THE ROLE OF GUT MUCOSAL IMMUNOLOGY IN HIV IMMUNOPATHOGENESIS

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Medical Science
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The role of gut mucosal immunology in HIV immunopathogenesis

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ABSTRACT

Progressive HIV infection is characterized by a rapid and profound depletion of CD4 T cells from the gut, structural deterioration of the intestinal epithelium, and microbial translocation into the gut mucosa and bloodstream. Circulating microbes are potent immune activators and the resulting inflammation is linked to HIV disease progression, serious non-AIDS events, and death. The gut is normally enriched with bacteria-specific Th17 cells and tissue protective Th22 cells that provide mucosal immune defense against invading microbes, however these cells may be preferential targets of HIV. Although HIV-infected elite controllers (ECs) suppress HIV to undetectable levels without antiretroviral therapy (ART), microbial translocation and chronic inflammation may increase serious non-AIDS occurrences and mortality.

My PhD work aims to elucidate the role of gut mucosal immunology in its protection against microbial translocation and immune activation in treated and untreated HIV infection. During chronic HIV infection, IL-22 producing gut lymphocytes, including Th22 cells, were profoundly depleted, epithelial integrity was compromised, and microbial translocation was increased. However, early infection was uniquely associated with increased IL-22 production by non-T cells and the preservation of epithelial integrity. The tissue protective role of Th22 cells was supported by in vitro studies demonstrating the capacity of IL-22 to prevent HIV-induced
epithelial damage. Sigmoid Th17 cells were also depleted during chronic HIV infection, but their pro-inflammatory polyfunctional capacity (co-production of IL-22, TNF-α and/or IFN-γ) was lost from the earliest stages. Although ART rapidly restored gut Th17 numbers, their functional reconstitution and resolution of microbial translocation was much delayed. ART-naïve ECs had normal levels of gut CD4 T cells, microbial translocation and immune activation, but soluble biomarkers of serious non-AIDS events (IL-6 and D-dimer) were elevated and a trial of short-term ART did not reduce these biomarkers.

These findings emphasize that gut immune changes during HIV infection are important contributors to microbial translocation and systemic inflammation, however gut independent mechanisms may also be involved. Novel therapeutic strategies to accelerate the restoration of gut immune function and to reduce chronic inflammation may have important benefit in people living with HIV.
I would like to start by thanking my supervisor Dr. Rupert Kaul for his guidance, mentorship, and dedication over the years, and for fostering the best environment for me to develop as a scientist. His patience, comprehensive support, and great sense of humor will be long remembered. In particular, I am grateful for the opportunities he has given me to build my network to kick-start my journey as a scientist. I would also like to thank my PhD advisory committee members, Dr. Mario Ostrowski and Dr. Kenneth Croitoru for their insights, thought provoking questions, and for their recommendations during my PhD program. Despite their extremely busy schedules, their availability went beyond the duty of committee members and I am thankful for their support in my future endeavors. I would also like to thank Drs. David O. Willer, Prameet Sheth and Kelly MacDonald from the University of Toronto HIV research team for their mentorship and career advice.

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LIST OF ABBREVIATIONS

AIDS – acquired immunodeficiency syndrome
APC – antigen presenting cells
ART – Antiretroviral therapy
C – constant region of HIV
CCL – CC motif chemokine
CCR5 Δ32 - CCR5 delta 32
CRP – C reactive protein
CTL – cytotoxic T cell
CYP – cytochrome P450
DC – dendritic cells
EC – elite controllers
GALT – gut-associated lymphoid tissue
GIT – gastrointestinal tract
HIV – human immunodeficiency virus
IBD – inflammatory bowel disease
ILC – innate lymphoid cells
ILF – isolated lymphoid follicle
INR – immune non-responders
LBP – LPS-binding protein
LPS – lipopolysaccharide
LTi cells – lymphoid tissue inducer cells
LTNP – long-term nonprogressors
MAdCAM-1 – mucosal addressin cell adhesion molecule 1
MFI – mean fluorescence intensity
MHC – major histocompatibility complex
MLN – mesenteric lymph nodes
NK cells – natural killer cells
NNRTI – non-nucleoside reverse transcriptase inhibitor
NRTI – nucleoside reverse transcriptase inhibitor
PAMPs - pattern-associated molecular patterns
PBMC – peripheral blood mononuclear cells
PCR – polymerase chain reaction
PI – protease inhibitor
PMA – phorbol 12-myristate 13-acetate
PPs – Peyer’s patches
PRR – pattern recognition receptor
RALDH – retinal dehydrogenase
$\rho_s$ – Spearman rank coefficient
SAMDH1 - SAM domain and HD domain-containing protein 1
sCD14 – soluble CD14
SCFA – short chain fatty acids
SFB – segmented filamentous bacteria
SIV – simian immunodeficiency virus
SNA – serious non-AIDS
T cells – T helper cells
T20 – Fuzeon/Enfuvirtide
Tcm – central memory T cells
Teff – effector T cells
Tem – effector memory T cells
TJ – tight junctions
TLR – toll like receptors
Tn cell – naïve T cell
Treg – regulatory T cells
V – highly variable region of HIV
WHO – World Health Organization
MANUSCRIPTS ARISING FROM THIS THESIS


CHAPTER 1

INTRODUCTION
1.1 The HIV pandemic

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS). Over 34 million people are infected with HIV worldwide and it has claimed nearly 46 million lives\(^1\). The highest number of HIV infections globally is concentrated in Sub-Saharan Africa with 68% of total infections (22.9 million cases), followed by South and South East Asia contributing over 12% of total infections (4.0 million cases; Figure 1.1)\(^1\). Of the two types of HIV that infect humans, HIV-1 (HIV) and HIV-2, the former is more virulent, has been responsible for the HIV pandemic\(^2\), and the focus of this dissertation.

Figure 1.1 Number of adults and children estimated to be living with HIV, 2011 by WHO.
1.1.1 HIV epidemiology in Canada

The number of Canadians living with HIV in 2011 was approximately 71,300 and consisted mostly men (76.7%). Almost half (46.7%) of people living with HIV in Canada are men who have sex with men (MSM), followed by those who contracted HIV from heterosexual contact from a non-HIV-endemic region (17.6%), intravenous drug use (16.9%), and through heterosexual contact from an HIV-endemic region (14.9%). Geographically, Ontario accounts for the highest proportion of cases (42.6%), followed by Quebec (21.5%), British Columbia (13.4%), Alberta (9.7%) and Saskatchewan (7.7%).

1.2 Biology of HIV

1.2.1 The virus

HIV is a positive, single-stranded RNA genome of 9.7 kilobases that belongs in the family Retroviridae and genus Lentivirus. It consists of two copies of RNA that codes for the virus’ nine genes (gag, env, nef, tat, vif, pol, rev, vpr, and vpu) that is enclosed in a conical capsid consisting of the p24 viral protein. The genes gag, env and pol carry the information required for the structural component of newly formed virus, whereas the other six genes are responsible for generating proteins that regulate the capacity of HIV to infect new cells, viral replication, and disease progression.

The capsid is further enclosed by the viral envelope, which is composed of a lipid bilayer membrane that is embedded with Env protein (Figure 1.2). Env is composed of three extracellular glycoprotein (gp) 120 molecules and three transmembrane molecules, gp41, that
anchors Env into the viral envelope. The gp120 is made of variable (V) and constant (C) regions, and the degree of variability in the variable 3 (V3) region influences the host co-receptor HIV interacts with. The gp120 and gp41 complex initiates the infectious cycle by allowing HIV attachment and fusion into target cells.

![Schematic diagram of HIV](image)

Figure 1.2  Schematic diagram of HIV. Taken from Karisson et al, Nature Reviews Microbiology (2008).

1.2.2 **HIV life cycle**

This section briefly discusses the various stages of the HIV life cycle (Figure 1.3), including HIV binding to its target cells and the steps involved in HIV replication. A comprehensive review of the HIV life cycle is beyond the scope of the topics covered in this thesis and can be found elsewhere⁵.
1.2.2.1 Virus binding and cell entry

HIV requires a host cell to begin its life cycle. This process is initiated by the interaction between HIV gp120 envelope protein and its primary receptor CD4 molecule on the host cell. Once HIV comes into close proximity of a target cell, the carboxyl terminal end of the gp120 binds to the CD4 molecule on the host, and then to one of two major HIV co-receptor CC chemokine receptor 5 (CCR5) or CXC chemokine receptor 4 (CXCR4). This interaction initiates cell entry where a conformational change in gp120 exposes the transmembrane envelope protein gp41, leading to the insertion of the N-terminal into the target cell membrane. Once the viral
membrane fuses with the host cell, viral RNA and key viral replication enzymes (reverse transcriptase, integrase and protease) are released into the cell cytoplasm\textsuperscript{2}.

1.2.2.2 Replication and transcription

Once HIV enters the host cell, the enzyme reverse transcriptase releases the single stranded RNA from viral proteins and copies it into a complementary DNA (cDNA) molecule. This is a highly error prone step (error rate of 1 in 1700) that may result in mutations that confer resistance to the host immune response\textsuperscript{8}. Reverse transcriptase also has ribonuclease activity and degrades the viral RNA, and has DNA polymerase activity that creates a sense DNA from the antisense cDNA; this allows for the formation of double-stranded viral DNA that is then transported into the cell nucleus. The viral enzyme integrase cleaves the two ends of viral DNA so that it can integrate into the host cell’s genome to form what is now referred as a provirus, and functions as a template for new virus transcription. At this stage, HIV may actively replicate or revert to latency in the host cell\textsuperscript{2,9}.

1.2.2.3 Proviral expression

During active viral replication, the integrated provirus is transcribed into mRNA, spliced into smaller fragments, and then exported from the nucleus into the cytoplasm. They are then translated into regulatory and structural proteins and packaged into new virus particles.

1.2.2.4 Viral assembly and budding
The assembly of new HIV virions takes place in the plasma membrane of the target cell. The Env precursor protein (gp160) is transported from the endoplasmic reticulum to the Golgi complex where it becomes cleaved by proteases to become the HIV envelope glycoproteins gp41 and gp120. These are transported to the plasma membrane of the host cell where gp41 anchors gp120 to the membrane. As the virion forms and buds from the host cell, other proteins such as Gag p55 and Gag-Pol also interact with the plasma membrane, along with the HIV genomic RNA. At this stage, the virus is still in its immature form as gag polyproteins, and the capsid and nucleocapsid proteins need to be further cleaved by proteases; only mature virions can successfully infect target cells.

1.2.2.5 Viral Latency

Resting CD4 T cells that become infected with HIV or HIV-infected cells that return to a resting state have restricted reverse transcriptase activity and can become latently infected (known as pre-integration latency). In the absence of an activation signal, the viral DNA loses its capacity to initiate productive infection and therefore the pre-integration latency is short-lived and the virus is degraded within hours to days. Another form of viral latency that is more clinically relevant occurs once a host cell with the integrated viral genome reverts back to a resting state (known as post-integration latency). These latently infected cells lay transcriptionally silent and serve as long-term viral reservoirs in an HIV-infected individual despite ART. Upon antigen stimulation of a latently infected cell, they become activated and produce virus. Of the 0.01% of CD4 T cells that carry the integrated HIV DNA, central and transitional CD4 memory T cells serve as the dominant cell subset that are latently infected. Viral latency poses a major challenge in achieving a cure for HIV. Latent viruses are
indistinguishable from the host DNA and are difficult to target; they evade the two primary defenses against HIV replication, the host immune system and ART.

1.2.3 HIV target cells

A variety of cells in the body express the HIV receptor CD4 and the co-receptors CCR5/CXCR4, including dendritic cells (DCs), macrophages and CD4 T cells\(^2\). Amongst these potential target cells, HIV preferentially infects activated CD4+ T cells\(^{15}\) because these cells transcriptionally upregulate HIV co-receptors and commonly co-expresses CCR5 and CXCR4 on their cell surface compared to quiescent CD4 T cells\(^{16}\). Although HIV can infect resting CD4 T cells, macrophages and DCs, they are less likely to propagate infection; a recently identified mechanism shows that SAM domain and HD domain-containing protein 1 (SAMHD1) blocks HIV replication by depleting the pool of nucleotides required for reverse transcription in these cells\(^{17}\). SAMHD1 converts nucleotide triphosphates to a nucleoside and triphosphate, which is required for HIV cDNA synthesis\(^{18,19}\). Interestingly, SAMHD1 is also found in activated CD4 T cells but it is believed that their varying enzymatic activities prevent depletion of nucleotide triphosphates\(^{20}\).

1.3 HIV clinical course

The clinical course of HIV infection is characterized by the interaction between a range of viral and host factors that determine the disease outcome of an HIV-infected individual. In this section, stages of HIV infection in a typical progressor, and subgroups of HIV-infected individuals known as long-term nonprogressors (LTNP) and elite controllers (ECs) are discussed.
1.3.1 Diagnosis

Once an individual is infected by HIV, the window period for detection by a clinical assay ranges from 9 days to 3-6 months\textsuperscript{21}. HIV infection can be identified either by detection of HIV-specific antibodies, HIV antigens, or HIV DNA in blood. The newest screening test is a 4\textsuperscript{th} generation HIV antibody and HIV p24 antigen ELISA that can detect infection up to 15 days post-infection with 100\% sensitivity\textsuperscript{22-24}. Following a positive screening test, a confirmatory Western blot for HIV antibody is required. If the screening test result is negative, the individual is either HIV-uninfected or the test may have been performed during the window period in which case a re-test is required. Polymerase chain reaction (PCR) is a molecular diagnostic test that detects the HIV RNA in very low levels (1-10 copies/ml) and can detect infection during the window period. However, it is costly and not routinely applied.

1.3.2 Acute/Early HIV infection

Majority of newly infected individuals (40-89\%) develop acute HIV syndrome within 1-4 weeks post-infection\textsuperscript{25,26}, with symptoms including fatigue (>70\% prevalence), skin rash (40-80\%), headache (32-70\%), gastrointestinal complications (30-60\%), and fever (>80\%)\textsuperscript{2,26}. In this early phase, there is limited anti-HIV immune response and HIV replicates vigorously with plasma viremia reaching its peak (up to $10^7$ copies/ml; Figure 1.4)\textsuperscript{27-29}. Plasma viremia gradually declines as HIV-specific CD8 cytotoxic T cell (CTL) responses arise and eventually reaches a steady state\textsuperscript{30}. Concomitant with the burst of plasma viremia, there is a rapid decline of CD4 T cells in the blood and mucosal tissues, specifically those expressing the HIV coreceptor CCR5. The loss of CD4 T cells is a consequence of HIV infection and direct killing by HIV, as well as bystander killing by apoptosis\textsuperscript{31,32}. In sites where there is an abundance of CCR5-expressing CD4
T cells, such as the gastrointestinal mucosa, the loss of CD4 T cells during acute/early infection is exaggerated (reviewed in section 1.4)\textsuperscript{2,33}.

Figure 1.4 The natural course of HIV infection. © Connie J Kim. Adapted from Goulder and Watkins, Nat Rev Immunol (2004).
1.3.3 Chronic HIV infection

The chronic asymptomatic stage may last up to 8-10 years without HIV medications. The duration of this phase is variable and the rate of disease progression depends on viral, host and external factors. Chronic HIV infection is characterized by a reduction in HIV replication followed by HIV stabilization to a level known as the viral set point; this viral set point predicts the subsequent rate of HIV disease progression (Figure 1.4)\textsuperscript{34}. The CD4 count returns to near-normal levels initially (500-700 cells/µl)\textsuperscript{35} but declines at a steady rate of about 25-60 cells/µl annually in the absence of ART\textsuperscript{36}.

1.3.4 AIDS

When the CD4 count falls below 200 cells/µl or upon the occurrence of AIDS related opportunistic illnesses, an individual is defined as having “AIDS”\textsuperscript{37}. A rapid rise in viremia and a reduction in HIV-specific immune response coincides with the emergence of opportunistic infections, such as *Pneumocystis carinii* pneumonia, esophageal candidiasis, and *Mycobacterium avium* complex disease\textsuperscript{38}. In addition to these infections, individuals with advanced HIV infection are at an increased risk of developing virus-induced cancers such as Kaposi’s sarcoma, non-Hodgkin lymphoma and cervical cancer, as well as non-infectious cancers such a lung, colon and ovarian cancers\textsuperscript{39}. In the absence of ART, the average survival time with AIDS is approximately 1-3 years\textsuperscript{40}.
1.3.5 **Long-term nonprogressors and elite controllers**

Long-term nonprogressors (LTNP) are a subgroup of HIV-infected individuals (2-15% prevalence) who maintain a normal blood CD4 count (500-1600 CD4 cells/µl) and lack symptoms related to HIV progression after 7-10 years without ART. These individuals often have blood viral loads under 10,000 copies/ml. Another group of HIV-infected individuals known as viral controllers and elite controllers (ECs) are able to spontaneously control HIV replication to low (50-2,000 copies/ml, 3.3% prevalence) or to undetectable levels (<49 copies/ml, <1% prevalence) respectively. There is some overlap between EC and LTNP and most ECs exhibit CD4 preservation and some LTNP have low/undetectable plasma viremia.

LTNP and EC are heterogeneous groups and their ability to control HIV and sustain normal CD4 counts is multifactorial. The potential mechanisms of spontaneous virus control include enhanced host immune responses, viral factors, and genetic factors. Infection with attenuated forms of the HIV virus with reduced viral fitness may be associated with viral control, however host genetic factors are also associated with viral control. Specifically, individuals with HLA B5701 and HLA B27 tend to control HIV replication, although possessing these alleles does not guarantee one’s EC or LTNP status. HLA B27 carriers form a stable complex with the Gag p24 epitope, a conservative core protein of HIV, and have a specific and stronger CTL response to HIV. A viral mutation that disrupts the interaction with HLA B27 has been associated with loss of viral control and disease progression. HLA-B5701 carriers control HIV by enhancing the function of anti-HIV CTL response, however the exact role of HLA-B5701 remains elusive. Another host genetic mutation that slows the rate of HIV disease progression is a deletion mutation known as CCR5 delta 32 (CCR5 Δ32) that impairs the ability of CCR5 tropic HIV to bind to CCR5 on CD4 T cells. Homozygotes of CCR5 Δ32 are highly resistant to HIV
infection, whereas heterozygotes have delayed progression to AIDS by an average of 2 years without ART compared to those lacking this allele\textsuperscript{51,52}. Host immune factors including innate immunity, polyfunctional CD4 and CD8 T cell responses, HIV-specific antibody responses are all immune factors that may characterize elite controllers but are not a common denominator amongst all\textsuperscript{53-55}.

1.4 Gastrointestinal tract

1.4.1 Gastrointestinal tract

The human gastrointestinal tract (GIT) is a 5-meter long tube-like structure that begins from the mouth and includes the esophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine, rectum, and terminates at the anus. The hollow center of the tube is known as the gut lumen and functions as the passageway for food and houses an assortment of microorganisms throughout the entire length of the GIT. The primary role of the GIT is to break down and absorb foods and liquids, however it is also the largest immune organ in the body,\textsuperscript{56} and this has significant implications for HIV transmission and pathogenesis\textsuperscript{57}. The rectal mucosa is highly susceptible to HIV invasion after sexual exposure to HIV, with one of the highest transmission probabilities compared to other sexual acts (1/20 to 1/300 per exposure event)\textsuperscript{58}. One contributing factor to this HIV invasion is that only a single layer of columnar epithelial cells separates the gut lumen from the rich bed of highly activated CCR5+ CD4 lymphocytes\textsuperscript{56,59}. These activated CD4 lymphocytes serve as major target cells after HIV infection and their rapid and near total depletion plays a central role in HIV disease progression. The focus of this dissertation will be on the immunopathological events within the gut mucosa that are believed to
drive HIV disease progression to AIDS and the development of serious non-AIDS (SNA) illnesses.

