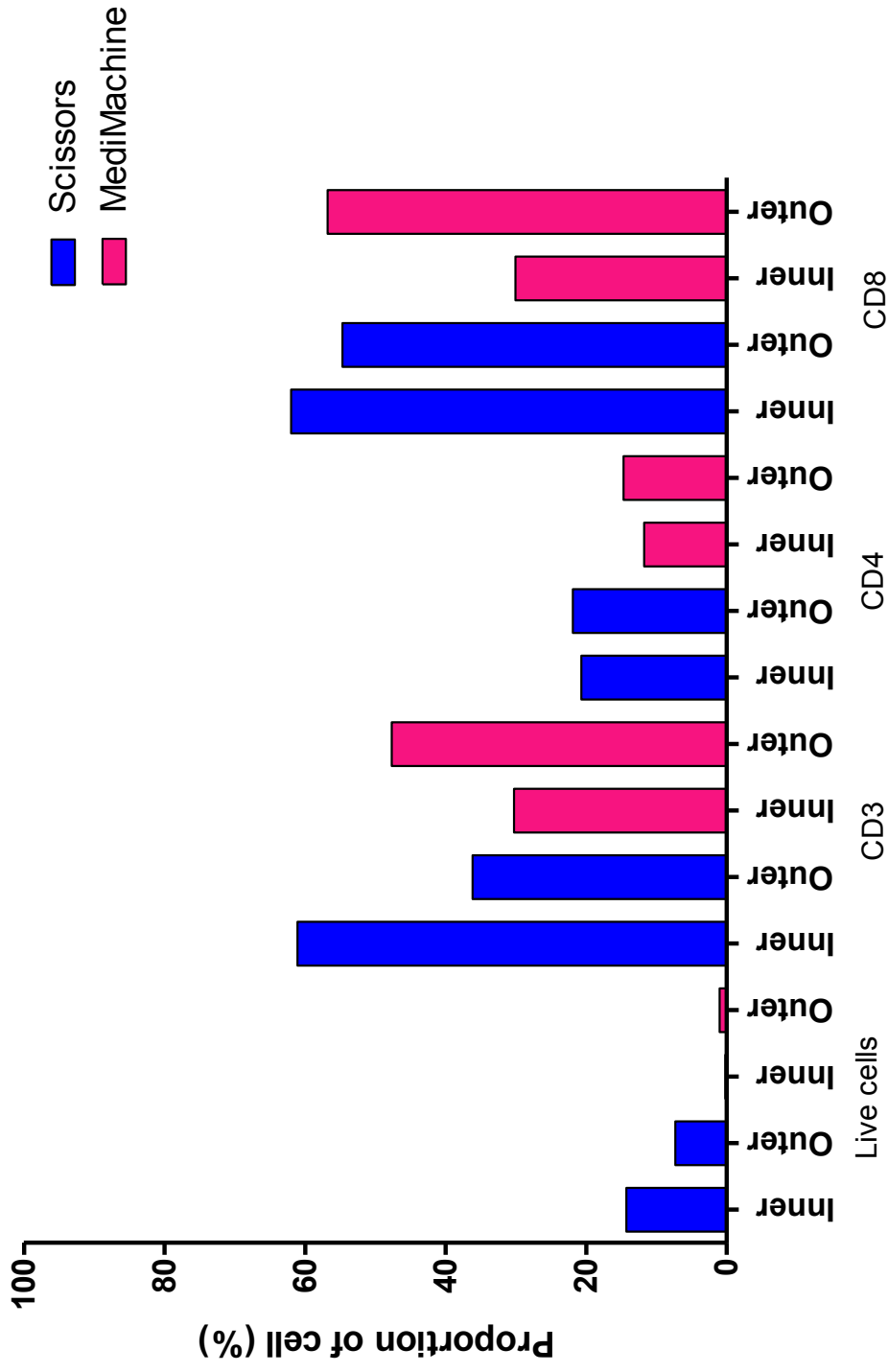
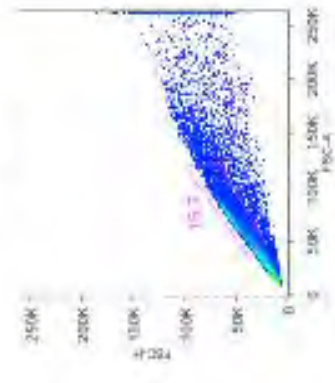


Figure 2.10: Proportion of cells (%) after tissue digestion at the optimal concentration. MediMachine vs Scissors

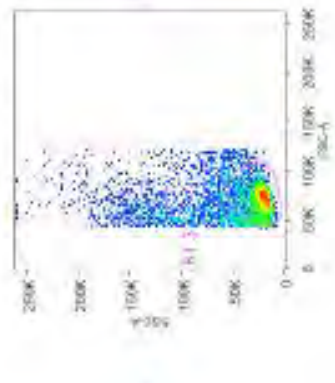
A

CHB009 Inner Fresh

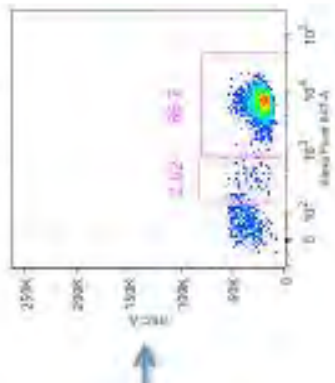
Singlets



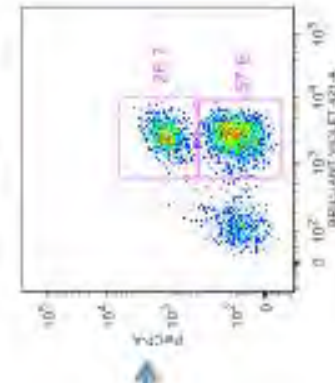
Lymphocytes



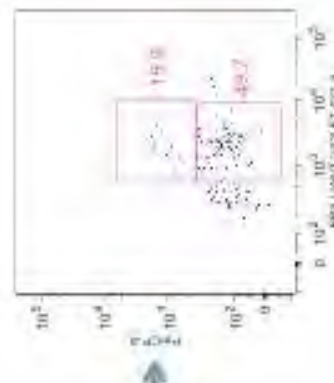
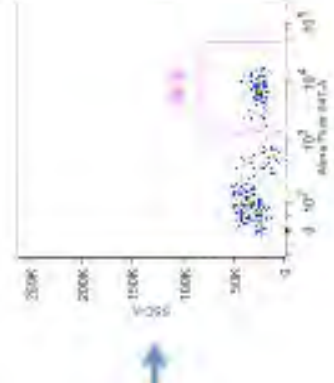
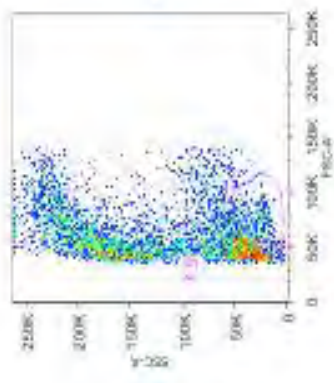
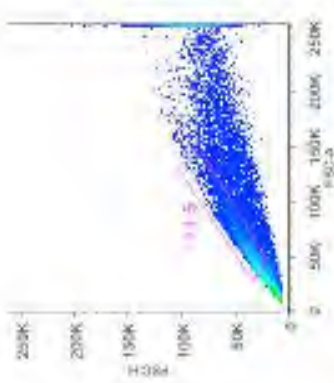
CD45+



CD4+/CD3+



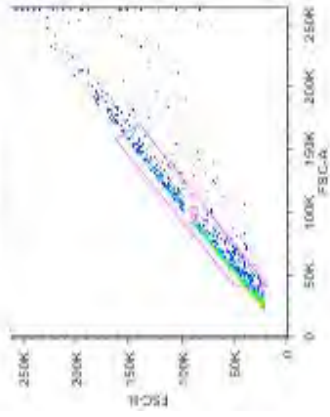
CHB009 Outer Fresh



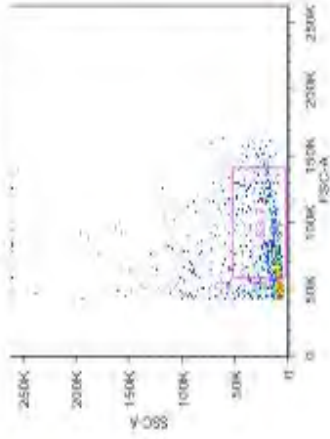
B

EDH082 Inner Frozen

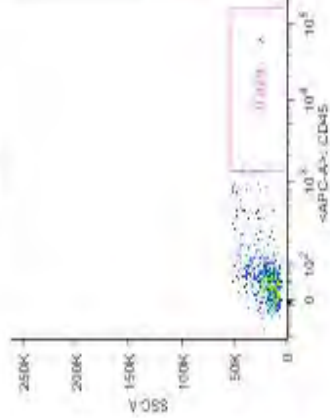
Singlets



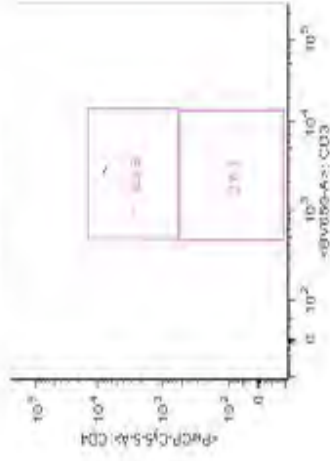
Lymphocytes



CD45+



CD4+/CD3+



EDH082 Outer Frozen

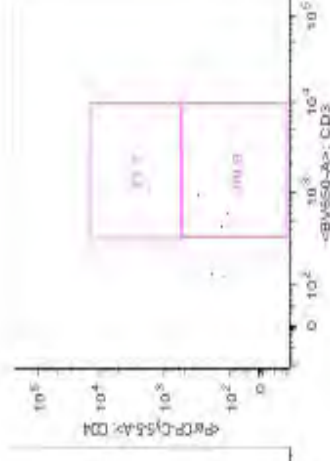
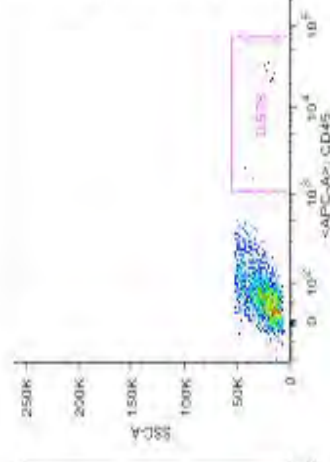
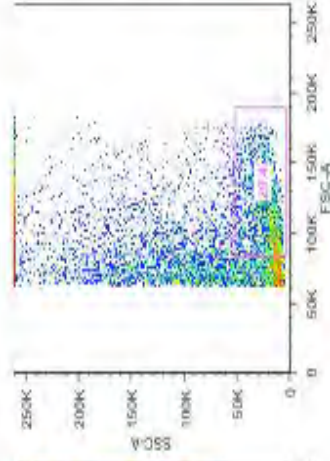
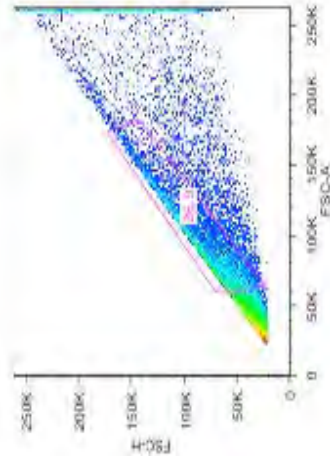


Figure 2.11: Comparison of processing fresh and frozen tissue. A) Fresh inner and outer tissue sample B) Frozen inner and outer tissue sample.

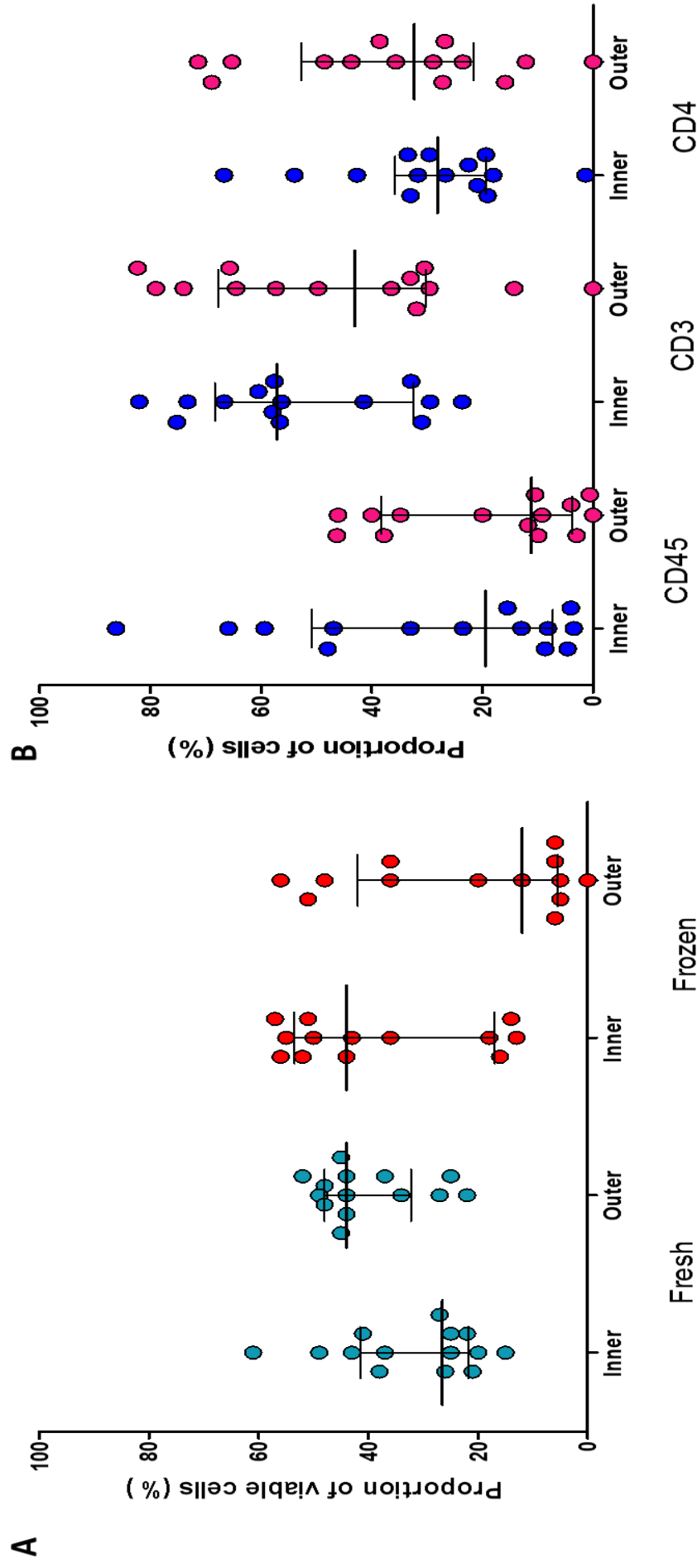


Figure 2.12: Proportion of cells after tissue digestion using a fresh versus a frozen sample. A) Percent of viable cells in the inner and outer foreskin from fresh and frozen tissue. **B)** Proportion of cells (%) of cell populations in the inner and outer foreskin of fresh tissue quantified by flow cytometry.

