EVALUATING CLINICAL AND IMMUNOLOGIC CORRELATES OF HIV AT MUCOSAL SITES

By

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ABSTRACT

HIV infects over 33 million people worldwide with a new infection occurring every 9 seconds. Sex is the primary mode of transmission and the majority of new infections occur during unprotected sexual contact between an HIV-infected individual and an uninfected sexual partner(s) since HIV infected individuals tend to shed virus in their genital secretions. The infectiousness of an individual is closely tied to the amount of virus in blood, which is closely associated with HIV levels shed in semen or vaginal fluid or rectal secretions. Although, Highly Active Antiretroviral Therapy (HAART) is associated with complete suppression of HIV RNA in blood to undetectable levels, the impact of HAART on semen HIV RNA levels is less clear.

I evaluated the correlation between systemic and mucosal HIV-specific CD8+ T cell immune responses and HIV RNA levels in blood and semen. Overall, there was a strong positive correlation between HIV RNA levels in blood and semen. Neither systemic nor mucosal (in semen) HIV-specific CD8+ responses were associated with HIV RNA levels in blood or semen, in fact CD8+ T cell immune responses in semen correlated with increased HIV RNA levels in semen. Furthermore, inflammatory cytokines (IL-6, and IL-8) CMV levels in semen were associated with increased semen HIV RNA shedding. HAART initiation was associated with complete suppression of HIV viremia, but a significant proportion of individuals on suppressive HAART continue to
shed HIV RNA in semen even after 6 months, and this isolated virus was infectious and often present at high levels (> 5000 copies/mL). Nevertheless, long-term HAART was associated with complete immune reconstitution of CD4+ T cells in the sigmoid colon of HIV-infected individuals on long-term therapy.

These findings demonstrate that neither systemic nor mucosal HIV-specific CD8+ responses, when assayed with IFN-γ production as an endpoint, were associated with reduced HIV RNA levels in blood or semen. Semen HIV RNA levels did correlate with local inflammatory cytokines and CMV reactivation. Furthermore, despite effective HAART a significant proportion of HIV-infected men continued to shed HIV RNA in semen. However, long-term completely suppressive HAART was associated with complete immune reconstitution of the sigmoid colon.
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LIST OF ABBREVIATIONS

3TC – Lamivudine
ABC – Abacavir Sulfate
ACD – Acid Citrate Dextran
AGM – African Green Monkey
AIDS – Acquired Immunodeficiency Syndrome
ANOVA – Analysis of variance
AP – Alkaline Phosphatase
APC – Allophycocyanin
ATZ – Atazanavir
AZT – Zidovudine
bDNA – Branched Deoxyribose Nucleic Acid Assay
CA – Capsid Gag protein
CBA – Cytometric Bead Array
CCR5 – CC Chemokine Receptor 5
CD – Cluster of Differentiation
CFSE – Carboxyfluorescein Succinimidyl Ester
CIRC – Canadian Immunodeficiency Research Collaborative
CMV – Cytomegalovirus
CRP – C-reactive proteins
C. trachomatis – Chlamydia trachomatis
CTL – Cytotoxic T cell
CXCR4 – CXC Chemokine Receptor 4
DC – Dendritic cell
DC-SIGN – Dendritic cell Specific Intracellular Adhesion Molecule-3 grabbing Non-integrin
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribose Nucleic acid
EFV – Efavirenz
EGRF – Epidermal Growth Factor Receptor
ELISA – Enzyme Linked Immunosorbent Assay
ELISPOT – Enzyme Linked Immunospot Assay
ENV – HIV envelope glycoprotein
ER – Endoplasmic Recticulum
FACS – Flow Cytometry
FBS – Fetal Bovine Serum
FCS – Fetal Calf Serum
FITC – Fluorescein isothiocynate
FTC – Emtricitabine
GAG – Group Specific Antigen
GALT – Gut Associated Lymphoid Tissue
CMV gB – CMV Glycoprotein B
CMV gM – CMV Glycoprotein M
GP160 – Envelope Glycoprotein 160
GP120 – Envelope Glycoprotein 120
GP41 – Envelope Glycoprotein 41
Group M – Major HIV strain
Group O – Outlier HIV strain
GUD – Genital Ulcer Disease
HAART – Highly Active Antiretroviral Therapy
HEPS – Highly Exposed Persistently Sero-negative
HHV-5 – Human Herpes Virus -5
HIV – Human Immunodeficiency Virus-1
HIV-2 – Human Immunodeficiency Virus -2
HLA – Human Leukocye Antigen
HSGP – Heparin Sulfate Glycoprotein
HSV-2 – Herpes Simplex Virus type2
IEL – Intestinal Epithelial Lymphocyte
IgG – Immunoglobulin G
IL – Interleukin
IE-1 – Immediate Early protein-1
IFN-γ – Interferon-gamma
IP-10 – Interferon Inducible Protein-10
KIR – Killer Immunoglobulin Receptors
LPV – Lopinovir
LTNP – Long Term Non-Progressor
M cells – Micro-fold cells
MA – Matrix Gag protein or p17
MALT – Mucosa Associated Lymphoid Tissue
MBL – Mannose Binding lectin
MCP – Macrophage Chemotactic Protein
MHC – Major Histocompatibility Complex
MHR – Major Homology Regions in the Gag Capsid protein
MIP-1β – Macrophage Inflammatory Protein 1-beta
MLMC – Maple Leaf Medical Clinic
MSM – Men who have Sex with Men
NEF – HIV Negative Factor
NES – Nuclear Export Sequence
*N. gonorrhea – Neisseria gonorrhea*
NK cell – Natural Killer cells
NLS – Nuclear Localization Signal
NRTI – Nucleoside Reverse Transcriptase Inhibitor
NNRTI – Non-Nucleoside Reverse Transcriptase Inhibitor
NVP - Nevirapine
p24 – Gag Capsid protein
p17 – Gag Matrix protein
p7 – Gag Nucleocapsid protein
PAMP – Pathogen Associated Molecular Pattern
PBMC – Peripheral Blood Mononuclear Cells
PCR – Polymerase Chain Reaction
PE – Phycoerythrin
PerCP – Perdinin Chrolophyll Protein
PHA - Phytohemagluttin
PI – Protease Inhibitor
PIC – Pre-integration Complex
POL – HIV Polymerase gene
PR – HIV Protease
PRR – Pattern Recognition Receptors
RRE – Rev Responsive Element
PP-65 – CMV Polyprotein-65
RANTES – Regulated Upon Expression Normally T cell Expressed and Secretes
REV – Regulator of Expression of Viral proteins
RPMI 1640 – Roswell Park Memorial Institute media
RRE – Rev Response Element
RNA – Ribonucleic Acid
RTC – Reverse Transcription Complex
RTV - Ritonovir
SAP – Serum amyloid protein
SEB – Staphylococcus Enterotoxin - B
SMC – Seminal Mononuclear Cells
SFU – Spot Forming Unit
SIV – Simian Immunodeficiency Virus
SLPI – Secretory Leukocyte Protease Inhibitor
SM – Sooty Mangabey
STI – Sexually Transmitted Infection
SQV – Saquinar
SVL – Semen Plasma Viral Load
TAR – Trans-activating Response element
TAT – Transactivator of Transcription
TBP – TAT Binding Protein
TCR – T-cell Receptor
TDF – Tenofovir
TGN – Trans-Golgi Network
TIP-47 – Tail Interacting Protein – 47
TLR – Toll-like receptor
TML – Toronto Medical Laboratories

TNF-α – Tumor necrosis factor – alpha

*T. palladum* – *Treponema palladum*

T Reg – T Regulatory Cells

UL – Unique Long Sequence

US – Unique Short Sequence

UNAIDS – Joint United Nations Program on HIV/AIDS

VDRL – Venereal Disease Research Laboratory test

VIF – Viral Infectivity Factor

VPR – Viral Protein R

VPU – Viral Protein U

WHO – World Health Organization
TABLE OF CONTENTS

ABSTRACT .............................................................................................................................. II

ACKNOWLEDGEMENTS ........................................................................................................ IV

LIST OF ABBREVIATIONS ................................................................................................... VI

TABLE OF CONTENTS .......................................................................................................... XII

TABLE OF FIGURES .............................................................................................................. XIX

MANUSCRIPTS ARISING FROM THIS THESIS .................................................................... XXI

THESIS OVERVIEW ................................................................................................................ XXIII

Chapter 1 ................................................................................................................................ xxiii

Chapter 2 ................................................................................................................................ xxiii

Chapter 3: ............................................................................................................................... xxiv

Chapter 4: ............................................................................................................................... xxv

Chapter 5 ................................................................................................................................ xxv

Chapter 6: ................................................................................................................................ xxvi

Chapter 7: ................................................................................................................................ xxvi

CHAPTER 1: INTRODUCTION .............................................................................................. 1

1.1 – HIV AROUND THE WORLD ......................................................................................... 2

1.2 – PROPOSED ORIGINS OF THE HUMAN IMMUNODEFICIENCY VIRUS (HIV) ............... 4

1.3 – THE HIV VIRAL LIFE CYCLE ...................................................................................... 6

1.3.1 – The HIV virus ......................................................................................................... 6

1.3.2 – HIV viral attachment and cellular entry .................................................................. 8

1.3.3 – Nuclear import and HIV integration ....................................................................... 9
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3.4 – HIV transcription and nuclear transportation</td>
<td>11</td>
</tr>
<tr>
<td>1.3.5 – Virion assembly</td>
<td>15</td>
</tr>
<tr>
<td>1.3.6 – Virion Release and Maturation</td>
<td>17</td>
</tr>
<tr>
<td>1.4 – NATURAL HISTORY OF HIV INFECTION</td>
<td>17</td>
</tr>
<tr>
<td>1.4.1 – Clinical manifestations of HIV infection</td>
<td>19</td>
</tr>
<tr>
<td>1.4.1.1 – Acute infection:</td>
<td>19</td>
</tr>
<tr>
<td>1.4.1.2 – Chronic or Latent HIV Infection</td>
<td>19</td>
</tr>
<tr>
<td>1.4.1.3 – Clinical AIDS</td>
<td>20</td>
</tr>
<tr>
<td>1.4.2 – Immunologic events following HIV infection</td>
<td>21</td>
</tr>
<tr>
<td>1.4.2.1 – Acute infection</td>
<td>21</td>
</tr>
<tr>
<td>1.4.2.2 – The GALT and acute infection.</td>
<td>21</td>
</tr>
<tr>
<td>1.4.2.3 – All roads lead to the GALT</td>
<td>23</td>
</tr>
<tr>
<td>1.4.4 – GALT depletion and Immune activation in HIV and SIV</td>
<td>24</td>
</tr>
<tr>
<td>1.4.5 – Immune activation: insights from the natural host of SIV</td>
<td>25</td>
</tr>
<tr>
<td>1.5 – UNDERSTANDING HIV TRANSMISSION</td>
<td>27</td>
</tr>
<tr>
<td>1.6 – HIV ACQUISITION AND THE GENITAL MUCOSA</td>
<td>31</td>
</tr>
<tr>
<td>1.6.1 – The Female Genital Tract</td>
<td>32</td>
</tr>
<tr>
<td>1.6.2 – The Male Genital Tract (MGT)</td>
<td>36</td>
</tr>
<tr>
<td>1.6.3 – The Rectal Mucosa</td>
<td>37</td>
</tr>
<tr>
<td>1.7 – HIV RNA SHEDDING IN GENITAL SECRETIONS</td>
<td>39</td>
</tr>
<tr>
<td>1.7.1 – Source of HIV RNA shed in Genital secretions</td>
<td>39</td>
</tr>
<tr>
<td>1.7.2 – HIV shedding and stage of HIV Infection</td>
<td>40</td>
</tr>
<tr>
<td>1.7.3 – Sexually Transmitted Infections and HIV</td>
<td>43</td>
</tr>
<tr>
<td>1.7.4 – Sexually transmitted infections and HIV acquisition</td>
<td>45</td>
</tr>
<tr>
<td>1.7.5 – Viral synergy: CMV and HIV</td>
<td>47</td>
</tr>
<tr>
<td>1.7.5.1 – Cytomegalovirus (CMV) – viral and host receptors</td>
<td>48</td>
</tr>
</tbody>
</table>
1.7.5.2 – CMV infection and latency ................................................................. 49
1.7.5.3 – Evidence for CMV and HIV synergy .................................................... 50
1.8 – HIGHLY ACTIVE ANTIRETROVIRAL THERAPY ........................................... 51
  1.8.1 – Major drug classes in HAART ............................................................... 53
    1.8.1.1 – Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTI/NtRTI) ........... 53
    1.8.1.2 – Non-nucleoside Reverse Transcriptase Inhibitors (NNRTI) ................................ 54
    1.8.1.3 – Protease Inhibitors (PI) .................................................................... 54
  1.8.2 - HAART and HIV decay dynamics in blood ............................................... 56
  1.8.3 - HAART and semen HIV RNA levels. ....................................................... 57
  1.8.4 - HAART and the GALT ...................................................................... 58
1.9 – THE HOST IMMUNE SYSTEM AND HIV ..................................................... 60
  1.9.1 – The Innate immune system .................................................................... 61
    1.9.1.1 – Pattern Recognition Receptors (PRR) .................................................. 61
    1.9.1.2 – Cellular innate mechanisms .................................................................. 63
    1.9.1.3 – Mucosal innate factors in the genital tract ............................................. 65
  1.9.2 – Adaptive Immune Responses .................................................................. 66
    1.9.2.1 – The T cell receptor (TCR) .................................................................... 67
    1.9.2.2 – Generating CD8+ T cell immune responses ......................................... 67
    1.9.2.3 – Peptide processing and loading on MHC class I complexes .................... 68
  1.9.3 – HIV-specific CD8+ T cell responses ....................................................... 70
    1.9.3.1 – Insights from the SIV model of infection .............................................. 70
    1.9.3.2 – CD8+ T cells and HIV viremia ............................................................ 71
    1.9.3.4 – HLA and CD8+ T cell immune response ............................................. 71
    1.9.3.3 – Long-Term HIV Non-Progression ....................................................... 72
    1.9.3.2 – Viral Escape – Evidence for CD8+ T cell immune pressure ..................... 73
  1.9.4 – HIV-specific CD8+ T cell responses in the genital mucosa ....................... 75
    1.9.4.1 – Characterizing CD8+ T cell in the genital mucosa .................................. 75
1.9.4.1 – Pre-existing HIV-specific CD8+ T cell immune responses in the genital tracts. ........................................ 76
1.9.4.2 – HIV shedding and HIV-specific immune responses in HIV infected individuals. ................................. 77

CHAPTER 2: ROLE OF SYSTEMIC IMMUNE RESPONSES ON SEMEN HIV SHEDDING ................................. 78

2.1 - INTRODUCTION ................................................................................................................................. 79
2.2 - METHODS ............................................................................................................................................... 80
  2.2.1 – Study Population. ......................................................................................................................... 80
  2.2.2 - Sample processing. ....................................................................................................................... 80
  2.2.3 - HIV-1 shedding in semen........................................................................................................... 81
  2.2.4 – Mapping HIV-specific CD8+ responses to HIV. ......................................................................... 81
2.3 - RESULTS ............................................................................................................................................... 82
2.4 - DISCUSSION .......................................................................................................................................... 83

CHAPTER 3: HIV-SPECIFIC CD8+ LYMPHOCYTES IN SEMEN ARE NOT ASSOCIATED WITH REDUCED HIV
SHEDDING ..................................................................................................................................................... 91

3.1 – ABSTRACT .............................................................................................................................................. 92
3.2 - INTRODUCTION ....................................................................................................................................... 93
3.3 - METHODS ............................................................................................................................................... 95
  3.3.1 - Study population .......................................................................................................................... 95
  3.3.2 - Sample processing. ....................................................................................................................... 95
  3.3.3 - HIV-1 shedding in semen ........................................................................................................... 96
  3.3.4 - Epitope mapping using IFN\(\gamma\) ELISpot .................................................................................... 96
  3.3.5 - Ex vivo stimulation and intracellular IFN\(\gamma\) staining .................................................................. 97
  3.3.6 - Cytometric bead array (CBA) .................................................................................................... 98
  3.3.7 - Measurement of innate immune factors. ...................................................................................... 98
  3.3.8 - Statistical analysis ....................................................................................................................... 99
CHAPTER 4: DISPROPORTIONATELY HIGH SEMEN SHEDDING OF HIV IS ASSOCIATED WITH
COMPARTMENTALIZED CYTOMEGALOVIRUS REACTIVATION.

4.1 – ABSTRACT

4.2 – INTRODUCTION

4.2 – METHODS AND MATERIALS

4.2.1 – Study population and STI screening.

4.2.2 – Sample processing.

4.2.3 – Viral shedding in blood and semen.

4.2.4 – Cytometric bead array (CBA).

4.2.5 – Statistical analysis.

4.3 – RESULTS

4.4 – DISCUSSION
CHAPTER 5: PERSISTENT HIV RNA SHEDDING IN SEMEN DESPITE EFFECTIVE ANTIRETROVIRAL THERAPY.

5.1 ABSTRACT .................................................................................................................. 139
5.2 INTRODUCTION ......................................................................................................... 140
5.3 METHODS .................................................................................................................. 141
  5.3.1 Study participants and design .................................................................................. 141
  5.3.2 Specimen collection and diagnostics ...................................................................... 142
  5.3.3 HIV sequence analysis and genotyping ................................................................. 142
  5.3.4 Antiretroviral drug levels in blood and semen plasma ............................................ 143
  5.3.5 HIV infectivity assays .......................................................................................... 144
  5.3.6 Statistical analysis ............................................................................................... 145
5.4 RESULTS ..................................................................................................................... 145
  5.4.1 Participant demographics ...................................................................................... 145
  5.4.2 Impact of effective HAART on HIV RNA levels in the blood and semen .............. 146
  5.4.3 Associations of isolated semen HIV RNA shedding ............................................. 147
  5.4.4 Viral characteristics of isolated semen viral isolates ............................................. 148
  5.4.5 Semen concentration of antiretroviral drugs ......................................................... 149
  5.4.6 Isolated semen shedding after very long-term suppression on HAART ................. 150
5.5 DISCUSSION ............................................................................................................... 151

CHAPTER 6: IMMUNE RECONSTITUTION IN THE SIGMOID COLON AFTER LONG-TERM HIV THERAPY .................................................. 167

6.1 ABSTRACT .................................................................................................................. 168
6.2 INTRODUCTION ......................................................................................................... 169
6.3 METHODS .................................................................................................................. 171
  6.3.1 Study Participants ............................................................................................... 171
6.3.2 Sampling Protocol.................................................................................................................. 171
6.3.3 Evaluating HIV-specific immune responses ex vivo.......................................................... 172
6.3.4 Measurement of proviral HIV DNA: ..................................................................................... 173
6.3.5 Statistical analysis.................................................................................................................. 174
6.4 RESULTS ..................................................................................................................................... 174
6.4.1 Study participants................................................................................................................... 174
6.4.2 CD4+ T-cell populations in the blood and the sigmoid colon .............................................. 175
6.4.3 Associations of HIV proviral load in CD4+ T-cells in blood and sigmoid colon ............... 176
6.4.4 HIV-specific T-cell responses............................................................................................... 177
6.4.5 Regulatory T-cell populations in blood and the sigmoid colon ........................................ 177
6.5 DISCUSSION ............................................................................................................................. 178

CHAPTER 7: DISCUSSION ................................................................................................................. 198

CHAPTER 8 – FUTURE DIRECTIONS ............................................................................................. 202
8.1 – Systemic HIV-specific CD8+ T cell responses and semen HIV RNA shedding .................... 203
8.2 – Evaluating mucosal γδCD8+ T cells and semen HIV RNA shedding .................................. 204
8.3 – CMV and HIV shedding in semen: understanding viral synergy ........................................ 205
8.4 – Investigating correlate of isolated semen HIV RNA shedding in HIV infected individuals on suppressive HAART .............................................................................................................. 207
8.5 – Isolated semen HIV shedding and CMV super-infection ..................................................... 209
8.6 – Reconstitution of CD4+ T cell subsets in the sigmoid colon of HIV-infected individuals on long-term HAART .......................................................................................................................... 210

CHAPTER 9: REFERENCES ................................................................................................................ 211
# Table of Figures

**Figure 1.1** – Schematic of an HIV virion ................................................................. 7

**Figure 1.2** – Overview of viral attachment and entry ......................................................... 9

**Figure 1.3** – HIV transcription ......................................................................................... 13

**Figure 1.4** – Natural course of HIV disease progression ....................................................... 18

**Figure 1.5** – HIV transmission risk and exposure ............................................................... 29

**Figure 1.6** – Potential sites of HIV entry in the female genital tract ...................................... 35

**Figure 1.7** – HIV sexual transmission and blood viral load .................................................. 42

**Figure 1.9** – HAART initiation and death rates - ................................................................. 52

**Figure 1.10** – Sites of HAART drug action ........................................................................... 55

**Figure 2.1** – Distribution of HIV-specific immune by HIV protein ....................................... 85

**Figure 2.2** – Magnitude of HIV-immune responses and semen HIV VL ................................. 86

**Figure 2.3** – Correlation between HIV levels in blood and semen .......................................... 87

**Figure 2.4** – Immune responses and semen HIV RNA levels ................................................ 88

**Figure 2.5** – HIV-specific immune response and HIV viral load in blood ............................... 89

**Figure 2.6** – Breadth of immune responses and blood HIV viral load .................................... 90

**Figure 3.0** – Strong positive correlation between semen HIV viral loads at two time-points ....... 112

**Figure 3.1** – Semen inflammatory cytokines and HIV shedding ............................................ 114

**Figure 3.2** – Expression of CD103 on CD8+ T cells in blood and semen ............................... 115

**Figure 3.3** – HIV-1 specific, IFNγ-producing CD8+ T-cells in semen ....................................... 119

**Figure 3.4** – HIV-1-specific, IFNγ-producing CD8+ T cells in semen and levels of HIV-1 RNA shedding ............................................................................................................. 121

**Figure 3.5** – Inflammatory cytokines in semen, increased HIV RNA and CD8+ T cells ............. 124

**Figure 4.1** – Disproportionate semen HIV shedding associated with increased CMV shedding in semen .................................................................................................................. 135

**Figure 4.2** – Correlation between levels of CMV and HIV in semen ....................................... 136
FIGURE 4.3: HIV RNA SHEDDING IN SEMEN IS STRONGLY ASSOCIATED WITH THE PRESENCE OF CMV DNA IN SEMEN. ... 137

FIGURE 5.1 – HIV LEVELS IN BLOOD AND SEMEN AFTER HAART INITIATION. .......................................................... 161

FIGURE 5.2 – PARTICIPANTS WITH UNDETECTABLE HIV RNA IN BLOOD AND SEMEN PLASMA, BY STUDY TIME POINT. .... 162

FIGURE 5.3 – COMPARING CONCENTRATIONS OF ANTIRETROVIRAL AGENTS IN BLOOD AND SEMEN PLASMA OF HIV-INFECTED PATIENTS FIVE MONTHS AFTER HAART INITIATION. .......................................................................................................................... 166

FIGURE 6.1 – PERCENTAGE OF T-CELLS EXPRESSION CD4 IN BLOOD AND THE SIGMOID COLON. ........................................... 183

FIGURE 6.2 – CD4 AND CD8 T CELL EXPRESSION IN BLOOD AND THE SIGMOID COLON. ....................................................... 184

FIGURE 6.3 – COMPARING CCR5 EXPRESSION ON CD4+ T CELLS IN BLOOD AND THE SIGMOID COLON. ................................. 185

FIGURE 6.4 – CCR5 EXPRESSION ON CD4+ T CELLS FROM BLOOD AND THE SIGMOID COLON. .................................................... 186

FIGURE 6.5 – BLOOD NADIR CD4 COUNT AND IMMUNE RECONSTITUTION OF CD4+ T CELLS IN BLOOD AND THE SIGMOID COLON ........................................................................................................................................ 188

FIGURE 6.6 – CORRELATION BETWEEN %CD4 AND HIV PROVIRAL DNA IN BLOOD. .............................................................. 189

FIGURE 6.7 – COMPARING EXPRESSION OF CD69 ON T CELLS IN BLOOD AND SIGMOID COLON MONONUCLEAR CELLS. ...... 190

FIGURE 6.8 – CORRELATING HIV PROVIRAL DNA WITH CD8+ T CELL IMMUNE ACTIVATION. ..................................................... 191

FIGURE 6.9 – CD8+ T CELL IMMUNE RESPONSE IN BLOOD AND THE SIGMOID COLON. ............................................................ 192

FIGURE 6.10 – MEASURING CD4-SPECIFIC IMMUNE RESPONSES IN BLOOD AND THE SIGMOID COLON. ................................. 193

FIGURE 6.11 – HIV PROVIRAL DNA IN CD4+ T CELLS AND HIV-SPECIFIC CD8+ T-CELL IMMUNE RESPONSES. ...................... 194

FIGURE 6.12 – REGULATORY T-CELL POPULATION IN BLOOD AND THE SIGMOID COLON. ......................................................... 196

FIGURE 6.13 – REGULATORY T CELLS IN SIGMOID COLON OF HIV-INFECTED LONG-TERM HAART AND HIV NEGATIVE INDIVIDUALS. .......................................................................................................................... 197

XX
MANUSCRIPTS ARISING FROM THIS THESIS


Thesis overview

Chapter 1:

This section provides a general overview of HIV including the HIV life cycle, the natural history of the disease, anti-HIV host immune responses, correlates of HIV transmission and the impact of HAART on transmission and disease progression. As the majority of my doctoral thesis focuses on sexual transmission of HIV, there are several sections dedicated to HIV and mucosal surfaces including common routes of sexual acquisition of HIV, understanding HIV transmission and determinants of HIV shedding in genital tract. The final section of the introduction focuses on better understanding the impact of starting HAART on HIV in blood, the genital tract and the gastrointestinal associated lymphoid mucosa.

Chapter 2:

This section describes a study evaluating the impact of systemic immune CD8+ T cell responses and their impact on semen HIV RNA levels. Since HIV RNA levels in blood correlate with HIV RNA levels in semen and as HIV-specific CD8+ T cell immune responses have been shown to reduce HIV viremia. We investigated if the magnitude and/or breath of HIV-specific immune responses in blood were associated with levels of HIV RNA shed in the semen of HIV infected therapy naive individuals. Our study concluded even though there was a strong positive correlation between HIV RNA levels in blood and semen, neither the breadth nor the magnitude of systemic HIV-specific
CD8+ T cell immune responses correlated with HIV RNA levels in blood or semen. Furthermore Gag-specific immune responses, that have been associated with systemic control of viremia in previous studies, did not correlate with HIV RNA levels in blood or semen plasma.

Chapter 3:

The first study to describe HIV-specific CD8+ T cell immune responses in the semen of HIV infected therapy naive men in an *ex vivo* fashion. We tested the hypothesis that semen HIV-specific CD8+ T cell immune responses might be associated with reduced levels of HIV RNA shedding in the semen of HIV infected therapy naive men. Overall, HIV-specific CD8+ T cell immune responses were stronger in semen compared to blood. However, neither HIV-specific CD8+ T cell immune responses in blood nor semen correlated with reduced HIV RNA levels in blood or semen plasma. Semen HIV RNA levels positively correlated with elevated concentrations of semen pro-inflammatory cytokines including IL6, IL8, IL12p40 and IFN-γ. We also evaluate the relationship between immune innate factors and semen HIV RNA shedding and found no correlation with SLPI and a positive correlation between lactoferrin and semen HIV RNA levels.
Chapter 4:

Cytomegalovirus reactivation has been associated increased HIV RNA shedding in the female genital tract however, the relationship between CMV and HIV in semen is unclear. In this study we evaluated the association between CMV DNA and HIV RNA levels in the genital tract of HIV-CMV co-infected therapy naive men. We observed a strong positive correlation between HIV RNA and CMV DNA levels in semen plasma. HIV-infected individuals with detectable CMV DNA in semen shed 10-fold more HIV RNA in semen compared to those without semen CMV shedding. Although generally HIV RNA levels in semen tended to be 10-fold lower than in blood, a proportion of HIV-infected individuals tended to shed more HIV RNA in semen compared to blood, described as “disproportionate” HIV shedders, were more likely to shed CMV in semen, suggesting that localized CMV reactivation may contribute to increased HIV transmission.

Chapter 5:

The impact of HAART on plasma HIV RNA levels has been well documented, but much less is known about the effect of therapy on semen HIV RNA levels. We enrolled HIV-infected men starting HAART, and followed participants prospectively for six months. Blood and semen samples were collected prior to HAART initiation and then at weeks 2, 4, 8, 12 16, 20 and 24 months on HAART. Blood and semen samples were also collected from individuals on long-term completely suppressive HAART. The
The majority of enrolees completely suppressed HIV RNA in both blood and semen plasma within 16 weeks of starting HAART. However, a significant proportion of individuals shed HIV RNA in semen intermittently despite complete suppression of HIV RNA in blood, a phenomenon we termed isolated semen HIV shedding. Isolated semen HIV shedding was independent of HSV-2 status, HAART regimen or semen plasma concentrations of HAART drugs.

**Chapter 6:**

HAART reduces HIV RNA levels in blood and the genital tract and is associated with reconstitution of important immune populations in blood and lymph nodes. The gastrointestinal associated lymphoid tissue (GALT) is the richest immunologic site in the body and HIV infection is associated with a dramatic loss of important immune populations in the GALT. Even though HAART restores CD4+ T cell populations in blood, the GALT has been described to be resistant to immunologic reconstitution. In this study we found that long-term completely suppressive HAART was associated with complete reconstitution of CD4+ T cell in the sigmoid colon of HIV infected individuals.

**Chapter 7:**

A brief outline of future directions to pursue to further our understanding and extend some of the findings presented in this doctoral thesis.
CHAPTER 1: Introduction
1.1 – HIV around the world

The Human Immunodeficiency Virus-1 (HIV), the etiological agent of the Acquired Immunodeficiency Syndrome, has claimed over 30 million lives in the last 25 years and at present infects 33.7 million people around the world\(^1\). In 2007 an estimated 6800/day or 2.5 million people around the world became newly infected with HIV and almost 2.1 million men, women and children died of AIDS and HIV-associated complications \(^1\). Should the current rate of new infections continue; the number of HIV-infected individuals will be well over 60 million by the year 2050\(^1\). Even more alarming is the expected loss of life over the next decade attributed to HIV, with some countries in sub-Saharan Africa predicted to lose almost a fifth of their adult population\(^1\).

With no potential vaccine candidates, the future of several countries with very high disease burdens appeared bleak at the turn of the decade. However, over the last few years the increased efforts of several countries in partnership with global health agencies, have embarked on public health projects promoting HIV prevention. Strategies include better educating the public about HIV and the introduction of wide-spread HIV testing initiatives. In several high disease burden countries including Kenya, Thailand and Cambodia, public health interventions have resulted in a downward trend in HIV prevalence \(^1\). Although these initiatives do not provide protection as complete as would be afforded by an efficacious vaccine, they are cost-effective strategies that poor and developing countries can deploy quickly to reduce HIV transmission rates, while the search for a vaccine or other potential therapeutic interventions continue.
A second more recent initiative undertaken by the WHO, UNAIDS and United States government has focused on providing universal access to first-line HIV treatment. HIV therapy improves the standard of living for those living with HIV and may reduce the risk of sexually transmitting HIV (see chapter 5); however, the costs of such a program are prohibitive for most countries in sub-Saharan Africa. Current estimates suggest that universal access to therapy in Africa will cost upwards of $10 billion annually, and that would be enough to provide only first-line HIV drugs to currently infected individuals. Even more sobering is that UNAIDS estimates that for every two people put on treatment five become newly infected with HIV, making it an unsustainable long-term solution to the HIV epidemic. However, if universal therapy was achieved it may reduce sexual transmission world-wide.