1.4.1.1 Gut flora

The microorganisms that reside along the entire length of gut lumen and the mucosa, called gut microbiota/flora/commensal bacteria, coexist “peacefully” with the surrounding mucosal immune system in a healthy equilibrium state. The large intestine is by far the most densely colonized with bacteria, with up to $10^{12}$ bacteria for every gram of gut content and contains greater than 100 times the frequency of bacteria compared to human genes. Four phyla dominate adult human intestine, and over 90% belong in the phyla Bacteroidetes (including \textit{Bacteroides}) and Firmicutes (including \textit{Clostridium}, \textit{Lactobacillus}, and \textit{Bacillus}), and a minor abundance of Proteobacteria (including \textit{Escherichia}) and Actinobacteria (including \textit{Bifidobacterium}; Figure 1.5). At lower taxonomical levels, there is great interindividual variation and it is estimated that only 35% of bacterial genes are common between individuals. These microbial communities in an individual are adaptable to various factors, such as diet, lifestyle, hygiene and immune status, as well as host genetics. Furthermore, they play important roles that benefit the host, including metabolic activity maintenance, immune development and prevention of pathogen invasion.
1.4.1.1 Gut flora and host immunity

The gut flora plays a profound role in shaping the gut immune system. It is established within days after birth, and throughout life they form a mutualistic relationship with the host and modulate immune development. For example, mice raised in a germ-free environment have reduced numbers of lymphocytes, lymphoid follicles and immunoglobulins, but following exposure to luminal bacteria they are greatly expanded. The introduction of a single commensal microbe, known as segmented filamentous bacteria (SFB), can induce the development of a single immune cell subset in germ-free mice. SFB adhere tightly to the surface of the gut epithelium, induce IL-17a and IL-22 production by CD4 T cells (known as Th17 and Th22 cells respectively), and their presence enhance resistance to pathogenic *Citrobacter rodentium* infection. Similarly, commensal *Clostridium* species can induce colonic T regulatory (Treg)
cells in mice. Inoculation of a purified mixture of Clostridium species from healthy human donors enriched Tregs in the colon and attenuated an animal model of gut inflammation.

Commensal bacteria can also directly regulate innate cell populations, including innate lymphoid cells and NK cells by signaling through immune cell receptors, like toll like receptors (TLRs).

Moreover, commensal bacterial metabolites such as short chain fatty acids (SCFA) can enhance Treg cell development through direct SCFA receptor interaction. In addition to the immunomodulatory role of commensal bacteria, they also restrict colonization of pathogenic bacteria by competing for their nutrients and attachment sites on the epithelium.

A deeper understanding of the relationship between commensal microbes and host immune system may provide unique abilities to modulate health and disease.

1.4.1.2 Layers of the GIT

Figure 1.6 Layers of the intestinal gut wall. Adapted from Gastrointestinal Physiology, McGraw-Hill (2006).
The GIT lining is composed of four layers that are present through the entire length of the GIT, including the mucosa, submucosa, muscularis, and the serosa (Figure 1.6). The composition of each layer differs to allow for optimal performance in their respective anatomical location. For example, the small intestinal epithelial cells in the mucosa have finger-like projections, called villi, which extend into the lumen to maximize the surface area for enhanced nutrient absorption. The intestinal crypt is another structure formed by the epithelium that is present in both the small and large intestine for water and electrolyte reabsorption. Since the large intestine does not function to absorb nutrients, it does not contain villi.

The innermost mucosa layer of the GIT is composed of three layers: a single layer of columnar enterocytes (epithelium), connective tissue (lamina propria), and a thin layer of muscularis (muscularis mucosae). The epithelium consists mostly of enterocytes, although specialized cells such as the mucous secreting goblet cells, intraepithelial lymphocytes, and antigen sampling microfold cells (M cells) can also be found. The lamina propria is composed of connective tissue and houses a large number of lymphocytes in the gut. The layer below the mucosa, known as the submucosa, is highly vascularized, glandular and has a relatively thick layer of connective tissue. Next, the muscularis layer is made up of an inner circular and an outer longitudinal layer of smooth muscle that contract to move food along the GIT. The outermost layer from the gut lumen, the serosa, functions as a protective barrier and is composed of avascular connective tissue and simple squamous epithelium. In healthy individuals, the small intestine contains the largest number of T lymphocytes, particularly concentrated in the lamina propria, Peyer’s patches (PPs) and isolated lymphoid follicles. The large intestine also houses many T lymphocytes, but instead of PPs, isolated colonic lymphoid follicles contain the majority of the lymphocytes.
1.4.2 Defense mechanisms in the GIT

The GIT has the largest mucosal surface area in the body that is in contact with over 300 different pathogenic and commensal bacterial species in the gut lumen. The gut mucosa has therefore developed multiple mechanisms to protect the host from this heavy load of organisms. These protective mechanisms include the mucous, epithelial cells, and the gut-associated lymphoid tissue (GALT), and can be categorized structurally as: epithelial and extra-epithelial protection, and immune defense within the lamina propria (Figure 1.7).
Figure 1.7 Defense mechanism in the intestinal mucosa. © Connie J Kim.
1.4.2.1 Epithelial and extra-epithelial mucosal protection

The GIT has multiple mechanisms to protect the mucosal barrier. Within the epithelium, goblet cells produce mucous that covers the epithelium, impedes bacterial attachment, and prevents bacteria from penetrating the gut mucosa. Next, the enterocytes and tight junction proteins form an impermeable physical barrier and lastly, epithelial cells secrete innate protective molecules into the lumen for additional antimicrobial defense.

1.4.2.1.1 Mucous layer

The mucous overlays the epithelium and provides the first line of defense against physical and chemical injury arising from food, microbes and microbial products (Figure 1.7i)\(^75\). Goblet cells are scattered along the epithelium and are the primary source of the protein MUC2, which forms the mesh-like network of viscous, permeable, and gel-forming mucous\(^76\). The mucous traps bacteria, thereby forming a chemical separation between the gut lumen and the epithelium\(^76,77\). The mucous itself is organized into two layers: the inner layer that is firmly attached to the epithelium and the loosely nonattached outer layer\(^75,78\). The inner layer is normally densely packed with MUC2 mucin and impermeable to bacteria\(^79\), whereas the outer layer’s mucin has a larger volume that in enriched with commensal bacteria\(^80\). The glycan molecules on MUC2 serve as nutrients and attachment sites for bacteria, thereby introduces a selection criteria\(^79\). In disease models of impaired intestinal permeability or “leaky” gut\(^81,82\), bacteria penetrate into the inner mucous layer in a Muc2 dependent manner\(^83\).
1.4.2.1.2 Enterocytes and tight junction proteins

The cellular component of the epithelium consists of a single layer of simple columnar enterocytes, along with goblet cells, intraepithelial lymphocytes and M cells (Figure 1.7, ii and iii). The epithelium has two main functions: 1) it provides a selective permeable barrier to allow for absorption of specific nutrients and fluids, while preventing undesirable solutes and pathogenic microorganisms from entering the body; and 2) it provides innate mucosal immunity against pathogenic gut flora\textsuperscript{84}. Enterocytes are held together by a series of intercellular junctional proteins known as the apical junctional complex, which regulate the paracellular movement of solutes. It is composed of tight junction (TJ) and adheren junction proteins (Figure 1.8). TJ proteins connect the actin cytoskeleton of neighbouring enterocytes, and are believed to be the key players in maintaining the gut barrier\textsuperscript{85}. TJ proteins include three transmembrane proteins (occludin, claudins, and junctional adhesion molecules) and three adaptor proteins (zonula occludens, ZO-1, ZO-2, and ZO-3).

Figure 1.8 Composition of the apical junctional complex, including tight junction proteins found in the epithelial cells. Taken from Aktories and Barbieri, Nat Rev Microbiol (2005).
1.4.2.1.3 Innate immunity

Intestinal epithelial cells contribute to the innate arm of the immune system by secreting a broad range of antimicrobial peptides, including defensins and cathelicidin, which form pores in the bacterial cell wall\(^{86}\). Enterocytes also express a wide range of pattern recognition receptors (PRRs) that can recognize highly conservative bacterial components, known as pattern-associated molecular patterns (PAMPs). Common PAMPs include bacterial components such as lipopolysaccharide (LPS), lipoproteins, flagellin, and unmethylated CpG-containing DNA\(^{87}\).

Upon ligation of PRRs, epithelial cells initiate the innate immune response that promote the secretion of pro-inflammatory cytokines, chemokines, co-stimulatory molecules and antimicrobial peptides\(^{88}\); collectively these molecules link the innate and adaptive arm of the immune system by promoting chemotaxis between immature DCs and memory T cells\(^{89}\).

Epithelial cells thereby respond rapidly to microbial invasion via the innate response but also play the important role of initiating the adaptive immune response.

1.4.2.2 The gut-associated lymphoid tissue

The gastrointestinal immune system can be functionally categorized into inductive and effector sites. The inductive site, known as gut-associated lymphoid tissue (GALT), includes the organized PPs in the small intestine, isolated lymphoid follicles (ILF) that are diffusely scattered in the lamina propria and the epithelium\(^{90}\). GALT is enriched with B and T cells, and antigen presenting cells (APCs) that function to differentiate, activate and migrate activated lymphocytes to the site of infection or the effector site.
1.4.2.2.1 Antigen presentation and cell trafficking

Antigen presentation is a process whereby APCs prime naïve T cells to recognize microbial antigens. In the gut mucosa, DCs are a common type of APC that innately recognizes PAMPs on microbial pathogens, processes them into epitopes and loads them on to major histocompatibility complex (MHC) class I or II molecules. In the case of extracellular pathogens, DCs load the epitopes onto MHC class II molecules and migrate to the draining mesenteric lymph node (MLN) via the lymphatic system, and present pathogenic antigens to naïve T cells. For intracellular pathogens, epitopes are presented on MHC class I molecules to CD8+ cytotoxic T cells (CTLs). Antigen presentation results in T cell differentiation and induces these activated T cells to express surface molecules, which enables them to migrate back to the site of infection. T cell differentiation is dependent on the type of pathogen and the cytokine milieu (reviewed in 1.4.2.2.2).

During antigen presentation, DCs imprint lymphocytes to express integrins, selectins, and chemokine receptors on their surface to facilitate homing to the site of the original infection. DCs do so by expressing the enzyme retinal dehydrogenase (RALDH1 and RALDH2) that metabolizes vitamin A into retinoic acid, which induces the expression of gut homing markers on T cells. Lymphocytes destined to traffic to the gut can be imprinted to express the gut-homing marker integrin α4β7, or the chemokine receptors CCR9 and CCR10. Mucosal addressin cell adhesion molecule-1 (MAdCAM-1), the main ligand for α4β7, is highly expressed on the surface of endothelial cells of the venules and enable cells to enter the gut tissue. CCR9 and CCR10 bind to the CC motif chemokine (CCL) 25 and CCL28, which are expressed on the epithelial cells of the small and large intestine, respectively. Once imprinted, lymphocytes migrate to their
respective homing sites and downregulate the expression of gut homing markers, while upregulating markers such as integrin αEβ7 that facilitate their retention in the gut\textsuperscript{97}.

1.4.2.2 Gut T cell subsets

During T cell activation, naïve T cells can expand in numbers as much as $10^{5}$-fold and differentiate into antigen specific effector T (Teff) cells\textsuperscript{98}. The pathogen and the cytokine milieu differentiate Teff cells into one of several lineages of T helper cells (T cells), including Th1, Th2, Th17 and regulatory T cells (Tregs). In general, their development towards one subset suppresses their differentiation to another subset (Figure 1.9)\textsuperscript{99}, although cells can exhibit functional plasticity where one differentiated subset can transition into another\textsuperscript{100}. T cell subsets can be characterized either by their cytokine profile or by their surface marker expression. Th1 cells respond to intracellular pathogens, produce IFN−γ, and express the transcription factors STAT4 and T-bet\textsuperscript{101}, and Th2 cells typically respond to helminth, produce IL-4 and express STAT6 and GATA3. Th17 cells express CCR6 and respond to extracellular bacteria and fungal infection by producing the cytokine IL-17a, Tregs have strong immunosuppressive properties that tightly regulate the inflammatory immune response, express the transcription factor FOXP3 and develop reciprocally to Th17 cells\textsuperscript{102}. There are two types of Tregs: natural Tregs develop from the thymus and exert their suppressive properties via cell contact, and induced Tregs develop from CD4 T cells and function in a contact independent manner and secrete the immunosuppressive cytokines IL-10 and TGFβ\textsuperscript{103}. More recently, Th22 cells that are similar to Th17 cell have been identified as another CD4 T cell subset\textsuperscript{104}. They are defined by their propensity to produce tissue protective IL-22 in response to bacterial and fungal antigens\textsuperscript{105}. 
Following clearance of pathogens, up to 95% of Teff cells die and the remaining cells become long-lived central memory T cells (Tcm) or effector memory T cells (Tem). Tcm reside predominantly in secondary lymphoid organs, produce IL-2, and proliferate extensively upon re-encounter of antigen. Conversely, Tem circulate in the periphery and do not proliferate to the extent of Tcm, but produce high levels of effector cytokines\textsuperscript{106}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Simplified representation of CD4 T cell differentiation and function. Naïve T cells (Tn cells) can differentiate into several CD4 lineages based on the dominant cytokine milieu (Th1, Th2, Treg, or Th17 cells). The dominant transcription factors, surface markers, and the effector cytokines of each CD4 T cell subsets are outlined. © Connie J Kim}
\end{figure}

In the intestine, the majority of the lymphocytes in the effector sites (lamina propria and the intraepithelial lymphocytes) are antigen-experienced Teff cells, however they are basally activated and express high levels of the HIV co-receptor CCR5 and CXCR4, making them ideal targets for HIV\textsuperscript{56,107}. 

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1.4.2.2.3 Gut innate lymphoid cells

Recent literature has shown that in addition to the adaptive lymphocytes in the gut mucosa, innate lymphoid cells (ILCs) are an important source of effector cytokines. ILCs lack cell lineage surface markers of the T cell, B cell, DCs or monocytes and do not respond in an antigen-specific manner. There are several types of ILCs, but lymphoid tissue inducer (LTi) cells and natural killer (NK) cells that produce IL-17a and IL-22 may be key players in providing first line of mucosal defense against microbes in the gut. These ILCs express the transcription factor RORγt but unlike Th17 and Th22 cells, their development is independent of microbiota. LTi cells are essential for lymphoid organogenesis during embryogenesis and are important regulators of lymphoid tissue architecture throughout life. Furthermore, ILCs produce IL-17a and IL-22 for antimicrobial immunity and tissue repair in response to IL-1β and IL-23. ILCs are a recently described immune subset and much effort is placed to characterize their physiological roles, their interaction with the adaptive immune system, and their impact on health and disease.

1.4.3 HIV and the GIT

The GIT is the largest immune organ that houses 40-60% of the body’s total lymphocytes, but is enriched with HIV target cells. Regardless of the route of HIV transmission, HIV traffics to the gut mucosa within days following infection where it rapidly replicates and depletes CD4 T cells. The large number of activated CD4 T cells propagates HIV production in the gut and following HIV infection their depletion is central to understanding mechanism of microbial translocation and HIV disease pathogenesis.
1.4.3.1 Microbial translocation

Microbial translocation is the process by which luminal microbes traverse the intestinal epithelium and into the blood stream. This process first entails local microbial translocation into the gut mucosa and subsequent penetration of the microbes into the circulation (called systemic microbial translocation). In a healthy state with normal intestinal barrier and gut immunity, basal levels of microbes that cross into the lamina propria are rapidly processed and removed without causing excessive inflammation\textsuperscript{112,113}. Local mechanisms that regulate microbial translocation in the gut involve the integrity of the epithelial barrier and the mucosal immune defense, such that a simultaneous loss of these factors may be a prerequisite for this phenomenon (Figure 1.10). If the bacteria in the gut persist, it can penetrate into blood circulation to cause systemic microbial translocation. Therefore, the underlying mechanism of local and systemic microbial translocation in HIV infection likely involves a combination of impaired epithelial integrity and mucosal immunodeficiency.
Increased translocation of bacterial products into the bloodstream during chronic HIV/SIV infection has been well documented, with over 45 reports of microbial translocation occurring during progressive HIV/SIV infection\textsuperscript{[14]}. The most widely used direct marker of microbial translocation is the plasma level of lipopolysaccharide (LPS), a component of the Gram-negative bacterial wall, while other direct markers include bacteria cell wall constituent peptidoglycan and conserved bacterial 16S rRNA gene\textsuperscript{[115-120]}. Indirect markers of microbial translocation are molecules that are involved in LPS clearance. Microbial translocation is associated with increased plasma levels of LPS-binding protein (LBP) and monocyte/macrophage activation marker soluble CD14 (sCD14)\textsuperscript{[121,122]}. 

Figure 1.10 Model of microbial translocation. © Connie J Kim
CD14 is expressed on blood monocytes and tissue macrophages and forms a complex with TLR4 and MD-2 to recognize LPS\textsuperscript{123}. Following LPS stimulation or activation of CD14+ monocytes/macrophages, membrane anchored CD14 is cleaved and able to bind LPS\textsuperscript{124,125}. Likewise, LBP is primarily produced by hepatocytes and can bind to circulating LPS. CD14 has a high affinity for the LPS-LBP complex and allow TLR4 on macrophages to recognize LPS\textsuperscript{126}. Although the LPS recognition cascade has been carefully identified, the precise biological activities of soluble factors (sCD14 and LBP) are incompletely understood and mainly serve as surrogate markers of microbial translocation in HIV studies\textsuperscript{127}. Importantly, TLR4 recognition of LPS results in the activation of a potent pro-inflammatory cytokine response to mediate bacterial clearance (including IL-6, TNF\(\alpha\), IL-1\(\beta\), IL-23 and type 1 IFN), and upregulate molecules that allow APCs to activate naïve T cells (including DC maturation molecules and co-stimulatory molecules)\textsuperscript{128,129}.

It is believed that the resulting potent innate pro-inflammatory response to microbial translocation in HIV-infected people is the major cause of the immune activation\textsuperscript{100,102,110} that has been associated with increased mortality and SNA conditions (neurocognitive and cardiovascular disease)\textsuperscript{130-137}. However, the direction of causality is unknown since most studies are correlative. Immune activation of blood CD8 T cells (measured by co-expression of molecules CD38 and HLA-DR) is a commonly used predictor for HIV disease progression to AIDS in ART-naïve individuals\textsuperscript{135}, and soluble markers of inflammation (IL-6 and sCD14) and coagulation (D-dimer and CRP) are important biomarkers of mortality and comorbidities in ART-experienced individuals\textsuperscript{138}. In particular, plasma levels of IL-6 and D-dimer were recently identified to be the best predictor of developing serious non-AIDS (SNA) conditions or death in an analysis of three large trial cohorts including 3766 individuals with controlled viral replication\textsuperscript{139}. 

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The onset of microbial translocation likely occurs during the chronic stage of HIV/SIV infection, following significant mucosal immunodeficiency and barrier impairment. In progressive SIV infection, microbial translocation into the lamina propria and to the draining lymph node was only observed during the chronic stages and not during acute or early stages, and increased plasma LPS levels was observed during chronic HIV infection.

1.4.3.2 HIV infection and mucosal CD4 T cells

Gut CD4 T cells are highly activated and express the HIV co-receptor CCR5, therefore are readily permissive to HIV/SIV infection. Over 60% of gut memory CD4 T cells are lost as early as 4 days post SIV infection and a similar acute phase CD4 depletion occurs in HIV infection. The mass depletion of gut CD4 T cells is a result of direct HIV infection and killing by HIV-specific CD8+ CTL, as well as by indirect death by cell activation induced death, and by a lack of CD4 T cell replenishment.

Within the CD4 subsets of the GIT, there is a preferential depletion of microbe responsive Th17 cells that are essential in providing mucosal immune defense and epithelial cell renewal, and an increase in Tregs that may prevent appropriate immune responses to invading microbes. Tregs develop reciprocally to Th17 cells and the mucosal Th17/Treg ratio is also reduced during HIV infection. It is hypothesized that the increase in immunoregulatory response and the loss of microbe responsive Th17 cells may impair the ability to clear invading pathogens. In HIV-infected individuals on ART, gut Th17 frequency correlates with reduced plasma LPS levels, and the loss of gut Th17 cells in pathogenic SIV infection promotes dissemination of pathogenic Salmonella typhimurium into the blood. The precise timing of gut Th17 depletion in HIV infection has not been well documented, but a recent sub-study in the
RV144 Thai trial showed a loss of sigmoid Th17/Treg balance during acute HIV-1 infection (about 25 days post infection)\textsuperscript{151}. Despite early and significant loss of CD4 and Th17 cells, plasma LPS levels were only increased during chronic HIV infection, demonstrating that the mechanism behind microbial translocation is incompletely understood.