Table 5: Methodology used to determine the optimal amount of antibody dilution required for optimal foreskin cell staining by immunohistochemistry

Antibody	Purpose	Method	Figure/s
CD4	To determine the optimal dilution	Protocol followed as per 2.6.1 with the following amendments: CD4 was used as the primary antibody at dilutions of 1:100; 1:200 and 1:400 (incubated overnight at 4°C) and detected using a secondary antibody, donkey-anti-mouse Cy3 (incubated for 30 min in the dark). There was no second primary antibody added.	2.13
Ki67	To determine if the use of Zenon kits or Alexa647 secondary antibody improved Ki67 detection	Protocol followed as per 2.6.1 with the following amendments: Alexa647 antibody: Ki67 was added as the primary antibody and incubated for 3 hours in the dark. Repeat wash step. A goat-anti-mouse secondary detection antibody was used to detect Ki67 and incubated for 1 hour in the dark. There was no second primary antibody added. Zenon kit: Ki67 was prepared in an eppendorf by adding 21µl of Ki67 antibody to 5µl of Zenon Alexa fluor 647 reagent A, mixed and incubated in the dark for 5 min. To ensure there was no non-specific binding, 5µl of Zenon blocking reagent B was added and incubated in the dark for 5 min. There was no second primary antibody added.	2.14
CCR5	To determine the optimal dilution	Protocol followed as per 2.6.1 with the following amendments: CCR5 was used as the primary antibody at dilutions of 1:5; 1:10 and 1:20 (incubated overnight at 4°C) and detected using a secondary antibody, donkey-anti-mouse Cy3 (incubated for 30 min in the dark). There was no second primary antibody added.	2.16
CD209	To determine the optimal dilution	Protocol followed as per 2.6.1 with the following amendments: CD209 was used as the primary antibody at dilutions of 1:150; 1:200 and 1:250 (incubated in the dark for 3 hours). There was no secondary detection antibody used as CD209 was a conjugated antibody to Alexa 647. There was no second primary antibody added.	2.15

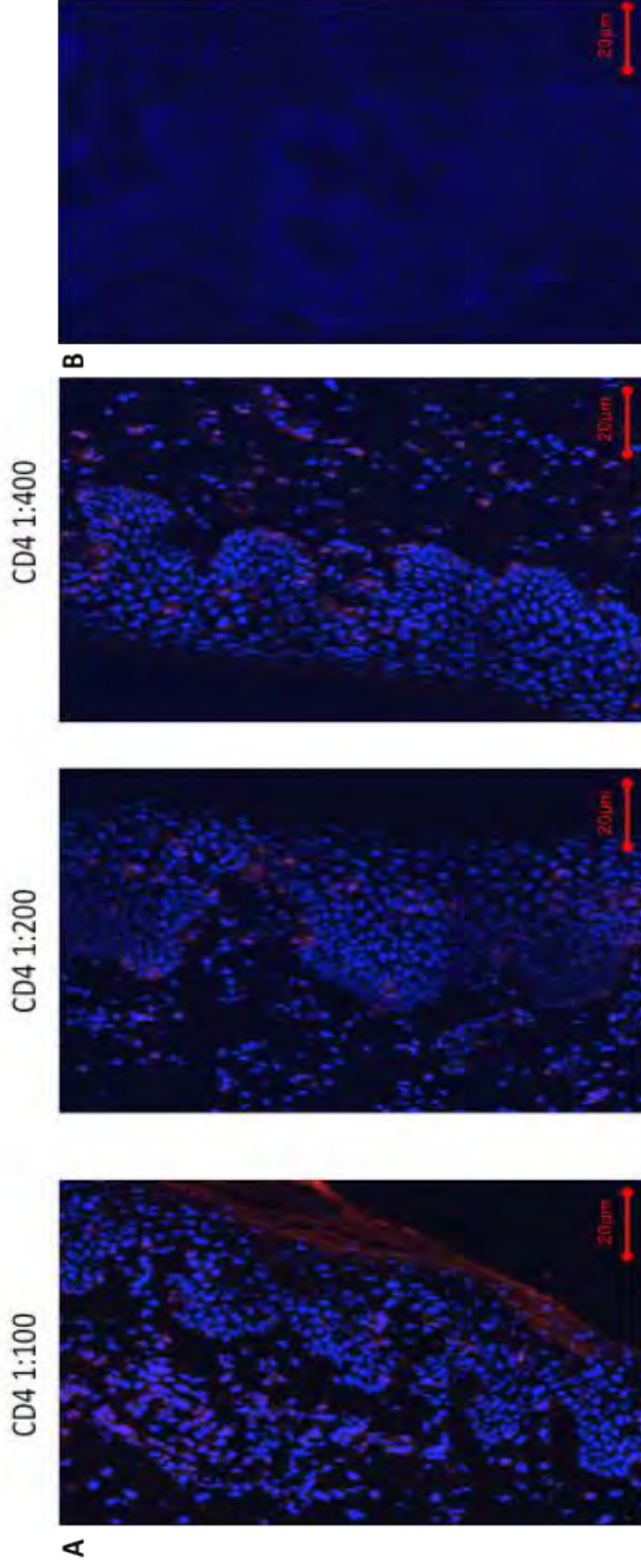
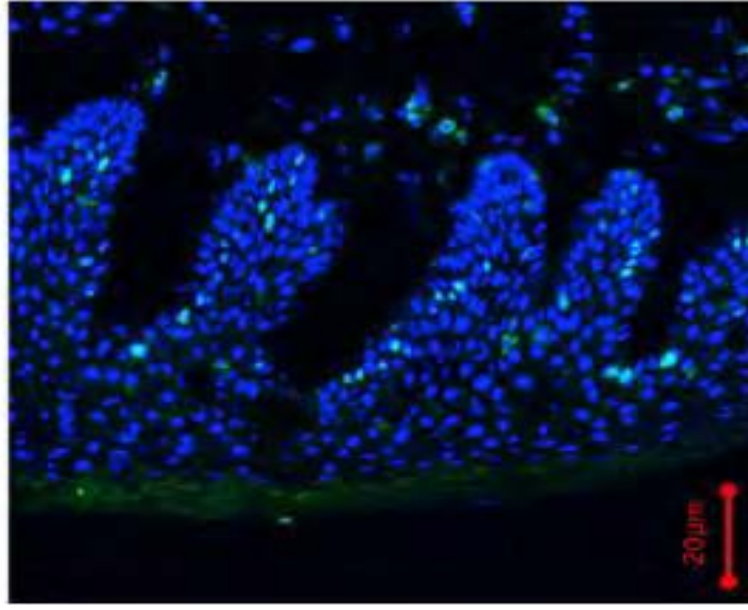


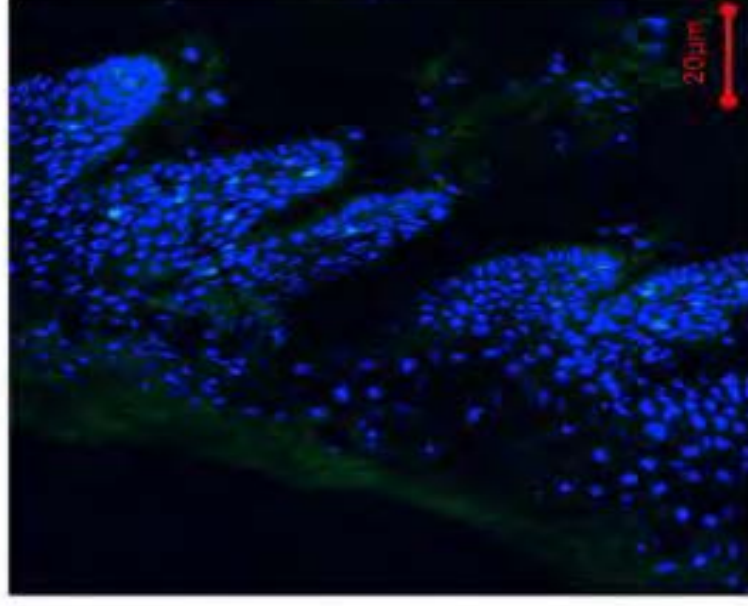
Figure 2.13: CD4 optimisation A) CD4 dilutions at various concentrations. B) Image of an isotype control

Ki67 Alexa 647



A

Ki67 Zenon Kit



B



Figure 2.14: Zenon kit vs secondary antibody **A)** Ki67 expression using Zenon kit vs Alexa 647 goat-anti-mouse secondary antibody. **B)** Image of an isotype control

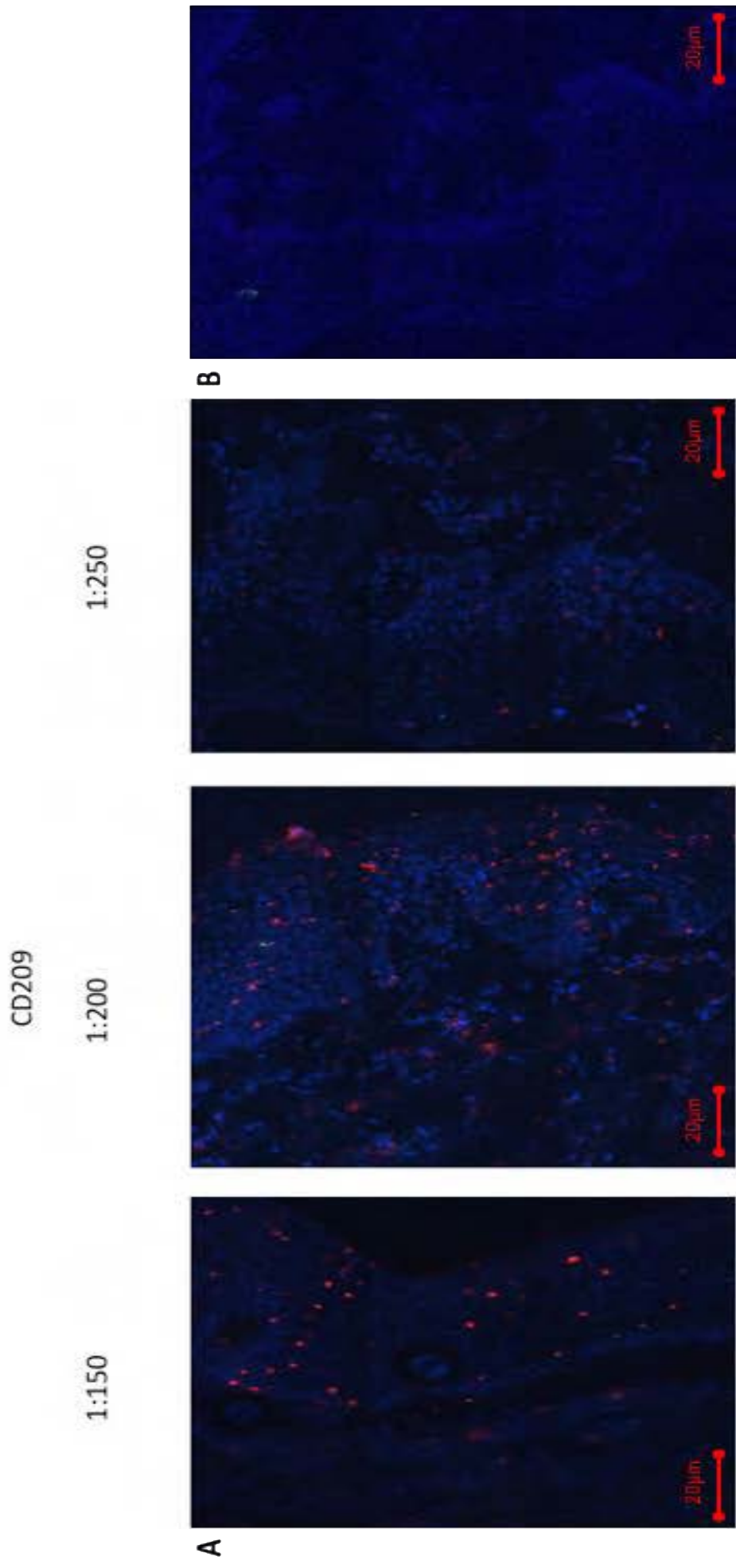


Figure 2.15: CD209 optimisation A) CD209 dilutions at various concentrations. B) Image of an isotype control

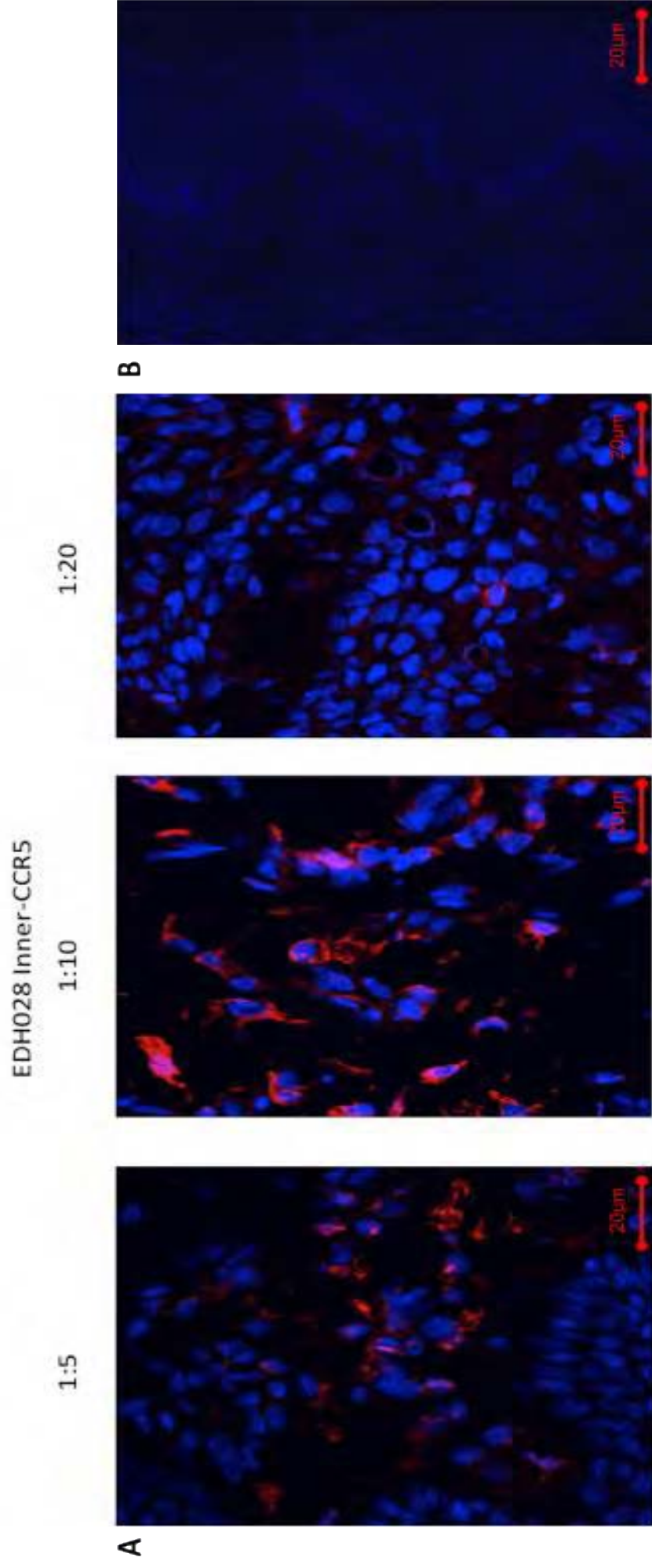


Figure 2.16: CCR5 optimisation. A) CCR5 dilutions at various concentrations. B) Image of an isotype control