It appears that while we wait for vaccine candidates to be developed and tested the most cost-effective and sustainable option is to implement several cost-effective interventions, both behavioural and biological, that have been proven to reduce HIV transmission rates and improve the health and standard of living of those already infected with the virus. To understand the dynamics of HIV transmission, more research is needed to better understand biological factors that determine how likely an HIV-infected individual is to sexually transmit HIV to his/her partners(s) (i.e. determinants of infectiousness).

As most HIV is transmitted sexually, my doctoral work focused on evaluating immunologic, virologic and clinical determinants of levels of HIV in semen, which is
used as a surrogate marker for HIV transmission. Specifically, my first two studies focused on identifying associations between HIV RNA shedding in semen and immune factors (Chapters 2 and 3), and viral co-infections (Chapter 4). The study described in Chapter 5 is a prospective one evaluating the impact of HIV therapy on HIV RNA levels in semen, and indirectly on sexual transmission of HIV (Chapter 5). The final data section focuses on evaluating the ability of long-term HIV therapy to restore mucosal population of immune cells in the sigmoid colon (Chapter 6).

1.2 – Proposed origins of the Human Immunodeficiency Virus (HIV)

The Human immunodeficiency virus (HIV) was identified as the etiological agent of Acquired Immunodeficiency Syndrome (AIDS) in 1983, when HIV was first isolated from AIDS patients by Luc Montanier, Françoise Barré-Sinoussi and Robert Gallo. For the discovery of HIV, the first human lentivirus, Drs. Montanier and Barre-Sinoussi shared the 2008 Nobel Prize in Physiology and Medicine. The HIV virus is a member of the genus Lentivirus, part of the Retroviridae family of viruses. Members of the Retroviridae family of viruses are retroviruses, meaning that they are RNA viruses that have to be reverse-transcribed into DNA before they can be incorporated into the host’s genome. Lentiviruses (lenti meaning ‘slow’) are characterized by a long duration of infection.
Five sero groups of species-specific lentiviruses have been identified (cow, sheep, primate, cats and cattle), all of which are believed to have co-evolved with their natural hosts\textsuperscript{13}. Although co-evolution of natural lentivirus species has not been observed in humans, zoonotic infections of primate retroviruses are frequently observed in human populations living in close proximity to non-human primates\textsuperscript{14, 15}. HIV is thought to have entered the human population through multiple zoonotic infections from non-human primates infected with the Simian Immunodeficiency Virus (SIV)\textsuperscript{14}. The multiple strains of HIV (HIV-1 and HIV-2) are a result of separate zoonotic events, as the most prevalent strain of HIV (HIV-1) is closely related to an SIV strain that infects Chimpanzees (SIV\textsubscript{cpz})\textsuperscript{16}, while HIV-2 is most closely related to one that infects Sooty Mangabeys (SIV\textsubscript{sm})\textsuperscript{17}.

Although the identification of HIV as an infectious disease and the causative agent of AIDS is relatively recent, in-depth phylogenetic analysis on global viral sequences have determined that HIV probably entered the human population sometime in the early 1930s\textsuperscript{17}. More recent evidence suggests that HIV crossed over into the human species even earlier, around the turn of the 20\textsuperscript{th} century\textsuperscript{18}. The virus and the illness went largely unnoticed until migrating to the United States sometime in the late 1960’s and early 1970’s\textsuperscript{17, 18}. The first cases of what we now know to be HIV infection presented in 1981 in San Francisco\textsuperscript{19}, when physicians started documenting rare opportunistic illnesses, such as Kaposi sarcoma and \textit{Pneumocystis carinii pneumonia} in young homosexual men; previously these had been observed only in highly immunosuppressed individuals\textsuperscript{20}.
1.3 – The HIV viral life cycle

Infection with the HIV virus leads to a progressive immune deficiency, and so the disease was aptly named the Acquired Immunodeficiency Syndrome (AIDS). The HIV virus is a complex retrovirus with nine genes (gag, pol, nef, vif, vpu, rev, tat, vpr and env) that encode 15-proteins. The common major genetic domains are the gag-pol-env genes which are synthesized as a single precursor. This section discusses the role of each of the HIV proteins and describes the HIV viral life cycle in detail.

1.3.1 – The HIV virus

The HIV virus is a spherical virus with approximately 72 spikes of the HIV envelope glycoprotein and a diameter of approximately 100nm. The virus has a lipid bilayer that surrounds the viral matrix (MA), which encapsulates a cone shaped capsid (CA) protein made of several hexametric rings (Figure 1.1). The cone shaped capsid surrounds the nucleocapsid (NC) core, which contains two single stranded positive sense RNA molecules and enzymatically active viral proteins that include; 1) protease (PR); 2) reverse transcriptase (RT); and 3) integrase (IN). The nucleocapsid also contains several accessory and regulatory viral proteins including Vif, Vpr and Nef along with cellular host factors (see Figure 1.1).
Figure 1.1 – Schematic of an HIV virion. A Cross-sectional diagram of an HIV virion with the viral envelope proteins (gp41 and gp120), the internal proteins (matrix and capsule proteins) and the viral RNA’s.
1.3.2 – HIV viral attachment and cellular entry

HIV virus entry into a host cells is mediated by interaction between the HIV gp120 glycoprotein and the host CD4- T cell molecule on the host cell \(^{22, 23}\). The CD4 molecule is the primary HIV receptor and is predominantly expressed on helper T cells and macrophages\(^{24, 25}\). The initial attachment between gp120 and CD4 is mediated by high affinity binding pockets between the surface gp120 virion glycoprotein and the host CD4 molecule \(^{12, 23, 26}\). Binding of gp120 to the CD4 molecule leads to a conformational change in the viral gp120 molecule that results in the exposure of additional binding sites \(^{27, 28}\). The host-virus complex is then thought to move along the cell membrane, almost as if the virus is ‘sliding’ across the cell surface, in search of additional co-receptors important for viral entry\(^{28, 29}\).

The two main co-receptors utilized by HIV to facilitate cellular entry are the chemokine receptors CCR5 and CXCR4 \(^{30, 31}\). Binding of the CD4-gp120 complex with either of the chemokine receptors results in a second conformational change in gp120, exposing gp41; a trans-membrane envelope protein of HIV\(^{32, 33}\). The N-terminus of gp41 has a fusion peptide that inserts into the host cellular membrane resulting in the final conformational change that results in the generation of 6 helical coil-coil structures\(^{32, 33}\). The 6-helical coil-coil domains behave like a spring bringing the virus and the host cell closer to one another, leading to the virion-host cell fusion\(^{32-35}\).
Figure 1.2 – Overview of viral attachment and entry. The schematic diagram illustrates viral docking and entry using the CD4 primary receptor and the conformation change that takes place following association with the CCR5 co-receptor.

1.3.3 – Nuclear import and HIV integration

After fusing with the plasma membrane, the virion enters the host cell cytoplasm and begins to un-coat, a process that is not well understood but is believed to involve the disintegration of the matrix (MA), and capsid (CA) proteins\textsuperscript{23, 34, 36}. When infecting new cells, a portion of the Gag MA dissociates from the newly infected cell exposing a
nuclear localization signal (NLS), which facilitates the transport of viral proteins to the cellular nucleus. The presence of an NLS is quite unique to HIV as most simple retroviruses (e.g. Murine leukemia virus) are only able to replicate in actively proliferating cells\textsuperscript{23, 37}. The presence of the NLS enables HIV to “home” to the nucleus and enables HIV to infect and replicate in non-dividing cells\textsuperscript{37, 38}.

The disassembly of the virion Gag CA and MA also results in the formation of a reverse transcription complex (RTC), made up of viral RNA and several viral proteins including reverse Transcriptase (RT), Gag MA and IN\textsuperscript{23, 34, 36, 39}. The RTC migrates through the cytoplasm towards the nucleus, while on its path to the nucleus the viral RNA is reverse transcribed into viral DNA by the viral RT, an enzyme that is stabilized by Viral Protein R (Vpr)\textsuperscript{40}. The association of Vpr with the RT enzyme is thought to stabilize the RT enzyme and reduce the rate of error during reverse transcription. Even though HIV reverse transcription is inherently error prone (1/10\textsuperscript{4} bases), the error rate is 4-fold higher in the absence of Vpr expression\textsuperscript{40, 41}. Reverse transcription of the HIV RNA results in the dissociation of IN molecule(s) from the RTC\textsuperscript{23, 37} and enables the viral genome to integrate into the host DNA. This complex of nucleic acids and viral proteins, after the HIV RNA is reverse transcribed to DNA, is termed the pre-integration complex (PIC)\textsuperscript{11, 34, 37}.

The PIC formed in the cytoplasm has to enter the nucleus to integrate with the host genome. Although the mechanisms of entry utilized by the PIC to enter the nucleus are not well understood, the current hypothesis is that the viral PIC may be imported into
the nucleus with the help of Vpr and IN proteins. In the nucleus the PIC integrates into the host genome as a single stranded viral DNA fragment, a process that is catalyzed by the viral IN protein. The integrated virus is now called a provirus and serves as the template from which new viral particles will be transcribed.

1.3.4 – HIV transcription and nuclear transportation

Transcription of HIV starts at the 5’ long terminal repeat (LTR) sequence, which functions like a transcriptional promoter. The LTR contains transcriptional promoters including an initiator sequence, a TATA box and three Sp1 sites. Despite the presence of several transcriptional promoters, the transcription rate of the HIV LTR is surprisingly low. To speed up the rate of transcription HIV uses a viral trans-activator protein (TAT) that stabilizes elongation of RNA polymerase II on the HIV provirus and increases the transcription rate of the viral genome. TAT enhances the rate of viral transcription by almost a 100-fold by binding to structured RNA elements, called trans-activating response elements (TAR). In the absence of TAT, although the RNA polymerase II initiates transcription of the HIV genome, it fails to elongate efficiently. In the presence of TAT, HIV transcription results in the generation of a large number of viral RNA’s containing introns.

Translation of intron containing RNA is extremely rare in the eukaryotic system, as in almost every other eukaryotic system mRNA splicing removes intron elements prior to nuclear export. However in the case of HIV, intron elements play an essential role in HIV assembly. HIV transcription results in the generation of three distinctly sized
RNA fragment: (1) an unspliced form, (2) a partially spliced form and (3) a multiply spliced RNA. The unspliced RNA fragments represent the mRNA transcripts from which the Gag and Gag-pol polyprotein precursors are synthesized, while the partially spliced mRNA encode the Env, Vif, Vpu and Vpr proteins\textsuperscript{42, 45}. The multiply spliced mRNA’s are translated into the Rev, Tat and Nef proteins\textsuperscript{34, 45} (Figure 1.3).
**Figure 1.3 – HIV Transcription.** CD4+/CCR5 medicated HIV binding and entry.

HIV, once inside the host cell, uncoats and undergoes reverse transcription in the cytoplasm before translocating to the cell nucleus where it integrates into the host DNA. Integrated proviral DNA now serve as a template for the transcription and translation of HIV proteins.
Both the unspliced and partially spliced viral transcripts encode important structural and enzymatic elements that are incorporated into each HIV virion. Therefore, the virus has to transport unspliced mRNA out of the nucleus. The HIV virus accomplishes this by using a novel shuttling protein called Regulator of expression upon transcription (REV). The Rev protein binds to an RNA responsive element (RRE) located in the Envelope (Env) gene, present in the incompletely spliced HIV RNA’s. The unique nature of the Rev protein is that it contains both a nuclear localization sequence (NLS) and a nuclear-export sequence (NES). The presence of an NLS and an NES enable REV to shuttle in and out of the nucleus. Rev first shuttles the unspliced and partially spliced mRNA forms out of the nucleus and releases them into the cytoplasm. The Rev protein, using the NLS sequence, then shuttles back into the nucleus and repeats the process. Since the translation of each form of the HIV RNA generates different HIV proteins, the production of infectious virions is dependent on attaining a balance between splicing RNA products and the REV-mediated transport of unspliced mRNA products out of the nucleus. If Rev does not shuttle enough unspliced mRNA out of the nucleus, only spliced mRNA forms are translated in the cytoplasm and will result in the deficiency of structural and enzymatic HIV proteins. The consequences of a deficiency (particularly of structural and enzymatic HIV proteins) have been observed in non-primate cells, resulting in inefficient viral replication.
1.3.5 – Virion assembly

Virion assembly is the final phase of the HIV life cycle that occurs in the host cell, and takes place at the plasma membrane.

After the export of all three forms of HIV mRNA fragments to the cytoplasm, the unspliced mRNA fragments are translated into three precursor proteins; the Gag precursor polyprotein Pr55\textsuperscript{Gag}, the Gag-Polymerase (Pol) complex and the Envelope (Env) protein\textsuperscript{42, 45, 50}. The Pr55\textsuperscript{Gag} polyprotein aggregates at the plasma membrane and forms the immature HIV capsid, which is made up of between 2000 – 5000 Gag precursor polyproteins\textsuperscript{42}. The multimerization of Gag-polyprotein molecules results in the formation of a spherical immature HIV capsid\textsuperscript{34, 36}. As the virion matures, the Gag-polyprotein is further processed by the viral protease (PR). Viral maturation occurs as the PR protease cleaves the Gag precursor protein resulting in the generation of four structural proteins; 1) Gag Matrix (p17 or MA), 2) Gag Capsid (p24 or CA), 3) Gag Nucleocapsid (p7 or NC) and 4) p6 protein\textsuperscript{28, 34}. Cleavage of Pr55 also results in the generation of two spacer proteins SP1 and SP2; however, their role in viral replication and/or viral assembly remains to be elucidated\textsuperscript{42}.

The MA, CA, and NC regions of Gag play important roles in facilitating virion assembly\textsuperscript{39, 51, 52}. The myristolation signals (or M domain) in the Gag MA protein is crucial for virion assembly, as deletion studies of all of MA except for the myristolation signal resulted in the production of infectious particles. Deletion of the M-domain resulted in aberrant targeting of Gag to other cellular compartments\textsuperscript{28, 34, 51, 53-55}. The Gag
CA protein contains a major homology region (MHR) at the C-terminus that is important for Gag-Gag association; mutations in the MHR result in defects in viral assembly. The Gag NC protein is also important for Gag-Gag and Gag-RNA interactions, as *in vitro* CA-NC fusion proteins appear to assemble more efficiently than CA proteins alone and mutations in the NC region of Gag interferes with RNA binding and subsequent incorporation into the virion.

The Envelope (Env) protein is co-translationally translocated into the endoplasmic reticulum (ER). The Env protein is synthesized as the precursor protein GP160, which undergoes oligomerization and glycosylation in the ER and then is transported to the trans-Golgi network (TGN). The Env precursor protein is then cleaved by various host factors localized near the TGN including the host cellular enzyme Furin. The proteolysis of gp160 results in the generation of two non-covalently associated glycoproteins; 1) a surface glycoprotein called gp120 (SU), and 2) a transmembrane protein called gp41 (TM). The gp120-41 protein complex then moves by vesicle transport to the plasma membrane and is subsequently incorporated into the budding virion. Although the mechanism of Env incorporation is not clear, it does appear that there is an interaction between the Gag MA protein and the cytoplasmic tail of gp41. The Gag MA-gp41 interaction is mediated by several host proteins including the tail interacting protein – 47 (TIP–47). This step is essential for incorporation of Env proteins into the virion as mutations in the Gag MA protein results in Env-deficient virion particles.
1.3.6 – Virion Release and Maturation

Following the co-localization and association between Gag precursor proteins and Env proteins the virus is released into the extracellular environment\textsuperscript{34, 60}. Virion molecules pinch off the host cell incorporating components of the plasma membrane. The p6 protein is thought to mediate the release of the HIV virion from the plasma membrane, as mutations in the p6 region of Gag results in the sequestration of HIV virions at the cellular membrane\textsuperscript{61}.

Once in the extracellular environment, the virus particle undergoes a maturation process that is critical for the generation of infectious virions. Maturation transforms the virus particle from a non-infectious particle with an electron translucent core in to an infectious particle with an electron-dense core\textsuperscript{50, 60}. Virion maturation occurs as the Gag precursor protein and the Gag-pol complexes are cleaved by viral proteases to produce the Gag proteins (MA, CA, NC and p6) and several accessory and regulatory enzymes including viral IN, RT, and PR proteins\textsuperscript{50}.

1.4 – Natural History of HIV Infection

The natural course of HIV infection is characterized by the unique interplay between the virus and several host factors described in detail in the following sections (in particular refer to sections 1.4.2 and 1.5). Below is a schematic diagram illustrating the
natural history of HIV outlining the various phases of infection and the interplay between HIV viremia and circulating CD4+ T cells in blood (Figure 1.4)

From *Immunity: The Immune Response in Infectious and Inflammatory Disease* by DeFranco, Locksley and Robertson

**Figure 1.4 – Natural course of HIV disease progression.** Primary (or acute) HIV infection is associated with a precipitous drop in absolute CD4+ counts (blue line) and a rise in HIV viremia (red line). After the initial acute phase of infection, which lasts between 6 – 9 weeks, HIV viremia drops to a lower level called the viral set-point. Establishment of a viral set-point typically results in an increase in absolute CD4 counts, which subsequently decline over the course of infection. A drop of CD4+ counts below
200/mm$^3$ is defined as clinical AIDS which is associated with a rapid rise in viremia and decline in absolute CD4+ counts.

1.4.1 – Clinical manifestations of HIV infection

1.4.1.1 – Acute infection:

The acute phase of infection occurs after infection with the HIV virus and is associated with a burst of viral replication, often reaching as high as $10^7$ copies/mL of HIV RNA in blood (Figure 1.4). The most common symptoms associated with acute HIV infection include malaise, fever, rash and headaches$^{62}$, however, almost half of all acutely infected individuals remain asymptomatic, exhibiting few or none of the general symptoms $^{63, 64}$. Due to the non-specific nature of the symptoms, even symptomatic individuals often ignore or attribute their symptoms to other more common afflictions. Current estimates predict that almost 40% of individuals infected with HIV (about 30% in Ontario) are unaware of their infection status $^1$.

1.4.1.2 – Chronic or Latent HIV Infection

Chronic or latent infection follows acute infection and is of variable duration (between 6 months to >20 years) depending on viral phenotypes$^{65}$, host genetics $^{66, 67}$, and other unknown factors. The chronic phase of infection begins with a rapid decline in HIV viremia from the acute peak (Figure 1.4) to a relatively stable level called the viral set-point (figure 1.4). The level of the viral set-point has been shown to correlate with the
rate of disease progression to AIDS\textsuperscript{68}. Typically, the chronic phase of infection is asymptomatic and is associated with a slow steady decline (mean time = 2-7 years), of the absolute CD4+ T cell count (Figure 1.4).

1.4.1.3 - Clinical AIDS

The occurrence of an opportunistic infections (OI) or fall of absolute CD4+ T cell counts in blood to below 200/mm\textsuperscript{3} is clinically referred to as AIDS\textsuperscript{69}. Clinically, progression to AIDS is defined by extreme immunodeficiency and coincides with a rapid rise in viremia and associated with increased incidences of opportunistic malignancies and infections\textsuperscript{69}. Although still unclear, it is believed that the myriad of opportunistic infections (\textit{Pneumocystis pneumonia}, CMV, EBV, and toxoplasma)\textsuperscript{70} associated with advanced HIV disease may be responsible for the constitutional symptoms reported by almost half of all individuals with advanced HIV disease (Figure 1.3). The most common constitutional symptoms include weight loss, fatigue, fever and sweating\textsuperscript{71}. Typically, without treatment of HIV with highly active anti-retroviral therapy the CD4 count will continue to drop, predisposing individuals to malignancies (including Kaposi Sarcoma and lymphomas), reactivation of otherwise clinically latent asymptomatic viruses (most notably Cytomegalovirus and Epstein Barr virus) and other infections (\textit{Pneumocystis pneumonia}, toxoplasmosis, cryptosporidiosis, infectious diarrhoea, and pneumococcal pneumonia). If untreated, AIDS will eventually lead to death.
1.4.2 – Immunologic events following HIV infection

1.4.2.1 – Acute infection

Unlike the non-specific clinical symptoms associated with acute HIV infection, immune events occurring early in infection are quite dramatic. The high viremia during acute infection facilitates wide-spread dissemination of HIV to different organs in the body, seeding various lymphoid organs that house high concentrations of target CD4+ T cells and macrophages\textsuperscript{72, 73}. Systemically, acute infection may be associated with a dip in circulating blood CD4+ T cells (Figure 1.4); however the loss of CD4+ T cells at mucosal sites, particularly the gastrointestinal associated lymphoid tissue (GALT), is much more profound\textsuperscript{74-77}.

1.4.2.2 – The GALT and acute infection.

The mucosal surface of the GI tract serves as a barrier against the microorganisms found in the human gastrointestinal system. The function of this barrier is to prevent the entry of bacteria and other enteric pathogens into the body while simultaneously facilitating the absorption of water and nutrients from the gut lumen. The majority of pathogens are either food or water borne and as such enter the body through the lower intestine, the GI tract houses the largest collection of lymphoid structures and is the largest reservoir of CD4+ T cells in the body.
The clinical GI manifestations of HIV were evident early in the epidemic. In fact as early as 1984, before the approval of the first commercial assay to test for HIV was available, it was documented that HIV infected individuals suffered from severe gastrointestinal abnormalities, including lymphocyte depletion, nutrient mal-absorption and damage to the epithelium.

As these early observations suggested, HIV infection results in the loss of a significant proportion of lymphocytes, particularly CD4+ T cells, housed in the GI mucosa. GALT CD4 T-cells are particularly susceptible to HIV as the majority of CD4+ T cells in the gut mucosa are activated. Activated CD4+ T cells are particularly permissive to HIV infection as activation results in the up-regulation of CCR5 on the cell surface. Almost 95% of memory CD4+ T cells in the GI tract expressing CCR5 are lost within two weeks of HIV or SIV infection. Furthermore in the SIV model, almost 60% of CD4+CCR5+ T cells express SIV RNA by day 10 of infection, and most of these (~80%) are eliminated by day 14 of infection. Flow-cytometry analysis has revealed that GALT resident CD4+ T cells harbour 13-fold higher HIV-1 viral DNA levels and 10-fold higher HIV-1 RNA levels than blood CD4+ T cells during acute infection.

Despite the virus’s proclivity for activated CD4+ T cells, a pronounced loss of resting CD4+ T cells is also observed in the GALT as HIV-1 RNA is detected in both activated and resting mucosal CD4+ T cells. The killing observed in the GALT is therefore thought to be mediated by direct target-cell infection, Fas-mediated viral killing
and bystander activation. In summary, following HIV infection, infected individuals lose the majority of their CD4+ T cells within a fortnight, an event that is not reflected in peripheral absolute CD4+ counts.

1.4.2.3. All roads lead to the GALT

The predilection of the virus for the gut mucosa was originally thought to be because of the large resident population of target CD4+ T cells in the GALT, but very little was known about the mechanism(s) employed by HIV to migrate to the target rich environment of the GALT. Regardless of the route of HIV or SIV acquisition, rapid depletion of GALT CD4+ T cells appears to be a central feature of SIV/HIV infection.

A recent report has shed some light on why HIV appears to preferentially target the GALT. The surface HIV glycoprotein gp-120 binds to and signals through a surface integrin on activated CD4+ T-cells and NK cells called α4β7, in a CD4-independent manner. Upon activation of CD4+ T cells, two otherwise independent integrins, α4 and β7 form a heterodimer that binds the HIV-gp120. Even though very little is known about the association between HIV and α4β7, it is clear that the physiological role of α4β7 as an important receptor in leukocyte trafficking to and retention in the GI mucosa plays a central role in HIV-mediated GALT damage.
1.4.4 – GALT depletion and Immune activation in HIV and SIV

The depletion of CD4+ T cell populations in the GALT appears to set the stage for the ongoing immune dysfunction observed in HIV infected individuals and in SIV infected Rhesus macaques (RM). Both SIV (in RM’s) and HIV infection are associated with diminished regenerative capacity of epithelial cells, compromised epithelial barrier and impaired absorptive function of epithelial cells isolated from the GI mucosa. Furthermore, gene expression profiling of GALT tissue demonstrated that HIV/SIV infection was associated with dysregulation of genes associated with T-cell homeostasis, growth factor production, cell cycle mediators and up-regulation of genes associated with apoptosis and necrosis.

The destruction of mucosal CD4+ T cells observed in HIV and SIV infection is associated with what has been described as a ‘leaky gut’, with compromised patency of the GI epithelial barrier. This exposes the host to bacterial products normally isolated within the gut lumen. HIV infected individuals have significantly higher concentrations of bacterial products in their blood plasma through a process called microbial translocation. Microbial translocation is defined as the translocation or transfer of bacterial products into the body, from the gut lumen, without overt bacteremia. In HIV infected individuals, microbial translocation of immune agonists including lipopolysaccharides (LPS) and proteoglycans (PGN), cell wall components of gram negative and gram positive bacteria respectively, may contribute to the systemic immune activation observed in HIV-infected individuals. Increased systemic levels of LPS in HIV infected individuals correlate with inflammatory markers on monocytes,
CD8+ T-cells, dendritic cells and B-cells. Furthermore, blood plasma from HIV-infected individuals added to lymphocytes in culture from HIV-negative individuals results in activation of T-cells, B-cells and monocytes. Systemic LPS levels tend to be much higher in HIV-infected individuals progressing to AIDS, compared to HIV controllers, pointing to a possible role of LPS-induced systemic immune activation as a driver of HIV disease progression.

1.4.5 – Immune activation: insights from the natural host of SIV.

Recent evidence suggests that the immune-pathogenesis of AIDS is related to the host immune response (e.g. immune activation) than to direct virus-mediated effects. Typically, the activation of host immunity following infection leads to control of pathogen replication. However, unlike other chronic and acute infections, HIV-infected individuals do not dampen down immune processes initiated in the acute phase of infection and these persist into the chronic phase of infection. The inability of HIV infected individuals to dampen or shut off immune activation after the acute phase may be exacerbated by the presence high HIV viremia and elevated concentrations of bacterial products in blood plasma, which are potent immune agonists. Immune activation leads to increased expression of activation (CD38) and apoptotic markers (FasL) on CD4+ and CD8+ T cells, elevated concentrations of pro-inflammatory cytokines (IL-6, IL-8 and TNF-α), and increased systemic CD4+ T cell turnover in HIV infected individuals.
Overall, systemic immune activation in HIV infected individuals may be a stronger predictor of HIV disease progression than HIV viral load or CD4 count\textsuperscript{68, 106, 107}. Along with the strong association between immune activation and HIV-1 disease progression, observations made in HIV-2 infected individuals and in natural hosts of SIV strongly suggest that immune activation may mediate HIV-1 disease progression\textsuperscript{108, 109}. The most compelling evidence that systemic immune activation may be closely tied to HIV disease progression comes from observations made in the natural host of SIV, sooty Mangabeys (SM). Although much like HIV infected individuals, SIV infection is SM’s is associated with high viremia, generation of SIV-specific CD8+ T cells immune responses and a rapid fall in mucosal and peripheral CD4+ counts, SM do not progress to or develop AIDS like illnesses\textsuperscript{110}. Unlike in HIV infected humans or SIV infected RM, a pathogenic primate model of SIV associated with progression to an AIDS like illness, the non-pathogenic SIV infection in SM’s has been shown to be associated more with the host-immune response to the virus rather than the virus itself\textsuperscript{110, 111}. Following SIV or HIV infection, immune response in SMs, RMs and humans are similar, and a sharp increase in viremia is accompanied by systemic immune activation\textsuperscript{110, 112, 113}. However, unlike the chronic immune activation that is observed in HIV infected individuals and SIV infected RM’s; SIV infected SM’s rapidly down-regulate systemic immune mechanisms including SIV-specific IFN-α production by plamacytoid dendritic cells\textsuperscript{111}.

Contrary to what is observed in SIV infected RM’s or in HIV infection, SIV infected SM’s have lower levels of CD4+ and CD8+ immune activation and no noticeable change in levels of T cell apoptosis\textsuperscript{110}. SIV infection in SM’s is also not
associated with elevated concentrations of LPS in blood plasma as SIV infected and uninfected SM’s have similar levels of LPS in their blood plasma\textsuperscript{75}. Down-modulation of immune responses and the ability to prevent/stop bacterial translocation despite high levels of viremia in SIV infected SM’s appear to be important in controlling SIV disease progression in the natural host of SIV. Furthermore, even in SIV infected SM’s a strong negative correlation has been observed between immune activation and peripheral CD4+ T cell counts, suggesting that immune activation has a negative impact on CD4+ T cell homeostasis even in the natural host\textsuperscript{114}. In SM’s, immune activation appears independent of SIV viral load and is dampened despite the presence of strong SIV-specific CD8+ T cell responses\textsuperscript{110}.

Along with the emerging role of systemic immune activation in HIV immunopathogenesis, localized immune activation (particularly in the genital mucosa) may play an important role in the acquisition and transmission of HIV (further discussed in section 1.7).

\section*{1.5 – Understanding HIV Transmission}

In 2007 according to the most recent HIV/AIDS data, HIV transmission occurred at the alarming rate of one new HIV infection every 10 seconds, which translates to almost 2.9 million new HIV infections annually\textsuperscript{115}. Almost 90\% of transmission occurs during sex\textsuperscript{116}. The probability of transmitting HIV through sexual contact is quite low,
ranging from a probability of 0.001% per coital act for female to male transmission and 0.005% for male to female transmission\textsuperscript{116}. Even high-risk behaviour like anal sex has a low probability of HIV transmission associated with it, between 0.001 – 1% per-contact (Figure 1.6), implying that sexual transmission of HIV is quite an inefficient process, something that may not seem apparent given the number of new infections that occur annually around the world (figure 1.5)\textsuperscript{116}. 
Figure 1.5 – HIV Transmission risk and exposure. Although the risk of transmitting HIV sexually is extremely small, it accounts for the majority of HIV infections world-wide.
The Reproductive Ratio (Ro) of an infectious disease is a mathematical formula describing the potential for spread of an infection in a population without pre-existing immunity\textsuperscript{117, 118}. The $R_o$ is the mean number of secondary cases resulting from a single infection in a population without pre-existing immunity. If the $R_o > 1$ the infectious disease should continue to spread within a population, while if the $R_o$ is $< 1$ it will not\textsuperscript{117, 118}. The reproductive ratio of an infectious disease is calculated using the following equation;

$$Ro = \beta Dc$$

In the case of HIV, the three determinants that influence the spread of HIV are

(1) $\beta$; efficiency of transmission – i.e. how likely an HIV-infected individual is to sexually transmit to his/her sexual partner(s) during a single contact (infectiousness);

(2) $D$; the duration an HIV-infected individual is infectious;

(3) $c$; the number of exposures during which transmission could occur (i.e. number of sex events or partners)\textsuperscript{117, 118}.

Anything that increases the reproductive ratio will result in an increase in the reproductive ratio of the infectious disease. On a population level, there are several biological factors including the stage of HIV infection, co-infection with sexually transmitted infections, and host genetics all of which will be discussed in detail in the following sections.
Along with biological factors, social determinants may also contribute to the spread of infectious diseases. For HIV these include 1) the number of sexual partners 2) the use to protection within a population and 3) cultural norms around sex and sexuality; such as the importance of preserving virginity, which may lead to alternate sexual practices (e.g. anal sex) in an attempt to preserve virginity. A recent mathematical model suggested that the HIV epidemic would be unable to sustain itself if a model of serial monogamy was followed (Ro = 1.09 in this case). However if high-risk social behaviour including random mixing of sexual partners within a population is included in the model the reproductive ratio almost doubles (Ro = 2.15), meaning that every HIV infected individual would infect 2 others, enough to sustain the growth of the epidemic.

Although social factors surrounding HIV are an intrinsic part of the HIV transmission equation, they are beyond the scope of this thesis. The section below focuses on some of the biological co-factors associated with sexual transmission and acquisition of HIV.

1.6 – HIV Acquisition and the Genital Mucosa

The majority of HIV is transmitted sexually as HIV infected individuals tend to shed cell free (HIV RNA) and cell-associated viral particles in their genital secretions. Even though not all the potential sites of entry or their relative contributions to HIV transmission or acquisition are known, understanding the immune-pathogenesis of HIV
transmission is pivotal in the development of more targeted and effective prevention interventions. Below is an overview of some of the known mechanisms utilized by HIV to enter and establish infection across the genital and rectal mucosa.