### 1.4.3.3 Epithelial integrity and HIV infection

HIV-infected individuals have mucosal abnormalities that may occur as early as the acute phase of HIV infection\textsuperscript{152}, including altered composition of epithelial tight junction proteins, decreased intestinal permeability, increased epithelial apoptosis and altered cytokine production\textsuperscript{140,153}. The causes of HIV-associated impairment of the gut epithelium are multifactorial, and include direct disruption of the tight junction proteins by HIV gp120, as well as exposure to pro-inflammatory cytokines in response to local inflammation\textsuperscript{154}. In chronic SIV infection, the multifocal breakdown of the epithelial tight junction protein is paralleled with leakage of microbial products into the adjacent mucosa and into the draining lymph nodes\textsuperscript{140}. However, the breakdown of the epithelial barrier alone is insufficient to cause systemic microbial translocation, and plasma LPS levels are only increased when epithelial breakdown is accompanied by mucosal immunodeficiency (see Figure 1.10)\textsuperscript{113}. Patients with active inflammatory bowel disease (IBD) also have gut inflammation, increased intestinal permeability and microbial translocation, however during clinical remission without gut inflammation, increased intestinal permeability is not accompanied by microbial translocation\textsuperscript{155}. Similarly, relatives of patient’s with IBD that are otherwise healthy also have increased intestinal permeability but do not have systemic microbial translocation\textsuperscript{156}. Thus, microbial translocation
into the bloodstream likely requires a simultaneous occurrence of breached epithelial barrier and an inability to clear invading pathogens by the mucosal immune system (Figure 1.10d).

1.5 Antiretroviral therapy

The advent of ART has drastically changed the mortality and morbidity associated with HIV infection. The blood CD4 T cell count is a clinical marker for HIV-infected patients that serve as an indicator of general immune function. It provides important information on when to start ART and to monitor the therapeutic response to ART. Past guidelines by World Health Organization (WHO) recommended that HIV medications should be taken once the CD4 count reaches 350 cells/µl, but in June 2013 the CD4 count threshold has been raised to 500 cells/µl due to improved long-term prognosis of early treatment. Standard ART consists of a combination of at least three antiretroviral agents from at least two different classes; each class targets a different step in the viral life cycle to minimize viral replication. These drug classes include nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), entry inhibitors, and fusion inhibitors. Typical ART regimen includes a cocktail of 2 NRTI with 1 NNRTI or 2 NRTI boosted with a PI. The mechanism of action and the drugs in each class are beyond the scope of this dissertation and can be found elsewhere. Instead, the classes of ART drugs will be briefly reviewed (Figure 1.11), and emphasis will be placed on clinical and immunological responses to ART and their limitations.
1.5.1 Classes of antiretroviral therapy

Figure 1.11 Life cycle of HIV and site the action of antiretroviral therapy by class (in red). Taken from Chen et al, Med J Aust (2007).
1.5.1.1 Reverse transcriptase inhibitors

Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) are the most commonly prescribed ART. They halt HIV DNA synthesis by inhibiting the activity of reverse transcriptase from extending the DNA chain\(^2\) (Figure 1.11). NRTIs are structural analogues of naturally occurring deoxynucleotides, except that they lack a 3’hydroxyl group and prevent the next deoxynucleotides from forming the proper bond to extend the viral DNA. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) stop HIV replication at a similar step but they bind to a less conserved pocket near the active site of reverse transcriptase to cause a conformational change\(^2\). This distorts the positioning of the deoxynucleotides that bind to the viral DNA, thereby inhibiting polymerization.

1.5.1.2 Protease inhibitors

HIV requires protease enzymes to cleave large polyproteins to smaller functional viral proteins (Figure 1.10). Protease inhibitors (PIs) function by binding to the active site of the viral protease enzyme and inhibiting the ability to produce functional smaller viral proteins, resulting in the production of non-infectious immature virions\(^2\).

1.5.1.3 Fusion inhibitors

The binding of HIV protein gp120 to the CD4 receptor and the subsequent conformational change of gp41 are necessary steps to initiate HIV fusion into the target cell. The fusion inhibitor, Enfuvirtidie/Fuzeon (T20) binds to the viral gp41 glycoprotein and prevents the virus
from integrating with the target cell membrane. T20 however is degraded by gastric acids and must be administered via subcutaneous injection, limiting its clinical use.

1.5.1.4 Integrase inhibitors

Integrase inserts the viral DNA into the host chromosomal DNA, and is another vital step of HIV replication. Integrase inhibitors prevent HIV replication by competing for the same cofactors (Mg2+ and Mn2+) that are required for integrase activities, thus dampening HIV replication.

1.5.1.5 CCR5 antagonists

CCR5 receptor antagonists function by binding to CCR5 on host target cells and blocking gp120 of an R5-tropic virus from associating with the co-receptor. Since HIV can also use the CXCR4 co-receptor, an HIV tropism test must be performed. Maraviroc was the first approved drug of this class to be used in combination with other ART for the treatment of R5-tropic HIV infection.

1.5.2 Antiretroviral drug delivery to the gut mucosa

Currently available antiretroviral drugs sufficiently reduce blood VL to undetectable levels, however ART drugs are not uniformly distributed throughout the body and certain anatomical sites maintain suboptimal drug concentrations (known as sanctuary sites). For example, the gut lymphoid tissue serves as a major site of HIV reservoir despite prolonged ART and retains
higher HIV RNA and DNA concentrations compared to blood\textsuperscript{165,166}. In SIV infection, the concentration of the drug 3TC (an NRTI) in the gut is the lowest amongst other ART drugs, and its level is correlated with reduced viral persistence in lymphoid tissues\textsuperscript{167}. This phenomenon may be due to ineffective drug delivery or increased expression of drug-metabolizing enzymes and transporters in the gut. For instance, the drug metabolizing agent cytochrome P450 (CYP) 3A, and the drug efflux pump P-glycoprotein are co-localized and expressed in abundance in the villus tip of the small intestine\textsuperscript{168,169}. This reduces the bioavailability of drugs that are substrates of CYP3A and P-glycoprotein, thus limiting the delivery of certain oral drugs\textsuperscript{169}. Other drugs such as darunavir (PI), ritonavir (PI), etravirine (NNRTI), and maraviroc (CCR5 antagonist) have better drug delivery to the gut and maintain higher drug concentrations in rectal tissue than blood\textsuperscript{170,171}. Raltegravir, an integrase inhibitor, reaches the highest drug concentration in the colon of all currently available ART, at a level 160-fold higher than the blood\textsuperscript{172}. Despite enhanced drug delivery to mucosal sites, viral reservoirs persist.

1.5.3 Clinical and immunological response to antiretroviral therapy

1.5.3.1 ART associated HIV viral decay

ART initiation reduces viral replication in three distinct phases (Figure 1.12)\textsuperscript{173}. The first phase of viral decay involves an exponential drop (up to 99\% reduction) in plasma viremia within the first two weeks of therapy, largely through elimination of free virus and loss of activated HIV infected CD4 T cells\textsuperscript{174,175}. The second phase (half life of 2 weeks) is a more gradual viral decay\textsuperscript{175}, which reflects viral clearance from long-lived HIV-infected cells such as macrophages\textsuperscript{176}, resting CD4 T cells\textsuperscript{177} and tissue bound HIV in peripheral lymphoid tissue\textsuperscript{13}. The
majority of individuals achieve blood viral control (<50 copies/ml) within 2 months of ART, but low levels of viral replication persist in the plasma and mucosal compartments\textsuperscript{178}, such as semen\textsuperscript{179,180}, gut and vaginal secretions\textsuperscript{181}. The third and final phase lasts for many years where the plasma viremia fluctuates between 2-50 copies/ml. It is believed that latent viral reservoirs provide a stable source of virus and mathematical models estimate that it would take over 60 years of suppressive ART to eliminate latent reservoirs\textsuperscript{182,183}.

With the goal of eliminating residual plasma viremia in HIV-infected individuals, ART “intensification” strategy was explored by adding a fourth ART drug to the regimen\textsuperscript{184,185}, but this method failed to reduce residual plasma viremia\textsuperscript{179,180}. Instead, timing of ART initiation may bear importance as ART administration during the first 6 months of infection was able to reduce HIV reservoirs compared to ART initiated in the chronic stages\textsuperscript{186}.

Figure 1.12 Schematic illustration of the decay dynamics of HIV after ART initiation. Taken from Kim and Perelson, PLoS Comput Biol (2006)
1.5.3.2 ART on blood CD4 T cells

ART restores CD4 T cells and drastically reduces mortality and morbidity\textsuperscript{187}. A major determinant of the degree to which ART can increase CD4 levels is having a higher nadir CD4 count, along with strict adherence to therapy. To this effect, the WHO has recently changed their guidelines and recommended that HIV-infected individuals initiate ART sooner (from 350 cells/\(\mu\)l to 500 cells/\(\mu\)l), based on work demonstrating that initiating ART during the earlier phases of infection improves CD4 restoration, and reduces mortality and morbidity\textsuperscript{157,188,189}.

1.5.3.3 ART on gut CD4 T cells

The impact of ART on immune reconstitution of the GIT mucosa is an important area of research because of the central role gut immunopathology plays on HIV disease progression and SNA conditions. In striking contrast to the reduction of blood HIV RNA levels and normalization of blood CD4 count post ART, gut HIV provirus levels persist\textsuperscript{164,166,190} and mucosal CD4 reconstitution is often incomplete and delayed\textsuperscript{191-193}. Slow gut CD4 reconstitution may be because the loss of CD4 T cells is more pronounced in the gut compared to blood, and therefore a greater degree of repair may be required\textsuperscript{111,143,192}.

Several studies have examined the ability of ART to reconstitute gut CD4 T cells in HIV-infected individuals. The duration of ART investigated ranges from 1 month to 7 years and includes a wide spectrum of CD4 recovery, from minimal to full CD4 T cell reconstitution\textsuperscript{143,194,195}. Although normalization of bulk CD4 T cells in the gut is pertinent in mucosal immune defense, the balanced proportion and function of each immune subset may bear more importance in reducing microbial translocation and decreasing immune activation. For
instance, full restoration of gut CD4 T cells after ART initiation does not equate to the proportional restoration of gut Th17 and Th1 cells\textsuperscript{106}. Slow reconstitution of mucosal Th17 cells on ART means that microbial translocation and systemic immune activation may remain elevated despite normalization of gut CD4 T cells. Indeed, Th17 cell reconstitution on ART is associated with reduced plasma LPS levels\textsuperscript{115,195}. It is clear that ART does not unanimously resolve gut immune dysregulation, and this may contribute to ongoing microbial translocation and persistent systemic immune activation.

Several strategies were explored to accelerate the reconstitution of gut CD4 T cells after ART initiation. Timing of ART initiation was hypothesized to be important, as initiating ART during the earlier stages may prevent further gut immunopathogenesis and therefore enhance reconstitution\textsuperscript{143}. In a non-human primate model, initiating ART as early as ten days post SIV infection failed to prevent mass CD4 depletion\textsuperscript{197}, but earlier treatment was associated with enhanced immune restoration in another SIV study\textsuperscript{198}. Furthermore, ART intensification by the addition of raltegravir to standard ART reduced the level of proviral HIV DNA and there was a trend towards increased CD4 T cells in the ileum\textsuperscript{199}. However, ART intensification with raltegravir in another study for 12, 48 or 96 weeks did not result in elevated CD4 T cell levels in the sigmoid colon\textsuperscript{199,200}.

1.5.4 Limitations of current standard ART

The advent of combination therapy has dramatically improved the survival and quality of life of the HIV-infected population; however, the life expectancy of an HIV-infected individual on ART remains 10-30 years below that of their age matched uninfected peers\textsuperscript{201}. Moreover,
increased immune activation and elevated microbial translocation despite effective ART may explain why HIV-infected individuals are at increased risk of developing SNA conditions, such as cardiovascular and neurocognitive diseases. Some factors contributing to increased rates of comorbidities and increased mortality in ART experienced individuals include residual viremia, delayed ART initiation, low nadir CD4 count, incomplete reconstitution of gut immune cells, ongoing microbial translocation, and persistent immune activation.

1.5.5 **Serious non-AIDS illnesses**

The reduction in AIDS related deaths with the advent of combination ART were met with increased deaths attributed to serious non-AIDS (SNA) conditions. Standard SNA events include cardiovascular disease, hepatic disease, renal disease and non-AIDS cancers, such as acute myocardial infarction, congestive heart failure, coronary artery disease, coronary revascularization, decompensated liver disease, deep vein thrombosis, diabetes mellitus, end-stage renal disease, peripheral arterial disease, pulmonary embolism, and stroke. In a large study following 6945 HIV-infected people from 1996 during the pre combination ART era to 2004 post-combination ART era, AIDS related mortality rates fell from 7.0 deaths/100 person-years from 1996 to 1.3 deaths/100 person-years in 2004. Conversely, the frequency of deaths due to SNA illnesses rose from 13.1% to 42.5% during this period. In HIV-infected individuals with higher CD4 counts (>350 cells/µl), death is more often related to SNA events than AIDS events. Since being on ART itself is associated with SNA, it is possible that SNA-related deaths may be attributed to prolonged use of ART due to their toxicity. However, Strategies for Management of Antiretroviral Therapy (SMART) study demonstrated that continuous ART
reduced mortality and opportunistic diseases compared to episodic ART treatment guided by CD4 count in a cohort of 5472 participants²⁰⁸.

A key underlying factor that may drive SNA events in ART experienced individuals is persistent systemic inflammation²⁰⁴, which is linked to microbial translocation from the gut¹¹⁶, inflammatory immune response form HIV, and co-infections. Inflammation that is associated with HIV lead to increased pro-inflammatory markers (IL-6, IL-8, C-reactive protein, etc), and other markers commonly associated with cardiovascular disease such as endothelial activation and damage (intercellular adhesion molecule, ICAM, and vascular cell adhesion molecule, VCAM) and coagulation factors (D-dimer)²⁰⁹-²¹¹. Amongst several activation markers that predict SNA events, recent analysis of 3766 ART-treated patients identified IL-6 and D-dimer as the best predictors of subsequent SNA conditions and death¹³⁹.
CHAPTER 2

A ROLE FOR MUCOSAL IL-22 PRODUCTION AND TH22 CELLS IN HIV-ASSOCIATED MUCOSAL IMMUNOPATHOGENESIS.

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2.1 Abstract

Interleukin-22 (IL-22) is a cytokine with epithelial reparative and regenerative properties that is produced by Th22 cells and by other immune cell subsets. Therefore we explored the hypothesis that disruption of the gut barrier during HIV infection involves the dysregulation of these cells in the gastrointestinal mucosa. Sigmoid IL-22 producing T cells and Th22 cells were dramatically depleted during chronic HIV infection, epithelial integrity was compromised, and microbial translocation was increased. These alterations were reversed after long-term antiretroviral therapy. While all mucosal IL-22 producing T cell subsets were also depleted very early during HIV infection, at these early stages IL-22 production by non-T cell populations (including NKp44+ cells) was increased and gut epithelial integrity was maintained. Circulating Th22 cells expressed a higher level of the HIV co-receptor/binding molecules CCR5 and α4β7 than CD4+ T cell subsets in HIV-uninfected participants, but this was not the case after HIV infection. Finally, recombinant IL-22 was protective against HIV and TNF-α-induced gut epithelial damage in a validated in vitro gut epithelial system. We conclude that reduced IL-22 production and Th22 depletion in the gut mucosa are important factors in HIV mucosal immunopathogenesis.
2.2 Introduction

Progressive HIV infection is characterized by a rapid and profound depletion of CD4 T cells from the gastrointestinal mucosa, as well as by structural deterioration of the gut epithelium and microbial translocation\textsuperscript{141,144,153,212}. Together, these factors may drive HIV disease progression. Despite tremendous survival benefit that is conferred by effective antiretroviral therapy (ART), microbial translocation and systemic immune activation may remain elevated in ART treated individuals, and contribute to an elevated risk of death owing to cardiac disease and neurocognitive dysfunction\textsuperscript{115,136,213,214}.

Gastrointestinal CD4 T cell depletion may be a necessary component of HIV mucosal immunopathogenesis, but this alone is insufficient to cause clinical immunodeficiency, as demonstrated by nonhuman primate models\textsuperscript{215,216}. However, during pathogenic HIV infection this mucosal CD4 T cell depletion is also accompanied by impaired epithelial barrier function and increased levels of microbial translocation\textsuperscript{116,152}. Recent literature indicates that the combination of structural epithelial deterioration and mucosal immunodeficiency are critical in driving HIV disease progression\textsuperscript{113,140,148,154}, yet little is known about how the epithelial barrier breaks down and why this leads to microbial translocation.

Nazli \textit{et al.} demonstrated \textit{in vitro} that HIV gp120 induces an innate TNF-\textalpha response by enterocytes, with subsequent breakdown of mucosal tight junction proteins\textsuperscript{154}. In pathogenic simian immunodeficiency virus infection, loss of colonic epithelial integrity was associated with increased lipopolysaccharide (LPS) levels in the lamina propria and draining lymph nodes, leading to bacteremia\textsuperscript{140}. Again, however, epithelial damage alone is insufficient to cause systemic microbial translocation, and plasma LPS levels were only elevated in humanized mice when epithelial breakdown was accompanied by simultaneous mucosal immunodeficiency\textsuperscript{113}. 
HIV is associated with mucosal depletion of specific T cell subsets, such as microbe-responsive Th17 cells, as well as with impaired neutrophil recruitment and phagocytic macrophage function, and this mucosal immune compromise may facilitate bacterial persistence in the gut mucosa and subsequent endotoxemia\textsuperscript{113,148,217,218}.

Interleukin-22 (IL-22) is a member of the IL-10 cytokine family that is produced by T cells, monocytes, DCs, natural killer (NK) cells, and innate lymphoid cells\textsuperscript{219}. IL-22 binds to a heterodimer consisting of IL-10Rβ-chain and IL-22R, signals through STAT3, and IL-22R is expressed predominantly on nonimmune cells of the skin, gut, and other organs\textsuperscript{220-222}. At mucosal surfaces IL-22 provides innate immune protection against bacterial and fungal infections, as well as promoting inflammation and enhancing epithelial proliferation and repair\textsuperscript{219,222,223}. In experimental colitis, IL-22 regulated immune homeostasis and promoted mucosal wound healing\textsuperscript{220,221}, and in murine \textit{Citrobacter rodentium} infection IL-22 regulated and maintained colonic lymphoid structure\textsuperscript{224}. While Th1 and Th17 cells commonly produce IL-22, recent studies have defined a Th22 CD4 T cell subset that produces IL-22 independent of IFN-γ and IL-17\textsuperscript{105,225,226}. These tissue reparative Th22 cells express IL-22 under the regulation of the aryl hydrocarbon receptor, and may share common surface markers with Th17 cells, such as the chemokine receptors CCR6 and CCR4, dipeptidylpeptidase IV CD26, and GPI-anchored protein CD90\textsuperscript{225-229}. Th22 cells have also been shown to express CCR10, potentially distinguishing them from the Th17 subset\textsuperscript{27,29,30}.

We hypothesized that mucosal IL-22-producing cells and Th22 cells have an important role in maintaining the gut mucosal barrier, and that their depletion during HIV infection contributes to epithelial damage and microbial translocation.
2.3 Methods

2.3.1 Participants

Study participants were recruited through the Maple Leaf Medical Clinic (Toronto, Canada) and provided written informed consent. The Research Ethics Boards at St. Michael’s Hospital, Toronto and the University of Toronto approved the study protocol. Participants were categorized into the following groups: HIV-uninfected controls (HIV-, n=8); ART-naive HIV-infected participants (HIV+, n=24) in early HIV infection (<7 months of HIV infection; N=12) and chronic HIV infection (>1 year of HIV infection; N=12); long-term ART treated individuals with an undetectable blood VL (<50 copies/ml) for at least 6 years on ART (HIV+ART, n=16).