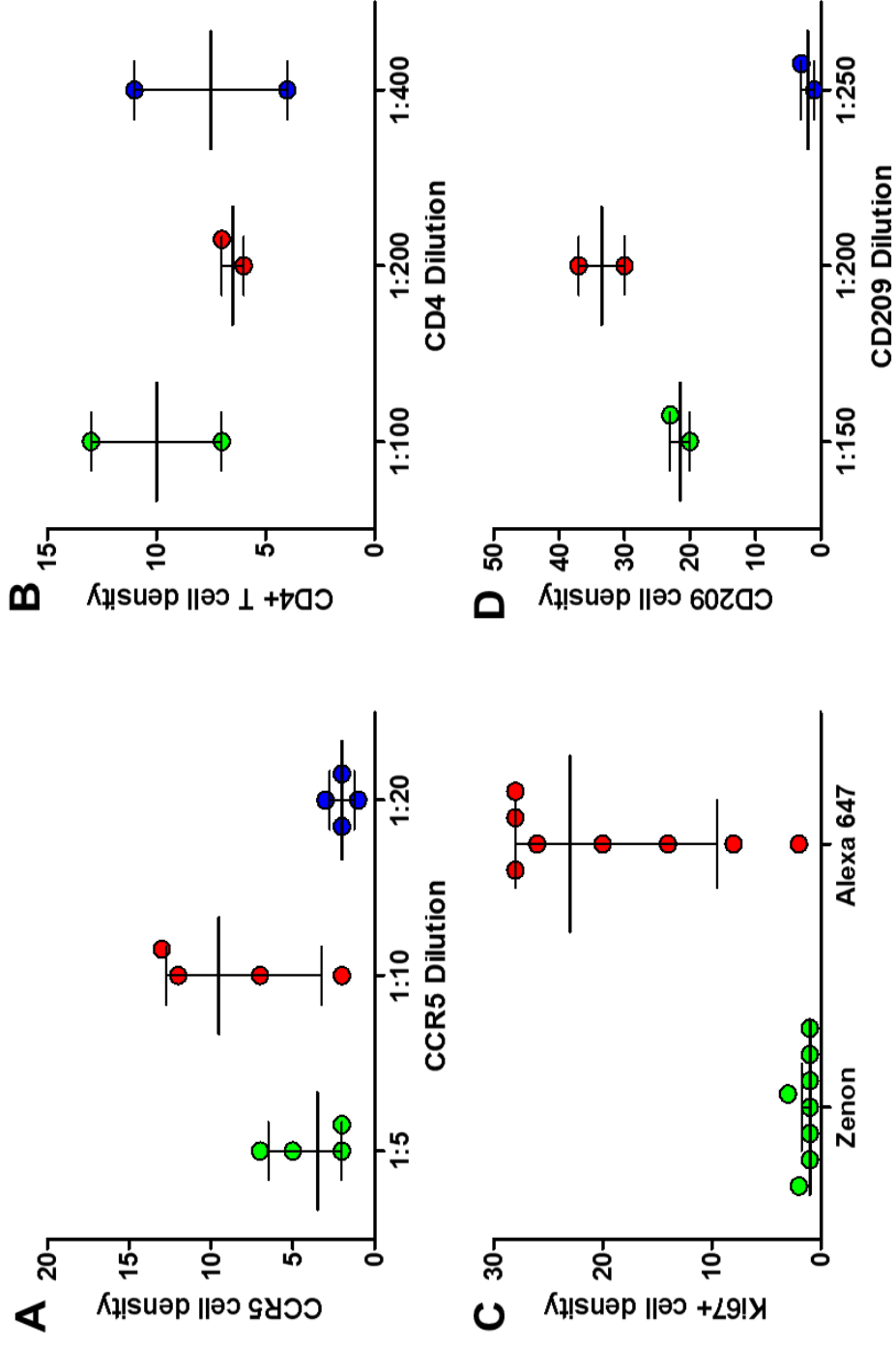


Figure 2.17: Cell density in various stains. A) CCR5 dilutions **B)** CD4 dilutions **C)** Ki67 using Zenon kit versus secondary antibody **D)** CD209 dilutions.

Optimisation Outcomes:

The first set of optimisation experiments comparing Collagenase A and B showed that collagenase A was able to yield a higher percentage of viable cells (39.3%) compared to that of collagenase B (18.5%) (Figure 2.5). Figure 2.6 shows that collagenase A had a higher yield of CD3⁺ and CD4⁺ T cells while collagenase B had a higher yield of CD8⁺ T cells.

The second set of optimisation experiments compared Collagenase A at concentrations of 62U/ml, 125U/ml, 250U/ml and 500U/ml using scissors and the BD medimachine to aid in digestion. The highest concentration (500U/ml) was most effective and yielded the highest percentage of viable cells, 16.7% and 14.9% for the BD medimachine and scissors respectively (Figure 2.7C and 2.7D) compared to 1.08% and 0% of viable cells at the lowest concentration (62U/ml) for BD medimachine and scissors respectively (Figure 2.7A and 2.7B). Figure 2.8 shows the yield of cells at various concentrations. At a concentration of 62U/ml, there were extremely low yields of cells in the inner foreskin when using both methods of mechanical digestion and low yields in the outer foreskin when using the medimachine. There were less than 40% of CD3 and CD4 cells and approximately 60% of CD8 in the outer foreskin when using scissors. At a concentration of 125U/ml, yields of viable, CD3, CD4 and CD8 cells in the inner foreskin were 0% regardless of mechanical digestion method used with low yields of live and CD3⁺ cells in the outer foreskin. At a concentration of 250U/ml, there were better yields of CD4 and CD8 cells using both mechanical digestion methods. Although, viable and CD3⁺ cell yield was still low. The use of scissors however, yielded more viable cells, CD3 and CD4 cells in the inner foreskin compared to use of the medimachine. At a concentration of 500U/ml, the yield of viable cells in the inner foreskin was slightly higher by use of the medimachine. The use of scissors however yielded approximately double the amount of CD3⁺ cells compared to the medimachine. The use of the medimachine yielded no CD8 cells in both the inner and outer foreskin and while the yield of CD4⁺ cells were 100%, this may be due to the low yield of CD3 cells and placement of the gate in FlowJo.

The third experiment was to optimised the tool used to aid in tissue digestion at the optimal concentration of collagenase A. Here the use of the medimachine

yielded 0.206% of viable cells compared to 14.3% of viable cells for scissors (Figure 2.9A and 2.9B). Figure 2.10 shows that the use of scissors is better suited in the digestion of tissue as there was a greater yield of viable and CD4 cells in the inner and outer foreskin, and a greater yield of CD3 and CD8 cells in the inner foreskin compared to the use of the medimachine.

The last set of optimisation was to determine whether using the optimised techniques above worked better for fresh or frozen samples. The use of fresh tissue showed a greater yield of CD3+ cells (57.6%) and (49.75%) in the inner and outer foreskin respectively compared to the use of frozen tissue where CD3+ cells accounted for 27.3% and 60.9% in the inner and outer foreskin respectively (Figure 2.11A and 2.11B). Figure 2.12A shows the yield of viable cells using the TC20. The median yield of viable cells in the inner foreskin from frozen tissue is higher than from the fresh tissue (44% and 26.5% respectively) while the median yield of viable cells in the outer foreskin from frozen tissue is lower than from fresh tissue (12% vs 44% respectively). The average amount of viable cells however, did not differ by much, with 32.14% and 38.85% of viable cells for fresh and frozen tissue in the inner foreskin respectively while the average yield of viable cells in the outer foreskin of fresh tissue was almost double the amount in frozen tissue (40.29% and 22.08% respectively). Another factor that may have contributed to the higher percent of viable cells in frozen samples may be due to the total amount of live cells used in the TC20 cell counter. The average amount of live cells used in fresh tissue samples were 2.26×10^6 cells in the inner foreskin and 2.07×10^6 cells in the outer foreskin compared to 2.95×10^5 cells in the inner foreskin and 3.02×10^5 cells in the outer foreskin of frozen tissue samples. Due to the low yield of cells experienced in some frozen samples where there was >80% of dead cells, we did not deem it logical to conduct flow cytometry on all of these samples. Figure 2.12B shows the percentage of CD45+, CD3+ and CD4+ cells in the inner and outer foreskin from fresh tissue samples. There is a higher yield of CD45 and CD3+ cells in the inner compared to the outer foreskin, while the outer foreskin has a higher yield of CD4+ T cells. There is a median yield of approximately 60% of CD3 cells from fresh samples.

Finally, we optimised the amount of antibody used for immunohistochemistry staining. The CD4 dilution worked best at 1:100 (Figure 2.13), the use of the Alexa647 goat-anti mouse secondary antibody allowed for better detection of KI67 than the Zenon kit (Figure 2.14), the CD209 stain worked best at 1:200 (Figure 2.15) and lastly, the CCR5 stain worked best at 1:10 (Figure 2.16) dilution. Figure 2.17 shows cell density of the various stains at different dilutions. There were 4 samples used in the optimisation of CCR5 (Figure 2.17A) with median cell densities of 3.5, 9.5 and 2.5 for the 1:5, 1:10 and 1:20 dilutions respectively. There were 8 samples for Ki67 (Figure 2.17C) with median cell densities of 1 and 23 for Zenon kit and Alexa647 respectively. There were 2 samples for CD4 with median cell densities of 10, 6.5 and 7.5 for 1:100, 1:200 and 1:400 dilutions respectively. There were 2 samples for CD209 with median cell densities of 21.5, 33.5 and 2 for 1:100, 1:150 and 1:200 dilutions. The best dilution was chosen based of brightness of fluorescence and median cell density yield per image.

CHAPTER 3: RESULTS

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3.1 Introduction

Confocal microscopy enables microscopic visualization of subsurface structures and the ability to section tissue pieces allows for the capturing of an intact tissue (Minsky 1957; Aspres *et al.* 2003). A tissue piece may also be imaged multiple times over a time period to examine any tissue changes due to the non-invasive nature of confocal microscopy (Aghassi *et al.* 2000). Confocal microscopy allows for development of specific techniques that enable the visualisation of structure, function and dynamics of the skin *in vivo* with the use of fluorescent markers (Anikijenko *et al.* 2001). Due to the development of these techniques, it has allowed studies to characterise the different layers of the skin such as the keratin, epidermal and dermal layers (Swindle *et al.* 2003).

A thick keratin layer of the epidermis has been thought to provide a more robust barrier of protection in the foreskin (Candi *et al.* 2005). Within the epidermal and dermal layer, immune cells such as LCs and CD4⁺ T cells have been observed in the foreskin and are potential targets for HIV-1 infection (Liu *et al.* 2014). LCs are thought to be the first immune cells to encounter HIV-1 (Patterson *et al.* 2002; Kawamura *et al.* 2005) and a small population of CD1a positive LCs are positive for CD4 expression (Lynch *et al.* 2003). The main target for establishing a productive HIV-1 infection, CD4⁺ T cells are also present in the dermis of the foreskin (Patterson *et al.* 2002; Donoval *et al.* 2006). There have been disparate findings with regards to the numbers of immune cells present in the foreskin. There were 4 groups that have found different results, Liu *et al.*, found a greater number of LCs and CD4⁺ T cells in the inner compared to the outer foreskin, Patterson *et al.*, and Lemos *et al.*, found more CD4⁺ T cells in the inner compared to the outer foreskin while McCoombe & Short found more CD4⁺ T cells in the outer compared to the inner foreskin. Donoval *et al.*, showed that the CD4⁺ T cells are found deep in the tissue and are therefore not likely the first cells to come into contact with HIV-1 and that STIs may increase the risk of HIV-1 infection, in particular the ulcerative STIs.

There may be some factors that are elevating the numbers of HIV-1 target cells that are present in the foreskin and one of these are STIs. Symptomatic STIs such as syphilis causes painless genital ulcerations, but is associated with the increase of target cells to the area of infection (reviewed by Kaul *et al.* 2008)

and asymptomatic STIs such as HSV, which is able to increase the numbers of CD4+ T cells expressing CCR5 in the foreskin (Shannon *et al.* 2014; Prodger *et al.* 2012). STIs have been strongly associated with the increased risk of HIV-1 infection (reviewed by Ward & Rönn 2010). The presence or history of a genital ulcerative diseases (GUD) such as NG, TV (reviewed by Fox & Fidler 2010), HSV-2, which is the most common GUD around the world (Morse 1999) and MG (Mavedzenge & Weiss 2009) is able to increase the per-act infectivity of HIV-1 five-fold compared to individuals with no STI (reviewed by Boily *et al.* 2009) due to inflammation, trauma and increased number of target cells to the site of infection (Shannon *et al.* 2014; Prodger *et al.* 2012; Johnson *et al.* 2009). A systematic review by Dunne *et al.*, found that $\geq 20\%$ of asymptomatic men have detectable HPV DNA in anogenital areas. HPV is one of the most prevalent STIs in the world and men with a HPV infection are three times as likely to contract HIV-1 compared to a HPV negative man (Rositch *et al.* 2014). Johnson *et al.*, showed that HSV-2 infection in men increases the CD4+ cell density in the foreskin. Donoval *et al.*, showed that there were a greater numbers of LCs in the foreskin of STI+ compared to STI- men whereas Hussain & Lehner., found no differences in the number of LCs and CD4+ cells in the foreskin.