1.6.1 – The Female Genital Tract

Between 30% and 40% of all new HIV infections are acquired across the female genital mucosa\(^1\). Therefore, a great deal of work has been done to better understand how the anatomy of the female genital tract relates to HIV acquisition at this site. As in vivo human studies are difficult to perform, several alternative models; including the use of non-human primate models, \textit{ex vivo} and \textit{in vitro} organ culture systems using cervicovaginal tissues obtained from human surgical samples have been used.

The lower female genital tract consists of the vagina, ectocervix and the endocervix, all of which have very different anatomic and physiological properties. Although the relative risk(s) of acquiring HIV at the various anatomical sites in the FGT remains unclear, it is generally believed that the majority of HIV is acquired through the endocervix. Unlike the endocervix, which is lined with a thin mono-layer of columnar epithelial cells, the vagina and the ectocervix have multilayered squamous epithelial cells (between 20 and 45 cells thick) lining the outside, which offers much better protection from pathogens including HIV \(^{120, 121}\). However, the vagina and ectocervix are prone to trauma, particularly micro-abrasions during sexual intercourse, which may compromise the patency of the epithelial lining and provide a portal of entry for HIV \(^{120-122}\).
Despite the thick lining of squamous epithelial cells surrounding the vaginal mucosa, vaginal acquisition of HIV has been demonstrated in vitro in the absence of sexual trauma and/or microabrasions\textsuperscript{123}. One possible mechanism for viral entry is hypothesized to be through specialized antigen presenting cells located in the vaginal mucosa and the skin called Langerhan cells (LC’s). LC’s are capable of directly binding HIV, and are found in abundance in the vaginal epithelial layers\textsuperscript{124, 125}. LC’s, like other dendritic cells, are immune sentinels that monitor the periphery often extending their processes across epithelial tight junctions into the vaginal lumen\textsuperscript{126}. Upon antigen recognition the vaginal LC’s home to the draining iliac lymph node and activate CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. Several in vitro studies using human tissue and in vivo studies carried out in the SIV model of infection at least two pathways of HIV-DC interaction have been suggested. HIV can either infect LC through a CD4-CCR5 mediated pathway\textsuperscript{127-129} or be internalized by LC through DC-SIGN or other C-type lectins, which facilitate binding and internalization of HIV\textsuperscript{130-132}. Upon activation LC’s migrate to draining lymph nodes, which are rich in HIV target cells thereby resulting in systemic dissemination of HIV\textsuperscript{128, 133, 134}. Although the role of LC in mediating HIV entry is not clear, it represents a potential mechanism of entry in the absence of vaginal trauma.

The endocervix is located proximal to the ectocervix in the female genital tract and is covered by a thin monolayer of simple columnar epithelial cells separating the lamina propria and the sub-mucosal layer from the cervical lumen. Although, it is unclear if the endocervix comes into direct contact with HIV following sexual intercourse, the endocervix presents an ideal site for HIV infection to occur as the cervical sub-mucosa is
rich in CD4+ cells, particularly the transformation zone\textsuperscript{135}. The transformation or transitional zone is a region of the cervix where the epithelial lining transitions from a thin layer of simple columnar cells (cervix) to the thicker stratified squamous epithelial cell lining (vagina)\textsuperscript{135}. The increased risk of acquiring HIV (and other sexually transmitted infections) observed in adolescent women\textsuperscript{136, 137} may be partly because premenarchal women have an enlarged transformation zone that extends into the vagina, which provides a larger surface area for contact with HIV during sexual intercourse\textsuperscript{138}. The single mono-layer of columnar cells of the transformation zone makes it particularly susceptible to HIV as it affords little protection to the target cells (CD4+ T cells and macrophages) in the sub-mucosa\textsuperscript{135}.

Although the epithelium in the cervix is not as thick as that of the vagina, endocervical epithelial cells are covered by a layer of mucus, which may serve to immobilize viral particles and prevent contact/entry with epithelial cells\textsuperscript{139}. Alternatively, mucous trapped virions may increase the exposure time of HIV to the cervical mucosa and therefore enhance HIV transmission at this site. Despite the differences in susceptibility to HIV between anatomical sites several recent studies have shown that all these sites (vagina, endocervix and ectocervix) are prone to infection by HIV\textsuperscript{134, 140-143}.
Figure 1.6 – Potential sites of HIV entry in the female genital tract.

Several potential sites of HIV entry in the female genital tract have been described and the above figure highlights the vaginal epithelium, the transformation zone and the cervix, all of which represent potential sites of HIV entry (designated by red arrows). The schematic illustrates the differences in the epithelial architecture at each anatomical site.
1.6.2 – The Male Genital Tract (MGT)

Although the surface area of the MGT exposed to virus is much smaller than the FGT, almost 70% of HIV infections in adult men are acquired through the penis, specifically the penile foreskin\textsuperscript{144, 145}. One of the earliest practices to protect men against sexually transmitted infections (e.g. syphilis) was the removal of the penile foreskin\textsuperscript{146}. Circumcision has been shown to reduce rates of some but not all STI’s\textsuperscript{147}, with the most dramatic reduction observed in HIV acquisition through the penis\textsuperscript{148}. The earliest suggestions that circumcision reduced the risk of acquiring HIV came from ecological studies in Africa where countries and regions, with similar sexual practices had very different rates of HIV prevalence\textsuperscript{148}. The most striking evidence was from Kenya, where HIV seroprevalence rates were four-times higher in western Kenya (Kisumu), where <20% of men are circumcised, compared to the rest of Kenya where >80% of men are circumcised\textsuperscript{115, 145}. Observations suggesting the potential link between circumcision and risk of HIV acquisition provided the impetus to conduct randomized clinical trials to investigate the relationship between circumcision and HIV. Large trials in both the Rakai district of Uganda\textsuperscript{149} and the Kisumu district in Western Kenya\textsuperscript{150} have recently demonstrated that circumcision reduced HIV acquisition rates by 50 – 60%\textsuperscript{149, 150}.

The increased risk of acquiring HIV through the foreskin is because the inner foreskin is lined with a thin layer of poorly keratinized epithelial cells\textsuperscript{144}, while the outer foreskin is covered with a thick layer of highly keratinized epithelial cells providing an almost impenetrable barrier to HIV\textsuperscript{151}. Detailed histological studies of adult foreskins
have suggested that although the inner and outer foreskin contain similar concentrations and numbers of CD4+ T cells co-expressing CCR5 and CXCR4, the inner foreskin contains more superficial Langerhan DC’s\textsuperscript{144, 152}. The thin layer of keratin over the epithelial monolayer and the superficial Langerhan DC’s make the inner foreskin particularly susceptible to HIV\textsuperscript{152}, as these cells are prone to infection and contain surface receptors that support viral attachment\textsuperscript{130}. Similar to the FGT, after activation LC’s home to local draining lymph nodes, which are rich in potential target cells for HIV including macrophages and CD4+ T cells. It is important to note that although the relative risk of acquiring HIV is much lower in circumcised men compared to their uncircumcised counterparts, it is still possible for circumcised men to acquire HIV through the penis, primarily through the urethera and penile salcus\textsuperscript{135}, and so safe-sex practices should always be followed during sexual encounters.

\textbf{1.6.3 – The Rectal Mucosa}

The rectal mucosa is highly susceptible to HIV as it contains none of the protective epithelial architecture (discussed above) found in the female and male genital tracts. The rectal mucosa consists of a single layer of columnar epithelial cells that separates the lumen from a very rich bed of activated lymphocytes that co-express high levels of HIV co-receptors CCR5+ and CXCR4+\textsuperscript{121, 153}. The thin epithelial lining and the permissive nature of the cells found in the sub-mucosa (section 1.4) makes it particularly susceptible to HIV, and as such anal sex is attributed with the highest risk of sexual transmission (Figure 1.6)\textsuperscript{116}. However, despite the high transmission risk associated with
anal sex, it is often overlooked when discussing HIV transmission, partly because the majority of HIV transmission world-wide occurs across the vagina. Although the number of studies evaluating the prevalence of anal sex in heterosexual couples are scant, a study in the United States involving 2,500 women between the ages of 19-29 found that 21.7% of heterosexual women practiced anal sex\textsuperscript{154}. Even more alarming was that two small studies carried out in Africa demonstrated that rates of anal sex in Africa is even higher than in North America, especially amongst sex workers\textsuperscript{155}, suggesting that anal sex may play a much larger role in fuelling the HIV epidemic than has been attributed to it\textsuperscript{156}. However, these small studies are highly controversial and the prevalence of anal sex within particular populations vary drastically between studies\textsuperscript{157}.

There are several factors that contribute to the susceptibility of the rectal mucosa to HIV including the increased risk of abrasions in anal tissue around the anus and inside the rectum, which would bring HIV virions into direct contact with activated immune cells found under the anal epithelium. Apart from physical tears, the thin epithelial lining in the rectal mucosa provides little protection against HIV; in fact, intestinal epithelial cells have been shown to transport HIV (primarily the CCR5-trophic strain) from the gut lumen into the sub-mucosa by transcytosis. This mechanism of entry brings HIV into direct contact with activated CD4+ T cells found just below the epithelial cell layer\textsuperscript{158,159}. The rectal mucosa also contains specialized cells called microfold cells (M cells), important for gut homeostasis (\textit{e.g.} nutrient absorption) and gastrointestinal immunity\textsuperscript{160}, which may facilitate HIV transport into the sub-mucosa from the gut lumen and make up between 5-15\% of cells of the gastrointestinal lymphoid tissue\textsuperscript{161}. M cells are immune
sentinels that help prime the gastrointestinal immune system by taking-up antigens from the gut lumen and presenting them to immune cells present in the underlying membrane\textsuperscript{161}. The ability of M cells to bind antigens from the gastrointestinal lumen and transport them to the sub-mucosa, making them an ideal conduit for HIV entry\textsuperscript{162, 163, 164}.

\textbf{1.7 – HIV RNA Shedding in Genital secretions}

\textbf{1.7.1 – Source of HIV RNA shed in Genital secretions}

Sexual transmission of HIV occurs because HIV infected men and women shed HIV in their genital secretions\textsuperscript{165, 166}. The source(s) of HIV RNA that is shed in genital secretions of men and women are not entirely clear.

Current evidence suggests that the majority of HIV shed in the female genital secretions originates primarily from the cervix and the upper genital tract\textsuperscript{167, 168}, although HIV RNA has been measured in the genital secretions of women that have undergone a hysterectomy\textsuperscript{169}. So, even though the majority of HIV RNA shed in female genital secretions may originate from the cervix, other sites must contribute to HIV shedding in the genital secretions of HIV-infected women.

Similarly, the source of HIV in the MGT has been difficult to pin-point, as the MGT is not accessible to direct sampling. However, studies carried out in HIV infected men suggest that the majority of cell free HIV RNA shed in semen plasma originates from the prostate and the bulbo-urethral gland\textsuperscript{167, 170, 171}. These sites are distal to the
seminiferous tubules as vasectomised men shed similar HIV RNA levels in seminal plasma as non-vasectomized men\textsuperscript{172}. Recent studies in acute and chronic SIV-infected non-human primates further confirmed earlier findings carried out in HIV infected men, demonstrating that CD4+ T cells in the prostate and the bulbo-urethral gland of non-human primates contained 100-fold more SIV RNA than CD4+ T cells isolated from other organs of the MGT including the testes and the epididymus\textsuperscript{173}.

HIV shed in genital secretions contains both cell free (as HIV RNA) and cell-associated virus (HIV proviral DNA)\textsuperscript{174}. Furthermore, even though the relative contribution of HIV RNA or cell associated HIV DNA to HIV transmission remains unclear, both cell-free\textsuperscript{175} and cell-associated\textsuperscript{176} virus have been shown to be infectious\textsuperscript{177}. Despite the strong correlation between HIV RNA levels in blood and genital secretions, several studies have documented compartmentalized sequences of HIV in genital secretions\textsuperscript{178, 179}, implying a local reservoir of replication. Typically, HIV RNA or HIV DNA is detected in the genital tracts of 10-20 \% of men and women on Highly Active Antiretroviral Therapy (HAART) despite undetectable viremia\textsuperscript{135, 180}, implying that the HIV virus is able to replicate within local organs of the male and female genital tracts.

1.7.2 – HIV shedding and stage of HIV Infection

The probability of sexually transmitting HIV to ones sexual partner(s) is closely tied to the amount of HIV RNA in blood (Figure 1.7)\textsuperscript{181, 182} and indirectly in genital secretions\textsuperscript{182, 183}. Modeling studies have shown that HIV sexual transmission follows a “U” shaped curve with rates of transmission being the highest very early and late in
infection\textsuperscript{184-186}. Although, the probability of transmitting HIV is highest early in infection and after progressing to AIDS, a lot more attention has been focused on the contribution of acute infection on the spread of the HIV epidemic. This is partly because, in addition to having a higher HIV viral load, acutely infected individuals are less likely to be aware of their infection status and may be less inclined to follow safe sex practices. Furthermore, individuals with advanced HIV disease are older than recently infected individuals and since age has been associated with reduced frequency of sexual contact and sexual partners\textsuperscript{185}, and therefore suggesting that acutely infected individuals probably have more sex and therefore would potentially contribute more to the transmission of HIV than those that have progressed to AIDS. The impact of acute infection on HIV transmission has been calculated, in longitudinal studies in sero-discordant couples, to be between 10 and 27 times higher following the first 5 months of infection than during chronic infection\textsuperscript{119,186} and 2-fold higher in individuals that progressed to AIDS\textsuperscript{187}. 

41
Figure 1.7 – HIV sexual transmission and blood viral load. The risk of sexually transmitting HIV from male to female and female to male is closely associated to HIV viral load in blood.
The chronic phase of HIV infection is typically asymptomatic and is when neutralizing antibodies and HIV-specific CD8+ T cell immune responses against HIV are generated (Figure 1.8). The generation of HIV-specific CD8+ T cells temporally coincides with a significant reduction in HIV RNA levels (Figure 1.8). Referring back to the reproductive ratio, even though the per coital risk of transmission (due to lower HIV RNA levels in blood and semen) during chronic infection is lower than during acute infection or AIDS (Figure 1.7), the chronic phase of infection is much longer. Therefore, despite a lower ‘β’; the ‘D’ and ‘c’ attributed to the chronic phase of infection are much larger, implying that chronically infected individuals may contribute substantially to the spread of HIV. A recent mathematical modeling study suggested that almost 70% of HIV transmission may be occurring in the chronic phase of infection.

1.7.3 – Sexually Transmitted Infections and HIV

After almost thirty years of researching the HIV epidemic, it has become increasingly evident that the spread of HIV globally is being assisted by several biological factors; one of the most important being sexually transmitted infections (STI). STI’s can be categorized into four categories (definitions from Galvin et al, 2004):

1) Systemic infections without mucosal disease: cytomegalovirus, HIV and hepatitis B virus
2) Genital ulcers: with Herpes simplex virus 1 and 2, *Treponema palladium* and *Heamophilus ducreyi*.

3) Mucosal inflammation: *Neisseria gonorrhoea, Chlamydia trachomatis* or *Trichomonas vaginalis*.

4) Changes in epithelial cells: Human pappillomavirus.

Epidemiological and cohort based studies have documented the synergistic relationship between HIV and STI’s, prompting the initiation of several clinical trials investigating the impact treating STI’s will have on HIV transmission and disease progression.

HIV-infected individuals co-infected with an STI tend to shed HIV RNA 2 – 3 times more frequently in their genital secretions, compared to HIV-infected individuals without an STI, making them more likely to transmit HIV during sex (increase in ‘β’)

A prospective study in Malawi found that HIV-positive men with urethritis were shedding 8-times (~ 0.46 log10 semen HIV RNA) more HIV RNA in semen compared to HIV-infected individuals without an STI. Antimicrobial therapy for the STI was associated with a drop in semen HIV RNA levels by 0.46 Log after two weeks of therapy. Furthermore, in the same study individuals that resolved their STI had comparable HIV RNA levels in semen to HIV-infected individuals without an STI. The impact of STI treatment on HIV RNA viral loads was also observed in the genital secretions of female sex-workers in Cote d’Ivoire. Antimicrobial therapy and resolution of an STI has been associated with a significant decrease in the proportion of
women shedding HIV RNA in genital secretions from 42% of women prior to STI treatment to 21% of women following treatment\textsuperscript{192}. Furthermore, STI treatment was also associated with a decrease in HIV RNA levels in genital secretions\textsuperscript{192, 195}.

Genital Ulcerative Disease (GUD) is associated with the greatest increase in frequency and concentration of HIV RNA shed in genital secretions\textsuperscript{196}. GUD’s also have a greater impact on HIV shedding as concentrations of HIV RNA shed in genital secretions was highest in individuals co-infected with a GUD compared to those infected with other non-ulcerative STI\textsuperscript{135, 195, 197}. Ulcers associated with an active GUD infection are also prone to bleeding during sex, further increasing the risk of transmitting HIV\textsuperscript{187, 198}. HIV RNA is also shed directly from ulcer, as HIV RNA has been isolated from the exudates of ulcers in co-infected women\textsuperscript{199}. In addition, subclinical HSV-2 reactivation in HIV infected individuals is also associated with increase HIV RNA shedding in the genital secretions of co-infected individuals, even in the absence of apparent ulcers\textsuperscript{200}. GUD’s, specifically \textit{Treponema palladium}, even in the absence of apparent ulcers have been shown to directly upregulate HIV gene expression in mononuclear cells, by enhancing the binding of nuclear factors (including NFkB) to the HIV promoter LTR region\textsuperscript{201}.

\textbf{1.7.4 – Sexually transmitted infections and HIV acquisition}

STI’s have been implicated in increasing the susceptibility to HIV, as HIV-negative individuals infected with an STI are 2-11 times more susceptible to HIV acquisition\textsuperscript{202}.
This is most marked for individuals with an ulcerative STI, which compromise the patency of the epidermis and mucosal barrier(s) and provides direct portals of entry for HIV. The most prevalent ulcerative GUD is HSV-2, with recent estimates suggesting that almost 25% of the adult population in the United States\textsuperscript{202} and as high as 70 – 80% of adults in some parts of eastern and southern Africa are infected with HSV-2\textsuperscript{200, 203}. The prevalence of HSV-2 and its ability to establish latent infection with episodic, often asymptomatic, reactivation is a cause for concern as an HIV-negative individual with a GUD is \textasciitilde{}3 fold (range 2.2 - 11.3 fold), more likely to contract HIV\textsuperscript{187, 193, 204, 205}. Data from the Mwanza trial in Tanzania also implicated \textit{Haemophilus ducreyi} (Chancroid) as an important factor associated with increased HIV acquisition, accounting for almost 25% of the HIV acquisition observed in that trial\textsuperscript{206, 207}.

Non-ulcerative STI’s like \textit{Neisseria gonorrhoea} and \textit{Chlamydia trachomatis} may also increase the risk of acquiring HIV, making individuals 3–4 times more susceptible to HIV infection because of inflammatory processes that are activated by STI infection(s)\textsuperscript{208}. Inflammation is the body’s response to a bacterial infection and typically results in recruitment of immune cells to the site of inflammation. Therefore, a localized infection, at the genital mucosa, leads to increased recruitment of immune cells like CD4+ T cells and macrophages to the genital mucosa. In one study HIV-negative women with a non-ulcerative STI had 2-times more cervical CD4+ T cell compared to women without an STI\textsuperscript{209}. Not only are there more CD4+ T cells present at the site of infection but also the inflammation at the site (due to the STI) activates CD4+ T cells, making them more permissive to HIV. Indeed a study conducted in HIV-negative women
infected with a non-ulcerative STI expressed three-fold more CCR5+ in CD4+ T cells in the genital tract compared to uninfected women²¹⁰.

The strong association between STI’s and increased HIV acquisition implies that interventions targeting STI’s may reduce the rate of HIV acquisition and transmission at a population level. However, several studies have documented that even though treatment of bacterial STI’s resulted in a significant reduction in the prevalence of bacterial STI’s with the cohort, azithromycin treatment was not associated with a reduction in the incidence of HIV-acquisition in female sex-workers²⁰⁵, ²¹¹ and in a community setting in Uganda²¹¹. Clinical trials examining the impact of therapy of STI’s have yielded mixed results with one early study showing a 40% reduction in HIV acquisition in a rural community in Tanzania²⁰⁷, while more recent studies showed no reduction in rates of HIV acquisition²⁰⁵, ²¹², ²¹³. In the more recent studies, although HSV-2 therapy reduced HIV RNA levels in blood²¹⁴ and the genital tract²¹⁵, it did not reduce the rate of sexual transmission of HIV²¹³.

1.7.5 – Viral synergy: CMV and HIV

The impact of symptomatic STI’s have been studied extensively (as mention in the previous section), the role of asymptomatic viral co-infections have been ignored despite their high seroprevalence in HIV-infected individuals and in the general population. The most prevalent herpes virus around the world is the Human Herpes virus-5 (HSV-5) or Cytomegalovirus (CMV), which infects almost 80% of the adult population worldwide and approximately 95% of HIV-infected individuals²¹⁶. CMV transmission
occurs from person to person, through contact with body fluids (urine, saliva, breast milk, blood, tears, semen, and vaginal fluids). CMV is a double stranded DNA human β-herpes virus with one of the largest viral genomes, producing more than 200 viral proteins in three overlapping phases; immediate early, early and late\textsuperscript{217, 218}. Similar to other herpes viruses, primary infection with CMV results in the onset of a persistent life-long infection characterized by periodic reactivation.

In the majority of cases (~ 92%), CMV infection in immune-competent adults is clinically asymptomatic and results in the onset of a latent infection that periodically reactivates without symptoms\textsuperscript{216}. Although, a small proportion of cases (< 8%), primary CMV infection in immune-competent individuals results in the onset of an infectious mononucleosis-like illness, followed by resolution and onset of individuals with diminished immunity or in immune-compromised individuals, such as AIDS, CMV reactivation can cause fulminant disease including retinitis, colitis, and encephalitis, which can become life-threatening if left untreated\textsuperscript{219}.

**1.7.5.1 Cytomegalovirus (CMV) – viral and host receptors**

A CMV virion houses a 235-kb DNA genome within a icosahedral protein capsid, which is surrounded by a protein layer called the tegument all of which are encapsulated by a viral envelope\textsuperscript{216}. The viral genome is divided into several internal repeat sequences termed unique long sequence (UL) and unique short sequence (US)\textsuperscript{220}. CMV is one of the most promiscuous viruses, and productive infection has been observed in almost every known vertebrate cell type including fibroblasts, smooth muscle cells, vascular endothelial cells, astrocytes, epithelial cells, trophoblasts, stromal cells and
hepatocytes\textsuperscript{221, 222}. CMV virions tether to ubiquitously expressed cell surface molecules including heparin sulphate proteoglycans (HSPG) and several cell surface integrins including $\alpha2\beta1$, $\alpha6\beta1$ and $\alphaV\beta3$\textsuperscript{223, 224}. Attachment to cell surface molecules is mediated primarily by two viral envelope proteins gB and gM, both of which contain integrin binding motifs\textsuperscript{224}. Following viral tethering via the various integrin receptors, CMV glycoprotein-B binds to a ubiquitous cellular receptor called Epidermal Growth Factor Receptor (EGFR) and facilitates CMV viral entry\textsuperscript{224}, although the role of EGRF in cellular entry of CMV has recently been contested\textsuperscript{222} and points towards yet-unknown surface receptor as the putative CMV entry receptor.

\textbf{1.7.5.2 – CMV infection and latency}

Viral attachment results in the fusion of the viral envelope with the host plasma membrane releasing the viral capsid and the tegument into the cellular cytoplasm\textsuperscript{216, 225}. CMV infection results in the onset of lytic infection or viral latency, which is largely dependent on tegument proteins. The tegument protein pp71 has been shown to be one of the most important determinants of whether infection becomes lytic or latent\textsuperscript{216}. Although the precise mechanisms still remain unclear, it appears that translocation of pp71 into the cell nucleus induces down-regulation of cellular repressive proteins and promotes the synthesis of immediate early proteins (IE)\textsuperscript{225}. The IE proteins are key mediators of the host cell environment and promote the expression of viral early genes that induce replication of viral DNA\textsuperscript{225}. Although the mechanism that results in the entry of latently infected cells to enter into lytic infection is still not clear, in vitro studies have suggested that inflammatory cytokines like IL-8 and IL-6 can induce reactivation of CMV from
latently infected cells\textsuperscript{226}. Furthermore, more frequent CMV reactivation is observed in immune compromised individuals such as in the elderly or in HIV-infected individuals suggesting that CMV replication is controlled by host immune responses\textsuperscript{226}.

1.7.5.3 – Evidence for CMV and HIV synergy

Even though the mechanisms of interaction between CMV and HIV are not completely understood, it is thought to be bidirectional in nature, as HIV appears to facilitate CMV replication and in turn CMV enhances the transcription of HIV virions\textsuperscript{227, 228}. The most convincing evidence for synergy between the two viruses comes from experiments done in cell culture that demonstrated that a cell line infected with CMV produced 20-times more HIV p24 protein compared to the same cell line in the absence of CMV\textsuperscript{227}. There are several possible mechanisms through which CMV may enhance HIV replication, as CMV could increase replication of HIV RNA by infecting the same cell and inducing HIV replication via the CMV IE protein, which has been shown to directly activate the HIV LTR\textsuperscript{229}. CMV may also indirectly stimulate the generation of cellular cytokines that, if released into the environment, would result in activation of the immune system and drive HIV replication in surrounding cells\textsuperscript{230}.

Apart from in vitro evidence for the synergy between CMV and HIV, there is a great deal of clinical evidence that points to the strong association between HIV and CMV. CMV-HIV co-infected individuals tend to shed CMV and HIV more frequently in their genital secretions compared to individuals that are mono-infected for either virus\textsuperscript{197, 231, 232}. Co-infected individuals also tend to progress 2.5 times faster to AIDS compared to HIV mono-infected individuals\textsuperscript{233}. Furthermore CMV/HIV infected infants born to co-
infected mothers have lower CD4+ T cell counts and higher HIV viremia compared to HIV infected infants born to CMV-negative mothers\textsuperscript{234}. CMV-shedding is also associated with increased concentrations of inflammatory cytokines in the female genital tract\textsuperscript{235}. Despite the number of studies carried out evaluating the role of CMV on HIV RNA in the genital tract, the impact that CMV reactivation has on levels of HIV RNA shed in the semen of co-infected men remains unclear. As the semen HIV level is a marker of how infectious an individual is, understanding the impact that sub-clinical reactivation of CMV on HIV RNA shedding in the semen of co-infected men is of substantial public health interest.

1.8 – Highly Active Antiretroviral Therapy.

The introduction of highly active anti-retroviral therapy (HAART) has transformed HIV from a fatal to a chronic disease, rapidly reducing mortality, morbidity and opportunistic infections associated with HIV infection (Figure 1.9)\textsuperscript{236,237}.

Conventional HAART is currently a cocktail of three antiretroviral drugs taken together, usually two nucleoside reverse transcriptase inhibitors (NRTI – section 1.8.1.1) with either a non-nucleoside reverse transcriptase inhibitor (NNRTI – section 1.8.1.2) or a protease inhibitor (PI- section 1.8.1.3)\textsuperscript{238}. HAART initiation results in a rapid drop in blood viremia, to below the limit of assay detection (> 50 copies/mL HIV RNA) often within two months on HAART in adherent individuals. The dynamics of HAART on systemic HIV RNA levels have been well documented, but very little is known about the
time it takes for HAART to reduce HIV RNA levels in the genital tract. As HIV shed in genital secretion is the principal mode of HIV transmission, understanding the impact of HAART on HIV levels in genital secretions provides insight into the impact HAART will have on the rates of global HIV transmission.

Figure 1.9 – HAART initiation and Death rates - Prospective data on the decrease in death rates attributable to HIV in a population before and after HAART initiation, historic data from 1995 to 2001. The figure demonstrates the temporal association between initiation of HAART and a dramatic decline in deaths per 100 person-years.
**1.8.1 – Major drug classes in HAART**

There are currently eight classes of antiviral drugs approved by the Food and Drug administration to be used for the treatment of HIV \(^{238}\). Four of the most commonly used drug classes that are incorporated in today’s effective HAART regimens are described below along with a brief overview of their mechanisms of action.

**1.8.1.1 – Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTI/NtRTI)**

NRTI’s were the first class of antiretroviral drugs developed and approved by the FDA for the treatment of HIV in 1987. NRTI’s are dideoxynucleotide analogues that target the HIV RT molecule and prevent HIV replication by incorporating into the new DNA molecule instead of a natural purine or pyrimidine (competitive inhibition). As NRTI analogues lack a 3’ hydroxyl group, once incorporated the next incoming deoxynucleotide cannot form a phosphodiester bond and the result is the premature termination of the synthesis of the proviral DNA molecule\(^{239}\). Prior to being incorporated, NRTI molecules have to be phosphorylated at the 4’ hydroxyl group by three cellular kinases to a tri-phosphate molecule\(^{239}\). A similar class of drug called the Nucleotide reverse transcription inhibitor (NtRTI) also works along the same mechanism of early chain termination, however molecules in this class already have one phosphate molecule at the 4’ position and so requires only two phosphorylation steps.
1.8.1.2 – Non-nucleoside Reverse Transcriptase Inhibitors (NNRTI)

A second class of antiviral molecules approved for treatment of HIV also targets the reverse transcriptase molecule. Non-nucleoside reverse transcriptase inhibitors (NNRTI) inhibit the action of RT using a different mechanism to that of NRTI’s. Molecules in this drug class prevent the action of RT by interacting with an allosteric non-substrate binding pocket found in HIV RT. NNRTI binding to HIV RT causes structural repositioning of amino acids located in the active site of RT. The structural repositioning of amino acids locks the active catalytic site into an inactive conformation and prevents RT from reverse transcribing the HIV RNA into DNA\textsuperscript{239, 240}. The binding pocket on HIV RT is also close to the substrate binding site and so NNRTI’s used in conjunction with NRTI’s result in a spatial and functional block of the HIV RT molecule\textsuperscript{239, 240}.

1.8.1.3 – Protease Inhibitors (PI)

Protease inhibitors, as the name suggests, are molecules that act as competitive inhibitors for the HIV protease (PR) molecule. HIV PR is a homodimeric aspartyl protease and plays a central role in the maturation of virus and assembly (see section 1.3)\textsuperscript{241}. Briefly, viral PR cleaves specific sites on the Gag-Pol polyprotein (p160) and in Gag (p55) generating viral structural elements that generate the viral matrix (MA), capsid (CA), and nucleocapsid (NC). PR mediated cleavage also generates functional enzymes including RT, IN and PR\textsuperscript{241}. Protease inhibitor molecules prevent the maturation of newly synthesized virions by mimicking and binding to the active site of PR on HIV gag polyprotein (see figure 1.7). The lack of protease processing arrests the maturation of
newly synthesized virions and results in the generation of immature virions unable to productively infect new cells \(^{241}\).

**Figure 1.10 – Sites of HAART drug action.** Illustrates the different stages of the HIV viral lifecycle and the mode of action of protease inhibitors, NNRTI and NRTI’s used in a HAART cocktail (depicted by a red X).
1.8.2 - HAART and HIV decay dynamics in blood

HIV production in blood is a dynamic cyclical process largely driven by activated CD4+ T cells and macrophages, whereby productively infected immune cells produce virions that then infect additional cells. Viral replication in CD4+ T cells is further facilitated by immune activation, as immune activation enhances viral replication and makes cells more susceptible to HIV infection\(^\text{242}\). HAART initiation results in a rapid reduction in HIV RNA accompanied by a systemic reduction in markers of immune activation and inflammation.