2.3.2 Cell isolation from blood and sigmoid colon

Peripheral blood was collected into Acid Citrate Dextran solution A (BD Bioscience) and PBMCs isolated by Ficoll-Hypaque density centrifugation\textsuperscript{115}. Sigmoid biopsies were sampled approximately 25-30 cm from the anal verge as previously described\textsuperscript{115}, weighed, and immediately placed into RPMI solution (RPMI-1640 media containing 100 U ml\textsuperscript{-1} penicillin, 100 µg ml\textsuperscript{-1} streptomycin, and 1x GlutaMAX-1; Invitrogen). Sigmoid mucosal mononuclear cells were isolated by two sequential Collagenase type II digestions at 0.5 and 1.0 mg ml\textsuperscript{-1} (Clostridiopeptidase A; Sigma-Aldrich) for 30 min each at 37°C on a shaking heated block. Mucosal cells were passed through a 100-µm filter and enumerated and the median cell yield was about 46.2 million cells/g tissue and was similar across study groups.
2.3.3 Flow cytometry immune studies

Isolated blood and sigmoid mononuclear cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 1 ng mL⁻¹) and ionomycin (1 µM mL⁻¹; Sigma), or negative control media alone for 6 hours at 37°C and 5% CO₂, with the last 5 hours including Brefeldin A (1 µm mL⁻¹). All stimulations occurred in RPMI solution supplemented with 10% fetal bovine serum (FBS). Cells were washed with ice cold 1% FBS-1x phosphate buffered saline, permeabilized, stained for 30 min with fluorochrome-labeled monoclonal antibodies and a viability dye (Invitrogen), and fixed in 2% paraformaldehyde. The following antibodies were used: CD3, CD4, CD8, HLADR, CCR5, IL-17a, IL-22, IFN-γ, CD49d, B7, and TNF-α (BD BioSciences; E-Bioscience; and Beckman Coulter). Cells were acquired on a FACSCanto II or LSR II (BD Systems). Data analysis was performed using Flow Jo analytical software v9.0.2 (Treestar), and flow cytometric gating was determined using media and/or all fluorescence minus one (FMO) controls, and an event count below 15 was not further sub-analyzed. Values for functional cell subsets were background-corrected.

2.3.4 Calculation of absolute number of sigmoid cell subsets

The absolute number of gut cells were determined by multiplying the percentage of live lymphocytes determined by flow cytometry to the number of gut cells per gram of tissue, as previously described¹¹⁷. Biopsies were pre-weighed before tissue digestion and enumerated and the total gut cell count/g tissue was determined by dividing the number of counted sigmoid cells to the total tissue weight. This value was then multiplied by the percent of cells in the live lymphocyte gate and multiplied by subsequent gates based on flow cytometric analysis.
2.3.5 Immunoﬂuorescence

Fresh sigmoid biopsies were embedded in OCT compound (Tissue-Tek) and flash frozen in an ice-cold bath of 2-methylbutane. Biopsies were sectioned (5 µm) using Leica 1035S cryostat (Leica Microsystems), ﬁxed in acetone, and air-dried. Sections were re-hydrated in PBS, blocked with PBS-T (PBS with 0.05% Tween-20; Sigma) supplemented with 4% BSA, 5% normal goat serum, and 10% normal human plasma for 30 minutes. Tight junction antibodies included anti-ZO-1 and anti-claudin-2 rabbit polyclonal antibodies (Zymed), and mouse monoclonal cytokeratin PE (Cedarlane) and T cell antibodies included anti-CD3 biotin (Biolegend), anti-CCR10 APC (Biolegend). Secondary antibodies included streptavidin FITC or goat anti-rabbit IgG AF488. Finally, slides were stained with DAPI (Invitrogen) and mounted with Acqueous Mounting Medium (R&D Systems). Images of stained sections were obtained by the use of a Leica DM-R digital ﬂuorescence microscope with Retiga EXi digital camera (Q Imaging) using OpenLab (Improvision). Images were processed using Adobe Photoshop CS5 (image crop/merge layers), ensuring that all panels were processed in the same manner.

2.3.6 In vitro T84 monolayer cytokine study

Human colon-derived crypt-like T84 epithelial monolayers were cultured as previously described\(^\text{154}\) in a 1:1 (vol/vol) mixture of 10% FBS supplemented DMEM and Ham’s F-12 medium at 37°C in 5% CO\(_2\) for 5-6 days until conﬂuency, pretreated with 10 ng/ml of recombinant human IL-17a, IL-22, both cytokines combined (R&D Systems), or with media alone, and then exposed to HIV strain ADA (R5 tropic; MOI =1; 10 ng/ml). In separate experiments, IL-22 and IL-17a were added individually or in combination with recombinant TNF-α apically and left on monolayers through the experiment. Controls included cultures that
were mock-treated with medium without HIV or TNF-α and positive controls included HIV or TNF-α alone; control conditions were devoid of cytokine. TER was measured using a volt ohm meter (EVOM; World Precision Instruments) prior to exposure, 24h and 48h, post-exposure to HIV or TNF-α and compared to mock infection. Studies were completed in three separate experiments and each experiment contained duplicate cultures for each condition.

Paraformaldehyde (4%) fixed monolayers was stained with rabbit anti-human ZO-1 (Zymed) in blocking solution for 1h at room temperature, followed by AF488 goat anti-rabbit IgG. All samples were imaged on an inverted confocal laser-scanning microscope (LSM 510) using standard operating conditions as previously reported154.

2.3.7 Microbial translocation markers

Assays were performed according to manufacturers’ instructions, in duplicate. Plasma samples were diluted 5X in endotoxin-free water, heat inactivated at 65°C for 15 minutes, and assayed to quantify LPS levels using the limulus amebocyte lysate assay kit (Cambrex). Commercially available ELISA kit was used to measure levels of sCD14 (R&D Systems).

2.3.8 Statistical analysis

Spearman’s rank-correlation, Wilcoxon, and Mann-Whitney U-tests were performed for all human studies and the One-way ANOVA with the Bonferroni post-hoc test was applied for the T84 in vitro study using IBM SPSS Statistics 18.0 for Mac (SPSS). P<0.05 was considered significant.
2.4 Results

2.4.1 Study participants for gut mucosal studies

Paired blood and sigmoid colonic biopsies were collected from 48 men in the following participant groups: HIV-uninfected (HIV-; n=8), HIV-infected, therapy-naïve (HIV+; n=24), and HIV-infected on long-term effective ART (HIV+ART; n=16). The HIV-infected, therapy-naïve group included participants during both early infection (<7 months infection, N=12) and chronic infection (>1 year; N=12). Criteria for early HIV infection were either documented seroconversion (N=6) or a known high-risk HIV sexual exposure followed by symptoms of seroconversion illness (N=6). All participants were HIV seropositive by the time of sample collection (data not shown). The ART-naive HIV-infected group had a median blood CD4 count of 390 cells/µl and a blood viral load (VL) of 27,451 RNA copies/ml. Among HIV-infected, ART-naïve participants the early-infected group had a higher CD4 count than those during chronic infection (P=0.026), but the blood VL was comparable. ART-treated participants had been on therapy for a median of 12.5 years, with an undetectable blood VL (<50 copies/ml) for no less than 6 years and a median CD4 count of 660 cells/µl.

2.4.2 T cell immune parameters in the blood and sigmoid colon

Immune parameters were assayed in freshly isolated mononuclear cells from the blood and sigmoid mucosa (Table 2.1). Untreated HIV infection was associated with preferential CD4+ T cell depletion and with an equivalent increase in immune activation (HLA-DR expression) in both the blood and gut mucosa. CCR5+CD4+ T cells were depleted within the sigmoid mucosa (P=0.004), but not in blood (P=0.151). While early HIV-infected participants tended to have a
higher %CD4 in blood (early, 36.8% vs chronic, 24.0%; P=0.078) and less blood CD4 activation than the chronic infection group (11.1% vs. 18.9%; P=0.027), mucosal CD4 depletion was comparable (17.2% vs. 13.4%; P=0.106). CD4 reconstitution was incomplete in the blood despite long-term ART (P=0.023), and also tended to be lower in the sigmoid mucosa (proportion, P=0.081; absolute number, P=0.086). Expression of the immune activation marker HLA-DR did not differ between ART-treated and HIV-uninfected participants.
Table 2.1  Descriptive immununological parameters of blood and sigmoid colon of the study cohort.

<table>
<thead>
<tr>
<th></th>
<th>HIV−</th>
<th>HIV+</th>
<th>HIV+ ART</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Blood CD4</td>
<td>63</td>
<td>(34–72)</td>
<td>(14–65)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(8–70)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*a,b,c</td>
</tr>
<tr>
<td>% Sigmoid CD4</td>
<td>58</td>
<td>(28–74)</td>
<td>(3–43)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*a</td>
</tr>
<tr>
<td>Abs sigmoid CD4</td>
<td>1.7</td>
<td>(0.4–6.0)</td>
<td>(0.1–2.8)</td>
</tr>
<tr>
<td>(10^6 cells/g tissue)</td>
<td></td>
<td></td>
<td>*a</td>
</tr>
<tr>
<td>% Blood CD8</td>
<td>33</td>
<td>(22–55)</td>
<td>(30–82)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*a</td>
</tr>
<tr>
<td>% Sigmoid CD8</td>
<td>27</td>
<td>(17–45)</td>
<td>(37–90)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>***a</td>
</tr>
<tr>
<td>Abs sigmoid CD8</td>
<td>0.9</td>
<td>(0.3–1.6)</td>
<td>(0.8–9.1)</td>
</tr>
<tr>
<td>(10^6 cells/g tissue)</td>
<td></td>
<td></td>
<td>***a</td>
</tr>
<tr>
<td>% Blood CCR5+ CD4 T cells</td>
<td>1.5</td>
<td>(0.1–7.0)</td>
<td>(0.1–15.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*a,b</td>
</tr>
<tr>
<td>% Sigmoid CCR5+ CD4 T cells</td>
<td>42</td>
<td>(36–67)</td>
<td>(6–71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*a</td>
</tr>
<tr>
<td>% Blood HLADR+ CD4 T cells</td>
<td>5.9</td>
<td>(2.3–20)</td>
<td>(5–46)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*a</td>
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<tr>
<td>% Sigmoid HLADR+ CD4 T cells</td>
<td>13</td>
<td>(5–39)</td>
<td>(10–56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*a</td>
</tr>
<tr>
<td>% Blood HLADR+ CD8 T cells</td>
<td>26</td>
<td>(11–36)</td>
<td>(11–86)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>***a</td>
</tr>
<tr>
<td>% Sigmoid HLADR+ CD8 T cells</td>
<td>33</td>
<td>(8–60)</td>
<td>(15–92)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>***a</td>
</tr>
</tbody>
</table>

Abbreviation: Abs, absolute.

Values indicate median (range).

Significance of $P \leq 0.05$ is indicated by *, and $P \leq 0.001$ is indicated by **.

*a=HIV− to HIV+; b=HIV+ to HIV+ ART; c=HIV− to HIV+ ART.
2.4.3 Preferential depletion of sigmoid Th22 cells during HIV infection and reconstitution on ART

Sigmoid Th22 cells were defined as CD4+ T cells producing IL-22 in the absence of IFN-\(\gamma\) or IL-17a, and Th17 and Th1 cells were defined by their capacity to produce IL-17a and IFN-\(\gamma\) respectively (Figure 2.1a). In HIV-uninfected donors, Th22 cells were enriched 8 fold in the sigmoid colon compared to blood (0.72% vs 0.09% respectively, \(P=0.012\)) and the following sigmoid CD4 T cell subsets were responsible for IL-22 production: Th1 cells (48.7% of CD4+ IL-22 production), Th22 cells (25.5%), and Th17 cells (27.3%). Most Th22 cells displayed an effector memory phenotype (CD45RO+CCR7-) in both the sigmoid mucosa (~85%) and blood (~70%; data not shown).

There was a substantial reduction in both the absolute number and frequency of Th22 cells in the sigmoid mucosa during untreated HIV infection compared to both HIV-uninfected (\(P=0.005\) and \(P=0.001\) respectively) and ART-treated groups (both \(P<0.001\); Figure 2.1b and 2.1c). Although there was substantial inter-individual heterogeneity in Th22 reconstitution after long-term ART, the degree of Th22 reconstitution was not associated with ART regimen or duration, or blood CD4 count (data not shown). The frequency and the absolute number of sigmoid Th1 cell subsets were comparable across the study groups. Unexpectedly, the mucosal Th17 frequency was comparable across study groups, although the Th17 cell number was depleted during chronic (not early) HIV infection (HIV-, 18.5 x 10\(^3\) cells/g tissue, chronic HIV+, 8.9 x 10\(^3\) cells/g tissue; \(P=0.045\); data not shown).

Immunohistochemical studies were performed on flash-frozen sigmoid biopsies to confirm Th22 depletion in HIV-infected participants. However, IL-22 expression was uncommon in the absence of PMA and ionomycin stimulation. Staining for CCR10, a recently identified
phenotypic marker of Th22 cells\textsuperscript{227}, we first confirmed that the rare mucosal CD3/IL-22 double positive cells did co-express CCR10, and then used CD3/CCR10 co-expression as a proxy for Th22 cells (data not shown). In HIV-uninfected sigmoid biopsies, clusters of CD3/CCR10 co-positive cells were often observed; HIV-infected biopsies contained far fewer co-staining cells, although single-staining cells for each marker were present (data not shown). Overall, there was clear preferential depletion of CD3/CCR10 co-staining (Th22) cells in the sigmoid colon during HIV infection, above and beyond the generalized mucosal CD4+ depletion.

The frequency of sigmoid Th22 cells correlated with an increased overall gut CD4 cell percentage (P=0.025; Spearman correlation coefficient, \( r_s =0.456 \)). In additional, the absolute number of sigmoid Th22 cells was associated with higher blood CD4 count (P=0.045; \( r_s =0.412 \)), a higher blood and gut CD4 percentage (blood, P=0.020; \( r_s =0.471 \); gut, P=0.017; \( r_s =0.482 \)). Neither the frequency nor the absolute number of mucosal Th22 cells were associated with blood VL.

In the blood compartment, frequency of Th1 and Th17 cells were comparable across the study groups, and Th22 cells were increased in ART-treated individuals compared to ART-naïve individuals (P=0.014; data not shown). Recent studies have identified CD26 and CD90 as surface markers that identify Th17 cells, including Th17 cells co-producing IL-22\textsuperscript{228,229}. However, while blood Th22 cells expressed high levels of CD26, similar to Th17 cells (Th22, 91.1% vs Th17, 77.9%), CD90 expression was infrequent on Th22 cells (Th22, 0.92% vs Th17, 6.56%; data not shown). Plasma levels of IL-22 were measured by a commercially available IL-22 ELISA kit (R&D Systems), but IL-22 concentrations were below the limit of detection (range, 0-6 pg/ml) in both the HIV-uninfected and HIV-infected groups.
Figure 2.1 Th22 cells were preferentially depleted in the sigmoid colon of the HIV-infected group and reconstituted in the ART treated group. (a) Gating strategy of sigmoid Th22 cells and representative flow cytometry plots. (b) Absolute numbers ($10^6$ cells/g tissue) and (c) frequency (%) of Th22 cells from Th22, Th1 and Th17 cell from the sigmoid colon.
2.4.4 IL-22 production by other mucosal cell subsets

Although Th22 cells were preferentially depleted from the sigmoid mucosa during HIV infection, these are not the only mucosal source of IL-22\(^{219}\). There was a dramatic reduction in the overall frequency of IL-22 producing gut lymphocytes, CD3+ cells, and CD4(-) T cells during all stages of untreated HIV infection, and as well, IL-22 producing capacity from bulk CD4+, Th1 cells, and Th17 cell was reduced (Figure 2.2). IL-22 production by all mucosal immune subsets was reconstituted after long-term ART.

Figure 2.2 Mucosal IL-22 production by various lymphocyte subsets was lost. IL-22 from (a) gut lymphocytes, (b) CD3+ T cells, (c) CD4(-) T cells, (d) CD4+ T cells, (e) Th1 cells, and (f) Th17 cells was depleted in HIV-infected but not ART-treated participants.
2.4.5 **IL-22 dysregulation during the early stages of HIV infection**

There was clear, preferential depletion of the mucosal Th22 subset during early HIV infection (Figure 2.3a and 2.3b) and total CD3+ T cell IL-22 producing capacity was also reduced during early HIV infection (P=0.002; Figure 2.3c). In addition, IL-22 production was reduced within bulk CD4+ T cells (HIV-, 2.1% vs. early HIV+, 0.7%; P=0.017), bulk CD4(-) cells (1.4% vs. 0.1%; P=0.001), Th1 (6.0% vs. 1.7%; P=0.005), and Th17 subsets (27.6% vs. 12.1%; P=0.021; data not shown). However, while we observed a dramatic reduction in IL-22 production capacity from all CD3+ T cell subsets during early HIV infection, this HIV stage was uniquely characterized by the enhanced production of IL-22 from a sigmoid population of IL-22+ CD3(-) cells (Figure 2.3d). A subset of these cells was CD4+ (median, 12.5%). In three ART-naïve HIV-infected participants we assessed the NK cell markers CD56 and NKp44; while most IL-22+ CD3(-) cells were CD56(-), a substantial subset expressed NKp44+ (16.7%, 45.0% and 77.8%; data not shown).
Figure 2.3 Early HIV-infected individuals had reduced sigmoid Th22 cells and IL-22 production capacity from CD3+ T cells, but increased IL-22 from CD3(-) subsets. (a) Absolute and (b) proportion of Th22 cells was depleted in early and chronic HIV-infected individuals, (c) as well as IL-22 depletion from bulk T cells (d). However, early HIV-infected individuals uniquely increased their capacity to produce IL-22 from CD3(-) lymphocytes.
2.4.6 **Sigmoid epithelial integrity at different stages of HIV infection**

We examined epithelial integrity in serially sectioned sigmoid biopsies by immunofluorescence analysis of tight junction proteins ZO-1 and claudin-2 (green), cytokeratin (red), and DAPI (blue). Epithelial damage was defined as ZO-1 reduction and increased pore-forming protein claudin-2 expression. Although heterogeneity was apparent in each group, biopsies from HIV-uninfected individuals showed continuous and robust expression of ZO-1 and minimal expression of claudin-2. Untreated chronic HIV infection was associated with multifocal disruption and reduced expression of ZO-1 and increased expression of claudin-2 in the epithelium. In contrast, epithelial integrity was maintained during early HIV infection. Three representative images of ZO-1 and claudin-2 are shown for each cohort: HIV-, early HIV+, and chronic HIV+ (Figure 2.4).
Figure 2.4 Immunofluorescence analysis of epithelial integrity of HIV-uninfected, and early and chronic HIV-infected participants. Sigmoid slides were stained with ZO-1 (green), claudin-2 (green), cytokeratin (red), and DAPI (blue) and imaged at 200X magnification. Representative images of three HIV-uninfected (ID 06, ID 02, and ID 08), three early HIV-infected (ID 09, ID 13, ID 14), and three chronic HIV-infected (ID 26, ID 25, ID 24) participants demonstrate (a) multifocal disruption and reduced expression of ZO-1 and (b) an increase in the pore-forming protein claudin-2 in chronic HIV infection, while early HIV-infected participants maintained gut integrity.
2.4.7 Plasma markers of microbial translocation during HIV infection

Plasma LPS and soluble CD14 (sCD14) are markers of microbial translocation that are increased during HIV infection\textsuperscript{116,134}. Plasma LPS was increased only during chronic, untreated HIV infection (P=0.025), but not in early HIV infection (P=0.817) or in long-term ART treated individuals (P=0.188; data not shown). sCD14 was elevated in all HIV-infected groups, regardless of disease stage (early, P=0.031; chronic, P=0.002) or treatment status (P=0.003; data not shown). Neither the frequency nor the absolute number of sigmoid Th22 cells were associated with plasma LPS or sCD14 levels.