3.2 Cohort Characteristics

We selected a subset of 28 age-matched STI+ and STI- individuals from the Edendale cohort for IHC staining. No men from the CHB cohort were selected for IHC staining. The median age of the men was 19 years (range 15-24). The subset consisted of 17 STI+ individuals (Figure 3.1) and 11 STI- individuals. The following STIs were screened for: *N. gonorrhoea* (NG), *C. trachomatis* (CT), *T. vaginalis* (TV), *M. genitalium* (MG), HSV-1 and HSV-2. CT was the most prevalent STI in the cohort, accounting for as much as 70% of STIs (Table 6). There were 3 individuals from the CHB site that were STI+ (7.3%) with 2 of the individuals testing positive for CT. We also tested for testosterone in the individuals from the EDH site to evaluate the differences in testosterone levels between age groups (Table 6).

Table 6: Cohort Characteristics from the Edendale and Chris Hani Baragwanath Site

	EDH	CHB
Number of participants (Total)	109	41
Median age (range)	19 (14-24)	18 (14-24)
Testosterone levels (nano mol/ litre)	21.7 (16.8-25.8) ^b	N/A
Number of STI+ individuals	17 (15.6) ^a	3 (7.3) ^a
<i>Chlamydia Trachomatis</i>	12 (70) ^a	2 (4.9) ^a
<i>Herpes Simplex Type 1</i>	0	0
<i>Herpes Simplex Type 2</i>	1 (5.9) ^a	0
<i>Mycoplasma Genitalium</i>	5 (29.4) ^a	1 (2.4) ^a
<i>Neisseria Gonorrhoea</i>	3 (17.6) ^a	0
<i>Trichomonas Vaginalis</i>	2 (11.7) ^a	1 (2.4) ^a
<i>Human Papillomavirus</i>	7 (36.8) ^a	0

^a Number of men (Percentage of total)

^b Median (Interquartile range)

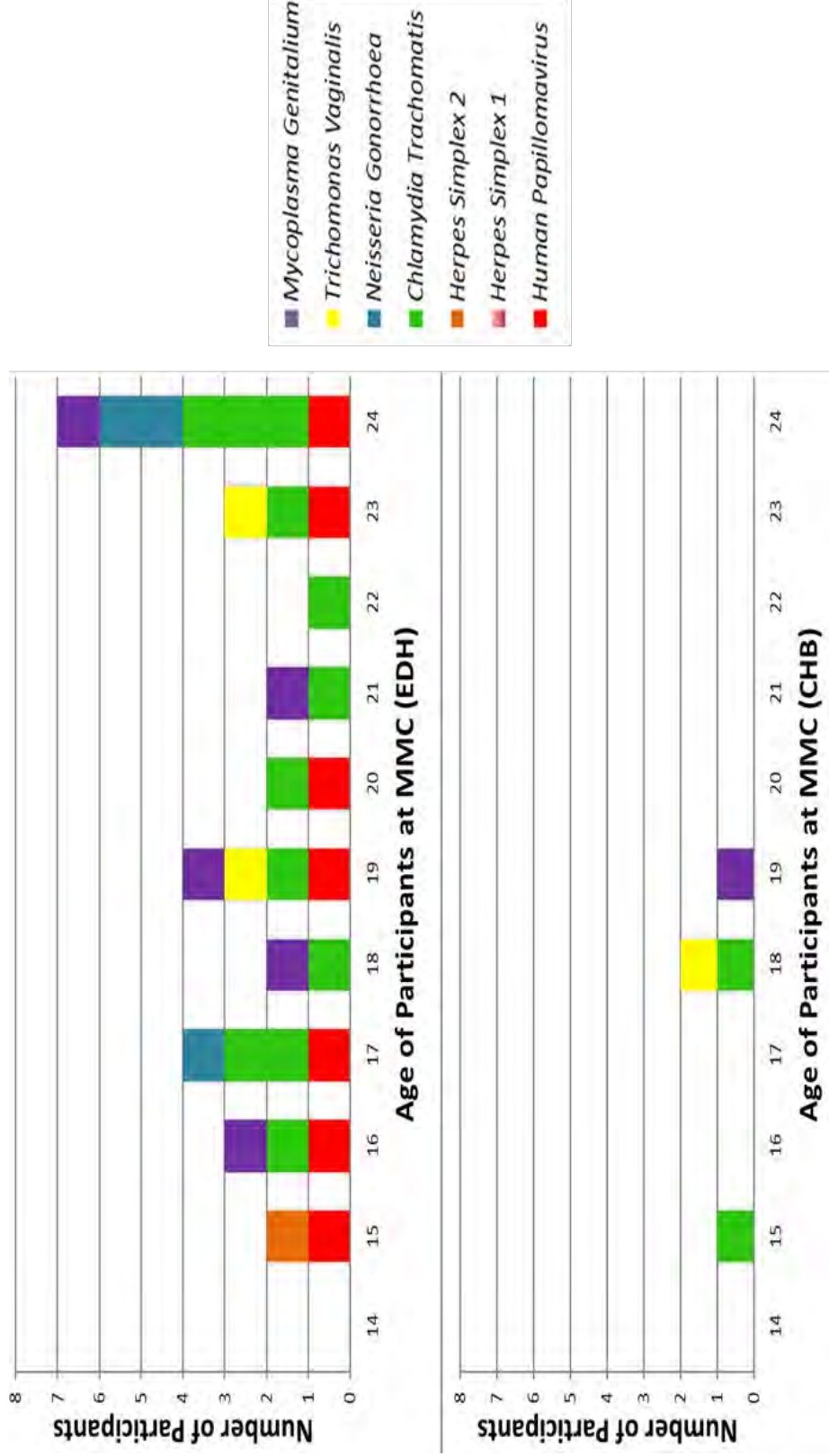


Figure 3.1: STI prevalence in young men between the ages of 14-24 at MMC. All STIs were measured by q-PCR except HPV which was measured using a Roche Linear Array Genotyping assay. The top represents the EDH site (17/150) where there is a higher prevalence of STIs compared to CHB (3/150) (bottom).

3.3 Differences in keratinisation levels between inner and outer foreskin

To investigate differences in the degree of keratinisation between the inner and outer foreskin, anti-filaggrin was used to stain the keratin layer in the subset of participants. Filaggrin was used as it is a filament-associated protein that binds keratin fibre's (Figure 3.2).

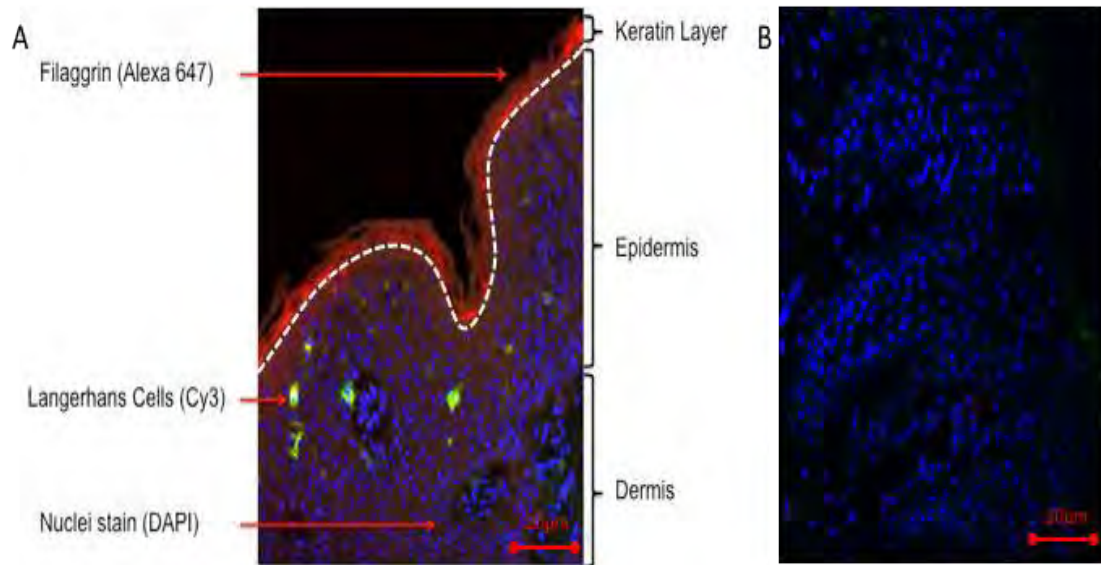


Figure 3.2: Annotation of image generated by confocal microscopy. A)

The keratin layer is shown by the filaggrin (Alexa 647) stain in red and is demarcated from the epidermis by the white dotted line. The LCs' cells represent the CD207 (Cy3) stain in green and the nuclei represents the dapi stain in blue. **B)** Image of an Isotype Control

In this dissertation, we compared the thickness of the keratin layer (8.3873 vs 6.674 μ m in the outer and inner foreskin, $p=0.0058$) and found that the outer foreskin was 1.712 μ m thicker than the inner foreskin, which is in agreement with findings by Patterson *et al.* 2002; McCoombe & Short 2006 and Ganor & Bomsel 2011 (Figure 3.3B). We then analysed the degree of keratinisation in context of asymptomatic STI status and found that there were no significant differences in the degree of keratinisation in the inner (6.8100 vs 6.5050 μ m in STI+ vs STI- men, $p=0.8538$) or outer (8.4282 vs 8.0780 in STI+ vs STI- men, $p=0.5647$) foreskin between participants (Figure 3.3A).

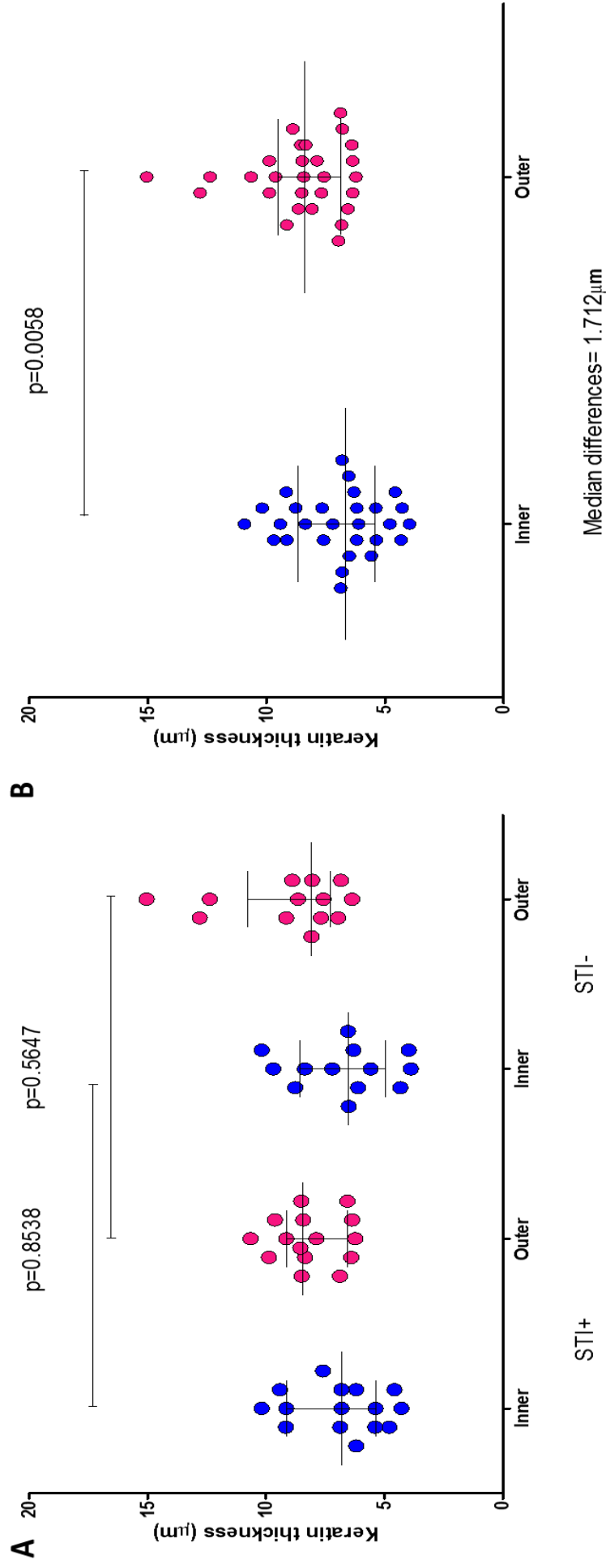


Figure 3.3: Degree of keratinisation in the inner and outer foreskin. A) Keratinisation in the inner and outer foreskin with regards to STI status (n=28; Inner, $p=0.8538$; Outer, $p=0.5647$ [Mann Whitney test] between STI+ and STI- men). **B)** Keratinisation in the inner and outer foreskin regardless of STI status (n=28; $p=0.0058$ [Mann Whitney test]).

3.4 Langerhans Cells

Langerin is a C-Type lectin expressed in LCs. In order to quantify the number of LCs, we used anti-langerin antibody to stain for langerhans cells present in the inner and outer foreskin. The LCs were found to be present in the epidermis of the inner and outer foreskin consistent with findings by Qin *et al.*, who also showed that the majority of LCs were found in the epidermis (Figure 3.4).

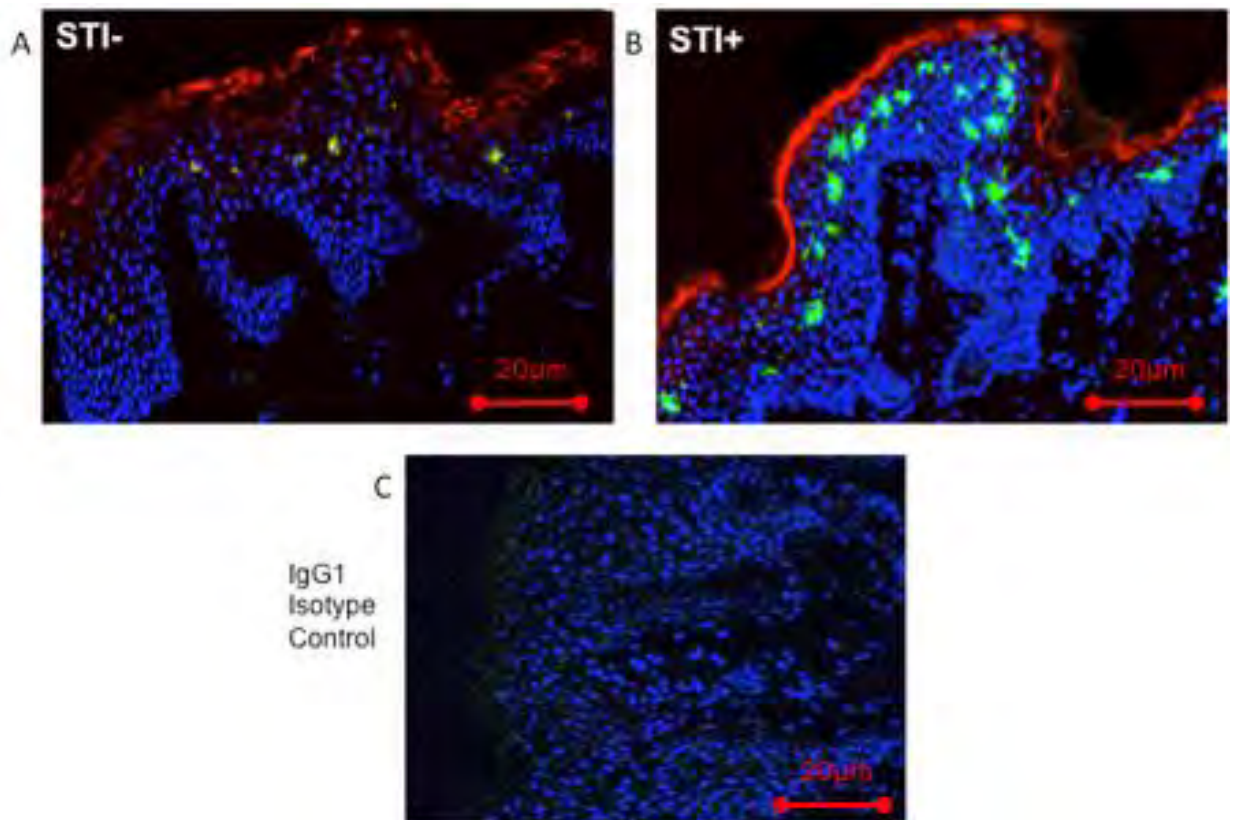


Figure 3.4: Confocal Images of Filaggrin and Langerhans' cell stains. A) Image of filaggrin (red), langerhans cell (green) and dapi (blue) staining in the inner foreskin of a STI negative participant. **B)** Image of filaggrin and langerhans cell staining in the inner foreskin of a STI positive participant. **C)** Image of an isotype control.