Decay of viremia after starting HAART is characterized by viral clearance and decay in three separate phases\(^\text{243, 244}\). The first phase occurs following the first two weeks of HAART initiation, and is characterized by a 2-log drop in HIV RNA concentrations in blood. The rapid drop is associated with preventing \textit{de novo} synthesis of HIV virions from productively infected CD4+ T–cells, which would typically replace the cleared virus from blood (half-life ~ 6 hours)\(^\text{104, 244, 245}\). The first phase of decay is accompanied by a dramatic drop in HIV viremia, with some studies showing that HIV viremia drops by almost 99% within two weeks of starting HAART\(^\text{244, 245}\). The second phase is much slower and is associated with the loss of chronically infected CD4+ T cells and macrophages with a lower activation state\(^\text{243, 246}\). After HIV RNA levels have fallen below the limit of assay detection, a very slow third phase of viral decay ensues and is thought to be mediated by the release of HIV RNA from resting CD4+ T cells and perhaps other sources, usually at levels below the limit of detection of most conventional
assays\textsuperscript{247, 248}. Although viral replication occurs at very low levels and intermittent blips do not represent the generation of resistance mutations\textsuperscript{249}, the latent viral reservoir is very stable in individuals on suppressive HAART, with current estimates suggesting that the half-life of the latent viral reservoir is \( \sim 44 \) months\textsuperscript{248, 250}.

### 1.8.3 - HAART and semen HIV RNA levels

The introduction of HAART has had a remarkable impact on people living with HIV, significantly enhancing their quality of life and preventing death. Apart from benefits to the individual starting therapy, HAART has also been shown to reduce HIV RNA levels in semen\textsuperscript{251, 252} and vaginal secretions\textsuperscript{253, 254}. Since the majority of HIV is transmitted sexually, several groups have suggested that wide-spread roll-out of HAART may be used as a way to reduce sexual transmission of HIV and prevent new infections\textsuperscript{255, 256}. In fact the Swiss Federal Commission for HIV/AIDS recently published an article stating that HIV-infected individuals on HAART with undetectable viremia for at least six-months, in the absence of an STI, are sexually non-infectious\textsuperscript{257}, a statement that has generated a great deal of controversy amongst individuals in the HIV prevention field\textsuperscript{258, 259}.

The WHO issued a statement that the assertion made by the Swiss Federal Commission for HIV/AIDS was premature, since the impact of starting HAART on HIV-RNA levels in semen requires further study\textsuperscript{258, 259}. Even though it is widely accepted that HIV-infected individuals on HAART shed significantly less HIV RNA in semen and are generally less infectious\textsuperscript{252}, several cross-sectional studies have documented that HIV-
infected men on HAART can shed both HIV RNA and HIV DNA in semen, despite undetectable blood VL\textsuperscript{177, 260, 261}. Furthermore, pharmacokinetic studies have shown that different classes of HIV drugs penetrate differentially into the male genital tract\textsuperscript{262-264}. Suboptimal concentrations of anti-retroviral drugs in the male genital tract may facilitate HIV shedding despite a blood VL < 50 copies/mL, and perhaps the generation of resistance mutations in viral populations, which have been found to be distinct from those in blood in some\textsuperscript{179, 265, 266} but not all studies\textsuperscript{267}.

In an effort to better elucidate the impact of HAART on HIV RNA levels in the semen of HIV infected men, we enrolled a cohort of HIV-infected therapy-naive men about to start HAART. Participants were followed prospectively, with blood and semen samples collected prior to HAART, every two weeks for the first month, and then monthly for 6 months after starting HAART. We also enrolled a cohort of men who had been on HAART with a blood VL < 50 copies/mL for at least 4 years and evaluated blood and semen HIV RNA levels. This study (Chapter 5) was aimed at better elucidating the impact of starting HAART on HIV RNA levels in semen, and the ability of HAART to prevent HIV shedding in semen of men on long-term completely suppressive HAART.

1.8.4- HAART and the GALT

Initiation of HAART is associated with suppression of plasma HIV RNA to below the limit of detection and accompanied by an increase in absolute blood CD4+ counts in peripheral blood. However the central role of the immune depletion of the GALT

58
(reviewed in sections 1.4.2.2 and 1.4.2.4) in HIV disease progression made it increasingly important to evaluate the impact of HAART on immune reconstitution in the GI mucosa.

Although HAART initiation is associated with normalization of systemic absolute CD4+ T cell counts, the GALT mucosa appears recalcitrant to immune reconstitution despite being on HAART\textsuperscript{268-270}. Several studies have demonstrated that HIV therapy results in incomplete and delayed restoration of GALT CD4+ T cell populations and remain significantly below CD4+ proportions observed in HIV-negative individuals\textsuperscript{268, 269}. Starting HAART has been demonstrated to be associated with a significant reduction in immune activation of CD8+ T cells\textsuperscript{89} and up-regulation of genes important for mucosal repair/regeneration and growth factor production\textsuperscript{91, 93}. HAART is also associated with reduced expression of pro-inflammatory genes and reduction of apoptotic cells in the GALT tissue of SIV infected RMs\textsuperscript{91}. Furthermore HIV infected individuals on HAART have significantly lower concentrations of bacterial products that translocate into peripheral blood from the gut lumen, demonstrating that HIV therapy enhances the patency of the GALT epithelial barrier\textsuperscript{96}.

Although it appears that the duration of HAART is unable to restore CD4+ T cell populations in the GALT to levels observed in HIV negative individuals, it had been hypothesized that HAART initiation during acute infection (early in infection) may preserve the gastrointestinal architecture and reduce the destruction of GALT CD4+ T cells\textsuperscript{270}. But recent studies in SIV infected RM’s have suggested that HAART initiation within 10 days of SIV infection failed to prevent the massive depletion of CD4+ T cells
in the GALT of SIV infected RMs\textsuperscript{271}. However, HAART initiation during acute infection was associated with better immune restoration of (almost two-fold more) GALT CD4+ T cells compared to individuals that initiated HAART later in infection\textsuperscript{91}, a phenomenon that has also been observed in the non-human primate model of HIV infection\textsuperscript{271}.

Despite all the studies carried out in HIV and SIV infections, it is still not clear if long-term completely suppressive HAART is able to restore CD4+ T cell populations in the GI mucosa. This is particularly important for over-all health as CD4+ T-cell proportions in the gastrointestinal mucosa have been linked to increased expression of genes involved in growth/repair and reduction in the expression of genes associated with inflammation, immune activation, and ultimately slower disease progression\textsuperscript{269}. Evaluating the impact of long-term completely suppressive HAART on immune CD4+ T cell populations would provide us with a better understanding of disease processes that influence/promote HIV disease progression, and the impact that therapy has on these processes.

1.9 – The Host immune system and HIV

The immune system is the body’s defence system and is essential for the clearance of invading pathogens from the body. The host immune system recognizes a plethora of antigens through several mechanisms. At its broadest, the immune system can be divided into two arms; the innate immune system and the adaptive immune system\textsuperscript{98}. 
1.9.1 – The Innate immune system

Innate immune responses are rapidly generated following antigen exposure but do not result in the generation of immunologic memory\textsuperscript{98}. The innate immune system is the body’s first line of inducible defence against infectious disease and is one of the earliest forms of immunity to have developed evolutionarily\textsuperscript{98}, as several human innate immune genes share substantial homology with immune mechanisms observed in insects and certain fungal species\textsuperscript{98}.

The innate immune system utilizes several immune mechanisms including pathogen specific receptors, immune proteins (complement and coagulation cascades), natural killer cells (NK), macrophages, mast cells, neutrophils and eosinophils\textsuperscript{272}. Innate immune responses shape the adaptive immune response through the release of cytokines, chemokines and cell surface co-stimulatory molecules that direct and facilitate homing of adaptive immune lymphocytes to sites of infection and inflammation\textsuperscript{273}. The interplay between the adaptive and innate immune systems still remain to be elucidated. Furthermore, recent findings have demonstrated the capacity of NK cells, a component of the innate immune system, to generate immunologic memory, blurring the lines between the adaptive and innate immunity\textsuperscript{274}.

1.9.1.1 – Pattern Recognition Receptors (PRR)

One mechanism for pathogen recognition utilized by the innate immune system relies on several families of pattern recognition receptors (PRR) that are expressed on the
surface of cells, within intracellular compartments and/or secreted into peripheral blood\textsuperscript{273, 275, 276}.

Toll-like receptors (TLR’s) represent the best known family of surface and intracellular receptors (twelve receptors of this family have been identified so far), and are expressed on the majority of immune cells including B-cells, macrophages, and NK cells\textsuperscript{275, 277}. Some TLR’s recognize several ligands, such as TLR2, which recognizes peptidoglycans and bacterial lipopolysaccharides, while others are more specific\textsuperscript{278}. Therefore, the twelve identified mammalian TLR’s recognize a plethora of ligands originating from viruses, bacteria, fungi, and parasites\textsuperscript{279}. TLR’s contain specialize motifs that recognize pathogen-associated molecular patterns (PAMP’s) that are unique to pathogens \textsuperscript{280}. Recognition of its corresponding ligand, activates TLR’s inducing the expression of host defence genes via two adaptor proteins (MyD88 or TRIF) that ultimately lead to the increased expression of anti-viral cytokines, chemokines, antimicrobial peptides, co-stimulatory molecules, MHC molecules and other factors central to the generation of effective host innate and adaptive immune responses\textsuperscript{273, 277}.

Secreted PRR’s include mannan-binding lectins (MBL), C-reactive proteins (CRP) and serum amyloid proteins (SAP)\textsuperscript{273}. All these secreted PRR’s are produced in the liver during acute and early phase infection \textsuperscript{278}. MBL’s recognize carbohydrate patterns commonly found on the surface bacteria, viruses, protozoa and fungi. Binding of MBL to cell surface carbohydrates results in the activation of the lectin pathway and the complement system \textsuperscript{275, 278}, both of which represent important mechanisms for host-
pathogen control they are beyond the scope of this thesis. CRP and SAP molecules bind to phosphocholine on the surface of pathogens, thus facilitating the recognition and ultimately pathogen phagocytosis.\textsuperscript{272, 273, 278}

### 1.9.1.2 - Cellular innate mechanisms

Although there are several molecules and immune cells that participate in the innate immune response, this section focuses on the most important innate cellular mechanisms that have been shown to be important in mounting anti-HIV responses.

Natural Killer (NK) cells are characterized by the absence of conventional antigen receptors such as immunoglobulin molecules or T-cell receptors (TCR) on the cell surface. NK cells are able to distinguish normal vs. abnormal expression of MHC class I molecules on somatic cells. As somatic cells express MHC class I molecules on their cell surface, NK cells are able to detect perturbations in the expression of MHC molecules on somatic cells, which are often a sign of viral infection or tumor transformation. NK cells, unlike other adaptive immune responses, recognize the absence of “self peptides” and not the presence of “non-self peptides”. Activation of NK cells is governed by a delicate balance between several activation and inhibitory killer immunoglobulin-like receptors (KIR) expressed on NK cells. KIR receptors bind to subgroups of HLA class I molecules and the ligation of inhibitory KIR’s by their ligands on healthy cells results in the inhibition of NK activation, protecting the cell from NK mediated lysis. Virus infection of a cell may result in decreased expression of
HLA class I molecules on the cell surface, a feature that is readily recognized by NK cells and results in NK-mediated lysis of the viral infected cell\textsuperscript{283}.

NK cells have long been thought of as a bridge between the innate and adaptive immune systems\textsuperscript{282}. Activated NK cells are capable of killing immature dendritic cells that have encountered antigen, despite normal surface expression levels of HLA class I molecules\textsuperscript{282}. However, upon encountering antigen(s), immature dendritic cells usually mature and upregulate HLA-class I expression, thereby becoming resistant to killing by activated NK cells\textsuperscript{282}. This process is thought to be important in determining the quality of the adaptive immune response generated\textsuperscript{282}, since dendritic cells that fail to mature following antigen exposure provide inadequate stimulation to T cells and lead to suboptimal generation of adaptive immune responses\textsuperscript{282}. Furthermore, recent reports have described properties of NK cells that are more typical of the adaptive immune system, including the ability to maintain immunologic memory\textsuperscript{274}. Recent adoptive transfer experiments of NK cells from CMV infected mice into naive animals resulted in a robust secondary expansion of NK cells up challenge by CMV and conferred protective immunity against murine CMV in these previously naive mice\textsuperscript{274}.

NK cells play a central role in controlling HIV replication, both through direct killing of infected cells and indirect (secretion of cytokines that suppress HIV replication) mechanisms\textsuperscript{284}. NK cells represent a major source of potent suppressive cytokines, including RANTES (regulated upon expression normal T cell expressed and secreted), MIP1\textalpha{} and MIP1\textbeta{} (macrophage inflammatory proteins)\textsuperscript{285} that indirectly suppress
replication of CCR5-trophic HIV strains. Ex vivo experiments have demonstrated an inverse correlation between NK cell function (direct and indirect) and viremia in HIV infected individuals.\textsuperscript{286}

**1.9.1.3 – Mucosal innate factors in the genital tract**

Most (> 99.5%) sexual encounters do not result in HIV transmission. This suggests that natural innate immune defenses play an important role in HIV protection. Several innate mucosal factors, including secretory leukocyte protease inhibitor (SLPI), lactoferrin, Elafin and RANTES are present in genital tract sections at concentrations that have been shown to prevent infection of activated CD4+ T cells \textit{in vitro}\textsuperscript{287-289}. Although the impact of these innate factors on HIV transmission or acquisition \textit{in vivo} is not clear, women who are highly exposed to HIV but remain persistently HIV sero-negative (HEPS) had 10-fold higher concentrations of RANTES in the genital mucosa compared to HIV-uninfected women at risk of becoming HIV infected\textsuperscript{290}. In addition, concentrations of SLPI in the vaginal mucosa have also been associated with a reduced probability of perinatal HIV transmission\textsuperscript{291, 292} and elevated SLPI levels in the saliva of breastfed infants have been correlated with reduced HIV acquisition through breast milk\textsuperscript{292, 293}. Concentrations of innate factors have also been directly implicated in reduce HIV acquisition, as elafin levels in the CVL of HIV-negative women were associated with reduced HIV acquisition in women in a prospective case-controlled trial\textsuperscript{289}. 

65
Although these and other mucosal innate factors have been associated with protection against HIV in vitro and in vivo studies, the impact of these innate factors on shedding of HIV RNA in the genital tract of infected individuals is unclear.

1.9.2 – Adaptive Immune Responses

The hallmark of the adaptive immune system is the generation of long-term immunologic memory following exposure to an antigen. The adaptive immune system developed much later on the evolutionary scale than innate immunity, and is thought to have originally developed in jawed vertebrates\textsuperscript{98}. The adaptive immune system can be divided into two major arms; the humoral arm (B-cells) and the cell-mediated arm (T-cells). The two branches of the adaptive immune system work in concert and result in the generation of neutralizing antibodies and cell mediate immunity against almost an infinite number of pathogens. Unlike the innate immune system, the adaptive immune system recognizes a specific antigenic sequence and generates antibodies and/or a T cell immune response targeting that epitope. Following activation, naive B and T cells mature into memory B and T cells, which will elicit a more robust and faster immune response upon antigen re-exposure\textsuperscript{98}. Although several facets of the adaptive immune system are elicited and involved in the control of HIV replication, this thesis focuses on the role and impact of HIV-specific CD8+ T cell responses on RNA levels in blood and semen plasma.
1.9.2.1 - The T cell receptor (TCR)

TCR’s are member of the immunoglobulin super family of proteins that are expressed on the surface of T cells and are responsible for recognizing foreign antigens bound to MHC molecules. Germ line DNA contains four TCR multi-gene families, each of which encodes one of the four T cell receptor chains (α, β, γ, δ). Two T cell receptors have been isolated to date, each a heterodimer made up of one α and one β chain (αβ T cell receptor) and the other made up of one γ and one δ chain (γδ T cell receptor). Each of the chains that make up the heterodimer through the combination of four polypeptides chains; the variable (V), diversity (D), joining (J) and constant (C) regions. All the regions are encoded on separate gene segments that recombine, which results in increased potential diversity of each of the chains. The αβ receptor is expressed on over 95% of peripheral blood T lymphocytes and is composed of highly variable domains with almost $10^{16}$ different possible combinations.

1.9.2.2 - Generating CD8+ T cell immune responses

The ability of T cells to recognize and mount immune responses against antigenic peptides relates to the specificity of the TCR that is expressed on the surface of T cells. The TCR interacts with MHC class I or II molecules that are bound to non-self peptides (TCR’s that recognize self peptides are eliminated during T cell haematopoiesis in the thymus). The TCR works in conjunction with two co-receptors, CD4 and CD8, that are constitutively expressed on T cells and determine if the TCR interacts with cells expressing MHC class I (CD8+) or MHC class II molecules (CD4+). However, prior to
the generation of a TCR-MHC complex, MHC molecules have to be loaded with antigenic peptides, a multistep process that ultimately determines the specificity of the host T cell immune response.  

1.9.2.3 – Peptide processing and loading on MHC class I complexes

The TCR of a CD8-expressing T cell interacts with the MHC I–peptide complex and is essential in mediating the anti-viral immune response. MHC class I molecules, present on all somatic cells in the body, are loaded with viral peptides that are generated within the cell during viral replication. The majority of peptides loaded on to an MHC class I molecule are between 8 – 11 amino acids long, and are generated by the proteosome. Proteosomes are ubiquitous proteases responsible for the degradation of proteins into peptides fragments of variable lengths (ranging from between 4 and 20 amino acids long) and are responsible for the majority of peptides that are loaded onto MHC class I molecules. Usually proteosomes diffuse freely in the cytoplasmic environment of the cell and find protein substrates randomly. While the exact length of generated peptides can vary, peptides that are too small to be loaded on MHC molecules are degraded, and peptides that are too long are often further trimmed by other proteases in the ER (ER-aminopeptidases; ERAP) and then loaded on MHC molecules. Following proteosomal degradation, viral peptides are transported from the cytoplasm to the site of MHC class I biogenesis – the endoplasmic reticulum (ER).

Transport of the majority of viral proteins from the cytoplasm into the ER is facilitated by an ER resident peptide transporter called transporter for antigen processing.
(TAP) protein\textsuperscript{298, 299}. TAP readily binds cytoplasmic proteins and transfers them to the ER lumen. The large protein fragments are trimmed further by ERAP and then loaded on to the MHC class I molecules in the ER\textsuperscript{298, 299}. ERAP’s are designed to trim the amino terminal of the protein while proteosomes are particularly efficient at cleaving amino acids at the carboxy-terminus\textsuperscript{300}. Although this process appears to be quite efficient, the majority of peptides generated will not bind to MHC complexes, due to length or sequence incompatibility. In addition, the majority of proteosome processed peptides do not efficiently bind to TAP, again an issue of length. Of the peptides that are the right size, sequence and bind efficiently to TAP and the MHC as initially processed, the peptides may end up in the wrong MHC loading complex\textsuperscript{296, 301}. The ER has several MHC loading complexes and as a single cell can express up to six MHC I alleles most MHC loading complexes express only four of them (at the most)\textsuperscript{296}. Therefore getting the peptide processed and loaded right is quite an undertaking and the majority of processed peptides never get presented on an MHC molecule. The peptides that are just right and are between 8 and 11 amino acids long are then loaded on to MHC molecules (at the right MHC loading complex) and the MHC-peptide complex is transported to the plasma membrane and expressed on the cell surface awaiting recognition by a CD8+ T cell\textsuperscript{298, 299}. A passing naive or effector (previously antigen experienced) CD8+ T cell, specific for the antigen expressed on the MHC I molecule of a somatic or antigen presenting cells, becomes activated.
1.9.3 – HIV-specific CD8+ T cell responses

Although, direct evidence (like in the SIV model of infection discussed below) demonstrating the ability of HIV-specific CD8+ T cell immune responses to control HIV viremia is not available, there is ample evidence in the literature suggesting the importance of CD8+ T cell immune responses in controlling HIV viremia and driving viral evolution.

1.9.3.1 – Insights from the SIV model of infection.

The importance of CD8+ T cell immune responses were first demonstrated in depletion studies carried out in the SIV infected non-human primates. Monoclonal antibody mediated depletion of CD8-expressing cells was associated with a rapid rise in SIV viremia in RM and the return of CD8+ cells coincided with a rapid reduction in SIV viremia. Antibody mediated depletion of CD8+ T cell in acute infection resulted in persistent high viremia and a delay in the establishment of a viral set-point until the re-emergence of CD8+ T cells. CD8+ T cells were also found to be important in chronically infected RM’s as again antibody-mediated depletion of CD8+ T cell after an established viral set-point resulted in a 1 – 4 log increase in viremia, and the re-emergence of CD8+ T cells was associated with a reduction to the previously established viral set-point. Although the use of CD8-depleting monoclonal antibodies also resulted in depletion of NK cells, which have been shown to be important in virus control, these early studies in the SIV model of infection provided strong evidence for the role of CD8+ T cell responses in early and chronic SIV infection.
1.9.3.2 - CD8+ T cells and HIV viremia

HIV-specific CD8+ T cell immune responses represent some of the earliest adaptive immune responses detected following HIV or SIV infection \(^{306}\). The first, and perhaps most obvious, evidence suggesting that CD8+ T cells play an important role in controlling HIV viremia was when a temporal association was observed between the emergence of CD8+ T cells and a $10^2$-$10^3$ fold reduction in viremia during acute HIV infection \(^{307, 308}\). Additional studies further demonstrated that HIV-specific immune responses appeared to be associated with reduced viremia in acute \(^{309, 310}\), but not chronic HIV infection \(^{311, 312}\).

More recent studies suggest that CD8+ immune responses against certain HIV proteins, notably Gag, is associated with lower viremia compared to immune responses against other HIV proteins \(^{313-315}\). In fact, the detection of immune responses against HIV Env and several of the accessory proteins in these studies was associated with increased viremia \(^{313}\). Despite the lack of direct evidence linking HIV-specific immune responses to reduced HIV viral replication \textit{in vivo}, several lines of evidence now suggest that HIV-specific CD8+ T cell immune responses place considerable immunologic pressure on the HIV virus \textit{in vivo} (discussed below) even during chronic HIV infection.

1.9.3.4 - HLA and CD8+ T cell immune response

A recent study examining whole genome associations of host control found that the six most significant protective determinants against HIV disease progression were found in the MHC region of the genome \(^{316, 317}\). Several HLA alleles HLA-B57, HLA-
B27, and HLA-B51 are associated with long-term non-progression and control of viremia. Furthermore, not only were certain HLA-types better than others, but individuals homozygous for HLA I alleles appear to progress more rapidly to AIDS compared to heterozygous individuals. This finding may relate to the ability of individuals with a heterozygous genotype to present a more diverse set of epitopes, resulting in a broader HIV-specific CD8+ immune response, making it more difficult for the virus to effectively escape from host CD8+ immune responses318, 319.

1.9.3.3 –Long-Term HIV Non-Progression

The most obvious place to look for evidence of host CD8+ immune control of HIV would be in long-term non-progressors. Long-term non-progressors (LTNP) are HIV infected individuals that progress to AIDS much slower than the majority of individuals without therapy 320. Most, but not all, LTNP’s share certain “protective” human leukocyte antigen (HLA) genotypes (discussed in more detail below) 321. HLA-genotypes determine which peptide sequences will be preferentially presented on MHC I molecules, consequently determining the epitope specificity of the HIV-specific immune responses 67, 322.

However, not all LTNP’s have protective HLA-types. Thus, there are other potential immune mechanisms that may be slowing disease progression. Recent advances in flow-cytometry and fluorescence technology have allowed for the evaluating of multiple parameters in a single population (or cell). Multi-parameter flow cytometry refers to simultaneous analysis of more than 4 analytes in a cell population has provided a
great deal of insight into better understanding and assessing the functionality of HIV-specific CD8+ T cell immune responses. LTNP’s appear to have more functional CD8+ T cell immune responses, a larger proportion of CD8+ T cells from LTNP’s produce several cytokines in response to antigenic stimulation, than individuals with a non-LTNP phenotype. Multi-parameter flow cytometry studies have demonstrated that LTNP’s have more (higher percentage) CD8+ T cell that produce multiple cytokines and antiviral factors in response to their cognate antigen, compared to non-LTNP’s. Furthermore, as certain HLA types are predictive of LTNP, this study compared functionality of a LTNP with and without a protective HLA type and found that the functionality of CD8+ T cell in these individuals was independent of their HLA type. Even though in this study, the overall HIV-specific CD8+ T cell immune response did not correlate with HIV viremia, the proportion of HIV-specific CD8+ T cells with the most functional profile inversely correlated with HIV viremia.

1.9.3.2 – Viral Escape – Evidence for CD8+ T cell immune pressure

Viral escape mutations were first described almost two decades ago when it was observed that variations in viral sequences resulted in the loss or reduced recognition of HIV by CD8+ T cell immune responses. Since then understanding the impact of host CD8+ T cell immune responses and their influence on the generation of escape mutations represents some of the most compelling evidence that HIV-specific CD8+ immune responses exert pressure and determine HIV evolution.
Escape mutations are seen as early as 2 months following infection with SIV. SIV Tat specific CD8+ responses are observed within 3 weeks of SIV infection, but begin to decline after the first 2 months of infection\textsuperscript{326}. The rapid decline of Tat specific immune responses coincides with the development of a mutation in the specific Tat epitope (SL8), a mutation that reduces the binding efficiency of the peptide-MHC complex \textsuperscript{326}. Similar to observation in SIV infected RM’s, the ability of CD8+ immune responses to influence HIV evolution has been demonstrated at both the individual \textsuperscript{316, 327, 328} and population level\textsuperscript{329}.

HIV escape mutations are also observed late following infection as is best demonstrated in the escape mutations that arise in HLA-B27+ LTNP. The control of viremia in HLA-B27+ LTNP is attributable to HIV specific CD8+ T cell epitopes that target functionally important region(s) of the HIV virus restricted by these protective HLA types. The CD8+ T cell immune response in HLA-B27+ individuals is centered around one Gag epitope \``KRWIILGLNK`` more commonly referred to as the KK10 epitope\textsuperscript{330}, a highly conserved epitope in HIV. HIV-specific CD8+ T cell immune responses in HLA-B27 individuals generate robust immune responses against the Gag KK10 epitope and a rise in viremia in these individuals is associated with the generation of an escape mutation at this epitope in HLA-B27+ individuals. Although HLA-B27+ individuals are LTNP, the generation of an escape mutation to the KK10 epitope is associated with rapid disease progression to AIDS. The late generation of the KK10 escape mutation is partly because the most common escape mutation (R264K) at this site incurs a large fitness cost and the virus must negotiate this fitness cost with additional
mutations called compensatory mutations. A compensatory mutation is a change in another section of the viral sequence that partially restores the fitness of the virus and is most often seen in B27-positive individuals where the R264K mutation is always preceded by a L268M compensatory mutation. Although compensatory mutations alleviate some of the fitness cost incurred by the virus as it mutates away from host CD8 immune response, viral mutants that incur a high fitness cost with that escape mutation often revert back to wild-type following transmission to an HLA-mismatched individual.

The generation of escape mutations, despite the fitness cost associated with these mutations have been shown in HIV infected individuals during all stages of HIV infection including acute and chronic infection. These findings provide strong evidence for the central role played by HIV-specific CD8+ T cell immune responses to controlling viremia at all stages of HIV infection.

1.9.4 – HIV-specific CD8+ T cell responses in the genital mucosa

1.9.4.1 – Characterizing CD8+ T cell in the genital mucosa

As the majority of HIV is transmitted sexually, the male and female genital tracts represent the initial sites of the majority of exposures to HIV and SIV. Perhaps the presence of HIV-specific CD8+ immune responses in the genital tract of uninfected individuals may be able to protect against infection following exposure. HIV-specific CD8+ T cell immune responses have been previously characterized in the genital tracts. CD8+ T cell have been detected in the vaginal mucosa of SIV-infected rhesus
macaques$^{338}$, HIV-infected women$^{339}$ and in the semen of HIV-infected men$^{340}$. In addition, the majority of CD8+ T cell immune responses expressed the $\alpha$$\beta$ TCR, similar to T cell populations in peripheral blood. Similar to evidence found in peripheral blood, CD8+ T cell population isolated from the vaginal and cervical mucosa of SIV-infected macaques and HIV-infected women were able to directly lyse SIV and HIV infected cells; respectively$^{338, 339}$. However, it is not clear if HIV-specific immune cells in the semen of HIV infected men are able to control HIV RNA shedding in semen.

1.9.4.1 – Pre-existing HIV-specific CD8+ T cell immune responses in the genital tracts.

The potential of genital tract immune responses to perhaps thwart SIV infection was originally demonstrated in a small study that evaluated the relationship between the presence of pre-existing cytotoxic CD8+ T cell in the lamina propria and protection after exposure to SIV$^{341}$. Briefly, Rhesus Macaques exposed rectally to limiting doses of two molecular SIV clones at baseline and then exposed to a heterologous SIV strain after several weeks$^{341}$ demonstrated that RM’s with a detectable Env-specific CD8+ T cell immune responses in the colonic mucosa were protected against a heterologous SIV strain (SIV/deltaB670)$^{341}$. This evidence suggested that mucosal CD8+ T cell immune responses may play an important in protecting the host against sexual acquisition of HIV. More evidence highlighting the importance of pre-existing CD8+ mucosal responses has since been published characterizing HIV-specific CD8+ T cell immune response in the cervix of HIV-infected$^{342}$ and Highly Exposed Persistently Seronegative (HEPS) women$^{343, 344}$. Indirect evidence for the importance of an inducible immune mechanism
was published as seroconversion of HEPS women commonly occurred following a break from sex work, and a break from sex work was associated with waning of CD8+ T cell responses \(^{344,345}\). Although prospective studies assessing the association between seroconversion and waning mucosal CD8+ T cell immune responses have not been evaluated, in part due to the difficulty associated with carrying out studies with limited cell numbers isolated from genital tract sampling, the indirect evidence linking mucosal HIV-specific immune responses and protection against infection is quite compelling and requires further study.

1.9.4.2 - HIV shedding and HIV-specific immune responses in HIV infected individuals.

The majority of HIV infected individuals shed HIV in their genital secretions \(^{166,232,346}\) and it is exposure to genital fluids containing virus during coitus that accounts for the majority of HIV transmission events world-wide. Although HIV-specific immune responses have been detected in the genital tracts of HIV-infected men \(^{340}\) and women \(^{339}\), very little is known about the associations between mucosal CD8+ immune responses and HIV RNA shedding in HIV infected individuals. CD8+ T cells isolated from mucosal sites have been shown to share HLA restriction patterns to systemic CD8+ T cell populations, suggesting that mucosal immune responses may influence HIV RNA levels shed in genital secretions \(^{347}\). Since genital HIV levels are key predictors of HIV transmission, we decided to investigate if HIV-specific CD8+ T cell immune responses correlated with HIV RNA shedding in blood and the semen of HIV infected therapy naive men.
CHAPTER 2: ROLE OF SYSTEMIC IMMUNE RESPONSES ON SEMEN HIV SHEDDING.
2.1 - Introduction

HIV-specific CD8+ T lymphocytes have been shown to play a critical role in host control of HIV replication. There is a clear temporal relationship between the development of HIV-specific CD8+ T cells and viral control during acute HIV infection\textsuperscript{307, 348}. Experimentally induced CD8+ T cell depletion in acute and chronically infected non-human primate models of HIV, using CD8+ monoclonal antibodies, resulted in a significant increase in plasma viral load (VL) and a return to steady state levels of virus that coincided with the return of SIV-specific CD8+ T cells immune responses\textsuperscript{302, 303}. In addition, epitope-specific CD8+ T cells place considerable evolutionary pressure on the virus in both primates and humans\textsuperscript{326, 349-353}. The magnitude of the systemic HIV-specific CD8+ T cell response has been inversely correlated with rates of disease progression\textsuperscript{354} and plasma VL in some studies\textsuperscript{310, 314, 355}, but not others\textsuperscript{311, 312, 356}. This may be because the inverse relationship only applies during acute HIV infection\textsuperscript{357-359}, or to CD8+ T cells recognizing specific HIV gene products, such as Gag\textsuperscript{313, 314}.