2.4.8 Circulating Th22 cells constitute a highly HIV-susceptible CD4+ subset.

Overall, there was dramatic depletion of Th22 cells and other IL-22+ cell subsets during untreated HIV infection. To investigate this further, levels of the HIV co-receptor CCR5 and the HIV-binding and gut-homing integrin α4β7 were compared between circulating bulk CD4+ memory (CD45RO+), Th1, Th17, and Th22 cells from HIV-uninfected (n=10) and ART-naive, chronically HIV-infected individuals (n=6). In contrast to the sigmoid mucosa, most IL-22 producing CD4+ T cells in the blood of HIV-uninfected donors were true Th22 cells (71.9%), with a lower proportion of IL-22 produced by Th1 (12.3%) and Th17 cells (14.8%).

In HIV-uninfected individuals CCR5 frequency was higher in Th22 cells than bulk CD4+ or Th1 cells, while similar to Th17 cells, and β7\textsuperscript{high} expression by Th22 cells was more frequent than by bulk CD4+ T cells but similar to Th1 cells and Th17 cells (Figure 2.5a and 2.5b). The mean fluorescence intensity (MFI) of CCR5 was at least 7.2 fold higher on Th22 cells than on any other subsets and the MFI of β7 was at least 1.8 fold higher than other subset (data not
shown). While CCR5 and $\beta^7_{\text{high}}$ expression were increased on all IL-22+ CD4 cells compared to bulk CD4 cells (both $P=0.005$), Th22 cells had particularly high CCR5 and $\beta^7$ MFI compared to total IL-22 producing CD4 cells ($P=0.009$ and $P=0.028$ respectively; data not shown).

Interestingly, no increased expression of CCR5 or $\beta^7_{\text{high}}$ expression was seen on blood Th22 cells after HIV infection (Figure 2.5C and 5D), and there was a selective HIV-associated reduction in the expression of both CCR5 and $\beta^7$ by Th22 cells in the context of untreated HIV infection (Figure 2.5e and 2.5f).
Figure 2.5 A higher frequency of circulating Th22 cells expressed the HIV co-receptor (CCR5) and binding molecule $\alpha_4\beta_7$ than other CD4 subsets in HIV-uninfected participants, but this phenotype was lost in HIV infection. Frequency of (a, c) CCR5+ and (b, d) $\beta_7^+$ CD4 subsets of HIV-uninfected and HIV-infected individuals. Expression of (e) CCR5 and (f) $\beta_7^{\text{high}}$ on Th22 cells were decreased in HIV-infected individuals compared to uninfected individuals while they were comparable in CD4 cells.
2.4.9 *In vitro effects of IL-22 on HIV and TNF-α-induced epithelial damage*

Based on these results we examined the ability of recombinant IL-22 and/or IL-17a to prevent HIV and TNF-α-mediated epithelial damage in an *in vitro* enterocyte system that has been previously validated\(^{154}\). T84 intestinal monolayers were pretreated with IL-17a, IL-22, or both cytokines for 2h, and HIV (ADA strain) added, or in a separate experiment cytokines were co-cultured with TNF-α for 24h and 48h, and transepithelial resistance (TER) was measured. By 24h, treatment with HIV or TNF-α alone had induced a profound reduction in epithelial integrity (P<0.001; Figure 2.6)\(^ {154}\). The TER reduction at 24h was completely abrogated by IL-22 alone (both P<0.001), and was attenuated to a lesser degree by IL-17a (HIV, P=0.051; TNF-α, P<0.01) or both cytokines combined (HIV, P=0.001; TNF-α, P<0.001; Figure 2.6a and 2.6b); at 48h, TER loss was effectively prevented by all three cytokine conditions (all P<0.001, data not shown). These TER findings were confirmed through immunofluorescence staining of the tight junction protein ZO-1 (Figure 2.6c). Overall, both exogenous IL-17a and IL-22 protected against HIV and TNF-α-induced epithelial barrier loss in this model system, with IL-22 demonstrating more rapid and complete protection.
Figure 2.6 T84 human intestinal monolayers were pretreated with IL-17a and/or IL-22 and HIV, or co-cultured with TNF-α for 24h, and transepithelial resistance (TER) measured. (a, b) IL-22 prevented TER loss more than IL-17a alone, however both IL-17a and IL-22 prevented TER loss in HIV and TNF-α induced epithelial damage. (c) Immunofluorescence staining of the cells with anti-ZO-1 (green) and nuclear dye propidium iodide (red) demonstrates the loss of tight junction in the HIV or TNF-α control condition and a robust presence of ZO-1 in the cytokine treated conditions.
2.5 Discussion

HIV infection is associated with the depletion and/or dysfunction of key mucosal effector cells and with direct damage to the epithelial barrier\textsuperscript{144,148,154}. While poorly understood, the simultaneous and synergistic occurrence of both factors is likely required for the development of microbial translocation and the systemic immune activation\textsuperscript{116}. These pathogenic events in the gastrointestinal mucosa are thought to play a central role in HIV pathogenesis and we now provide the first evidence that gut IL-22-producing and Th22 cells link these two aspects of HIV mucosal immunopathogenesis. IL-22-producing cells and Th22 cells were preferentially depleted from the gut mucosa during all stages of untreated HIV infection, and the increased production of IL-22 by CD3(-) mucosal immune cells (some of which were NKp44+) appeared to compensate for this loss during early HIV infection. However, during chronic infection all lymphocytic mucosal sources of IL-22 were depleted, gut epithelial integrity was compromised, and there was evidence of systemic microbial translocation. Finally, the protective and reparative role of IL-22 was confirmed in a well-defined \textit{in vitro} enterocyte model, where exogenous IL-22 abrogated HIV-induced epithelial damage.

The mechanism(s) underlying the preferential loss of sigmoid IL-22-producing and Th22 cells in HIV infected individuals was not determined in this translational study. Circulating Th22 cells expressed a highly HIV-susceptible phenotype, manifested by an increased expression of the HIV co-receptor CCR5 and HIV-binding integrin $\alpha 4\beta 7$, and past studies have demonstrated that these markers are a good predictor of mucosal cell death and HIV gp120 binding\textsuperscript{218,230,231}. However, expression of these surface markers only offers a partial explanation of IL-22 production loss, since CD4(-) T cells and CD3(-) non-T cells that would not be directly infected by HIV also demonstrated a reduced capacity to produce IL-22. Furthermore, we were not able
to sort these functionally defined cells to examine provirus levels. Other possible mechanisms of reduced mucosal IL-22 production could include an inability of mucosal dendritic cell subsets to differentiate IL-22 producing cells, or a local deficiency in other immune signals necessary for IL-22 production. In the skin where IL-22 production has been studied in greater detail, Langerhans cells were able to differentiate Th22 polarized cells\textsuperscript{212}. Therefore, future studies of mucosal dendritic cell subsets and IL-22 production during HIV infection may be of value.

Th22 cells are not the only source of IL-22, and in our study other important sources of IL-22 in the gut mucosa included the Th1 and Th17 CD4+ subsets, as well as NK22 cells\textsuperscript{219,233}. Non-T-cell lineage-negative innate lymphoid cells, such as NKp44+/CD56+ cells and lymphoid tissue-inducer (LTI)-like cells, are well-described and important mucosal sources of IL-22\textsuperscript{219,225}. We were unable to define whether IL-22 producing cells or Th22 cells play an equivalent role in maintaining the gut barrier. However, the protective effect of recombinant IL-22 in our in vitro epithelial damage model and in other mucosal studies suggest that this cytokine has an important in vivo role regardless of the cell source\textsuperscript{220-222}.

While we did observe depletion of Th17 numbers in the sigmoid mucosa in the sigmoid mucosa of chronically HIV-infected participants, the degree of depletion was lower than expected and did not extend to a reduced Th17 proportion, as has been previously observed\textsuperscript{115,118,148}. The reason for this is not known. One possibility relates to differences in the participants themselves. For example, recent studies in rhesus macaques demonstrated substantial variability in Th17 frequencies between animals (>5-fold), and that the size of Th17 cell compartment before SIV infection was an important predictor of SIV clinical outcome\textsuperscript{234}. Whether such preexisting differences might explain variation in mucosal Th17 populations in humans is unknown. Notwithstanding the lower-than-expected Th17 depletion observed, the association of HIV
infection with IL-22 depletion and reduced Th22 proportions and numbers in this cohort was clear and unambiguous.

A major consequence of HIV-induced gut epithelial damage is an influx of microbial products into the submucosa and subsequently into the blood, and circulating LPS may play an important role in driving HIV disease progression by increasing systemic immune activation. Previous studies have suggested that endotoxemia requires both the presence of structural barrier damage and mucosal immunodeficiency. In keeping with this, plasma LPS levels were only elevated in our chronic HIV-infection group, where mucosal IL-22 depletion and epithelial structural compromise were present simultaneously. In contrast, epithelial integrity was maintained during early HIV infection (despite mucosal immune compromise) and plasma LPS concentrations were normal. These data suggest that both mucosal structural damage and mucosal immune compromise must both be present to result in significant microbial translocation. The lack of an association between mucosal IL-22 production and plasma LPS levels in our study is likely to reflect the presence of multiple intermediate steps between microbial translocation and actual endotoxemia, including microbe-responsive Th17 cells, neutrophils, and phagocytic macrophages/monocytes in the gut mucosa, liver and blood.

In conclusion, our results suggest that IL-22 production and Th22 cells within the gut mucosa have an important role in the maintenance of mucosal epithelial integrity. Their preferential depletion during HIV infection may be important in mucosal immunopathogenesis, and suggests a possible avenue for novel HIV therapeutics.
CHAPTER 3

MUCOSAL TH17 CELL FUNCTION IS ALTERED DURING HIV INFECTION AND IS AN INDEPENDENT PREDICTOR OF SYSTEMIC IMMUNE ACTIVATION

3.1 Abstract

Mucosal Th17 cells maintain the gut epithelial barrier and prevent invasion by luminal bacteria through a delicate balance of immunosuppressive and pro-inflammatory functions. HIV infection is characterized by mucosal Th17 depletion, microbial translocation and immune activation. Therefore, we assessed the function of blood and sigmoid Th17 cells during both early and chronic HIV infection, as well as the impact of short- and long-term antiretroviral therapy (ART). Th17 cells were defined as IL-17a+ CD4 T cells, and their functional capacity was assessed by the coproduction of the inflammatory cytokines IL-22, TNF-α and IFN-γ, as well as the immunoregulatory cytokine IL-10. Gut Th17 cells had a much greater capacity to produce pro-inflammatory cytokines than those from the blood, but this capacity was dramatically reduced from the earliest stages of HIV infection. Immunoregulatory skewing of mucosal Th17 cell function, characterized by an increased IL-10/TNF-α ratio, was uniquely seen during early HIV infection, and was independently associated with reduced systemic immune activation. ART rapidly restored mucosal Th17 cell numbers; however normalization of mucosal Th17 function, microbial translocation and mucosal/systemic immune activation was much delayed. These findings emphasize that strategies to preserve, or to more rapidly restore mucosal Th17 function may have important therapeutic benefit.
3.2 Introduction

Protective immunity and immune regulation is provided by the balanced function of various CD4 T cell subsets, and their depletion in HIV and SIV infection leaves the host susceptible to a range of opportunist infections that define AIDS\textsuperscript{235}. The progression of HIV and SIV infection is mediated by a rapid depletion of gastrointestinal CD4 T cells, followed by a deterioration of the gut epithelium and increased microbial translocation\textsuperscript{116,140,236}. IL-17a-producing Th17 cells are an important subset of CD4 T cells that maintain the gut mucosa by inducing proliferation of epithelial cells\textsuperscript{237}, promoting antibacterial defensin production\textsuperscript{238}, and recruiting neutrophils in the context of bacterial invasion\textsuperscript{239,240}. Th17 cells are highly susceptible to HIV and are preferentially depleted in the gut mucosa\textsuperscript{115,118,148}, whereas HIV infection is associated with an increase in mucosal immunosuppressive regulatory CD4 T cells (Treg) that develop reciprocally to Th17 cells\textsuperscript{115,118}. These alterations in CD4 T cell subsets impair mucosal protection against luminal bacteria and may lead to microbial translocation into the systemic circulation\textsuperscript{118}. Increased levels of circulating bacterial products, such as lipopolysaccharide (LPS) and peptidoglycan, elicit potent pro-inflammatory innate immune responses and drive persistent immune activation\textsuperscript{116,130,241}. Importantly, HIV-infected individuals have elevated systemic immune activation, and in an antiretroviral therapy (ART)- naïve individual the degree of this activation is the best predictor of HIV disease progression to AIDS\textsuperscript{135}. Furthermore, despite the significant survival benefits of ART, gut immune reconstitution is often incomplete, and ongoing immune activation contributes to persistent neurocognitive and cardiovascular dysfunction\textsuperscript{115,136,192,213}.

Although murine Th17 cell differentiation has been well defined, human Th17 cell development is distinct and incompletely understood\textsuperscript{240,242}. Several studies indicate the necessity
of TGF-β together with a pro-inflammatory cytokine such as IL-6, IL-21, IL-1β or IL-23 for human Th17 development, although a common differentiation pathway has not been identified\textsuperscript{240,243,244}. In mice, the cytokine milieu in which Th17 cells differentiate can lead to polarization of subsequent Th17 function. Specifically, stimulation with TGF-β and IL-6 generated IL-17a- and IL-10-producing CD4 T cells with a reduced proinflammatory capability\textsuperscript{245-247}. However, the addition of IL-23 led to the expansion of IL-17a+ CD4 T cells that produced an abundance of proinflammatory cytokines such as IL-22, IFN-γ, and TNF-α\textsuperscript{242,245}. These pro-inflammatory Th17 cells have been associated with autoimmune diseases such as inflammatory bowel disease, psoriasis and rheumatoid arthritis\textsuperscript{248-250}, but they play an important role in defense against microbial translocation from the gut\textsuperscript{237,238,251,252}. For example, although a key role of Th17 cells is to recruit neutrophils to the site of bacterial invasion, human neutrophils do not express IL-17a receptor, instead, they depend on the coproduction of IFN-γ and TNF-α by Th17 cells\textsuperscript{239}. Interestingly, a recent study demonstrated that Th17 cell functionality was pathogen-specific: human Th17 cells primed with the bacteria \textit{Staphylococcus aureus} produced IL-17a and IL-10, whereas those primed with the fungus \textit{Candida albicans} produced IL-17a and IFN-γ under the regulation of IL-1β\textsuperscript{252}. Therefore the co-production of specific cytokines by mucosal Th17 cells may be a critical determinant of their ability to maintain gut mucosal defense.

The ability of HIV-specific CD8+ T cells to manifest multiple functions, including the co-production of perforin and different cytokines, is central to host virus control\textsuperscript{48,253}. Polyfunctional virus-specific T cells are associated with improved control of HIV replication and with delayed HIV disease progression\textsuperscript{48,254,255}. In addition, elite HIV non-progressors who maintain normal CD4 T cell counts and an undetectable HIV blood viral load (VL) in the absence of ART demonstrate more polyfunctional and more complex HIV-specific CD8 T cells in the blood and
rectal mucosa than do HIV-infected non-controllers\textsuperscript{48,256}. Although recent studies of the Th17 subset have also demonstrated considerable functional diversity, the relevance of this polyfunctionality to gut mucosal defense and the impact of HIV infection on this function have not been explored. Therefore we investigated the functional profile of blood and sigmoid colonic Th17 cells during HIV infection and ART, as well as the relationship of these parameters with microbial translocation and host immune activation.
3.3 Methods

3.3.1 Study participants

Sixty study participants were recruited through the Maple Leaf Medical Clinic (Toronto, Canada) and provided written informed consent. The Research Ethics Boards at the University of Toronto and St. Michael’s Hospital, Toronto, Canada, approved the study protocol. Participants belonged to one of the following study groups: HIV-uninfected controls (HIV-, N=9); ART-naïve HIV-infected participants in early and chronic phases of infection (early HIV+, N=24; chronic HIV+, N=12), and long-term ART treated aviremic participants (HIV+ART, N=15). A subset of early (N=6) and chronic (N=5) HIV-infected individuals was followed longitudinally after a median of 12 months after ART initiation.

3.3.2 Cell isolation from blood and sigmoid colon

Peripheral blood was collected into Acid Citrate Dextran solution A vacutainer tubes (BD Bioscience) and PBMCs were isolated by Ficoll-Hypaque density centrifugation. Sigmoid biopsies were collected approximately 25-30 cm from the anal verge as previously described\textsuperscript{115}, immediately placed into RPMI solution (RPMI-1640 media containing 100 U ml\textsuperscript{-1}, penicillin, 100 μg ml\textsuperscript{-1}, streptomycin, and 1x GlutaMAX-1; Invitrogen), and weighed. Mucosal tissue mononuclear cells were isolated by two sequential Collagenase type II digestions at 0.5 and 1.0 mg ml\textsuperscript{-1} (Clostridiopeptidase A; Sigma-Aldrich) for 30 min each on a shaking heated block at 37°C. After tissue digestion, cells were passed through a 100-μm strainer. The median cell number was 45.2 million cells/g of tissue (range: 21.0-105.5 million cells/g) and it did not differ with regard to HIV or treatment status.
3.3.3 Flow cytometry

Isolated cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 1ng/ml) and ionomycin (1 μM/ml; Sigma), or with media alone for 6 hours at 37°C, with the last 5 hours containing Brefeldin A (1 μm/ml) in RPMI 1640 solution supplemented with 10% fetal bovine serum (FBS). Cells were washed with 1% FBS-1x phosphate buffered saline, permeabilized, stained for 30 min with fluorochrome-labeled monoclonal antibodies and a violet or aqua LIVE/DEAD viability dye (Invitrogen), and fixed in 2% paraformaldehyde. Antibodies included CD3, CD4, CD8, HLA-DR, CD38, IL-17a, IL-22, IFN-γ, IL-10 and TNF-α (BD BioSciences; eBioscience; and Beckman Coulter). Cells were acquired on a FACSCanto II (BD Systems) and analyzed using FlowJo analytical software v9.0.2 (Treestar). Flow cytometric gates were set based on media alone and/or all fluorescence minus one control, and values for functional cell subsets were background-corrected where applicable. Dead cells and doublets were excluded based on live/dead staining and side scatter cell-granularity properties. Th17 cells were defined as IL-17a+ of CD4 T cells and Tregs were defined as FOXP3 and CD25 dually positive CD4 T cells. The absolute number of gut cells was determined by multiplying the percentage of live lymphocytes determined by flow cytometry to the number of gut cells/g of tissue, as previously described. Th17 cell polyfunctionality analysis was generated by Boolean gating analysis using FlowJo software and a minimum event number of 50 was set for Th17 polyfunctionality analyses. SPICE software v5.22 (National Institute of Allergy and Infectious Diseases/National Institutes of Health) was used to graph these data using the average value for each of the cytokine category.
3.3.4 **Microbial translocation markers**

Assays were performed according to the manufacturers’ instructions, in duplicate. Plasma samples were diluted 5X in endotoxin-free water, heat inactivated at 65°C for 15 minutes, and assayed to quantify LPS levels using the limulus amebocyte lysate assay kit (Cambrex). A commercially available ELISA kit (R&D Systems) was used to measure levels of soluble CD14 (sCD14).

3.3.5 **Statistical analysis**

The Spearman rank-correlation test, Mann-Whitney U-test, and Wilcoxon signed-rank test were performed using IBM SPSS Statistics 20.0 for Mac (SPSS) and Th17 polyfunctionality analysis was performed using SPICE Student t-test (v5.22). Linear regression analyses were used to measure independent predictors of immune activation. The dependent variable (blood CD38+DR+CD8+ T cells) was logit transformed, and backward stepwise linear regression analyses were performed for multiple independent covariates using SPSS. Values of p<0.05 were considered significant.
### 3.4 Results

#### 3.4.1 Participant clinical and immune characteristics

Sigmoid colon biopsies and peripheral blood were collected from 60 participants: HIV-uninfected individuals (HIV-, N=9); ART-naïve individuals during early (early HIV+; N=24) and chronic (chronic HIV+; N=12) stages of HIV infection; and long-term ART-treated individuals (long-term ART; N=15). Early HIV-infected individuals either had documented seroconversion (N=12) or a known high-risk HIV sexual exposure followed by symptoms compatible with seroconversion illness within the past 7 months (N=12); all were IgG seropositive and the median duration of infection was 4 months. Participants on long-term ART had been treated for a median of 13 years (range, 6-20 years), with an undetectable blood VL for at least 6 years. Participant clinical and immune characteristics are outlined in Table 3.1.