There were no significant differences in Langerhans cell density (2.5 vs 3.5 cells/mm² in inner vs outer foreskin; Figure 3.5A). We then analysed the Langerhans' cell density in context of the asymptomatic STI status. There was a significantly higher number of LCs present in the foreskin of men with STIs in both the inner (3.3 vs 0.3 cells/mm² in STI+ vs STI- men, p<0.0017) and outer (4.4 vs 0.6 cells/mm² in STI+ vs STI- men, p<0.0005) foreskin. (Figure 3.5B).

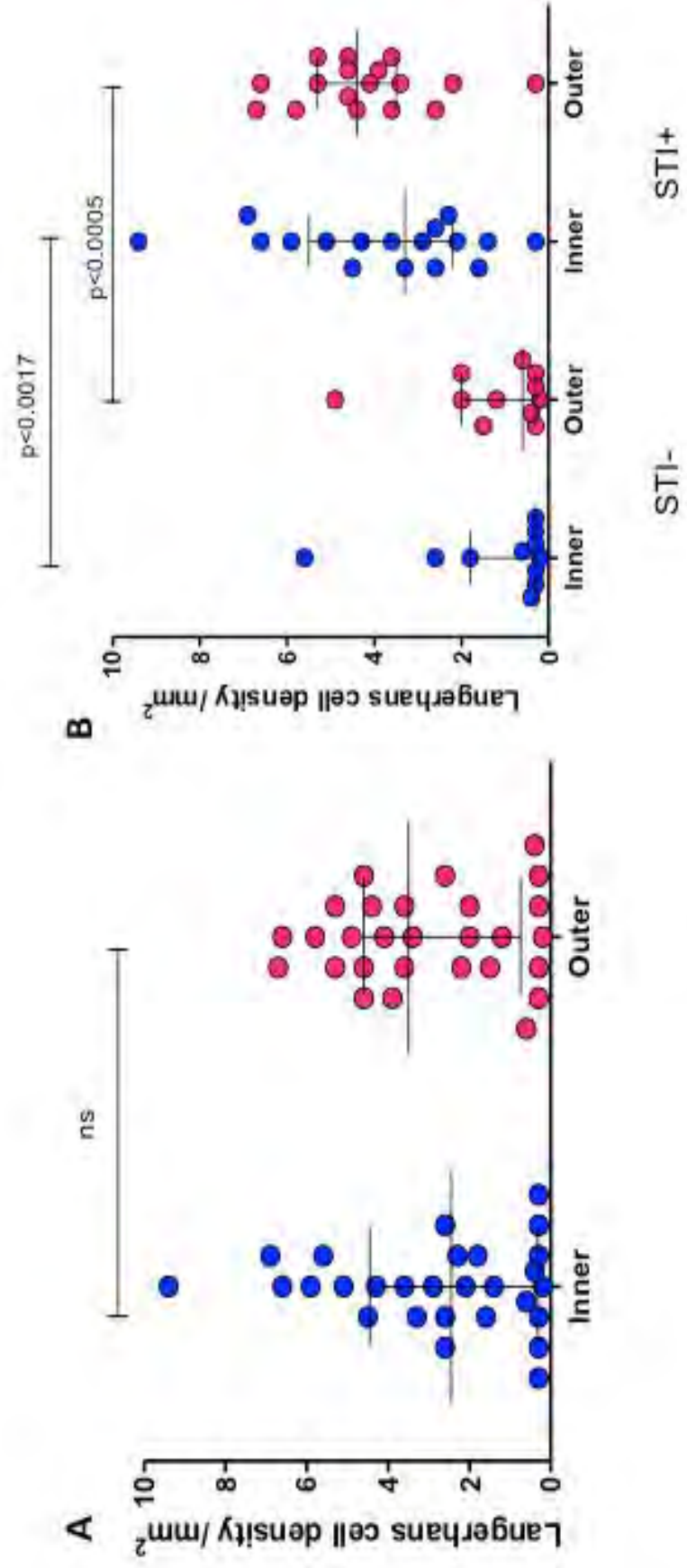


Figure 3.5: Langerhans cell density in the inner and outer foreskin. A) LC density in the inner and outer foreskin regardless of STI status (n=28; p= non-significant [Mann Whitney test]) **B)** LC density in the inner and outer foreskin of STI+ and STI- individuals (n=28; Inner, p<0.0017; Outer, p<0.0005 [Mann Whitney test]).

3.5 CD4 and Ki67

CD4+ T cells are a major target for HIV-1 infection and Ki67 is a proliferation marker. We used anti-CD4 and anti-Ki67 to stain the inner and outer foreskin tissue to determine the density of CD4+ T cells and Ki67+ cells present in the inner and outer foreskin tissues (Figure 3.6).

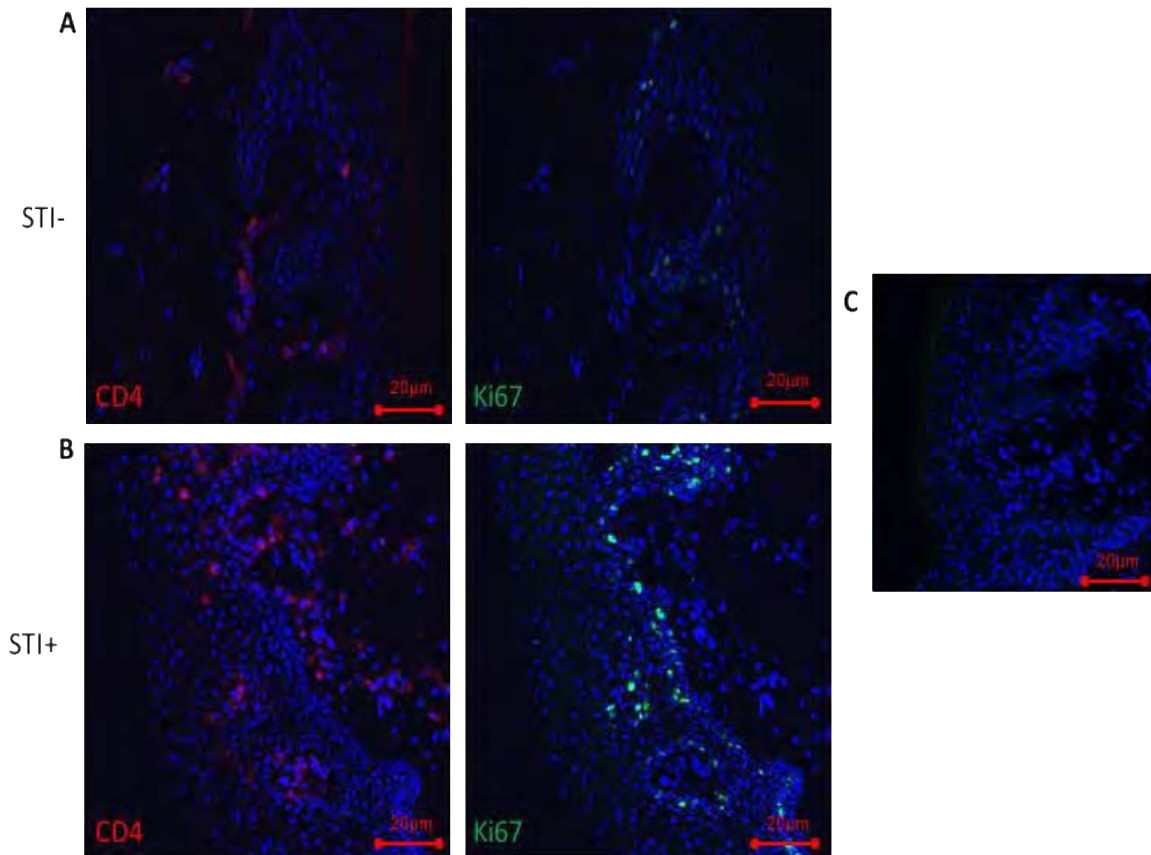


Figure 3.6: Confocal images of a STI negative and positive individual that have been split into CD4 and Ki67. A) STI- images of CD4 (left) and Ki67 (right) B) STI+ images of CD4 (left) and Ki67 (right) C) Image of isotype control.

There were no significant differences in the number of CD4 (4.4 vs 2.7 cells/mm² in inner and outer foreskin; Figure 3.7A) and Ki67 (3.8 vs 3.2 cells/mm² in inner and outer foreskin; Figure 3.7A) cells. We then analysed the CD4 and Ki67 cell densities in context of asymptomatic STI status. We found no significant differences in the number of CD4 cells in the inner (4.9 vs 4.3 cells/mm² in STI+ and STI- men, p=0.5402) and outer (2.2 vs 3.5 cells/mm² in STI+ and STI- men, p=0.9624) foreskin. There were no significant differences in the number of Ki67 cells in the inner (3.5 vs 3.8 cells/mm² in STI+ vs STI- men, p=0.7233) and outer (1.6 vs 3.8 cells/mm² in STI+ vs STI- men, p=0.5394) foreskin. (Figure 3.8B and Figure 3.8B respectively). Although there were no significant differences in the number of CD4 T cells in the inner and outer foreskin, CD4 T cells were present in high numbers in the epithelial tissue indicating that these cells are able to move to non-lymphoid tissue. The Ki67+ cells present in the foreskin are proliferating cells that are not CD4+ T cells as shown by figure 3.9.

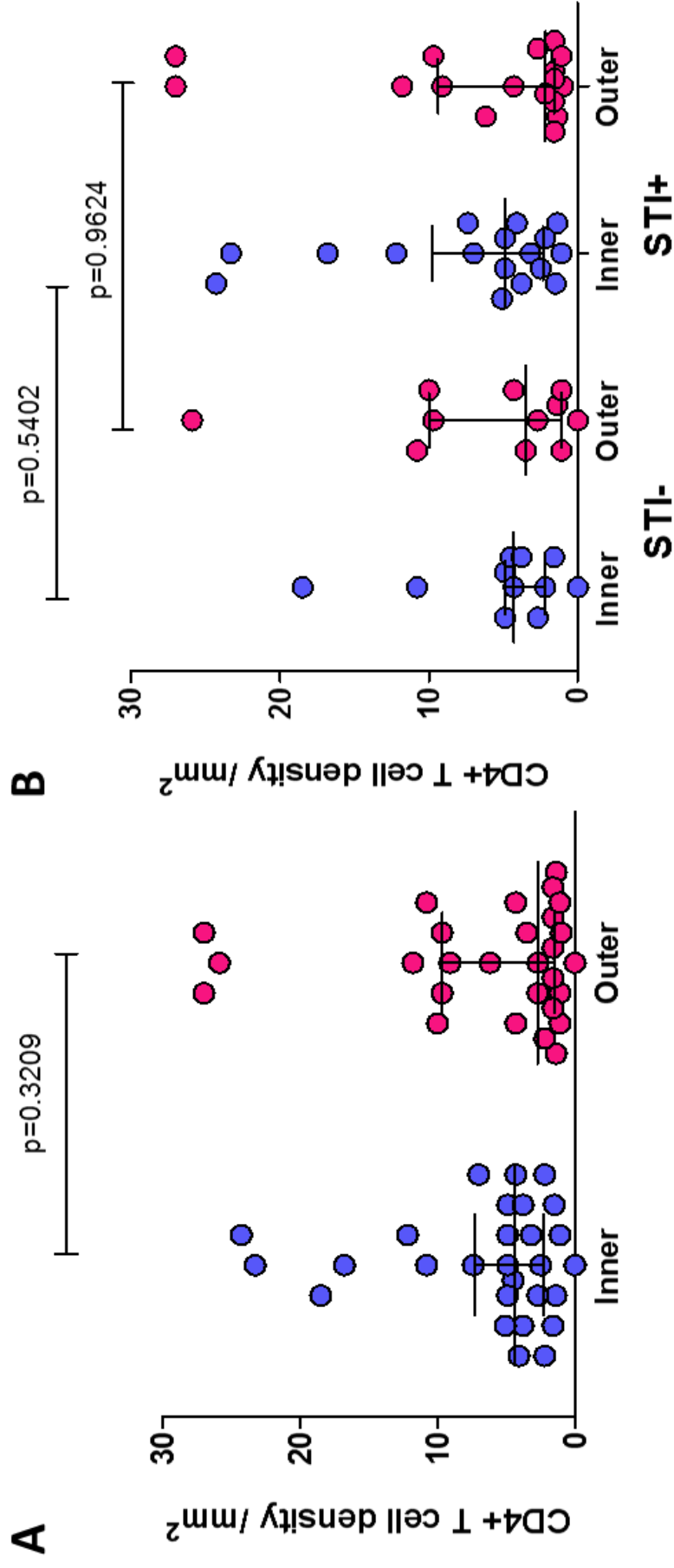


Figure 3.7: CD4+ T cell densities in the inner and outer foreskin of STI- and STI+ individuals. A) CD4+ T cell density in the inner and outer foreskin regardless of STI status (n=28; p=0.3209 [Mann Whitney test]). **B)** CD4+ T cell density in the inner and outer foreskin of STI+ and STI- individuals (n=28; Inner, p=0.5402; Outer, p=0.9624 [Mann Whitney test]).