HIV plasma VL is strongly associated with the probability of sexual HIV transmission\textsuperscript{116}, likely because plasma VL correlates with the amount of virus shed in semen\textsuperscript{360-363} and female genital tract secretions\textsuperscript{166, 364, 365}. HIV-specific CD8+ T cells can be detected in the semen\textsuperscript{340} and the female cervix\textsuperscript{342, 344, 366} of HIV-infected individuals. Furthermore, the specificity, function and ontogeny of HIV-specific CD8+ T cells in the genital tract of infected individuals are identical to T-cells found in the blood\textsuperscript{347}. We
therefore decided to study the relationship between systemic HIV-specific CD8+ T cell immune responses and HIV shedding in the male genital tract.

2.2 - Methods

2.2.1 – Study Population.

Antiretroviral therapy-naive men with chronic HIV-1 infection were recruited through the Canadian Immunodeficiency Research Collaborative (CIRC), Toronto, Canada. Clinicians were asked to enrol participants not expected to start antiretroviral therapy within the next two years. All subjects provided informed, written consent. The study protocol was approved by Research Ethics Boards at the Mount Sinai Hospital, the University Health Network, Toronto, and at the University of Toronto.

2.2.2 - Sample processing.

Paired blood and semen samples were collected from 20 chronic HIV infected treatment-naïve men enrolled through the Canadian Immunodeficiency Research Collaborative Inc (CIRC), Toronto, Canada. Semen samples were collected by masturbation into a dry sterile container, and immediately transported to the clinic at room temperature; blood was collected into acid citrate dextran. A first-void urine sample was screened for the presence of leukocytes (Bayer Diagnostics, Puteaux Cedex, France), and for infection by either Neisseria gonorrhoeae or Chlamydia trachomatis (Amplicor CT/NG assay, Roche Diagnostic, Quebec). Any participant with a positive result was excluded from analysis. Seminal plasma was isolated by centrifugation (~850g / 10 min),
and peripheral blood mononuclear cells (PBMC) were obtained through Ficoll-Hypaque
density centrifugation.

2.2.3 - HIV-1 shedding in semen.

Blood and semen RNA viral load (VL) was measured using the Versant HIV-1
RNA 3.0 assay (bDNA; Bayer Diagnostics, Puteaux Cedex, France) which has been
validated as a robust assay to measure HIV RNA in semen, and RT-PCR based assays
have been found to be inhibited by semen constituents in previous studies \(^{367}\). HIV RNA
viral loads were measured directly in blood and seminal plasma.

2.2.4 – Mapping HIV-specific CD8+ responses to HIV.

IFN\(\gamma\) ELISpot assays were set up using PBMC at \(1\times10^5\) per well, as previously
described\(^{368, 369}\). A matrix of 756 15-mer peptides overlapping by 11 amino acids,
spanning the clade B HIV-1 genome (AIDS Research and Reference Reagent Program,
Division of AIDS, NIAID, NIH) was established, with each peptide appearing uniquely
in 2 separate pools at a final working concentration of \(2\mu\text{M}\). HIV-specific CD8+ T cell
responses were counted using an automated ELISpot counter (Cellular Technology Ltd,
Cleveland, OH). A positive HIV-specific CD8+ response was defined as an HIV specific
response that was at least (1) two-fold higher than background (PBMC + 2\(\mu\text{m DMSO}\)),
and (2) \(\geq100\) spot forming units (SFU)/ \(10^6\) cells. All positive responses were confirmed
using individual 15-mer peptides.
2.3 - Results

No inhibition was evident when assaying seminal plasma viral load. There was a trend to an inverse relationship between peripheral blood CD4+ T cell count (mean 574 cells /mm$^3$: range 350-850 cells /mm$^3$) and the HIV VL in both semen and plasma (P=0.2 for both). VL in seminal and blood plasma were closely correlated (r=0.5; P=0.03; Figure 2.3), although viral levels in blood were approximately ten-fold higher than in semen (mean, 4.3 vs 3.3 log10 copies /ml; P<0.001, paired t-test). Study participants could be divided into three sub-groups, based on their semen viral load$^{370}$ (a) 3/20 (15%) had no detectable virus in semen (≤ 50 copies /ml) (b) 3/20 (15%) had high levels of HIV in semen (≥ 0.6 log$_{10}$ blood VL), and (c) 70% (14/20) of individuals had detectable semen VL < 0.6 log$_{10}$ blood VL. In one individual, the semen VL was ten-fold higher than in blood (5.3 vs 4.3 log10 copies /ml) in the absence of a detectable sexually-transmitted infection or urethral inflammation.

Systemic CD8+ IFNγ responses were detected in all participants. A total of 159 epitopes were recognized (mean 8 epitopes per participant, range 2-20 epitopes), and all were confirmed to be CD8+ T cell mediated by flow cytometry with intracellular cytokine staining. Responses focused on Gag (37% of epitopes), Pol (20%), Nef (20%), Env (13%), and less commonly on Tat, Rev, Vpr, Vpu and Vif (figure 2.1). The mean total magnitude of CD8+ T cell IFNγ responses for each participant was 6,776 SFU/10$^6$ PBMC (range 1,135–25,955 SFU/10$^6$ PBMC). Neither semen nor blood VL correlated with the total HIV-specific CD8+ response magnitude (semen: r=0.1, P=0.6; blood:
r=0.3, P=0.3) or the response breadth, defined as the total number of HIV epitopes recognized in an individual (semen: r= -0.03 P= 0.9; blood: r=1.0, P=0.7; Figure 2.2). While there was an inverse association between both the magnitude and breadth of Tat-specific responses and semen HIV shedding (r = -0.5 for both; P= 0.02; P= 0.03 respectively), only 3/20 participants responded to Tat, limiting our power to draw firm conclusions, and significance was lost when correcting for multiple comparisons. No association was seen between the magnitude or breadth of Gag-specific CD8+ T cell responses and VL at either site (semen: r=0.06, P=0.8; blood r=-0.09, P=0.7; Figure 2.1).

2.4 - Discussion

In conclusion, we have confirmed that there is a direct correlation between HIV VL in the semen and blood, and that the VL in semen tends to be approximately ten-fold lower than in blood. A subset of HIV-infected therapy naive men tended to shed more HIV RNA in semen compared to blood (super-shedders). Super-shedders are of particular interest as they may be more likely to transmit HIV to their sexual partner(s). These individuals merit further investigation as to why they are shedding more semen HIV. Understanding the mechanism that may be responsible for super shedding of HIV is particularly important from a public health standpoint as such individuals could pose an increased HIV-1 transmission risk116.
Furthermore, a better understanding of the immune correlates of viral shedding in semen will be important to inform the development of immunotherapeutics, topical microbicides and rational public health policy and interventions. However, we found no association between systemic HIV-specific CD8+ T cell responses and semen HIV VL, or blood plasma VL. In addition, no association was seen between Gag-specific systemic responses and viral levels in either blood or semen. These results show that systemic HIV-specific CD8+ T cell IFN\(_\gamma\) responses are not directly associated with viral levels in the blood or semen compartments. Elucidating the immune correlates of HIV semen shedding will be important in directing strategies to reduce sexual transmission.

Although these findings suggest that systemic HIV-specific CD8+ T cell immune responses are not associated with HIV RNA shedding in semen, more recent findings suggest that IFN-\(\gamma\) may not be the best surrogate marker to use when evaluating HIV-specific CD8+ immune responses. In addition, several reports have found that HIV-specific immune responses correlate with reduced HIV RNA levels in blood during acute, but not chronic, infection. As this study is cross-sectional and was done in a cohort of chronically infected individuals it is unable to evaluate if HIV-specific CD8+ T cell immune responses control HIV RNA shedding in semen during acute, but not chronic, infection as has been suggested by a recent study in SIV infected rhesus macaques\(^{371}\).
HIV-specific CD8+ T cell immune responses described as a percentage of the overall responses. The majority of responses were found to be against Gag, Pol, Env and Nef, with only a small proportion of responses targeting accessory proteins.
Neither the magnitude of overall HIV-specific immune responses ($r^2 = 0.3$, $p = 0.3$) nor the magnitude of Gag-specific immune responses ($r^2 = 0.36$, $p = 0.88$) correlated with semen HIV RNA levels.

**Figure 2. Magnitude of HIV-immune responses and semen HIV VL**

Linear regression of HIV-specific immune responses and semen HIV RNA viral load.
Figure 2.3– Correlation between HIV levels in blood and semen. Positive correlation between HIV levels in blood and semen plasma of HIV infected therapy naïve men ($r^2 = 0.5$, $p = 0.03$).
Figure 2. 4– Immune responses and semen HIV RNA levels.

Strong HIV-specific Gag and overall immune responses in blood were detected in blood using ELISpot. No correlation was observed between the breadth of total responses or Gag-specific immune responses with semen levels of HIV RNA.
Figure 2. 5–HIV-specific immune response and HIV viral load in blood.

HIV-specific CD8+ T cell immune responses to the entire HIV genome or to the Gag gene were detectable, no correlation was observed between the magnitude of total (entire HIV genome) or Gag-specific immune responses to HIV RNA levels in blood.
Figure 2.6– Breadth of immune responses and blood HIV viral load.

The breadth of HIV-specific CD8+ T cells to the entire HIV genome or Gag did not correlate with HIV blood RNA levels.
CHAPTER 3: HIV-SPECIFIC CD8+ LYMPHOCYTES IN SEMEN ARE NOT ASSOCIATED WITH REDUCED HIV SHEDDING
3.1 – Abstract

Sexual contact with HIV-infected semen is a major driving force behind the global HIV pandemic. Little is known regarding the immune correlates of virus shedding in this compartment, although HIV-1-specific CD8+ T cells are present in semen. We collected blood and semen from 27 chronically HIV-infected, therapy-naive men without common sexually transmitted infections or urethral inflammation and measured HIV-1 RNA viral load and cytokine/chemokine levels in both compartments. HIV-1 RNA levels were 10-fold higher in blood than semen, but discordantly high semen shedding was associated with higher semen levels of the pro-inflammatory cytokines IL-6, IL-8, IL-12, and IFN-γ. Virus-specific CD8+ T cell epitopes were mapped in blood by IFN-γ ELISPOT, using an overlapping HIV-1 clade B peptide matrix, and blood and semen CD8_ T cell responses were then assayed ex vivo using intracellular IFN-γ staining. HIV-specific CD8+ responses were detected in 70% of semen samples, and their frequency was similar to or higher than blood. There was no correlation between the presence of virus-specific CD8+ T cells in semen and levels of HIV-1 RNA shedding. Among participants with detectable CD8+ IFN-γ semen responses, their relative frequency was not associated with reduced HIV-1 RNA shedding, and their absolute number was correlated with higher levels of HIV-1 RNA semen shedding \( (r = 0.6; \ p = 0.03) \) and of several pro-inflammatory cytokines. Neither the presence nor the frequency of semen HIV-specific CD8_ T cell IFN-γ responses in semen correlated with reduced levels of HIV RNA in semen.
3.2 - Introduction

The HIV-1 pandemic has claimed over 20 million lives, and 42 million people are currently infected\textsuperscript{372}. Given the scale of the global pandemic and the fact that most transmission is sexual, HIV-1 sexual transmission is surprisingly inefficient, with a transmission risk of 0.1-1% per sexual contact\textsuperscript{116}. When sexual transmission of HIV-1 does occur, it takes place in two broad steps: first, the virus must be shed in the genital fluids (semen or cervico-vaginal secretions) of the infected partner; second, the virus must cross the mucosal epithelium of the uninfected partner and establish persistent infection\textsuperscript{142, 373, 374}. Previous investigations have found a strong association between levels of virus in blood and the probability of sexually transmitting HIV-1\textsuperscript{181}, and this likely relates to the fact that the amount of virus in the genital secretions tends to correlate with levels in the blood plasma\textsuperscript{135, 361, 370}. Despite the correlation between levels of virus in the blood and genital tract, some individuals may shed disproportionately high or low levels of HIV-1 in the semen in comparison to blood, and this phenomenon could have profound public health implications\textsuperscript{361, 375}. The immune correlates of HIV-1 shedding in semen, particularly in such “discordant” shedders, are poorly understood.

Systemic HIV-specific CD8+ T cells responses are clearly important in host HIV-1 immune control, as evidenced by their temporal association with viral control in humans and animal models, and by the strong immune selection pressure that they place on the virus\textsuperscript{336, 353, 354}. However, the direct association between the presence/frequency of systemic HIV-specific CD8+ T cells and the blood VL is less clear, with different groups
reporting an inverse correlation\textsuperscript{310, 311}, a positive correlation\textsuperscript{312}, or no correlation at all\textsuperscript{342, 375}. HIV-specific CD8+ responses are present in the genital tract of both HIV-infected, and exposed uninfected subjects\textsuperscript{339-341, 344, 347, 366}, but their role in controlling levels of viral shedding at mucosal surfaces, or in mediating protection against infection, has not been elucidated. Nonetheless, in animal models it is mucosal, not systemic, CD8+ lymphocytes that have been shown to be most critical in mediating protection against mucosal viral challenge\textsuperscript{376, 377}. Furthermore, after vaginal SIV infection the earliest virus-specific CD8+ T cells detected are in the vaginal mucosa\textsuperscript{76}, and HIV-1 is known to replicate preferentially within the mucosal lymphoid tissue of the gut\textsuperscript{74, 378}. These observations suggest that mucosal CD8+ T cell responses may be important in the local control of mucosally-acquired HIV-1 infection, and perhaps in controlling levels of viral shed in genital secretions and therefore infectiousness.

Although CD8+ T cell responses place a strong immune pressure on HIV-1, excessive systemic immune activation is associated with higher levels of plasma viremia, and with more rapid disease progression. Negative prognostic markers of immune activation include elevated expression of CD38, and increased levels of inflammatory cytokines such as interleukin 6 (IL6) and tumor necrosis factor alpha (TNF\(\alpha\))\textsuperscript{379-381}. In addition, high levels of HIV-1 RNA in the gut mucosa of HIV-1-infected men taking antiretroviral therapy are associated with gastrointestinal mucosal cytokine activation\textsuperscript{367}, presumably during increased local HIV-1 replication. However, the impact of the systemic or local (genital tract) cytokine milieu on levels of HIV-1 in semen is not known. In order to study the role of host immune factors in HIV-1 semen shedding, we
have assayed ex vivo CD8+ T cell responses in semen for the first time, and have examined the association between these mucosal CD8+ responses, the local and systemic cytokine milieu and levels of HIV-1 RNA in the semen of HIV-1-infected, therapy-naive men.

3.3 - Methods

3.3.1 - Study population.

Antiretroviral therapy-naive men who have sex with men (MSM) with chronic HIV-1 infection were recruited through the Canadian Immunodeficiency Research Collaborative (CIRC), Toronto, Canada. Clinicians were asked to enroll participants not expected to require antiretroviral therapy within the next two years. All subjects provided informed, written consent. The study protocol was approved by Research Ethics Boards at the Mount Sinai Hospital, the University Health Network, Toronto, and at the University of Toronto.

3.3.2 - Sample processing.

At the recruitment visit (Visit 1) 24 ml of venous blood was collected into acid citrate dextran for CD8+ epitope mapping. At two subsequent visits, paired blood and semen specimens were collected within an hour of each other. Semen samples were collected by masturbation into a dry sterile container at Visit 2, and into 10 ml of sterile RPMI containing antibiotic and antimycotic at Visit 3. Samples were processed within 2 hours of collection. A first-void urine sample was screened for the presence of leukocytes.
using a standard urine dipstick (Bayer Diagnostics, Puteaux Cedex, France), and for infection by either Neisseria gonorrhoeae or Chlamydia trachomatis using the Amplicor CT/NG assay (Roche Diagnostic, Quebec, Canada). Any participant with urethral leukocytes, gonorrhea or chlamydia was excluded from analysis. Seminal plasma was isolated by centrifugation at 850g for 10 minutes, and the semen cell pellet was then resuspended in 10ml of sterile R10 medium. Peripheral blood mononuclear cells (PBMC) and seminal fluid mononuclear cells (SFMC) were then isolated by Ficoll-Hypaque density centrifugation. Blood and seminal plasma were immediately frozen at –86oC.

3.3.3 - HIV-1 shedding in semen.

Blood and semen RNA viral load (VL) was measured using the Versant HIV-1 RNA 3.0 assay (bDNA; Bayer Diagnostics, Puteaux Cedex, France), since RT-PCR based assays have been found to be inhibited by semen constituents in other studies. Viral loads were either measured directly in blood and seminal plasma, or in the supernatant of semen samples collected into RPMI. In the latter case, semen viral load was corrected for the appropriate dilution factor.

3.3.4 - Epitope mapping using IFNγ ELISpot.

HIV-1-specific CD8+ T cell responses were mapped in blood using an IFNγ ELISpot assay, as previously described. PBMC were incubated at 1x105 per well with a matrix of 756 15-mer peptides, overlapping by 11 amino acids, spanning the entire clade B HIV-1 genome (obtained through the AIDS Research and Reference Reagent
Program, Division of AIDS, NIAID, NIH). Each peptide appeared uniquely in two separate matrix pools, at a final working concentration of 2µM. All responses detected using the matrix pools were confirmed using individual 15-mer peptides. Response frequencies were calculated using an automated ELISpot counter (Cellular Technology Ltd, Cleveland, OH), and a positive response was defined as an HIV peptide specific response (1) at least two-fold higher than background (PBMC + 2 µm DMSO), and (2) =100 spot forming units (SFU)/million cells. All responses were confirmed to be CD8+ mediated using IFNγ intracellular cytokine staining.

3.3.5 - Ex vivo stimulation and intracellular IFNγ staining.

A pool of all responding epitopes mapped in blood was used to examine HIV-specific IFNγ responses in blood and semen. Briefly, 1x10^6 blood mononuclear cells were incubated for 1 hr at 37°C in 5% CO2 with media alone, staphylococcus enterotoxin B (SEB, 3 µg/ml), or the HIV epitope pool (each peptide at 10µg/ml); semen mononuclear cells were split in two vials and incubated with media alone or the HIV epitope pool. Brefeldin A (Becton Dickinson Immunocytometry systems, San Jose, CA) was then added (1µg/mL), and samples were incubated for 5 hrs, permeabilized, and stained with combinations of CD3-FITC, CD3-PerCP, CD8-PE, CD8-PerCP, CD4-PE, CD69-FITC, CD103-PerCP, CD3-APC and IFNγ-APC (Becton Dickinson Immunocytometry Systems, San Jose, CA). Samples were acquired using a FACSCalibur flow cytometer (BD Systems), and data analysis performed using FlowJo analytical software (Treestar Inc. Ashland, OR). A positive response was defined as an HIV-
specific response (1) at least two-fold higher than the unstimulated control; and (2) a CD8+ HIV-1-specific response frequency of \(=0.05\%\).

3.3.6 - Cytometric bead array (CBA).

Cytokine levels in blood and semen plasma were measured using CBA (Becton Dickinson Immunocytometry Systems, San Jose, CA), according to manufacturer’s instructions. Cytokines/chemokines assayed were IL2, IL4, IL5, IL10, TNF\(\alpha\), IFN\(\gamma\), IL1\(\beta\), IL8, IL6, IL12p70, Regulated on Activation Normal T-cell Expressed and Secreted (RANTES), Monokine Induced by IFN\(\gamma\) (MIG/CXCL9), Macrophage Chemotactic Protein (MCP1/CCL2), and Interferon Inducible Protein-10 (IP10/CXCL10). Blood and semen plasma samples were incubated for 3 hrs at room temperature with a mixture of antibody-coupled beads (50 \(\mu\)L/sample) and PE-conjugated secondary antibodies (50 \(\mu\)L/sample) against each cytokine. Samples were then washed with 2% paraformaldehyde (PFA), resuspended in 150 \(\mu\)L of PBS wash buffer and analyzed using flow cytometry.

3.3.7 - Measurement of innate immune factors.

Levels of secretory leukocyte protease inhibitor (SLPI) and lactoferrin were measured in seminal plasma by ELISA (Quantikine Human SLPI kit, R&D Systems, Minneapolis, MN; and Enzyme Immunoassay for Human Lactoferrin, Oxford Biomedical Research, Oxford, MI) according to manufacturer’s instructions, after dilution at 1:64,000 and 1:3,000 in diluent buffer. ELISA plates were read in a standard
96-well Thermomax reader (Molecular Devices, Woking, UK) at 450nm using 570nm as a reference.

3.3.8 - Statistical analysis.

SPSS 11 for the Macintosh OS X (SPSS Inc, Chicago) was used for statistical analysis. Comparisons between the blood and semen of study subjects were performed using the paired samples t-test. The Mantel-Haenszel chi-square test with calculation of likelihood ratios and confidence intervals was used to compare dichotomous variables between study groups, and comparison of means between groups was performed by one-way analysis of variance (ANOVA). Linear association of continuous variables was assessed using the Pearson correlation coefficient. Independent associations of HIV-1 RNA shedding were examined in a multivariable linear regression model.

3.4 - Results

3.4.1 - Participant demographics.

Twenty-seven chronically HIV-1-infected, therapy-naive MSM consented to take part in the study. Their median CD4+ T cell count was 550 /mm3 (range; 120-1260 /mm3). All participants had been HIV-1 infected for at least six months. No participant had clinical urethritis or genital ulcer disease, or laboratory evidence of infection by T. pallidum, C. trachomatis, or N. gonorrhoeae. A first-void urine dipstick for leukocytes (a screen for urethral inflammation) was negative for all participants.
3.4.2 – HIV-1 RNA viral load in blood and semen plasma.

The median blood plasma viral load was 20,302 copies /ml plasma (range; <50 to 401,448 copies). HIV RNA was detected in the semen of 21/27 participants (78%), and there was no evidence of PCR inhibition in any assay. Levels of HIV-1 RNA in semen were highly variable, with a median value of 758 copies /ml semen plasma (range, <50-210,350 copies). Only one participant had an undetectable viral load in blood, and this individual also had no virus detected in semen. Six participants (22%) had an undetectable semen viral load, and their blood viral load was quite variable, with a median of 9,893 copies /ml plasma (range, <50-76,014 copies). As reported in a preliminary analysis, semen viral load was positively correlated with HIV-1 levels in blood plasma (pearson correlation coefficient, r = 0.4; P=0.02), although semen levels of virus were over ten-fold lower than in blood (3.1 vs 4.3 log10 copies /ml, P<0.001, paired samples t-test). There was an inverse correlation between blood CD4+ T cell counts and the RNA viral load in blood (r = -0.5; P=0.02), but not semen (r = -0.3, P=0.2).

Although HIV-1 RNA levels in blood and semen were correlated overall, some participants clearly demonstrated disproportionate shedding of HIV-1 RNA in semen. To evaluate the role of semen immune factors on discordant HIV-1 shedding; participants were divided into three groups: Group 1 had an undetectable semen viral load (non-shedders; N=6); Group 2 had a semen viral load < 60% that of blood plasma (concordant shedders; N=17); and Group 3 had a semen viral load ≥ 60% that in blood plasma (discordant shedders; N=4).
3.4.3 - Systemic HIV-specific CD8+ responses and semen HIV RNA.

Peripheral blood HIV-specific CD8+ responses were screened using the IFNγ ELISpot, and HIV-1-specific responses were detected in 27/27 participants. Most epitopes fell within HIV-1 Gag (33%), Pol (26%), Env (17%) or Nef (6.7%). Individuals responded to a mean of 8.6 epitopes (range; 3-20 epitopes), and the mean total magnitude (sum) of HIV-1-specific CD8+ responses was 6,833 spot forming units (SFU)/million cells (range; 770-25,955 SFU). All IFNγ responses were confirmed to be CD8+ T cell mediated using intracellular cytokine staining and flow cytometric analysis. As noted in a preliminary report\textsuperscript{384}, there was no association between the frequency of systemic (blood) HIV-1 specific CD8+ IFNγ responses and the RNA viral load in either blood ($r=0.3$; $P=0.1$) or semen ($r=0.2$; $P=0.4$). In addition, there was no association between the breadth or specificity of blood CD8+ T cells responses and viral load in either compartment. The weak inverse association previously described between the magnitude and breadth of HIV-1 Tat-specific responses and semen HIV-1 shedding was no longer apparent with the increased sample size ($P=0.8$ and $P=0.9$, respectively).

3.4.4 - Semen pro-inflammatory cytokines and HIV-1 RNA shedding.

Cytokine/chemokine levels were assayed directly in semen and blood plasma using the cytometric bead array. Semen levels of several pro-inflammatory cytokines were associated with disproportionate shedding of HIV-1. In particular, disproportionate semen HIV-1 RNA shedding was associated with increased levels of IL6 ($P=0.008$; Figure 3.1a), IFN-γ ($P=0.04$), IL-12 ($P=0.008$), and IL-8 ($P=0.02$; Figure 3.1b). No
association was seen between HIV-1 RNA shedding in semen and the other cytokines or chemokines assayed in semen, and there was no correlation between levels of cytokines/chemokines blood plasma and the HIV-1 RNA viral load in blood or semen.

3.4.5 - Ex vivo measurement of HIV-specific CD8+ T lymphocytes.

Based on the association of local inflammatory cytokines, including IFNγ, with HIV semen VL, we went on to examine the association of HIV-specific, IFNγ-producing CD8+ T cells in semen with HIV shedding. At a follow-up clinic visit, semen samples were collected from 20 participants by masturbation into 10ml of sterile RPMI, in order to preserve lymphocyte viability (with correction for the resulting dilution factor when reporting semen RNA viral load and cytokine levels).

Semen mononuclear cells were split into two equal aliquots, one used as a negative control and the second sample to evaluate semen HIV-specific responses assayed by IFNγ intracellular staining, after short-term ex vivo stimulation with a pool of HIV-1 epitopes previously mapped in blood. Cells were permeabilized and stained cells were acquired using flow cytometry. The median number of gated CD3+ T lymphocytes acquired per preparation was 2,862 cells (range; 476-70,030 T cells). Expression of CD103 is a marker for mucosal lymphocytes in both the male and female genital tracts, and the gastrointestinal tract\(^{340, 383, 385}\). CD103+ expression was measured for 12 subjects, and was higher in semen than blood CD3+ T cells (4.4% vs. 0.1%; \(P=0.003\), paired samples t-test; Figure 3.2). CD8+ T cells constituted a higher proportion of T cells in the semen than in blood (69.9% vs. 52.9%; \(P<0.001\), paired samples t-test), as has also been
described in the semen of HIV-uninfected men. Seminal CD8+ T cells were also more activated than those in blood, with much higher levels of spontaneous IFNγ release (1.3% of semen vs. 0.05% of blood CD8+ T cells; P=0.005, paired samples t-test; Figure 3.3b).

HIV-1-specific CD8+ IFNγ T cell responses were detected in the peripheral blood of all participants (20/20), and a response to the same pool of HIV-1 epitopes was detected in most semen samples (14/20; 70%; Figure 3b for representative example and summary data). Virus-specific CD8+ responses were present in semen at a significantly higher frequency than blood (2.6% vs 0.7% of CD8+ T cells; P=0.04). There were no detectable HIV-1-specific CD8+ responses in the semen of 6/20 participants (30%) despite a strong CD8+ response in blood (Figure 3.3c and Figure 3.3d, lower panel: representative example). In 4/6 cases this may have been due to a low numbers of gated T cell events (<1000 gated cells), but in 2/6 cases there was no semen response seen despite relatively high numbers of gated CD3+ T cells (mean, 5.5x10^3 gated T cells). Lack of response was unlikely to be due to anergy of semen T cells, since SEB stimulation induced a robust IFNγ response in the semen of both an HIV responder (4% of CD3/CD8+ T cells) and an HIV non-responder (11.5% of CD3/CD8+ T cells). The frequency of HIV-1-specific IFNγ CD8+ T cells in blood was similar in participants with and without CD8+ responses detected in semen (0.7% vs. 1.3% of CD8+ T cells, respectively; P=0.3). No HIV-specific responses were seen in the semen of 5 HIV
uninfected controls after incubation with a pool of the 8 HIV epitopes most commonly recognized by infected participants.

3.4.6 - Semen HIV-1-specific CD8+ T cell responses and HIV-1 RNA.

Semen levels of HIV-1 RNA at the time of the semen CD8+ assay, a mean of 177 days after the baseline assessment (range; 73-313 days), were highly correlated with HIV-1 RNA levels measured at enrolment (r=0.7; P=0.001; Figure 3.0), confirming the robust nature of our semen VL assay. There was no association between the presence/absence of detectable HIV-1-specific CD8+ T cell IFNγ responses in semen and the semen HIV-1 RNA load: the mean semen VL was 4.0 log10 RNA copies /ml in non-responders (N=6), and 3.7 log10 copies /ml in responders (N=14; P=0.6). Among participants with a detectable semen CD8+ response (N=14), no association was seen between the relative frequency of semen HIV-specific CD8+ T cells and the semen viral load, when the frequency of HIV-1-specific CD8+ T cells was expressed as a percentage of all CD8+ T cells (r=-0.2; P=0.5; Figure 3.5a).

Since the total number of T cells in semen varied widely between study participants, the absolute number of HIV-1-specific CD8+ T cells in a given semen sample was also calculated for each participant, and this absolute number was then transformed into a logarithmic scale. When analysed in this fashion, there was a positive correlation between the semen CD8+ T cell response and levels of HIV-1 RNA, so that a higher absolute number of HIV-1-specific, IFNγ-producing CD8+ T cells in semen was associated with higher semen HIV-1 RNA shedding (r=0.6; P=0.03; Figure 3.4b).
the total number of CD3+ T cells in each semen sample was calculated, and then transformed into a logarithmic scale, there was a trend to a positive correlation with the semen HIV-1 RNA load \((r=0.5; P=0.07)\). The absolute number of HIV-1-specific CD8+ T cells was closely related to the total number of CD3+ T cells in a given sample \((r=0.8; P=0.001)\).

Participants were again divided into three groups based on the concordance of HIV-1 RNA levels in blood and semen, as described earlier: non-shedders \((N=5)\), concordant shedders \((N=8)\) and discordant shedders \((N=7)\). There was no significant association between discordant HIV-1 semen shedding and the frequency of semen CD8+ T cell responses (percentage of semen CD8+ T cells 2.5, 2.0 and 1.8, respectively; \(P=0.8\)), or the absolute number of virus-specific CD8+ T cells (log10 absolute number of responding cells 1.6, 1.8 and 2.0; respectively, \(P=0.6\)).

3.4.7 - CD8+ T cell responses in semen and local inflammation.

Since our baseline analysis had found that discordant HIV-1 semen shedding was associated with higher local levels of the pro-inflammatory cytokines, we examined the relationship between semen CD8+ T cell responses, pro-inflammatory cytokines and semen HIV-1 RNA shedding. Levels of several pro-inflammatory cytokines in semen positively correlated with higher HIV-1 RNA shedding, IL6 \((r=0.4; P=0.05)\) and IL8 \((r=0.5; P=0.04; \text{Figure 3.6b and 3.6c})\). In addition, there was a positive correlation between the log10 absolute number of HIV-1-specific semen CD8+ T cell IFN\(\gamma\) responses and local levels of the pro-inflammatory cytokines TNF\(\alpha\) \((r=0.5; P=0.02)\), IL6
(r=0.6; P=0.006) and IL8 (r=0.5; P=0.02; Figure 3.6b). The absolute number of HIV-1-specific, IFNγ-producing CD8+ T cells in semen was therefore associated with both increased semen HIV-1 RNA shedding, and increased levels of pro-inflammatory cytokines in semen (Figure 3.6 a – c).

3.4.8 – Soluble innate factors in semen and HIV-1 RNA levels.