Absolute and relative CD4 T cell numbers were depleted in the blood and sigmoid colon during both early and chronic HIV infection, and long-term ART restored all parameters except for the absolute number of CD4 T cells in the sigmoid mucosa. The number of Th17 cells, defined as CD4 T cells producing IL-17a after mitogen stimulation, was decreased in the sigmoid mucosa during chronic HIV infection (P=0.002), with a similar trend during early HIV infection (P=0.075). Immune activation was defined by the coexpression of HLA-DR and CD38 by CD8+ T cells, and was substantially increased in the blood and sigmoid mucosa during both early and chronic stages of HIV infection (data not shown). Immune activation during early and chronic HIV infection was comparable in both the blood and gut (P=0.473 and P=0.754 respectively). Plasma lipopolysaccharide (LPS) levels, a marker of microbial translocation, were increased during the chronic stage of HIV (P=0.025), but not the early HIV stage (P=0.817), whereas
plasma levels of sCD14 were increased during both early and chronic stages (early HIV+, P=0.031; chronic HIV+, P=0.002; data not shown).

Table 3.1 Clinical and immune characteristics of study participants.

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<th>HIV- (n = 9)</th>
<th>Early HIV+ (n = 24)</th>
<th>Chronic HIV+ (n = 12)</th>
<th>Long-Term ART (n = 15)</th>
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<td>33 (28-40)</td>
<td>33 (26-44)</td>
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<td>Blood CD4 count (/ml)</td>
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<td>440 (242-688)**</td>
<td>275 (210-435)**</td>
<td>700 (400-770)</td>
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<td>Blood VL (c/µl)</td>
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<td>29,628 (6,688-113,687)</td>
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<td>Blood CD4 T cells (%)</td>
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<td>38 (32-48)*</td>
<td>24 (21-35)**</td>
<td>48 (34-56)</td>
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<tr>
<td>Gut CD4 T cells (%)</td>
<td>58 (45-67)</td>
<td>21 (15-28)**</td>
<td>13.4 (10-16)**</td>
<td>49 (41-55)</td>
</tr>
<tr>
<td>Abs gut CD4 T cells (10^6 cells/g tissue)</td>
<td>1.7 (1.1-3.4)</td>
<td>0.7 (0.4-1.1)*</td>
<td>0.4 (0.2-1.1)*</td>
<td>0.9 (0.3-1.5)*</td>
</tr>
<tr>
<td>Blood Th17 cells (%)</td>
<td>0.3 (0.2-1.0)</td>
<td>0.6 (0.4-1.4)</td>
<td>0.7 (0.4-1.5)</td>
<td>0.5 (0.3-1.0)</td>
</tr>
<tr>
<td>Gut Th17 cells (%)</td>
<td>2.1 (1.1-3.1)</td>
<td>2.3 (1.2-4.5)</td>
<td>1.3 (0.9-2.4)</td>
<td>3.0 (1.3-4.4)</td>
</tr>
<tr>
<td>Abs gut Th17 cells (10^2 cells/g tissue)</td>
<td>4.0 (1.7-6.7)</td>
<td>1.7 (0.1-3.3)</td>
<td>0.9 (0.5-1.5)*</td>
<td>1.9 (1.2-5.8)</td>
</tr>
<tr>
<td>Blood Tregs (%)</td>
<td>1.0 (0.9-1.5)</td>
<td>1.8 (1.0-3.1)*</td>
<td>1.4 (0.7-2.3)</td>
<td>0.3 (0.1-0.7)</td>
</tr>
<tr>
<td>Gut Tregs (%)</td>
<td>2.4 (1.5-4.2)</td>
<td>4.8 (3.9-6.4)*</td>
<td>4.4 (2.3-6.6)</td>
<td>1.6 (0.8-3.3)</td>
</tr>
</tbody>
</table>

All comparisons are made to HIV(-) group; significance of P≤0.05 is indicated by *, and P≤0.001 is indicated by **.

Abbreviations: Abs, absolute number; Th17 cells were defined as IL-17a producing CD4 T cells and Treg cells were defined as CD4 T cells co-expressing FoxP3 and CD25; N/A, not applicable.

Values indicate median (IQR).
3.4.2 Reduced functional capacity of sigmoid Th17 cells during untreated HIV infection

The functional capacity of Th17 cells was assessed by the co-production of the pro-inflammatory cytokines IL-22, IFN-γ and/or TNF-α, as assessed through Boolean gating. First, we compared the function of Th17 cells from the sigmoid colon and blood in HIV-uninfected individuals. Gut Th17 cells had a much greater capacity to produce pro-inflammatory cytokines than did those from the blood (P=0.018; Figure 3.1a), with higher frequencies of triple cytokine-producing (P=0.006), and dual TNF-α and IL-22 coproducing Th17 cells (P=0.002; data not shown). However, pro-inflammatory cytokine production capacity by sigmoid Th17 cells was dramatically reduced during both the early and chronic stages of HIV infection (HIV-uninfected versus early, P<0.001 and HIV-uninfected versus chronic, P=0.019; Figure 3.1b). Although there was substantial heterogeneity within study groups (Figure 3.1c), the mean number of pro-inflammatory cytokines produced per Th17 cell was substantially reduced during both early and chronic HIV infection (HIV-uninfected, 1.21 cytokines; early HIV+, 0.70 cytokines; and chronic HIV+, 0.73 cytokines; Figure 3.1d). This reduction was primarily due to a decrease in triple and dual TNF-α and IL-22 cytokine-producing Th17 cells (Figure 3.1e). Sigmoid Th17 polyfunction was reduced by a similar amount during early and chronic HIV infection (Figure 3.1b, P=0.416 and Figure 3.1d, P=0.542). HIV infection was not associated with changes in the pro-inflammatory function of blood Th17 cells (data not shown). Although CD8+ T cells were also documented to produce IL-17a (called Tc17 cells)\textsuperscript{228,258}, a subset analysis including one HIV-uninfected control and one ART-treated individual demonstrated that sigmoid colonic CD4 T cells produced an abundance of IL-17a compared with CD8 T cells (median, CD4: 1.17\% vs. CD8: 0.15\%).
Figure 3.1 Polyfunctionality of mucosal Th17 cells, as measured by the co-expression of IL-22, IFN-γ, and/or TNF-α, was reduced in untreated HIV infection. (a) Sigmoid Th17 cells in HIV-uninfected individuals were much more polyfunctional than blood Th17 cells, but (b) this was profoundly reduced during all stages of untreated HIV infection. (c) Heatmap style plot demonstrate the vast heterogeneity within each study group, however (d) the mean number of sigmoid Th17 function per cell was reduced in early and chronic HIV infection. (e) Each possible combination of IL-22, IFN-γ, and TNF-α production by Th17 cells is shown; horizontal bars indicate the median and box plots depict the interquartile range. Th17 functional analysis was only available in a subset of study participants in each group and was based on cell availability.
3.4.3 Immunoregulatory skewing of mucosal Th17 function

Because the capacity of mucosal Th17 cells to produce pro-inflammatory cytokines was dramatically reduced during HIV infection, we next assessed production of the immunoregulatory cytokine IL-10. In HIV-uninfected participants, mucosal Th17 cells most commonly produced the pro-inflammatory cytokine TNF-α (median, 69.7%), whereas production of IL-10 was rare (0.3%); however, there was immunoregulatory Th17 skewing during untreated HIV infection (TNF-α median, 43.4% and IL-10 median, 3.5%; Figure 3.2a). Immunoregulatory skewing was calculated by the ratio of immunoregulatory (IL-10+) Th17 cells to pro-inflammatory (TNF-α+) Th17 cells, and was particularly increased during early HIV stages (P<0.001; Figure 3.2b). The IL-10/TNF-α Th17 ratio was comparable between HIV-uninfected group and the chronic HIV-infected or long-term ART treated group (P=0.676 and P=0.387 respectively), but with some interindividual heterogeneity. Mucosal IL-10+ Th17 cells did not co-express the Treg marker FOXP3+; the frequency of IL-10 production by gut Th17 cells or bulk CD4 T cells did not correlate with mucosal Tregs in ART-naïve individuals (Spearman rank coefficient, P=0.237, $r_s$ = -0.205 and P=0.255, $r_s$ = -0.195 respectively). Although IL-10+ sigmoid Th17 cells did not typically coproduce other proinflammatory cytokines, a small fraction coproduced IFN-γ and TNF-α (<5% for each).

We hypothesized that immunoregulatory mucosal Th17 skewing would be associated with reduced mucosal immune activation during untreated HIV infection, including both early and chronic stages. An increase in the mucosal Th17 immunoregulatory ratio was associated with reduced CD8+ T cell immune activation in both the gut (P=0.034, $r_s$ = -0.383) and blood (P=0.002, $r_s$ = -0.518; Figures 3.2c and 3.2d). Furthermore, an increased immunoregulatory Th17
ratio correlated with reduced plasma LPS levels ($P=0.002, r_s=-0.612$; Figure 3.2e) and a lower blood HIV VL ($P=0.026, r_s=-0.376$; Figure 3.2f). No associations were seen with plasma sCD14 levels ($P=0.538, r_s=-0.142$).

We also assessed IL-10 production by gut Th1 cells (IFN-$\gamma$+ CD4 T cells). Again, early HIV infection was associated with increased IL-10 by gut Th1 cells compared with the HIV-uninfected group (median, 5.43% vs. 2.15%, $P=0.001$) and chronic HIV infection (2.72%, $P=0.020$); however the level of IL-10 production by gut Th1 cells was not associated with blood ($P=0.121, r_s=-0.275$) or gut immune activation ($P=0.820, r_s=-0.042$), or with plasma LPS levels ($P=0.159, r_s=-0.297$) in ART-naïve individuals.
Figure 3.2 The ratio of immunoregulatory Th17 cells (IL-10+) and pro-inflammatory Th17 cells (TNF-α+) was increased in early HIV-infected individuals. (a) In HIV-uninfected controls, sigmoid Th17 cells commonly produced TNF-α while IL-10 production was scarce. However this was reversed in untreated HIV infection where IL-10 production by sigmoid Th17 cells was increased and TNF-α was reduced. Unstimulated media control of CD4 T cells was used for gating purposes. (b) Immunoregulatory skewing of sigmoid Th17 cells (calculated by the ratio of IL-10+ Th17 cells to TNF-α+ Th17 cells) was unique to early HIV infection. (c-d) IL-10/TNF-α Th17 ratio was associated with reduced gut and blood immune activation (HLA-DR and CD38 co-positive CD8 T cells), (e) with reduced plasma LPS levels, and with (f) reduced blood VL in all HIV-infected, ART-naïve individuals, including early and chronically infected individuals. Immune activation markers were available in N=22 early and N=9 chronic HIV-infected participants, and plasma LPS and sCD14 levels were available in N=12 early and N=11 chronic HIV-infected participants.
3.4.4 Cytokine production by bulk CD4 T cells in the gut mucosa

Given that HIV infection and immune activation were associated with the altered function of gut Th17 cells, we also evaluated the frequency and number of bulk CD4 T cells producing the cytokines IL-10, IFN-γ, TNF-α and IL-22 in the gut. Similar to gut Th17 cells, bulk CD4 T cells producing IL-10 were also elevated during early HIV infection, but not during chronic HIV infection (P<0.001 and P=0.477 respectively), whereas the absolute numbers remained unchanged compared to HIV-uninfected individuals (Figure 3.3a). The proportion of IFN-γ producing CD4 T cells, referred to as Th1 cells, was comparable between HIV-uninfected and HIV-infected individuals, but their absolute numbers were reduced during early HIV infection (P=0.001) and a similar trend followed during the chronic stage (P=0.088, Figure 3.3b). TNF-α producing CD4 T cells were reduced in proportions and numbers only during early HIV infection (P=0.039 for both comparisons, Figure 3.3c), whereas IL-22 producing CD4 T cell numbers and proportions were reduced in both early and chronic stages of HIV infection (Figure 3.3d).

Plasma LPS and sCD14 levels were not associated with the proportion of any of the cytokines produced by bulk gut CD4 T cells in ART-naïve individuals (LPS, P>0.214 and sCD14, P>0.443, data not shown), as well as blood immune activation (P>0.269). However, there was a trend of IL-10 producing CD4 T cells correlating with reduced blood immune activation (P=0.097, r_s=-0.294) and TNFα producing CD4 T cells were associated with increased gut immune activation (P=0.007, r_s=0.467).
Figure 3.3 Proportion and number of bulk gut CD4 T cells producing cytokines. (a) IL-10, (b) IFN-γ, (c) TNF-α, and (d) IL-22 production by CD4 T cells in HIV-uninfected, and early and chronic HIV-infected individuals.
3.4.5 **Independent association of systemic immune activation**

Systemic immune activation is a well-defined predictor of HIV disease progression and was elevated to comparable levels during early and chronic untreated HIV infection (data not shown). In our participants, systemic immune activation was associated with the blood VL and mucosal immune activation, and was inversely correlated with the blood CD4 count, the frequency of IL-10+ gut Th17 cells and the mucosal immunoregulatory Th17 ratio (Table 3.2). Therefore we performed a stepwise multivariate linear regression model to define the best clinical and immune variables that were independent predictors of systemic immune activation. In this model, only the blood CD4 count and the mucosal immunoregulatory Th17 ratio were independently associated with systemic immune activation (P=0.002 and P=0.004 respectively, Table 3.2).

**Table 3.2** Univariate and multivariate linear regression of variables correlated with systemic immune activation in untreated HIV-infected individuals.

<table>
<thead>
<tr>
<th></th>
<th>Univariate Linear Regression</th>
<th>Multivariate Stepwise Linear Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstandardized B Coefficient</td>
<td>95% CI</td>
</tr>
<tr>
<td><strong>Clinical parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>−1.243</td>
<td>−0.027 to 0.050</td>
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<tr>
<td>Blood CD4 count (/ml)</td>
<td>−0.002</td>
<td>−0.003 to −0.001</td>
</tr>
<tr>
<td>Blood viral load (c/ml)</td>
<td>4.54 × 10^−6</td>
<td>2.0 × 10^−6 to 7.0 × 10^−6</td>
</tr>
<tr>
<td>Plasma LPS (EU/ml)</td>
<td>0.829</td>
<td>−0.707 to 2.364</td>
</tr>
<tr>
<td>Plasma sCD14 (µg/ml)</td>
<td>−0.522</td>
<td>−1.772 to 0.729</td>
</tr>
<tr>
<td><strong>Mucosal parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-DR+CD38+ CD8 T cells (%)</td>
<td>0.025</td>
<td>0.008 to 0.042</td>
</tr>
<tr>
<td>Th17 cells (%)</td>
<td>0.039</td>
<td>−0.153 to 0.232</td>
</tr>
<tr>
<td>IL-10+Th17 cells (%)</td>
<td>−0.070</td>
<td>−0.121 to −0.019</td>
</tr>
<tr>
<td>TNF-α Th17 cells</td>
<td>0.003</td>
<td>−0.018 to 0.025</td>
</tr>
<tr>
<td>IL-22 Th17 cells</td>
<td>−0.001</td>
<td>−0.026 to 0.025</td>
</tr>
<tr>
<td>IFN-γ Th17 cells</td>
<td>−1.234</td>
<td>−0.069 to 0.026</td>
</tr>
<tr>
<td>IL-10/TNF-α Th17 cell ratio</td>
<td>−2.162</td>
<td>−3.646 to −0.678</td>
</tr>
</tbody>
</table>

*Indicates variables that were not independently associated with blood immune activation and were eliminated from the stepwise linear regression model. b indicates blank values.

1 Dependent variable (blood immune activation) was logit transformed.
3.4.6 Impact of long-term ART on mucosal Th17 number and function

In participants on long-term ART with complete gut CD4 reconstitution (P=0.183), blood immune activation and plasma LPS levels were comparable to HIV-uninfected controls (P=0.156 and P=0.188 respectively; data not shown). In addition, mucosal Th17 numbers (P=0.355), relative frequency (P=0.180), and function (P=0.111) did not differ from HIV-uninfected individuals (Figure 3.4a-3.4c). There was complete restoration of triple cytokine-producing Th17 cells in the sigmoid mucosa, and of dual IL-22 and TNF-α co-producing Th17 cells (data not shown). The production of TNF-α+ by mucosal Th17 cells exceeded that of HIV-uninfected controls (HIV-, 69.7% versus ART, 88.0%; P=0.002), however the immunoregulatory mucosal IL-10/TNFα Th17 ratio in the long-term ART group was similar to that in HIV-uninfected individuals (P=0.319). Long-term ART had similar cytokine production by bulk CD4 T cells in the gut (IL-10, IFN-γ, TNF-α and IL-22) compared with HIV-uninfected individuals (data not shown), with the exception of gut Th1 cell numbers being less than those of HIV-uninfected controls (HIV-, 0.293 versus ART, 0.104 cells/g tissue, P=0.042).

We next dichotomized the ART treated group based on the degree of gut CD4 reconstitution to determine whether effective CD4 restoration positively impacted the function of gut CD4 cells and HIV disease progression. High gut CD4 reconstitution was defined as ART-treated individuals with CD4 T cell frequency above the median (>48.8%). Although the level of CD4 reconstitution in the gut did not impact blood or gut immune activation (HLA-DR+ on CD8 T cells, P>0.563), sCD14 levels were greater in individuals with low CD4 reconstitution (low, 2.34 versus high 1.93 μg/ml; P=0.049) and a similar trend followed with plasma LPS levels (low, 1.47 versus high, 1.32 EU/ml; P=0.105). Those with high CD4 reconstitution on ART had similar proportion and number of gut Th17 cells (P=0.643 and P=0.418 respectively), and similar
Th17 polyfunction (P=0.682; Figure 3.4d). Furthermore, the level of gut CD4 reconstitution on ART did not impact the relative and absolute number of IL-22 (both P=0.248), IL-10 (P=0.431 and P=0.189 respectively), IFN-γ (Th1 cells; P=0.298 and P=0.165 respectively) or TNF-α (both P=0.115) production by bulk CD4 T cells in the gut mucosa.

Figure 3.4 Reconstitution of sigmoid Th17 cell number and their function after long-term ART. The (a) absolute and (b) relative frequency of sigmoid Th17 cells and (c) their polyfunctional capacity in long-term ART treated individuals were comparable to HIV-uninfected controls. (d) Sigmoid Th17 polyfunction after long-term ART was similar between those with high CD4 reconstitution and low CD4 reconstitution (based on gut CD4 reconstitution above or below the median value of 48.8% in ART group).
3.4.7 Impact of short-term ART on mucosal Th17 cells and immune activation

We then assessed these mucosal parameters in a subset of HIV-infected individuals (N=11) who initiated ART during chronic (N=5) and early (N=6) stages of HIV infection, with repeat sampling after a median of 12 months. The blood VL became undetectable at a median of 4 months (range, 1-6 months) after ART initiation. Gut and blood CD4 T cell frequencies increased after short-term ART (paired analysis: gut, P=0.003 and blood, P=0.006), although the CD4 T cell frequency remained below the level of HIV-uninfected controls at both sites (gut, 39% versus 58% P=0.009 and blood, 36% versus 62% P=0.030).