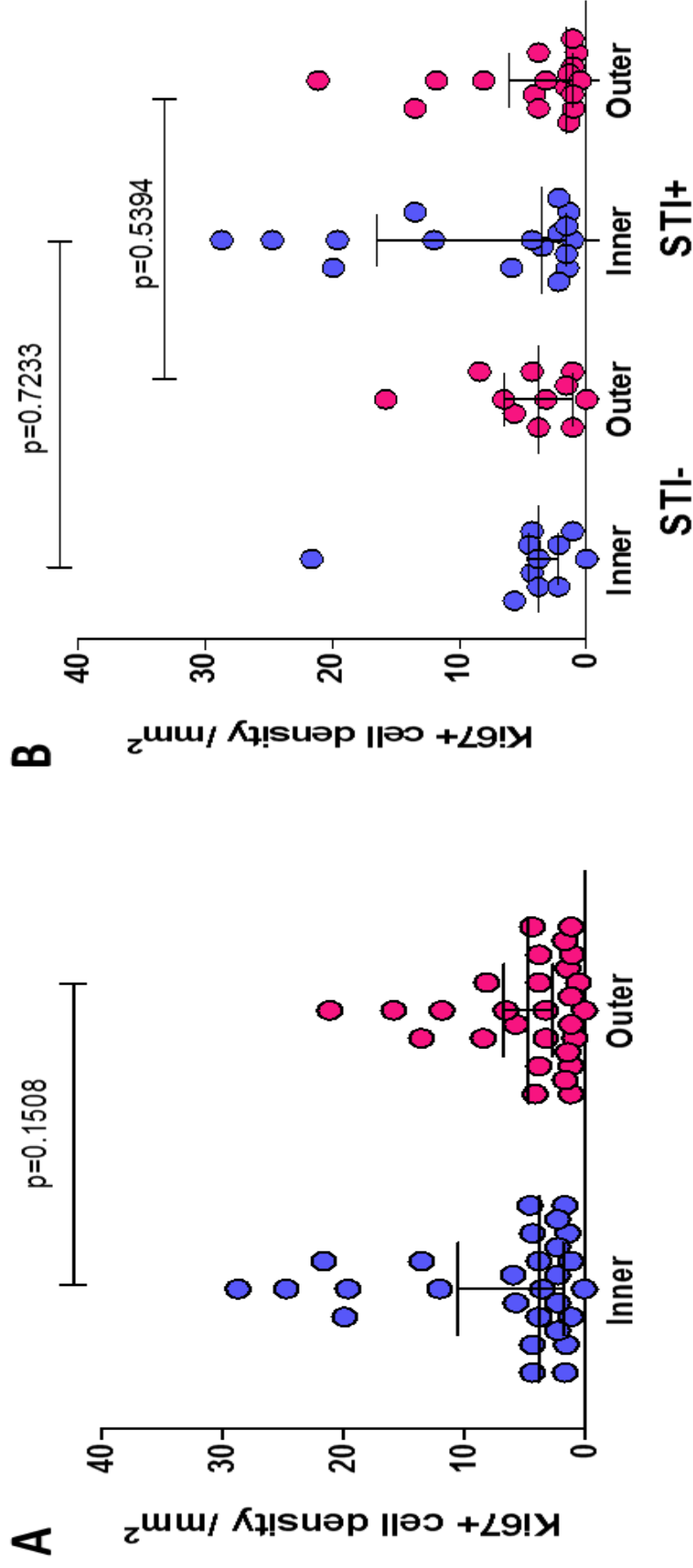


Figure 3.8: Ki67 cell density in the inner and outer foreskin of STI- and STI+ individuals. A) Ki67 cell density in the inner and outer foreskin regardless of STI status (n=28; p=0.1508 [Mann Whitney test]). **B)** Ki67 cell density in the inner and outer foreskin of STI+ and STI- individuals (n=28; Inner, p=0.7233; Outer, p=0.5394 [Mann Whitney test]).

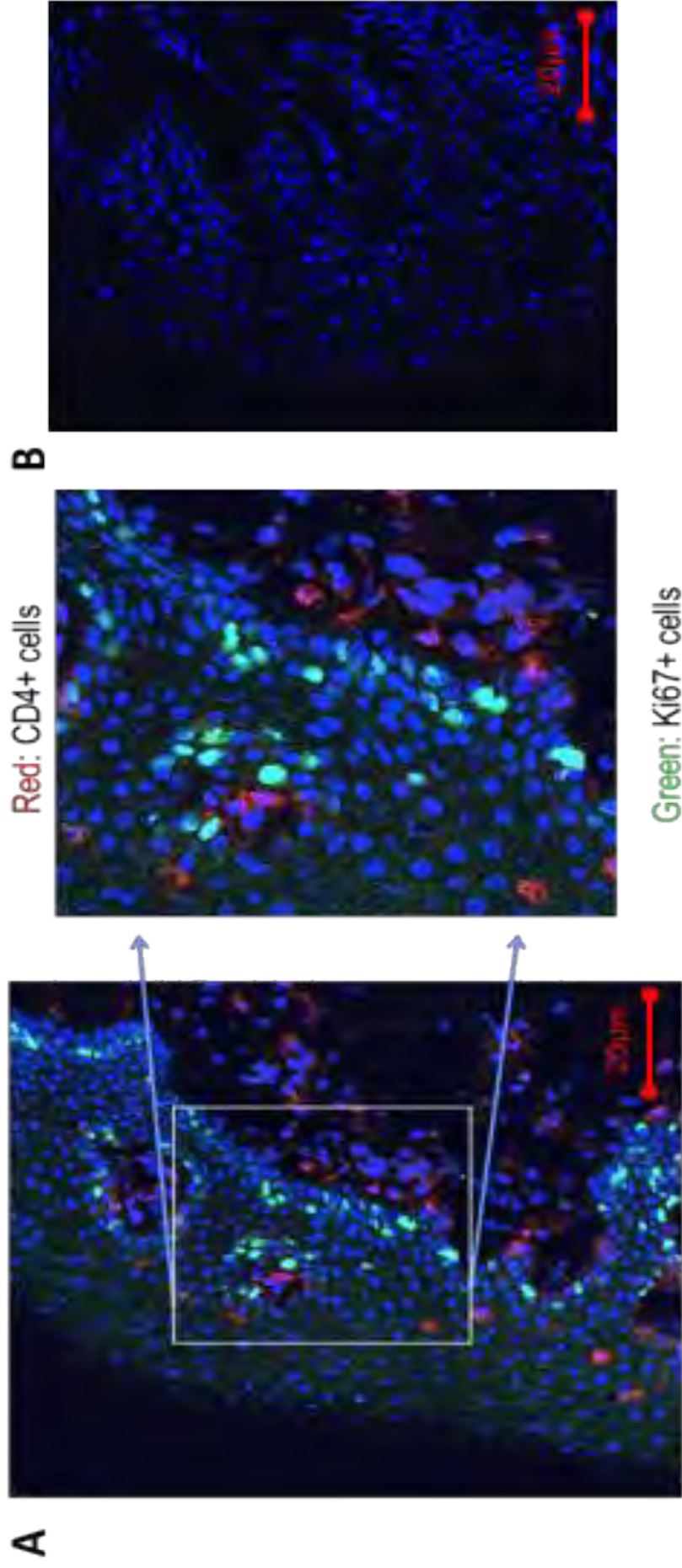


Figure 3.9: Anti-CD4 and anti-Ki67 stain. A) Original image of CD4 and Ki67 stain (left) and a magnified image of a section with CD4 and Ki67 cells (right). **B)** Image of an isotype control

3.6 Distance of Immune cells from epithelium

Immune cells are present in the epidermis and dermis of the foreskin and have the potential to migrate. We therefore quantified the distance of LCs present in the inner and outer foreskin from the epithelium (Figure 3.2).

In this dissertation, we found a significant difference in the distance of LCs from the epithelium (99.19 vs 75.25 μm in the inner and outer foreskin, $p < 0.0001$; Figure 3.10A) with a median difference of 23.922 μm . Next we analysed the distance of LCs in context of STIs and found that there were no significant differences between the inner foreskin of STI+ and STI- individuals. We did however find that median distance of LCs was significantly closer to the epithelium in the outer foreskin (68.19 vs 83.608 μm in STI+ vs STI- men, $p = 0.0385$; Figure 3.10B) with a median difference of 15.418 μm , suggesting that these cells are closer to the epithelium in the outer foreskin of STI+ individuals. The position of these cells could predispose them to a greater risk of infection during an HIV-1 transmission event. Next, we measured the distance of CD4+ cells from the epithelium in the inner and outer foreskin (168.5 μm and 155.1 μm respectively) regardless of STI status and found no significant difference between the inner and outer foreskin (Figure 3.11A). Lastly, we measured the distance of CD4+ cells in the inner and outer foreskin of STI+ and STI- individuals. There were no significant differences in the distance of CD4+ cells from the epithelium in the inner and outer foreskin of STI+ compared to STI- individuals (Figure 3.11B). Although there were no significant differences observed in the distance of CD4+ cells from the epithelium, the CD4+ T cells are further away from the epithelium compared to LCs and are therefore less likely the first immune cells to be infected by an HIV-1 transmission event.

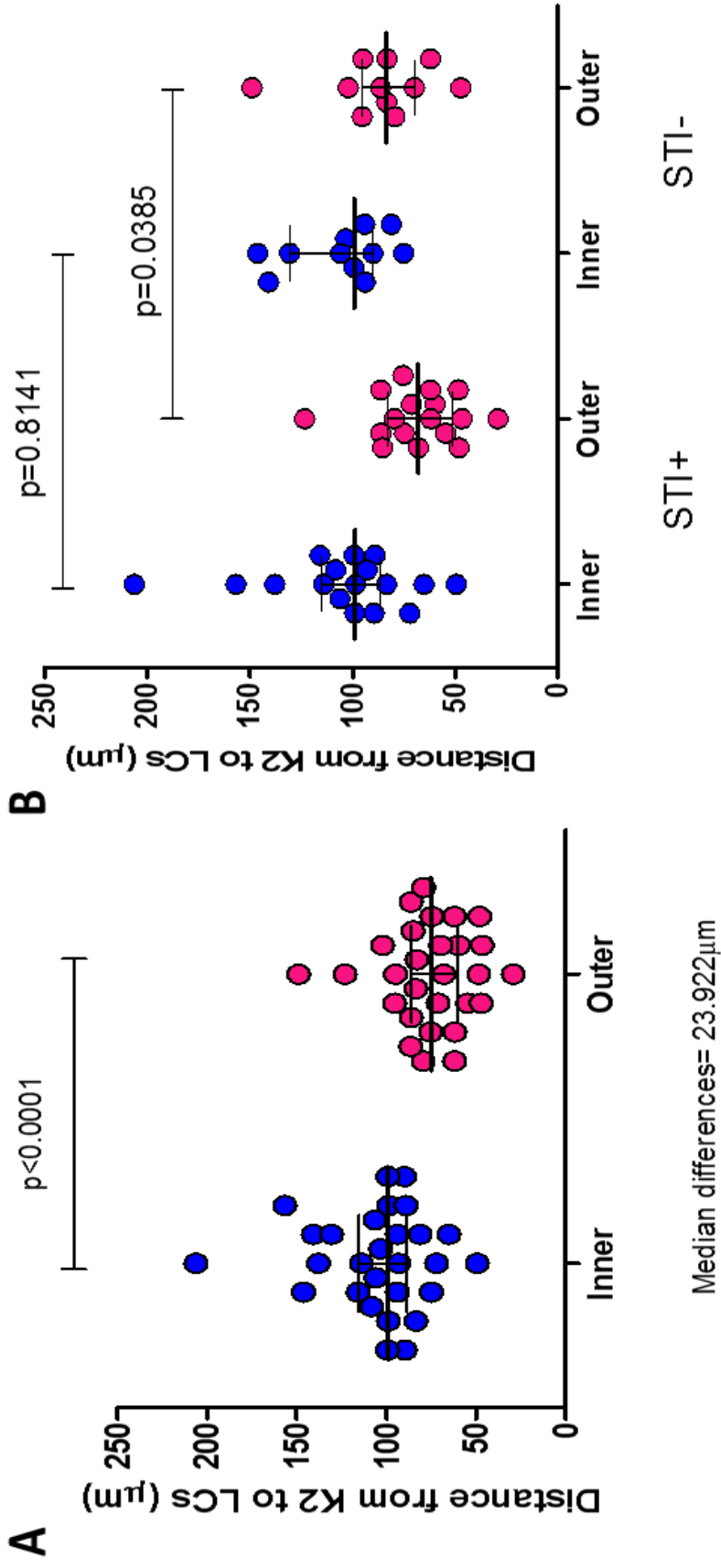


Figure 3.10: Langerhans cell distance from epithelium (K2) of foreskin. A) Distance of LCs from K2 between the inner and outer foreskin regardless of STI status (n=28; $p < 0.0001$ [Mann Whitney test]). **B)** Distance of LCs from K2 between the inner and outer foreskin in STI+ and STI- individuals (n=28; Inner, $p = 0.8141$; Outer, $p = 0.0385$ [Mann Whitney test]).