Both SLPI and Lactoferrin were detected in the semen of all participants. The mean level of SLPI was 29.0 µg/ml (median 27.3 µg/ml, range 1.1-72.5 µg/ml), and of lactoferrin was 168.4 ng/ml (median 169.2 ng/ml, range 5.1-566.4 ng/ml). There was no association between SLPI levels and semen HIV-1 RNA detection (33.1 µg/ml in HIV-1 shedders vs. 22.3 µg/ml in non-shedders; P=0.3), semen HIV-1 RNA levels (r=0.24; P=0.3), or any of the pro-inflammatory cytokines. Although lactoferrin levels did not differ based on the detection of HIV-1 RNA (195.1 ng/ml in HIV-1 shedders vs. 115.3 in non-shedders; P=0.3), there was a positive correlation between lactoferrin and levels of HIV-1 RNA (r=0.54; P=0.01). Concentrations of Lactoferrin also tended to be higher in discordant shedders (115.3 ng/ml in non-shedders, 145.0 ng/ml in concordant shedders, 259.6 ng/ml in discordant shedders; P=0.07). No association was found between levels of lactoferrin and any semen cytokine/chemokines.
3.5 – Discussion

Although contact during sex with semen containing HIV-1 is the most common means of HIV transmission, little is known about the immune correlates of HIV-1 shedding in semen. There is no question that systemic levels of virus are an important predictor of semen virus load $^{135, 197, 355, 361, 387, 388}$, but our study confirms the observation that discordance between levels of virus in the blood and semen is relatively common $^{361}$. Understanding the immune basis of HIV-1 semen shedding, and particularly of discordant shedding, is therefore important for the development of both immunotherapeutics and rational public health policy. Virus-specific CD8+ T cell responses are critical in the control of HIV-1 by an infected person $^{353, 354}$, but there is controversy as to whether there is a direct correlation between the frequency of systemic virus-specific CD8+ T cells and HIV viremia $^{311, 312, 314, 389}$. Furthermore, the optimal assay technique to measure these responses is also unclear $^{390}$. HIV-1-specific CD8+ T cells are present in the mucosa of the female cervix $^{340, 347}$, in semen $^{341}$ and in the gastrointestinal mucosa $^{391, 392}$. However, previous assay techniques have not allowed precise quantitation of responses in semen, and have not attempted to correlate mucosal response levels with shedding of HIV-1.

For the first time, we have been able to assay functional CD8+ responses directly ex vivo in the semen of HIV-1 infected men, and to examine their association with semen virus load. HIV-1-specific, IFN-$\gamma$-producing CD8+ T cells were present in the semen of most participants, and the frequency of these mucosal responses exceeded that measured
in blood. However, no association was seen between the detection of semen CD8+ IFN\(\gamma\) responses and the presence or levels of HIV-1 RNA shedding in semen. Indeed, among those with a detectable CD8+ semen response, higher absolute numbers of virus-specific CD8+ T cells in semen correlated with increased levels of HIV-1 RNA. The fact that CD8+ responses were also associated with higher semen pro-inflammatory cytokines, including IL-6, IFN-\(\gamma\) and IL-8, suggests a possible mechanism for this observation.

Although limited semen lymphocyte numbers necessitated screening semen responses using epitopes that had been mapped in blood, this should not affect our results, since the ontogeny and antigen specificity of mucosal HIV-specific CD8+ T cells closely mirror that of blood 339, 341, 344, 391. These observations do not prove that HIV-1-specific CD8+ T cells are themselves driving inflammation and increased HIV-1 levels in semen. Our study shows a clear association between semen inflammation, HIV-1 RNA shedding and CD8+ T cell responses, but we cannot demonstrate causation. Increased levels of inflammatory cytokines in the blood have been associated with higher viral load in blood plasma, and in the gastrointestinal mucosa with higher HIV-1 levels in the mucosal tissues of the gut 367. It is therefore possible, and perhaps more intuitive, that inflammatory cytokines in semen might induce viral shedding, and that the increased virus-specific CD8+ T cell numbers are secondary to inflammation and/or the resultant higher levels of virus. The absolute number of HIV-1-specific CD8+ T cells in semen tended to mirror the total number of CD3+ T cells, so that any inflammatory process recruiting CD3+ T cells might increase both HIV-1 shedding and local numbers of CD3+ T cells, including HIV-1-specific CD8+ T cells. If both HIV-1 semen shedding and
CD8+ T cell responses driven by pro-inflammatory cytokines, then it will be important to identify the cause of local semen inflammation. The semen cytokine milieu was not simply a reflection of systemic cytokine levels, since there was no significant correlation of pro-inflammatory cytokine levels in semen and blood. Men were screened for gonorrhea and chlamydia, as well as for urethral inflammation due to other factors, so classical sexually transmitted infections should not have been responsible.

Several innate mucosal factors, including SLPI and lactoferrin, have anti-HIV activity and are found at high levels at many mucosal surfaces. Lactoferrin has been described to block HIV-1 replication in T cells, to block dendritic cell uptake of HIV-1 by binding to DC-SIGN, and may reduce breast milk transmission of HIV-1\textsuperscript{393}. SLPI has direct anti-HIV activity, is induced in the oral mucosa by HIV-1, and levels of SLPI in the female genital tract and breast milk have been correlated with reduced mother-child transmission of HIV-1\textsuperscript{291, 293, 394}. We did not find that either factor was associated with reduced HIV-1 semen levels: indeed, lactoferrin was associated with increased shedding. This may reflect different levels or effects of these factors at different mucosal sites, or could reflect induction of lactoferrin as an antiviral defence, in response to higher local levels of HIV-1.

Our cytokine bead array analyses involved the measurement of levels of 14 different cytokines/chemokines in semen: without correction for multiple comparisons, one might expect at least one significant association with shedding, using a 2-sided error of 0.05. However, the hypothesis of an association between HIV-1 RNA levels and
semen inflammation was generated at the first study visit, and was then confirmed at a second, separate visit, making it extremely unlikely that this represents a chance finding. In addition, an association arising through chance alone would be unlikely to result in the repeated clustering of associations with only the pro-inflammatory cytokines, and not with the other cytokines/chemokines screened.

Although this cross-sectional study cannot prove that seminal HIV-1-specific CD8+ responses in semen increase semen HIV-1 RNA levels, there was clearly no association with reduced virus levels. Whether the induction or boosting of semen CD8+ responses by therapeutic vaccines or other immunotherapeutics would affect semen shedding or transmission cannot be addressed in this setting. Although the association of semen CD8+ responses with higher HIV shedding might imply that this will not be a useful therapeutic strategy, our use of IFN-γ production as a means of identifying HIV-1-specific CD8+ T cells might bias to finding an association between inflammatory cytokines and CD8+ responses. Future studies might consider alternate means to measure semen responses, including MHC class I peptide tetramers, antigen-specific proliferation, expression of the lytic marker CD107, or the production of alternate cytokines/chemokines, particularly IL2 or MIP-1β. The ability of CD8+ T cells to proliferate in response to cognate epitope, rather than to produce IFN-γ, has been linked to enhanced viral control in HIV-1-infected long-term non-progressors (53). Similar studies may also be useful in elucidating the role of semen CD8+ T cells in control of HIV-1 shedding and transmission. Nonetheless, our results do imply that when
therapeutic HIV vaccines are developed that boost HIV-1-specific CD8+ T cell immunity, the impact of such vaccines on semen CD8+ T cell responses and levels of HIV shedding should be carefully monitored.
Figure 3.0 – Strong positive correlation between semen HIV viral loads at two time-points. Semen HIV RNA levels were evaluated an average 177 days apart, a strong positive correlation was observed between HIV RNA levels in semen between the two time-points.
Figure 3. 1 - Semen inflammatory cytokines and HIV shedding.

Participants (N=27) were divided into three shedding groups: non-shedders (Group 1; N=6) had an undetectable semen viral load; concordant shedders (Group 2; N=17) had a semen viral load <60% that in blood plasma; and discordant shedders (Group 3; N=4) had a semen viral load =60% that in blood plasma. There was a stepwise association between discordant HIV-1 RNA shedding and semen levels of several pro-inflammatory cytokines, including IL6 (a; P=0.008) and IL8 (b; P=0.02).
Figure 3. 2 - Expression of CD103 on CD8+ T cells in blood and semen.

Expression of the mucosal marker CD103 (aEß7) was measured on CD3/CD8+ T cells from the blood and semen of 12 participants, and was more common on semen T cells (P=0.003; paired samples t-test).
C.

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OM 125

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OM 129

CD8+ cells

Peripheral blood mononuclear cells

IFNg+
D.

Medium          HIV Peptides

OM 125

OM 129

CD8+ cells

Semen mononuclear cells

IFNg+
Figure 3. 3 - HIV-1 specific, IFNγ-producing CD8+ T-cells in semen.

Ex vivo HIV-specific CD8+ IFN-γ responses were detected in the blood of 20/20 and the semen of 14/20 HIV-infected (A), therapy naive men, after short term stimulation with a pool of HIV epitope peptides mapped in blood. High IFN-γ background (B) and strong HIV-specific responses are demonstrated in a representative semen responder OM125 (C and D; upper panels). No semen responses were demonstrated in 6/20 participants despite strong blood responses, as demonstrated in OM129 (C and D; lower panels).
Figure A: Scatter plot showing the relationship between semen HIV-specific CD8+ responses (%) and Log10 semen HIV RNA (copies/mL). The line of best fit indicates a negative correlation.

Figure B: Scatter plot showing the relationship between Log10 (absolute CD8 Responses) and HIV Semen RNA (copies/mL). The correlation coefficient is r = 0.6; p = 0.03.
Figure 3. 4 - HIV-1-specific, IFNγ-producing CD8+ T cells in semen and levels of HIV-1 RNA shedding.

In participants with an HIV-1-specific CD8+ T cell response in semen, (A) there was no correlation between the frequency of these responses and the semen HIV-1 RNA load, when CD8+ responses were expressed as a percentage of all CD8+ T cells (Figure a; r=-0.2; P=0.5). (B) When CD8+ T cell responses were expressed as log10 absolute number of semen HIV-specific CD8+ T cells, there was a positive correlation between CD8+ responses and the level of semen HIV-1 RNA (Figure b; r=0.6; P=0.03).
A. Log10 absolute number semen CD8 responses (%) vs. IL-8 in semen (log10 pg/mL).

r = 0.4, p = 0.05

B. Semen HIV RNA (log10 copies/mL) vs. IL8 in semen (Log10 pg/mL).

r = 0.4, p = 0.04
C.

$\text{Semen HIV RNA (Log10 copies/mL)}$

$r = 0.5, p = 0.04$

$\text{IL6 in semen (Log10 pg/mL)}$
Figure 3.5 - Inflammatory cytokines in semen, increased HIV RNA and CD8+ T cells. Increased semen levels of several inflammatory cytokines correlated with HIV-specific CD8+ T cell responses in this compartment (IL8 is shown in Figure 4a; r=0.4; P=0.05), and also with Semen viral load (4b, 4c and 4d).
CHAPTER 4: Disproportionately high semen shedding of HIV is associated with compartmentalized cytomegalovirus reactivation.
4.1 – Abstract

Semen transmission of human immunodeficiency virus (HIV) drives the global pandemic. HIV loads are generally lower in semen than in blood, but semen loads may be disproportionately high in a subgroup of men. HIV loads in semen exceeded those in blood in 9 (35%) of 26 of antiretroviral therapy–naive men, and disproportionately high shedding was strongly associated with compartmentalized semen cytomegalovirus (CMV) reactivation (odds ratio [OR], 10.5; \( P = 0.01 \)). Overall, 17 of 26 participants were shedding CMV in semen. Semen levels of HIV and CMV were closely correlated \((r = 0.5; \ P = 0.01)\), independently of blood HIV load and CD4+ T cell count. Prevention of CMV reactivation warrants further study as a possible strategy to reduce semen shedding of HIV.
4.2 - Introduction

Semen shedding of HIV is the major driving force behind the global pandemic, but our understanding of the immune control and correlates of HIV semen shedding is rudimentary. The probability of HIV transmission during a single sex act is low, from 0.1 – 1%, and is strongly associated with HIV levels in the blood of the infected partner(s). Although blood viral load is generally reflective of HIV levels in genital secretions, this is not always the case, as several previous studies have shown that a small proportion of individuals tend to shed disproportionately more HIV in genital secretions compared to blood. The inability of blood viral load to reliably predict genital shedding is very important from a public health perspective. Local genital factors also increase HIV shedding, particularly the presence of sexually transmitted infections, which may explain the strong epidemiologic associations between STIs and HIV transmission. HIV levels have been shown to increase as much as ten-fold in the semen of men with bacterial urethritis, regardless of whether the infection is symptomatic or asymptomatic, and fall after urethritis therapy.

Human cytomegalovirus (CMV) and Herpes simplex virus type 2 (HSV-2) are extremely prevalent, ranging from 60–70% for CMV and between 20–30% for HSV-2, and the prevalence is even higher in HIV infected individuals. Both of these viral infections cause life-long latent infections characterized by episodic genital reactivation that is generally asymptomatic. CMV infection may be a cofactor in HIV disease progression, and co-culture of CMV from semen has been associated with HIV
shedding\textsuperscript{197}. HSV-2 infection has been associated with higher HIV plasma viral load\textsuperscript{396}, and asymptomatic genital reactivation may increase genital HIV levels even further \textsuperscript{397}. The extremely high population prevalence of these infections means that a better understanding of their role in HIV genital shedding and transmission is warranted. We therefore studied the impact of these co-infections on levels of HIV semen shedding in a cohort of antiretroviral-naive men\textsuperscript{384}.

4.2 – Methods and Materials

4.2.1 – Study population and STI screening.

Chronically (>6 months) HIV infected, antiretroviral therapy-naive men were recruited through the Canadian Immunodeficiency Research Collaborative (CIRC), Toronto, Canada. A first-void urine sample was screened for leukocytes using a standard urine dipstick (Bayer Diagnostics, Puteaux Cedex, France), and for infection by Neisseria Gonorrhoea or Chlamydia trachomatis using the Amplicor CT/NG assay (Roche Diagnostic, Quebec, Canada). Participants with urethral leukocytes, gonorrhoea or chlamydia, were excluded, since urethritis is known to increase HIV shedding\textsuperscript{135}. All subjects provided informed, written consent. The study was approved by Research Ethics Boards at the University of Toronto, the Mount Sinai Hospital, and the University Health Network, Toronto, Canada.
4.2.2 – Sample processing.

Paired blood and semen specimens were collected within an hour of each other. Semen samples were collected by masturbation into 10 mL of sterile RPMI containing antibiotic and antimycotic, and blood into acid citrate dextran (ACD). All samples were processed within 2 hours of collection. Seminal plasma was isolated by centrifugation at 850g for 10 minutes. Viral loads were either measured directly in blood and seminal plasma, or in the supernatant of semen samples collected into RPMI. In the latter case, semen viral load was corrected for the appropriate dilution factor.

4.2.3 – Viral shedding in blood and semen.

Blood and semen plasma HIV-1 viral loads (VL) were measured using the Versant HIV-1 RNA 3.0 assay (bDNA) (Bayer Diagnostics, Puteaux Cedex, France). Blood serology was performed for Herpes simplex type 2 (HSV-2) IgG (Kalon Biological, Aldershot, UK) and CMV IgG (AxSYM CMV IgG assay, Abbott Laboratories, Abbotts Park, IL). Semen shedding of CMV and HSV-2 DNA was assayed using the Cobas Amplicor PCR assay (Roche Diagnostic Systems, Branchburg, NJ).

4.2.4 – Cytometric bead array (CBA).

Cytokine levels in blood and semen plasma were measured using CBA (Becton Dickinson Immunocytometry Systems, San Jose, CA), according to manufacturers’ instructions. Cytokines/chemokines assayed were IL2, IL4, IL5, IL10, TNFα, IFNγ, IL1β, IL8, IL6, IL12p70, Regulated on Activation Normal T-cell Expressed and Secreted
(RANTES), Monokine Induced by IFNγ (MIG/CXCL9), Macrophage Chemotactic Protein (MCP1/CCL2), and Interferon Inducible Protein-10 (IP10/CXCL10). Plasma samples were incubated for 3 hrs at room temperature with antibody-coupled beads (50 μL/sample) and PE-conjugated secondary antibodies (50 μL/sample), washed and resuspended in 150 μL of 2% paraformaldehyde, and analyzed by flow cytometry.

4.2.5 – Statistical analysis.

SPSS 11 for the Macintosh OS X (SPSS Inc, Chicago) was used for statistical analysis. The Mantel-Haenszel chi-square test with calculation of likelihood ratios and confidence intervals was used to compare dichotomous variables between study groups, and comparison of means between groups was performed by one-way analysis of variance (ANOVA). Linear association of continuous variables was assessed using the Pearson correlation coefficient. Independent associations of HIV semen shedding were examined in a multivariable linear regression model that included those significant on univariate analysis (P<0.05) or previously reported to be associated with HIV shedding.

4.3 – Results

26 chronically HIV-1-infected, therapy-naive participants were enrolled. The median CD4+ T cell count was 574 /mm³ (range; 120-1260 /mm³), and the median blood plasma viral load was 21,400 copies /ml plasma (range; <50 to 824,732 copies). As previously reported, blood HIV RNA levels exceeded semen (log10 VL blood=4.3; log10 VL semen=3.7; P<0.01) and levels of virus in these two compartments were
significantly correlated (r=0.5, P=0.02). However, semen levels of HIV RNA exceeded blood in 9/26 participants (35%), and these individuals were classified as “disproportionate” HIV shedders.

All participants were CMV IgG seropositive (26/26), and 17/26 (65%) were actively shedding CMV in their semen. CMV blood antigenemia was not detected in any participants. Men shedding CMV in semen had a lower blood CD4 T cell count (509 vs. 696/µl; P=0.04), but there was no difference in blood HIV viral load (4.5 vs. 3.9 log10 copies/ml; P=0.2). Participants were divided into 3 groups based on CMV shedding pattern: non-shedders (9/26; 31%), low-level shedders (<20,000 copies DNA/ml, 5/26; 19%) and high-level shedders (≥20,000 copies DNA/ml, 11/26; 42%). Semen HIV levels were ten-fold higher in men shedding any CMV (log10 HIV RNA 4.1 vs. 3.1; P=0.02), and there was a stepwise correlation between semen HIV viral load and the level of CMV shedding (non-shedders, HIV log10 VL=3.1; low level shedders, 3.4; high level shedders, 4.3; P=0.02). In addition, semen levels of HIV and CMV were directly correlated (r=0.52; P<0.01; Figure 4.2). CMV was detected more frequently in the semen of disproportionate HIV shedders (9/9 vs. 8/17; LR=10.0, P=0.002, Figure 4.1). In addition, CMV was present in the semen at high levels in most disproportionate HIV shedders (7/9 vs 5/17; LR=10.5, P<0.01), so that mean semen CMV levels were fifty-fold higher in the group with disproportionate HIV shedding (5.4 vs. 3.7 log10 CMV DNA; P<0.01; Figure 4.2).
Shedding of both CMV and HIV in semen were associated with elevated levels of IFN\(\gamma\) in both the blood (HIV, \(P=0.01\); CMV, \(P=0.04\)) and semen (HIV, \(P=0.02\); CMV; \(P=0.02\)). No other semen cytokines/chemokines were associated with CMV shedding, although the semen pro-inflammatory cytokines IL6, IL8 and TNF\(\alpha\) were associated with increased HIV shedding\(^{398}\). Levels of several cytokines in blood were correlated with CMV shedding, including IL12 (\(P=0.04\)), IL6 (\(P=0.02\)), IL1 (\(P=0.02\)), TNF\(\alpha\) (\(P=0.06\)), IL4 (\(P=0.01\)) and IL8 (\(P<0.001\)). However, after correction for multiple comparisons, only blood IL8 levels remained associated with semen shedding of CMV (\(P=0.01\)).

In a univariate analysis, shedding of HIV RNA in semen was correlated with a higher blood plasma HIV VL (\(r=0.5\); \(P=0.02\)) and with higher levels of semen CMV DNA shedding (\(r=0.52\); \(P<0.01\)). In order to confirm that the association of HIV in semen with CMV shedding was a true compartmentalized effect, rather than simply a reflection of increased systemic immune suppression, logistic regression analysis was performed to identify independent associations of HIV RNA semen shedding. The model included absolute blood CD4+ T cell count, log\(10\) blood plasma HIV VL, and log\(10\) semen CMV VL. Only semen CMV viral load remained independently associated with HIV shedding (\(P=0.04\); Figure 4.3), while the association with blood plasma HIV VL did not (\(P=0.1\)).

Eight participants (8/26; 31%) were HSV-2 IgG seropositive. HSV-2 infection was associated with a lower absolute CD4 T cell count in blood (440 vs. 614/ul; \(P=0.05\)), but not with differences in the HIV VL in blood (4.1 vs. 4.3 log\(10\) copies/ml, respectively; \(P=0.6\)) or semen (3.9 vs. 3.6 log\(10\) RNA copies/ml; \(P=0.5\)). However, HIV
disproportionate shedders were more likely to be HSV-2 IgG seropositive (5/9 vs. 3/17; OR=3.8, P=0.05). Semen CMV shedding tended to be more common in HSV-2 co-infected men (7/8 vs 10/18; LR=2.9, P=0.1), and CMV semen levels were higher (5.3 vs. 3.8 log10 DNA copies/ml; P=0.03). However, HSV-2 serostatus was not independently associated with HIV shedding or disproportionate HIV shedding in multivariable models that included CMV shedding. There were no differences in semen or blood cytokine/chemokine levels based on HSV-2 IgG serostatus.

4.4 – Discussion

The strongest independent risk factor for HIV sexual transmission is the blood plasma viral load of the infected partner\textsuperscript{181}, and a log10 increase in the blood plasma viral load increases the per-sex-act transmission rate three-fold\textsuperscript{185}. This is probably because levels of HIV in the blood correlate quite closely with levels in the genital tract (semen or vaginal secretions)\textsuperscript{364, 384}. However, some individuals shed disproportionately high amounts of HIV in the genital tract\textsuperscript{364, 370, 384}, and genital shedding may persist despite undetectable blood HIV RNA levels on antiretroviral therapy\textsuperscript{364}. Elucidating the mechanism of disproportionate semen shedding of HIV is of obvious importance for the design of public health strategies to reduce HIV sexual transmission.

We have confirmed that the presence of CMV in semen is associated with an increased likelihood of HIV shedding\textsuperscript{197}, and for the first time have shown a direct, quantitative correlation between semen levels of HIV and CMV. More importantly, we have demonstrated that disproportionate shedding of HIV in semen is driven by local
CMV reactivation in the male genital tract. This appears to be a truly compartmentalized effect, rather than a reflection of systemic immunosuppression, since it was independent of both peripheral blood CD4+ T cell counts and HIV viral load. Studies in the female genital tract suggest that HIV replication in the context of CMV reactivation may be induced via the pro-inflammatory cytokine IL-8\textsuperscript{235}. Although we previously found that HIV shedding was associated with the semen pro-inflammatory cytokines IL8 and IFN\textgreek{g}\textsuperscript{398}, in this study we found that CMV shedding was only associated with IFN\textgreek{g} in semen, albeit with a host of inflammatory cytokines in blood, most strongly IL8.

Genital herpes is clearly a major co-factor in HIV sexual transmission on a global level\textsuperscript{397}. However, HSV-2 was not present the semen of any of our HIV/HSV-2 co-infected participants, perhaps due to the selection of men with high CD4 T cell counts, and this lessened our ability to study the effect of HSV-2 infection on HIV shedding. Although HSV-2 sero-status was associated with disproportionate HIV semen shedding, this did not remain significant in multivariable analysis. Nonetheless, further studies are warranted, perhaps within selected groups that are shedding HSV-2.

We conclude that the local reactivation of CMV in the male genital tract was associated with a ten-fold increase in semen HIV viral load, and may drive the phenomenon of disproportionate HIV shedding. Given that a ten-fold increase in blood HIV viral load is associated with a three-fold increase in sexual transmission\textsuperscript{181,185}, a ten-fold increase in semen HIV levels might reasonably be expected to have an even more significant impact on HIV transmission.
Figure 4.1: Disproportionate semen HIV shedding associated with increased CMV shedding in semen.

HIV infected individuals that were shedding disproportionately higher HIV RNA in semen compared to blood were shedding significantly more CMV DNA in semen.
Figure 4.2: Correlation between levels of CMV and HIV in semen.

A positive spearman’s rank correlation between levels of HIV RNA and CMV DNA in the semen of HIV infected therapy naive individuals.
Figure 4.3: HIV RNA shedding in semen is strongly associated with the presence of CMV DNA in semen.

HIV infected individuals that were shedding CMV DNA in semen were shedding almost 10-fold more HIV RNA in semen compared to HIV-CMV infected individuals that were not shedding CMV in semen.
CHAPTER 5: Persistent HIV RNA shedding in semen despite effective antiretroviral therapy.
5.1 Abstract

The semen of an HIV-infected man is the most common mode of HIV transmission. Highly Active Antiretroviral Therapy (HAART) often results in an undetectable blood HIV RNA viral load (VL), and it has been suggested that there may be no risk of sexual transmission in this context. However, the impact of effective HAART on HIV levels in semen requires further study. We performed a prospective, longitudinal study of semen and blood HIV RNA levels after HAART initiation, and a cross sectional study in men on long-term effective HAART. Effective HAART was defined as the achievement of an undetectable blood VL. In 25 participants followed prospectively after HAART initiation, the blood VL was consistently undetectable by week 16. Isolated semen HIV RNA shedding was detected in 12/25 (48%) participants despite effective HAART, and at a high level (>5,000 copies/ml) in 4/25 (16%). Isolated semen shedding was detected at 19/116 (14%) visits with an undetectable blood VL, and was associated with the pre-therapy semen VL. Semen isolates did not contain drug resistance mutations, and were infectious in vitro. In 13 participants with prolonged suppression of the blood VL (median; 126 months) isolated semen HIV shedding persisted in 4/13 (31%). These findings suggest that although effective HAART often eliminated HIV RNA from the semen, isolated HIV semen shedding was common, even after extremely prolonged suppression of the blood VL. Public health messages and policy must be tailored carefully to reflect this reality.
5.2 Introduction

There were an estimated 2.5 million new HIV infections in 2007, and most resulted from sexual contact with the semen of an infected man. The level of HIV RNA in blood is a critical determinant of the probability of sexual HIV transmission, likely because this tends to correlate with virus levels in the genital tract. Highly Active Antiretroviral Therapy (HAART) will often suppress both blood and genital HIV RNA levels to below the limit of detection, and therefore may reduce HIV transmission at a population level. Based on these observations, it has recently been stated that individuals receiving effective HAART (i.e.: those with an undetectable blood HIV RNA viral load (VL) on therapy) are sexually non-infectious.

The latter statement has caused controversy in the HIV prevention field. A model-based analysis demonstrated the potential for increased HIV incidence in the absence of condom use, particularly among men who have sex with men, despite substantial and consistent reductions in the genital VL on HAART. In addition, the impact of therapy on genital tract virus levels appears to be quite heterogeneous, since semen HIV shedding may be present despite HAART. While the mechanisms for this phenomenon are poorly defined, discordant HAART effects in blood and semen could include compartmentalization of drug-resistant viral strains in the genital tract, incomplete semen penetration of antiretroviral drugs, or genital mucosal inflammation resulting from localized co-infections or other causes. Based on these concerns, the Joint United Nations Programme on HIV/AIDS (UNAIDS) has
stated that “more research is needed to determine … the association between the viral load in blood and the viral load in semen”1.

In order to address these issues, we have performed a prospective, longitudinal study to define the impact of HAART initiation on HIV RNA levels in the blood and semen more precisely. Our results strongly suggest that, while HAART is likely to reduce HIV sexual transmission at a population level, individual counselling must continue to emphasize the importance of safe sex.

5.3 Methods

5.3.1 - Study participants and design.

HIV-infected men were recruited through an HIV primary care clinic (the Maple Leaf Medical Clinic, Toronto, Canada). Participants with no evidence of urethritis or other active sexually transmitted infection (STI) at baseline were enrolled. All HIV-infected men starting HAART were therapy-naïve and have a detectable blood HIV RNA viral load prior to starting therapy. Simultaneous blood and semen samples were collected at baseline and then prospectively at weeks 2, 4, 8, 12, 16, 20 and 24 after HAART initiation. All participants provided informed, written consent and the study protocol was approved by the Research Ethics Board at the University of Toronto. The HAART regimens used were selected by individual physicians, based upon current clinical guidelines406 and the results of baseline antiretroviral resistance screening (VirtualPhenotype™, Virco, Bridgewater, NJ, USA).
5.3.2 Specimen collection and diagnostics.

Semen samples were collected by masturbation into a sterile container containing 10 mL of RPMI with penicillin/streptomycin, and blood was collected into acid citrate dextran\textsuperscript{15}. Seminal and blood plasma were isolated by centrifugation, and HIV RNA load measured using the Versant HIV-1 RNA 3.0 assay (bDNA; Bayer Diagnostics, Puteaux Cedex, France). Seminal plasma VLs were multiplied by 6 to correct for dilution during sample collection, as previously described \textsuperscript{398, 405}. A semen VL >5,000 RNA copies/mL was defined as “high level”, since the semen VL tends to be approximately ten-fold lower than blood\textsuperscript{384, 398} and individuals with a blood VL >50,000 RNA copies/mL have been demonstrated to be most infectious\textsuperscript{181}. A first-void urine sample was screened for infection by \textit{Neisseria gonorrhoea} or \textit{Chlamydia trachomatis} (Amplicor CT/NG assay, Roche Diagnostic, Quebec, Canada), and syphilis serology performed. Blood serology was performed for Herpes simplex type 2 (HSV-2) IgG (Kalon Biological, Aldershot, UK) and human cytomegalovirus (CMV) IgG (AxSYM CMV IgG assay, Abbott Laboratories, Abbotts Park, IL).

5.3.3 HIV sequence analysis and genotyping.

RNA was extracted from blood/semen plasma using the Total RNA Isolation System Kit (Promega, Madison, Wisconsin, USA)\textsuperscript{407} and cDNA was synthesized using RT-PCR and the SuperScript\textsuperscript{TM} III First-Strand Synthesis System (Invitrogen, Carlsbad, CA)\textsuperscript{408}. A 1.6kb fragment encompassing the protease and reverse transcriptase regions of HIV-1 \textit{pol} was amplified with nested PCR primers and the XL PCR Kit containing rTth DNA
polymerase enzyme (Applied Biosystems, Foster City, CA). To determine unique variants, serial four-fold cDNA dilutions were performed and each dilution was PCR amplified. The endpoint dilution was defined as the last dilution cDNA yielding a positive PCR. Multiple aliquots of the end point dilution were subjected to amplification, yielding detectable PCR products in 30-40% of the aliquots. Both strands of the PCR products were then sequenced for each unique variant. Computational analysis was performed using the Bioedit software (available online; http://www.mbio.ncsu.edu/BioEdit/bioedit.html) for sequence alignment, and MEGA version 4.0 (http://www.megasoftware.net) for phylogenetic analysis. Phylogenetic trees were constructed after gap-stripping of columns and employing the neighbor-joining method. Genotypic drug resistance was determined by submitting the sequences obtained to the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu).

5.3.4 Antiretroviral drug levels in blood and semen plasma.

Antiretroviral drug concentrations were determined by High Performance Liquid Chromatography (HPLC). Prior to analysis, protein was removed from the sample by precipitation with 0.1% acetic acid in methanol (Sigma, Oakville, Canada) followed by centrifugation at 3000 rpm for 5 minutes. One hundred microliters of a 2µg/mL internal standard, 6,7-dimethyl-2,3-di(2-pyridyl)-quinoxaline (DMDPQ) (Aldrich, Milwaukee, WI), were added to 250 µL of supernatant and the concentrations of efavirenz, lopinovir, atazanavir, ritonavir, abacavir, zidovudine, and lamivudine were determined using HPLC-MS/MS. The gradient mobile phase consisted of: A, 5mM ammonium acetate, pH
4.15 with 5% methanol; B, pure methanol; and C, 0.1% acetic acid (Sigma, Oakville, Canada). All solvents and reagents were HPLC grade. A Hewlett Packard-1100 HPLC system using a Supelco (Supelcosil™ ABZ + Plus) 15cm x 4.6mm, 3µm C18 column was coupled to a PE Sciex API-2000 LC/MS/MS triple quadruple mass spectrometer equipped with a turbo ion spray source. Analyst software V 4.11 was used as the system controller and integrator. Seminal plasma drug concentrations were multiplied by 6 to correct for dilution during sample collection, as previously described\textsuperscript{398,405}.