During untreated chronic HIV infection, plasma LPS levels were elevated and sigmoid Th17 cell function and numbers were lost. After short-term ART, both the frequency and the number of gut Th17 cells were reconstituted (paired analysis, both P=0.043; Figure 3.5a and 3.5b); interestingly, the frequency of gut Th17 cells exceeded that of HIV-uninfected individuals (P=0.020), whereas their numbers were comparable (P=0.549). Their functional capacity was not altered (P=0.898); this remained lower than both the HIV-uninfected group (P=0.049) and long-term ART group (P=0.002; Figure 3.5c). In addition, the IL-10/TNF-α Th17 ratio was not altered by short-term ART (P=0.815, data not shown). Although there was a reduction in systemic immune activation (paired analysis, P=0.043, Figure 3.5d and 3.5e), as well as a trend toward reduced mucosal immune activation (P=0.080, Figure 3.5e) after ART initiation, gut immune activation remained significantly elevated compared with HIV-uninfected participants (30.9% versus 14.4%, P=0.009) and a similar trend followed in the blood (10.3% versus 2.6%, P=0.076). Neither plasma LPS nor sCD14 levels changed after ART initiation (paired analysis, P=0.175 and P=0.313 respectively; data not shown).
Because ART treatment during the chronic stage of HIV infection did not reconstitute gut Th17 cell function or immune activation in the short-term, we next explored the impact of initiating ART during the early stages of HIV infection. Early initiation of ART reduced immune activation in the blood and gut (paired analysis, both P=0.028), however they still remained higher than in HIV-uninfected individuals in the blood (13.3% versus 2.6%, P=0.045) and a similar trend followed in the gut (31.0% versus 14.4%, P=0.100). Moreover, short-term ART during early infection did not enhance the functionality of gut Th17 cells (P=0.391, data not shown).
Figure 3.5 Numerical and functional profile of sigmoid Th17 cells and level of immune activation soon after initiating ART during chronic stage (N=5) of HIV infection. Short-term ART initiated during chronic HIV infection normalized (a) the relative and (b) absolute number of sigmoid Th17 cells, (c) however, their polyfunctional capacity was not different than their respective ART-naïve baseline time points. (d) Blood immune activation was reduced in all individuals soon after ART initiation, and (e) gut immune activation was reduced in 4 of the 5 individuals but remained greater than HIV-uninfected controls. #, indicate paired analysis.
3.5 Discussion

Progressive HIV infection is characterized by a preferential loss of mucosal Th17 cells, a CD4 T cell subset that plays an important role in mucosal defense against potentially pathogenic microbes from the gut lumen\textsuperscript{115,148,150}. In the absence of HIV, mucosal Th17 cells produce several cytokines with a range of effector functions\textsuperscript{252,259}. Because HIV infection is associated with a dramatic reduction in the polyfunctional capacity of HIV-specific CD8+ T cells\textsuperscript{260,261}, we examined the effector functions of mucosal Th17 cells and their relationship with HIV-associated microbial translocation and immune activation. In the absence of HIV, Th17 cells in the gut mucosa expressed a much wider array of functions than did those from blood. However, this polyfunction was dramatically reduced from the very earliest stages of HIV infection (<7 months of infection), even before the mucosal Th17 number had been significantly reduced. Although ART quickly increased Th17 numbers, restoration of their polyfunctional capacity was only apparent after a prolonged period of treatment. Interestingly, among ART-naive participants, there also was skewing of gut Th17 cells towards an immunoregulatory phenotype (increased IL-10/TNF-\(\alpha\) ratio), which was only seen during the early stages of HIV infection. Moreover, in a linear regression model that included key mucosal and clinical parameters, only the gut immunoregulatory Th17 ratio and CD4 count were independent predictors of systemic immune activation. Therefore, the mucosal immunoregulatory skewing of Th17 cells may play an important role in maintaining the mucosal barrier during HIV infection.

In addition to IL-17a, Th17 cells produce several other effector cytokines: TNF-\(\alpha\) and IFN-\(\gamma\) recruit neutrophils to the site of infection\textsuperscript{239}, and IL-22 promotes epithelial renewal and enhances the expression of antimicrobial peptides by epithelial cells\textsuperscript{237,238}. Our results suggest that the HIV-associated loss of Th17 cell effector functions in the gut mucosa may be causally
related to the increased translocation and systemic dissemination of luminal bacteria\textsuperscript{150}. However, increased systemic microbial translocation (i.e. plasma LPS levels) was only apparent during chronic HIV infection, whereas a dramatic impairment of Th17 function was apparent very early after HIV acquisition. We hypothesize that this delayed impact of Th17 functional impairment on microbial translocation may be because a loss of epithelial integrity is also needed for microbial translocation to occur, and this integrity is only compromised later in HIV infection, coincident with the loss of mucosal IL-22 production capacity\textsuperscript{140,236}.

Although the polyfunctional capacity of mucosal Th17 cells was reduced during untreated HIV infection, this was not directly associated with microbial translocation or systemic immune activation. Instead, the only mucosal immune parameter that was independently associated with systemic immune activation in our study was the immunoregulatory skewing of Th17 cells (increased IL-10/TNF-\(\alpha\) ratio). The latter was most apparent during the early stages of HIV infection, and was also inversely correlated with mucosal immune activation, microbial translocation, and the blood VL. HIV infection was associated previously with an increase in IL-10 production by several cell subsets, but their role in HIV disease progression or protection has not been delineated\textsuperscript{262-265}, and the impact of IL-10 producing mucosal Th17 cells on HIV pathogenesis has not been explored. Our data suggest that although retaining the overall polyfunction of mucosal Th17 cells may be important, maintaining a fine balance between the pro-inflammatory and immunoregulatory functions of mucosal Th17 cells may even be more critical.

However, this is a cross-sectional study and the direction of causality is unclear. Immunoregulatory skewing of gut Th17 cells may either be protective against, or the result of, mucosal microbial translocation and inflammation. Bacteria such as \textit{S. aureus} were shown to
induce human blood Th17 cells that co-produce IL-10\textsuperscript{252}, and so the immunoregulatory Th17 phenotype might simply reflect the colocalization of translocated luminal bacteria. Thus, Th17 cells within the mucosa may have little impact on epithelial integrity. In contrast, the self-regulating expression of IL-10 by Th17 cells was important in preventing Th17-induced colitis in a murine model\textsuperscript{245}; therefore, these cells might be playing a direct role in the reduced levels of mucosal and systemic inflammation that we observed in individuals with skewed gut regulatory Th17 cells. IL-10 deriving from Th17 cells in the gut mucosa may function synergistically with other Th17-associated cytokines to fine tune and balance the pro-inflammatory capacity of Th17 cells; however further studies, likely in animal models, are needed to determine the direction of causality.

Our study cannot define the mechanism behind the loss of gut Th17 cell polyfunction. It is possible that polyfunctional Th17 cells are more susceptible to HIV infection, which may impact their cell number or functionality\textsuperscript{148}; thus slow HIV clearance in the gut mucosa after ART initiation may hinder and delay functional reconstitution of Th17 cells. Moreover, Th17 development and/or recruitment may be affected by local or systemic factors during HIV infection. This will be an important area for future research. In addition, our assays measured a limited repertoire of Th17 functions, specifically the production of cytokines IL-17a, IL-22, IL-10, TNF\textgreek{a} and IFN-\gamma and were unable to assess the functionality of IL-17a producing CD8 T (Tc17 cells). It is likely that HIV is also associated with alterations in the production of other cytokines or the performance of additional Th17 functions. Future studies will need to address this, as well as the precise timing of Th17 functional restoration in relation to ART, which our study can only narrow to somewhere between 1 and 13 years.
In summary, we show that HIV infection causes dramatic alterations in the functional capacity of mucosal Th17 cells. These alterations occur even earlier in the HIV disease course than do the previously described reductions in Th17 number, and are less readily reversed after the initiation of effective ART. Their association with mucosal and systemic immune activation and microbial translocation suggests that strategies to preserve, or to more rapidly restore, mucosal Th17 function will have important therapeutic benefit.
CHAPTER 4

IMPACT OF ANTIRETROVIRAL THERAPY IN HIV-INFECTED ELITE CONTROLLERS; GUT IMMUNOLOGY, MICROBIAL TRANSLOCATION AND SERIOUS NON-AIDS BIOMARKERS

Unpublished work (submitted).
4.1 Abstract

Serious non-AIDS (SNA) conditions are increased in HIV-infected individuals, and are associated with microbial translocation and elevated plasma levels of inflammatory cytokine IL-6 and coagulation biomarker D-dimer. Despite undetectable viremia, HIV-infected elite controllers (ECs) are at increased risk of SNA conditions. We examined the impact of short-term antiretroviral therapy (ART) on gut immunology and SNA biomarkers. Blood and sigmoid biopsies were collected at baseline, 6 months after ART initiation and 3 months after ART discontinuation in four ECs, and as well as HIV-uninfected and chronically HIV-infected participants. CD4 T cell subsets (regulatory, Th1, Th17 and Th22 cells), Th17 cell functionality and CD8 T cell were assessed, as well as plasma markers of microbial translocation and SNA biomarkers. Prior to ART, ECs and HIV-uninfected controls had similar proportions of gut CD4 T cells, regulatory T cells, Th1 cells and Th22 cells and activated CD8+ T cells; polyfunctional gut Th17 cells were actually increased and plasma markers of microbial translocation were comparable. However plasma levels of IL-6 and D-dimer were elevated despite ART compared to HIV-uninfected individuals. EC demonstrated no evidence of gut immune dysfunction or increased microbial translocation, but plasma IL-6 and D-dimer levels were elevated despite ART. This suggests an independent mechanism for SNA pathogenesis in ECs.
4.2 Introduction

Serious non-AIDS (SNA) conditions are increased in HIV-infected individuals despite antiretroviral therapy (ART), and are important contributors to morbidity and mortality\cite{210,266}. These SNA conditions include cardiovascular, hepatic, psychiatric and renal diseases\cite{267,268} that are associated with chronic inflammation and abnormal coagulation\cite{269}. A recent study combining data from three large cohorts of ART-experienced HIV-infected individuals showed that plasma levels of pro-inflammatory cytokine IL-6 and coagulation biomarker D-dimer were the best predictors of subsequent SNA events and death\cite{139}.

Gut microbial translocation is a hallmark of progressive HIV infection\cite{114}. Circulating microbial products are potent triggers of the innate immune system and a major driver of chronic inflammation\cite{116,130}. During progressive HIV infection, mucosal CD4 T cell depletion and impairment of the gut epithelial barrier are simultaneous processes that allow for microbial translocation\cite{140,143,148,236}. Gut CD4 T cells that produce the cytokines IL-22 (Th22 cells) and IL-17a (Th17 cells) normally maintain epithelial integrity and provide mucosal defense against invading pathogens\cite{238}, but are preferentially depleted soon after HIV infection\cite{115,148,236,270,271}. The functional capacity of gut Th17 cells assessed by the coproduction of inflammatory cytokines determine the ability of Th17 cells to protect the gut mucosa from microbial pathogens; however this is drastically reduced very early in HIV infection\cite{270}. Furthermore, the level of immunoregulatory gut Th17 cells that produce the anti-inflammatory cytokine IL-10 during HIV infection is associated with reduced systemic immune activation and decreased microbial translocation; therefore gut immunoregulatory Th17 cells may have tissue protective roles during HIV infection\cite{270}.
Elite controllers (ECs) are a subset of HIV-infected individuals who maintain undetectable blood viral load in the absence of ART\textsuperscript{43}, but they are also at an increased risk of SNA conditions\textsuperscript{272}. One important contributor to chronic inflammation in ECs may be microbial translocation and gut immune damage\textsuperscript{116}. Increased plasma levels of microbial product lipopolysaccharide (LPS) and macrophage/monocyte activation marker soluble CD14 (sCD14), as well as increased plasma levels IL-6 and D-dimer have also been implicated in SNA development in ECs\textsuperscript{116,139,269,273,274}. Low-level HIV replication is also seen in ECs and may contribute to SNA\textsuperscript{275,276}. ECs have abnormal gut lymphoid structures with increased collagen deposits and fibrosis, both which indicate chronic immune activation and end-organ damage\textsuperscript{277,278}. Although gut CD4 T cell and Th17 cell numbers may be preserved in ECs\textsuperscript{257}, other CD4 subsets and the function of Th17 cells have not been described. We investigated the impact of short-term ART on SNA biomarkers, gut immunology and microbial translocation in ECs.
4.3 Methods

4.3.1 Participants

Four ECs were recruited based on their ability to suppress blood viremia below 50 copies/ml in the absence of ART over the preceding 5 years from the Maple Leaf Medical Clinic (Toronto, Ontario). Standardized ART regimen of tenofovir/emtricitabine and raltegravir was administered for 9 months; blood and sigmoid biopsies were collected at baseline, 6 months after ART initiation, and 3 months after ART discontinuation. HIV-uninfected and ART-naïve chronically HIV-infected participants with limited clinical information were recruited for baseline comparisons. All participants provided written informed consent, and the University of Toronto and St. Michael’s Hospital Research Ethics Boards approved the study protocol.

4.3.2 Tissue and blood cell isolation

Venipuncture blood was collected into Acid Citrate Dextran solution A (BD Biosciences), and peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density centrifugation. Sigmoid biopsies were collected and processed as previously described\textsuperscript{270}. Briefly, biopsies were collected about 25 cm from the anal verge and immediately placed into RPMI solution (RPMI 1640 containing 100 U/ml penicillin, 100ug/ml streptomycin and 1X GlutaMAX-1; Invitrogen). Pre-weighed sigmoid biopsies were processed by two sequential Collagenase type II (Sigma-Aldrich) digestions for 30 min each on a shaking heated block at 37°C, passed through a 100 µm strainer, and counted.
4.3.3 **Flow cytometry**

Isolated cells from blood and sigmoid colon were stimulated with PMA (1 ng/ml) and ionomycin (1 µM/ml; Sigma) or with media alone for 6 h at 37°C, with brefeldin A (1 µm/ml) for the last 5 h in RPMI 1640 solution supplemented with 10% FBS. Cells were washed, permeabilized and stained with fluorochrome-labeled antibodies, aqua LIVE/DEAD viability dye (Invitrogen), and fixed (CD3, CD4, CD8, HLA-DR, CD38, IL-17a, IL-22, IFNγ, IL-10 and TNFα; BD BioSciences, eBioscience and Beckman Coulter). Cells were acquired on FACSCanto II (BD Systems) and analyzed using FlowJo v9.0.2 (Treestar). Dead cells and doublets were excluded from analysis and positive responses were background corrected where applicable. Th17 polyfunctionality analyses and data graphing were performed using SPICE software v5.22 (NIAID).

4.3.4 **Plasma markers of microbial translocation, inflammation and coagulation**

Limulus amebocyte lysate assay kit (Cambrex) was used to detect plasma LPS levels and a commercially available ELISA kits were used for sCD14 (R&D Systems), CRP (R&D Systems) and D-dimer (Imuclone). Custom designed 7-plex ELISAs (Meso Scale Discovery) were used for cytokine and chemokine detection.
4.3.5 **Statistical analysis**

The Mann-Whitney *U* test and Wilcoxon signed-ranked test were performed using IBM SPSS Statistics 20.0 for Mac (SPSS), and Th17 polyfunctionality analysis was performed using SPICE Student *t* test (v5.22). *P* values <0.05 were considered significant.
4.4 Results

4.4.1 Participant characteristics

The median duration of HIV infection in ECs was 18.5 years (range: 5-23 years) and the median duration of undetectable plasma viremia was 9 years (range: 5-14, data was unavailable for one participant). All ECs had normal levels of CD4 count at baseline (median, 605/µl; range, 490-710/µl) and all participants had plasma viremia below 20 copies/ml with the exception of one EC who had a viral blip of 88 copies/ml. All participants maintained normal CD4 counts and undetectable viremia throughout the duration of the study (data not shown).
Table 4.1 Baseline levels of cellular and soluble markers of microbial translocation, coagulation and inflammation in blood

<table>
<thead>
<tr>
<th></th>
<th>HIV-</th>
<th>HIV+</th>
<th>EC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median values (range)</td>
<td>Sample size</td>
<td>variation was dependent on sample availability (HIV- and HIV+, N=6-12; EC, N=4)</td>
</tr>
<tr>
<td>HLA-DR+ CD38+ on CD8 T cells, %</td>
<td>2.55 (1.5-6.2) N=7</td>
<td>24.4 (17.9-45.8)* N=10</td>
<td>6.0 (1.6-9.6)# N=4</td>
</tr>
<tr>
<td>LPS, EU/ml</td>
<td>1.23 (1.19-1.41) N=8</td>
<td>1.55 (1.36-1.97)* N=12</td>
<td>1.26 (0.89-1.61) N=4</td>
</tr>
<tr>
<td>sCD14, μg/ml</td>
<td>1.48 (1.22-1.72) N=8</td>
<td>2.06 (1.82-2.37)* N=12</td>
<td>1.46 (0.94-1.80)# N=4</td>
</tr>
<tr>
<td>D-dimer, ng/ml</td>
<td>98.84 (55.42-220.90), N=11</td>
<td>166.2 (55.42-889.4)* N=10</td>
<td>146.3 (115.1-225.8)* N=4</td>
</tr>
<tr>
<td>CRP, mg/ml</td>
<td>501.2 (369.4-6104) N=6</td>
<td>1621.0 (348.7-8483) N=6</td>
<td>821.3 (347.5-1245) N=4</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>1.05 (0.62-2.39) N=8</td>
<td>1.86 (0.92-7.56) N=12</td>
<td>1.93 (1.55-5.88)* N=4</td>
</tr>
<tr>
<td>IL-17a, pg/ml</td>
<td>1.47 (0.95-2.62) N=8</td>
<td>2.09 (0.82-10.76) N=12</td>
<td>1.28 (0.73-1.51)# N=4</td>
</tr>
<tr>
<td>MIP-1β, pg/ml</td>
<td>52.02 (19.63-224.2) N=8</td>
<td>34.22 (24.81-54.28)* N=12</td>
<td>49.66 (30.87-82.51) N=4</td>
</tr>
<tr>
<td>IP-10, pg/ml</td>
<td>129.1 (83.36-316.8) N=8</td>
<td>295.5 (154.7-229.2)* N=12</td>
<td>160.3 (50.16-215.1)# N=4</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>0.92 (0.46-65.37) N=8</td>
<td>1.68 (0.85-5.55)* N=12</td>
<td>0.74 (0.44-1.59)# N=4</td>
</tr>
<tr>
<td>IL-10, pg/ml</td>
<td>0.65 (0.35-1.40) N=8</td>
<td>1.53 (0.79-3.38)* N=12</td>
<td>0.52 (0.36-0.82)# N=4</td>
</tr>
</tbody>
</table>

Sample size variation was dependent on sample availability (HIV- and HIV+, N=6-12; EC, N=4)

*, P<0.05 EC and HIV+ compared to HIV-

#, P<0.05 EC compared to HIV+
4.4.2 **Cellular and soluble markers of microbial translocation and SNA biomarkers in blood**

Blood levels of immune activation (CD38+HLA-DR+ on CD8 T cells), and plasma markers of microbial translocation (LPS), monocyte activation (sCD14), inflammation (IL-6, IL-17a, MIP-1b, IP-10, TNF-a and IL-10) and coagulation (CRP and D-dimer) were measured and compared to HIV-uninfected and chronic HIV-infected individuals (Table 4.1). CD8 T cell activation, plasma levels of microbial translocation (LPS and sCD14), plasma inflammatory markers TNF-a, MIP-1b, IL-17a, IP-10 and IL-10, and CRP were similar between EC and HIV-uninfected controls. However, plasma levels of IL-6 and D-dimer were elevated in ECs (IL-6 median values: HIV-, 1.05 pg/ml and EC, 1.93 pg/ml and D-dimer values: HIV-, 98.84 ng/ml and EC, 146.3 ng/ml; Table 4.1).