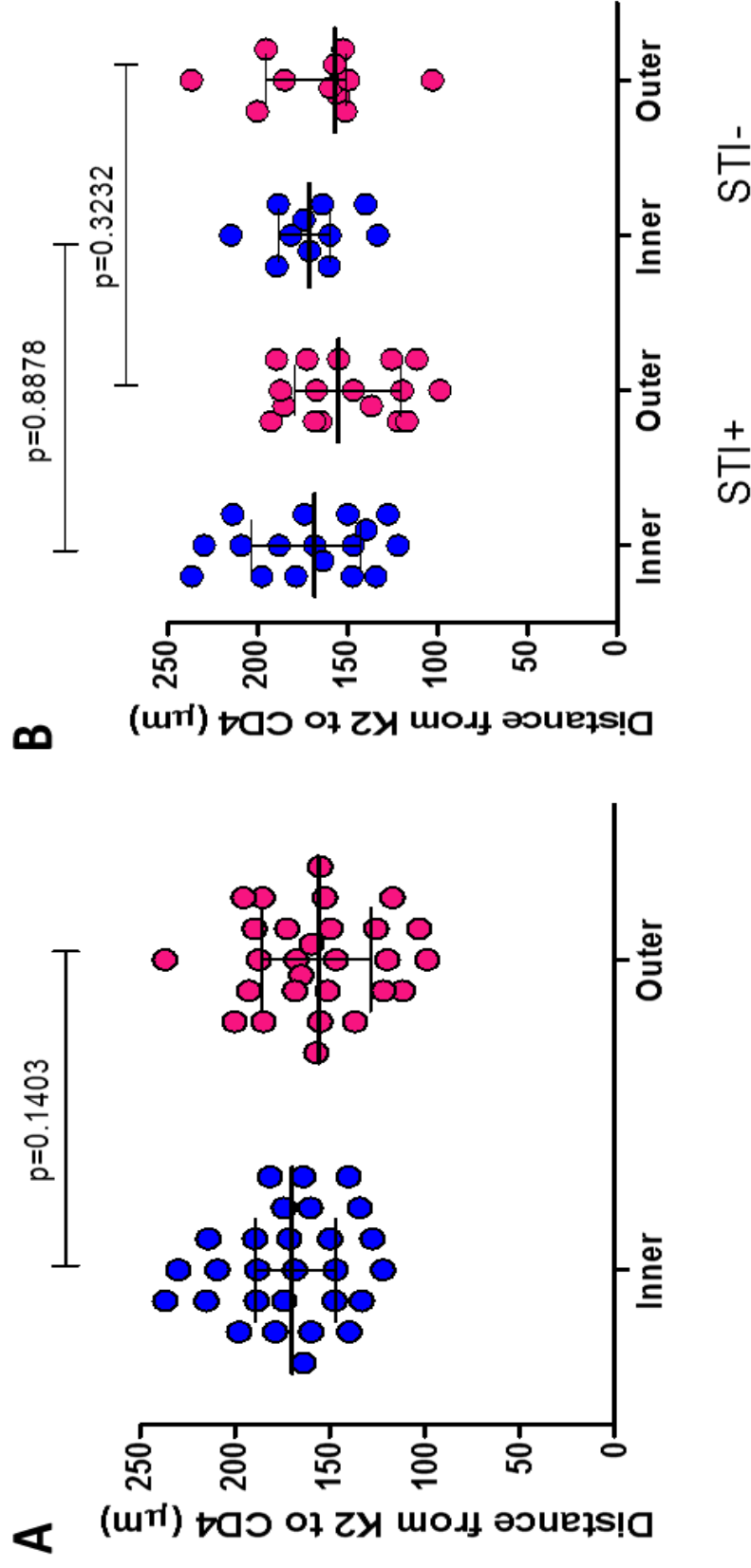


Figure 3.11: CD4 distance from epithelium (K2) of foreskin. A) Distance of CD4 from K2 between the inner and outer foreskin regardless of STI status (n=28; p=0.1403 [Mann Whitney test]). **B)** Distance of CD4 from K2 between the inner and outer foreskin in STI+ and STI- individuals (n=28; Inner, p=0.8878; Outer, p=0.3232 [Mann Whitney test]).

3.7 Discussion

There are up to 80% of adults that are infected with HIV-1 through genital mucosal surface exposure to the virus (UNAIDS 2010). The sexual transmission of HIV-1 in men are acquired mainly through the penis (Boily *et al.* 2009). Direct mucosal disruption leading to a compromised barrier integrity (reviewed by Ward & Rönn 2010) and the thinner keratinised layer of the foreskin is believed to play a role in HIV-1 acquisition as HIV-1 is able to enter the foreskin easily (Ganor *et al.* 2010). Other factors that influence the risk of HIV-1 acquisition such as STIs, especially ulcerative STIs (reviewed by Ward & Rönn 2010) and a larger foreskin surface area (Kigozi *et al.* 2009) may increase the number of target cells present in the foreskin, which in turn may impact HIV-1 acquisition. Furthermore, young people between the ages of 14-24 bear an enormous burden of the HIV-1 pandemic and at least 40-50% of all new HIV-1 infections among adults may occur in the young population (Unaid 2015). Therefore, the aim of this study was to examine the immunological cellular composition and levels of keratinisation in the inner and outer foreskins after medical male circumcision as well as to characterise the number and position of LCs and CD4+ T cells within the inner and outer foreskin in young men between the ages of 14-24.

de Vincenzi & Mertens., proposed that keratinisation of the penis may provide an impermeable barrier to HIV-1, thus making it an unlikely source for primary infection unless it has been compromised by lesions, micro-trauma as well as inflammation. The variability of the keratin thickness between individuals may vary depending on epithelial cell differentiation and regulation of structural protein expression levels which is dependent on both physical and environmental factors (Dinh *et al.* 2012). Mechanical stress such as the act of sexual intercourse may introduce abrasions in the keratin layer and alter keratinisation of the foreskin. Other factors that may impact keratinisation is the type of environment the foreskin is exposed to. The outer foreskin is exposed to an aerobic environment whereas the inner foreskin has an anaerobic environment (O'Farrell *et al.* 2006; Price *et al.* 2010). The results of this dissertation show that the outer foreskin keratin layer was significantly thicker by 1.712 μm than the inner foreskin. These results are consistent with findings by Patterson *et al.*, McCoombe & Short as well as Ganor & Bomsel who also found the keratin layer in the outer foreskin to be thicker than that of the inner foreskin. In contrast, two studies that have found no differences in the thickness of the keratin layer between the inner

and outer foreskin (Dinh *et al.* 2010; Dinh *et al.* 2012). The differences in keratin thickness that are seen by the different study groups could be due to ethnic origin or sexual behaviour. The subpreputial epithelium that covers the inner foreskin comprises of moist epithelia which may make it more susceptible to HIV-1 infection due to the thinner, more permeable keratin layer as seen by the findings reported in this dissertation and findings by recent studies (McCoombe & Short 2006; Patterson *et al.* 2002; Anderson, J. A. Politch, *et al.* 2011), whereas the thicker keratin layer in the outer foreskin may provide a more robust barrier to HIV-1 viral entry compared to the inner foreskin. There are currently no studies that look at the environmental effect on the degree of keratinisation in the inner and outer foreskin. This may be important due to the change of the penile environment upon circumcision and its effects on keratinisation of the glans of the penis. The thicker keratinised layer may provide protection against HIV-1 acquisition, although, the complete removal of the foreskin would eliminate the susceptibility of less keratinised layer as seen in the inner foreskin.

The foreskin has been shown to harbour many target cells for HIV-1 infection. These cells include LCs and CD4+ T lymphocytes which are targets for HIV-1 infection (Hussain & Lehner 1995; Donoval *et al.* 2006; McCoombe & Short 2006; Patterson *et al.* 2002). The results in this study show that there were no significant differences in the number of LCs, CD4+ and Ki67 cells present in the inner and outer foreskin, where the LCs were mostly present in the epithelium of the foreskin, consistent with findings by Donoval *et al.* Although there were no significant differences in the number of LCs and CD4+ T cells in the inner and outer foreskin, these cells play a critical role in HIV-1 infection. CD4+ T cells are known targets of HIV-1 and the presence of these immune cells in the foreskin may elevate the risk of HIV-1 acquisition. (Prodger *et al.* 2012), showed that the proportion of CD4+CCR5+ T cells were up to four times higher in the foreskin compared to the blood. This could potentially enhance susceptibility to HIV-1 infection. LCs have been shown to express low levels of CD4 and are susceptible to infection with HIV-1 (Kawamura *et al.* 2003; Turville *et al.* 2002; Zaitseva *et al.* 1997). The productive infection of LCs may allow the transfer of the virus to CD4+ T cells thus increasing the risk of HIV-1 infection (Sarrami-Forooshani *et al.* 2014; Kawamura *et al.* 2003; de Witte, Nabatov & Geijtenbeek 2007; reviewed by McDonald 2010). Although, a study by de Witte, Nabatov, Pion, *et al.*, in 2007 showed that langerin acts as a natural barrier to HIV-1 transmission in LCs, this was only possible

if langerin was not blocked by an antibody or mannan, suggesting that LCs play a protective role in HIV-1 infection as well as serve as targets for HIV-1 infection. These experiments have been conducted *in vitro* and the relative importance *in vivo* needs to be investigated. In addition, a study by Geijtenbeek *et al.*, shows that DC-SIGN is able to recruit and facilitate HIV-1 infection of CD4+ T cells *in vitro*.

One of the major factors increasing HIV-1 susceptibility is a concomitant infection with an STI. In this study we tested all participants for a panel of 6 asymptomatic STIs (*N. gonorrhoea*, *C. trachomatis*, *T. vaginalis*, *M. genitalium*, HSV-1 and HSV-2) and a subset for HPV infection. This allowed us to investigate our results in the context of asymptomatic STI status. We found that asymptomatic STIs have no significant impact on the degree of keratinisation in the inner or outer foreskin, suggesting that underlying local inflammation caused by STIs may not be playing any role in keratin deposition in the foreskin.

Next, we focused on LCs, CD4+ and Ki67+ cells. In the literature, there are contradictory results regarding the numbers of HIV-1 target cells in the inner and outer foreskin, with some studies reporting more target cells in the inner foreskin (Ganor *et al.* 2010; Patterson *et al.* 2002; Donoval *et al.* 2006; Fischetti *et al.* 2009; Liu *et al.* 2014) while others reporting more cells in the outer foreskin (McCoombe & Short 2006; Qin *et al.* 2009). We found that there was a significant difference in the number of LCs present in the inner and outer foreskin of STI+ compared to STI- individuals, this was similar to findings by Donoval *et al.*, who had observed a higher number of langerhans cell in individuals with an asymptomatic STI compared to those without a STI. We found that LCs were predominantly present in the epidermis of the foreskin. This is not surprising as these are the first immune cells to come into contact with invading pathogens (Patterson *et al.* 2002; Kawamura *et al.* 2005) and LCs have dendrite like appendages that sample the environment for antigen presentation to T cells. This suggests that the presence of an STI may play a role in the number of LCs found in the epidermis of inner compared to the outer foreskin. Furthermore, the type of STI present had no effect on the number of LCs present in the foreskin. We found no significant differences in the number of CD4+ and Ki67 cells present in the inner and outer foreskin of STI+ and STI- individuals similar to findings by Donoval *et al.*, who had also reported no differences in the number of CD4+ T cells regardless of STI status. However, a study by Lemos *et al.*, found that there were a higher number of

CD4+ cells in the inner compared to the outer foreskin, although there was no significant difference. Although we saw no differences in the number of CD4+ T cells, these cells were present in high numbers with clear clumping around vasculature. CD4+ T cells are the primary targets for HIV-1, the presence of these cells at high densities in the foreskin as shown by our study suggests that this may increase the risk of HIV-1 infection in men. The high density and clumping of CD4+ T cells in the dermis that we observed may make them easier targets for productive infection with HIV-1 upon antigen presentation by a LC carrying a HIV-1 virion. The presence of CD4+ T cells in high numbers also suggests that these cells are able to move to non-lymphoid epithelial tissue. This study also shows that the proliferating cells are not CD4+ T cells.

Lastly, we looked at the position of LCs and CD4+ T cells within the foreskin tissues assayed as the position of these cells within the epithelium may make them more susceptible to infection upon HIV-1 entry. The position of these cells may place them at a greater risk of being infected with HIV-1, this also implies that in the presence of a STI, LCs are more concentrated near the epidermis of the outer foreskin. The high numbers of LCs in the epidermis also poses an increased risk of HIV-1 infection. A study by Ganor *et al.*, of an *in vitro* model showed that the inner foreskin was preferentially infected with HIV-1 infected cells. The formation of viral synapses and HIV-1 particle budding was followed by internalisation by LCs which migrated to the dermal layer and formed LC-T cell conjugates where HIV-1 was transferred to T cells. We postulate based on *in vitro* models in two studies that HIV-1 is able to preferentially infect LCs and CCR5 expressing CD4+ T cells in the inner foreskin (Patterson *et al.* 2002) and that R5 specific HIV-1 strains infects foreskin as well as other tissues such as the glans, urethral tissue and meatus equally (Fischetti *et al.* 2009). Although the presence of these cells increases the number of targets for HIV-1, two studies have shown the protective effect of LCs. The study by Sarrami-Forooshani *et al.*, used an *ex vivo* tissue model and showed that LCs transmitted R5 specific HIV-1 and restricted transmission of X4 specific LCs. Taken together, LCs may play an important role in the protection and prevention of transmission of HIV-1 to T cells. This however is limited to a low viral load as a higher viral load at the foreskin may overwhelm the protective function of LCs and lead to infection (Ganor *et al.* 2010). It is therefore important to develop models in which the early events in HIV-1 infection in the male

genital tract are characterised as well as identify the roles as well as susceptibility to infection of CD4⁺ T cells and LCs in the genital tract. In this study, there were no significant difference between the distances of CD4⁺ T cells from the keratin layer to the position they were found within the inner compared to the outer foreskin. We did however find a significant difference in the distances of LCs from the outer keratin layer in the foreskin regardless of STIs. We measured the distances of LCs in context of STI status and found no significant differences between the inner foreskin of STI⁺ and STI⁻ individuals. However, there was a significant difference in the distances of LCs to the outer keratin layer in the outer foreskin of STI⁺ compared with STI⁻ individuals. There was a pronounced significant reduction in the median distance of LCs in the outer foreskin of STI⁺ individuals, suggesting that these cells have moved closer to the epithelium. Our group has shown a positive correlation between CCL27 and LCs (unpublished data), this may explain the recruitment of LCs to the epithelium of the foreskin making them susceptible to HIV-1 infection.

The presence of a STI may enhance the number of HIV-1 target cells present in the foreskin tissue, therefore, this creates a greater risk for HIV-1 acquisition and therefore onward transmission to sexual partners. Co-infection with an asymptomatic STI has been identified as a primary driver of inflammation in the genital tract and is involved in the recruitment of HIV-1 target cells to the foreskin tissue (Prodger *et al.* 2014; Freeman *et al.* 2006) and HIV-1 transmission has been shown to be elevated in circumstances where individuals have an asymptomatic and symptomatic STI infection (reviewed by Vermund 2007; reviewed by Ward & Rönn 2010). Although we saw differences between the control and STI⁺ group, some of the control participants may have had an asymptomatic STI that was not screened for. This may explain some of the high numbers of LCs and CD4⁺ T cells seen in STI⁻ individuals. Fleming & Wasserheit., have shown that STIs, predominantly ulcerative STIs increases the risk of HIV-1 infection. The presence of asymptomatic STIs may be elevating the risk of HIV-1 acquisition in males and this may be a result of recruitment of immune cells due to inflammation in the foreskin, therefore the immune cells present serve as targets for HIV-1 infection. Asymptomatic STIs may be overlooked as the factor driving foreskin inflammation in the MGT and a study by Lemos *et al.*, highlights the risk of inflammation in the foreskin. Lemos *et al.*, show that the inflammatory milieu in the inner foreskin increases the density of CCR5⁺ as well as CD4⁺CCR5⁺ cells in the

epidermis. This event may position the HIV-1 target cells in the epidermis where HIV-1 entry and infection is facilitated. It is clear that infection with an asymptomatic STI may likely increase the risk of HIV-1 acquisition. However, the effects of asymptomatic vs symptomatic STIs on cell numbers need to be investigated in order to determine which of these types of STIs pose a greater risk. CT was the most prevalent STI amongst the STI+ group with many individuals positive for CT across all age groups in addition to other STIs and CT is representative of the most common bacterial STI globally (Cook *et al.* 2005). Immunologically, CT increases HIV-1 shedding in the genital tract due to the recruitment of HIV-1 target cells such as CD4+ T cell due to the inflammatory response against CT (Fleming & Wasserheit 1999; Buckner *et al.* 2016), thereby increasing the chance of HIV-1 infection in CT+ individuals. HPV is the most common sexually transmitted virus and it is most prevalent amongst young individuals after their sexual debut (Burchell *et al.* 2006; Dunne *et al.* 2006). In our cohort, there were a total of 7 in the STI+ and STI- group that tested positive for HPV. HPV has been shown to increase the risk of HIV-1 acquisition in men that have a recently cleared or persistent HPV infection (Rositch *et al.* 2014). Rositch *et al.*, showed that the immune-regulatory mechanism that is associated with HPV clearance may also facilitate HIV-1 acquisition. This suggests that even though a man is being treated for the HPV infection, during this time he is still at an increased risk of HIV-1 acquisition. This study suggests that STI status may play a vital role in the number of HIV-1 target cells present in the foreskin, furthermore circumcision has been shown to protect against HPV by up to 35% (Tobian *et al.* 2009; Tobian *et al.* 2010).