5.3.5 HIV infectivity assays.

Blood and semen plasma samples were layered on a 20% sucrose solution in a micro-centrifuge tube and centrifuged at 20,000g for 1 hour at 4°C. The supernatant was carefully aspirated leaving ~20uL in the tube. The pellet was resuspended with 100uL of R10 media (RPMI media + 10% FBS + 1% penicillin/streptomycin + 1% L-glutamine). The sucrose-purified virus was added to ViroMag (VM) magneto-infection beads (1:20 v/v) and incubated at room temperature for 15 minutes. After incubation, the magnetized virus was added to 1 million CD4+ enriched (Easysep, Stemcell Technologies, Vancouver, BC) target cells which had been activated for 48 hours with anti-CD3/anti-CD28 (ebiosciences, San Diego, CA) in a 24 well plate. The cell-virus suspension was centrifuged for 2 minutes at 460g and then placed on a magneto-infection plate for 15 minutes at 37 °C and 5% CO\textsubscript{2}. The plate was then incubated for 10 days at 37°C and 5% CO\textsubscript{2}. After 10 days of incubation, the cell supernatant was sucrose purified, re-incubated with ViroMag magneto-infection beads and re-incubated for 10 days with fresh CD4+.
enriched activated target T cells. Concentrations of p24 antigen in the cell supernatant were assayed using the Retro-Tek p24 HIV-1 antigen ELISA (Zeptometrix, Buffalo, NY).

5.3.6 Statistical analysis.

SPSS 12 for windows XP was used. Comparisons between blood and semen were performed using a non-parametric Wilcoxon Signed Ranks paired t test. Dichotomous variables were compared between groups by Chi squared tests. Continuous variables were compared between groups using the Mann Whitney non-parametric test.

5.4 Results

5.4.1 Participant demographics

Twenty-five therapy-naïve participants were enrolled in the prospective study of HAART initiation (Table 6.1), with a median absolute CD4+ T cell count of 213 cells/mm³ (range; 60-590/mm³), and a blood HIV RNA VL of 50,000 copies/mL (range; 120 – >500,000 copies/mL). The median semen HIV VL prior to therapy initiation was 3,834 RNA copies/mL (range; <300 – 86,856 copies/mL), and high-level semen shedding (>5,000 RNA copies/mL; see above) was present in 13/25 participants (52%). All participants started HAART within 48 hours of enrolment and provided paired blood and semen samples at each of the eight scheduled time points, for a total of 200 study visits. No C. trachomatis infection, N. gonorrhoeae infection, syphilis or clinical STI/urethritis were detected at any time point. The HSV-2 seroprevalence was 9/25 (36%), and all
participants were CMV seropositive. No major resistance mutations to any of the three main antiretroviral drug classes were detected in blood genotypes performed prior to therapy.

5.4.2 Impact of effective HAART on HIV RNA levels in the blood and semen.

All participants had achieved an undetectable blood viral load (<50 RNA copies/ml) by week 16, thereby meeting the definition of effective HAART, and all but two participants had suppressed semen VL to undetectable levels (<300 RNA copies/ml) by the same time point (Figure 5.1a). One of these participants never suppressed semen virus despite consistently effective HAART (Figure 5.1b). In the other, blood VL was undetectable by week 4, but rebound was seen at week 16 despite initial suppression, and the participant admitted to suboptimal medication compliance; after appropriate counseling, complete suppression was achieved in both the blood and semen compartments.

Although semen HIV RNA levels initially became undetectable in 24/25 (96%) participants, there was substantial inter-individual heterogeneity in the consistency of semen VL suppression. Specifically, semen HIV shedding despite an undetectable blood viral load (isolated semen HIV shedding) was detected during at least one visit for 12/25 (48%) participants (Table 5.2). This occurred after the initial achievement of an undetectable semen VL in 9/12 (75%) participants. Overall, isolated semen HIV RNA shedding was detected during 19/116 (16.4%) study visits on effective HAART, and was present at a high level during 5/19 of these visits (26%; Figure 5.1b-d for representative

146
examples). High-level isolated semen HIV RNA shedding was observed at any time in 4/25 (16%) participants, with peak semen HIV RNA levels ranging from 6,672–16,026 RNA copies/mL. While the proportion of participants at any given time point with an undetectable blood HIV VL reached 100% by six months, the proportion with undetectable semen virus appeared to plateau at just over 80% (Figure 5.2).

Phylogenetic tree analysis including reference strains in the Los Alamos HIV Sequence Database (http://hiv-web.lanl.gov/content/hiv-db/mainpage.html) showed that baseline sequences were unique and of subtype B origin (data not shown). Sequences derived from the blood plasma and semen of each patient were closely related but distinct. There was no evidence of super-infection or recombination.

5.4.3 Associations of isolated semen HIV RNA shedding.

No association was seen between isolated semen HIV RNA shedding and specific antiretroviral agents or classes (Table 5.1). Just over half the participants were taking a boosted protease inhibitor (PI; 13/25; 52%), most commonly lopinavir or atazanavir, and the remainder were taking a non-nucleoside reverse transcriptase inhibitor (NNRTI; efavirenz in 10/12 cases). All participants were also taking a dual nucleoside reverse transcriptase inhibitor backbone, most commonly tenofovir/FTC or abacavir/3TC. Seven (7/12) participants with isolated HIV shedding were taking an NNRTI vs. 5/13 of those without (p=0.32); of those participants with high-level isolated semen shedding, 1/4 was taking an NNRTI (p=0.31).
Clinical parameters routinely tested prior to HAART initiation were not useful in predicting subsequent isolated semen shedding (Table 5.3). Specifically, the pre-therapy blood VL did not differ between groups (medians; 4.65 and 4.50 log10 RNA copies/ml in men with vs. without isolated shedders, respectively; p=0.86), nor did the baseline CD4+ T cell count (medians; 195 vs. 230/mm³; p=0.97). The seroprevalence of HSV-2 in ever and never isolated shedders was similar (5/13 vs. 4/12; p=0.57), and all participants were CMV infected. However, although not standard clinical test, the baseline semen HIV RNA load was ten-fold higher in those participants with subsequent isolated semen shedding (median 4.42 vs. 3.41 log10 RNA copies/ml; p=0.03).

5.4.4 Viral characteristics of isolated semen viral isolates.

Sequence based screening of HIV RNA in blood for antiretroviral resistance mutations was performed at baseline for all participants. In addition, semen HIV isolates were tested at baseline and on therapy for three out of four participants with high-level isolated semen shedding (>5,000 HIV RNA copies/ml). No major or minor drug resistance mutations to any of the three major drug classes (nucleoside reverse transcriptase inhibitors, protease inhibitors or non-nucleoside reverse transcriptase inhibitors) were detected in the blood or semen HIV isolates from these individuals, either before or during HAART therapy. Limitations in PCR amplification did not permit analysis of semen HIV isolates with lower viral loads and in 1/4 participant with high-level isolated semen shedding.
Infection assays using enriched CD4+ T cells were performed to determine whether semen HIV RNA detected on suppressive HAART was infectious in vitro. Infectivity assays were set up for three participants using semen plasma from time points with high-level (>5,000 RNA copies/mL) isolated semen shedding. Despite the substantial toxicity of seminal plasma on target cells in this in vitro system, high-level p24 production was apparent in 1/3 (33%) participants, demonstrating the infectious potential of isolated semen viruses. Although in vitro infection was only demonstrated in 1/3 participants from HIV RNA from isolated semen shedding episode, infection assays were particularly difficult to perform. Semen plasma reduced cell CD4+ T cell activation and viability and we observed that even baseline (prior to HAART) semen samples were unable to cause productive infections in vitro.

5.4.5. Semen concentration of antiretroviral drugs.

Concentrations of antiretroviral drugs were measured in paired blood and semen plasma samples for 19/25 (76%) study participants. Blood and semen samples were collected within 2 hours of each other, at varying intervals after medication dosing. In participants with isolated HIV RNA semen shedding, drug concentrations were measured in both compartments at the visit corresponding with the peak semen HIV RNA level; in participants with consistently suppressed semen virus, drug concentrations were measured at the 5 month visit. All antiretroviral drugs were readily detected in the blood plasma. Concentrations of efavirenz were significantly lower in semen than in blood plasma (mean 0.3 vs. 1.29 mg/L respectively; p<0.001) and semen levels of the protease
inhibitors were generally very low or undetectable, while 3TC concentrations were >100-fold higher in semen relative to blood (mean 5.8 vs. 0.044 mg/L respectively; p=0.01). There was no difference in semen 3TC concentrations between individuals with isolated semen shedding and those without (5.17 vs. 4.67 mg/L respectively; p>0.9). Although semen levels of abacavir, efavirenz and the protease inhibitors were generally low (Figure 5.3), when compared to suppressed controls, neither the levels in semen, nor the ability to detect drug in semen (i.e.: detectable vs. non-detectable) were associated with isolated semen shedding (Figure 5.3).

5.4.6 Isolated semen shedding after very long-term suppression on HAART.

Participants in this prospective study were only followed for six months after HAART initiation. However, it has been suggested that men may be sexually non-infectious after more than six months of complete suppression of the blood VL. To address this issue specifically, we examined semen HIV RNA levels in a group of men who had been on uninterrupted HAART with an undetectable blood HIV viral load for at least 4 years, and who have been shown to have unusually complete normalization of HIV-associated immune defects in both the blood and gastrointestinal mucosa. Semen samples were collected at a single time point from 13 such individuals without STIs or evidence of urethral inflammation. Despite a median duration of complete blood VL suppression of 82 months (range; 48-216 months; Table 5.4), isolated semen HIV RNA shedding was detected in 4/13 participants (31%) at a median level of 564 RNA
copies/ml (range, 336-828 HIV RNA copies/ml). Although isolated shedding was common, high level isolated virus was not detected in any participant on long-term completely suppressive HAART.

While, isolated semen shedding was seen in association with both NNRTI-based and boosted PI-based HAART regimens (Table 5.4), these was an association with HSV-2 seropositivity: 4/8 HSV-2 infected participants demonstrated isolated semen shedding, vs. 0/5 HSV-2 uninfected (p=0.03). No participant had clinical evidence of HSV-2 reactivation, although microbiological screening for HSV-2 reactivation was not performed

5.5 DISCUSSION

Blood HIV levels predict the likelihood of HIV sexual transmission\textsuperscript{181} and tend to correlate with virus levels in the genital tract. However, while the suppression of HIV blood VL on HAART is likely to correlate with a substantially reduced probability of sexual transmission\textsuperscript{6, 399, 400}, data are lacking to support the recent statement that such individuals are sexually non-infectious\textsuperscript{257}. Our prospective study confirms earlier reports that effective HAART substantially reduces HIV semen RNA levels\textsuperscript{260, 410}. Just over half of our study participants never had detectable semen HIV RNA after suppression of blood viremia to undetectable levels, and this reduction in semen virus levels would be expected to have a substantial impact on HIV sexual transmission at a population level. However, isolated HIV RNA shedding was detected in the semen of almost half of participants, despite suppression in blood, and often at high enough levels to pose a
potential significant transmission risk. Although semen plasma had toxic effects in our \textit{in vitro} PBMC infectivity assay system, precluding detailed analysis of relative semen infectivity, we did demonstrate that HIV RNA detected during one episode of isolated shedding was associated with \textit{in vitro} infectiousness.

Standard pre-therapy clinical and laboratory investigations were not useful in predicting which participants would develop isolated semen shedding after HAART initiation: blood VL and CD4 count were well matched between groups, and neither CMV nor HSV-2 infection status were predictive. The only robust predictor of the phenomenon was the pre-therapy HIV RNA semen viral load, which was ten-fold higher in participants with subsequent isolated semen shedding. However, semen HIV RNA VL testing is only available as a research tool, and not as a tool for clinicians or their patients. Furthermore, it should be noted that semen shedding despite effective HAART was observed in one individual with a pre-therapy semen VL of just 384 copies/ml. Although not associated with isolated shedding in the prospective study, HSV-2 infection was a significant risk factor for low-level semen virus in the small, cross-sectional study of participants with very long-term suppression of blood virus. Globally, the majority of HIV-infected people are co-infected by HSV-2, and HIV/HSV-2 co-infection substantially enhances HIV sexual transmission\textsuperscript{200}. Therefore, despite the relatively low level of semen virus detected, the potential for HSV-2 to drive ongoing HIV sexual transmission despite effective HAART may merit further study.
Isolated semen shedding was not associated with specific antiretroviral agents. Tenofovir, which has excellent semen penetration[^411], was included in the regimen of most participants with semen HIV detected despite effective HAART. In all cases this was combined with 3TC or FTC, as well as with at least one other recommended first line agent, most commonly efavirenz or a boosted protease inhibitor (lopinavir or atazanavir). Although NNRTIs and boosted PIs had low semen levels, as has been previously described[^265, 412-414], concentrations of 3TC in semen were on average 100 fold higher in semen than blood. Furthermore, the phenomenon of isolated semen HIV RNA shedding while on effective HAART was not associated with inter-individual differences in antiretroviral semen penetration, and so there was no suggestion that switching to an alternate antiretroviral agent(s) would prevent isolated semen shedding.

Semen HIV shedding while taking effective HAART was not generally related to a persistent lack of antiretroviral activity in this compartment. In all but one participant with isolated shedding, there had been initial reduction of semen HIV RNA to undetectable levels. It is also unlikely that medication non-compliance played a significant role, since (in addition to self-reported compliance) the blood VL remained persistently undetectable after initial suppression in all but one participant, perhaps suggesting reactivation of latent virus rather than active replication, similar to the phenomenon of blood plasma viral blips[^248]. In many cases isolated HIV shedding was detected after initial complete suppression of semen HIV RNA, suggesting that isolated semen HIV shedding may be related to reactivation of latent viruses (CMV) or increase
in levels of inflammation (IL8, IL6), which have been demonstrated to be elevated in HIV infected individuals \cite{415,416} and enhance HIV replication in vitro\cite{416}.

No HIV antiretroviral resistance was detected during isolated semen shedding. However, the time to development of resistance on a non-suppressive regimen is quite variable\cite{417}, and participants were only followed up to 24 weeks in our prospective study protocol. In HIV infected women, compartmentalized viruses may be present within the blood and genital tract, with subsequent recombination\cite{418}. Therefore, whether isolated shedding of drug resistant isolates might develop over time, and whether this might lead to a failure of blood VL suppression, are important questions that will require further study.

This longitudinal, prospective study clearly demonstrates the potential for HIV transmission through semen despite attaining an undetectable blood HIV viral load on HAART. While effective antiretroviral therapy is likely to substantially reduce HIV transmission at a population level, we believe that health care providers must continue to provide HIV-infected individuals with a strong and consistent message regarding the need for ongoing safe sex in this context.
Table 5.1. Characteristics of study participants initiating HAART.

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<th>Subject</th>
<th>Baseline CD4+ T cell count (/mm³)</th>
<th>Baseline blood HIV VL (RNA copies/mL)</th>
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</table>

*Participant demonstrated subsequent isolated HIV semen shedding (Table 1b for details).

**Antiretroviral abbreviations: ABC, abacavir; 3TC, lamivudine; FTC, emtricitabine; RTV, ritonavir; ATZ, atazanavir; EFV, efavirenz; AZT, zidovudine; LPV, lopinavir; TDF, tenofovir; NVP, nevirapine; SQV, saquinavir.
Table 5.2. Longitudinal HIV RNA levels in blood and semen plasma of participants with isolated semen viral shedding†

<table>
<thead>
<tr>
<th>ID#</th>
<th>Sample</th>
<th>baseline</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
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</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>002</td>
<td>Blood</td>
<td>190,532</td>
<td>488</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>870</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>&lt;50</td>
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<tr>
<td></td>
<td>Semen</td>
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<td>&lt;300</td>
<td>&lt;300</td>
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<td>&lt;300</td>
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<tr>
<td>004</td>
<td>Blood</td>
<td>&gt;500,000</td>
<td>5,135</td>
<td>&lt;50</td>
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<td>&lt;50</td>
<td>&lt;50</td>
<td>&lt;50</td>
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<td>Semen</td>
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<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
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<tr>
<td>005</td>
<td>Blood</td>
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<td>970</td>
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<tr>
<td></td>
<td>Semen</td>
<td>82,836</td>
<td>447,660</td>
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<td>Semen</td>
<td>13,230</td>
<td>20,685</td>
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<tr>
<td>007</td>
<td>Blood</td>
<td>39,564</td>
<td>333</td>
<td>119</td>
<td>61</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>&lt;50</td>
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<tr>
<td></td>
<td>Semen</td>
<td>384</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
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<td>Blood</td>
<td>53,585</td>
<td>715</td>
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<td></td>
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<td>52,494</td>
<td>19,296</td>
<td>900</td>
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<td>&lt;50</td>
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<tr>
<td>009</td>
<td>Blood</td>
<td>120</td>
<td>68</td>
<td>&lt;50</td>
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<td>&lt;50</td>
<td>&lt;50</td>
<td>&lt;50</td>
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<tr>
<td></td>
<td>Semen</td>
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<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
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<td>012</td>
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<td>24,085</td>
<td>163</td>
<td>714</td>
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<td>&lt;50</td>
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<tr>
<td></td>
<td>Semen</td>
<td>31,242</td>
<td>1,488</td>
<td>1,518</td>
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<td>&lt;50</td>
<td>&lt;50</td>
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<tr>
<td>015</td>
<td>Blood</td>
<td>406,990</td>
<td>1,722</td>
<td>556</td>
<td>265</td>
<td>503</td>
<td>&lt;50</td>
<td>&lt;50</td>
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<tr>
<td></td>
<td>Semen</td>
<td>86,856</td>
<td>4,206</td>
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<tr>
<td>021</td>
<td>Blood</td>
<td>54,495</td>
<td>274</td>
<td>52</td>
<td>51</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>&lt;50</td>
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<tr>
<td></td>
<td>Semen</td>
<td>22,386</td>
<td>564</td>
<td>576</td>
<td>1,512</td>
<td>2,688</td>
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<td>Blood</td>
<td>4,882</td>
<td>280</td>
<td>108</td>
<td>74</td>
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<td>&lt;50</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td></td>
<td>Semen</td>
<td>32,352</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
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<td>&lt;300</td>
</tr>
<tr>
<td>025</td>
<td>Blood</td>
<td>31,507</td>
<td>383</td>
<td>357</td>
<td>85</td>
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<td>&lt;50</td>
<td>&lt;50</td>
<td>&lt;50</td>
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<td>Semen</td>
<td>846</td>
<td>1,164</td>
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<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
</tr>
</tbody>
</table>

† Time points with isolated semen viral shedding are indicated in bold.

* Upper limit of detection in blood was 500,000 copies/mL

** Lower limit of detection in blood was 50 HIV RNA copies/mL.

*** Limit of detection in semen was 300 HIV RNA copies/mL.
Table 5.3. Pre-therapy semen viral load as a predictor of subsequent isolated semen HIV RNA shedding.

<table>
<thead>
<tr>
<th></th>
<th>Any shedding (Medians)</th>
<th>No shedding (Medians)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 T cell count (/mm$^3$)</td>
<td>195</td>
<td>230</td>
<td>0.97</td>
</tr>
<tr>
<td>Blood HIV VL (RNA copies /ml)</td>
<td>4.65</td>
<td>4.50</td>
<td>0.86</td>
</tr>
<tr>
<td>Semen HIV VL (RNA copies /ml)</td>
<td>4.42</td>
<td>3.41</td>
<td>0.03</td>
</tr>
<tr>
<td>Protease inhibitor based therapy</td>
<td>4/10</td>
<td>7/11</td>
<td>0.64</td>
</tr>
<tr>
<td>HSV-2 infected</td>
<td>5/13</td>
<td>4/12</td>
<td>0.57</td>
</tr>
</tbody>
</table>

* Mann Whitney test.
Table 5.4. Characteristics of study participants on long-term effective therapy.

<table>
<thead>
<tr>
<th>ID</th>
<th>CD4+ count*</th>
<th>Duration HAART (months)</th>
<th>Duration blood VL undetectable (months)</th>
<th>HAART regimen</th>
<th>HSV-2 serostatus</th>
<th>Semen RNA VL (copies/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>540</td>
<td>132</td>
<td>120</td>
<td>ABC/EFV/3TC</td>
<td>Positive</td>
<td>378</td>
</tr>
<tr>
<td>2</td>
<td>440</td>
<td>86</td>
<td>75</td>
<td>ABC/3TC/RTV/SQV</td>
<td>Positive</td>
<td>&lt;300</td>
</tr>
<tr>
<td>3</td>
<td>970</td>
<td>192</td>
<td>103</td>
<td>ABC/3TC/SQV/LPV</td>
<td>Negative</td>
<td>&lt;300</td>
</tr>
<tr>
<td>4</td>
<td>730</td>
<td>168</td>
<td>102</td>
<td>ABC/3TC/SQV/RTV</td>
<td>Positive</td>
<td>&lt;300</td>
</tr>
<tr>
<td>5</td>
<td>560</td>
<td>180</td>
<td>112</td>
<td>TDF/3TC/EFV</td>
<td>Negative</td>
<td>&lt;300</td>
</tr>
<tr>
<td>6</td>
<td>970</td>
<td>120</td>
<td>115</td>
<td>ABC/3TC/RTV/SQV</td>
<td>Positive</td>
<td>750</td>
</tr>
<tr>
<td>7</td>
<td>710</td>
<td>116</td>
<td>54</td>
<td>TDF/3TC/LPV/RTV</td>
<td>Negative</td>
<td>&lt;300</td>
</tr>
<tr>
<td>8</td>
<td>580</td>
<td>198</td>
<td>57</td>
<td>3TC/EFV/LPV/RTV</td>
<td>Positive</td>
<td>&lt;300</td>
</tr>
<tr>
<td>9</td>
<td>610</td>
<td>216</td>
<td>82</td>
<td>ABC/3TC/ZDV/TDF/EFV</td>
<td>Positive</td>
<td>&lt;300</td>
</tr>
<tr>
<td>10</td>
<td>580</td>
<td>102</td>
<td>70</td>
<td>EFV/AZT/3TC</td>
<td>Positive</td>
<td>828</td>
</tr>
<tr>
<td>11</td>
<td>590</td>
<td>85</td>
<td>69</td>
<td>ABC/3TC/ATV/RTV</td>
<td>Negative</td>
<td>&lt;300</td>
</tr>
<tr>
<td>12</td>
<td>780</td>
<td>51</td>
<td>49</td>
<td>ABC/3TC/LPV/RTV</td>
<td>Negative</td>
<td>&lt;300</td>
</tr>
<tr>
<td>13</td>
<td>490</td>
<td>114</td>
<td>48</td>
<td>AZT/3TC/RTV/SQV</td>
<td>Positive</td>
<td>336</td>
</tr>
</tbody>
</table>

*Absolute blood CD4+ T cell count
Figure 5.1 – HIV levels in blood and semen after HAART initiation.

Median levels of HIV RNA in the blood and semen of all 25 participants (Figure 1a), demonstrating that complete viral suppression below the level of detection in each compartment was the rule. However, this masked significant inter-individual heterogeneity. One participant had sustained semen shedding despite effective HAART (Figure 1b). Other individuals developed isolated semen shedding of HIV RNA despite initial semen suppression, both at low levels (<5,000 RNA copies/ml; Figure 1c) and high levels (≥5,000 HIV RNA copies/ml; Figure 1d).
Figure 5.2 – Participants with undetectable HIV RNA in blood and semen plasma, by study time point. The proportion of study participants with HIV RNA levels below the limit of detection in blood (< 50 copies/mL) and semen (< 300 copies/mL) up to 24 weeks after HAART initiation.
A.

![Graph showing concentrations of drugs in blood plasma](image)

**Concentrations of drugs in blood plasma (µg/mL)**

- **Efavirenz**
  - Shed: p=0.5
  - No shed

- **Ritonavir**
  - Shed: p=1.0
  - No shed

- **Lamivudine**
  - Shed: p=0.1
  - No shed

**Isolated semen HIV shedding (shed/no shed)**
B.

**Concentrations of drugs in semen plasma (μg/mL)**

- **Efavirenz**
  - Shed: [Data Points]
  - No shed: [Data Points]
  - p = 0.29

- **Lamivudine**
  - Shed: [Data Points]
  - No shed: [Data Points]
  - p = 0.84

*Isolated semen HIV shedding (shed/no shed)*
Figure 5.3 – Comparing concentrations of antiretroviral agents in blood and semen plasma of HIV-infected patients five months after HAART initiation. Similar levels of antiretroviral drugs in blood (A) and the semen (B) of HIV infected individuals on completely suppressive HAART. Drug levels were quite varied in blood and semen plasma, as some drugs (EFV) were found in higher concentration in blood while others (3TC) were found at much higher levels in semen plasma (C). Drug concentrations in blood or semen were not associated with isolated semen HIV shedding.
Chapter 6: Immune reconstitution in the sigmoid colon after long-term HIV therapy

© Mucosal Immunology 2008; 1(5) 382 – 388.
6.1 Abstract

Early and profound CD4+ T-cell depletion in gut-associated lymphoid tissue (GALT) may drive HIV-1 (HIV) immune-pathogenesis, and GALT immune reconstitution on highly active antiretroviral therapy (HAART) may be suboptimal. Blood and sigmoid colon biopsies were collected from HAART-treated individuals with undetectable blood HIV RNA levels for ≥4 years, and from uninfected controls. HIV proviral levels and T cell phenotype/function were examined in blood and sigmoid colon. CD4+ T-cell proportions in the sigmoid, including those expressing CCR5, were higher than in blood. HIV proviral load did not correlate with CD4+ reconstitution at either site, but in the sigmoid was correlated with CD8+ T-cell immune activation. Colonic Gag-specific T-cell responses were common, but were not associated with proviral load or immune activation. In this select study population, long-term HAART was associated with complete CD4+ T cell reconstitution in sigmoid colon. However, colonic immune activation may drive ongoing HIV replication.
6.2 Introduction

HIV-1 (HIV) infects almost 32 million people worldwide\textsuperscript{115}. Although HIV infection is characterized by progressive immunodeficiency at a systemic level, HIV is usually acquired across a mucosal surface (the genital or gastrointestinal mucosa), and mucosal events are increasingly recognized as critical to HIV pathogenesis\textsuperscript{419}. CD4+ T-cell levels in blood serve as reliable prognostic markers for HIV clinical outcome, but recent studies have shown that the most profound and early CD4+ depletion occurs in the gastrointestinal associated lymphoid tissue (GALT), which houses up to 80% of CD4+ T-cells in the body\textsuperscript{79,420,74,76}. In striking contrast to the gradual depletion of CD4+ T-cells observed in peripheral blood, almost 90% of GALT CD4+ T-cells are depleted within two weeks of HIV infection\textsuperscript{74,82,86,83} and it has been hypothesized that these mucosal events may pave the way for progressive immune dysfunction and death\textsuperscript{75}.

Highly Active Antiretroviral Therapy (HAART) rapidly reduces the level of plasma HIV viremia, and is accompanied by a more gradual reconstitution of blood CD4+ T-cell populations and dramatic improvements in life expectancy\textsuperscript{421}. Prior studies have suggested that CD4+ T-cell depletion in the gastrointestinal tract may not be reversed to the same degree as in blood\textsuperscript{268,270}. Although such studies generally evaluated the impact of relatively short term HAART (<4 years)\textsuperscript{268,270}, recent work demonstrated incomplete reconstitution in the GALT of the small intestine even after prolonged, effective therapy\textsuperscript{422}. This suggests either that immunologic reconstitution in the gastrointestinal tract takes longer than in blood, or that HAART is simply unable to
reconstitute gastrointestinal CD4+ T-cell populations. Since depletion of gastrointestinal CD4+ T-cells has been associated with reduced expression of genes important for digestive functions, reduced nutrient absorption, and increased mucosal expression of genes associated with inflammation/immune activation, this may have important clinical consequences.

HIV infection is associated with increased immune activation in the GALT. Systemic immune activation has been associated with a higher HIV blood viral load and faster disease progression to AIDS. Regulatory T-cells (CD4+CD25+) that suppress immune activation have been associated with lower HIV viremia and higher peripheral CD4/CD8 T-cell ratios. HIV-specific cellular immune responses also play an important role in host immune control. Specifically, CD8+ T-cell immune responses targeting HIV Gag have been associated with lower HIV RNA plasma viral load and delayed disease progression to AIDS. Strong HIV-specific CD8+ T-cell responses may be present in the gastrointestinal mucosa of HIV-infected individuals, although their role in local (mucosal) HIV immune control and/or immuno-pathogenesis is not clear.

We hypothesized that, due to the absence of local HIV replication, long-term suppressive HAART would be associated with complete immune reconstitution in the sigmoid colon and a loss of HIV-specific CD8+ T-cell responses in both blood and the colonic mucosa. To address this hypothesis, we have examined the phenotype and
function of T cells from the blood and sigmoid colon of HIV-infected men on long-term, completely suppressive HAART.

6.3 Methods

6.3.1 Study Participants

HIV infected and uninfected controls were recruited through the Canadian Immunodeficiency Research Collaborative at the Maple Leaf Medical Clinic in Toronto, Canada. All HIV-infected participants had been on uninterrupted HAART for at least 48 months with an undetectable blood HIV viral load (< 50 copies/mL). All subjects provided informed, written consent. The study protocol was approved by Research Ethics Boards at the University Health Network, Toronto, the University of Toronto and St. Michael’s Hospital, Toronto.

6.3.2 Sampling Protocol

Blood was obtained by venipuncture and collected into Acid Citrate Dextran solution A (BD Bioscience, La Jolla, CA). Plasma viremia was determined using the Versant HIV-1 RNA 3.0 assay (bDNA; Bayer Diagnostics, Puteaux Cedex, France). Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation as previously described. Between 20-25 biopsies were obtained from the recto-sigmoid, approximately 25–30 cm from the anal verge. Biopsies were immediately placed into RPMI containing 100 U/mL penicillin, 100μg/mL streptomycin and 1X GlutaMAX-1 (Invitrogen, Carlsbad, CA). Colonic mucosal mononuclear cells (CMMC) were isolated
by digestion with 0.5-1.0 mg/mL of Collagenase type II (Clostridiopeptidase A, Sigma-Aldrich, St Louis, MI) for 30 minutes on a stirring heating block. The digested cell suspension was filtered through a 100 µm filter and enumerated.