4.4.3 **Baseline gut immunology**

We next analyzed the number and function of gut CD4 T cell subsets in ECs. The proportion of bulk CD4 T cells, regulatory T cells (FOXP3+CD25+ CD4 T cells), Th1 cells (IFNγ+ CD4 T cells) and Th22 cells (IL-22+IFNγ-IL-17a- CD4 T cells) were similar in HIV-uninfected controls and ECs (P=0.120, P=0.258, P=0.203, and P=0.888 respectively). However, the frequency of Th17 cells was increased in ECs (P=0.045; Figure 4.1a). The function of gut Th17 cells was assessed, based on their ability to produce the pro-inflammatory cytokines TNFα, IL-22 and IFNγ, and the regulatory cytokine IL-10. Immunoregulatory skewing of gut Th17 cells, defined as increased IL-10 production, was increased in ECs compared to uninfected controls (P=0.030; Figure 4.1b). ECs also maintained high levels of polyfunctional gut Th17
cells, comparable to HIV-uninfected individuals (P=0.946), with similar IL-22, TNFα and/or IFNγ production (Figure 4.1c and 4.1d).
Figure 4.1 Gut Th17 cells in ECs, HIV-uninfected controls and chronically infected individuals. (a) Frequency of Th17 cells and (b) IL-10 producing Th17 cells in the gut were increased in ECs compared to HIV-uninfected individuals. (c) Polyfunctional capacity of gut Th17 cells was similar to the HIV-uninfected controls and higher than chronically HIV-infected participants. (d) Each combination of IL-22, IFNγ and TNFα production by Th17 cells is shown; horizontal lines indicate the median and box plots depict the interquartile range.
4.4.4 Short-term ART and gut Th17 cells

We next evaluated the impact of ART on gut Th17 cells and their function. Th17 frequency and polyfunctionality were unchanged after 6 months of ART (P=0.255, Figure 4.2a), but discontinuation of ART unexpectedly reduced their functional capacity compared to baseline (P=0.021); a similar trend was observed compared to 6 months post-ART (P=0.072; Figure 4.2a). Th17 polyfunctional analysis on individual participants across three sampling time points demonstrated the reduction in the frequency of triple cytokine producing Th17 cells, while an increase in IL-17a only producing cells. Immunoregulatory skewing by gut Th17 cells (IL-10+ Th17 cells) was further increased after ART initiation in all study participants (Figure 4.2b), and decreased after ART cessation in three participants.
Figure 4.2  Impact of short-term ART on gut immunology. (a) Sigmoid Th17 polyfunctionality was reduced after 3 months of ART discontinuation, whereas (b) IL-10 producing Th17 cells were increased after ART initiation. Grey box indicates period of ART administration and dark lines show the median values.
4.4.5 **Plasma levels of IL-6 and D-dimer after ART**

ECs had increased levels of IL-6 and D-dimer compared to HIV-uninfected controls at baseline, and we hypothesized that ART initiation would reduce these biomarkers. However, neither ART initiation nor discontinuation in ECs had any significant impact on IL-6 and D-dimer plasma levels (Figure 4.3a and 4.3b).

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**Figure 4.3 Impact of short-term ART on SNA biomarkers.** Plasma levels of (a) D-dimer and (b) IL-6 were not reduced after ART initiation and discontinuation. Grey box indicates period of ART administration and dark lines show the median values.
4.5 Discussion

SNA conditions are a cause of mortality and morbidity in HIV-infected individuals and are elevated despite effective ART\textsuperscript{279}. Microbial translocation from the gut lumen and mucosal immune deficiencies are a major cause of chronic inflammation in progressive HIV infection. HIV-infected ECs who maintain an undetectable blood viral load in the absence of ART are also at increased risk of SNA conditions and death, and have increased levels of SNA biomarkers (IL-6 and D-dimer)\textsuperscript{272,274}. In this regard, ART in ECs have been implicated to reduce SNA events and mortality\textsuperscript{139,267,273,274}. Since gut immune deficiency and microbial translocation are major causes of inflammation in HIV-infected individuals, we investigated the impact of short-term ART on gut immunology, microbial translocation and SNA biomarkers in ECs. ECs had normal proportions of gut CD4 T cells, regulatory T cells, Th1 cells and Th22 cells, and increased proportion of polyfunctional Th17 cells. Despite normal gut immunology and no evidence of microbial translocation in ECs, SNA biomarkers were elevated at baseline, and remained so after short-term ART. This implies that increased SNA events in ECs may be independent of gut microbial translocation.

Since microbial translocation is a major cause of immune activation in HIV infection\textsuperscript{116} and elevated in ECs\textsuperscript{116,273}, we hypothesized that microbial translocation and gut immune defects may contribute to SNA events in ECs. However, our cohort of ECs had elevated levels of SNA biomarkers despite normal gut immunology and no evidence of microbial translocation. It is possible that low-level viral replication in ECs may contribute to inflammation and SNA events, however short-term ART in these ECs diminished low-levels of viral replication while SNA biomarkers remained elevated\textsuperscript{275,276}. Therefore, these data suggest that SNA conditions in ECs may be an independent of microbial translocation and low-level viral replication. Although ECs
had a higher number of highly polyfunctional gut Th17 cells at baseline, we unexpectedly observed a reduction in Th17 function after 3 months of ART discontinuation. The reason for this is unknown, however another study involving these participants demonstrated a reduction in HIV-specific CD8 T cell response even after ART discontinuation²⁷⁵.

ART in ECs have been previously studied and there has been clinical and immunological benefit with increased CD4 counts²⁸⁰, reduced viral replication²⁷⁵,²⁷⁶ and decreased immune activation²⁷⁵,²⁷⁶,²⁸¹. While these studies suggest benefit to ECs, our cohort of ECs had normal CD4 counts and similar levels of CD8 T cell activation compared to HIV-uninfected participants. Although our study shows that ECs have persistently elevated SNA biomarkers, our sample size was limited and the duration of ART was relatively brief. Therefore a larger study with longer duration of ART is required to confirm these findings.
CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS
5.1 Discussion

Of the 34 million people infected with HIV world wide, 8 million people have access to life saving ART\textsuperscript{282}, yet this population still experiences higher morbidity and mortality than their age-matched HIV-uninfected peers\textsuperscript{201,283}. While ART rapidly normalizes blood CD4 counts and reduces blood viremia to undetectable levels, gut HIV reservoirs persist and mucosal CD4 T cell reconstitution is slow and incomplete\textsuperscript{182,192}. The aim of my doctoral work was to define the role of gut mucosal immunology in HIV immunopathogenesis, with the overarching goal of developing novel therapeutic approaches to improve the quality and duration of life of the HIV-infected population.

Microbial translocation is an important cause of systemic immune activation\textsuperscript{114}. It is a consequence of two simultaneous events in the gut, namely CD4 depletion and mucosal barrier impairment. These mucosal changes are slow to normalize after ART, and I therefore became interested in understanding how structural and immune changes in the gut mucosa led to microbial translocation in the context of HIV infection and ART. In chronic HIV infection, all IL-22 producing gut lymphocytes were profoundly depleted and this was associated with reduced epithelial integrity and microbial translocation. Interestingly, there was increased IL-22 production by non-T cells during early HIV-infection and this was associated with preserved epithelial integrity and normal levels of microbial translocation. In a validated \textit{in vitro} model of HIV-induced epithelial damage, IL-22 prevented HIV and TNF-\textalpha-induced epithelial damage, confirming their tissue protective capacity. Sigmoid Th17 cells too were lost during chronic HIV infection, however their proinflammatory polyfunctional capacity was greatly reduced from the earliest stages. Although ART initiation restored gut Th17 numbers rapidly, their functional reconstitution and resolution of microbial translocation was much delayed. Since gut immune
Deficiencies are a major cause of systemic inflammation and SNA events, we next studied the impact of ART on ECs. ART-naïve ECs had normal levels of gut CD4 T cells, microbial translocation and immune activation, however soluble biomarkers of SNA (IL-6 and D-dimer) were elevated and a trial of short-term ART did not normalize them.

These studies, while novel, had several limitations. Our studies were primarily observational, and so it was difficult to provide mechanistic insights or determine the direction of causality. Moreover, we were unable to determine the root cause of gut Th17 and Th22 cell depletion during HIV infection, although data suggests that these cells may be preferential targets of HIV\textsuperscript{148,284} and/or their development may be halted due to a depletion in DCs that differentiate Th17 and Th22 cells\textsuperscript{271}. Despite these shortcomings, the studies presented in this dissertation suggest that enhancement of mucosal IL-22 and IL-17a levels and/or function during HIV infection could be a novel area for therapeutic intervention. A therapeutic intervention should selectively increase IL-22 and IL-17a to biologically relevant concentrations that are restricted to IL-22 and IL-17a deficient sites; excessive levels of these pro-inflammatory cytokines may damage mucosal tissues or cause systemic inflammation\textsuperscript{285-287}.

EC are an unusual group of HIV-infected individuals who spontaneously control viral replication to undetectable levels, but markers of microbial translocation and SNA biomarkers (IL-6 and D-dimer) may be elevated\textsuperscript{273,288}. We hypothesized that short-term ART may reduce SNA biomarkers and microbial translocation. Although ECs in this cohort had normal gut immunology and normal levels of microbial translocation, IL-6 and D-dimer levels were elevated, and short-term ART did not reduce these biomarkers. It is possible that low levels of viral replication in ECs may contribute to increased SNA biomarkers, however two studies
showed that ART-initiation in ECs diminished viral replication entirely, suggesting independence of these phenomenon.

5.2 Future directions

My doctoral work primarily focused on defining gut immunology and its relationship to HIV immunopathogenesis. These findings provide new information that may guide the development of novel therapeutics for HIV-infected individuals. One possible avenue is by manipulating the gut microbial environment to enhance gut immune reconstitution in HIV-infected individuals. Furthermore, it will be important to build on the current knowledge of gut immunology as it pertains to HIV disease pathogenesis and therapy.

5.2.1 Microbial manipulation to enhance gut immunology

The advent of combination ART has dramatically reduced mortality and SNA events in HIV-infected individuals, but they have greater adverse health outcomes. My doctoral work, along with others, demonstrated that one major area of ART limitation is the slow and incomplete reconstitution of gut immunology. This results in persistent microbial translocation, ongoing immune activation, and increased mortality and comorbidity. There is growing evidence that the gut microbiota influences the development of mucosal immune cells and vice versa. For example, segmented filamentous bacteria (SFB) regulated CD4 T cell development in the gut, particularly mucosal Th17 cells and Th22 cells in mice, and a carefully selected mixture of Clostridia bacteria strains induced the development of colonic Tregs. Therefore, the bacteria present in the gut lumen that interact with epithelial and mucosal immune cells may be critical in
developing normal gut immunology. This suggests that manipulating the gut microbiota may accelerate the normalization of gut immune cells, such as Th17 and Th22 cells in HIV-infected individuals.

![Schematic diagram of gut dysbiosis and immunopathogenesis in HIV infection.](image)

**Figure 5.1** Schematic diagram of gut dysbiosis and immunopathogenesis in HIV infection. © Connie J Kim.

The gut microbiome of an HIV-infected individual is different to that of the uninfected population. At the phylum level, HIV infected individuals have a reduced abundance of Bacteroidetes and Firmicutes and an increased abundance of Proteobacteria (Figure 5.1)[290,291]. Prospective fecal sampling of chimpanzees pre- and post-SIV infection demonstrated that SIV accelerated the rate of change in the gut microbial composition with higher frequencies of
pathogen containing bacterial genera\textsuperscript{292}. However there is no bacterial species that is consistently associated with HIV infection and this may be a reflection of varied sampling site and the microbiome analysis technique. The most common and easily obtainable samples for gut microbiome analysis include fecal samples and anal swabs, while tissue biopsies are cumbersome to collect. Fecal samples and anal swabs can be self-collected and provide general information about the passing luminal microbiota, but may not provide adequate information about the microbiota that adhere to or are intimately associated with the epithelium. Bacteria adhered to the mucosa may have greater mucosal immune impact compared to the passing microbiota. In two separate SIV studies, the fecal microbiome of SIV-infected macaques was similar to that of uninfected animals, and ART had minimal impact\textsuperscript{293,294}. \textit{Lactobacillales} levels from anal swabs of HIV-infected individuals correlated with reduced microbial translocation, but HIV-uninfected controls were not compared in this study\textsuperscript{295}. However, dysbiosis of the gut microbiota obtained from rectosigmoid tissues during HIV infection was associated with loss of Th17 cells and increased inflammation\textsuperscript{290}. Therefore, sampling location and analytical methodology in microbiome analysis will be important, and bacteria that are most closely associated with the mucosal tissue and immune cells may bear more importance than the passing microbiome.

Since the gut microbiome can profoundly alter the gut immune system, manipulating the gut microbiome may be a novel area of immunotherapeutics. Through diet, lifestyles or intake of natural health products such as probiotics and prebiotics, the gut immune system may be restored more rapidly in HIV-infected individuals\textsuperscript{296,297}. The use of complementary and alternative therapies is common among people living with HIV, with estimates of use ranging from 16-60\%\textsuperscript{298,299}. Probiotics are live bacteria that may confer health benefits to the host, and prebiotics are non-digestible foodstuffs that stimulate the growth of beneficial gut bacteria; when combined
together they are known as synbiotics\textsuperscript{300}. There is growing evidence that the use of probiotics, prebiotics and/or synbiotics may reduce gut inflammation and improve gut health in several randomized clinical trials including patients with ulcerative colitis and pouchitis\textsuperscript{300-302}. In a recent study, synbiotic administration of ART with 5 months in SIV-infected pigtail macaques improved mucosal Th17 function, increased gut CD4 numbers and reduced fibrosis in lymphoid tissues, although systemic inflammation or microbial translocation markers were not altered\textsuperscript{293}. The gut microbiome of humans differs drastically from that of nonhuman primates\textsuperscript{294} and a proof-of-concept is required before a controlled human study can be justified. Therefore I hypothesize that the co-administration of synbiotics for 1-year in HIV-infected individuals starting ART will accelerate gut mucosal immune reconstitution and reduce biomarkers of microbial translocation and inflammation.

To begin to determine the therapeutic effects of synbiotics in the HIV context, I propose to use the probiotic VSL\#3 and prebiotic inulin, in conjunction with conventional ART, to reduce HIV-associated inflammation. VSL\#3 is one of the highest potency probiotic products, containing 450 billion live bacteria per sachet, and inulin is one of two prebiotic products that meet the strict definition of a prebiotic – a selectively fermented ingredient that allows for the change in composition and/or activity in the gut microflora that confers benefits the host\textsuperscript{303}. In a SIV study applying a similar synbiotic blend, Klatt et al. only observed mucosal immunological benefits when synbiotics were co-administered with ART for 5 months\textsuperscript{293}. It is plausible that a longer duration of synbiotic administration with ART may have reduced systemic inflammation and microbial translocation. In a double-blind placebo controlled randomized pilot trial (1:1), I hope to investigate the ability of synbiotics (VSL\#3 and inulin) to reduce immune activation in HIV-infected men starting ART in a 1-year prospective study. The primary objective of the
study would be to elucidate the differences in CD8 T cell immune activation (HLA-DR and CD38 co-expression) at year one in the placebo versus synbiotic arm. The secondary objectives would include a similar analysis in the following parameters: i) plasma markers of microbial translocation, including LPS and sCD14; ii) frequency of blood and gut CD4 T cells; iii) gut immune cell subset numbers and function, including Th1, Tregs, Th17 and Th22 cells, and mucosal CD8 T cell immune activation; iv) in vivo intestinal permeability test using a lactulose-mannitol urine test; v) microbiome changes from the sigmoid biopsy and anal swabs; vi) plasma biomarkers of SNA events (IL-6 and D-dimer), and vii) safety, adherence, and tolerability of the synbiotics. The in vivo permeability test will entail drinking a sugar juice containing a known amount of lactulose and mannitol before going to bed at night, and collecting a first-void urine sample the following morning. Mannitol is readily absorbed in the GIT but lactulose absorption is restricted in the small intestine except in the context of increased gut permeability, therefore higher lactulose/mannitol ratio indicates gut leakiness. The microbiome analysis entails extracting genomic material from the anal swabs and sigmoid tissues, and using universal 16s primers to amplify microbial-specific products. Pyrosequencing of the hyper variable 16s rRNA gene in combination with sample-specific barcode sequencing will enable full characterization of microbial communities present from phylum to genus level. Based on preliminary data from my PhD work, mean values and the standard deviation of blood CD8 T cell immune activation from HIV-uninfected individuals (mean, 3.73%; SD, 4.01%) and HIV-infected individuals after 1-year ART (mean, 20.92%; SD, 14.59%), a sample size of 40 (20 in each arm) will power a detection of a minimum difference of 4.87% between the two study groups (alpha, 0.5 and power 80%).

If the efficacy of synbiotics in the proposed phase 2 clinical trial is demonstrated, I propose to examine a similar question in HIV-infected individuals on ART who have persistently
suboptimal CD4 count recovery (<350 cell/µl) despite undetectable viremia, known as immune nonresponders (INRs)\textsuperscript{158}. INRs constitute up to 25\% of HIV population on ART\textsuperscript{158} and there is evidence in this group of increased immune activation, microbial translocation and tissue fibrosis\textsuperscript{293,304,305}. I propose to delineate the mechanism behind the increased inflammation in INR, and to assess the benefit of synbiotic treatment in this group.

\textbf{5.2.2 Innate lymphoid cells and HIV}

Innate lymphoid cells (ILC) have recently been shown to accumulate in mucosal surfaces and play important functions in lymphoid tissue formation, tissue repair, antimicrobial immunity and inflammation (briefly described in 1.4.2.2.3)\textsuperscript{109}. ILCs lack rearranged antigen receptors and react rapidly to a wide range of antigens. Importantly many ILC subsets that have been identified to parallel the effector cytokine profile of specific CD4 T cell subsets\textsuperscript{306}. Three ILC groups have been identified to date: group 1 ILCs (ILC1) comprise of NK cells that mirror Th1 cells and produce type 1 cytokines (IFN\textgamma and TNF\alpha), group 2 ILCs (ILC2) produce type 2 T cell cytokines (IL-4 and IL-13), and group 3 ILCs (ILC3) produce cytokines that define cells of the Th17 and Th22 lineage\textsuperscript{110,306}. Common ILC3s include cells previously named lymphoid tissue-inducer cells (LTi), NK22 cells, ILC22 and ILC17 cells, and ILC3s are defined by their propensity to produce IL-17a and IL-22. ILC3s also express the hormone receptor ROR\gamma t and are stimulated by the cytokines IL-23 and IL-1\beta to provide mucosal immune protection\textsuperscript{109}. Unlike Th17 and Th22 cells, ILC3s do not typically express the HIV receptor CD4 and therefore may not be depleted following HIV infection\textsuperscript{109}. As shown in Chapter 2 of my dissertation, it is possible that ILCs may provide a secondary source of IL-17a and IL-22 when Th17 and Th22 cells are depleted, thereby providing important host mucosal defense during early stages HIV infection. However,
it is also possible that HIV infection may alter or impair the function of all cells producing IL-17a and/or IL-22. Although human studies involving mucosal levels of ILCs are limited, SIV macaque studies revealed that mucosal innate levels of CD3(-)CD8(high) lymphocytes that produce IL-17a were depleted following SIV infection\textsuperscript{307}. ILCs interact closely with commensal bacteria but there is conflicting data as to how commensal bacteria influence ILC development\textsuperscript{308}. Since ILC3s respond to IL-23 and IL-1β, bacteria may indirectly influence the development and functionality of ILC3s. It will be important to investigate ILCs in the context of HIV infection and ART, and also to incorporate ILC3s in the synbiotic clinical trial I previously proposed.

5.2.3 **Th17 and Th22 functionality in HIV and ART**

Although I showed changes in gut Th17 functionality, a maximum of four effector cytokines were assessed and I was unable to investigate the functionality of gut Th22 cells. Furthermore, it is unknown how the functionality of Th17 and Th22 cells are lost. It will be important to assess a wider repertoire of pro-inflammatory and immune-suppressive cytokines, surface marker expression, and signaling molecules to gain a comprehensive understanding of the changes in these cells after HIV infection and ART initiation. One method of analyzing a wider depth of mucosal cell functionality will be to perform microarray analysis on isolated Th17 (CCR6+CCR10-) and Th22 cells (CCR6+CCR10+) from the gut mucosa based on their surface markers\textsuperscript{105,309}. The benefit of using surface markers to define Th17 and Th22 cells is that their ability to produce IL-17a and IL-22 can be measured, but this technique may capture non-Th17 and/or Th22 cells that express these surface markers. Alternatively, a strict functional definition of Th17 and Th22 cells may allow for a pure isolation of these cells through the use of a secretion assay detection kit manufactured by Miltenyi Biotec. This assay can isolate IL-17a producing
cells from a culture of activated CD4 T cells. However, IL-22 secretion assay detection kit is currently unavailable.