In summary, these results show that the outer foreskin is more keratinised than the inner foreskin and asymptomatic STIs may play a role in elevating the risk of HIV-1 acquisition by increasing the number of HIV-1 target cells present in the inner and outer foreskin.

CHAPTER 4: DISCUSSION AND CONCLUSION

Circumcision has been shown to decrease HIV-1 infection by up to 60% and this is possibly due to the removal of potential target cells as well as an inflamed environment that recruits possible HIV-1 target cells to the foreskin surface yet, globally, there are 70% of the men that remain uncircumcised (WHO & Unaid 2007). In areas of South Africa like Kwa-Zulu Natal, there is a misconception about the protective effect of circumcision and communities may not have a scientific understanding of this and would therefore engage in increased risky sexual behaviour (Milford *et al.* 2016). In mainly the Eastern Cape of South Africa, there are currently, 52.2% of males that undergo traditional circumcision while 51.6% of males aged 14-24 undergo medical male circumcision (Shisana, O, Rehle, T, Simbayi LC, Zuma, K, Jooste, S, Zungu N, Labadarios, D, Onoya 2014). Circumcision that occurs in non-clinical setting can result in the death of an individual (Peltzer *et al.* 2008). Furthermore, traditional circumcision does not fully remove the foreskin and there is need for re-circumcision at a medical facility which could result in the excessive removal of skin and deepened wounds (Wilcken *et al.* 2010). A study by Hoffman *et al.*, in South Africa found that 26% of individuals circumcised for improvement in hygiene and health benefits, 37% for cultural or religious reasons, 11% were children circumcised due to parents' decision and 26% did not provide any reason for circumcision. There were 71.8% of men that knew of at least one health benefit of circumcision while 28.2% knew of none, although only had any knowledge of the reduced risk in HIV-1 (Hoffman *et al.* 2015). South Africa has reached a total of 1.8 million MMCs by 2014 and the continued scale up will help achieve the 80% target of 4.3 million MMCs by 2016 (WHO 2015). Circumcision has also been shown to reduce the chances of acquiring STIs which in turn reduces the chances of HIV-1 acquisitions. WHO and UNAIDS have already called for the scale up of circumcision as a preventative measure against HIV-1 in men. Circumcision has also been shown to have protective effects for female partners, such as reduced risk of cervical cancer, decrease in HPV infection, BV and TV in female partners (Tobian *et al.* 2010). If there is a 60% protection against HIV-1 acquisition conferred through circumcision, conversely, it means it is not effective in 40% of men. There has been speculation that the route of HIV-1 entry may be through the epithelium of glans and the urethral opening in the penis. It is difficult to obtain fresh samples of these areas in order to study these tissues, however more research into these areas needed to determine the possible HIV-1 target cells present and their role in HIV-1 acquisition. There is not much knowledge of STI/HIV-1 acquisition and transmission in

adolescents, therefore circumcision in adolescents aged 14-24 is of utmost importance as this particular population of boys and young men are at a higher risk of a STI due to their sexual activities, this in turn may elevate their risk of HIV-1 acquisition and transmission. A study profiling the humoral response in the penis demonstrated that local and systemic antibodies contribute to the foreskin immune response and that there was selective antibody isotype penetration to the foreskin epidermis, particularly at the inner foreskin (Lemos *et al.* 2015). These findings may contribute to vaccine studies that have a protective effect against STIs (Lemos *et al.* 2015). Discussing sexual intercourse is to a certain degree a taboo topic amongst many societies in southern Africa, the practice of circumcision and knowledge about STIs and HIV-1 may impact safer sexual practices. Circumcision in this group of young men can decrease the risk of contracting and transferring STIs as well as provide a protective effect against the acquisition of HIV-1, thereby lowering the prevalence of HIV-1 in this vulnerable group of young men.

This dissertation has examined some immune properties of the foreskin from young males aged 14-24 years who elected to undergo medical male circumcision. It is known that boys in this group demonstrate biological and psychosocial changes such as puberty and social development that take place and will affect the lives of these adolescents and signifies the move from childhood to adulthood (UNAIDS/UNICEF 2015). AIDS is the leading cause of death among adolescents in Africa with 120 000 deaths in adolescents in 2013 and the second most common cause of death among adolescents around the world (UNAIDS/UNICEF 2015). This is also the age of many adolescents' sexual debut and engagement in risky sexual behaviour such as multiple sexual partners, sexual intercourse while under the influence of drugs or alcohol and having unprotected sex (Shisana, O, Rehle, T, Simbayi LC, Zuma, K, Jooste, S, Zungu N, Labadarios, D, Onoya 2014). There are currently only 24.3% in this age group that have knowledge about HIV-1 transmission and prevention in South Africa (Shisana, O, Rehle, T, Simbayi LC, Zuma, K, Jooste, S, Zungu N, Labadarios, D, Onoya 2014). The aim of this thesis was to optimise the techniques used to determine the type of immune cells present in the foreskin by flow cytometry and immunofluorescence so that the impact of an asymptomatic STI on the numbers of immune cells present in the inner and outer foreskin could be investigated.

In Chapter 2, as part of method development, we optimised a protocol to isolate viable cells from the foreskin of men. We found that the use of fresh tissue with collagenase A at a concentration of 500U/ml to digest the foreskin tissue and the use of scissors to macerate the foreskin tissue was best suited to liberate and isolate viable cells. A major limitation to the optimisation was the limited availability of resources such as the medimachine which was brought to our lab as a demo and the low viability of cells retrieved from frozen samples. It was therefore not possible to repeat experiments in triplicate as there was limited time in which to use the machine. Flow cytometry analysis on all the samples was not possible due to the low amount of viable cells isolated from foreskin tissue in stored samples. Furthermore, flow cytometry analysis was limited due to lack of fresh foreskin samples available after optimisation of the foreskin cell isolation method. Therefore, an accurate comparison using fresh compared to frozen tissue to isolate viable cells from the foreskin tissue was not possible. For future considerations, these experiments should be performed in triplicate on fresh samples received in the lab. A portion from the same fresh sample should be frozen and stored for a week before applying the same protocol to those samples.

There were a few other limitations to this study, not all possible STIs were tested for and a subset of the cohort was tested for HPV. The effects of HPV alone could not be examined as the majority of the individuals with an HPV infection also had a STI. Furthermore, there were low DNA yields from swabs, therefore these samples were not tested for HPV and lastly, it was not feasible to test for HPV in all of the individuals. Individuals with other asymptomatic STIs such as *syphilis*, *hepatitis A*, *B* and *C* may have had an influence on the number of HIV-1 target cells present in the foreskin. Foreskins from the EDH site arrived within 24 hours of circumcision, the delay in processing of the foreskin sample may have had potential effects such a degradation of enzymes and change in genes (Grizzle *et al.* 2010). We did not investigate comparisons in transportation time, however, we were aware that any optimisation of Flow cytometric methodology should be assessed using the 2 h samples from the Johannesburg site. For the IHC results, all data were generated using the 24 h samples from the Edendale site. Furthermore, in order to determine the effects of collagenase digestion on cytokine profile, it would be best to compare the various tissue digestion methods and thereafter look at the cytokine profiles. Staining of

images using IHC was limited to two colours as we were only able to use the red and blue lasers. Optimisation for CCR5 was only possible using cell lines (M4T, GXR) and a few tissue samples from participants. Furthermore, due to time constraints and CCR5 antibody availability, CCR5 staining was not extended to the subset of participants. The staining is currently being carried out by collaborators at Northwestern University in Chicago. IHC staining was not done blinded, i.e. tissue site (inner/outer) and participant status (STI+/-) was known and this could have introduced bias. The sample size may not be indicative of the prevalence of STIs in this community. Although an average of five images were taken per tissue at widely distributed positions per tissue section, this is not necessarily representative of the total size of the inner or outer foreskin. Each image taken had varying number of immune cells present in each tissue section, therefore imaging of the entire foreskin may reveal greater numbers of HIV-1 target cells in the foreskin. The drawing of the lines used to measure keratin thickness was by hand and not computer generated, regardless, human error cannot account for the median difference of 1.712 μ m found between the inner and outer foreskin.

For future considerations, we should compare the tissue from the glans and urethra of a circumcised penis by use of tissue biopsies of the foreskin tissue to determine whether the foreskin truly enhances susceptibility to STIs and HIV-1 infection. The use of STI and HIV-1 negative tissue samples from individuals from these regions can be used and infected with different types of STIs and HIV-1 using a model to culture the different types of tissue using a transwell method similar to that by Ganor *et al.*, The amount and type of immune cells infected can then be quantified in each type of tissue to determine which type of tissue is more susceptible to infection with a STI and HIV-1. Next, investigate the tissues with different types of STIs (that was previously infected) by infecting them with HIV-1 using the same model in order to determine the impact of HIV-1 acquisition in the presence of STIs by quantifying the amount of immune cells infected with HIV-1. This would help evaluate the effect of STIs and HIV-1 on the number of cells infected, effect of different STIs on HIV-1 acquisition, the role of the keratin layer in the inner and outer foreskin and whether the glans, foreskin or urethral tissue is more susceptible to infection. Furthermore, the characterisation of different subsets of potential target cells in the different types of tissue should be evaluated and quantified in order to determine if an increased number of target cells

increases infectivity. However, this method would be difficult as fresh tissue samples from the glans and urethra of the penis would be difficult to obtain. Samples should be transported to the lab within 4 hours of circumcision and all experiments should be conducted on fresh tissue as far as possible. The three randomised control trials that were conducted in SA showed circumcision reduced HIV-1 acquisition in heterosexual males regardless of surgical procedure (Tobian *et al.* 2010), although MMC methods should be standardised in studies as well sites where the study is being conducted.

A bigger sample number and complete screening for asymptomatic STIs should be included when recruiting patients. A study conducted in the Gauteng province in South Africa on the quality of STI case management services found that general knowledge about STIs was sub-optimal among providers without STI training (Ham *et al.* 2016). The findings by Ham *et al* indicated that the responses about STI case management did not meet standards by WHO and South African Department of Health (SADH). This highlights the need for additional STI education and training in this department. Lastly, although screening for asymptomatic STIs is costly, it cannot be emphasized enough that South Africa revise their current treatment guidelines for STIs and include treatment of asymptomatic STIs in their syndromic STI management programmes.

In conclusion, this study has contributed to research on the MGT in South Africa. Further optimisation of the foreskin cell isolation method is required in order to isolate a higher percentage of viable cells from the foreskin. The flow cytometry panel can then be expanded in order to identify the different types of immune cells that are present in the foreskin. The use of immunohistochemistry to determine the density and position of HIV-1 target cells in this study may be used to identify possible mechanisms of HIV-1 entry and infection in the foreskin. Triple stains using immunohistochemistry and imaging of entire sections of foreskin tissue at a time would allow for better quantification of the different types and position of immune cells present in the foreskin. Lastly, although this study did not test for all possible STIs, the treatment of asymptomatic STIs is needed as this study has shown the increased number of HIV-1 target cells in STI+ compared to STI- participants. L. F. Johnson *et al.* 2011., estimated that more than 50% heterosexually transmitted HIV-1 in the early stages of the South African pandemic is owed to STIs. The syndromic management of STIs in South Africa has reduced the STI prevalence in both men and women (Johnson *et al.* 2011), however, there is no treatment plan in place to treat asymptomatic STIs which

may be driving inflammation and recruitment of cells to the foreskin, thereby increasing the chance of HIV-1 acquisition.

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APPENDIX

Table S1: IDL algorithm and cell density formula applied to analyse images for immunohistochemistry

Parameter	Algorithm/Formula applied
CD207Filaggrin	tissue_measure, '*Maximumintensityprojection*.lsm', n=3, i=[1,1,0], d=[[1,0],[0,1],[2,0]], h=1, l=['K1', 'K2', 'LC'], ver=1
CD4Ki67	tissue_measure, '*Maximumintensityprojection*.lsm', n=3, i=[1,0,0], d=[[1,0],[2,0]], h=1, l=['K', 'Ki67', 'CD4'], ver=1
Cell Density	*N _c /A; A= N _i x l ₁ x l ₂ x p ²

* N_c = number of cells; A = Area Surveyed; N_i = number of images; l₁ = y pixel size; l₂ = x pixel size; p² = pixel size in μm squared

Table S2: List of reagents used in the laboratory

Reagent	Composition	Supplier	Details
1 x RPMI-1640 Medium (RPMI)	200mM L-glutamine, indicator	Sigma Aldrich	500ml- Cat. No.: R8758
1 x Phosphate buffered Saline (PBS)	0.138M NaCl, 0.0027M KCl (pH 7.2)	Sigma Aldrich	500ml-Cat. No. D8537
Bovine Serum Albumin (BSA)	-	Santa Cruz Biotechnology	100g-Cat. No. sc-2323
BD Facs lysing solution	-	BD Bioscience	Cat. No. 349202
Dimethyl Sulfoxide (DMSO)	-	Sigma-Aldrich	Cat. No. D2650
Biochrom AG Fetal Bovine Serum (FBS)	-	Scientific Group	100ml- Cat. No.:S0613
Ficoll- Histopaque®	-	Sigma-Aldrich	500ml-Cat. No. 10771
Collagenase solution	1g Life collagenase + 56ml RPMI	*	
Cytofix/cytoperm	10% Facs lysing solution + 90% RO water	*	
Cell fix solution	1% cell fix + 99% RO water	*	
Permwash solution	10% permwash + 90% PBS	*	
R10	90% RPMI + 10% FBS	*	
R15	85% RPMI + 15% FBS	*	

Table S2: List of reagents used in the laboratory continued

Reagent	Composition	Supplier	Details
1% wash buffer	1% FBS + 99% PBS	*	
Freezing media	20% DMSO + 80% FBS	*	
2% BSA	2% BSA + 98% PBS	*	

*Mixed in the laboratory