### 6.3.3 Evaluating HIV-specific immune responses ex vivo

HIV Gag-specific CD4+ and CD8+ T-cell specific immune responses were measured in blood and the sigmoid colon, using modifications of a previously described protocol\textsuperscript{398}. One million CMC and PBMC were incubated in media with 2% DMSO (negative control), or stimulated using Staphlococcus enterotoxin-B (SEB, positive control; 3µg/mL), or Gag Clade-B peptide pool (1µg/mL/peptide) for 1 hour at 37°C and 5% CO\textsubscript{2}. No co-stimulatory molecules were used. Clade-B Gag pool was made up of 122 15-mer overlapping peptides encompassing the entire Gag-gene. Brefeldin A (1µg/mL) was added, and samples incubated for another 5 hours at 37°C and 5% CO\textsubscript{2}. Samples were washed with 1% FBS-PBS, permeabilized, and stained with fluorochrome-labelled monoclonal antibodies specific for CD8, CD3, TNF-α and IFN-γ (Becton Dickinson Immunocytometry Systems, San Jose, CA). Samples were acquired using a FACSCalibur flow cytometer (BD Systems), and data analysis performed using Flow Jo analytical software version 7.2.2 (Treestar Inc. Ashland, OR). A positive response was defined as background-corrected cytokine production in the HIV Gag well exceeding 0.05% of gated cells, and background levels of cytokine production by at least two-fold.
6.3.4 Measurement of proviral HIV DNA:

CD4+ T-cells were isolated from PBMCs using a column-based cell separation technique (StemCell Technologies, Vancouver, BC, Canada) as previously described \(^{429}\). To determine the proviral HIV DNA copies per million CD4+ T-cells, genomic DNA was isolated using the Puregene DNA isolation kit according to the manufacturer’s specifications (Gentra, Minneapolis, MN). 1µg of DNA was then used as template for real-time PCR in an iCycler (Bio-Rad, Hercules, CA). The amplification reaction was carried out in triplicate using 0.5 µM primers, 0.2µM fluorescent probe, 0.8mM dNTPs, 5mM MgCl\(_2\), and 2.5U Platinum Taq Polymerase (Invitrogen, Carlsbad, CA) in 50µl total volume. The following primers were used: 5’GGTCTCTCTTGAGTTTAGACCAGAT-3’ (5’ primer) and 5’-CTGCTAGAGTTCTTCACACTG-3’ (3’ primer) along with the fluorescent probe 5’-6FAM-AGTAGTGTGCTGACCTGGGT-TAMRA-3’. PCR conditions consisted of a denaturation step at 95°C for 3 min followed by 45 cycles of 15 sec at 95°C and 1 min at 59°C. Serially diluted ACH-2 DNA was also subjected to the above PCR to obtain standard curves. Approximately 200,000 CD8-depleted cells were lysed in 10mM Tris-HCl pH8 containing 100µg/ml proteinase K (Roche Applied Science, Indianapolis, IN) for one hour at 56°C followed by heat inactivation of the enzyme. Real-time PCR specific for human β-actin DNA (Applied Biosystems, Foster City, CA) was carried out on the above cell lysates in order to determine the exact copy number of cells per µl of cell lysate. Serially diluted ACH-2 DNA was also subjected to the above PCR to obtain standard curves. Finally, real-time PCR specific for HIV DNA was carried out as described above and the copy number of HIV DNA per 1×10^6 CD4+
T-cells was calculated based on the results obtained from FACS and PCR experiments. This protocol was performed by Dr. Tae-Wook Chun in the laboratory of immune regulation at the National institute of Health.

6.3.5 Statistical analysis

SPSS 14 for Windows XP (SPSS Inc, Chicago, IL) was used for statistical analysis. Intra-individual comparisons of T-cell populations in blood and sigmoid colon were performed using the paired samples t-test. The Mantel-Haenszel chi-square test with calculation of likelihood ratios and confidence intervals were used to compare dichotomous variables between study groups, and comparison of means between groups was performed by the Mann Whitney U non-parametric test. Linear association of continuous variables was assessed using the Spearman’s rank correlation coefficient.

6.4 Results

6.4.1 Study participants

The study population consisted of 23 HIV infected men on long-term HAART and 3 HIV uninfected controls (Table 1). The mean duration of prior HAART was 120 months (range, 51-216 months), and the mean duration of viral suppression below the level of detection was 89 months (range, 49 – 130 months). Therapy had been initiated a mean of 28 months (range, 2-108 months) after the first HIV positive ELISA. For those participants with data available, the mean nadir CD4+ T cell count had been 183/mm³ (range, 32-360/mm³).
6.4.2 CD4+ T-cell populations in the blood and the sigmoid colon

The proportion of CD4+ T-cells in the sigmoid colon of highly-suppressed participants was significantly higher than in peripheral blood (mean, 56% vs. 45%, respectively; Paired t-test p = 0.001; Figure 6.1 and 6.2), and was similar to that seen in HIV uninfected controls (mean, 56% vs. 64%; p = 0.14; Figure 6.1 and 6.2). However, blood CD4+ T-cell proportions in blood remained lower in HIV infected participants (45% vs. 65% respectively, p = 0.008; Figure 6.1).

Earlier studies described incomplete reconstitution of CD4+ T-cells co-expressing CCR5+ in the recto-sigmoid despite HAART\textsuperscript{268}. CCR5+ expression was assayed in 10/23 (43%) HIV infected participants on long-term HAART, with similar levels to HIV negative controls seen in both blood (mean %CD4+CCR5+: 1.8 vs. 3.8 respectively; p = 0.15; Figure 6.3 and 6.4) and sigmoid colon (mean %CD4+CCR5+: 47% vs. 53% respectively, p = 0.53; Figure 6.3 and 6.4).

HIV-infected participants had been on suppressive HAART for different lengths of time (range: 51 – 216 months, Table 1), and had started at different stages of infection. Therefore, the impact of these differences on CD4+ T-cell immune reconstitution was examined. The prior duration of HAART did not correlate with CD4+ T-cell reconstitution in blood (r=0.16; p=0.50) or sigmoid colon (r=0.14; p=0.55), although those treated for >10 years tended to have higher colonic CD4+ T-cell proportions than those treated for <5 years (58% vs. 50%, respectively; p=0.065). Pre-therapy nadir CD4+
T-cell counts were available for 15/23 (65%) participants, and did not correlate with immune reconstitution in blood (Figure 6.5a) or the sigmoid colon (Figure 6.5b).

6.4.3 Associations of HIV proviral load in CD4+ T-cells in blood and sigmoid colon

HIV RNA was undetectable in blood of all HIV infected individuals, and was not measured in sigmoid colon. HIV proviral DNA was assayed in the blood of all participants and in the sigmoid colon of 11 of 23 individuals (48%). Although there was a negative correlation between proviral HIV DNA levels and CD4+ T-cell proportions in blood (r = -0.41, p = 0.04, Figure 6.6), no such correlation was observed in the sigmoid colon (r = 0.31, p = 0.39). Since CD4+ T-cell populations in blood and sigmoid were isolated using different techniques, we were unable to directly compare proviral HIV DNA levels between these two compartments.

Expression of the early activation marker CD69 was much higher in colonic than peripheral blood T-cells, as expected, on both CD4+ T-cells (40.7% vs. 0.33%, p<0.001, Figure 6.7) and CD8+ T-cells (35.4% vs. 0.7%, p < 0.001, Figure 6.8). The same applied to expression of HLA-DR by colonic CD4+ T cells (3.2% vs. 0.35%, respectively; p<0.001). However, levels of CD69 and HLA-DR expression on colonic CD4+ and CD8+ T-cells in HAART treated individuals did not differ from HIV negative controls (data not shown). Colonic HIV proviral DNA levels were correlated with increased mucosal CD8+ T cell immune activation, as reflected by CD69+ expression (r=0.7, p=0.02, Figure 6.8).
### 6.4.4 HIV-specific T-cell responses

HIV Gag-specific T-cell responses were measured in blood and the sigmoid colon of 16/23 (70%) participants. IFN-γ and/or TNF-α immune responses against HIV Gag were detected in the colon of 7/16 (44%) and in the blood of 10/16 (63%) patients (Figure 6.9 and 6.10). There was no association between CD4/CD8+ T cell ratio and the presence or absence of HIV Gag-specific T-cell responses in either compartment (sigmoid, 50% vs. 55%, p=0.46; blood, 45% vs. 48%, p=0.5). Likewise, no association was seen between CD4/CD8+ T-cell ratio and the strength of HIV-specific T-cell responses, using either IFNγ or TNFα as a functional output. Neither CD4+ nor CD8+ T-cell responses in the blood or the sigmoid colon were associated with HIV proviral DNA levels at their respective sites (p>0.4 for both, Figure 6.11).

### 6.4.5 Regulatory T-cell populations in blood and the sigmoid colon

Regulatory T-cells were defined as CD4+ T-cells expressing both FoxP3 and CD25+ were significantly increased in sigmoid colon relative to blood in HIV-infected participants (mean, 1.2% vs. 0.2% of CD4+ T-cells; Paired t test p=0.002, Figure 6.12) and HIV-negative controls. Regulatory T-cell proportions were much lower in the blood of HIV-infected individuals on long-term HAART than in HIV-uninfected controls (mean, %CD4+CD25+FoxP3+; 0.18 vs. 1.12, respectively; p=0.009) (Figure 6.12; representative dot plot), but in contrast to previous reports430 did not differ in the sigmoid colon (0.56% vs. 0.88%, respectively; p=0.49) (Figure 6.13). T regulatory populations in the sigmoid colon did not correlate with mucosal immune activation, although in blood
there was a positive correlation with CD4+ T-cell expression of HLA-DR (r=0.57; p=0.02). There were no associations in either compartment between regulatory T-cell populations and duration of therapy or time to initiation of therapy (p > 0.1 for both).

6.5 Discussion

The early damage inflicted by HIV at the level of the host gastrointestinal mucosa may set the stage for subsequent systemic inflammation and progressive immune dysfunction\textsuperscript{74,81,93}. Despite the dramatic effects of HAART on blood CD4+ T-cell counts and host survival, it has been unclear to what degree antiretroviral therapy is able to correct these mucosal immune defects, since significantly reduced CD4/8+ T-cell ratios persist despite effective therapy\textsuperscript{268,269}. It is therefore encouraging that, in this selected cohort of HIV infected men on long term suppressive HAART, CD4+ T-cell reconstitution in the sigmoid colon actually exceeded that observed in the blood, with CD4+ T-cell proportions in the colonic mucosa of HAART-treated men approximating those of HIV-uninfected controls. Furthermore, similar degrees of reconstitution were observed in the proportion of colonic CD4+ T-cells expressing the HIV co-receptor and activation marker CCR5. However, although increased CD4+ T-cell proportions in the gastrointestinal mucosa have been linked to increased expression of genes involved in growth/repair and reduction in the expression of genes associated with inflammation, cell activation, and ultimately slower disease progression\textsuperscript{269}, the clinical implications of our findings are not known.
The highly-selected participants in this study had unusually complete virus suppression on HAART, but results from this subgroup clearly demonstrate that normalization of colonic CD4+ T-cell proportions is possible on HAART. Prior studies have found persistent depletion of CD4+ T-cells in the recto-sigmoid colon, particularly CD4+ cells expressing CCR5, after three years or more of HAART\textsuperscript{268}. Our positive findings might relate to the increased duration, and perhaps to increased completeness, of viral suppression in our study population. However, a very recent study found that GALT immune reconstitution was incomplete in the terminal ileum (lower small intestine) of a similar highly-suppressed patient population\textsuperscript{422}. While small intestinal biopsies were not collected in our study, this suggests the possibility that there may be differential immune reconstitution in the mucosa of the large vs. small intestine after long term viral suppression on HAART. Confirmation of this hypothesis will require further studies with sampling of both the large and small intestinal mucosa. Furthermore, although we found no statistical difference between CD4+ T cell percentages between HIV-negative and HIV-infected individuals on long-term HAART, we do acknowledge that levels of CD4+ T cells were a little lower in HIV-infected individuals and perhaps the difference would have been statistically significant if we had more HIV-negative individuals enrolled in our study.

HIV preferentially infects and replicates in activated CD4+ T-cells. This may underlie the viral predilection for the gastrointestinal mucosa, where the number of activated, proliferating CD4+ T-cells is high\textsuperscript{268}. Levels of immune activation may be elevated in both blood and colonic mucosa of HIV-infected, therapy-naïve individuals,
based on expression of HLA-DR\textsuperscript{268}. Immune activation in the colon of our participants on long-term HAART, as assayed by expression of HLA-DR and the activation marker CD69, was similar to uninfected controls. Despite this, the size of HIV proviral reservoir in the colon was correlated with the degree of CD8+ T-cell immune activation, implying that even a “normalized” level of immune activation in the gut mucosa may be sufficient to support low level virus replication despite HAART.

In HAART-naïve individuals, HIV Gag-specific CD8+ T-cell responses in blood have been associated with reduced viremia and slower disease progression\textsuperscript{313}. HIV-specific CD8+ T-cell responses are also detectable at mucosal sites, including the gut\textsuperscript{391}, cervix\textsuperscript{342,366} and semen\textsuperscript{398,340}, although their clinical significance at these mucosal sites is less clear. In our highly-suppressed participants, Gag-specific CD8+ (and to a lesser degree CD4+) T-cell immune responses were often detectable in the blood and colonic mucosa, but neither their presence nor their magnitude was associated with the degree of CD4+ T-cell reconstitution or the HIV latent proviral DNA load at either site. However, we examined only a limited repertoire of T-cell immune functions (production of TNF-\(\alpha\) and IFN-\(\gamma\)), and the immune techniques used did not permit us to determine the clonal diversity of the HIV-specific T-cell immune responses observed. Specifically, responses may have represented the preservation or reconstitution of a clonally diverse T-cell population, or the re-expansion of an oligo-clonal response from a small reservoir of memory effector T-cells. In addition, cell numbers did not permit detailed mapping of the antigen specificity of the responses seen, a factor that may impact host immune control of virus. Nonetheless, since blood HIV-specific CD8+ responses wane on HAART, in the
absence of antigenic stimulation\textsuperscript{358}, the detection of responses in our participants implies that there is ongoing low level viral replication in blood and sigmoid colon despite long term suppressive therapy.

In summary, levels of CD4+ T-cell reconstitution in the sigmoid colon of this selected group on highly-suppressive antiretroviral therapy actually exceeded those observed in blood, with CD4/CD8+ ratios comparable to uninfected controls. However, the persistence of HIV-specific CD8+ T-cell responses in both the blood and recto-sigmoid, suggests that there may be ongoing low level HIV replication in the gastrointestinal mucosa despite prolonged undetectable viremia. Although complete normalization of CD4+ T-cell proportions in the sigmoid colon can be achieved on long-term suppressive HAART, the clinical importance of such normalization remains unclear, and will be an important area for further study.
Table 6.1- Study participant characteristics of HIV-infected individuals on long-term suppressive HAART.

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<th>Time on HAART (months)</th>
<th>Nadir CD4 Count (/mm$^3$)</th>
<th>Duration of complete viral suppression* (months)</th>
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*The sensitivity of viral load assays varied over time, from a threshold of >1000 RNA copies/ml (1995) to >50 RNA copies/ml (currently).
Figure 6.1 – Percentage of T-cells expression CD4 in blood and the sigmoid colon. CD3+CD4+ T cells in blood and the sigmoid colon in HIV infected individuals on long-term HAART and HIV-negative individuals.
Figure 6.2 – CD4 and CD8 T cell expression in blood and the sigmoid colon. Representative dot-plot of CD3+CD4+ T cells in blood (left panels) and the sigmoid colon (right panels) in HIV negative (bottom row) and HIV positive individuals on long-term suppressive HAART (top row).
Figure 6.3 – Comparing CCR5 expression on CD4+ T cells in blood and the sigmoid colon

Proportion of CD4+ T cells in blood and the sigmoid colon of HIV-infected individuals on long-term HAART and HIV negative individuals co-expressing CCR5.
Figure 6.4 – CCR5 expression on CD4+ T cells from blood and the sigmoid colon. Representative example of CD4+ T cells co-expressing CCR5 in blood (left column) and the sigmoid colon (right column) in HIV-infected individuals on long-term suppressive HAART (top row) and HIV-negative individuals (bottom row)
Figure 6.5 – Blood nadir CD4 count and immune reconstitution of CD4+ T cells in blood and the sigmoid colon. No correlation between nadir CD4 counts prior to starting HAART and level of immune reconstitution in blood (A) and the sigmoid colon (B) in HIV-infected individuals on long-term completely suppressive HAART.
Figure 6.6 – Correlation between %CD4 and HIV proviral DNA in blood. A negative spearman correlation between proportion of CD3+CD4+ T cells and number of copies of pro-viral HIV DNA in blood CD4+ T cells in HIV-infected individuals on long-term completely suppressed HAART.
Figure 6.7 – Comparing expression of CD69 on T cells in blood and sigmoid colon mononuclear cells. The percentage of CD4+ and CD8+ T cells co-expressing the early activation marker CD69 in blood and the sigmoid colon of HIV-infected individuals on long-term suppressive HAART.
Figure 6.8 – Correlating HIV proviral DNA with CD8+ T cell immune activation. Spearman rank correlation between CD8+ T cell expressing CD69+ and HIV proviral DNA of CD4+ T cells in the colonic mucosa.
Figure 6.9 – CD8+ T cell immune response in blood and the sigmoid colon. Gag-specific CD8+ T cell immune responses in the sigmoid colon (top row) and blood (bottom row) in HIV-infected individuals on long-term completely suppressive HAART. IFN-γ and TNF-α as functional outputs of an immune response.
Figure 6.10 – Measuring CD4-specific immune responses in blood and the sigmoid colon. Gag-specific CD4+ T cell immune responses in the sigmoid colon (top row) and blood (bottom row) in HIV-infected individuals on long-term completely suppressive HAART, IFN-γ and TNF-α as functional outputs of an immune response.
Figure 6.11 – HIV proviral DNA in CD4+ T cells and HIV-specific CD8+ T-cell immune responses. The presence of a Gag-specific CD8+ T-cell immune response in the sigmoid colon was not associated with reduced HIV proviral DNA copy numbers in enriched CD4+ T cells isolated from the sigmoid colon of HIV-infected individuals on long-term suppressive HAART.
A. 

![Bar chart showing %CD4+ T cells expressing CD25+Foxp3+ in Sigmoid Colon and Blood, with p = 0.002.]
B.

Figure 6.12 – Regulatory T-cell population in blood and the sigmoid colon. Proportions of CD4+CD25+Foxp3+ T-regulatory cells were significantly higher in the sigmoid colon compared to peripheral blood in HIV-infected individuals on long-term completely suppressive HAART.
Figure 6.13 – Regulatory T cells in sigmoid colon of HIV-infected long-term HAART and HIV negative individuals. Similar proportion of T-regulatory cells (CD4+CD25+Foxp3) in the sigmoid colon of HIV-infected individuals on long-term HAART and HIV negative participants.
Chapter 7: Discussion
The majority of HIV is transmitted sexually, and so understanding the correlates of HIV RNA levels in semen is important from a public health perspective. The focus of my doctoral work was to identify immunologic and clinical correlates of HIV RNA levels in semen with the overall aim to inform novel immunotherapeutic and clinical interventions to reduce sexual transmission of HIV.

As HIV-specific CD8+ T cell immune responses have temporally associated with control of HIV viremia during acute infection\cite{Koup,1994#526}, I was interested to see whether HIV-specific CD8+ T cell immune response in blood and semen were associated with lower HIV RNA levels in semen. We found that neither systemic nor mucosal HIV-specific IFN-\(\gamma\) CD8+ T cell immune responses correlated with HIV RNA shedding in semen, in fact the latter were associated with increased levels of semen HIV RNA, implying that perhaps boosting up mucosal or systemic HIV-specific CD8+ T cell immune responses in HIV-infected individuals through a therapeutic vaccine may not reduce transmission. However, our study had several limitations including the fact that HIV RNA and HIV-specific CD8+ T cells in semen originate from different organs/components of the male genital tract at varying levels\cite{Coombs,2006#3893}\cite{Shevchuk,1998#3324; Le Tortorec,2008#4399; Roulet,2006#3886}. HIV RNA in the male genital tract has been isolated from the prostate\cite{Coombs,2006#3893}, the bulbourethral gland\cite{Coombs,2006#3893} and the testes\cite{Shevchuk,1998#3324; Le Tortorec,2008#4399; Roulet,2006#3886}. Therefore it is possible that HIV-specific CD8+ T cells be associated with lower HIV RNA levels in some organs, but not in others. Thus, the compartmental nature of semen, with contribution of fluids and cells
from different organs, may have confounded my attempts to evaluate the role of HIV-specific CD8+ T cell immune responses in reducing HIV RNA levels in the male genital tract. In addition, IFN-γ production may not be the best surrogate marker for assessing the functionality of HIV-specific CD8+ T cell immune responses. Recent finding suggest that poly-functional HIV-specific CD8+ T cells may be better predictors of the quality of HIV-specific CD8+ T cells in HIV infected individuals {Betts, 2006 #3636}. Overall, my findings suggest that boosting up IFN-γ specific CD8+ immune responses with a therapeutic HIV vaccine is not a promising strategy to reduce sexual transmission of HIV.

I next evaluated the correlation between common viral co-pathogens and semen HIV RNA levels. We found a strong positive correlation between levels of HIV RNA and CMV DNA in semen, and disproportionately high HIV RNA shedding in semen was associated with CMV reactivation (Chapter 4). Although, the cross-sectional nature of our studies made it impossible to determine the direction of causality, suggesting that asymptomatic reactivation of this near ubiquitous pathogen may alter the infectiousness of an HIV infected individual. A logical next step could be treat CMV and evaluate the impact that has on HIV RNA levels in semen. In fact these studies have been recently undertaken within our research group and preliminary results suggest that treating CMV has an impact of semen HIV RNA levels, suggesting that CMV reactivation was promoting HIV RNA replication in semen.

Perhaps the most intuitive way to reduce HIV levels in semen and subsequently reduce sexual transmission is HAART, which is associated with complete suppression of
HIV RNA in blood. However, a better understanding of the impact of HAART on HIV RNA levels in semen is needed. We found that in the majority of individuals HAART was associated with complete suppression of HIV RNA in semen, but found incomplete suppression of semen HIV RNA in a significant proportion of men {Sheth, 2009 #4982}. These results suggest that HAART will reduce sexual transmission of HIV at a population level. However, as some HIV-infected individuals on therapy continue to shed infectious HIV RNA in their semen, the individual risk of transmitting HIV is unlikely to be zero. Despite our data suggesting the impact of HAART on HIV transmission, data from clinical trials examining the impact of therapy on HIV transmission in the real world are urgently needed. In the meantime it is imperative that studies continue to identify immune and viral correlates of HIV shedding in semen in hopes that better understanding of correlates can lead to the development of novel immunotherapeutic interventions as well as better inform public health policy.
Chapter 8 – Future Directions
As with most scientific undertakings, these results generated more questions as well as answers. The development of more sophisticated techniques and a better understanding of the immune system over the last few years will enable future studies to revisit old questions with more powerful tools and further some of my findings to better inform the scientific community about potential mechanisms and inform public health policy. In this section I have suggested a brief description of future projects that can be undertaken to further some of the conclusions that were reached over the course of my doctoral work.

8.1 –Systemic HIV-specific CD8+ T cell responses and semen HIV RNA shedding

My early work demonstrated that HIV-specific CD8+ T cell immune responses in blood or semen did not correlate with HIV RNA levels in either compartment in HIV infected therapy naive men. However, all of these studies were carried out using IFN-γ production as a surrogate marker of HIV-specific CD8+ T cell antiviral activity. Recent developments in the field have suggested that using IFN-γ as a surrogate marker in the evaluation of HIV-specific immune responses may be incomplete. Although immune correlates of HIV protection or progression remains elusive, in studies comparing immune responses in progressors vs. long-term non-progressors (LTNP), non-progression of HIV was associated with more functional HIV-specific CD8+ T cell responses, and with focus on HIV-gag. Analysis of HIV-specific immune responses and their ability to reduce HIV RNA shedding in semen using multi-parameter flow cytometry and
functional assays would provide more definitive answers around the quality of HIV-specific immune responses in semen as they relate to HIV shedding.

8.2 Evaluating mucosal γδCD8+ T cells and semen HIV RNA shedding

Studies performed for my PhD centered on investigating the associations of αβ CD8+ T cells and HIV RNA shedding in the semen of therapy-naive men. However a subset of CD8+ T cells that express γδ chain TCR are thought to play an important role in mucosal immunity. γδ CD8+ T cell make up almost 10% of CD8+ T cells in peripheral blood but are particularly enriched in the gastrointestinal associated lymphoid tissue and the genital mucosa. The importance of γδ CD8 T cells in mucosal immunity has been demonstrated in experiments carried out in SIV infection studies in RM. RM’s resistant to SIV infection following rectal SIV exposure had 10-fold more γδ CD8+ T cells in the rectal mucosa compared to RM’s that became infected with SIV following rectal challenge. Depletion studies using monoclonal antibodies against γδ T cells in SIV infected RM resulted in a four-fold reduction in anti-SIV CD8 suppressor activity in vivo. γδ CD8+ T cell depletion was also associated with reduction in several anti-HIV factors including RANTES, MIP1α and MIP1β – all of which have direct anti-HIV and anti-SIV activity. Furthermore, γδ CD8+ T cells have also been shown to have antiviral and more importantly direct anti-HIV activity. However, despite the enrichment of γδ CD8+ T cells in the genital tract, very little is known about their ability to influence HIV RNA shedding in the genital tract of HIV infected men and women. It would be particularly interesting if future projects would investigate the anti-HIV activity
of γδCD8+ T cells from the male and female genital tracts *ex vivo* to establish their anti-HIV killing activity. Furthermore, as γδCD8+ T cell have been implicated in thwarting SIV infection in RM’s, evaluating these T cell populations in HEPS individuals would be interesting. In addition, the relative enrichment of γδCD8+ T cells in the genital tract environment may influence HIV RNA shedding in the genital secretions of HIV infected men and women.

### 8.3 –CMV and HIV shedding in semen: understanding viral synergy

CMV reactivation was associated with disproportionately high semen HIV RNA shedding in HIV-infected, therapy-naive men (Chapter 4). Furthermore, CMV reactivation is a strong predictor of HIV RNA shedding in the male and female genital tracts, and levels of CMV DNA positively correlates with semen HIV RNA levels. As understanding mechanisms of viral synergy may lead to the development of more effective ways to prevent transmission, it would be particularly interesting to evaluate the impact CMV immunity and drug suppression would have on HIV RNA shedding in semen.

CMV-specific CD8+ and CD4+ protect both HIV-negative and HIV positive individuals from CMV disease. CMV-specific immune responses have demonstrated the ability to control CMV viremia. It would be particularly interesting to examine if CMV specific CD8+ and CD4+ T cell responses are associated with either reduced CMV shedding, or more importantly with reduced HIV shedding in the semen of therapy-naive co-infected men. The presence of immune responses against CMV
proteins pp65 and IE-1 have been associated with robust protection against CMV disease and so future investigations can perhaps focus on immune responses against these two CMV proteins and CMV DNA shedding in semen. Furthermore, as virus specific immune responses are detectable in semen, and often stronger than blood\textsuperscript{398}, measuring CMV-specific immune responses in semen and evaluating the correlation between CMV-specific immune responses in semen and CMV DNA (and HIV RNA) shedding in semen would be valuable to understand the immune associations of CMV on sexual transmission of HIV.

A more direct method to evaluate the impact of CMV suppression on HIV RNA in semen would be to therapeutically suppress CMV reactivation. I propose enrolling HIV-CMV co-infected men into a placebo-controlled double blinded study evaluating the impact of CMV therapy on CMV DNA levels (and HIV RNA levels) in semen.

Valgaciclovir is a potent DNA polymerase inhibitor, a pro-drug formulation of ganciclovir, commonly used as first-line treatment for CMV disease\textsuperscript{438}. The low bioavailability of oral ganciclovir led to the development of valganciclovir, which has been demonstrated to have 10-fold higher absolute bioavailability than valganciclovir, with similar efficacy and safety\textsuperscript{438, 439}. Valganciclovir prevents CMV replication as it substitutes valganciclovir triphosphate (in place of deoxyguanosine triphosphate), thereby inhibiting DNA synthesis\textsuperscript{438, 440} and preventing CMV disease.

I propose carrying out a prospective double-blinded placebo controlled study to evaluate the impact of CMV therapy on HIV RNA levels in semen. The study would
enrol HIV-CMV co-infected men and then measure HIV RNA and CMV DNA levels in blood and semen samples at baseline (prior to any interventions), while on valganciclovir or placebo and then after completion of their regimen (valganciclovir or placebo).

This double blinded placebo controlled study would elucidate the impact of CMV reactivation on HIV RNA levels in semen. The prospective nature of this therapeutic intervention would enable us determine causality, and better understand if CMV reactivation directly induced HIV RNA shedding in semen or if CMV reactivation a consequence of semen HIV RNA shedding. It would also help in better understanding if CMV therapy has any long-term effects on HIV RNA levels or if the effect (if any) is transient. A better understanding of the contribution of ubiquitous viral pathogens like CMV on HIV RNA shedding in semen would be of great interest and will help make more informed and targeted public health interventions.

8.4 – Investigating correlate of isolated Semen HIV RNA shedding in HIV infected individuals on suppressive HAART.

In HIV-infected individuals, starting HAART results in a rapid reduction of HIV RNA levels in blood and semen (Chapter 5). However, HAART was not associated with a reduction in levels of CMV DNA in blood or semen plasma (Sheth PM, unpublished results). In our most recent study (Chapter 5) we observed isolated semen HIV RNA shedding in men with no detectable viremia. In fact, isolated semen HIV shedding was also observed in HIV infected men who had been on completely suppressive HAART with >4 years of completely suppressed viremia (Sheth PM, AIDS 2009 in press).
Although we evaluated several co-factors including HSV-2 sero-status, absolute CD4 count, blood viral load and semen concentrations of HAART drugs none of them were associated with isolated semen HIV shedding.

We hypothesize that CMV reactivation may be an important factor contributing to isolated semen HIV shedding in co-infected men on HAART. Understanding factors that may be associated with isolated semen HIV RNA shedding in semen would be particularly useful and informative when tailoring prevention policy for treated men. The project would involve measuring CMV DNA levels in semen plasma of men on completely suppressive HAART with and without episodes of isolated semen HIV shedding. This initial investigation would attempt to examine if CMV reactivation in semen would be associated with isolated semen HIV RNA shedding.

We also observed that inflammatory cytokines (particularly IL-6, IL-8 and IL12p40) in semen plasma were associated with levels of HIV RNA in semen plasma (Chapter 4). As several inflammatory cytokines have been shown to activate the HIV LTR directly, perhaps completely suppressive HAART in HIV infected individuals does not completely suppress inflammation in genital tract of HIV infected men. Inflammatory cytokines (IL6, IL-8, IL12p40, IP-10) and βchemokines (RANTES, MIP1β and MIP1α) should be measured in the blood and semen plasma of HIV-infected individuals on completely suppressive HAART with and without isolated semen HIV RNA shedding in semen using a multiplex ELISA assay or the previously used CBA assay.
8.5 - Isolated semen HIV shedding and CMV super-infection

A more in-depth study to examine potential mechanisms associated with CMV reactivation and isolated semen HIV RNA shedding would shed light on both the phenomenon of isolated semen HIV shedding and disproportionate shedding of HIV RNA in the semen of co-infected therapy naive men. Pre-existing CMV infection is not protective against CMV infection from additional CMV strains and is quite common in HIV infected individuals. Therefore, it would be interesting to evaluate the impact of CMV super-infection on HIV RNA shedding. Although the association between multiple strains of CMV and HIV has not been examined, CMV reactivation has been associated with increased HIV shedding and infection with multiple strains of CMV has been shown to correlate with increased CMV viral loads. Furthermore, a positive correlation has been observed between bacterial vaginosis and multiple CMV strains in the vaginal wash of women attending an STD clinic. Again the majority of studies have been carried out in women and the impact of CMV super-infection on HIV RNA levels in semen is unclear and should be an important next step in better understanding correlates of HIV RNA shedding in semen.
8.6 – Reconstitution of CD4+ T cell subsets in the sigmoid colon of HIV-infected individuals on long-term HAART

In 2008, our group published the first report investigating the impact long-term completely suppressed HAART (>4 years) had on CD4+ T cell in the sigmoid colon (Chapter 6) 409. We observed that long-term completely suppressive HAART was associated with complete reconstitution of CD4+ T cells in the sigmoid colon, to levels observed in HIV-negative individuals. However, recent studies have identified a functionally distinct CD4+ T cell population that has been implicated in controlling and reducing bacterial translocation across the gut mucosa 444 in HIV infected individuals.

Although long-term HAART is associated with complete immune reconstitution of CD4+ T cell in the sigmoid colon, the ability of HAART to restore key CD4+ subsets, particularly IL-17 producing CD4+ T cells (referred to as Th17 cells) remains unclear. Since elevated levels of proviral HIV DNA may be due to low level viral replication in the GALT, and as immune activation is a strong predictor for HIV disease progression, reconstitution of these immune populations may have important implications in better understanding HIV pathogenesis.


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